Tuning SpyTag–SpyCatcher mutant pairs toward orthogonal reactivity encryption†

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Genetically encoded covalent peptide tagging technology, such as the SpyTag–SpyCatcher reaction, has emerged as a unique way to do chemistry with proteins. Herein, we report the reactivity engineering of SpyTag–SpyCatcher mutant pairs and show that distinct reactivity can be encrypted for the same reaction based on protein sequences of high similarity. Valuable features, including high selectivity, inverse temperature dependence and (nearly) orthogonal reactivity, could be achieved based on as few as three mutations. This demonstrates the robustness of the SpyTag–SpyCatcher reaction and the plasticity of its sequence specificity, pointing to a family of engineered protein chemistry tools.

Introduction

In recent years, covalent peptide tagging technology has attracted numerous research interests as a “molecular super-glue” possessing “infinite” affinity to protein partners.1–5 Based on isopeptide-forming pilin subunits,6 a few reactive pairs have been developed, including isopeptag-N and pilin-N,7 SpyTag and SpyCatcher,8 SnoopTag and SnoopCatcher9 and SdyTag and SdyCatcher.10 Such reactions are considered genetically encodable, allowing one to program the post-translational modification of proteins in cells and expand the protein backbone topology beyond the linear configuration.11–13 Not only have cyclic, tadpole-like and other branched proteins been prepared,11 but direct cellular syntheses of protein catenanes and star proteins have also been demonstrated.13,14 Covalent tagging is thus an “iron grip” for synthetic biology, offering tremendous opportunities in applications such as preparing all-protein-based hydrogels,14 making “living” materials,15 engineering synthetic vaccines,16 controlling protein cellular locations7 and membrane protein activity18 and enhancing sortase efficiency.16 The extraordinary reactivity of SpyTag–SpyCatcher chemistry is reminiscent of “click” chemistry in materials science.19 Nevertheless, unlike functional groups in synthetic molecules, whose reactivity is defined mostly by their intrinsic properties, the reactivity of such peptide–protein pairs is instead defined by sequences and the resulting folded structures. Such a prerequisite precludes its application in nonaqueous environments or other denaturing conditions, but it also promises a family of peptide–protein reactive pairs with diverse features, distinct specificity and stimuli-responsive reactivity based on the same structural scaffold.20 In other words, even for the same reaction, the reactivity might be encoded orthogonally. In this contribution, we report the rational design and directed evolution of SpyTag-SpyCatcher reactive pairs toward orthogonal reactivity with sequences of high similarity (Fig. 1). We show that valuable features, including high selectivity, reversed temperature dependence and (nearly) orthogonal reactivity, could be achieved on the SpyTag–SpyCatcher scaffold with as few as three mutations.

Results and discussion

Evolving the reactive pairs

Directed evolution is a powerful technique in protein engineering to improve protein stability and to adapt proteins to new substrates, as well as to create new protein functions.21–24 The key lies in the generation of genetic diversity and the identification of protein variants with the desired properties.25 To date, no methods have been developed to direct the evolution of chemically reactive peptide–protein pairs. This is non-trivial, as this is a two component system and it is not easy to distinguish chemical bonding from strong physical association under the normal conditions used for screening. It was reported that the SpyTag–SpyCatcher reaction allowed the in vivo cyclization of an elastin-like protein (ELP) (AB).21 We envisioned that
few mutations as possible. If we make an analogy between the SpyCatcher (BVA) partially restores the reactivity with AW
screening results). Finally, we found that a F77V, F94A mutant found to yield almost exclusively the linear product, indicating
isoleucine (Ile3) to abolish its reactivity. The I3W mutant was round, we introduced mutations onto SpyTag at the position of
directed evolution (see Fig. S1 and Table S1†). Using this strategy, we aimed to achieve orthogonal
reactivity. The mutant’s reactivity toward SpyCatcher drops upon increasing the size of the side chains. Eventually, the yield between Aω-GFP and B is merely ~39%. While BVA retains full reactivity with A-GFP, the yield drops to 47% for Aω-GFP and 14% for Aω-GFP, but rises again to 60% for Aω-GFP. The trend of reactivity is consistent with the in vitro cyclization results, but the yields seem a little higher because we purposely used a higher concentration of reactants and an excess of B or BVA to

In vitro reactivity assay
To gain a full picture of the reactivity profile between the SpyTag mutants and the final SpyCatcher mutant (BVA), the SpyTag mutants were individually fused with a green fluorescent protein (Aω-GFP), where X stands for the mutated amino acid (see Fig. S2 for the sequences and S3 for the mass spectra†). SpyCatcher (B) and the final mutant, BVA, were also cloned and expressed separately (see Fig. 2 and S4 for the sequences and S5 for the mass spectra†) for in vitro reactivity assay with Aω-GFP.

The reaction was carried out in PBS at pH 7.4, at different temperatures, at a concentration of 40 μM for Aω-GFP and 80 μM for B or BVA. The kinetics were followed by taking aliquots at designated times and the yields were determined by SDS-PAGE and gel densitometry (Fig. 3 and S6–S12†). In general, the SpyTag–SpyCatcher reaction remains the best of its kind. Mutations on either side seem to compromise the reactivity. The kinetics toward B are slowed down slightly from A-GFP to Aω-GFP, more significantly from A-GFP to Aω-GFP, and dramatically from A-GFP to Aω-GFP. For BVA, the trend is similar, but the kinetics are much slower than those for B. Surprisingly, its reactivity is almost nullified toward Aω-GFP, but is restored toward Aω-GFP, even though the side chain of Trp is considerably larger. Specifically, we compared the results after reaction at 4 ºC for 5 hours, as shown in Fig. 3A. SpyTag behaves like a master key with excellent reactivity toward both B and BVA. The mutants’ reactivity toward SpyCatcher drops upon increasing the size of the side chains. Eventually, the yield between Aω-GFP and B is merely ~39%. While BVA retains full reactivity with A-GFP, the yield drops to 47% for Aω-GFP and 14% for Aω-GFP, but rises again to 60% for Aω-GFP. The trend of reactivity is consistent with the in vivo cyclization results, but the yields seem a little higher because we purposely used a higher concentration of reactants and an excess of B or BVA to

the ratio between cyclized (c-AB) and linear (l-AB) proteins, as shown in SDS-PAGE, would represent their reactivity under the conditions of expression (i.e. 16 ºC in Escherichia coli) (Fig. 1A). Although this is not a high throughput method, it still provides a convenient way to screen the variants’ reactivity.

To begin with, we targeted the key residues within 5 Å around SpyTag in the complex (PDB: 4MLS). SpyTag is shown in pink and SpyCatcher is shown in green. The catalytic isopeptide triad is shown as pale sticks, the three key mutation sites are colored orange, and the other key residues in the hydrophobic pocket are colored blue. (C) By considering SpyTag as the key and SpyCatcher as the lock, we aimed to find a master key that can open all locks (i.e. forming covalent isopeptide bonds) and a pair of orthogonal sub-keys/sub-locks.

isopeptide bonds), and a pair of orthogonal sub-keys/sub-locks for selective functionalization (Fig. 1C).

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To begin with, we targeted the key residues within 5 Å around SpyTag in the complex (PDB: 4MLS) as the protein–protein interaction interface, which is critical for tuning the specificity and efficiency of the reactive pairs (Fig. 1B). To generate genetic diversity, we performed sequential focused site-saturation mutagenesis on each of them. In the first round, we introduced mutations onto SpyTag at the position of isoleucine (Ile3) to abolish its reactivity. The I3W mutant was found to yield almost exclusively the linear product, indicating poor reactivity (Fig. S1†). Based on this, we constructed a library of variants with site-saturation mutagenesis on SpyCatcher, and screened for restored reactivity in the subsequent rounds of directed evolution (see Fig. S1 and Table S1† for typical screening results). Finally, we found that a F77V, F94A mutant of SpyCatcher (BVA) partially restores the reactivity with Aω (Table S1†). Using this strategy, we aimed to achieve orthogonal reactivity encryption within the same protein scaffold with as few mutations as possible. If we make an analogy between the SpyTag–SpyCatcher reaction and the “lock-and-key” concept, we hope to find a master key that opens all locks (i.e. forming isopeptide bonds), and a pair of orthogonal sub-keys/sub-locks for selective functionalization (Fig. 1C).
drive the reaction forward. The possibility to manipulate the reactivity by the experimental conditions is an advantage for \textit{in vitro} experiments. Interestingly, the temperature dependence of the reactivity is completely opposite for B and BVA (Fig. 3B and S6†). This is best demonstrated in their reactions with A\textsubscript{W}-GFP. For B, the yield increases and plateaus at higher temperatures, but for BVA, the yield is promoted at lower temperatures. This suggests that they may have different mechanisms for reconstitution and reaction. From the current data, we deduced that (i) BVA exhibits selective reactivity with A, but not A\textsubscript{Y}, whereas A\textsubscript{Y} reacts well with B; (ii) at lower temperatures, A\textsubscript{W} would preferentially react with BVA, while A\textsubscript{Y} would selectively react with B, which is close to orthogonal.

To examine the selectivity of these mutant pairs, we designed a series of experiments. In the first one, elastin-like proteins (E) bearing A\textsubscript{Y} (or A\textsubscript{W}) at the N-terminus and A (or A\textsubscript{Y}) in the middle of the chain were designed (A\textsubscript{X1-EA\textsubscript{X2}}E), as well as their nonreactive controls, where the reactive Asp in either one of the SpyTag variants was changed to Ala (A\textsubscript{X1-EEA\textsubscript{X2}}E or A\textsubscript{X1-EA\textsubscript{X2}}E) (Fig. S13–S16†). There are two possible pathways for their reaction with SpyCatcher and its mutant. Depending on the first reaction site, this would lead to type I and II intermediates (Fig. 4A). If selectivity permits, sequential functionalization could be performed on one protein scaffold to give distinct diadducts. By design, the nonreactive mutants will trap the reaction at different intermediate states as controls. The second design was to use a BVA-functionalized cyan fluorescent protein (CFP-B\textsubscript{VA}) to selectively fish out the right tag from a pool of telechelic proteins containing different SpyTag mutants (such as A\textsubscript{X}-GFP and SUMO-A\textsubscript{X}) (the “tag-fishing” experiment is shown in Fig. 4B and S17–S20†). Finally, to rigorously test the orthogonality, we mixed multiple reactive proteins in one pot and determined the product distribution (Fig. 4C).

**Fig. 3** (A) SDS-PAGE analysis of the reaction products of A\textsubscript{X}-GFP and B or BVA at 4 °C for 5 hours; (B) yields after reaction at 4, 16, 25 and 37 °C for 5 hours.

**Fig. 4** Experimental design to test the reaction selectivity and orthogonality: (A) the sequential reaction of A\textsubscript{X1-EA\textsubscript{X2}}E with B or BVA may proceed through two pathways; (B) BVA could selectively react with A-GFP or SUMO-A but not A\textsubscript{Y}-GFP or SUMO-A\textsubscript{Y}; (C) orthogonal reactions lead to only two corresponding products from a complex mixture of reactive proteins.

**Fig. 5A** shows the reaction products between A\textsubscript{X1-EA\textsubscript{X2}}E and B or BVA at 4 °C at 15 μM each for 12 hours. By comparison with the control reactions, it is evident that B reacts efficiently with both A and A\textsubscript{Y} while BVA only reacts with A, but rarely with A\textsubscript{Y} (Fig. S21†). The reaction using a BVA-functionalized ELP (EB\textsubscript{VA}) gives similar results (Fig. S21†). Although previous model experiments show slight residual reactivity between BVA and A\textsubscript{Y} (Fig. 3), it seems that this is inhibited tremendously in the presence of A due to the distinct kinetic difference between A and A\textsubscript{Y}, even though A\textsubscript{Y} is placed at a more accessible terminal site. This feature should be useful for controlling the functionalization sites and preparing proteins with complex topology. As an example, we sequentially reacted A\textsubscript{Y}-EAE firstly with BVA and then with B. The size exclusion chromatography (SEC) overlay of the products at two stages clearly shows the sequential progression of the reactions (Fig. 6), and the identity of the products was corroborated by MALDI-TOF mass spectra and SDS-PAGE analysis (Fig. S22 and S23†). This excellent...
selectivity was also reflected in the "tag-fishing" experiments. We used CFP-BtA to react with AY-GFP and/or SUMO-AY at 4 °C in PBS buffer (pH 7.4) in a 1 : 1 molar ratio at a concentration of 30 μM each for 5 hours. The SDS-PAGE analysis clearly shows that no products formed from a mixture of CFP-BtA with any AY-containing protein, whereas a mixture of CFP-BtA with any SpyTag-containing protein yielded the corresponding products (Fig. 5B and S24A†). The ability to distinguish the reactivity of peptide tags that differ by only one amino acid is remarkable.

**Tuning toward orthogonal reactions**

The ability to encode information about chemical reactions into protein sequences greatly adds to the diversity of reactions and reactivity control. Even for the same type of reaction, there may be multiple, mutually orthogonal ways for reactivity encryption. While reactive pairs developed from completely different domains are intrinsically orthogonal to each other, orthogonality may also arise from the same ancestor domain.

Since AY and BtA possess opposite reactivity to B and BtA, we proceeded to examine how orthogonal they are to each other. We firstly reacted AWEAYE with B or BtA under identical conditions. The cross-reaction of AYEAWE (left) or AWEAYE (right), firstly with BtA and then with B. The products at each stage were characterized by MALDI-TOF mass spectra and SEC (Fig. 6, S22 and S23†). The results show that the reaction of AYEAWE with B gives almost exclusively the type I product and very little di-adducts, indicating a very high selectivity (Fig. S21C†). On the other hand, for BtA, the selectivity for AY over AW is excellent. No products form from the reaction between BtA and AY at all, while the yield from the reaction between BtA and AWEAYE when AW is at a terminal site, and poor for AYEAWE when AW is in the middle (Fig. S21C†). The good selectivity encouraged us to also pursue sequential functionalization on AWEAYE, firstly with BtA and then with B. The products at each stage were characterized by MALDI-TOF mass spectra and SEC (Fig. 6, S22 and S23†). The results are similar to the previous case, except that the intermediate is a type I mono-adduct in this case instead of a type II mono-adduct. The two intermediates have different retention volumes, with type I being smaller (11.9 vs. 12.2 mL). This is consistent with the expanded linear-chain-like topology of the type I mono-adduct and the compact star-like topology of the type II mono-adduct, confirming again the highly selective functionalization.

A strict evaluation of the orthogonality would be from multiple reactants in one pot. It was hoped that the reactions would proceed in parallel in one pot without mutual interference. We thus mixed AY-GFP, SUMO-AY, CFP-BtA and B at a concentration of 30 μM for the SpyCatcher variants and 60 μM for the SpyTag variants at 4 °C. Control reactions were run by
mixing only two reactive proteins under identical conditions (Fig. 5C, lanes 2–5). A time course for the one-pot reaction was recorded (Fig. 5C, lanes 6–10). This clearly shows that the major products are the desired products, CFP-B\(_{VA}\):AW-GFP and B:SUMO-A\(_{V}\). There is only very little cross-reacted product of B\(_A\):AW-GFP appearing at the late stages (~6 hours). No cross-reacted product of CFP-B\(_{VA}\):SUMO-A\(_{V}\) was observed (Fig. S24†).

Therefore, A\(_A\)/B\(_A\) and A\(_V\)/B could be viewed as two mutant pairs with nearly orthogonal reactivity at shorter times and lower temperatures. Even considering the product distribution after 10 hours of reaction, B still has ~9.1-fold selectivity for SUMO-A\(_{V}\) over A\(_A\)/B\(_A\), and A\(_V\)/B-GFP still exhibits ~5.5-fold selectivity for CFP-B\(_{VA}\) over B. Considering that the two reactive pairs only differ by three amino acids, this result is impressive. It is also worth noting that the reactivity may vary depending on the location of the reactive domains and the 3D structure of the fusion proteins, which may or may not promote the selectivity of the reactions.

To understand the reaction selectivity, we built structural models of the mutant pairs using RosettaDesign (Fig. S25†). When the I3W mutation is introduced into RosettaDesign, the bulky side chain of Trp clashes with the original residues on B, which leads to a calculated binding free energy as high as 534.99 REU (Rosetta energy units, an arbitrary energy unit based on the Rosetta score function). By changing the two Phe residues on B to smaller residues (Val and Ala), B\(_A\) could better accommodate the Trp residue of A\(_W\), as demonstrated by the much lower calculated binding free energy of ~36.14 REU for A\(_W\)/B\(_A\). On the other hand, the I3Y mutation in SpyTag retains its reactivity with SpyCatcher by forming good hydrophobic interactions between A\(_V\) and B with a packing score of 0.758, while the interactions between A\(_V\) and B\(_A\) are significantly deteriorated, as reflected by the much lower packing score of 0.683. Although the detailed molecular mechanism affecting the reactivity remains to be illustrated by techniques like crystallography, the trend predicted by computational studies is generally consistent with the experimental results. Aided by computational studies and rational design, we are continuing to improve the orthogonality and reactivity of the mutant pairs along this line.

**Conclusions**

In summary, we have developed mutants of SpyTag-SpyCatcher reactive pairs with engineered reactivity and selectivity through directed evolution. With only two mutations, B\(_A\) exhibits distinct reactivity profiles compared to B. The inverse temperature dependence of the reactivity suggests that they may adopt different reconstitution mechanisms. SpyTag is like a master key, reactive with both B and B\(_A\), A\(_V\) is like a sub-key that only “opens” the lock B rather than B\(_A\). In contrast, A\(_W\) possesses dramatically reduced reactivity with B but still exhibits decent reactivity with B\(_A\) at lower temperatures. The reactivity between A\(_V\)/B and A\(_W\)/B\(_A\) is thus close to orthogonal with some minor cross-reaction between A\(_W\) and B. Although the promiscuity is not completely removed, the ability to engineer reactivity with minimum sequence variation (as few as three mutations) opens up new avenues in the ever-expanding “iron grip” toolbox for synthetic biology. It also promises a family of genetically encoded and engineered peptide–protein chemistry tools, much like what the fluorescent protein family has offered to the community.

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