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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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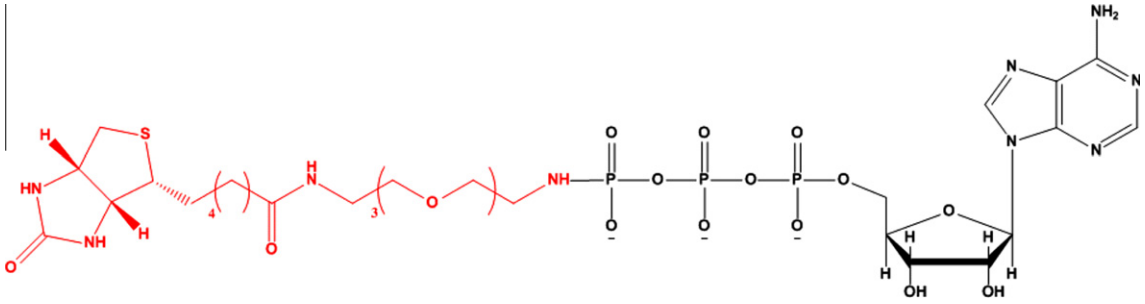

Fig. 1. Chemical structure of ATP-γ-Biotin-LC-PEO-amine.

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.

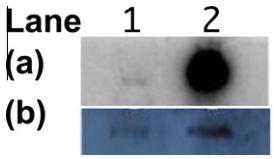

Fig. 2. Biotinylation by ATP-biotin is not an accurate representation of kinase activity levels. Lane 1 contains histidine kinase HahK while Lane 2 contains tyrosine kinase MbSrc4. Autoradiography detection of radiolabeled proteins generated during a kinase assay performed using ATP-^{32}P shows MbSrc4 to be much more active than HahK, giving a large, intense band on the autoradiogram (panel a). SA-HRP Western blotting of otherwise identical reactions performed using ATP-biotin instead of ATP-^{32}P do not reflect this difference in activity level (panel b). This experiment shows that incubation of a kinase with ATP-biotin and detection of biotinylation with SA-HRP Western blotting reports on protein concentration, while incubation of a kinase with ATP-^{32}P and detection of radiolabeling with autoradiography is indicative of kinase phosphorylation activity.

3.2. ATP-biotin can biotinylate non-kinase proteins

In order to investigate our hypothesis that kinase biotinylation by ATP-biotin is not kinase catalyzed, we decided to incubate ATP-biotin and ATP-^{32}P with a panel of proteins, some with and some without kinase activity. These proteins included HahK (an active histidine kinase) as well as a HahK point mutant, HahK-H257A, in which the site of histidine autophosphorylation has been mutated to an alanine, which should render it inactive to autophosphorylation. We also examined the ability of ATP-biotin to biotinylate two gas-binding heme proteins, commercially available horse heart myoglobin, and partially purified H-NOX (heme-nitric oxide/oxygen binding domain), neither of which has any known enzymatic activity. Equimolar amounts of each of these four proteins were incubated with ATP-biotin or ATP-^{32}P. We found that each of these proteins was biotinylated (Fig. 3), but only wild-type HahK was radiolabeled (Fig. 4). As only wild-type HahK is an active kinase, this result is consistent with the conclusion that ATP-^{32}P is a kinase substrate, and that radiolabeling with ATP-^{32}P is accurately reporting on kinase-catalyzed phosphorylation, but ATP-biotin is able to non-enzymatically modify proteins.

This unexpected result prompted us to try further kinase activity controls. ATP-biotin and ATP-^{32}P were incubated with wild-type HahK without the addition of $MgCl_2$. Magnesium is required by kinases for ATP binding and kinase activity [18]. Both biotinylation and radiolabeling of HahK were observed in the presence of magnesium. However, we again observed biotinylation (Fig. 3), but not radiolabeling (Fig. 4), of HahK in the absence of magnesium, where no kinase activity is expected.

In all of our experiments, SA-HRP Western blots were imaged using photographic film as well a Typhoon imager. While not all of the contaminating bands are visible using the Typhoon (it automatically adjusts the contrast and background to the brightest bands on the gel), the film indicates that, in addition to the test proteins, every protein contaminant in every reaction sample is biotinylated (Fig. 3) but not radiolabeled (Fig 4). This cannot be explained by non-specific binding of the antibody SA-HRP, because these proteins are not detected by the same antibody in experiments without ATP-biotin (Fig. 3). The unexpected biotinylation also cannot be explained by the presence of a contaminant with kinase activity, because the same protein samples are not radiolabeled (Fig. 4).

Taken together, these results strongly support our conclusion that protein biotinylation by ATP-biotin occurs via a non-kinase catalyzed mechanism, in contrast to protein radiolabeling by ATP-^{32}P, which occurs via kinase-catalyzed phosphorylation.

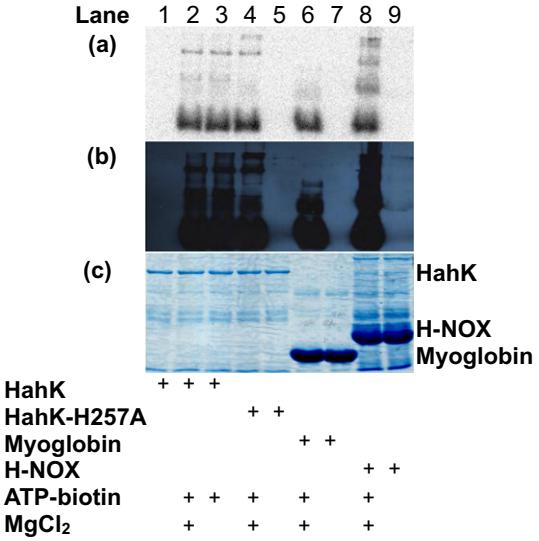

Fig. 3. ATP-biotin biotinylates proteins that lack kinase activity. A panel of proteins, some, but not all of which, have kinase activity (HahK, HahK-H257A, H-NOX, and myoglobin), were incubated with ATP-biotin in a series of kinase activity assays. Three identical sets of experiments were performed and analyzed using three different SDS–PAGE-based assays, from top: (a) SA-HRP Western blot of gel imaged by Typhoon imager; (b) SA-HRP Western blot of gel imaged on photographic film; and (c) Coomassie stained gel. Western blot images (panels a and b) shows thabiotin is biotinylated, as detected by SA-HRP [HahK (lane 2), HahK-H257A (lane 4), myoglobin (lane 6), and H-NOX (lane t each protein incubated in the presence of ATP8)]. These same proteins are not detected by SA-HRP in the absence of ATP-biotin, indicating they are not natively biotinylated [HahK (lane 1), HahK-H257A (lane 5), myoglobin (lane 7), and H-NOX (lane 9)]. Biotinylation is also observed for HahK incubated with ATP-biotin in the absence of Mg^{2+} (lane 3), which is required for kinase activity. Furthermore, every protein contaminant (see Coomassie-stained gel, panel c) is detected by SA-HRP upon incubation with AP-biotin. The major band on the gel front of the Western blots is excess ATP-biotin. Relative positions of HahK (55 KDa), myoglobin (17 KDa) and H-NOX (22 KDa) are indicated on the Coomassie stained gel. This experiment demonstrates that ATP-biotin can biotinylate proteins in the absence of an active kinase.

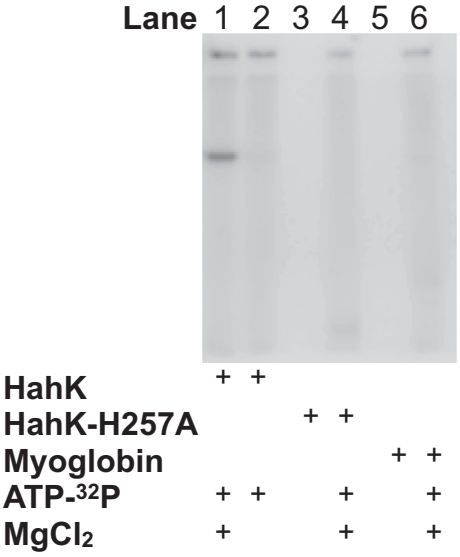

Fig. 4. Kinase assays performed with ATP-^{32}P reflect kinase activity. A panel of proteins, some, but not all of which, have kinase activity (HahK, HahK-H257A, and myoglobin), were incubated with ATP-^{32}P in a series of kinase activity assays and radiolabeling with ^{32}P was detected by autoradiography. HahK is radiolabeled when incubated with ATP-^{32}P (lane 1), but not in the absence of Mg^{2+} (lane 2). Radiolabeling is not observed for the proteins that lack kinase activity [HahK-H257A (lane 4), myoglobin (lane 6)]. These data demonstrate that only proteins with kinase activity are radiolabeled with ATP-^{32}P.

4. Discussion

Under the conditions used in our experiments, we observe biotinylation of any protein upon incubation with ATP-biotin, regardless of kinase activity. We observe biotinylation, but not radiolabeling, of myoglobin, H-NOX, an inactive kinase point mutant, and active kinases in the absence of magnesium. Each of these experiments indicates that ATP-biotin is capable of biotinylating proteins via a non-kinase catalyzed mechanism. Furthermore, the biotinylation levels we observe are correlated with protein concentrations, rather than kinase activity levels (Fig. 2).

There have been several reports in the literature using ATP-biotin as a probe for kinase activity [7,19]. In these studies, an external peptide or protein was used as the phosphorylation substrate (rather than kinase autophosphorylation) and the concentration of the kinase used was much, much lower than the concentration of the substrate peptide. In fact, the kinase concentration was too low to be visualized on a Coomassie stained gel. Hence, even if these kinases were getting non-enzymatically biotinylated during the kinase reaction, they would not have been detected. We used autophosphorylating kinases, so by definition, the kinase and its substrate are the same molecule, thus the kinase concentration is high enough to be detected by Western blot. Nonetheless, the reported results using ATP-biotin are consistent with the conclusion that peptide biotinylation is occurring, at least partially, via phosphorylation. For full-length protein substrates and cell lysates, however, the data is less conclusive [7]. Based on this study, we conclude it is very likely that at least some of the biotinylated proteins detected in these assays have been biotinylated via a kinase-independent mechanism.

A closer look at the ATP-biotin structure (Fig. 1) reveals that there are two reactive groups, an acid labile phosphoramidate bond between the gamma-phosphate and the linker, and an amide bond in the middle of the linker, in addition to the phosphodiester bonds. All of our reactions were performed at an alkaline pH, which should prevent hydrolysis of the phosphoramidate bond. However, we propose that proteins are able to react at one of these sites to be biotinylated without phosphorylation. The exact mechanism has not been elucidated. We attempted to use MS/MS to detect ATP-biotin modified peptides from myoglobin. These studies failed, however, either because the biotinylated peptides did not fly under the conditions we used for mass spectrometry, or because non-enzymatic biotinylation with ATP-biotin is stochastic and non-specific. If the latter, then most protein molecules would not biotinylated, and among those that are biotinylated, each would be modified at a different site, thus there would not a large enough population of a particular biotinylated peptide for quantification.

In the last few years, there have been some major advances in the study of histidine phosphorylation. Antibodies have been raised towards phosphohistidine using a triazole epitope [12], aiding in the detection and enrichment of this elusive moiety. Thiophosphorylation using an ATP-γ-S has emerged as a useful tool to generate stable phosphohistidine analogues and develop tags for histidine phosphorylation [13]. These developments have opened a variety of new tools to study histidine phosphorylation and two-component signaling in bacteria. We envision the use of a biotin tag to enrich for these proteins followed by acid cleavage of the histidine phosphate to isolate histidine kinases from cell lysates. The currently available ATP-biotin conjugate, however, has been shown in this report to be capable of biotinylating proteins via a mechanism independent of kinase catalysis. Our work highlights the importance of exhaustive control experiments during tool development. It would be interesting to combine more recent techniques, like thiophosphorylation, to synthesize novel biotin or other affinity tags for enrichment of proteins containing phosphohistidines.

Acknowledgments

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