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Method for the Synthesis of Mono-ADP-ribose Conjugated Peptides

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Abstract: ADP-ribosylation is an important post-translational modification involved in processes including cellular replication, DNA repair, and cell death. Despite these roles, the functions of ADP-ribosylation, in particular mono-ADP-ribosylation, remain poorly understood. The development of a technique to generate large amounts of site-specific, ADP-ribosylated peptides would provide a useful tool for deconvoluting the biochemical roles of ADP-ribosylation. Here we demonstrate that synthetic histone H2B tail peptides, incorporating aminoxy or *N*-methyl aminoxy functionalized amino acids, can be site-specifically conjugated to ADP-ribose. These peptides are recognized as substrates by the ADP-ribosylation biochemical machinery (PARP1), can interact with the ADP-ribose binding proteins macroH2A1.1 and PARP9, and demonstrate superior enzymatic and chemical stability when compared to ester-linked ADP-ribose. In addition, the incorporation of benzophenone photo-cross-linkers into these peptides is demonstrated to provide a means to probe for and enrich ADP-ribose binding proteins.

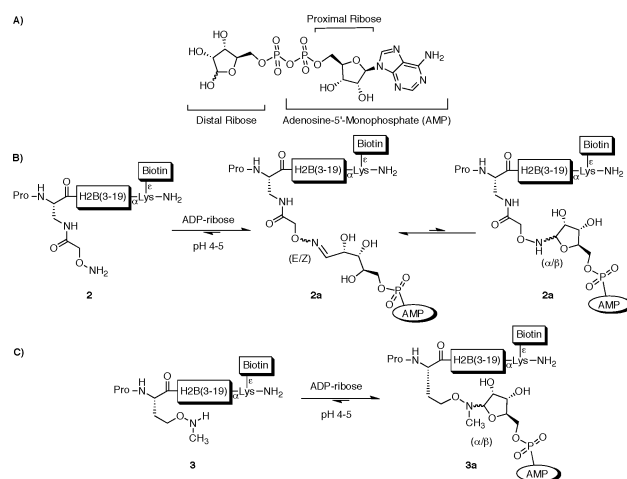
ADP-ribosylation involves the transfer of ADP-ribose (ADPR) from β -NAD⁺ onto protein substrates.¹ A family of enzymes termed poly(ADP-ribose) polymerases (PARPs), which generate protein-linked ADPR monomers (mono-ADP-ribosylation) or polymers (poly-ADP-ribosylation), catalyze this reaction. ADP-ribosylation occurs on several residues including glutamic acid where the attachment is through an ester linkage.¹ The functional roles of ADP-ribosylation, in particular mono-ADP-ribosylation, are poorly understood. The study of ADP-ribosylation has proven difficult for many reasons. For example, the ester-linked ADPR generated by PARPs is unstable at basic pH ($t_{1/2} < 1$ h pH 7.5^{1b}), and endogenous enzymes rapidly cleave poly-ADP-ribose polymers (e.g., poly(ADP-ribose) glycohydrolase; $t_{1/2}$ 0.6–6 min^{1b}). There is also a lack of commercial antibodies specific for mono-ADP-ribose, mono-ADP-ribose protein linkages, or poly-ADP-ribose branching.¹ The ability to site-specifically attach ADPR to peptides/proteins would provide a useful tool for studying the biochemical effects of this modification. Such a technique, using a protected mono-ADP-ribosylated asparagine building block for peptide synthesis, has recently been described.² In this report, we demonstrate the use of aminoxy-functionalized amino acids to enable site-specific attachment of ADPR onto peptides. Using this technology, we show that an ADP-ribosylated version of the histone H2B tail can interact with the ADPR binding protein, macroH2A1.1 (mH2A1.1), and demonstrate that incorporation of photo-cross-linkers into these peptides improves their ability to detect ADPR binding proteins.

We set out to develop a general strategy for the synthesis of mono-ADPR-peptide conjugates. In principle, a chemoselective ligation approach would allow precise control over the ADPR conjugation site using readily accessible building blocks. This would enable large amounts of ADPR conjugated peptides to be synthe-

sized for biochemical studies. Further, the chemical approach would allow the incorporation of linkers featuring improved chemical and enzymatic stability, when compared to enzymatic ADP-ribosylation. Uses for such a method would include the investigation of protein interactions, the potential to generate site-specific poly-ADP-ribosylated peptides through a templated initiator ADPR residue,³ and the generation of ADPR conjugated proteins using expressed protein ligation.⁴

Under physiological conditions, ADPR (Scheme 1A) reacts with lysine and arginine side chains to form imine-linked species, which degrade to give a mixture of products.⁵ While this process does not provide a route to stable, site-specific ADPR-peptide conjugates, it suggested to us that oxime ligation might prove suitable for this purpose. Oxime ligation is performed at \sim pH 4.5, a pH at which peptide lysine and arginine residues are protonated and unreactive. In addition, oxime ligation is compatible with all natural amino acid functional groups, and the oxime linkage is significantly more stable to hydrolysis compared to imines or the native ADPR ester linkage.⁶ Ligation of reducing carbohydrates through an oxime, using an aminoxy group, generates mainly ring-opened carbohydrates, with a small amount of the ring-closed form (Scheme 1B).⁷ Conversion of the aminoxy group to a secondary alkoxyamine, through *N*-methylation, results in the attached carbohydrates adopting a ring-closed form exclusively (Scheme 1C).^{7b} Based on these observations, we decided to investigate if aminoxy and *N*-methyl aminoxy functionalized peptides could undergo site-specific ligation reactions with ADPR.

Scheme 1. Structure of (A) ADPR and Its Ligation to (B) Aminoxy and (C) *N*-Methyl Aminoxy Functionalized Peptides



Histone H2B was selected as a model for ADPR conjugation as it has been reported to be mono-ADP-ribosylated on glutamate residue 2 in rat liver.⁸ In preliminary studies, we found this modification can also occur on glutamate-2 of human H2B *in vitro* using recombinant PARP10 (Figure S1), which has been shown to

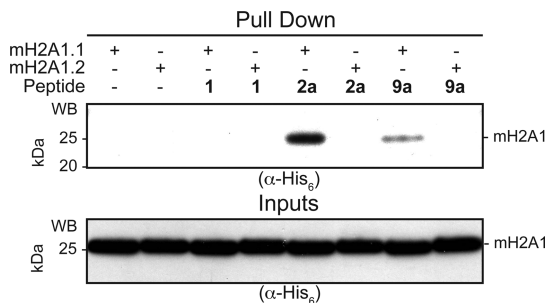


Figure 2. Binding of synthetic biotinylated peptides to mH2A1.1. Indicated peptides were incubated with either mH2A1.1 or, as a control, mH2A1.2. Peptides were then immobilized using streptavidin beads and captured complexes analyzed by SDS-PAGE followed by Western blotting against the His₆-tag on the protein. Equal loading of peptides was shown by RP-HPLC.

of peptide **8a**, but not **7**, with HeLa S3 nuclear lysates doped with mH2A1.1 followed by UV irradiation, resulted in the generation of a robust cross-link to the *macro* domain (Figure 3). This effect was observed at two *macro* domain concentrations, differing by 5-fold. At the higher concentration, peptide **8a** was able to pull down both cross-linked mH2A1.1 and non-cross-linked mH2A1.1 (upper and lower bands, respectively, in the α -His blot of the precipitated material, Figure 3). Since the mH2A1.1 *macro* domain can form dimers,¹⁶ these data suggest that the cross-linked species may coprecipitate interacting proteins in their native state. In addition, PARP9, a known ADP-ribose binding protein,^{13b} could be enriched from Farage nuclear lysates after cross-linking with peptide **8a**, but not with **7** (Figure S10). Based on these observations, incorporation of photo-cross-linkers and ADPR into peptides could be useful for enriching or probing for ADPR binding proteins.

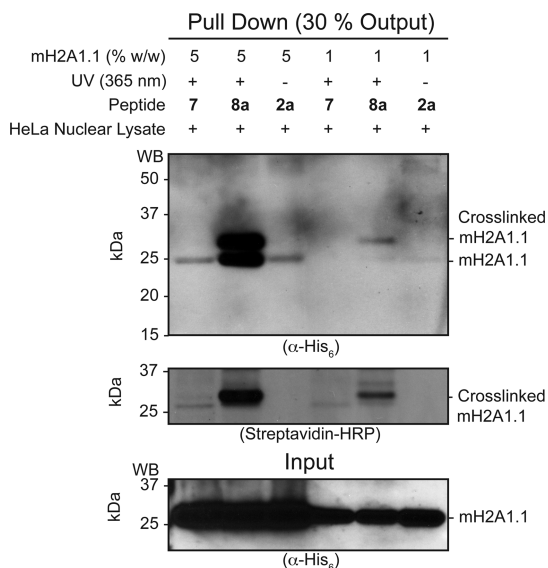


Figure 3. Photo-cross-linking studies. Indicated peptides were incubated with HeLa cell nuclear lysates doped with mH2A1.1 at two concentrations. Mixtures were then irradiated with UV light (or not in the case of peptide **2a**) and then immobilized using streptavidin beads. Captured complexes were analyzed by SDS-PAGE followed by Western blotting against mH2A1.1 (α -His₆) or the peptide (streptavidin-HRP).

Research into ADP-ribosylation has proven difficult due to the paucity of tools available for studying this modification. Several β -NAD⁺-based chemical probes have been described.¹⁷ These

analogues, if recognized by ADP-ribosylating enzymes, offer the potential to detect and enrich ADP-ribosylated proteins. The sites of modification, however, depend on the enzyme used, and unstable ADPR-protein linkages may be generated. In contrast, the methodology described in this study enables multimilligram amounts of highly pure, stable, and site-specific ADPR-peptide conjugates to be generated. The ADPR-peptide conjugates can bind to the mH2A1.1 *macro* domain, the first demonstration that a *macro* domain can engage ADPR when linked to a peptide. Moreover, the ADPR-peptide conjugates could be poly-ADP-ribosylated by PARP1, and incorporation of photo-cross-linkers allowed the capture of ADPR-binding proteins from lysates. Consequently, this technique may be useful for identifying and enriching ADPR binding proteins and may enable the generation of site-specifically poly-ADP-ribosylated peptides and proteins for biochemical assays.

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Supporting Information Available: Experimental procedures, characterization data, complete ref 9b, and supporting figures. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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