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Chemically reprogramming the phosphotransfer reaction to crosslink protein kinases to their substrates

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Abstract: **The proteomic mapping of enzyme–substrate interactions is challenged by their transient nature. A method to capture interacting protein kinases in complexes with a single substrate of interest would provide a new tool for mapping kinase signaling networks. Here, we describe a nucleotide-based substrate analog capable of reprogramming the wild-type phosphoryl-transfer reaction to produce a kinase-acrylamide-based thioether crosslink to mutant substrates with a cysteine nucleophile substituted at the native phosphorylation site. A previously reported ATP-based methacrylate crosslinker (ATP-MA) was capable of mediating kinase crosslinking to short peptides but not protein substrates. Exploration of structural variants of ATP-MA to enable crosslinking of protein substrates to kinases led to the discovery that an ADP-based methacrylate (ADP-MA) crosslinker was superior to the ATP scaffold at crosslinking *in vitro.* The improved efficiency of ADP-MA over ATP-MA is due to reduced inhibition of the second step of the kinase–substrate crosslinking reaction by the product of the first step of the reaction. The new probe, ADP-MA, demonstrated enhanced *in vitro* crosslinking between the Src tyrosine kinase and its substrate Cortactin in a phosphorylation site-specific manner. The kinase–substrate crosslinking reaction can be carried out in a complex mammalian cell lysate setting, although the low abundance of endogenous kinases remains a significant challenge for efficient capture.**

Keywords: protein kinases; signaling networks; chemical genetics; mass spectrometry; kinase-substrate interactions; phosphorylation

Introduction

Posttranslational modifications (PTMs) of proteins are critical modulators of cellular function. Advances in proteomics have accelerated the rate of discovery of PTMs; however, proteomic profiling generally cannot capture the transient enzyme–substrate interactions that place and remove PTMs. Consequently, much effort has been made to deorphan PTMs by developing tools to identify the enzymes that regulate them. Activity-based protein profiling (ABPP) describes a class of chemical tools which profile the functional status of enzymes in a given family.[1] ABPP probes covalently target the active site of

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enzymes for their detection or purification. Substrate ID describes a set of tools for the capture of the downstream substrates of a given enzyme.[2–4] While ABPP and substrate ID are powerful tools for assessing the functional status and output of enzymes, we sought a way to not only identify active kinases but also to capture the kinases as they bind to a specific substrate, a method we term substrate-based protein profiling (SBPP).

To capture the transient kinase–substrate complex, we have designed SBPP probes which form two covalent bonds: an amide bond with a lysine residue in the kinase active site and a thioether bond with a cysteine residue at the phosphorylation site in the protein substrate (Fig. 1). We previously reported a dialdehyde crosslinker that could crosslink peptide substrates with low efficiency for their detection by western blot, but could neither crosslink protein substrates nor mediate crosslinking in complex cell lysates.[5,6] We next adapted an existing kinase-based ABPP method to convert a conserved lysine in all kinases into an affinity-dependent and substrate-specific electrophile in the active site. This next-generation kinase–substrate crosslinking probe was based on an ATP scaffold and converted the conserved kinase catalytic lysine into an electrophilic methylacrylamide for the reaction with the introduced cysteine mutation in the substrate phosphorylation site.[7] This approach significantly improved the reaction such that crosslinking to a model peptide could be observed in cell lysate. However, the *in vitro* yield of kinase–peptide crosslinking with this probe in a model system was limited to about 30% and we were unable to achieve crosslinking of protein kinases with protein substrates.

Here, we explore the structure–activity relationship of the ATP-MA crosslinker and identify an ADP-based probe that yields quantitative crosslinking between kinase and peptide *in vitro*. We further demonstrate crosslinking between the model kinase Src and its protein substrate Cortactin *in vitro*. We explore the biochemical basis for improved crosslinking based on the use of an ADP- versus ATP-methacrylate probe. We further immunoprecipitate the crosslinked kinase–protein substrate complex from cellular lysate to specifically detect the product of this reaction by western blot and LC–MS/MS.

Results and Discussion

We first sought to improve the yield of Src crosslinking to a cysteine containing peptide (Src-tide) from that observed (ca. 30%) with ATP-MA.[7] We explored the initial step of lysine acylation with methacrylate [Fig. 1(B)] by attaching the acyl group on the β- rather than the γ-phosphate position of the nucleotide (i.e., using ADP rather than ATP). We envisioned that this modification might enhance the rate of the first step of the reaction or possibly result in acylation of a different lysine residue in the Src active site which might then be better positioned for the cysteine attack on the methylacrylamide [Fig. 1(C)].

We synthesized the ADP-based methacrylate crosslinker ADP-MA and developed an improved purification protocol for its isolation. Using a two-step protocol in which an anion exchange column was first used to remove unreacted starting material, followed by purification on a styrene-divinylbenzene solid support column[8] to separate positional isomers resulted in a robust isolation protocol for both ADP-MA and ATP-MA.

Using the model kinase Src, we characterized the kinetics of the first step of the reaction, the formation of methylacrylamide in the kinase active site [Fig. 1(A, B)]. We monitored the formation of Src-MA by LC–MS and found the k_{inact}/K_I of ADP-MA to be 45 ± 2 M^{-1} s^{-1} at 4°C and that of ATP-MA to be 129 ± 8 M^{-1} s^{-1} (Fig. 2). The correlation between k_{obs} and [ADP-MA] remained linear at all probe concentrations tested (up to 50 μM), from which we inferred that K_I is greater than 50 μM. This lower bound for K_I is consistent with the previously reported K_m of ATP for Src (51–169 μM).[9–15] The k_{inact}/K_I of ADP-MA and ATP-MA are approximately an order of magnitude higher than that of general covalent lysine-targeted kinase inhibitors such as 5′-fluorosulfonylbenzoyl adenosine,[16] but three to five orders of magnitude lower than covalent kinase inhibitors such as those targeting the epidermal growth factor receptor (10^5–10^7 M^{-1} s^{-1}),[17] which modify cysteine, a more nucleophilic amino acid.

We next set out to determine if a protein rather than a peptide substrate could be crosslinked to Src, which had not been possible using previously reported SBPP probes.[5–7] We selected the actin regulator Cortactin as a model Src protein substrate. When phosphorylated by Src, Cortactin promotes actin polymerization by recruiting Arp2/3 complex proteins to the actin filament, playing a role in cellular migration and invasion.[18–20] We expressed FLAG-tagged Cortactin (apparent molecular weight: 90 kDa) mutants in which the native cysteine residues were mutated to serine (Cyslite) and the phosphorylation sites Y421, Y466, Y486, and Y489 were each mutated to cysteine (unless otherwise noted, all Cortactin constructs tested are Cyslite). We found that ADP-MA mediated Src (apparent molecular weight: 40 kDa) crosslinking to each of these mutants *in vitro*, with a distinctive molecular weight shift in the gel consistent with the sum of the masses of the two proteins [Fig. 3(A)]. We observed that crosslinking to the cysteine mutants at the position of Src phosphorylation was more robust than crosslinking to the native cysteines present in wild-type Cortactin [Fig. 3(A)], similar to observed crosslinking preference for substrate peptide over non-substrate peptides [Fig. S1(A,B)].

We next compared ADP-MA and ATP-MA in the Src/Y421C FLAG-Cortactin crosslinking reaction. ADP-MA demonstrated more robust crosslinking than

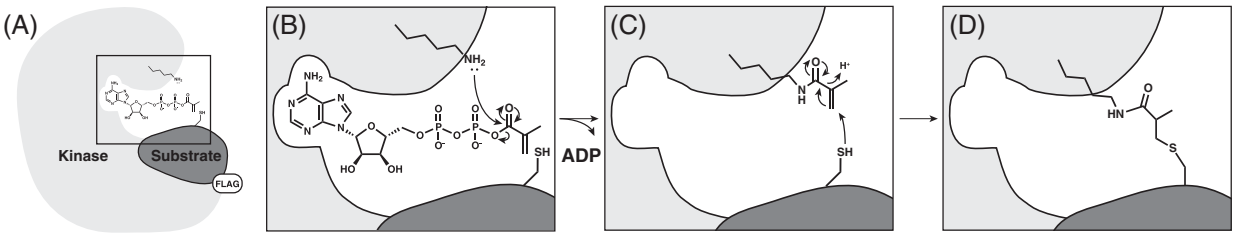


Figure 1. Schematic representation of the kinase–substrate crosslinking reaction mediated by ADP-MA.

ATP-MA [Fig. 3(B)], which was unexpected considering the slightly faster labeling of Src kinase by ATP-MA. Given that both probes share a common mechanism in the second step of crosslinking, we were intrigued by this difference in efficiency and tested a series of hypotheses to explain these results.

The distinct orientation of the reactive acylphosphate by ATP-MA and ADP-MA in the kinase active site led us to consider the possibility that the two probes labeled different lysines in the Src active site.[21] Both probes singly labeled Src by whole protein LC–MS (Fig. S2). Using trypsin and Glu-C double protease digestion followed by LC–MS/MS analysis, we found that both probes primarily modified the catalytic Lys 295 [Fig. 4(A,B)]. This selectivity is similar to previous observations using the ABPP kinase probes ATP-desthiobiotin and ADP-desthiobiotin.[22,23]

Given that both probes predominantly produce the same Src-K295-MA product [Fig. 4(B)], we tested additional components of the reaction to see if they were mediating the differences in crosslinking efficiency. In particular, we examined the nucleotide products ADP and ATP, which may occupy the kinase active site and influence the second step of the reaction [Fig. 1(C)]. Using ADP-MA (150 μM) and ATP-MA (150 μM), we found that at this lower probe concentration (150 μM) [Fig. 5(A), Lanes 2 and 9] compared with 500 μM used in Figure 3(B), both probes were capable of mediating crosslinking of Src to Y421C FLAG-Cortactin. We found that ATP and ADP inhibited the protein crosslinking reaction [Fig. 5(A)]. AMP and adenosine did not inhibit the crosslinking reaction, consistent with their weaker affinity for the nucleotide binding site [Fig. 5(A)].

Having determined that nucleotide (ATP or ADP) binding to the kinase could limit the crosslinking step, we wondered if the addition of EDTA would reduce the inhibitory activity of ATP by chelating Mg^{2+}. The addition of EDTA resulted in a modest improvement in crosslinking by ATP-MA in the presence of ATP [Fig. 5(A)]; however, we found that EDTA also reduces the efficiency of the first step of the reaction (Fig. S3), complicating the use of EDTA to enhance crosslinking.

To isolate the effect of reaction components on the second step of the crosslinking reaction [Fig. 1 (C)], we pre-labeled Src with 50 μM ADP-MA or ATP-MA and verified that Src-MA was formed to completion by LC–MS before initiating crosslinking by the addition of Y421C FLAG-Cortactin. Robust crosslinking was observed whether ADP-MA or ATP-MA was used to form the electrophilic Src-MA intermediate [Fig. 5(B), Lane 5 vs. 9]. When nucleotide or SBPP competitors (ATP 5 mM, ADP 5 mM, ATP-MA 250 μM, or ADP-MA 250 μM) were included in the reaction with Y421C FLAG-Cortactin, potent inhibition was observed with ATP and ATP-MA [Fig. 5(B), Lanes 7/10 and 8]. In contrast, ADP and ADP-MA exhibited less potent inhibition of crosslinking [Fig. 5(B), Lanes 6/11 and 12]. The addition of EDTA did not diminish crosslinking efficiency when the first step had proceeded to completion [Fig. 5(B), Lanes 13 and 14]. These results demonstrate that high concentrations of ATP (the product of kinase modification by ATP-MA) or high concentrations of ATP-MA inhibit the final step of the kinase–substrate crosslinking reaction. The inhibition by ATP-MA at high concentration (250 μM) was consistent with the poor crosslinking observed in Figure 3(B) at 500 μM concentration.

Having identified ADP-MA as an improved SBPP probe for substrate–kinase crosslinking, we next explored whether the reaction could be observed in a complete cell lysate in the presence of competing proteins. We tested a range of concentrations (1.67 mM, 167 μM, and 16.7 μM) of both ATP-MA and ADP-MA to examine concentration-dependent effects in the presence of other nucleotide binding proteins in the lysate. We found that both ATP-MA and ADP-MA mediated crosslinking of Y421C FLAG-Cortactin to Src, but the highest concentration of ATP-MA showed poor crosslinking (Fig. 6), consistent with the inhibitory effect of ATP-MA observed in Figures 3(B) and 5 (B). We concluded that at high-concentration crosslinking by ADP-MA is more efficient than ATP-MA, but both ADP-MA and ATP-MA may have utility for capturing kinase–substrate complexes in cellular lysate.

The non-peptidic crosslink between Src and Y421C FLAG-Cortactin contains a thioether bond [Fig. 1(D)] which is potentially reversible via a retro-Michael reaction. To test if "uncrosslinking" might be contributing to lower yields, we tested several protein denaturation conditions prior to SDS-PAGE. We found that the standard protocol of boiling protein samples with SDS and a reducing agent before running protein gels interfered with detection of the crosslinked product (Fig. S4). Our findings are consistent with the literature precedent for the retro-Michael addition of nucleophiles to β-alkylthiol

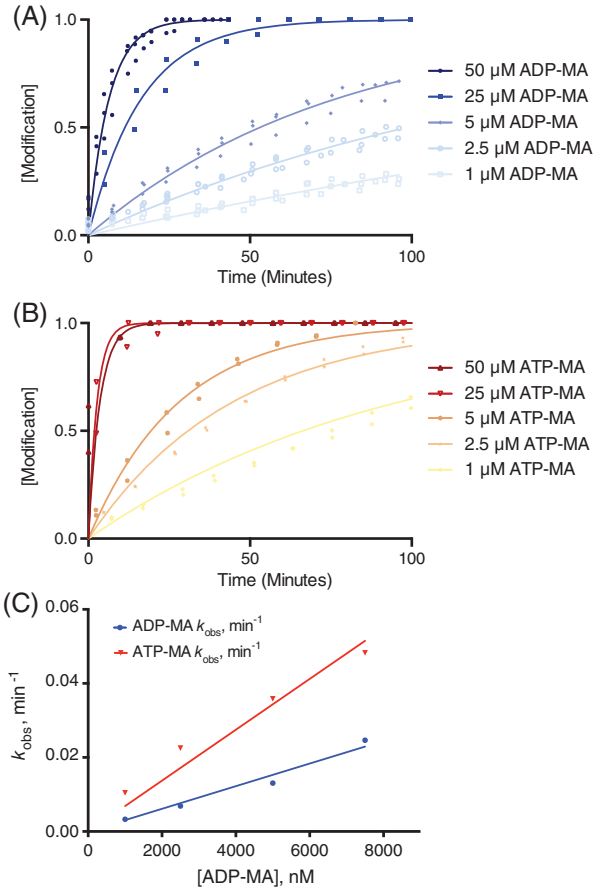


Figure 2. Characterization of covalent kinetics of reaction of Src with ADP-MA by LC–MS at 4°C. (A) Time course of formation of Src-MA with ADP-MA. (B) Time course of formation of Src-MA with ATP-MA. (C) k_{obs} versus [ADP-MA] and [ATP-MA]. All conditions were tested in duplicate or triplicate.

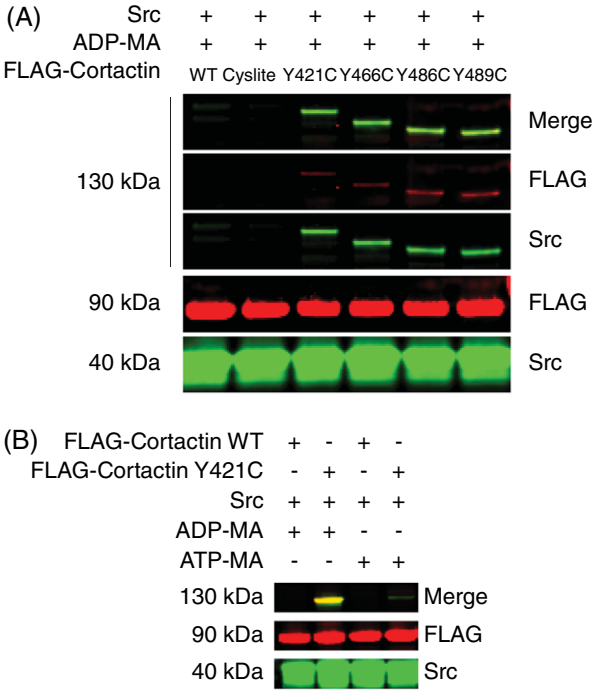


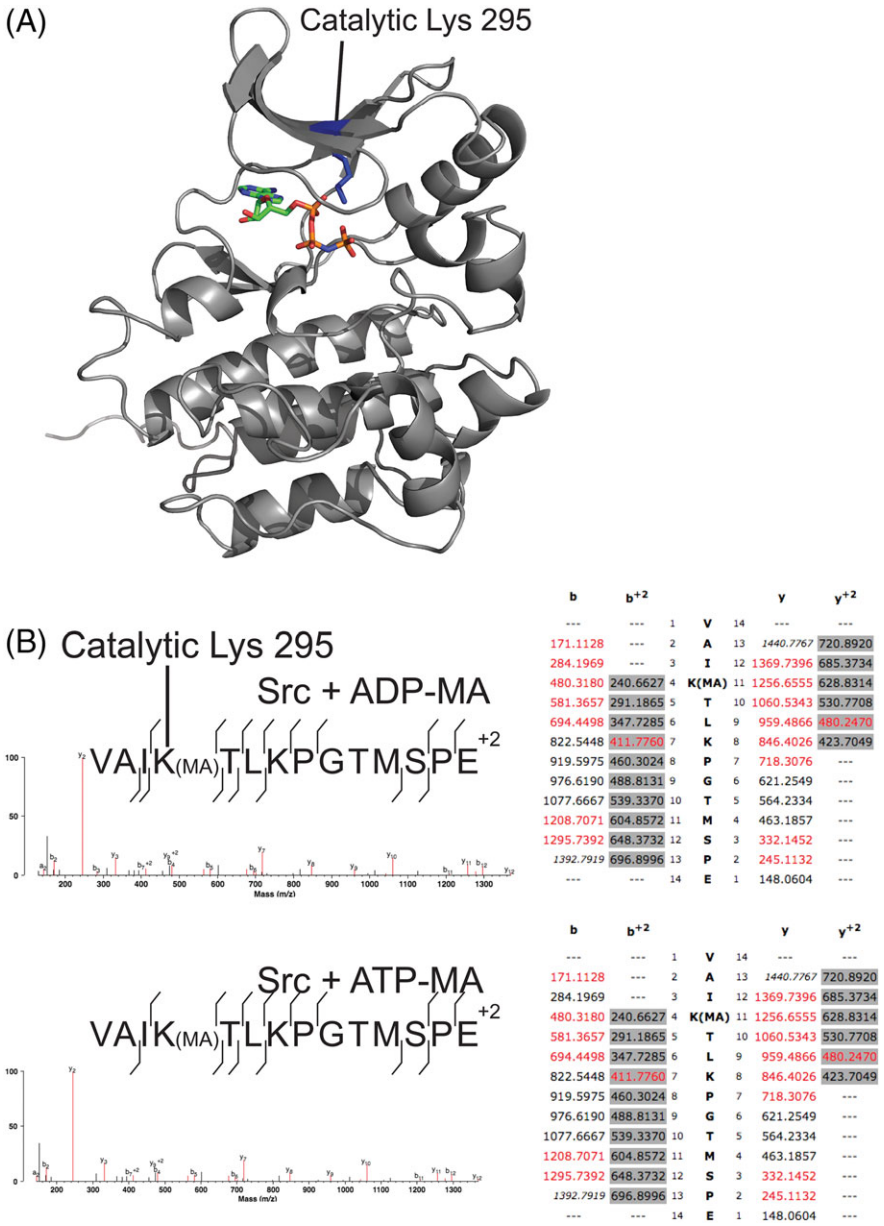
Figure 3. (A) Western blot analysis of 16 μM ADP-MA mediated *in vitro* crosslinking of 2 μM Src and phosphorylation site to 1.5 μM WT and Cys mutant FLAG-Cortactin. (B) Comparison of 0.5 mM ADP-MA and ATP-MA mediated *in vitro* crosslinking with 3.5 μM Src and 2 μM protein substrate Y421C FLAG-Cortactin.

amides when heated.[24] In the subsequent studies, we omitted the heating step following the addition of SDS and reducing agent to samples prior to sample loading.

LC–MS/MS identification of the kinases interacting with a known substrate requires enrichment from lysate. We established a mass spectrometry-based method to identify kinases crosslinked to Y421C FLAG-Cortactin following ADP-MA-mediated SBPP. We first transiently transfected HEK 293T cells with FLAG-tagged WT or Y421C Cortactin and prepared desalted cellular lysates. All lysates were supplemented with 2 μM purified Src kinase domain before incubating in the presence or absence of ADP-MA. We then immunoprecipitated FLAG-tagged substrate and substrate–kinase complexes, washed, and eluted with 3×-FLAG peptide before visualizing by western blot and silver stain (Fig. 7). We observed the formation of a Src/Y421C FLAG-Cortactin band at 130 kDa that was enriched by immunoprecipitation and labeled by both anti-FLAG antibody and anti-Src antibody (Fig. 7) in the Y421C FLAG-Cortactin + ADP-MA sample only, but not in controls (WT FLAG-Cortactin + ADP-MA and Y421C FLAG-Cortactin + no crosslinker). We performed silver stain, in-gel digest, and MS/MS on the excised 130 kDa region from two replicate samples of 0.65 μM Src and Y421C FLAG-Cortactin with no crosslinker or with 5 μM ADP-MA. By LC–MS/MS, we identified Src and Cortactin in the ADP-MA samples (Supplementary Material).

To apply SBPP for the identification of kinase-substrate interactions, we turned to the challenge of capturing endogenous levels of kinase(s) from ADP-MA-treated lysates prepared from Y421C FLAG-Cortactin expressing cells followed by immunoprecipitation, on-bead digestion, and LC–MS/MS analysis. When no exogenous kinase was added, we did not observe statistically significant enrichment of protein kinases after averaging MS/MS data across three biological replicates compared with control conditions (WT + ADP-MA and Y421C FLAG-Cortactin + no crosslinker) (Fig. S5). The most likely reason for this finding is a low yield of crosslinking that is below the sensitivity of detection by MS/MS when only low levels of endogenous protein tyrosine kinases are present.[25]

To determine whether the first or the second step of the SBPP reaction was limiting the ability to capture endogenous kinases, we turned to a competition reaction with an ABPP probe. We found that pretreatment with ADP-MA (15 μM) could compete with a high-affinity kinase focused ABPP probe, XO44, for the kinase active site lysine in lysate, indicating that the first step of the reaction takes place [Fig. S6



b	b+2				y	y+2
---	---	1	V	14	---	---
171.1128	---	2	A	13	*1440.7767*	720.8920
284.1969	---	3	I	12	1369.7396	685.3734
480.3180	240.6627	4	K(MA)	11	1256.6555	628.8314
581.3657	291.1865	5	T	10	1060.5343	530.7708
694.4498	347.7285	6	L	9	959.4866	480.2470
822.5448	411.7760	7	K	8	846.4026	423.7049
919.5975	460.3024	8	P	7	718.3076	---
976.6190	488.8131	9	G	6	621.2549	---
1077.6667	539.3370	10	T	5	564.2334	---
1208.7071	604.8572	11	M	4	463.1857	---
1295.7392	648.3732	12	S	3	332.1452	---
1392.7919	696.8996	13	P	2	245.1132	---
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b	b+2				y	y+2
---	---	1	V	14	---	---
171.1128	---	2	A	13	*1440.7767*	720.8920
284.1969	---	3	I	12	1369.7396	685.3734
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1295.7392	648.3732	12	S	3	332.1452	---
1392.7919	696.8996	13	P	2	245.1132	---
---	---	14	E	1	148.0604	---

Figure 4. (A) Lys modification by ADP-MA and ATP-MA was modeled onto Src kinase in complex with AMP-PNP (PDB file 2SRC). Catalytic Lys (blue). (B) LC–MS/MS fragmentation of ADP-MA and ATP-MA labeled Src Glu-C/tryptic peptide containing catalytic Lys.

(A,B)].[26] This suggested that the nucleophilic cysteine attack of the methylacrylamide may benefit from a more reactive electrophilic moiety. We tested additional thiol-reactive groups including the more reactive unsubstituted acrylate (ADP-AA) to see if increased reactivity could improve crosslinking efficiency. However, we observed an unexpected intact ADP-AA modification of Src via a cysteine in the Src active site. We found that Michael addition by the Src cysteine competed with the formation of the Src-acrylamide in the presence or absence of cysteine containing substrate (data not shown). It appears that further enhancement of electrophile reactivity compromised the ability to form the desired thiol-reactive acrylamide in the kinase active site.

We tested additional protein kinase–substrate pairs to explore the ability of SBPP probes to mediate additional crosslinking reactions. ADP-MA and ATP-MA mediated the crosslinking of non-Src family kinases to their protein substrates *in vitro*, including the unrelated Ser/Thr kinases SRPK2 to S637C Rbm20 and CDK4 to S780C Rb, respectively [Fig. S7(A,B)]. ATP-MA produced higher yield of SRPK2-Rbm20 crosslinking than ADP-MA, suggesting that both SBPP probes are useful when attempting crosslinking with new kinase substrates. We additionally confirmed that both probes modify a divergent protein kinase, Aurora A, and confirmed labeling of the catalytic Lys in Aurora A by trypsinization followed by LC–MS/MS analysis [Fig. S8(A,B)].

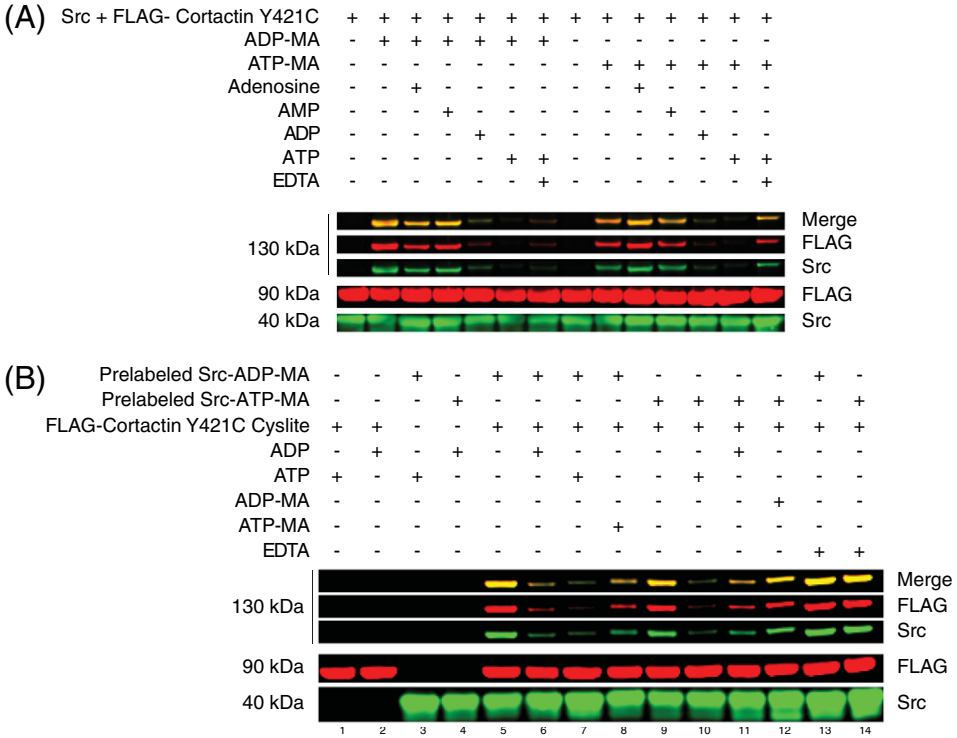

Figure 5. (A) 150 μM ADP-MA and ATP-MA mediated *in vitro* crosslinking of 2 μM Src and FLAG-Cortactin Y421C in the presence of 2.5 mM nucleotide and 10 mM EDTA. (B) Src was prelabeled with ADP-MA or ATP-MA and incubated with Y421C FLAG-Cortactin and nucleotide *in vitro*. ATP, ADP, and ATP-MA interfered with crosslinking. EDTA improved prelabeled Src-MA crosslinking. Conditions for crosslinking were the following: prelabeled: Src 3 μM, ADP-MA/ATP-MA 50 μM, Cortactin 1 μM, 18 h, 4°C in HEPES 25 mM pH 7.4, NaCl 150 mM, $MgCl_2$ 1 mM, with the addition of 250 μM ADP-MA or ATP-MA or 5 mM ADP or ATP or 10 mM EDTA.

In conclusion, in the present study, we report ADP-MA, a new SBPP probe capable of crosslinking exogenously added protein kinases in cell lysates from cells expressing mutated protein substrates. This crosslinking reaction displays preference for introduced phosphorylation site cysteine over native cysteines in the Src substrate Cortactin, as well as preference for substrate Cys-peptide over non-substrate Cys-peptides. We found that ADP-MA was more efficient at Src-Cortactin crosslinking *in vitro* than ATP-MA. This effect is due to a decreased interference with the second step of crosslinking by the product ADP relative to ATP. In the case of SRPK2 crosslinking to its substrate Rbm20, ATP-MA was preferred to ADP-MA, highlighting the importance of testing multiple probes for new substrate and kinase pairs. We additionally found that these differences in efficiency were attenuated when crosslinking was carried out in cellular lysate, likely due to the presence of many competing nucleotide binding proteins, suggesting that both probes may have utility in future experiments. Finally, we established an SBPP method to purify and profile kinase–substrate protein complexes from lysate, which may have future applications in functional studies for unbiased identification of the kinases interacting with a substrate phosphorylation site.

The transient nature of kinase–substrate complexes not only poses a challenge to their *de novo* identification but also to their high-resolution structural characterization. Relatively few structures of kinase–substrate phosphorylation complexes exist in the literature, and even fewer depict protein rather than peptide substrates.[27–31] A recent structure of the kinase PINK1 in complex with its substrate P-Ubiquitin required mutation of the kinase to stabilize binding to Ubiquitin as well as a llama antibody raised against the chemically crosslinked PINK1/Ub complex to aid in stabilization for X-ray structure determination. The SBPP probes described here produce a specific intramolecular crosslink at the phosphorylation site which could aid in isolation of 1:1 kinase:substrate complexes, improving homogeneity in samples used for cryo-EM or crystallography.

In the present study, we found that ADP-MA and ATP-MA did not yield detectable levels of crosslinked kinase–substrate product at endogenous concentrations of kinase. We conclude that the rate of the second step of the reaction, cysteine attack, is limited to a relatively narrow range, hindering further optimization.[32] However, bio-orthogonal reaction pairs are highly selective and their rate constants of reaction span over six orders of magnitude, representing a highly tunable set of tools.[33] Thus, one promising avenue for future exploration is the introduction of bio-orthogonal functional groups into the kinase active

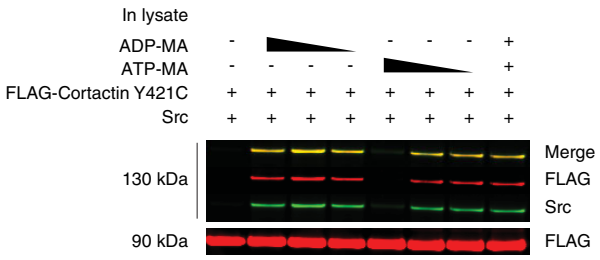

Figure 6. Crosslinking of 2 μM Src and transiently transfected FLAG-Cortactin Y421C in lysate mediated by ADP-MA and ATP-MA gradient (1.67 mM, 167 μM, and 16.7 μM), final lane 167 μM each.

site with a modified SBPP probe and by the introduction of a complementary group into the substrate using unnatural amino acid mutagenesis. This approach would circumvent the problem that increasing the reactivity of the thiol-reactive electrophile resulted in off-target reactivity with a native cysteine in the kinase active site. We hope to leverage expansions in the genetic code and bioorthogonal chemistry in future efforts to address this important problem of identifying the kinases interacting with a substrate phosphorylation site.

Materials and Methods

Src-Cortactin crosslinking in vitro *and in lysate*

Src kinase domain (*Gallus gallus*, Residues 251–533) was expressed in BL21 cells as previously described.[34] FLAG-tagged WT, WT Cyslite, and Cys phosphorylation site mutant Cortactin were overexpressed and purified from HEK293T cells (Supplemental Methods). Purified Cortactin was incubated with Src and ADP-MA or ATP-MA overnight at 4°C in HEPES 50 mM pH 7.4, NaCl 150 mM in 15–20 μL. Specific conditions are listed under each figure. In lysate, 2 μM Src and ADP-MA gradient (1.67 mM, 167 μM, and 16.7 μM) was added to lysate and incubated for 16 h at 4°C and immunoprecipitated as above.

Preparation of ADP-MA and ATP-MA-labeled kinase for LC–MS/MS analysis

To 7 μg of kinase (Src and Aurora A) in 20 μL of HEPES 25 mM pH 7.4, NaCl 150 mM, 15 equivalents ADP-MA or ATP-MA was added. Samples were incubated for 4 h at 4°C, labeling was verified by LC–MS, and loading buffer was added and samples were run on a 4–12% gradient gel for 1 h at 180 V using MOPS buffer. Gel was washed with distilled water three times and stained with SafeStain Coomassie dye (Thermo Scientific, Waltham, MA). Bands corresponding to kinase were cut out for in-gel tryptic digest. Src samples were additionally dried in a GeneVac (SP Scientific, Ipswich, UK) for 1 h and rehydrated with 0.75 μL 1 mg/mL Glu-C and incubated at 37°C for 4 h before purification with C18 zip tip (Millipore Sigma, St. Louis, MO) before LC–MS/MS analysis.

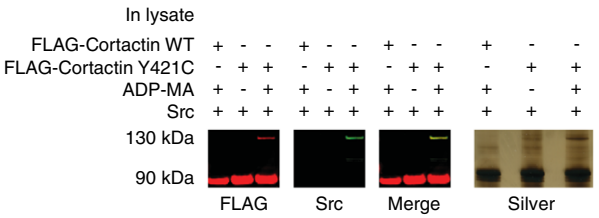

Figure 7. Western blot analysis and silver stain of ADP-MA mediated Src-Y421C FLAG-Cortactin crosslinking in protein lysate with 2 μM exogenously added Src.

LC–MS/MS analysis of ADP-MA and ATP-MA-labeled Src

On the Orbitrap Velos (Thermo Scientific), data-dependent acquisition mode was used to switch between MS and MS/MS and the top six precursor ions with a charge state of 2+ or higher were fragmented by HCD. Peak lists were searched on Protein Prospector using a mass tolerance of 20 ppm for precursor masses and ± 0.6 Da for fragment masses with cysteine carbamidomethylation as a fixed modification and missed cleavage and C_4H_4O modification of Lys as a variable modification. The digestion was specified as Trypsin + Glu-C with three maximum missed cleavages allowed. The UniProt protein identifiers for Src and Cortactin were specified (P00523 and Q14247, respectively).

XO44 chase experiment

Approximately, 20×10^6 293T cells were harvested and lysate was made as above. Hundred microgram of lysate in 15 μL final volume was used per sample. Samples were incubated overnight at 4°C with water, ADP-MA, or ADP at 15 μM. After overnight incubation, 2 μM XO44 was added and samples were warmed to room temperature and incubated for 10 min. A master mix of click reagents was made: 20% SDS, 5 mM TAMRA-N_3, 50 mM TCEP, 1.7 mM TBTA, 50 mM $CuSO_4$, and 10% DMSO. To 10 μL of each sample, 2.125 μL click reaction master mix was added and incubated for 1 h at room temperature in the dark. Loading dye was added and samples were run on a 4–12% gradient gel with MOPS buffer for 1 h at 200 V. The gel was removed and washed for 1 min with 10 mL 25 mM Tris pH 8, 10% methanol buffer and rinsed three times with 10 mL water before imaging on a Typhoon FLA 9500 (GE Healthcare, Chicago, IL) instrument.

LC–MS analysis and kinetics assays

Samples were analyzed on a quadrupole time-of-flight LC/MS instrument (Waters Xevo G2-XS QTof, Milford, MA). For kinetics samples, to 200 μL of 100 nM Src in HEPES 25 mM pH 7.4, NaCl 150 mM, $MgCl_2$ 1 mM buffer at 4°C, ADP-MA or ATP-MA was added to 1–50 μM. Sequential 7 μL LC–MS samples were taken of the unquenched reaction mixture. Time of analysis was measured by the instrument. Data were processed automatically using MaxEnt software with a range of

30–40 kDa. For Mg^{2+}/EDTA samples, buffer contained 10 mM Mg^{2+} or EDTA and ADP-MA or ATP-MA was added to 25 μM concentration.

Peptide crosslinking and kinase assay

Src 2 μM and ADP-MA 7.5 μM and FLAG-peptide gradient were incubated at 4°C for 2 h in MHBS. Peptides were tested at 0, 5, 25, and 125 μM concentration. Loading dye was added and samples were run on a 12% gel with MOPS buffer for 1 h at 180 V and transferred to nitrocellulose and visualized with Dylight 680 conjugated FLAG monoclonal antibody on a LiCor instrument (LiCor, Lincoln, NE). Kinase assay was as previously described.[35]

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Conflict of Interest

The authors declare no conflict of interest.

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