Protein Modification

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Rhodium(II) Metallopeptide Catalyst Design Enables Fine Control in Selective Functionalization of Natural SH3 Domains**

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Abstract: *Chemically modified proteins are increasingly important for use in fundamental biophysical studies, chemical biology, therapeutic protein development, and biomaterials. However, chemical methods typically produce heterogeneous labeling and cannot approach the exquisite selectivity of enzymatic reactions. While bioengineered methods are sometimes an option, selective reactions of natural proteins remain an unsolved problem. Here we show that rhodium(II) metallopeptides combine molecular recognition with promiscuous catalytic activity to allow covalent decoration of natural SH3 domains, depending on choice of catalyst but independent of the specific residue present. A metallopeptide catalyst succeeds in modifying a single SH3-containing kinase at endogenous concentrations in prostate cancer (PC-3) cell lysate.*

"Chemical" posttranslational modification of natural proteins is a remarkable challenge, the products of which find use in diverse fields. Biological posttranslational modification is exquisitely selective, functions in complex aqueous environments hostile to many synthetic catalysts, and achieves chemoselectivity based on subtle structural factors. In contrast, selective chemical catalysis on natural proteins is an unsolved problem, largely because chemical reactions are driven by inherent functional-group reactivity and, as such, struggle to achieve selectivity in biological environments. We describe a molecular-recognition approach to site-specific modification with rhodium(II) metallopeptide catalysts, allowing independent modification of different residues on a natural SH3 domain, depending on choice of catalyst.

The polyfunctional nature of proteins makes them poor substrates for traditional catalysis: there are almost no unique functional groups. As a result, most site-specific modification[1,2] relies on engineering and recombinant introduction of a uniquely reactive group or sequence. A variety of creative and effective tagging methods are known,[3] including unnatural amino acids and canonical sequences that enable selective chemical[4–6] or enzymatic reactions.[7–9] But known methods do not extend to natural proteins, apart from special cases, such as N-terminal functionalization,[10,11] the fortuitous presence of a unique surface-exposed cysteine thiol, or enzymes (e.g. kinases) that lend themselves to mechanism-based covalent inhibition.[12]

Relatively few research groups have confronted selective modification of natural proteins, even though the alternative engineered approaches have limitations, which may include studies limited to a model organism and scale restrictions. Some pioneering work with small-molecule natural products[13,14] has established the ability of molecular recognition to overcome inherent substrate reactivity, such as polyol substrates in catalytic acylation.[15] However, proteins represent a more demanding and complex challenge. Among recent efforts,[16–19] the idea of catalytic acylation has been extended to directed lysine modification on cell surfaces with a designed DMAP-conjugated (DMAP = 4-dimethylaminopyridine) ligand,[20] and $[Ru(bpy)_3]^{2+}$ (bpy = 2,2′-bipyridine) complexes have been used for selective tyrosine modification of carbonic anhydrase (CA) by a single-electron-transfer oxidation mechanism.[21] Based on previous studies,[22,23] we reported rhodium(II) metallopeptides for modification of designed polypeptide targets, with the peptide ligand serving as a molecular recognition fragment and the rhodium(II) center capable of catalyzing proximity-driven functionalization of a remarkably wide range of side chains (Figure 1), roughly 10 different amino acids constituting ca. 40% of protein sequence space.[24–26] Importantly, relatively weak metallopeptide–protein interactions (K_d = 1–20 μM) were sufficient to template selective catalysis at biologically relevant concentrations. Among the key advantages, at least conceptually, the rhodium metallopeptide approach is not limited to designed substrates and could be amenable to natural protein targets, provided a ligand for the protein in question could be discovered, synthesized, and conjugated to a rhodium(II) complex (Figure 1a).

However, numerous limitations and problems prevented us from extending this proof-of-concept result to any real extent. We were able to demonstrate a single example of modification of a purified, natural SH3 domain (Fyn) at Trp119, by designing metallopeptides based on the short proline-rich PPII sequences (see Figure 1b for examples) that bind SH3 domains with modest affinity (ca. 1 μM, see Figure S14 in the Supporting Information for affinity data for metallopeptides employed in this work).[27,28] However, the initial catalysts studied (**S2E^{Rh}, R5E^{Rh}**) were sluggish, inefficient, and poorly controlled, providing incomplete modification and mixtures of mono- and bis-functionalization (Figure 2d). To a real extent, mixtures of modification site and number defeats the purpose of developing site-specific methods. Furthermore, we reported Fyn modification with

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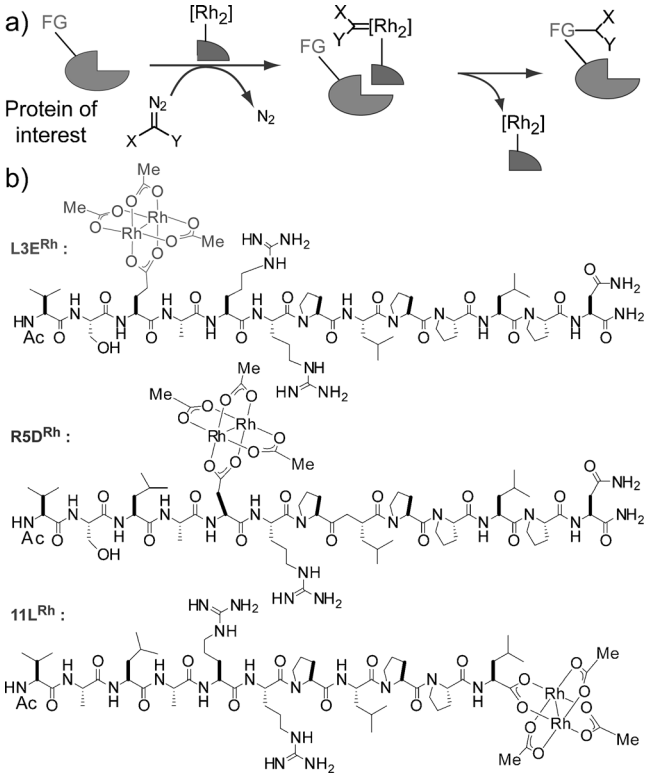

Figure 1. a) Metallopeptide–protein interaction as basis for proximity-driven modification of a natural protein target (FG = functional group). b) Structures of metallopeptides **L3E^{Rh}**, **R5D^{Rh}**, and **11L^{Rh}** derived from SH3 domain binding peptide VSL12 (VSLARRPLPPLPP), showing placement of rhodium(II) core.

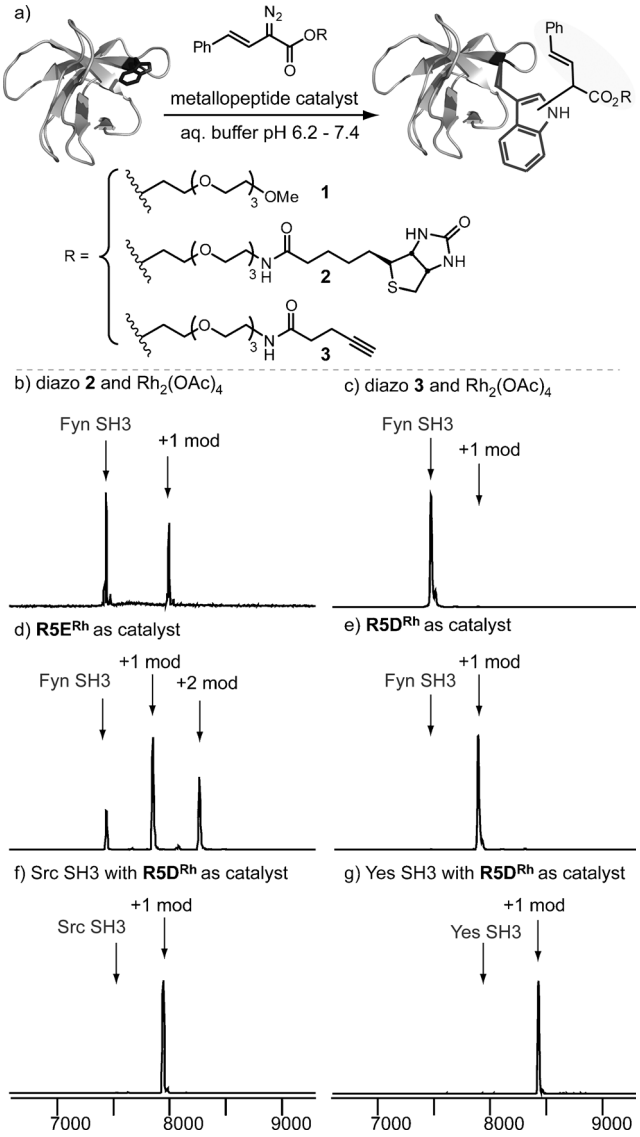

Figure 2. a) Modification reaction conditions: 10 μM SH3 domain protein, 5 μM catalyst, 500 μM diazo, TBHA (*N*-(*tert*-butyl)hydroxylamine) buffer pH 6.2. MALDI-MS spectra of end point analytes after 5 h reaction at room temperature. Modification occurs at Fyn Trp119 (or the homologous Trp residue) for all metallopeptide reactions, by MS-MS analysis. b) Fyn SH3 modification with biotin diazo **2** and $Rh_2(OAc)_4$; c) Fyn SH3 modification with diazo **3** and $Rh_2(OAc)_4$; d) Fyn SH3 modification with diazo **3** and **R5E^{Rh}** metallopeptide; e) Fyn SH3 modification with diazo **3** and **R5D^{Rh}** metallopeptide; f) Src SH3 modification with diazo **3** and **R5D^{Rh}** metallopeptide; g) Yes SH3 modification with diazo **3** and **R5D^{Rh}** metallopeptide.

a "dummy," unfunctionalized diazo compound (**1**). For conjugates with actual utility, we used a bifunctional biotin-diazo reagent **2**, which was very effective in designed coiled-coil systems.[29] Curiously, catalytic modification of *natural* SH3 domains with diazo **2** was slow and uncontrolled: lysate-based reactions required elevated diazo concentrations and led only to indiscriminant protein labeling. Reactivity was observed even with the negative control ($Rh_2(OAc)_4$) (cf. Figure 4b,c). The rhodium metallopeptides based on the VSL12 peptide ligand[30,31] have affinity for Fyn and other Src-family SH3 domains, such as Lck, Src, and Yes, and indiscriminant reactivity persisted with other Src-family members (data not shown).

Two alterations dramatically improve modification efficiency and enable useful functionalization in complex environments: 1) optimization of metallopeptide structure and 2) development of a new bifunctional diazo reagent and corresponding new protein blot imaging protocol. Optimization of sequence and rhodium placement on the SH3-binding peptide led to a new metallopeptide, **R5D^{Rh}**, a variant of the parent VSL12 peptide (Figure 1b). This catalyst has a shorter side chain (aspartate vs. glutamate) connecting the rhodium(II) center to the peptide, presumably with some accompanying decreased conformational freedom. This subtle change results in an extremely efficient catalyst that is both more active and also more selective: Complete conversion to a single product is observed (Figure 2e). No modification is observed with $Rh_2(OAc)_4$ (Figure 2c). Perhaps most surprisingly, the exquisite selectivity of the **R5D^{Rh}** catalyst is retained across other members of the Src SH3 family (Figure 2f,g). MS-MS sequencing has consistently proven that Fyn Trp119 (or the corresponding tryptophan residue in homologous proteins) is the site for all Src-family modifications catalyzed by **R5D^{Rh}** (see Supporting Information for details). As with Fyn, the previous-generation catalyst provided mixtures of zero, one, and two modifications for these other domains as well. Metallopeptide structural optimization now allows

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production of monodisperse samples of modified Src-family SH3 domains.

The observation that subtle changes in metallopeptide sequence could have a strong effect on catalyst selectivity led us to pursue changes in metallopeptide structure in the hopes of modifying other residues. For example, the Lck SH3 domain contains, in addition to Trp97, and two histidine residues (His70, His 76) near the peptide-binding pocket. As anticipated, metallopeptide **R5D^{Rh}** also promotes modification of Trp97 (homologous to Fyn Trp119, see Figure 3a). Histidine is a poor substrate for rhodium-catalyzed modification: In our coiled-coil work, we estimated that tryptophan was more reactive by > 2 orders of magnitude.[24] Nonetheless, working from the Lck structure, we designed the metallopeptide **11L^{Rh}**, positioning the rhodium center near His70, that induces efficient modification of the targeted histidine residue (Figure 3a; reactivity information for other metallopeptides tested during this study is available in Supporting Information). His70 was unambiguously identified as site of modification with **11L^{Rh}** by MS-MS methods (Figure S3). For optimal reactivity at His70, it was necessary to conduct the reactions at elevated pH (7.4) to minimize histidine protonation. A clear trend of increasing rate and conversion with increasing pH was observed (Figure S2), in contrast to tryptophan modification examples, where we and others[22,23] observe increasing pH leads to a decrease in rate and conversion.

The second histidine (His76) in the Lck SH3 domain could also be targeted for modification. With a different catalyst, **L3E^{Rh}** (Figure 1), we observed clean modification of this other histidine instead, albeit at lower rates. Thus, by judicious choice of catalyst, we demonstrated site-specific modification of a wide range of Src-family SH3 domains and showed that functionalization could be directed to three different sites around the peptide-binding interface, all with an identical reagent and without recourse to mutagenesis.

With an efficient means of controlling the site of modification at several different points on the surface of SH3 domains, we addressed the vexing lack of selectivity in reactions of natural domains with the bifunctional biotin-diazo **2** (Figure 4b, nonselective labeling). We quickly verified that the pendant biotin structure in diazo **2** was responsible for anomalous non-selective reactivity; it now seems likely that the anomalous reactivity of the biotin-diazo reagent, **2**, is due to interactions of the thioether group with a metallocarbene intermediate. (We have previously observed effects of intramolecular axial ligands on rhodium(II) reaction selectivity in other contexts.)[32,33] In our hands, even biotin itself accelerates some tryptophan modification reactions, the mechanistic details of which are not entirely clear. Among a few alternative functional diazo reagents examined, an alkyne-diazo (**3**) provided selective reactivity similar to simple diazo **1**, as judged by mass spectrometry.

Successful modification and analysis of SH3 domains in *E. coli* cell lysate required optimization of our visualization method. While the alkyne-diazo reagent **3** solved issues of reaction selectivity, visualizing the modified proteins in lysate was challenging, especially for small protein domains, where separation from small-molecule reagents was difficult. Using a fluorogenic azide, these issues were solved by performing copper-catalyzed azide–alkyne coupling reactions directly on the surface of a PVDF transfer membrane after a typical protein gel. The "fluorogenic," or "turn-on" reagent, 3-azido-

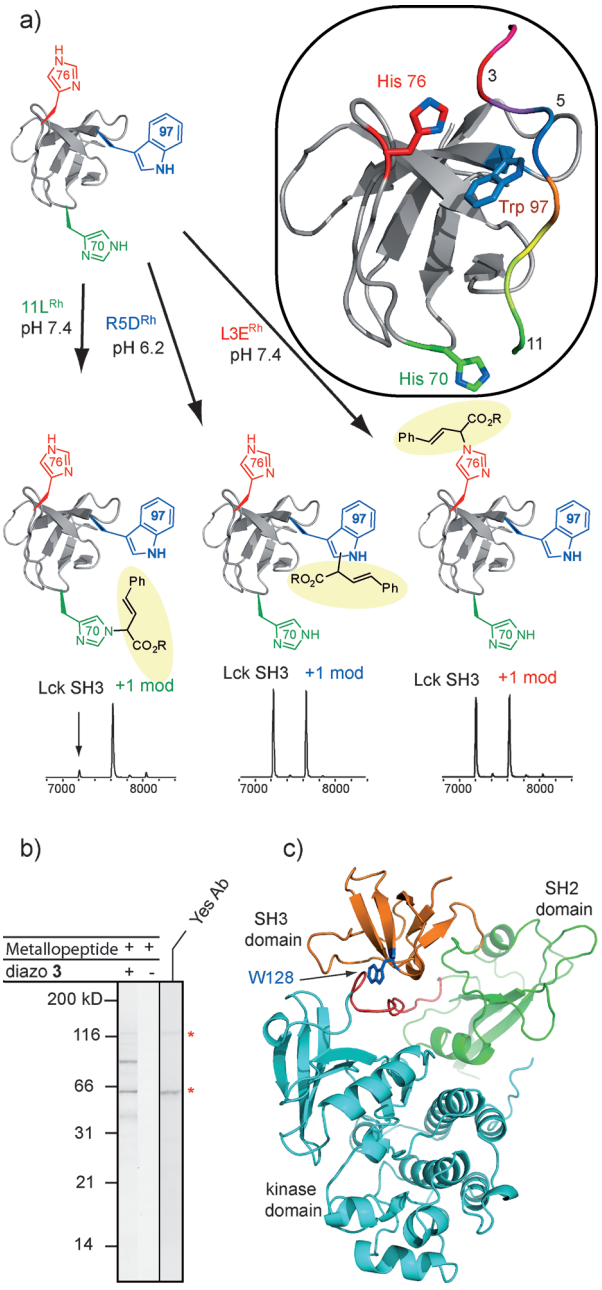

Figure 3. a) Lck SH3 domain site-specific modification reactions catalyzed by **11L^{Rh}**, **R5D^{Rh}**, and **L3E^{Rh}**. MALDI-MS spectra (bottom) shows conversion at 5 h at room temperature. His modification reactions carried out in Tris buffer pH 7.4 with diazo **3**. Biotin (≈900 μM) was added to modification of Trp97. Inset: Structure of Lck SH3 domain bound to a peptide ligand (PDB ID: 2IIM) used for design of metallopeptides targeting different residues. b) Human PC-3 cell lysate subjected to modification reaction catalyzed by rhodium(II) metallopeptide (**R5D^{Rh}**, 15 μM). For experimental details, see Supporting Information. c) The enzyme resting state of Src-family kinases contains an interaction between the SH3 domain (orange) and a loop region near the kinase domain (cyan), effectively burying the SH3 peptide-binding motif. Shown here for Src (PDB ID: 2SRC).

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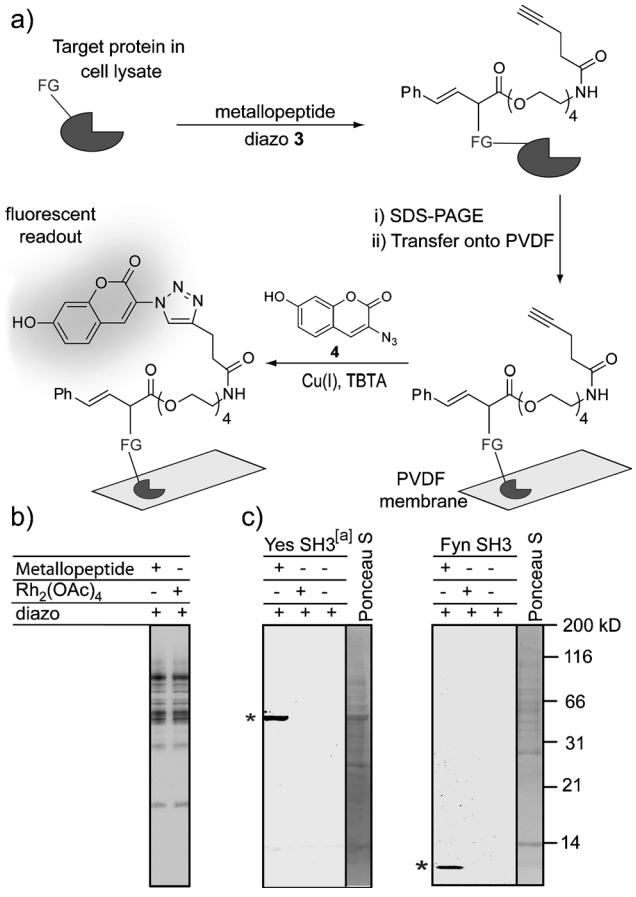

Figure 4. Modification of natural SH3 domains in *E. coli* lysate. Target protein concentration (2 μM), metallopeptide (20 μM), and diazo (500 μM) were added to lysate. a) Conceptual representation of fluorogenic analysis of blotting membranes containing lysate based modification reaction products (PVDF = polyvinylidenedifluoride blotting membrane, TBTA = *tris*[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine). b) Western blot of analysis of lysate-based modification with biotin-diazo **2**, showing non-selective reactivity. Visualization with Avidin-Hrp. c) Blots of modification reactions using diazo **3** and visualized as outlined in (a). * Denotes target protein band. Total protein loading shown using Ponceau S staining. [a] Yes SH3 expressed as fusion with maltose binding protein.

7-hydroxycoumarin (**4**)[34]—non-emissive ("dark") until it reacts with alkyne—allowed direct visualization of SH3 domain modifications upon bathing a transfer membrane in a solution of azide **4** and a copper catalyst. Although conceptually straightforward, chemical reactions on blotting membranes is little utilized,[35] and we are not aware of other use of fluorogenic reagents in this context. Gratifyingly, this chemical blotting of lysate reactions produced an image with a single fluorescent band. Control reactions with $Rh_2(OAc)_4$ or without catalyst had no observable fluorescence (Figure 4d). It worth noting that we successfully tagged and analyzed a protein sample with molecular weight <10 kDa (Fyn SH3 domain), a significant result given the challenges of such small proteins.

We next examined the human tumor cell line, PC-3,[36] which expresses the full-length Yes kinase, a member of the Src family (Figure 3b). The PC-3 whole lysate was subjected to modification conditions and analyzed. A fluorescent band at 60 kDa appeared, corresponding to Yes kinase and confirmed by conventional western blot with a Yes antibody. Interestingly, a second major band at 80 kDa was also visualized. This band, like Yes, was absent from the negative control, $Rh_2(OAc)_4$, indicating that it binds to the PPII metallopeptide catalyst or co-assembles with Yes kinase. Modification of natural levels of full-length Yes kinase is especially noteworthy because the targeted SH3 domain[37] is inaccessible in the kinase resting state.[38] It remains bound to a loop region near the kinase domain (Figure 3c). Peptides and small molecules of modest affinity (≈ 1 μM) for SH3 domains, such as the **R5D^{Rh}** catalyst, typically display IC_{50} values of >1 mM for binding to the full kinase, because small peptides do not effectively out-compete the intramolecular loop interaction.[39–41] Catalytic activity of the metallopeptide at only 15 μM indicates that catalyst–substrate assembly need not be the predominant species in solution to achieve efficient modification.

In a departure from traditional residue-selective chemistries, the promiscuity of metallocarbene intermediates allows specific decoration of a protein surface that does not depend on the presence of specific residues. In reactions with purified protein, catalyst design overrides inherent chemical reactivity, selecting for His70 or His76 modification rather than reactions at the more reactive Trp97 residue, despite a significantly higher inherent reactivity (>2 orders of magnitude more reactive) for tryptophan relative to histidine.[24] Custom tailored-catalysts may deliver modification of virtually any protein of interest, especially given advances in methods for ligand discovery.

Developing chemistry to tackle the complex, functional-group-rich environment of natural proteins remains a challenge. Despite what is now decades of effort to overcome this limitation and develop selective reactions that rival enzymatic transformations, successful examples are rare—such as recent examples with complex natural products[13,14]—and are just beginning to touch the surface of what is possible. The results of Yes kinase from PC-3 tumor cells indicate that specific modification reactions, in lysate and at natural abundance, are possible. Furthermore, the fluorogenic chemical blot method improves sensitivity, allows improved detection of small proteins, and, in initial studies, appears broadly robust with a variety of fluorophores and conjugation chemistries.

Keywords: bioorganic chemistry · diazo compounds · homogeneous catalysis · protein modifications · rhodium

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