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Catalytic Protein Modification with Dirhodium Metallopeptides: Specificity in Designed and Natural Systems

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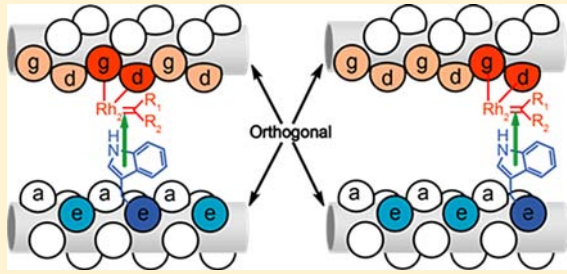
Supporting Information

ABSTRACT: In this study, we present advances in the use of rhodium(II) metallopeptides for protein modification. Site-specific, proximity-driven modification is enabled by the unique combination of peptide-based molecular recognition and a rhodium catalyst capable of modifying a wide range of amino-acid side chains. We explore catalysis based on coiled-coil recognition in detail, providing an understanding of the determinants of specificity and culminating in the demonstration of orthogonal modification of separate proteins in cell lysate. In addition, the concepts of proximity-driven catalysis are extended to include modification of the natural Fyn SH3 domain with metallopeptides based on a known proline-rich peptide ligand. The development of orthogonal catalyst−substrate pairs for modification in lysate, and the extension of these methods to new natural protein domains, highlight the capabilities for new reaction design possible in chemical approaches to site-specific protein modification.



INTRODUCTION

Chemical modification of proteins is an important tool in diverse fields. Protein-based therapeutics often exhibit improved efficacy and pharmacodynamics upon attachment of molecules such as oligo(ethylene glycol).[1] Chemical biology relies on access to proteins with diverse functionality including small-molecule dyes, protein ligands, and reactive functional groups. Single-molecule and other biophysical measurements of protein structure and function also rely heavily on protein modification for surface immobilization or attachment of reporter molecules.

Chemical reactions to achieve protein modification generally rely on residue-selective chemistry. Examples of these methods include classical functionalization of lysine and cysteine as well as more recent methods targeting other amino acids such as tyrosine and tryptophan.[2−5] However, proteins contain many copies of most reactive side chains, and so reaction typically results in an ensemble of products containing multiple modifications at different sites on the protein surface. These heterogeneous protein populations create difficulties in biophysical measurements, especially single-molecule spectroscopy. Moreover, residue-selective methods are limited to purified protein rather than modification of a target protein in a complex mixture.

To combat these shortcomings, tagging sequences have been developed that can be incorporated in a recombinant protein to allow modification with either chemical reagents or enzymes that form chemical linkages within specific sequences.[6−13] While powerful, both enzyme- and reagent-based methods have limitations, and we believe that there could be a unique role for designed, transition-metal catalysis approaches to sequence-specific modification. Enzyme-like reactivity in a designed metal catalyst could allow development of protein modification methods with the attributes of small-molecule reactions—tolerance of varied or denaturing reaction conditions and straightforward application to new systems—and enzymes—high turnover and reactivity based on molecular recognition that overrides inherent chemical reactivity. In addition, current methods for site-specific protein modification require access to recombinant, "tagged" protein. Methods to directly modify natural proteins in a site-specific manner would be a significant new capability relative to current methods.

We initially reported a strategy for modification of polypeptide chains based on rhodium(II) metallopeptide catalysts (Figure 1).[14−16] Our approach is predicated on the idea that molecular recognition and transient assembly, which are commonly used to template covalent cross-linking or dimerization,[17−20] could be used for proximity-driven transition-metal catalysis.[21,22] By comparing tryptophan modification within a designed coiled coil to that in a random control sequence, we were able to observe large rate enhancement (>10^3) relative to nonselective background catalysis from modest affinity interactions (10−50 μM K_d).[14] More significantly, a wide variety of amino-acid side chains could be efficiently modified within a coiled coil assembly, reactions that were not observed at all with the simple $Rh_2(OAc)_4$ catalysis.[15] The peptide coils could be fused to recombinant

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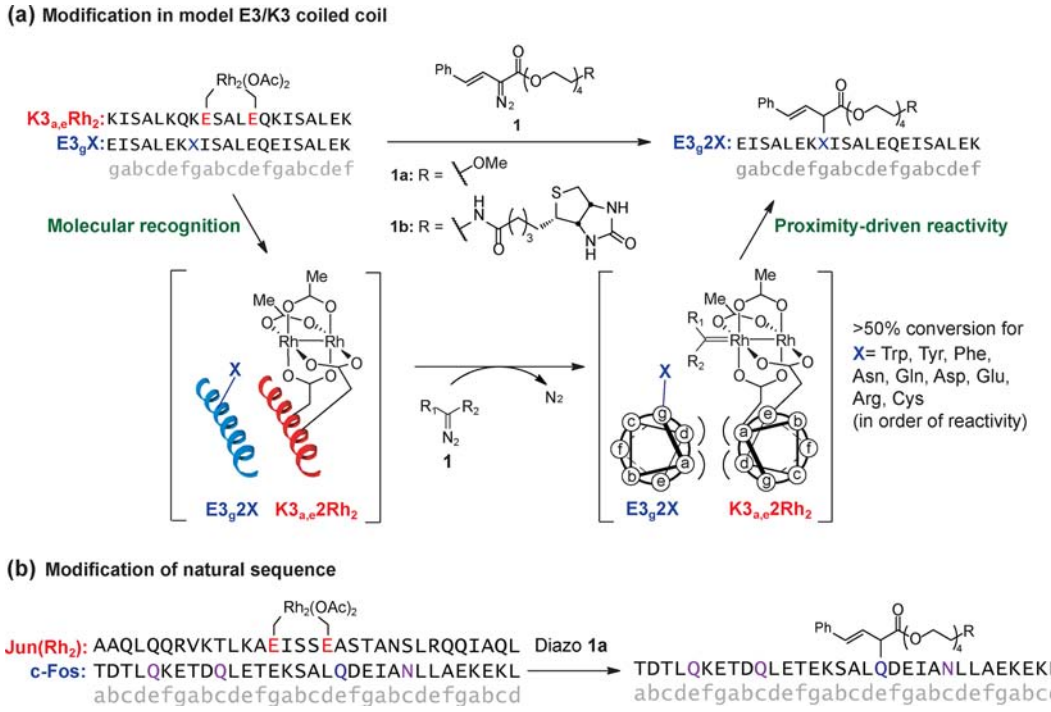

Figure 1. Proximity-driven modification of (a) designed (E3/K3) and (b) natural (c-Fos/Jun) coiled coils catalyzed by rhodium(II) metallopeptides.

proteins and used as a tag for protein modification in cell lysate.[16] This site selectivity is an example of catalysis that overrides inherent chemical reactivity, a key capability of natural metalloenzyme reactivity that is difficult to achieve with traditional transition-metal catalysis. Efforts to design[23,24] catalytically active metalloproteins or metallopeptides have demonstrated diverse reactivity, including electron transfer,[25,26] enantioselective catalysis,[27−37] and acceleration of fundamental reactions such as saponification or carbonic anhydrase-like activity.[38−40] Limited success with designed catalysts that exhibit enzyme-like selectivity has been reported, including examples of DNA cleavage[39,41−47] and cyclodextrin-based molecular recognition.[48] In a complementary approach, organocatalytic methods for catalyzing selective reactions on complex substrates have been reported for peptide,[49,50] protein,[21,22] or secondary metabolite[51,52] substrates.

In this manuscript, we explore the extent to which established ideas for ground-state peptide−peptide and peptide−protein assembly can be used as the basis for designing selective transition-state assemblies leading to enzyme-like chemical catalysis. Our investigations focus on two distinct areas: (1) discovery and specificity of new designed coiled coils that exhibit orthogonal reactivity and enable orthogonal labeling of multiple proteins and (2) use of proximity-driven metallopeptide catalysis for modification of the natural Fyn SH3 domain. The modular nature of rhodium(II) metallopeptides—combining peptide-based molecular recognition and rhodium(II) catalysis—makes them amenable to new substrate structures and new recognition elements. Coiled coils in particular allow control over precise placement of both the rhodium(II) center and the reactive residue in the coiled coil (Figure 1a), and moving these reactive partners to new positions on a coiled-coil scaffold is a straightforward way to design new catalysts with orthogonal reactivity. Beyond designed coiled coils, we have been eager to extend the ideas of proximity-driven catalysis to modify completely natural protein targets. To this end, we examined the Fyn SH3 domain,[53,54] a prototypical SH3 domain. Fyn is a protein−tyrosine kinase signaling protein and has been implicated in tumor development due to its role in regulating cell growth. Short peptide sequences that bind to SH3 domains have been reported. The binding interface tolerates significant variation in peptide sequence, facilitating design of metallopeptide catalysts. Successful modification of the Fyn SH3 domain would be a powerful advance in the search for a general approach to site-specific protein modification that is applicable to natural proteins.

RESULTS AND DISCUSSION

Specificity in Catalysis of Coiled Coils: Results. To assess specificity, we examined small changes in substrate positioning within the designed coiled-coil template. Properly designed metallopeptide−substrate pairs position the rhodium center near to a specific reactive residue (Figure 2). The sequence of the coiled coils used in these studies contains three repeating "heptads" of seven amino acids (labeled *abcdefg*). The E3/K3 coiled coil has been well studied[55−58] and reviewed.[59,60] An NMR structure established formation of parallel heterodimers,[61] and the sequences have been utilized widely by a variety of groups in various applications.[62−67] We previously established that appending a chelating rhodium(II) center within the K3 coil produces the expected 1:1 dimer structure with only small changes in stability, and our previous modification results demonstrate the expected parallel alignment.[14,15] Proximity-driven modification is observed at position *e* or *g*, the two side chains located near opposite faces of the dimerization interface. An appropriately designed metallopeptide will position a rhodium catalyst near either the *e* or the *g* position of the complementary coil. Thus, for each catalyst, there is a "facial match" and a "facial mismatch" substrate that positions a reactive side chain on the opposite face of the coiled coil (Figure 2b). Because the coiled coil is composed of three

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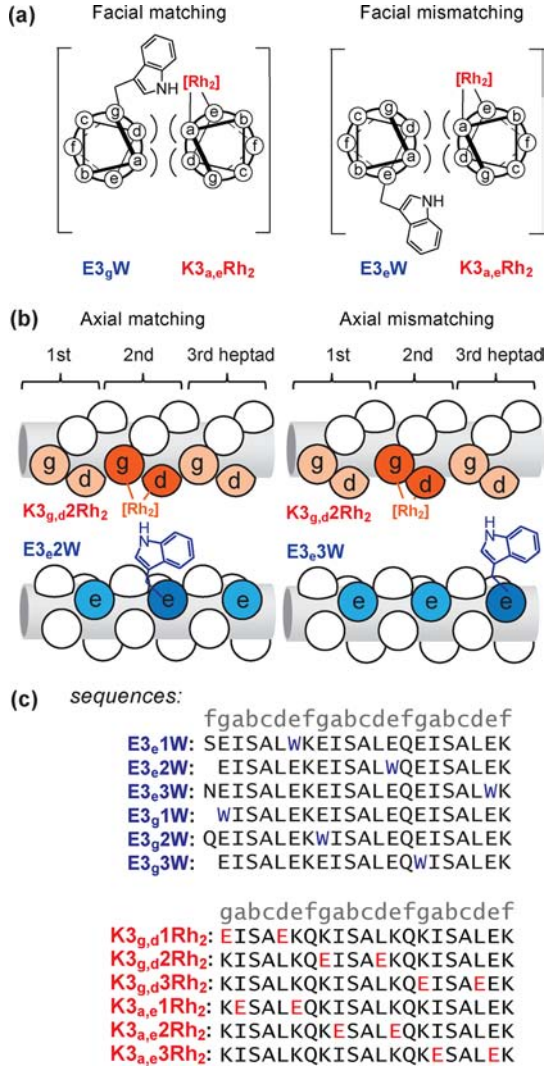

Figure 2. Conceptual illustration of axial and facial match/mismatch in coiled coils, and sequences for six E3 substrates and six K3 rhodium(II) metallopeptides. (a) Axial matched case, $K3_{g,d}2Rh_2$ and $E3_e2W$, and axial mismatched case, $K3_{g,d}2Rh_2$ and $E3_e3W$. (b) Example of a simple facial mismatch. (c) Sequence of all coil peptides. For substrate peptides, site of modification (W) is shown in blue. For metallopeptides, site of rhodium attachment is shown in red. Nomenclature: the lowercase letter indicates the facial position of the reactive site (*g* or *e* for tryptophan and *a,e* or *g,d* for dirhodium) and number following (1, 2, or 3) indicates the axial location along the helix.

heptads, the location of a reactive residue could also be misaligned along the axis of the coil (Figure 2a, termed "axial mismatch"). We examined tryptophan modification in our selectivity studies because tryptophan is significantly more reactive than other amino acids, simplifying analysis. For modification reactions with 25 μM substrate, low metallopeptide loading (2 mol %, 500 nM) and modest diazo concentration (750 μM) result in negligible ($\leq$5%) modification of residues other than tryptophan.

We prepared all six possible combinations of substrate (E3W) and metallopeptide ($K3Rh_2$) coils (all possibilities of 3 axial and 2 facial positions). Most metallopeptides formed the expected coiled coils with a matched tryptophan-containing peptide. Thermal unfolding studies using circular dichroism (CD) produced melting curves that demonstrated stability (T_m = 45−62 °C or $K_{d(app)}$ = 1−40 μM) similar to the parent E3/K3 coil ($K_{d(app)}$ = 9 μM[58]). The $K3_{g,d}1Rh_2$ metallopeptide was the lone outlier, exhibiting no evidence of coiled-coil assembly and no reactivity toward any of the substrate peptides. This unique case is consistent with previous studies noting that changes to the N-terminal amino acid—attached to rhodium in our case of $K3_{g,d}1Rh_2$—have a large impact on coiled-coil stability.[68] We examined the relative reaction efficiency of perfectly matched catalysts by determining product formation at short reaction times. Excepting the aforementioned $K3_{g,d}1Rh_2$ metallopeptide, all catalysts did catalyze tryptophan modification of properly designed substrates with significant rate acceleration relative to the control catalyst, $Rh_2(OAc)_4$ (Table 1, entries on diagonal with white background). We

Table 1. Relative Modification Efficiency for All Possible E3/K3 Assemblies[a]

entry		a	b	c	d	e	f	
		$E3_e1W$	$E3_e2W$	$E3_e3W$	$E3_g1W$	$E3_g2W$	$E3_g3W$	facial selectivity[c]
1	$K3_{g,d}1Rh_2$	no reaction[b]						
2	$K3_{g,d}2Rh_2$	0.02	**0.83**	0.03	0.01	**0.17**	0.05	4.9
3	$K3_{g,d}3Rh_2$	0.04	0.03	**0.33**	0.04	0.03	**0.16**	2.1
4	$K3_{a,e}1Rh_2$	**0.10**	0.04	0.04	**0.23**	0.05	0.01	2.3
5	$K3_{a,e}2Rh_2$	0.03	**0.12**	0.06	0.02	**0.72**	0.01	6.0
6	$K3_{a,e}3Rh_2$	0.02	0.04	**0.13**	0.01	0.01	**0.27**	2.1

[a]Relative modification efficiency for each substrate/catalyst pair determined by conversion at t = 4 h . Peptides, E3W (columns a−f) catalyzed by various metallopeptides, $K3Rh_2$ (rows 1−6) are presented. Reaction conditions: substrate peptide (25 μM), metallopeptide (0.02 equiv, 0.50 μM), pH 6.2. Background color: white, matched; green, facial mismatch; red, axial mismatch; blue, axial and facial mismatch. Modification ratio = (1mod + 2(2mod))/(total peptide) where 1mod and 2mod are the amount of singly- and doubly-modified peptide, respectively. [b]Metallopeptide $K3_{g,d}1Rh_2$ does not form coiled coils and provided no modification (Figure S1, Supporting Information). [c]Facial selectivity: ratio reactivity for matched substrate to the second most reactive substrate in a row.

observed the highest reactivity for the two matched cases (Table 1, entries 2b and 5e), in which the tryptophan and rhodium center were on the central heptad, where helix fraying—previously demonstrated by a variety of experimental methods—is minimized.[57,69−73]

Mismatched substrates were uniformly less reactive (Table 1, background colors indicate the type of mismatch examined). In every case, the second largest modification rates (Table 1, green entries) corresponded to catalyst/substrate pairs that are appropriately axially matched yet placed the reactive tryptophan group on the opposite face of the coiled coil from the rhodium catalyst. From the perspective of both catalysts and substrates (i.e., along rows or along columns), this axially matched, facially mismatched case proved to be the most reactive mismatched pair, with reactivity (green entries, 10−17% conversion) well above the baseline of other off-target modification (1−5%). We define a term of "facial selectivity" to describe the ratio of conversion of the matched case to that of the most reactive mismatched substrate in each row. The two metallopeptides with rhodium in the central heptad (rows 2 and 5) had higher facial selectivity than those with rhodium in either the first or the last heptad. The same trend is observed from the perspective of substrate (down a column). All substrate/

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catalyst pairs that are mismatched in the axial direction show poor catalytic reactivity (generally < 5% conversion).

With information from the comparative single-substrate reactivity measurements in hand, we moved to analyze competitive selectivity between pairs of two different substrates. We tested a variety of combinations. For each mixture of substrates chosen, matched catalysts for each of the two peptides were examined independently (Table 2, Figure 3). We

Table 2. Competitive Modification of Coiled Coils[a]

		"e" face			"g" face		
		$E3_e1W$[b]	$E3_e2W$	$E3_e3W$	$E3_g1W$	$E3_g2W$	$E3_g3W$
"e" face	$E3_e1W$[b]		--	--	--	--	--
	$E3_e2W$			>99:1 17:83		94:6 3:97	
	$E3_e3W$				60:40 50:50	98:2 <1:99	
"g" face	$E3_g1W$					94:6 <1:99	
	$E3_g2W$						>99:1 11:89
	$E3_g3W$						

axial differences
facial differences
both differences

[a]Catalytic selectivity of a selection of two-peptide mixtures with two different orthogonal catalysts. Each entry presents two selectivity ratios achieved with each of two orthogonal metallopeptides designed for one of the two peptide substrates. Taken together, the two ratios are a measure of selectivity and orthogonality for a given set of catalyst/substrate pairs. Top ratio is selectivity for the peptide along the row; bottom ratio is selectivity for the peptide down the column. Color indicates the difference in tryptophan positioning between the two peptide substrates: facial orientation, green; axial position, red; both facial orientation and axial position, blue. Conditions: substrate peptides, 25 μM each; metallopeptide, 4–8 mol %; 25 °C; 4 h. [b]The $E3_e1W$ catalyst does not form coiled coils or catalyze modification at an appreciable rate.

tested a range of peptide mixtures, including those having facial (green) or axial (red) differences in tryptophan positioning, as well as those with both facial and axial differences (blue). In almost all cases, kinetic selectivity was quite good—better even than that expected from conversion ratios in individual reactivity measurements (cf. $E3_e2W$–$E3_g2W$ Table 1, entries 2e and 5b and Table 2, green entry). In general, mismatched reactivity declines significantly in the presence of matched substrate. We did find one competition reaction (Table 2, $E3_e3W$–$E3_g1W$) that was far less selective than expected from individual kinetic experiments, which predicted selectivity in excess of 90:10.

Specificity in Catalysis of Coiled Coils: Discussion. The primary conclusion of our reactivity and competition studies is that designing metallopeptides based on established coiled-coil assembly principles allows creation of rhodium metallopeptide catalysts that exhibit a significant preference for modification of an intended target peptide (Table 1). In competition experiments (Table 2), five of six mixtures examined achieved acceptable levels of selectivity, most in excess of 9:1. Fundamentally, this result demonstrates that proximity-driven catalysis allows site-specific catalysis that overrides inherent functional-group reactivity. The ability to distinguish between substrates with a single change in amino-acid sequence represents the successful design of enzyme-like selectivity, as enzymes routinely distinguish substrates with small sequence

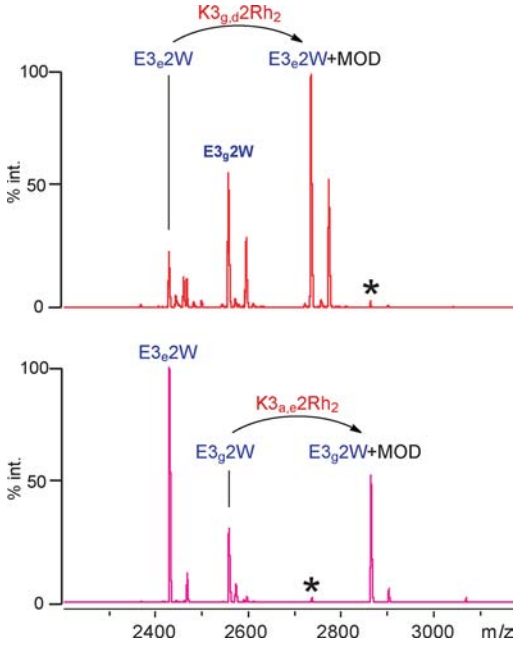

Figure 3. MALDI–MS spectra for the competition experiment between peptides $E3_e2W$ and $E3_g2W$ (Table 2, entry 1). Catalysts $K3_{g,d}2Rh_2$ (top) and $K3_{a,e}2Rh_2$ (bottom) were employed to modify either peptide in the mixture. Undesired cross reactivity is labeled with a black star. Conversion ratios of 94:6 and 97:3 were observed for the top and bottom reactions, respectively.

differences. Importantly, catalysis can be directed based on broad self-assembly principles of weakly interacting molecules without knowing the precise molecular details of the assembly or accounting for alternative assemblies that are minor components in solution. This property is essential for designing catalysts to modify natural proteins (see below).

Despite an extensive history of work directed at the design, analysis, and application of heterodimeric coiled coils such as the E3/K3 coils, many details of coiled-coil assembly remain challenging to assess. Alternative orientations and topologies can be accessible, typically as minor components, in the dynamic assemblies of short peptides employed here, which have K_d values in the range of 20 μM.[74] Subtle changes in sequence or experimental conditions can alter the equilibria of monomers, dimers, and higher order assemblies. In this series of experiments, we synthesized six different metallopeptide coils based on a very short (21-mer) parent coil with weak heterodimerization stability. While proximity-driven catalysis succeeds as expected when described in broad strokes, the details point to significant additional complexity. The fact that some reactivity (0.10–0.17 conversion, Table 1, green entries) was observed with facially mismatched substrates was initially unexpected. In a parallel coiled coil, it is impossible to position the rhodium center anywhere near the tryptophan substrate, which lies on the opposite face of a coiled coil (i.e., Figure 2b). Partial unfolding may explain aspects of slight but unexpected reactivity, such as facial mismatches in the terminal heptads (Table 1, entries 3f, 4a, and 6c) that can be explained by the reported existence of fraying in terminal heptad residues.[57,69–73] Antiparallel assembly is one possible explanation for mismatched reactivity in the central heptad (Table 1, entries 2e and 5b), but this explanation is inconsistent with the lack of reactivity observed in other cases where antiparallel assembly should also facilitate modification (Table 1, entries 3d, 4f, and

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6a). Thus, antiparallel assembly does not appear to be a significant contributor to even minor reactivity.

The reactivity of substrates with tryptophan mismatched in the axial direction (regardless of the facial orientation) was uniformly low (Table 1, red and blue entries). Bringing the tryptophan residue into proper position for catalysis in these cases is possible by offsetting the peptides with a free heptad dangling from both ends of the assembly. Offset assemblies with "sticky ends" are known and may even be the predominant species with proper sequence design,[75] but examples of successful "sticky ended" systems typically require peptides longer than 21 amino acids.[76] It is a significant success of this study that offset assemblies do not compromise catalytic selectivity.

The lone nonselective result in competition studies (Table 2, $E3_e3W-E3_g1W$) identifies the limits of using observable, ground-state conformations of binary mixtures as a proxy for transition states of catalytic reactions in complex mixtures. In this case, the presence of matched substrate led to an *increase* in mismatched reactivity. This breakdown in designed reaction specificity, which must include molecules of the "matched" substrate in the catalytic pathway leading to "mismatched" reactivity, is further evidence that the common understanding of a heterodimeric ground-state assembly is not necessarily sufficient to define the pathways of catalytic processes. Understanding reactivity driven by supramolecular assembly requires an understanding of dynamics and transient, low-population states that is difficult to examine by traditional methods.

Orthogonal Protein Modification in Lysate. Encouraged by the selectivity observed in competition experiment results for peptide substrates, we moved to examine the orthogonality of substrate/catalyst pairs for protein modification. We previously demonstrated selective modification of a recombinant maltose binding protein in cell lysate.[16] In addition to a maltose binding protein-$E3_g2W$ fusion (MBP-$E3_g2W$, 45 kDa) produced previously, we utilized an *E. coli* vector to express recombinant glutathione *S*-transferase (GST) with an orthogonal peptide sequence $E3_e2W$ at the C terminus (GST–$E3_e2W$, 28 kDa). We subjected a 1:1 mixture of lysates from expression of MBP-$E3_g2W$ and GST-$E3_e2W$ to the appropriate metallopeptide, and the results were analyzed by SDS-PAGE and biotin-specific western blot. As expected, a single band in each reaction demonstrated the high specificity and orthogonality made possible by appropriate catalyst design (Figure 4). In general, reaction selectivity might be expected to decrease in more demanding environments such as lysate (where, for example, significantly higher metallopeptide loading is required). However, in our studies, excellent selectivity is observed in the lysate modification.

Modification of the Fyn SH3 Domain. The SH3 domain from the protein Fyn is a significant and valuable test for extending the ideas of designed proximity-driven catalysis to natural targets. Fyn and other members of the tyrosine kinase family are important therapeutic targets for cancer treatment. SH3 domains bind relatively weakly to their target sequences—making them difficult to study with traditional techniques—and SH3 domains exhibit promiscuous and overlapping sequence specificities. From a structural perspective, SH3 domains are tertiary structures significantly larger (~7.5 kDa) than the coiled coils studied previously. SH3 domains recognize and bind to short proline-rich, PPII-helix sequences, most commonly containing a Pro-Xaa-Xaa-Pro motif. The peptide-

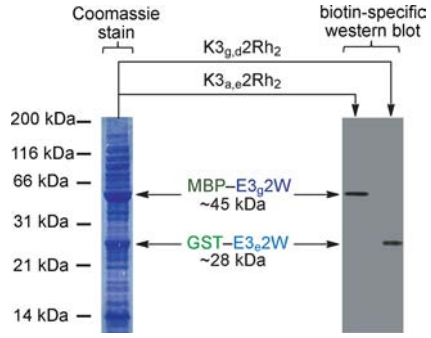

Figure 4. Orthogonal chemical biotinylation of two different proteins in cell lysate with designed metallopeptide catalysts. Recombinant fusions MBP-$E3_g2W$ and GST-$E3_e2W$ were expressed in *E. coli*. Reaction conditions: proteins, ~1.0 μM for each; metallopeptide, 2.0 equiv, 2.0 μM; biotin diazo **1b** (100 μM) in aq PBS buffer (0.10 M, pH = 7.2); total reaction volume, 20 μL; 4°C; 16 h. MBP = Maltose-binding protein, GST = glutathione *S*-transferase.

binding pocket of SH3 domains contains several conserved aromatic residues, which are expected to be reactive toward rhodium(II) catalysis (Figure 1a).

Starting from a known 12-mer peptide ligand, VSL12 (VSLARRPLPPLP, reported K_d = 0.60 μM),[77] we designed a series of metallopeptides (Figure 5a). We used structural information and previous binding studies to incorporate rhodium(II) into the peptide at positions near the binding interface that were deemed least likely to adversely affect binding. We made four variants of VSL12: $S2E^{Rh}$, $L3E^{Rh}$, $R5E^{Rh}$, and a C-terminal extension, $13D^{Rh}$. The $13D^{Rh}$ variant that had a rhodium(II) center positioned distal to the binding interface was synthesized as a negative control. We chose monocarboxylate peptides bound to rhodium at a single amino acid because the extended PPII helix conformation of the bound peptide is not compatible with bridging glutamates. Synthesis of the requisite metallopeptides proceeded smoothly by direct metalation of the peptide with $Rh_2(OAc)_3(tfa)$ under conditions developed previously for reaction of bridging bis-carboxylate metallopeptides with $Rh_2(OAc)_2(tfa)_2$. In our hands, the stability of monodentate metallopeptides, Rh(peptide)$(OAc)_3$, is not materially different from chelating, bis-carboxylate metallopeptides, Rh(peptide)$(OAc)_2$. Isothermal titration calorimetry (ITC) was used to assess the affinity of the metallopeptides. ITC measurements verified submicromolar binding (K_d = 0.65 μM) of VSL12 to the Fyn SH3, and three of the metallopeptides ($S2E^{Rh}$, $R5E^{Rh}$, and $13D^{Rh}$) bound Fyn SH3 with comparable affinities (K_d = 0.24–0.76 μM, see Supporting Information). The fourth metallopeptide, $L3E^{Rh}$, had a somewhat lower affinity (K_d = 5.14 μM) that did not affect catalytic activity under our working conditions (see below). The validity of ITC affinity measurements for metallopeptide binding was verified by fluorescence microscale thermophoresis (MST), which gave similar K_d values.

Gratifyingly, the three designed metallopeptides turned out to be efficient catalysts for modification of Fyn SH3 domain (Table 3, entries 1–3). Negligible modification was seen with a small-molecule catalyst, $Rh_2(OAc)_4$ (Table 3, entry 7), consistent with a proximity-driven mechanism. While the designed metallopeptides all exhibited efficient modification, the control metallopeptide, $13D^{Rh}$, with an improperly positioned rhodium(II) center, exhibited minimal modification similar to $Rh_2(OAc)_4$ (Table 3, entry 6). The absence of

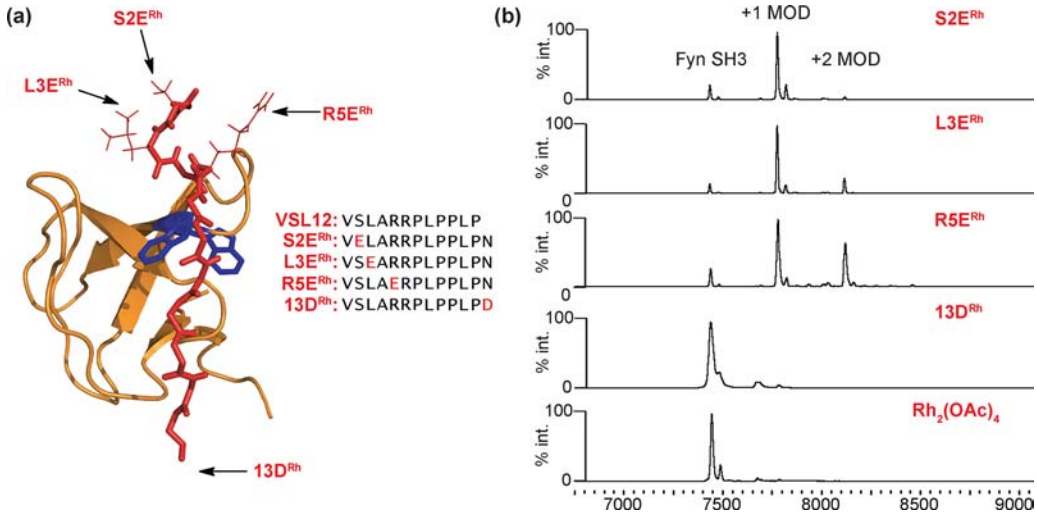

Figure 5. (a) Model of the interaction between VSL12 (red) and Fyn SH3 domain (orange). Figure adapted from PDB ID 1QWF and PDB ID 1A0N.[77,78] See Supporting Information for details. Arrows indicate the sites of amino-acid substitution for attachment of rhodium in the metallopeptides examined. Tryptophan residues in the binding pocket are shown in blue. (b) MALDI–MS spectra for the Fyn SH3 modification. See Table 3 for details.

Table 3. Fyn SH3 Domain Modification with Metallopeptides Based on the VSL12 Peptide[a]

entry	catalyst	notes	conversion, %[b]
1	S2E^{Rh}		93
2	L3E^{Rh}		96
3	R5E^{Rh}		85
4	R5E^{Rh}	+10 μM VSL12	38
5	R5E^{Rh}	+50 μM VSL12	1
6	13D^{Rh}		<1
7	$Rh_2(OAc)_4$		<1

[a]Conditions: Fyn, 10 μM; rhodium catalyst, 5 μM; diazo **1a** (250 μM) for 5 h at 25 °C in pH 7 buffer. [b]Conversion measured by MALDI–MS, calibrated against an internal standard. See Supporting Information for spectra and details.

proximity-driven reactivity with metallopeptide 13D^{Rh} is also consistent with the low cross-reactivity seen in axial-mismatch coiled coils and demonstrates the selectivity of modification possible with this approach. Mixtures of single and double modification at a single tryptophan residue have been observed previously,[3,5,14,15] and the R5E^{Rh} catalyst also produced mixtures of single and double modification (Figure 5b). Under the conditions examined, the S2E^{Rh} catalyst, on the other hand, was efficient in furnishing only single-modification products at high substrate conversion. Reactions with L3E^{Rh} produced intermediate levels of double modification. Thus, there are subtle differences in activity and selectivity among the designed metallopeptide catalysts. The presence of double-modification products with some metallopeptides implies that singly modified SH3 domains retain their secondary structure and peptide-binding capability, an important observation for future applications. Native ligand (VSL12) added to the reaction mixture results in dose-dependent inhibition of the modification reaction, consistent with competitive inhibition of protein binding (Table 3, entries 3–5). We also performed competitive modification reactions with a tryptophan-containing coil peptide, E3$_e$2W, chosen because it has an easily accessible tryptophan residue. We found that both Fyn SH3 and the control peptide were modified only sluggishly and in trace amounts in the presence of $Rh_2(OAc)_4$. Upon treatment with the R5E^{Rh} catalyst, >80% conversion of the Fyn SH3 was observed, with only trace modification of the control peptide, similar to the levels observed with the simple small-molecule catalyst, $Rh_2(OAc)_4$ (see Supporting Information for details).

To investigate the site of Fyn SH3 modification, we performed trypsin digestion and LC–MS/MS studies on Fyn SH3 that had been modified in reactions with the L3E^{Rh} catalyst using a higher substrate/catalyst ratio to avoid double modification (Figure 6a). We observed a digest fragment, Phe32–Arg46, that contained a modification with diazo **1a**. The fragment sequence includes the two tryptophan residues, Trp42 and Trp43. Because the two tryptophan residues are in neighboring positions, it is difficult to conclusively establish the site of modification. However, fragmentation of the Phe32–Arg46 ion led to the observation of several daughter ions (Figure 6b), including the y-4 ion without modification and the y-5 ion with modification, supporting a conclusion that modification occurs predominantly at Trp42. This finding is consistent with structural models of the Fyn SH3 domain, in which a β strand positions the neighboring Trp42 and Trp43 side chains in opposite directions, with the Trp42 indole extending toward the peptide-binding site and the Trp43 indole directed toward a hydrophobic core of the SH3 domain (Figure 6c).

■ CONCLUSION

These studies demonstrate two important capabilities: design of coiled coils with orthogonal catalytic reactivity for protein modification in lysate and extension of template-driven catalysis to modification of a natural SH3 domain. Site-specific modification is an important tool in protein science. Site specificity addresses issues of ensemble averaging and polyfunctionalization that accompany traditional residue-selective chemical modification.[7−13] Protein modification tools become more powerful when multiple different functionalities can be attached on a single protein. For example, FRET-based measurements of protein folding and dynamics are possible when two fluorophores are attached. In addition, measurements of surface-bound proteins are more readily

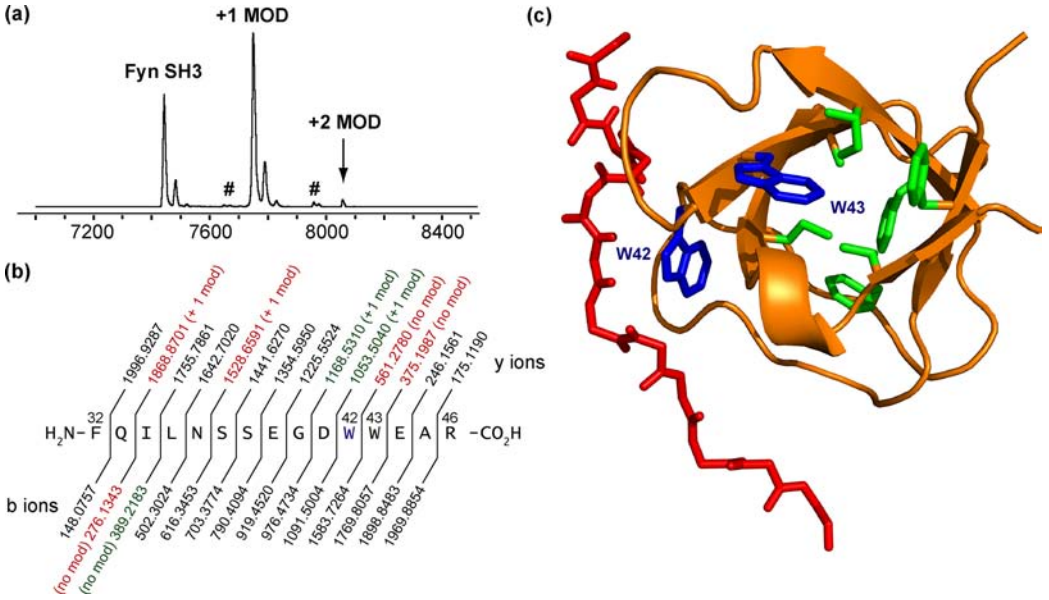

Figure 6. MS analysis of crude Fyn SH3 modification used for LC–MS/MS analysis of modification site. Conditions: Fyn, 20 μM; L3E^{Rh}, 5 μM; diazo **1a** (500 μM) for 5 h in pH 7 buffer. (b) LC–MS/MS analysis of Fyn SH3 modified by action of the L3E^{Rh} catalyst. Sequence of the Fyn SH3 trypsin digest product, Phe32–Arg46 ($[M + H]^+ = 2143.9754$), with expected y and b ions, assuming modification of Trp42. Expected daughter ions shown in colored text were matched to within 15 ppm in MS/MS fragmentation of the $z = 2$ ($m/z = 1072.4845$, green text) or $z = 3$ ($m/z = 715.3273$, red text) ion of the Phe32–Arg46 peptide. See Supporting Information for spectra. (c) Model of the interaction between VSL12 (red) and the Fyn SH3 domain (orange). Hydrophobic residues packed with Trp43 are shown in green.

accomplished if surface anchoring sites can be independently controlled and spatially segregated from FRET dyes or other functionality. This report presents new strategies for orthogonal, independent modification events under a single set of reaction conditions.[79,80] The method described requires only canonical amino acids, and so the substrate proteins are easy to produce in preparative quantities and easily studied in different host organisms. Finally, extending the ideas that have enabled development of enzyme-like catalysts to natural protein targets beyond simple coiled-coil dimers is a significant advance that clearly distinguishes this work from tag-based protein modification strategies. The three different designed metallopeptides all efficiently modify the Fyn SH3 domain, indicating that the metallopeptides have enough molecular flexibility that it is not necessary to conduct a burdensome search for an "ideal" orientation to apply these ideas to new protein systems. In principle, this approach is limited only by our ability to discover sequences that bind, even quite weakly, to a protein of interest. Fortunately, modern screening and computational methods have greatly accelerated the search for new protein ligands.

Understanding the reactivity, rather than just structure, of transient molecular assemblies is a fundamental challenge in chemistry and enzymology. This paper presents a study of reactivity and specificity in a designed metallopeptide catalyst that uses a coiled-coil assembly as a simple model for enzymatic substrate recognition. Our reactivity and specificity studies demonstrate our ability to design substrate recognition that leads to productive and selective catalysis.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details, characterization of peptide–peptide and peptide–protein assemblies, mass spectroscopy analysis of modification reactions, and characterization of peptides and metallopeptides. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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