

# Concepts of Catalysis in Site-Selective Protein Modifications

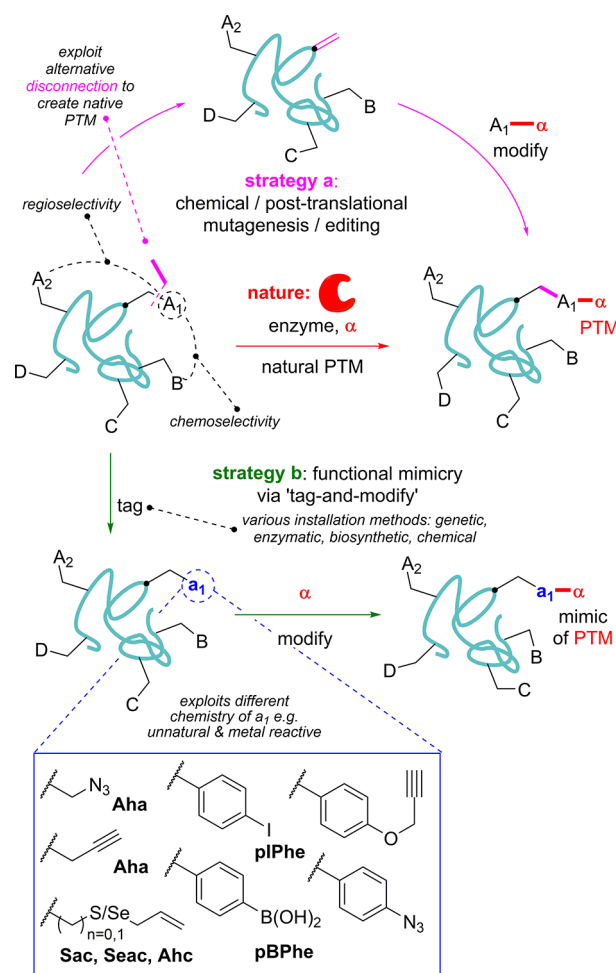
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**ABSTRACT:** The manipulation and modulation of biomolecules has the potential to herald new modes of Biology and Medicine through chemical “editing”. Key to the success of such processes will be the selectivities, reactivities and efficiencies that may be brought to bear in bond-formation and bond-cleavage in a benign manner. In this Perspective, we use select examples, primarily from our own research, to examine the current opportunities, limitations and the particular potential of metal-mediated processes as exemplars of possible alternative catalytic modes and manifolds to those already found in nature.

## INTRODUCTION

Principles of catalysis rightly dominate the logic of chemical and biological transformations. Beyond the immediate advantages of efficiencies in time and yield, ever-present in chemical thinking, there are also immediate strategic advantages that nature exploits extensively. Among these are critical features of control, feedback and diversification achieved by adding (and sometimes dynamically removing) available structural features to proteins after or during expression. Such elaboration of structure can powerfully modulate function. These are dominated in nature by post-translational modifications (PTMs, Figure 1) achieved largely through enzymatic side-chain alterations ranging from the small (methylation, acetylation, phosphorylation) to the complex (glycosylation, peptidylation). Cascades of catalysis allow molecular amplification of such on-protein marks or signals in biology to drive the most fundamental physiologically relevant processes: signaling (e.g., via activatory, cytoplasmic phosphorylation cascades at Ser/Thr/Tyr), folding and energy storage (e.g., via oscillatory glycosylations-deglycosylations at Tyr/Asn), protein degradation (e.g., via deactivatory, intracellular ubiquitylation-proteolysis at N-termini-/Lys/backbones) and even wound responses (e.g., via self-regulatory, intravascular proteolytic (thrombolytic/genic) cascades at backbone zymogenic sites). All transmit and amplify molecular information signals at efficiencies and turnover numbers (and often frequencies) that are currently inaccessible to even the best synthetic or chemical methods and do so in selectivity modes that cannot yet be considered. Yet the catalytic principles involved are immediately accessible and, if correctly applied both benignly and selectively, “new-to-nature” catalysis could interface with and exploit such natural catalytic modes in a striking manner: this highlights a powerful strategic opportunity for the control of biology and physiology via abiotic, catalytic post-translational chemistry. Many such catalyst modes may be considered; we see particular strategic



**Figure 1.** Protein modification strategies to make or mimic natural post-translational modifications (PTMs). Tag examples chosen here focus on use of metal-mediated catalysis (see also Figure 2).

merit in those that are metal-mediated and this review will focus primarily on these, while, at the same time, considering them only to be illustrative.

Key principles emerge. To study or modulate a specific biological event mediated by a modified protein then needed functionalization should likely occur precisely at a given position of interest with excellent site-selectivity. This site-selectivity arises, in essence, from both chemo- and regioselectivity. This may prove challenging not only given the density of potentially competing functional groups in proteins

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but also the high copy-number of certain reactive amino-acids (issues of chemo- and regio-selectivity, respectively). Some generalities exist, however, that can aid strategy. For example, many such competing groups are hard Lewis bases. Therefore, use of more rare (low copy number) soft Lewis bases, such as naturally found in cysteine (Cys), can provide a (partly) chemically distinct “handle” (e.g., with “soft” electrophiles). Alternatively, more distinct (“orthogonal”) unnatural amino acids, some with highly conditional reactivity, can be used. When these functional groups are site-selectively positioned, as a “tag” (in a first step), they can then be chemoselectively addressed (“modified” in a second step) in a two-step “tag-and-modify”<sup>1</sup> approach that results in site-selective installation of chosen alterations typically as non-native mimics (Figure 1). We find the origins of such an approach in methods first delineated by Wilchek et al.<sup>2</sup> and developed by seminal contributions of Koshland, Bender, Lowe, Jones and Hilvert among others.<sup>3–6</sup> Notably, the variety of bond-forming processes employed in such approaches still remains limited and few allow native or closely mimicking modifications;<sup>7</sup> most synthetic bioconjugations are still far from “traceless” or “native” (Figure 1).

Although not essential for “tag” positioning, ready integration with protein expression can exploit forms of “codon reassignment”. This can allow “genetic control” of the insertion of unnatural amino acids through the reassignment<sup>8,9</sup> of sense codons (e.g., via use of Met-auxotrophs to reassign the Met codon) or of nonsense codons (e.g., via use of compatible synthetase-tRNA pairs in the suppression of stop, typically amber, codons). In this way, simple alteration of codons in a gene sequence can code for “tag” position. If combined with mutation, in the case of sense codons, to “free-up” those codons (e.g., by converting all Met codons to those for near-isosteric Ile) then even greater positional freedom for the “tag” may be achieved. This is one way in which direct interfacing of “tags” with living systems can be considered [as “stumps” smuggled into biology for further chemical “grafting”] and, of course, those systems may be used to readily prepare proteins for *in vitro* use. This said, some thought must be given to the intolerance of “ribosomal filtering” for many structures, which despite continued advances, limits the diversity of “tags” and prevents the direct incorporation of many of the more complex PTM side-chains (e.g., glyco-amino acids). For example, most of the engineered systems used to suppress stop codons still typically generate closely resembling “tags” to the their parent systems (lysine- or tyrosine-like) in their chemical and structural properties.<sup>9</sup>

The choice of both the transformation used to “modify” and any associated catalyst should make reference to biology in choosing not only appropriate conditions but function: what rates or processes provide (likely complementary) value in interrogating or modulating biological activity? Biologically ambient conditions are apparent: at or below 37 °C, (part) aqueous solvent (with only trace organic) at moderate (buffered) pH 6–8, often dense and/or at high ionic strength. Furthermore, although not essential, ready correlation of structural change with functional outcome benefits from near complete conversions. This may be aided by the resulting product showing stability in aqueous media and biology (e.g., toward hydrolysis or catabolism). In this regard, metal-catalyzed (e.g., carbon–carbon or carbon–heteroatom) bond-forming reactions throw up strong candidates, in part

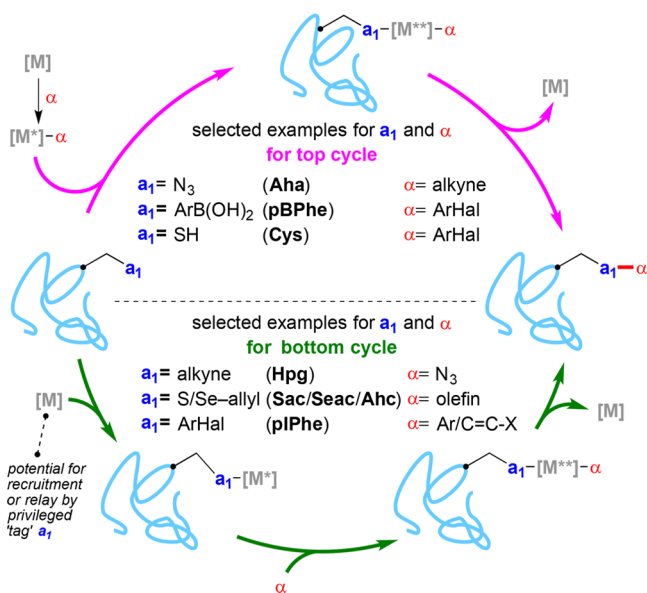
due to their complementarity to (and hence nonprocessability by) natural bond-breaking.

Some strong catalytic candidates that are highly efficient in their processing of small molecules appear to fit-the-bill, including some that even display good moisture-compatibility or even act in aqueous solutions. However, limitations arise not only in catalyst solubility but reagent/substrate solubility and the potential for protein-as-ligand engagement. The latter leads potentially to sequestration, poisoning, degradation (induced proteolysis) or simply confounded reaction analysis (e.g., suppressed MS ionization). It is of interest that this may not necessarily affect reactivity or selectivity in a negative manner.<sup>10</sup>

Perhaps foremost among these challenges are those of protein concentration. Indeed, compared to typical small molecule transformations (M–mM), concentrations of protein are  $\mu\text{M}$ –nM: higher levels drive protein (co)aggregation and even within most protein crystals these are estimated to be only mM.<sup>11</sup> A perhaps overgeneralized analysis of enzymatic catalysis highlights that nature solves these issues by using substrate recruitment to Michaelis complexes to drive effective molarities in a manner that contrasts with the likely nonsaturative modes of many small molecule catalysts. Evolutionary pressure is therefore often brought to bear upon  $K_M$  driven by available/intracellular concentrations.<sup>12,13</sup> By contrast, iterative design “pressures” upon small molecule catalysts focus on turnover (numbers and frequencies).

Effective rate may need to be key in biological contexts (e.g., to trap transient events or to minimize competing degradation) and, as a result, in synthetic protein alterations relatively high concentrations of catalysts are often used. In addition, unlike many other convergent modes of synthesis, one reaction partner, i.e. the protein substrate, is normally (but see radiolabeling below) strategically far more important. Therefore, the need to drive conversion of the protein to completion leads also regularly to excesses of modifying reagent as well as catalyst. While arguably inefficient, this proves feasible due to ready separation of product from small-molecule reagents and catalysts using a variety of what might be termed “size-exclusion” methods (often gel permeation- or dialysis-based); in this sense, the protein itself is acting also as a “purification hook” and some strategic similarity with solid-phase methods exists. It may also be argued therefore that a primary focus of the field, to date, has been to engender and exploit selectivity (via enhanced relative rate) rather than reactivity per se. Given this use at often superstoichiometric levels, it also raises fundamental questions about the true strategic role of the catalytically competent intermediates that are being exploited (catalysts or reagents?). This too then gives rise to necessary considerations of the required order of recruitment of substrates to catalyst centers (perhaps driven by excess or relay). That, in turn, then requires consideration of which (e.g., metalated) intermediates should then be protein-bound (or not, Figure 2) and hence a consideration of the “tag” type required in the protein, i.e. “tag orientation” (Figure 2).

In this Perspective, we aim to use select metal-mediated manifolds as examples that not only illustrate these limitations and considerations but highlight their functional potential through examples that focus on mimicry of biologically relevant PTMs (Figure 1), as well as some mimicry of the modes of natural catalysis (cycles and cascades of nested selectivity) that introduce and remove such PTMs.

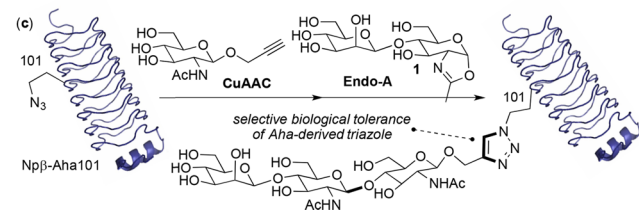
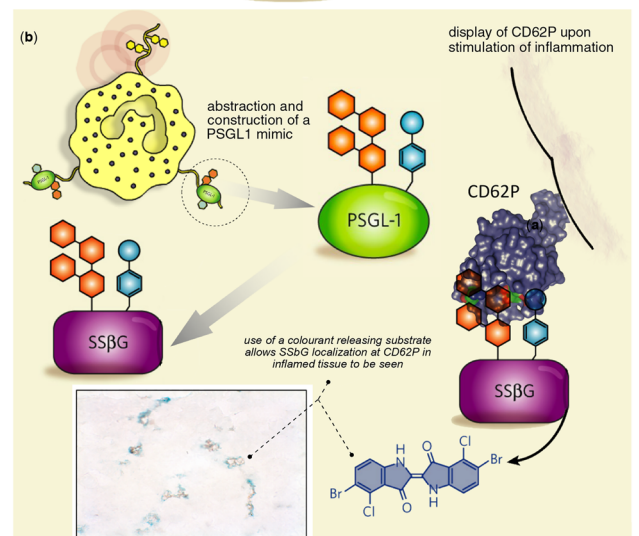
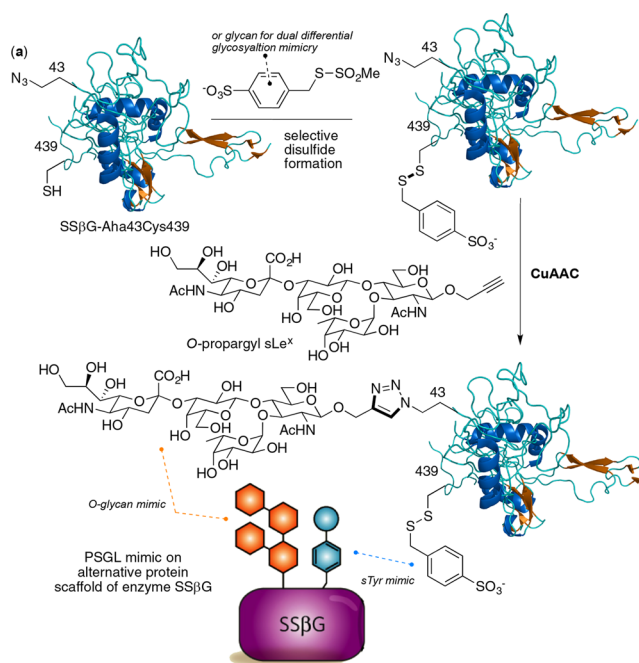


**Figure 2.** Protein modifications using metal-mediated catalysis. Tag orientation determines cycle and hence reactivity and selectivity.

## ■ COPPER-MEDIATED AZIDE-ALKYNE ADDITION

The early promise of generating functional effectiveness as well as the clear appetite for such conjugation methods is perhaps no better illustrated than by the now near-classical, transition-metal-catalyzed variants of Huisgen–Dimroth–Michael 1,3-dipolar-cycloadditions between azides and alkynes (CuAAC). These form triazoles with increased (1,3) regioselectivity<sup>17,18</sup> as well as sufficient rate acceleration to suggest compatibility under biologically benign conditions (water with a range of pH and oxygen). When coupled with ready “tags” such as Met-analogues homopropargylglycine (Hpg)<sup>19</sup> azidohomoalanine (Aha)<sup>20</sup> or via amber codon suppression with Tyr analogues such as *p*-propargyloxyphenylalanine<sup>21</sup> these allowed early lessons in on-protein efficiency and biological function to be learnt. When compared at identical protein sites azido-“tags” proved almost an order of magnitude more efficient (under pseudo-first order conditions) than similar alkynyl (e.g., Aha *cf* Hpg).<sup>14</sup> These observations are consistent with recent mechanistic observations<sup>22</sup> implicating alkyne as a key protodemetalating species in both mono- and bis-copper(I) cycles and with the greater effectiveness of generating an excess of cuprated-alkyne to react with low concentration protein azides: this highlights the need for strategic considerations, i.e. an azido “tag” > alkynyl “tag” orientation (Figure 2). Initial suggestions of nonspecific toxicity from observations in cell-surface labeling studies<sup>23</sup> have more recently been attributed to forms of oxidative damage at Cys, Met and even His via oxygen-derived ROS.<sup>24</sup> These highlight the utility of high-purity Cu(I) reagent, ligands that stabilize Cu(I)/(II) and outcompete nonspecific protein sites as well as the use of sacrificial ligands as oxidative “buffers”.

The compatibility of this optimized chemistry in complex protein assembly and the utility of 1,3-triazole as a tolerated link motif for functional mimicry was illustrated through sequential protein modifications combining triazole chemistry with kinetically controlled disulfide formation (Figure 3a).<sup>14</sup> These allowed site-selective multisite attachment as well as differential modification attachment with essentially complete conversion to test mimicry of dual PTM patterns, including



**Figure 3.** CuAAC combined with other modifications allows either (a) dual display of different PTM mimics<sup>14,15</sup> or (c) nested selectivity in sequential catalysis.<sup>16</sup> (b) Such mimics function even *in vivo* allowing a PSGL1-mimic to visualize CD62P as a marker of inflammation. [Adapted from reference 14 with permission from SpringerNature.]

dual differential glycosylation<sup>15</sup> or sulfo-Tyr/glycosylation patterns.<sup>14</sup> In this way, creation of a mimic of the white-blood cell-surface protein P-selectin-glycoprotein-ligand-1 (PSGL-1) was accomplished bearing sTyr-like and sialyl-Lewis-x-like PTM moieties; these engendered comparable binding properties to the mimic as the wild-type to cognate receptor CD62P and so created a protein that could function *in vivo* to visualize inflammation (Figure 3b).<sup>14</sup> This chemistry

also proves sufficiently robust to create virus-decoy particles at extremes of valency, creating protein constructs bearing even up to 1,620 glycans.<sup>25</sup> These latter glycodendriprotein particles displayed striking, sugar-mediated binding function, thereby preventing mammalian cell infection by Ebola pseudotyped virus through competitive blockade of the DC-SIGN receptor with IC<sub>50</sub>s in the nanomolar to picomolar range. Rates of reactions for such on-protein CuAAC are also high enough to allow ready use of common radioreagent [<sup>18</sup>F]-FDG as a prosthetic radiolabel in proteins, despite its short half-life.<sup>26</sup>

Apparent considerations of linker-dependent compatibility with biological function were highlighted in later model systems in a demonstrated but select tolerance with subsequent enzymatic processing (here glycosylation, Figure 3c). This allowed overall chemo-enzymatic modification in a manner that, in part, mimics the sequential glycosylation processes of post-translational O-glycosylation in the creation of synthetic glycoproteins. Thus, combination of a mutually compatible synthetic Cu(I)-driven GlcNAc attachment cycle with the use of an unnatural oxazoline substrate **1** in a transglycosylation cycle processed by the hydrolytic endoglycosidase Endo-A allowed overall attachment of a Man-GlcNAc-GlcNAc trisaccharide site-selectively into proteins (Figure 3c). Importantly, the Aha-derived N-triazole-linked GlcNAc-protein proved to be far the most efficient substrate for Endo-A, allowing even selection of one product triazole variant over another from mixtures. Albeit in a short sequence, this biomimetically highlighted the role of a precursor (here Cu(I)-driven) cycle in determining a pathway guided (or filtered) by a subsequent (here Endo-A-driven) cycle. Such catalytic sequences may also be inverted (e.g., Endo-S then CuAAC).<sup>27</sup> Such layers of selectivity derived from sequential catalytic cycles we postulate will prove critically powerful in application to more complex environments (see also below PdCC on GYG for another selective metal-then-enzyme catalysis sequence).

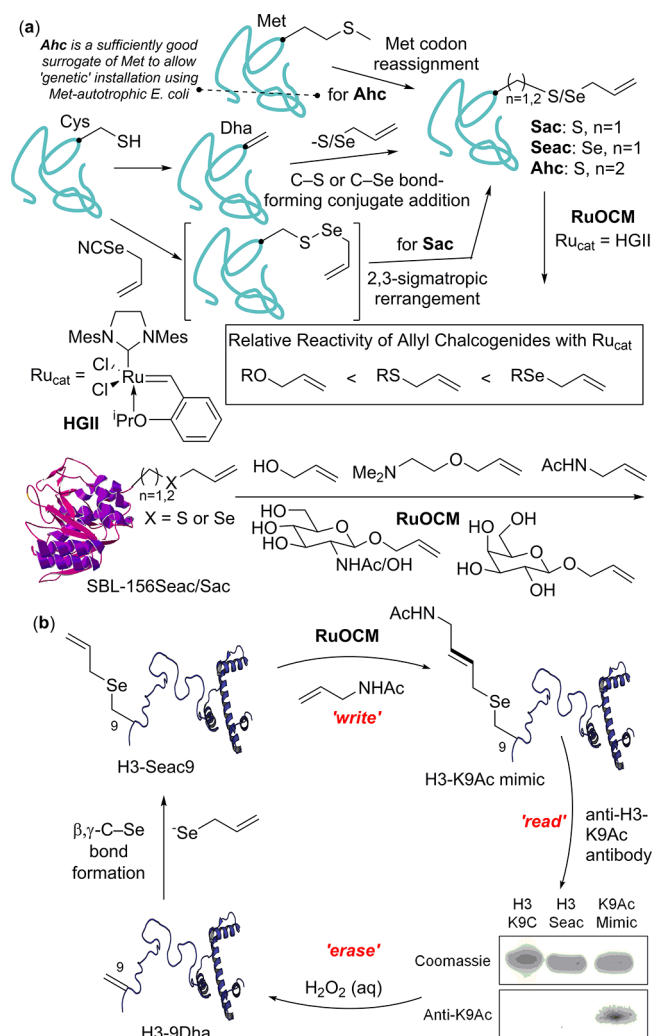
## ■ RU-MEDIATED OLEFIN CROSS-METATHESIS

Ready C–heteroatom bond-formation to generate heterocycles may prove attractive in proteins, particularly in high-stability motifs (e.g., triazoles via C–N) that display apparent functional compatibility, as those above (Figure 3). However, there will undoubtedly be need for more subtle attachment methods that lack such relatively bulky heterocyclic “scars”. This dictates more discreet motifs, of which high stability C=C bonds could prove useful and versatile candidates. Ru-mediated olefin cross-metathesis (OCM) therefore provided an excellent possibility in its known air and moisture stability, strong chemoselectivity for a nonproteinogenic functional group and useful tolerance for other functional groups. Indeed, such use of olefins as reactive tags in proteins has been suggested for more than two decades.<sup>28</sup>

Successful application of OCM in proteins, hinged on what were initially counterintuitive observations. In our hands<sup>29</sup> and those of others, simple olefins provided, e.g. by tags such as homoallylglycine, fail. This failure and the subsequent solution illustrates additional useful principles in catalysis related to tag orientation (Figure 2). While semitolerant of moisture, necessary Ru alkylidenes eventually decompose in protic solvents. Reaction with protein at a low concentration therefore, in effect, becomes a reaction controlled by relative decomposition rates vs those of protein substrate recruitment. Reactive relaying methods have been powerful tools in

enabling OCM but the potential of ligand relaying by some types of motifs had perhaps been overlooked. Among these, chalcogenic (e.g., S(II) thioether) motifs had been, in fact, identified as poisoning to certain Ru-mediated CM processes.<sup>30</sup> However, screening of olefinic thioethers in amino acids side-chains<sup>29</sup> revealed that chalcogenic Ru-coordination ability could prove wholly beneficial in aqueous OCM, if positioned correctly: allylic chalcogens proved highly effective.<sup>30</sup> As a result allylchalcogen protein “tags” for protein OCM have emerged that can be installed chemically, such as S-(Sac) and Se-(Seac) allylcysteine variants, using a variety of methods that include either nucleophilic conjugate addition C<sub>β</sub>–S<sup>29</sup> or C<sub>β</sub>–Se<sup>31</sup> bond formation from unnatural amino acid precursor dehydroalanine (Dha) or via 2,3-sigmatropic C<sub>β</sub>–S bond formation from Cys-derived selenenylsulfide precursors (Figure 4a).<sup>32</sup> More recently, we have shown that the homologated allylsulfide “tag” residue S-allylhomocysteine (Ahc) can also be installed genetically as a Met surrogate (Figure 4a).<sup>33</sup>

The recruiting ability of the chalcogen heteroatoms in these side-chains toward readily available Hoveyda–Grubbs second generation catalyst (HGII) proved to be a critical determinant



**Figure 4.** (a) RuOCM is enabled by various modes of chemical (for Sac, Seac)<sup>29,31,32</sup> or genetic<sup>33</sup> (for Ahc) allylchalcogen tag installation as privileged motifs. (b) RuOCM may be embedded in mimics of write–read–erase cycles.<sup>31</sup>

in choice and exploration of appropriate catalytic cycle and “tag” orientation (Figure 2); this enables efficient OCMs in a variety of proteins<sup>29,31,33,34</sup> at rates dictated by on-protein rate constants of the order of  $10^{-1} \text{ M}^{-1} \text{ s}^{-1}$  (and therefore comparable in rate with faster CuAAC reactions). Effective Ru-mediated ring-closing metathesis in proteins with allylic oxygen as the heteroatom in olefinic tags has also been observed.<sup>35</sup> It should be noted that not all proteins are compatible with *t*-BuOH cosolvent that is typically required for sufficient HGII dissolution. Nonetheless, RuOCM has allowed attachment of a variety of different olefin-derived moieties including PEGylation, fluorophores and, perhaps more relevantly, allylamines that provided access to modified Lys-mimetics (see below, Figure 4b). Computational analyses<sup>31</sup> suggest a reaction profile in which the Ru-carbene intermediate after “Se-relay” is enhanced in stability over that from “S-relay” and is also accessed through a lowered transition state. The enhancing effect of linker-extended side-chains to allow greater accessibility<sup>34</sup> also appears consistent with the key role of such chalcogen-mediated recruitment.

Notably, addition of  $\text{MgCl}_2$  as a source of competitive hard Lewis acid has proven crucial to prevent nonproductive chelation of Ru by nonspecific amino acid residues in proteins.<sup>29</sup> This too is illustrative of broader principles: absolute avoidance of likely protein–metal coordination is not at all necessary; productive reactivity may still be gained simply through effective competition to generate a sufficiently available catalyst pool. Again, we suggest that such dynamic, competitive principles are reflective of those exploited in complex biological media.

Such protein–RuOCM has various potential benefits of reversibility (as yet unrealized in proteins) of  $\text{C}=\text{C}$  bond formation and of putative biomimicry. In one such demonstration of the latter, installation of Seac (from Dha) into histone H3 at the relevant K9 site enabled the use of allylacetamide as an olefinic reagent (that is typically sluggish in CM) to install (“write” in the language of epigenetics) a mimic of the side-chain K9Ac (Figure 4b). The resulting protein mimic of histone H3-K9Ac was successfully “read” by anti-LysAc antibodies. It could then be removed (or “erased”) through oxidative Cope-type selenoxide elimination to reaccess the Dha precursor (and so go “full-circle”). As for Cu-mediated methods, this biomimetic *in vitro* ‘write–read–erase’ cycle, in part mediated by abiotic Ru-catalysis, again suggests that relevant exploration of biology will prove possible through insertion of chemical catalysis into sequences that mimic nature.

## ■ PD-MEDIATED CROSS-COUPLING

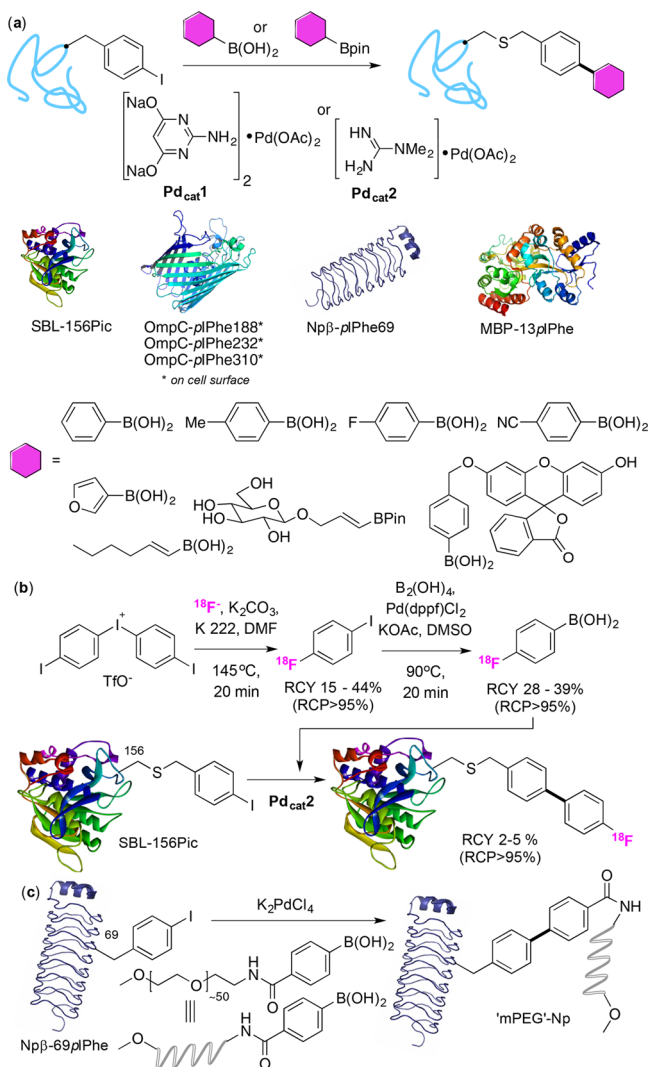
This use of an abiotic metal as an effective and selective inducer of function raises the potential of other synthetically powerful metals as similar triggers in Biology. None has been more powerful in small molecule chemistry than Pd.<sup>36</sup> Pioneering early use of Pd in proteins<sup>37</sup> (see also below and reviews<sup>38–40</sup>) had revealed low conversions and limitations in proteins caused, in part, variously by the nature of the Pd source, protein loss, need for cosolvents and attempted use of phosphine-based ligands that are typical in small molecule cross-coupling (CC). As for CuAAC, considerations of tag-to-reactant orientation are mechanistically pertinent (Figure 2). Installation of the reductively susceptible partner, e.g. arylhalide, as the “tag” in, e.g. *p*-iodophenylalanine (*p*IPhe), carries advantages of not only good incorporation efficiency

(*p*IPhe is one of the best incorporated amber suppressors<sup>41</sup>) but in principle allows putative flexibility toward different CC manifolds (see also below). It also, however, necessitates productive interception of palladated-protein intermediates over competitive side processes such as proto-depalladation, a potential disadvantage when considering tag orientation (Figure 2). Conversely, incorporation of *p*-boronophenylalanine (*p*BPhe) as “tag”, while bringing with it the potential to use, e.g.  $\text{Ar}[\text{Pd}]\text{-Hal}$ , intermediates in excess in Suzuki–Miyaura CC to side-step such side-reaction, may then become limited by other steps, e.g. poor transmetalation by (perhaps internally chelated) *p*BPhe. Indeed, for Suzuki–Miyaura CC, the latter appears to be limiting giving only moderate observed conversions at high reaction temperatures ( $70^\circ\text{C}$ ) likely incompatible with retaining function in most proteins.<sup>42</sup>

These prior limits for both “orientations” of tags suggested to us the necessity for catalyst variation with an emphasis on protein utility. Thus, use of disulfide-compatible phosphine-free ligands, in the creation of fully water-soluble palladium-pyrimidine (pre)catalysts enabled on-protein Suzuki–Miyaura CC using chemically installed arylhalides as “tags” with essentially full conversion for a wide variety of boronic acid partners under mild reaction conditions (Figure 5a).<sup>43</sup>

Fuller strategic generality was then realized by also creating “tags” through amber suppression with *p*IPhe.<sup>44</sup> This allowed not only coupling via “genetic installation” *in vitro* but also application to increasingly complex protein structures and contexts, including those in cellular and living contexts. Effective CC on living (*E. coli*) cell surfaces enabled not only the labeling of cellular subpopulations but also highlighted sufficient nontoxicity as well as the ability to “switch” the functional states of cell-surface proteins, such as the ion channel OmpC.<sup>45</sup> This illustrated the utility of the strategy of “tag-and-modify” in which Pd acts as an abiotic, nontoxic trigger of that switching in living systems. Extension of the method<sup>46</sup> also allowed the content of sugars displayed on living cell-surfaces to be so heavily “overwritten” that the global interaction of cells with corresponding sugar-binding lectin proteins could be determined simply by Pd-mediated chemistry; this allowed a form of glycolyx engineering on living cells.

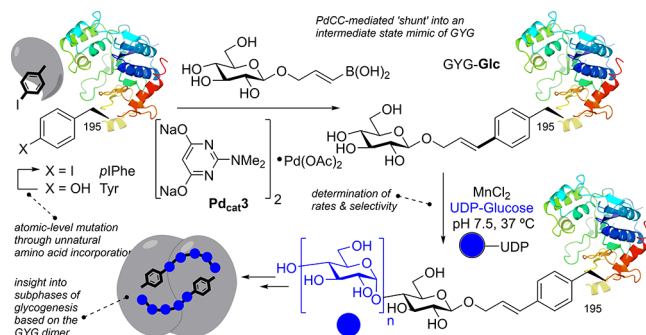
As well as the triggering and modulation of living biology, the Suzuki–Miyaura CC has also proven to be a useful testing ground for biotechnologically relevant challenges. As noted above, strategic focus on the “value” of protein product allows excess of reagent/catalyst in many *in vitro* settings (and this may override or complement considerations based on catalytic cycle design, Figure 2). One key exception is found in radiolabeling, where low concentrations of reagent and short half-lives enforce further constraints. Suzuki–Miyaura CC-mediated  $^{18}\text{F}$ -radiolabeling of proteins using the prosthetic reagent [ $^{18}\text{F}$ ]4-fluorophenylboronic acid necessitated the development of new catalytic systems. Screens of alternative pyrimidine- and guanidine-based ligands at different substrate concentrations identified dimethylguanidine as suitable for lower boronic acid concentrations, allowing generation of  $^{18}\text{F}$ -labeled protein in 5% RCY, despite the short ( $t_{1/2} \sim 110$  min) half-life of  $^{18}\text{F}$  (Figure 5b).<sup>47</sup> PEG-ylation is a widely adopted method for the modulation of protein pharmacokinetics. Notably, exploration of Suzuki–Miyaura conditions not only allowed precise PEG positioning in proteins but also revealed unexpected interactions with glycol moieties. These in turn led to the development of a self-liganding system using a methyl-



**Figure 5.** (a) Benign PdCC allows attachment of various groups using pyrimidine- or guanidine-based ligands including (b) use at low concentrations of  $^{18}\text{F}$ -prosthetic reagent and (c) self-liganding PEGylation reagents.

“PEG2K”-derived boronic acid and Pd without need for external ligand (Figure 5c),<sup>48</sup> a result that was subsequently exploited for intracellular Pd-delivery.<sup>49</sup>

The activity and compatibility of these systems has more recently allowed applications to address increasingly more complex questions in biology. The formation of glycogen, which is a primary energy storage molecule of many organisms, provides an intriguing challenge (Figure 6). Glycogen particles are initiated from a seed-core protein glycogenin GYG that autoglucosylates one of its own residues, an anchor Tyr at site 195, to start the polysaccharide. This self-modification means that, intriguingly, GYG is not strictly a catalyst and its glucosyltransferase enzymatic state (reactivity and selectivity) is potentially altered at each individual, unisolable step. Probing this unusual mode of self-modulation has therefore proven strikingly difficult to address through traditional biochemical means. Instead, through the construction of an “OH  $\rightarrow$  I” (Tyr  $\rightarrow$  pIIPhe195) mutation (via amber suppression), an “off” state of GYG could be generated that allowed Pd-mediated shunting into active mimics that were representative of these intermediate states (Figure 6).<sup>50</sup> This



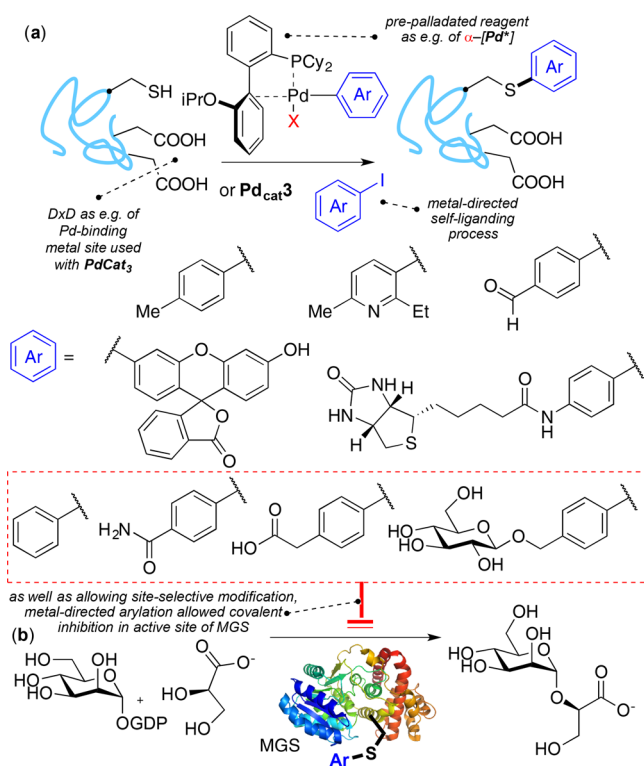
**Figure 6.** Use of PdCC-mediated “shunting” allows access to mimics of intermediate states found in the initiation of glycogen.<sup>50</sup>

Pd-mediated switching “on” of GYG was accomplished despite GYG’s own dependence on transition metal Mn, toward which Pd at extreme concentrations is inhibitory. In this way chemical (here PdCC) cycles were again (see above) inserted sequentially with subsequent biocatalytic (here GYG-autoglucosylation) steps (a triggered cascade), albeit nested inside that catalyst. It revealed three distinct subphases in “glycogenesis”, prime–extend–refine, the second of which appears to be another example of a retentive  $\text{S}_{\text{N}}1$ -like glycosylation, displaying striking rates and even plasticity toward the sugars that it utilizes. This highlights that novel synthetic chemical approaches (e.g., bond-making in biology) may have the power to probe the mechanisms of biology more deeply than previously possible.

These demonstrated advantages of such activation modes seemingly compatible with even such “delicate” systems coupled also with a wide variety of commercially available, potential coupling partners (boronic acids, silanes, halogenated aromatic systems, alkynes etc.) suggests a likely proliferation of modes of abiotic-metal-mediated reactivity adapted for Biology aided by the use of appropriate strategies and/or catalyst systems discussed above. Indeed, in the past decade Heck<sup>37,51</sup> and Tsuij–Trost<sup>52</sup> reactions; and Sonogashira CCs, (using pyrimidine- or PEG-based ligands) have emerged. In addition Pd-mediated manipulations of peptides have elegantly facilitated the ligation assembly of peptides and proteins.<sup>56,57</sup>

While “tag” selection (and orientation, Figure 2) is a key strategic tool in the recruitment and exploitation of such catalytic systems in such biomolecule methods, its sometime dependence on less-familiar methods (e.g., unnatural amino acid incorporation) has seen a continued interest in application to native residues. Francis’s early prescient work had highlighted the potential of heteroatomic nucleophiles in intercepting palladated intermediates,<sup>52</sup> by exploiting Tyr-OH. This concept has more recently also been extended to the exploitation of Cys-SH as a usefully selective native heteroatom. Thus, intermolecular<sup>58</sup> and pseudointramolecular<sup>59</sup> S-arylation have been elegantly demonstrated using preformed aryl palladium ligand oxidative addition complexes (Figure 7a).

This exploitation of native residues coupled with the known utilization by some proteins of transition metals raises an exciting possibility as to whether such CC could be directed through a self-protein-liganded manifold by exploiting native metal-binding sites. This “guiding” of a suitably reactive metal-complex was recently exploited in regioselective protein functionalization (Figure 7a,b). In metal-dependent mannosyl-glycerate synthase (MGS) a variety of arylation events were



**Figure 7.** (a) S-Arylation of native Cys is enabled either by use of pre-palladated oxidative-addition complexes<sup>58,59</sup> or metal-binding-site directed methods.<sup>10</sup> (b) Active site directed S-arylation allows effective covalent inhibition of the enzyme.<sup>10</sup>

guided to a Cys adjacent to the enzyme's DxD metal-binding motif allowing not only effective site-selective S-arylation modification with added aryl iodide reagents but also reagent-dependent covalent inhibition of the enzyme (Figure 7b).<sup>10</sup> Given the recent resurgence of covalent inhibition in enzymes that bear conserved active-site Cys, this raises the clear potential of this method even in eventual "catalytic therapies".

## SUMMARY, LESSONS AND OUTLOOK

The interfacing of catalysis with bioconjugations brings with it unique considerations and opportunities. Common to both chemo- and bio-catalysis are issues of recruitment of protein to catalyst center that may be limited by or that may, indeed, take advantage of (e.g., through relaying in a tag-determined order to recruit as a form of 'Michaelis-complex mimicking') different cycle steps. Both can be aided by not only mechanistic analysis but also by protein-specific considerations, such as tag-orientation (Figure 2) or use of preformed/excess of catalyst+reagent. In such cases the corresponding alkylidene (RuOCM) or oxidative addition complex (PdCC) may be being used at levels that make them more like highly selective reagents rather than catalysts. Nonetheless, they are also saturated catalyst states used effectively, akin to how biology accomplishes transformations. They also perhaps point the way toward future engineering of saturation (again akin to biological optimization of  $K_M$ ) in chemical systems to improve their effectiveness while maintaining turnover. Use of preformed saturated catalyst states<sup>58</sup> might also rescue (by better sequestering) biologically troubling (e.g., phosphine) ligands in such systems.

For example, our discovery of the effectiveness of chalcogen-relayed RuOCM in proteins we find to also be illustrative of a

different strategic thinking that may prove useful in catalytic protein chemistry inasmuch as we were not the first to attempt on-protein OCM. Yet our somewhat antithetic use of privileged tags (Sac, Seac, Ahc), which may be viewed as an atypical, perhaps limiting, mode of "substrate engineering" in many methodological studies, was instead highly effective in proteins in its enabling of reactivity, selectivity and function by driving effective protein recruitment.

Other considerations of solubility and nonspecific metal-protein interaction<sup>60</sup> may require competition (e.g., from other metals as by Mg(II) in RuOCM<sup>29</sup>) or scavengers (e.g., as for 3-mercaptopropionic acid in PdCC<sup>44</sup>) and altered substrates (e.g., lipid-boronic acid rather than boronate ester, where the acid was a needed "solubility handle" that was discarded during PdCC<sup>43</sup>). In this context it should be noted that at high concentrations some exogenous thiol-based scavengers may prove toxic or act as reductants, potentially disrupting, e.g. intramolecular disulfides in proteins. Nonetheless, concentrations for scavenging are typically well tolerated. Moreover, residual or competitive metals do not necessarily lead to loss of structure and/or function<sup>50,58,61</sup> (e.g., as high as 6% Pd<sup>58</sup> in some functional protein samples).

One may view all of these as biomimetic strategies, required in the "systems chemistry" that is Biology. While we have focused this review largely on metal-mediated process, we have also noted examples of its combined use with biocatalysis, often also being applied in unnatural modes. The principles of ordering, orientation, selectivity and mimicry set out here in many parts apply also to solely biocatalytic strategies.<sup>62</sup> Indeed, one might consider the exploitation of Ubq-mediated proteolysis in so-called Protacs<sup>63</sup> or the repurposing of proteases to target their degradation to chosen proteins<sup>64</sup> as examples of man-made, "new-to-nature" modes of hydrolytic protein modification.

Increasing translation into more complex living systems will hinge on proper exploitation of the selectivity of such manifolds and it is perhaps this, rather than the inherent efficiency of catalysis, that is a primary benefit. The corresponding interfacing or separation of these manifolds with/from those that exist in nature becomes an intriguing challenge in chemical control. The future, therefore, will lie in other catalytic modes via perhaps other (even endogenous) metals and tags, as well as nonmetal small molecule catalysts<sup>65</sup> or those that drive electron transfer/redox (metal or nonmetal).<sup>66,67</sup> If these can be tamed for benign use *in vivo* and combined with multiple strategies of selectivity (even beyond chemo-/regio-/stereo-) then such catalytic protein (and other biomolecule) editing could prove widely powerful in biology and physiology.

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### Notes

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