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A Cell-Permeable ATP Analogue for Kinase-Catalyzed Biotinylation**

*Ahmed E. Fouda and Mary Kay H. Pflum**

Abstract: *ATP analogues have been powerful compounds for the study of kinase-catalyzed phosphorylation. However, the cell impermeability of ATP analogues has largely limited their use to in vitro lysate-based experiments. Herein, we report the first cell-permeable ATP analogue, ATP–polyamine–biotin (APB). APB is shown to promote biotin labeling of kinase substrates in live cells and has future applications in phosphoprotein purification and analysis. More generally, these studies provide a foundation for the development of additional cell-permeable ATP analogues for cell-signaling research.*

Living cells respond to extracellular conditions through signaling cascades, which are mediated by a variety of protein modification reactions. One ubiquitous protein modification that regulates many metabolic and cell-signaling pathways is kinase-catalyzed protein phosphorylation (Figure 1 A).[1] Alteration of pathways involving kinases and phosphorylation can lead to various diseases, such as Parkinson's disease,[2] cancer,[3] and diabetes mellitus.[4] Therefore, studying protein kinases and their phosphorylated substrates is critical to understand cell-signaling pathways in both diseased and healthy cells.

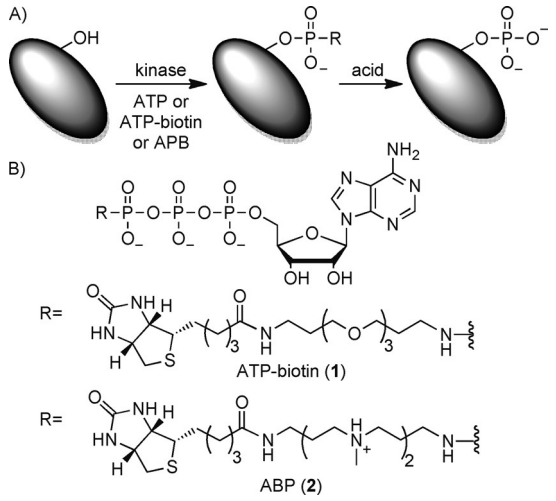

Figure 1. A) Kinase-catalyzed phosphorylation of a protein substrate with B) ATP (R = O^-) or the ATP analogues ATP–biotin (**1**) or APB (**2**).

With over 500 kinases and potentially thousands of phosphoproteins,[5] multiple complementary approaches are necessary to monitor the complex cellular phosphoproteome. One powerful approach exploits analogues of the universal cosubstrate of kinases, adenosine-5′-triphosphate (ATP; Figure 1 B). Multiple ATP analogues have been employed in kinase research, including base-modified,[6] sugar-modified,[7] and triphosphate-modified analogues.[8] Our laboratory and other groups have utilized γ-phosphate-modified ATP analogues to label kinase substrates for subsequent purification and analysis.[8e–j,9] For example, ATP–biotin (**1**; Figure 1 B) is promiscuously accepted as a cosubstrate by protein kinases to phosphorylbiotinylate substrates.[9b,d,10] After kinase-catalyzed biotinylation with ATP–biotin, the biotin group facilitates the analysis of phosphoproteins using various commercial streptavidin-conjugated reagents.[9b,11] Unfortunately, owing to the impermeability of ATP analogues,[12] ATP–biotin has been used in vitro only.[9d] The ability to utilize ATP–biotin in living cells would promote the study of protein kinases under more physiologically relevant conditions. Herein, we describe the first cell-permeable ATP–biotin analogue for live-cell kinase-catalyzed biotinylation.

Previous reports documented neutralizing the negative charge of phosphate groups to promote the cell permeability of compounds such as bisphosphonates[13] and phosphoinisitols.[12,14] Building on these precedents, we replaced the poly(ethylene glycol) PEG linker of ATP–biotin with a polyamine linker to create ATP–polyamine–biotin **2** (APB; Figure 1). Polyamines are known cell-delivery vehicles for anionic nucleic acids.[15] In the case of APB, the polyamine linker will be positively charged under physiological conditions to partially neutralize the triphosphate charge and promote cell permeability. We chose spermine as the linker because its size mimics the original PEG linker in ATP–biotin (Figure 1). Furthermore, we used methylated spermine to avoid possible side reactions of the nucleophilic secondary amines.

To computationally analyze the kinase compatibility of APB, docking studies with the PKA kinase crystal structure[16] were performed using the Autodock program.[17] Nearly identical PKA binding was observed with APB (Figure 2 A), ATP–biotin (Supporting Information, Figure S1 B), and ATP (Figure S1 C). The biotin group protrudes from the active site while the triphosphate is positioned in close proximity to the co-crystallized peptide. The α-phosphate of APB is 3.8 Å away from the catalytic amino acid K72, as compared to 3.7

[*] A. E. Fouda, M. K. H. Pflum
Department of Chemistry, Wayne State University
5101 Cass Avenue, Detroit, MI 48202 (USA)
E-mail: pflum@chem.wayne.edu
Homepage: http://chem.wayne.edu/pflumgroup/

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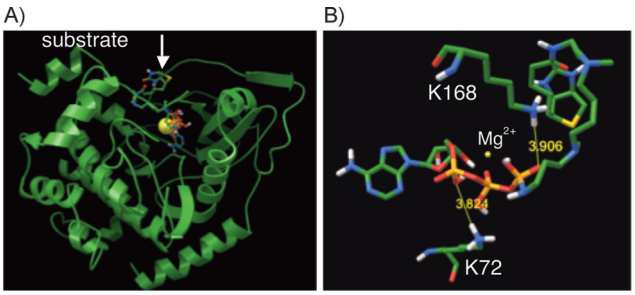
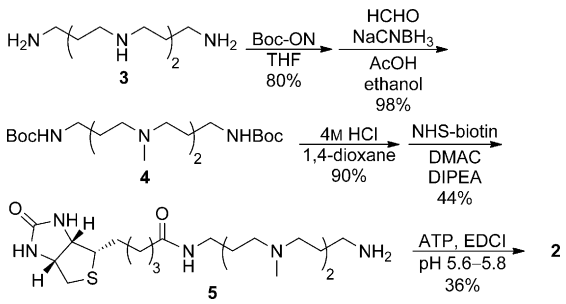


Figure 2. A) Docking of APB into the crystal structure of the catalytically active site of PKA kinase co-crystallized with peptide substrate (PDB No. 4DH1)[16] using Autodock 4.2.[17] The arrow points to the solvent-exposed biotin group. B) Enlarged view of the interaction of APB with the catalytic Mg^{2+} metal center (yellow orb) and the amino acids K72 and K168. The γ-phosphate of APB is positioned in close proximity to K168 while the α-phosphate lies near K72. The APB atoms are color-coded (carbon green, hydrogen gray, nitrogen blue, oxygen red, phosphorus orange). For clarity, the atomic radius of Mg^{2+} was reduced to 0.5 Å.

and 3.2 Å with ATP or ATP–biotin, respectively (Figure S1 E and F), suggesting that the three ATP molecules bind similarly in the active site. In contrast, the γ-phosphate of APB is 3.9 Å from K168 (Figure 2 B), as compared to 2.4 Å with both ATP and ATP–biotin (Figure S1 E and F). The docking studies suggest that APB is a potential kinase cosubstrate owing to similar active-site binding. However, the long distance between the γ-phosphate of APB and K168 suggests that APB may be a less efficient cosubstrate than ATP or ATP–biotin.

To experimentally test APB as a kinase cosubstrate, this compound was first synthesized from commercially available spermine (Scheme 1). Spermine (**3**) was protected at the primary amines followed by reductive amination to give Boc-protected methylated spermine (**4**).[18] After deprotection,[18] the NHS ester of biotin (Figure S2) was synthesized as reported[19] and coupled to give polyamine–biotin (**5**).[20] Finally, **5** was coupled with ATP to obtain APB (**2**).[21]

To determine its compatibility with kinase-catalyzed biotinylation, APB was incubated with PKA kinase and a full-length protein substrate, myelin basic protein (MBP). Biotinylation was visualized after SDS-PAGE gel separation, transfer to a PVDF membrane, and staining with a streptavidin–Cy5 conjugate (Figure 3 A). Biotinylation was observed



Scheme 1. Synthesis of ATP–polyamine–biotin **2** (APB). Boc-ON = 2-(*tert*-butoxycarbonyloxyimino)-2-phenylacetonitrile, DIPEA = diisopropylethylamine, DMAC = dimethylacetamide, EDCI = 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride.

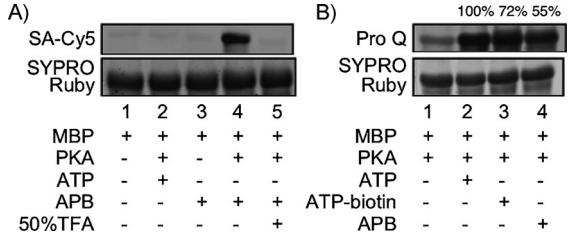


Figure 3. Kinase-catalyzed biotinylation with APB. A) Myelin basic protein (MBP) was incubated with or without PKA kinase in the presence of either ATP or APB. The labeling mixtures were separated by SDS-PAGE and visualized with SYPRO Ruby total proteins stain (bottom) or streptavidin–Cy5 (top). Trifluoroacetic acid (TFA; 50% final concentration) was added after biotin labeling (lane 5). Full gel images are shown in Figure S10. B) Quantitative analysis of MBP phosphorylation was performed in the presence of PKA and ATP (lane 2), ATP–biotin (lane 3), or APB (lane 4). TFA was used to cleave the biotin group and produce the same phosphoprotein product with all analogues. The reaction mixtures were separated by SDS-PAGE and visualized with SYPRO Ruby stain (bottom) or ProQ diamond phosphoprotein stain (top). The percentage phosphorylation was calculated by comparison with ATP (set as 100%). The gels are representative of at least three independent trials.

only in the presence of kinase (Figure 3 A, compare lanes 3 and 4). Furthermore, MBP biotinylation was lost in the absence of APB (Figure 3 A, lane 2), in the presence of the kinase inhibitor staurosporine (Figure S11, compare lanes 2 and 3), or upon incubation with acid (Figure 3 A, lane 5) because of cleavage of the phosphoramidate bond (Figure 1 A). To further confirm kinase-catalyzed biotinylation with APB, a mass-spectrometric (MS) study was performed. In this case, the PKA peptide substrate kemptide was incubated with APB and PKA before MALDI-TOF MS analysis. The biotinylated kemptide product was observed only in the presence of the APB cosubstrate (Figure S12, m/z 1332.469 $[M+H]^+$). The combined gel and MS analyses confirmed that APB is a kinase cosubstrate.

To investigate the efficiency of biotinylation using APB, both quantitative conversion and kinetic studies were performed. For quantitative conversion studies, APB, ATP–biotin, and ATP were separately incubated with MBP and PKA, followed by cleavage of the phosphoramidate bond with acid to produce phosphoprotein products with all ATP analogues (Figure 1 A), which allowed for quantitative comparison. The reaction mixtures were then separated by SDS-PAGE with phosphoproteins visualized by ProQ diamond stain (Figure 3 B), as previously reported.[9d] Quantification showed 55 ± 6% conversion with APB compared to ATP (Figure 3 B, lane 4), whereas ATP–biotin showed 72 ± 7% conversion compared to ATP (Figure 3 B, lane 3). Biotinylation was less efficient with APB than with ATP–biotin, as predicted by the docking studies. However, the observed quantitative analysis confirmed that APB was a kinase cosubstrate. Next, kinetic studies were performed by incubating APB or ATP (0.5–100 μM) with PKA and the kemptide peptide substrate. APB showed a reduced k_{cat}/K_M ratio ($0.25\ s^{-1}\,\mu M^{-1}$) compared to ATP ($0.52\ s^{-1}\,\mu M^{-1}$; Figure S13). However, the kinetics are similar to those observed with other ATP analogues used for kinase studies, including the γ-phosphate-modified ATP analogue ATP–dansyl,[22] or the

base-modified analogues N6-benzyl-ATP or N6-(2-phenethyl)-ATP.[6b,23] Overall, both quantitative conversion and kinetic studies confirmed that APB is an efficient kinase cosubstrate with conversions and kinetics similar to those of other known ATP analogues.

To analyze the compatibility of APB with cellular kinases, HeLa cell lysates were incubated with APB, followed by SDS-PAGE analysis. Biotinylation of proteins was detected in the APB reaction (Figure 4A, lane 4), showing the promiscuity of cellular kinases for APB. Similar levels of labeling were observed with APB and ATP–biotin (Figure 4A, lane 4 vs. 7). In a control experiment, heat-denatured lysates generated low levels of biotinylation with both APB and ATP–biotin (Figure 4A, lanes 3 and 6), which confirmed the kinase dependence of biotinylation. Acid treatment also reduced biotinylation (Figure 4A, lane 5), which indicated labeling via the phosphoramidate bond in APB (Figure 1A). These studies in lysates further established the compatibility of APB with a range of cellular kinases and substrates, similar to ATP–biotin.[9b,d]

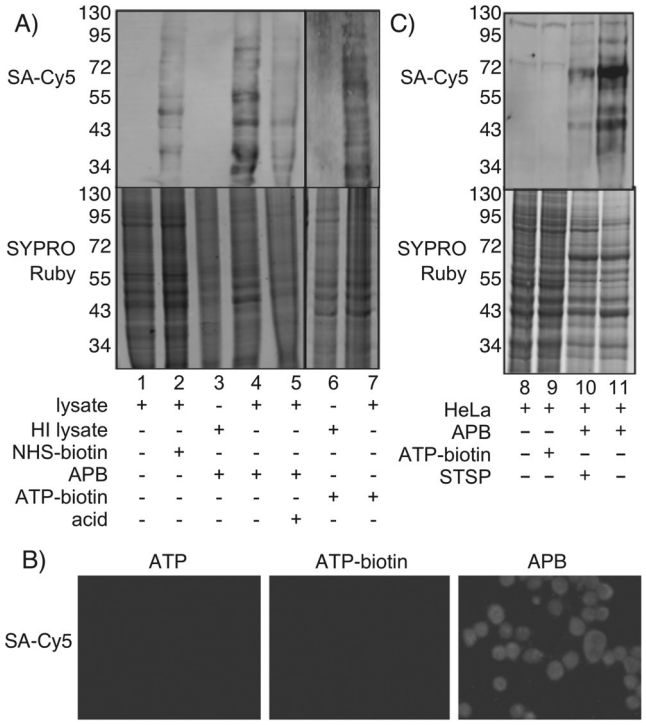


Figure 4. A) Kinase-catalyzed biotinylation of HeLa cell lysates (lysate) or heat-inactivated Hela cell lysates (HI lysate) with APB and ATP-biotin. Acid (TFA, 50% final concentration) was added after biotin labeling to cleave the biotin tag (lane 5). NHS–biotin (Figure S2) was used to assess nonspecific biotinylation. B) Fluorescence microscopy images of Hela cells after treatment with ATP, ATP–biotin, or APB, fixation, and visualization with SA-Cy5 for detection of biotin (white). Enlarged images and DAPI nuclear staining are shown in Figure S14. C) In cellulo kinase-catalyzed biotinylation with ATP–biotin or APB in HeLa cells. As a control, the kinase inhibitor staurosporine (STSP) was pre-incubated with cells to prevent kinase catalysis (lane 3). Reaction mixtures (A and C) were separated by SDS-PAGE and visualized with streptavidin–Cy5 (SA-Cy5; top gel) or SYPRO Ruby total protein stain (bottom gels). The gels are representative of at least three independent trials.

Having confirmed the kinase compatibility of APB in vitro, we next sought to test kinase-catalyzed biotinylation in live cells. As a first step, fluorescence microscopy was used to confirm the cell permeability of APB. Hela cells were incubated with APB, followed by washing, fixation, and visualization with streptavidin–Cy5 to observe biotin. Cells treated with APB showed fluorescence corresponding to the presence of biotin (Figure 4B). As controls, untreated cells (Figure S14) or cells incubated with ATP or ATP–biotin showed no biotin signal (Figure 4B), which indicated that the polyamine linker in APB was required to promote cell permeability. These microscopy studies confirmed that APB was is cell-permeable and validated the use of polycationic groups to enhance the permeability of ATP analogues.

With the cell permeability of APB confirmed, live-cell biotinylation was performed. HeLa cells were incubated with APB, washed to remove excess analogue, lysed, and then analyzed by SDS-PAGE. Cells incubated with APB showed protein biotinylation (Figure 4C, lane 11), which is consistent with cell permeability. Biotinylation was absent with ATP–biotin under the same conditions (Figure 4C, lane 9), further confirming that the polyamine linker is necessary to enhance cell permeability. Also, pre-treating cells with the kinase inhibitor staursporine reduced biotinylation (Figure 4C, lane 10), indicating that the labeling is kinase-dependent. As a final control, ATP–biotin was incubated with HeLa cells in the presence of lysates containing kinase activity, and no biotin signal was observed (Figure S15A), which assured that biotinylation was independent of cell-surface protein labeling. Treatment with APB was accompanied by a modest loss of total protein (Figure 4C, lanes 10 and 11, bottom gel) compared with the controls (Figure 4C, lanes 8 and 9, bottom gel), which is similar to the levels of protein loss observed in previous cell-permeability studies,[24] including experiments with widely used cationic permeabilization reagents.[25] To assess APB cytotoxicity, a dose-dependent cell viability assay was performed. APB showed a cytotoxicity EC_{50} value of 19 ± 1 mM (Figure S16A). Importantly, 96 ± 1% cell viability was observed with the 5 mM concentration of APB used in the cell labeling assay (Figure S16B). The cell-based studies showed that APB is cell-permeable and nontoxic at low mM concentrations, with cell penetration and labeling dependent on the polyamine linker.

To assess the quality of biotinylation in the various labeling reactions, lysates were incubated with the non-specific biotinylation reagent, NHS–biotin (Figure S2). The biotinylated protein bands that were observed with NHS–biotin treatment were different to those of the kinase-catalyzed reactions with APB or ATP–biotin (Figure 4A and C, compare lane 2 to lanes 4, 7, and 11), suggesting kinase-selective biotinylation of both lysates and cells. Importantly, a comparison of APB labeling reactions in lysates and cells revealed different biotinylated protein products (Figure 4A and C, lanes 4 vs. 11), which indicated that in cellulo labeling is distinct from labeling in lysates. We speculate that the difference in live-cell versus lysate labeling may be due to compartmentalization inside the cell, which suggests that in cellulo labeling studies will better interrogate the phosphoproteome for cell-signaling studies.

In conclusion, we have reported the first cell-permeable ATP analogue compatible with kinase-catalyzed biotinylation. APB acted as a cosubstrate with protein kinases in vitro and in cellulo. While the percentage conversion and kinetic efficiency was lower than for ATP or ATP–biotin in vitro, APB was able to label phosphoproteins in live cells. Importantly, different biotinylated proteins were observed in cellulo compared to the lysate studies, which argues that labeling in cellulo will better reflect the cellular phosphoproteome. These results are the foundation for future work using APB and kinase-catalyzed biotinylation as methods to identify and isolate phosphoproteins from cells, which will enhance cell-signaling research. More generally, these studies have established that cationic groups attached to ATP analogues promote cell permeability, which provides a general strategy for the development of other ATP analogues for live-cell labeling studies.

Experimental Section

APB (**2**) was synthesized and characterized by ^{1}H, ^{13}C, and ^{31}P NMR spectroscopy, UV spectroscopy, and ESI and MALDI spectrometry, as discussed in the Supporting Information (Figures S3–S8). For in-cell kinase-catalyzed biotinylation, Hela cells were grown in 12 well plates for 2 days in growth media (F-12 containing 10% FBS, 9 units penicillin, and 9 units streptomycin). The growth medium was removed, replaced with fresh growth medium containing APB (5 mM) or ATP–biotin (5 mM), and incubated at 37°C for 1 hour in a CO_2 incubator to allow for kinase-catalyzed labeling. As a control, staurosporine (1 μM) in fresh growth medium was added to the cells for one hour before adding APB. After washing the cells, the reaction mixtures were separated by SDS-PAGE and visualized with SYPRO Ruby total protein stain. Where indicated, the gel was stained with ProQ Diamond Phosphoprotein stain (Invitrogen), or the proteins were transferred onto a PVDF membrane (Immobilon-P, Millipore) and visualized with the streptavidin–Cy5 reagent (Life Technologies) to detect biotinylated proteins. Detailed experimental procedures and data can be found in the Supporting Information.

Keywords: ATP · biotinylation · cell permeability · enzymes · kinases

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