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Exploring Kinase Cosubstrate Promiscuity: Monitoring Kinase Activity through Dansylation

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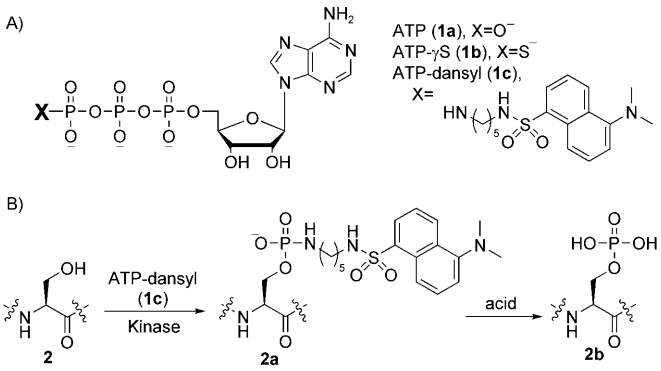
Kinases catalyze protein phosphorylation, which is a key event in cell signalling. Importantly, kinases are targeted by a number of drugs in clinical trials for cancer, rheumatoid arthritis, and immunosuppression.[1] Because kinases and protein phosphorylation play fundamental roles in disease, methods to monitor kinase activity and substrates are needed.

We recently described a phosphoprotein-labelling reaction that couples kinases with an analogue of the adenosine 5′-triphosphate (ATP, **1a**) cosubstrate. Specifically, ATP conjugated to biotin through the γ-phosphate (ATP-biotin) served as a kinase cosubstrate, which allowed phosphoprotein biotinylation in vitro and in cell lysates.[2] The studies with ATP-biotin raise the possibility that cellular kinases generally tolerate γ-phosphate-modified ATP analogues (Scheme 1A) as cosubstrates. Cosubstrate promiscuity is documented with multiple protein-modifying enzymes, including farnesyltransferase,[3] transglutaminase,[4] galactosyltransferase,[5] *N*-acetyltransferase,[6] DNA methyltransferase,[7] and phosphopantetheinyl transferase (PPtase).[8] To explore kinase cosubstrate promiscuity, we hypothesized that additional γ-phosphate-modified ATP analogues would be compatible with kinase-catalyzed labelling.



Scheme 1. Kinase-catalyzed dansylation. A) General structures of γ-phosphate-modified ATP analogues. B) ATP-dansyl (**1c**) acts as a kinase cosubstrate to enable phosphorylation-dependent dansylation of peptides and proteins. Incubation with acid (2 N HCl or 50% TFA) cleaves the dansyl label.

We describe here a kinase-catalyzed dansylation reaction for fluorophore labelling of phosphopeptides and phosphoproteins; this reaction substantiates the cosubstrate promiscuity of kinases. Specifically, a commercially available γ-phosphate-modified ATP analogue, ATP-dansyl (**1c**; dansyl = 5(-dimethylamino)naphthalene-1-sulfonyl), was coupled with three kinase substrate peptides containing serine (**3**), threonine (**4**), or tyrosine (**5**), and three kinases, cAMP-dependent protein kinase (PKA), casein kinase II (CK2) and Abelson tyrosine kinase (Abl). In all cases, ATP-dansyl served as a kinase cosubstrate, converting each peptide to its corresponding dansylated phosphopeptide (Scheme 1B), as assessed by MALDI-TOF MS (Table 1). To determine the efficiency of the dansylation reaction, we employed quantitative MS analysis (Scheme S1 in the Supporting Information). After incubation with the corresponding kinase and either ATP (**1a**) or ATP-dansyl (**1c**), the phosphopeptide products were isotopically labelled through esterification as previously described,[2,9] and the dansyl group was removed with acid (Scheme 1B). By comparing the two isotopically differentiated phosphopeptides using MALDI-TOF MS, the ATP-dansyl reactions demonstrated 81–91% conversion compared to the ATP reactions (Table 1 and Figures S1–S3 in the Supporting Information). The data indicate that kinase-catalyzed dansylation is compatible with the three natural hydroxyl-containing residues and three kinase enzymes, similar to results previously observed with ATP-biotin.[2]

Table 1. MALDI-TOF MS data of peptides **3**–**5** after incubation with ATP-dansyl and PKA, CK2, or Abl kinase.

Peptide substrate	Kinase	Dansylated peptide Calcd	Obsd[d]	Conversion[e]
(**3**) LRRT**S**IIGT	PKA	1411.73[a]	1412.06	91%
(**4**) RRREEE**T**EEE	CK2	1851.66[b]	1851.55	81%
(**5**) EAI**Y**AAPFAKKK	Abl	1731.87[c]	1731.51	87%

[a] Calculated mass $[M-H]^-$. [b] Calculated mass $[M+K+Fe]^+$. [c] Calculated mass $[M-H]^-$. [d] Observed mass based on MALDI-TOF MS analysis. [e] Percentage conversion was determined using quantitative MS by comparing to ATP phosphorylation (100%). See Figures S1–S3 in the Supporting Information.

We next tested compatibility of the kinase-catalyzed dansylation reaction with a full-length protein substrate. Full-length β-casein was incubated with ATP-dansyl (**1c**) and CK2, trypsin-digested, and analyzed by quantitative MALDI-TOF MS analysis. Dansylation of the β-casein peptide (FQpSEEQQQ**pT**EDELQDK) occurred with 54% conversion compared to the ATP reaction (Figure S4 in the Supporting Information). The data indicate that kinase-catalyzed dansylation is compatible with a full-length protein substrate.

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To characterize the efficiency of the dansylation reaction, kinetics measurements were obtained with the kemptide peptide (LRRASLG) and PKA using an enzyme-coupled assay (Figure S5 in the Supporting Information).[10,11] ATP demonstrated K_M and k_{cat} values consistent with previous reports.[12,13] ATP-dansyl (**1c**) displayed a similar K_M compared to ATP (Table 2).

Table 2. Kinetic constants of PKA and kemptide with ATP, ATP-γS or ATP-dansyl as the cosubstrate.[a]

Cosubstrate	K_M [μM]	k_{cat} [s^{-}]	k_{cat}/K_M [s^{-1} mM^{-}]
ATP	24 ± 5	3.9 ± 0.5	160
ATP-γS	266 ± 19	6.5 ± 0.7	24
ATP-dansyl	23 ± 2	0.41 ± 0.05	18

[a] Based on three independent trials with standard error shown.

The data indicate that the γ-phosphate modification does not interfere significantly with kinase binding. In contrast, ATP-dansyl displayed a 9.1-fold reduced k_{cat} value compared to ATP; this reduction suggests that phosphoramidate transfer with ATP-dansyl is not as rapid as phosphate transfer with ATP, or the dansyl group at the γ-phosphate interferes with substrate binding or the catalytic mechanism. While ATP-dansyl is a less efficient cosubstrate than ATP, dansylation with ATP-dansyl demonstrated catalytic efficiency (k_{cat}/K_M) similar to thiophosphorylation with ATP-γS (**1b**- Table 2).[12] Because ATP-γS is documented for phosphoprotein labelling,[14–16] the kinase-catalyzed dansylation occurs with catalytic efficiencies appropriate for future applications.

Having established the kinase-catalyzed dansylation reaction, we developed a kinase activity assay in which dansylation of a fluorophore-labelled peptide substrate promotes fluorescence resonance energy transfer (FRET; Figure 1 A). Specifically, the Abl substrate peptide **5** (Table 1) was synthesized with a 5(6)-carboxy-X-rhodamine (ROX) fluorophore attached at the amino terminus (ROX **5**, Figure 1 A). The expectation was that kinase-catalyzed dansylation would position the two fluorophores appropriately for FRET (peptide **6**, Figure 1 A). To test the assay, the ROX **5** peptide was incubated with ATP-dansyl (**1c**) in the presence and absence of Abl kinase and FRET was monitored. In the presence of recombinant Abl, a 30 ± 5% increase in emission fluorescence was observed compared to the reaction without kinase activity (Figure 1 B, columns 1 and 2; Table S1 and Figure S7 in the Supporting Information), which indicates that the FRET assay is sensitive to kinase activity. The 30% increase is consistent with previous FRET-based kinase assays in which 10–60% signal changes are reported.[17–19] To further confirm the quantitative nature of the FRET assay, kinetics measurements were performed. The catalytic efficiency (k_{cat}/K_M) was similar with ATP-dansyl whether using the enzyme-coupled or FRET assay (Table 3). Consistent with results observed with PKA, ATP-dansyl displayed a 6.2-fold reduced k_{cat} value compared to ATP (Table 3, Figure S6 in the Supporting Information). The combined data indicate that the FRET assay based on kinase-catalyzed dansylation reproducibly and quantitatively monitors kinase activity.

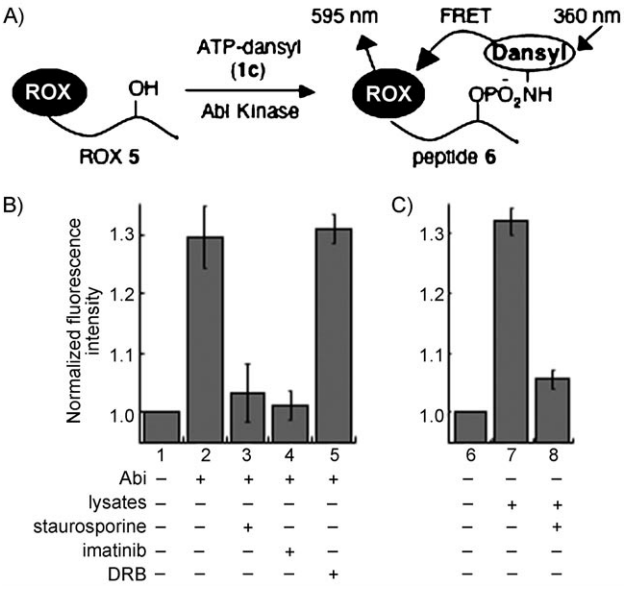

Figure 1. FRET-based kinase activity assay. A) The ROX-peptide **5** (ROX-**5**, Table 1) was dansylated by Abl and ATP-dansyl to create peptide **6**; B) and C) Normalized fluorescence intensity at 595 nm after excitation at 360 nm with ROX **5**, ATP-dansyl, and the indicated reaction components in B) 84- or C) 96-welled plates, respectively, where "lysates" indicates HeLa cell lysates. The average of three trials with standard error is shown.

Table 3. Kinetic constants of Abl with ATP or ATP-dansyl as the cosubstrate.[a]

cosubstrate	K_M [μM]	k_{cat} [s^{-1}]	k_{cat}/K_M [s^{-1} mM^{-1}]
ATP[b]	51 ± 7	6.9 ± 0.8	130
ATP-dansyl[b]	115 ± 5	1.1 ± 0.1	9.6
ATP-dansyl[c]	39 ± 5	0.28 ± 0.10	7.2

[a] Based on three independent trials with standard error shown. [b] Determined using the enzyme-coupled assay with peptide **5**. [c] Determined using the FRET assay with ROX **5** peptide.

Kinases are the targets of multiple small-molecule inhibitor drugs. As a result, identification of inhibitors of kinase activity is an important area of pharmaceutical research. To determine if the FRET assay is appropriate for drug screening, known kinase inhibitors were preincubated with Abl before addition of the ROX **5** peptide and ATP-dansyl. While a 30% increase in emission fluorescence was observed in the absence of inhibitor (Figure 1 B, column 2), no increase in emission fluorescence was observed in the presence of staurosporine, a general kinase inhibitor[20] (Figure 1 B, column 3; Table S1 in the Supporting Information). Similar results were seen with imatinib (STI-571; Figure 1 B, column 4), which has preference for Abl[20] and is used clinically to treat chronic myeloid leukemia.[21] In contrast, the emission fluorescence was similar in the absence or presence of 5,6-dichloro-1-β-D-ribofuranosyl benzimidazole (DRB), an inhibitor with preference for CK2 kinase[22] (Figure 1 B, compare columns 2 and 5). These results indicate that the FRET assay is sensitive and selective for Abl kinase inhibitors, and thus, it is appropriate for drug screening.

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Fluorescence-based kinase assays have been used to detect kinase activity in mammalian cells and lysates for drug design and disease characterization.[23–29] To further assess the utility of the FRET assay, we monitored Abl kinase activity in HeLa cell lysates. In this case, a $32 \pm 2\%$ increase in emission fluorescence was observed compared to the reaction without lysates (Figure 1C, columns 6 and 7; Table S2 in the Supporting Information). Similar to experiments with recombinant Abl, staurosporine inhibited kinase activity and resulted in a loss of fluorescence emission (Figure 1C, column 8; Table S2). These studies indicate that the FRET assay is capable of monitoring cellular kinase activity and is compatible with drug screening using lysates. Significantly, these studies establish that cellular kinases accept ATP-dansyl to label substrates, which corroborates the cosubstrate promiscuity of kinases.

In summary, we have established a kinase-catalyzed dansylation reaction with ATP-dansyl as a cosubstrate. The reaction is compatible with peptide or full-length protein substrates and kinases from cell lysates. In addition, the kinase-catalyzed dansylation reaction is appropriate for use in FRET-based kinase activity and inhibition assays. Because kinases are the targets of a variety of clinical drugs, the FRET assay can be applied towards monitoring kinase activity in diseased states and facilitating drug screening efforts. Combined with the kinase-catalyzed biotinylation reaction,[2] the results establish that cellular kinases tolerate γ-phosphate-modified ATP analogues as cosubstrates. With the critical role of kinases in signalling and disease, these studies pioneer development of chemical tools monitoring kinase activity and protein phosphorylation.

Experimental Section

All kinase-catalyzed dansylation reactions were performed as previously described.[2] In this case, to create a dansylated phosphopeptide product, ATP-dansyl (2 mM, Affinity Labeling Technologies Inc., Lexington, KY, USA) was used after evaporation of the methanol storage solvent. All MALDI-TOF MS and quantitative data was generated as previously described.[2] For the FRET assay, ATP or ATP-dansyl (0.6–1 mM), peptide (35–100 μM), and either Abl kinase (20 units μL^{-1}) or HeLa cell lysates (32 μL of 6 mg mL^{-1}) were combined in the manufacturer's kinase buffer and incubated at 30 °C for 2 h. The reactions were transferred to a 96- or 384-well plate and the fluorescence signal due to FRET emission at 595 nm was observed after excitation at 360 nm using a GENios Plus Fluorimeter (Tecan, Männedorf, Switzerland). In reactions containing kinase inhibitors, inhibitor (a final concentration of 30 μM for staurosporine and DRB or 1 mM for imatinib) was preincubated for 10 min with kinase prior to reaction. Kinetics experiments with the enzyme-coupled assay[30,31] were performed with kemptide (1.29 mM, LRRASLG) in the presence of ATP-dansyl (5–250 μM), ATP-γS (5–2000 μM), or ATP (5–2000 μM) with PKA (2.4 units μL^{-1}). In the reactions with Abl, peptide **5** (1.29 mM) was incubated with Abl (1.6 units μL^{-1}) under identical conditions. Absorbance at 360 nm was taken every minute for 20 min. In the case of the FRET kinetics analysis, Abl (1.6 units μL^{-1}) and ROX-peptide **5** substrate (1.25 mM) were incubated with ATP-dansyl (5–250 μM) in provided manufacturer's buffer in a 384-well plate format. Measurements of FRET were taken every two minutes for 30 min, using an excitation wavelength of 360 nm and an emission of 595 nm. Additional experimental details, MALDI-TOF MS and FRET data, and kinetics measurements are available in the Supporting Information.

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