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Expedient Synthesis of a Modular Phosphate Affinity Reagent

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Abstract

Isolation and identification of phosphorylated macromolecules is essential for the deconvolution of most biological regulatory networks. Koike and coworkers recently reported the application of a dinuclear zinc-(pyridylmethyl)amine complex to phosphate-specific affinity purifications, and gave it the shorthand name “phos-tag”. This complex is valuable for studying phosphorylation because it binds selectively to phosphate dianion in the presence of acidic functional groups at physiological pH, and because the binding is largely independent of molecular context. These properties of phos-tag recommend it for applications in phosphoproteomics, metabolomics and nucleic acid biology. The catch has been that the molecule is difficult to make, and prohibitively expensive to buy. Here we describe an efficient and inexpensive synthesis of a phos-tag derivative with a versatile alkyne handle. The alkyne handle allows for attachment of phos-tag to alkyl azides via the copper(I)-catalyzed azide-alkyne cycloaddition reaction (“click chemistry”). We characterize the phosphate binding behavior of the new phos-tag derivative in a variety of experimental assays, including its conjugation to a fluorescent reporter, to acrylamide gels, and to sepharose chromatography resin. The synthesis we report should enable a broader use of phos-tag for phosphate-related biochemistry, as both an analytical and as a preparative reagent.

Communication

Phosphorylation plays a central regulatory role in many biological networks (1-3). Understanding such networks requires the detection and purification of different phospho-isoforms of proteins, nucleic acids, lipids, sugars and small metabolites. Existing work has focused primarily on proteins, and multiple technologies for phospho-peptide enrichment exist (4). Importantly, however, most of these techniques require harsh acidic conditions to suppress polyvalent binding of carboxylic acids (4). The necessity for acidic pH, which can induce chemical decomposition or denaturation, limits the utility and generality of most known phosphate affinity reagents.

Phos-tag is a phosphate-binding metal chelate complex that operates under milder conditions (4,5). It contains an organic core consisting of two bis(pyridylmethyl)amine moieties attached to the first and third carbon atoms of an isopropanol molecule (Scheme I). The core chelates a pair of divalent metal cations (Cd^{2+} , Co^{2+} , Cu^{2+} , Fe^{2+} , La^{2+} , Mn^{2+} , Ni^{2+} and Zn^{2+} in literature reports) (6-11). Some of these metals in turn coordinate tightly to the non-bridging oxygen atoms of phosphate. At physiological pH and salt concentration, phosphate monoester dianion binds to phos-tag(Zn^{2+}) with nanomolar affinity, while sulfate dianion, acetate, chloride and phosphate monoanion bind with dissociation constants that are 5,000 to 800,000-fold weaker (12). Phos-tag has been used successfully to produce phospho-specific fluorescent dyes, to retard phosphorylated macromolecules in acrylamide gels, to tag or

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Supporting Information Available: materials, detailed synthetic procedures, characterization of synthesized molecules and experimental protocols. This material is available free of charge via the Internet at <http://pubs.acs.org>.

isolate phosphorylated compounds for mass spectrometry and to enrich phosphorylated peptides and proteins by column chromatography (11,13-17).

Currently, tethered phos-tag is available from a single commercial source in a limited number of formats at a cost of ~\$50,000/g (18). A single synthesis of phos-tag with an immobilization handle is reported in the patent literature (19). The published synthesis proceeds from the cost-limiting reagent 6-methyl nicotinate (\$371/g) in a 38% reported yield over four steps. The steps generate product mixtures, and four flash column purifications are required. This cumbersome synthetic scheme, and the expense of the commercially available material, practically limits the use of phos-tag for preparative applications and in novel experimental formats. Here we present a simple, inexpensive and convergent synthesis of a phos-tag ligand bearing a versatile alkyne handle for single-point attachment to solid supports and to reporter molecules.

An exploration of the literature for synthetic strategies to the unsubstituted phos-tag ligand (Scheme 1) yielded two prevalent synthetic routes: alkylation of 1,3-diamino-2-propanol with methylpyridines (20) and addition of one or two bis(pyridylmethyl)amine units to epichlorohydrin (21,22). In our hands, the alkylation route generated a mixture of products that were difficult to separate. The epichlorohydrin route, on the other hand, promised greater synthetic control. It could be carried out in two steps with sequential addition of a first and a second bis(pyridylmethyl)amine unit. Addition of unsubstituted bis(pyridylmethyl)amine followed by a singly-functionalized bis(pyridylmethyl)amine would generate a single phos-tag product with a chemically accessible handle (Scheme 1).

A search of commercially available pyridylmethyl derivatives led to 2-formyl, 5-bromopyridine (Scheme 1). Reductive amination of this compound with 2-aminomethylpyridine provides an asymmetric, singly brominated bis(pyridylmethyl)amine. Sequential addition of unsubstituted bis(pyridylmethyl)amine and then the brominated bis(pyridylmethyl)amine to epichlorohydrin produces a singly brominated phos-tag derivative. Finally, Sonogashira coupling to (trimethylsilyl)acetylene, followed by base-mediated deprotection, converts the bromine group to a terminal alkyne group (23,24).

The synthesis proceeds in four steps from five commercially available reagents. The overall yield from the cost limiting reagent, 2-formyl, 5-bromo pyridine (\$19/g), is 31%. Thus the cost per gram of the product amounts to approximately \$60. The alkyne handle is particularly useful because it can be linked chemoselectively to azide-containing partners by the copper-mediated alkyne-azide cycloaddition reaction (colloquially called the “click” reaction). The resulting linker consists of a triazole.

A first question is whether triazole-linked phos-tag binds to phosphate monoesters with the same affinity as the known amide-linked phos-tag. To address this question, we measured the dissociation constant (K_D) of phos-tag(Zn^{2+})-triazole-aminomethylcoumarin (**5a**) from fluorescein-phosphoethanolamine using a published fluorescence assay [Fig. 1 and (25)]. The fluorescence emission of the aminomethylcoumarin donor chromophore (emission maximum at 445 nm) is suppressed by increasing concentrations of the fluorescein acceptor (absorption maximum at 490 nm). The bimolecular dissociation curve that best fits the data has a K_D of 47 ± 10 nM, comparable to the 70 nM K_D measured for the dissociation of phostag(Zn^{2+})-amide-aminomethylcoumarin from fluorescein-phosphoserine (25). The data suggest that the binding properties of phos-tag are largely unperturbed by the triazole linker.

We next checked whether triazole-linked phos-tag is a practical phosphate-affinity reagent in standard electrophoretic and chromatographic applications. For electrophoresis experiments, the phos-tag alkyne was coupled to an azido-acrylamide moiety (**5b**) and incorporated into native 15% acrylamide gels supplemented with zinc. We compared the

migration of two double-stranded PCR amplicons of 200 base-pair length that differed by a single 5'-phosphorylation (Fig. 2A). Compound **5b** at 700 nM shifted the phosphorylated amplicon such that it migrated with an apparent size of 275 base pairs, while the amplicon lacking a terminal 5'-phosphate migrated as a 200mer. The two amplicons migrated identically in a gel containing zinc but lacking compound **5b**.

For phosphate-affinity chromatography experiments, we coupled phos-tag alkyne to azide-derivatized NHS-sepharose at a final substitution level of 5 mM. The phos-tag sepharose resin (**5c**) was placed into 50 μ L volume spin columns and loaded with Zn^{2+} . We compared the retention of the poly-phosphorylated protein β -casein to the retention of the acidic protein bovine serum albumin (BSA). The use of the highly acidic protein BSA [net charge of -11 at the pH 7.4 used for the separations (26)] represents a challenging control. Acidic peptides and proteins are commonly co-purified in phospho-specific purifications, presumably through weak polyvalent interactions of carboxylic acids with the affinity reagent. Nevertheless, the triazole-linked phos-tag(Zn^{2+}) resin cleanly separated β -casein from BSA (Fig. 2B), consistent with previous reports for amide-linked phos-tag(Zn^{2+}) (17). Thus, triazole-linked phos-tag works well for purification of poly-phosphorylated proteins from mixtures.

Previously published phos-tag affinity separations of simple protein mixtures have all used poly-phosphorylated proteins as the model phosphorylated species (5,17). To explore the selectivity for mono-phosphorylated proteins, we measured the retention of a variant of RNase H engineered to include a single substrate recognition site for protein kinase A. The mono-phosphorylated RNase H bound to the resin but was eluted by citrate wash buffer. It remained bound during washes with 1M NaCl and could then be eluted by addition of phosphate (Fig. 2C). However, BSA was not fully cleared under these milder wash conditions, and some BSA eluted in a phosphate-dependent manner. Alternate wash solutions (1 M sodium acetate and 1 M ethylene diamine) and loading the phos-tag with an alternate metal (Mn^{2+} instead of Zn^{2+}) failed to abolish binding of BSA while retaining complete binding of mono-phosphorylated RNase H.

Although phos-tag(Zn^{2+}) binds to phosphate dianion 16,000-fold more tightly than it binds to acetate (12), in our hands phos-tag sepharose resin cannot cleanly separate a mono-phosphorylated protein from the acidic protein BSA. Additionally, the tri-carboxylic acid citrate at 100 mM concentration competitively displaces mono-phosphorylated protein from phos-tag sepharose resin, whereas the mono-carboxylic acid acetate at 1 M concentration does not. A possible explanation is that multiple weak interactions of carboxylates with the phos-tag sepharose resin are comparable in strength to the interaction with a single phosphate monoester. Increasing the mean distance between phos-tag ligands would suppress such multivalent binding, and could be accomplished by reducing the phos-tag substitution level on the resin and by ensuring an even spatial distribution of the ligand. This remedy would presumably improve the specificity with which mono-phosphorylated proteins could be isolated, at the cost of decreased resin binding capacity. Notably, β -casein remains bound to phos-tag sepharose at high concentrations of citrate. Thus, isolation of poly-phosphorylated proteins from complex mixtures is possible using highly substituted resins.

When synthesized as described, phos-tag alkyne is roughly three orders of magnitude less expensive than commercially available phosphate-affinity reagents or supports. It recapitulates the published properties of the amide-linked molecule. Our data suggest that phos-tag alkyne can be substituted directly for the commercially available phos-tag, with similar performance. The substantial cost reduction achieved by the new synthesis allows phos-tag to be considered as a practical reagent for preparative protein purification. The

versatility of the alkyne linkage handle should also facilitate new applications of the phos-tag chelate core.

Summary

Deconvoluting biological regulatory networks depends on isolating and identifying phosphorylated macromolecules. Koike and coworkers recently reported a di-nuclear zinc-pyridylmethylamine complex, “phos-tag”, that binds phosphate di-anion selectively in the presence of acidic functional groups at physiological pH. Here we describe an efficient and inexpensive synthesis of a phos-tag derivative with a versatile alkyne handle. The new ligand functions comparably to known phos-tag derivatives, and should enable a broader application of phos-tag in analytical and preparative biochemistry.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Scheme 1.

Synthesis of modular phos-tag ligand. Percentages describe representative yields of given reaction. M: 2+ metal ion. TMOF: trimethyl orthoformate. MeOH: methanol. DIEA: diisopropylethylamine. TMSA: (trimethylsilyl)acetylene. TEA: triethylamine. DMF: dimethylformamide. DCM: dichloromethane.

Figure 1.

Triazole-linked phos-tag binds phosphate monoesters with an affinity comparable to amide-linked phos-tag. **A.** Overlaid emission spectra of phos-tag(Zn^{2+})-triazole-aminomethylcoumarin (**5a** at 170 nM concentration, λ_{ex} =345 nm, 25 °C) with increasing concentrations of fluorescein-phosphoethanolamine. The aminomethylcoumarin fluorophore has an emission maximum at 445 nm, and the fluorescein has an emission maximum at 524 nm. **B.** Plot of triplicate FRET data with error bars indicating ± 1 standard deviation. The fraction of bound phos-tag(Zn^{2+})-triazole-aminomethylcoumarin is plotted versus fluoresceinphos-phoethanolamine concentration (0, 25, 50, 75, 100, 125, 150, 175, 200, 250, 300, 400, 600, 800, and 1200 nM). The calculated bimolecular dissociation curve with a K_D of 47 nM is shown as a solid line. Phos-tag(Zn^{2+})-amide-aminomethylcoumarin is reported to bind fluorescein-phosphoserine with a K_D of 70 nM (25).

Figure 2.

Detection and purification of phosphorylated macromolecules with the triazole-linked phos-tag. **A.** Phospho-specific DNA gel shift. A conventional 15% native DNA acrylamide gel (left) is compared with an identical gel containing 700 nM phos-tag acrylamide (right). Both gels were cast with 100 μ M zinc nitrate. The samples include a 100 base-pair DNA ladder (L), a 200mer DNA with a single 5'-phosphate modification (P), and an identical 200mer DNA with two 5'-hydroxyl termini (C). The migration of the phosphorylated DNA is retarded in the phos-tag-containing gel. **B.** Phospho-specific protein purification. Load (L), flow-through (FT), wash and elute fractions from a phos-tag sepharose spin column were analyzed on 15% Laemmli gels stained with colloidal coomassie blue. β -casein (a poly-phosphorylated protein) is separated away from bovine serum albumin (BSA, a non-phosphorylated acidic protein). The wash buffer was 100 mM citrate, 100 mM Tris-acetate pH 7.4. Elution was accomplished by addition of 100 mM sodium phosphate to the wash buffer. **C.** Laemmli gel as in **B** showing the partial separation of a mono-phosphorylated RNase H derivative (engineered to contain a single substrate recognition site for protein kinase A) away from BSA. The wash buffer was 1 M sodium acetate, 100 mM Tris-acetate pH 7.4. Elution was accomplished by addition of 10 mM sodium phosphate to the wash buffer.