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Unexpected biotinylation using ATP-γ-Biotin-LC-PEO-amine as a kinase substrate

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ABSTRACT

Protein phosphorylation is the most widely studied post-translational modification. Reversible protein phosphorylation is implicated in the regulation of a broad range of cellular processes. As such, there is extensive interest in simple and sensitive procedures for the isolation and detection of phosphorylated proteins. Synthetic analogues of ATP, with a biotin linked to the gamma-phosphate of ATP, have been reported to biotinylate kinase substrates in a kinase-catalyzed reaction. This could be an extremely attractive and versatile method for affinity enrichment of phosphorylated proteins. However, as we report here, the commercially available biotin-ATP analogue, ATP-γ-Biotin-LC-PEO-amine, is capable of biotinylating proteins independent of kinase activity. In fact, we demonstrate that this reagent is capable of non-specifically biotinylating any protein. Although the mechanism of biotinylation is not known, this report uncovers a flaw in a commercially available reagent and also highlights the importance of control experiments when developing new biochemical tools to study enzyme activity.

1. Introduction

Protein phosphorylation is the most common post-translational modification, found in cell signaling pathways from eukaryotes to proteobacteria [1]. Due to its prominent role in cell biology and biochemistry, protein phosphorylation is the subject of an enormous body of literature as well as significant ongoing research efforts. In addition to understanding the role of phosphorylation in particular pathways or proteins, there are now considerable efforts to map and characterize all protein phosphorylation sites, leading to new areas of inquiry such as phosphoproteomics, kinomics, and phosphoregulation.

This great interest in protein phosphorylation has led the development of a number of tools for the study of phosphoproteins, including, antibodies [2,3], phosphate-specific stains [4,5], and mass spectrometry [6]. Adenosine triphosphate analogues, modified at the gamma-phosphate are now also commonly used to get a handle on protein phosphorylation [7–9]. Structural analyses have revealed that the ATP binding sites of kinases are partially solvent exposed, allowing for modifications at the gamma-phosphate that are catalytically transferred to the protein during enzymatic phosphorylation. One such analogue that has recently been used to modify the peptide substrates of several kinases is ATP-γ-Biotin-LC-PEO-amine (ATP-biotin; Fig. 1). It has also been reported that ATP-biotin can modify the full-length protein substrates of serine/threonine and tyrosine kinases via a kinase-catalyzed mechanism [7].

Generally speaking, all of these techniques have been developed for kinase-catalyzed *O*-phosphorylation of tyrosine, serine and threonine residues. However, several other phospho-amino acids, such as *N*-phosphates on histidines, *S*-phosphates on cysteines, and acyl-phosphates on glutamic and aspartic acid residues, are also recognized as of significant biological importance. In bacteria, for example, the primary mode of signal transduction is by phosphotransfer between histidine and aspartate residues, often referred to as two-component signaling [10]. The first step of signal transduction occurs by autophosphorylation of a conserved histidine residue on a histidine kinase. Mechanisms of histidine phosphorylation are not well understood and have been a challenge to study due to the acid lability of the phosphoramidate bond. Until recently [11–13], there were no tools for the detection and isolation of phosphohistidines [14].

Our primary goal in initiating this study was to investigate the applicability of ATP-biotin for the enrichment of phosphohistidine containing proteins from cell lysates. Bacterial histidine kinases differ from the serine/threonine and tyrosine kinases used in previous studies in that they autophosphorylate; the histidine residue is contained on a phosphotransfer domain within the same protein. Biotinylation of histidine kinases would provide a useful affinity

Abbreviations: ATP-biotin, ATP-γ-Biotin-LC-PEO-amine; ATP-^{32}P, ATP-γ-^{32}P; SA-HRP, Streptavidin–horseradish peroxide conjugate antibody; H-NOX, heme-nitric oxide/oxygen binding domain; MbSrc4, Src-family tyrosine kinase from *Monosiga brevicollis*; HahK, H-NOX-associated histidine kinase; HahK-H257A, HahK point mutant H257A.

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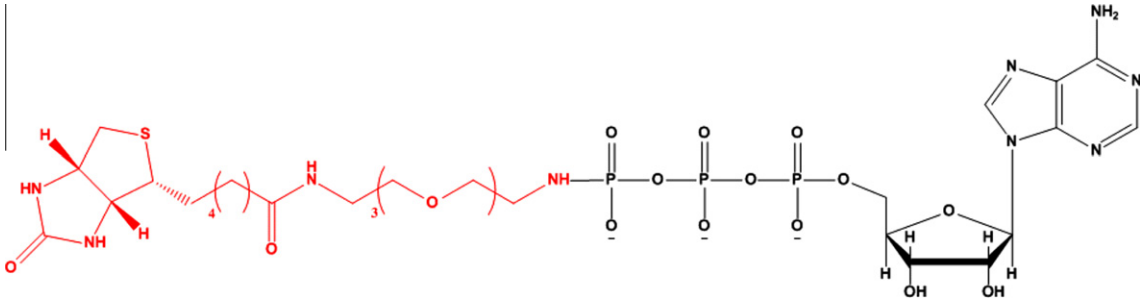

Fig. 1. Chemical structure of ATP-γ-Biotin-LC-PEO-amine.

tag for the identification and isolation of bacterial signaling proteins.

However, in this report we describe our experiments indicating that biotinylation of proteins using commercially available ATP-biotin occurs either in part, or entirely, via a non-kinase catalyzed mechanism. While the precise mechanism of this protein biotinylation remains unknown, our work highlights the necessary controls needed while developing methods to study kinase activity. Possible mechanisms for the non-enzyme-specific biotinylation point to flaws in the design of linker between ATP and biotin.

2. Materials and methods

2.1. Materials

ATP-γ-Biotin-LC-PEO-amine (ATP-biotin) was purchased from Affinity Photoprobes (#AB09). The streptavidin-horseradish peroxide conjugate (SA-HRP) used for Western blotting was purchased from Fisher Scientific. ATP-γ-^{32}P (ATP-^{32}P) was purchased from Perkin Elmer. ATP was purchased from Promega. Immobilon HRP substrate was purchased from Millipore. Photographic film was purchased from Kodak. DC Assay was purchased from Bio-Rad. All reagents were purchased in their highest available purity and used as received.

2.2. Proteins

Histidine kinase HahK from *Pseudoalteromonas atlantica,* HahK point mutant H257A (HahK-H257A), and H-NOX from *P. atlantica* were expressed and purified as previously described [15]. Src-family tyrosine kinase from *Monosiga brevicollis* (MbSrc4) was obtained as a gift from Professor Todd Miller, Stony Brook University. Horse heart myoglobin was purchased from Sigma. Protein concentrations were measured using the BioRad DC Assay.

2.3. ATP-biotinylation

ATP-biotinylation was performed using a modified version of previously described method [7]. Briefly, 5 or 10 μM HahK or MbSrc4 was incubated with 5 mM $MgCl_2$ and 1 mM ATP-biotin (after evaporation of methanol storage solvent) in reaction buffer containing 50 mM Tris–HCl, 300 mM NaCl, and pH 8. The reaction was allowed to proceed at ambient temperature for 30 min. Biotinylated proteins were detected by Western blotting. In experiments exploring the biotinylation of non-kinases, the concentration of HahK-H257A was 10 μM and myoglobin and H-NOX were each 50 μM.

2.4. Western blotting

Protein samples were separated by SDS–PAGE and then electrotransferred to nitrocellulose membrane. The membrane was blocked overnight in a 1% casein solution in PBS with 0.01% Tween-20 at 4 °C. Before incubation with antibody, the membrane was blocked for an additional 1 h at ambient temperature. The membrane was then incubated with 0.03% SA-HRP for 1 h at ambient temperature. This was followed by two washes in blocking solution and two washes in PBS supplemented with 0.01% Tween-20. Each wash was 15 min in duration. The membrane was then incubated with freshly prepared HRP substrate for 1 min before being exposed to photographic film. The blot was also visualized using a Typhoon Imager to detect chemiluminescence.

2.5. Kinase activity assay

HahK and MbSrc4 autophosphorylation were assayed using ATP with trace ATP-γ-^{32}P. Briefly, 5 or 10 μM kinase was incubated with 5 mM $MgCl_2$, 1 mM ATP and 10 μCi γ-^{32}P-ATP. Reactions were carried out at ambient temperature and quenched after thirty min by the addition of SDS–PAGE loading dye and boiling for 5 min at 95 °C. Proteins were separated by SDS–PAGE and dried (Bio-Rad Gel Drier Model 583) before exposure to an autoradiography screen for 12–16 h. Gel images were obtained using a Typhoon Imager.

3. Results

3.1. Biotinylation by ATP-biotin is not an accurate representation of kinase activity levels

Experiments using ATP-γ-Biotin-LC-PEO-amine (ATP-biotin) as a substrate for HahK (an autophosphorylating histidine kinase) and MbSrc4 (an autophosphorylating tyrosine kinase) reveal that both proteins are biotinylated, as detected by Western blotting with SA-HRP. Furthermore, biotinylation levels of the two proteins (assayed at the same concentration) are comparable (Fig. 2). In contrast, when the same experiment was performed using ATP with trace ATP-γ-^{32}P (ATP-^{32}P) as a substrate, both proteins are radiolabeled, but MbSrc4 accumulates much more phosphate than HahK (Fig. 2). This is not an unexpected result, because the kinetics of MbSrc4 ($k_{cat}/K_m = 2.9 \times 10^5$ min^{-1} M^{-1}) [16] indicate that it is a very active tyrosine kinase, much more active than kinetically characterized histidine kinases ($k_{cat}/K_m = 1.2 \times 10^3$ min^{-1} M^{-1}) [17]. Furthermore, phosphorylated tyrosine is much more stable than phosphorylated histidine [14], which allows for greater accumulation of radiolabeled phosphate on the protein. Therefore, while the ATP-^{32}P kinase assay reflects kinase activity levels, the ATP-biotin assay appears to be reflective of protein concentration. Possible explanations of this would include slower kinetics of MbSrc4 autophosphorylation using ATP-biotin than ATP-^{32}P, instability of phospho-biotinylated tyrosine, or that biotinylation by ATP-biotin is not kinase-catalyzed.