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## Specific Modulation of Protein Activity Through a Bioorthogonal Reaction

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### Abstract

Unnatural amino acids with bioorthogonal reactive groups have the potential to provide a rapid and specific mechanism for covalently inhibiting a protein of interest. Here, we use unnatural mutagenesis to insert an azide (Z) into the target protein at positions such that a “click” reaction with an alkyne modulator (X) will alter protein function. This bioorthogonal reactive pair can engender specificity of X for the Z-containing protein even if the target is otherwise identical to another protein, allowing for rapid target validation in living cells. We demonstrate our method using inhibition of the *E. coli* enzyme aminoacyl transferase by both active site occlusion and allosteric mechanisms. This “clickable magic bullet” strategy should be generally applicable to protein inhibition, within the limits of unnatural amino acid mutagenesis.

### Keywords

chemical genetics; bioorthogonal chemistry; unnatural amino acids; covalent inhibitor; drug discovery

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Most pharmaceuticals are designed to function as molecular entities acting on specific biological targets, what Erlich referred to as “magic bullets” (MBs).<sup>[1]</sup> A key early event in the development of any pharmaceutical is target-validation: confirmation that modulation of a particular gene or protein will improve a disease phenotype.<sup>[2]</sup> In modern drug discovery efforts, validating a target often begins with genetic deletion of the protein product. This can be accomplished either with RNA interference (RNAi) or the breeding of knock out animal models.<sup>[3]</sup> However, there are limitations to these two technologies, and in many cases greater understanding of biological pathways can be attained with small molecules of high target specificity, even if they do not ultimately become drugs.<sup>[4]</sup> The field of Chemical Genetics encompasses an array of strategies to link genotypes and phenotypes using small molecules in model systems.<sup>[5]</sup> Here, we present a method designed to attain small molecule specificity rapidly and simply using a combination of genetic manipulation and molecular design.

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Supporting information for this article is given via a link at the end of the document.

Our strategy is simple: use unnatural amino acid mutagenesis to insert bioorthogonal functional groups (Z in Figure 1) into a “magic bullet” target (MBT) protein at positions such that reaction with a small molecule partner bearing a complementary reactive group (X in Figure 1) will alter protein function. In this work, we will use azidophenylalanine (Azf) as the Z group and dibenzocyclooctynes (DBCOs) as X groups, taking advantage of the strain-promoted cycloaddition of Azf and DBCO to rapidly and specifically attach X to the MBT protein.<sup>[6]</sup> We note that our general strategy is equally applicable to other bioorthogonal “click” chemistry reactive pairs such as cycloalkene/tetrazine.<sup>[7]</sup> Protein modulation will be specific, provided that the reactions are truly chemoselective and only the MBT protein contains the targeting group Z. The position of the Z targeting moiety can be easily scanned throughout the protein sequence to identify positions for which the MBT protein has wild type (WT) activity prior to reaction with an X molecule. These MBT mutants will be taken forward to studies in which they are reacted with X molecules and the phenotype of the modified protein is evaluated.

The two previous Chemical Genetics experiments most relevant to our work are the “bump-and-hole” strategy employed by Schreiber, Shokat, and others; and the tethering approach of Erlanson and Wells.<sup>[8]</sup> In the “bump-and-hole” method, a bump must be added to the small molecule in a way that precisely matches a hole generated in the binding pocket of the target enzyme.<sup>[9]</sup> To be implemented well, this strategy generally requires prior structural knowledge and a deep binding pocket like an active site. Tethering uses reaction with an exogenous Cys thiol to covalently attach small molecules to the surface of proteins.<sup>[8a, 10]</sup> These molecules can act as allosteric modulators, so they do not need to reside in active sites.<sup>[11]</sup> Moreover, the Cys thiol can be scanned across the surface of a protein in a screening protocol, so that careful, structure-based design is not necessary. However, since many proteins contain Cys, specificity derived from tethering is not easily retained in lysates or *in vivo*. Our method shares many features with these two strategies, but the ability to scan Z through a protein sequence should make it much more broadly applicable than the bump-and-hole strategy and the chemoselectivity of “click” reactions should allow it to be applied *in vivo*, unlike the tethering strategy. Strategies similar to our own using tetracysteine motifs and bisarsenical compounds have been described, but these methods have not been broadly applied.<sup>[12]</sup> Finally, it should be noted that unnatural amino acids, including bioorthogonal reactive handles, have been used extensively to probe protein structure and function, particularly in membrane protein work by Sakmar, Wang, and Dougherty.<sup>[13]</sup> However, none of these previous studies made use of the reactive handle as a general tool for perturbing function as we do here.

To incorporate Azf into the MBT proteins site-specifically, we can employ unnatural amino acid mutagenesis techniques developed by Schultz and coworkers.<sup>[14]</sup> These methods allow one to insert an unnatural amino acid by using a “21st” aminoacyl tRNA synthetase (aaRS) which charges a tRNA<sub>CUA</sub> that recognizes the UAG stop codon. Two plasmids are transformed into *E. coli*: pAzfRS (previously referred to as pDULE2<sub>Azf</sub>), encoding Azf aaRS and tRNA<sub>CUA</sub>, and a plasmid encoding the MBT protein with the UAG mutation.<sup>[15]</sup> After growth in the presence of Azf, the MBT protein is either purified for *in vitro* studies, or reacted with DBCO reagents *in vivo*.

To show proof-of-concept, we have used the MB strategy to inhibit the *E. coli* aminoacyl transferase (AaT) protein.<sup>[16]</sup> This protein functions in the N-end rule pathway by tagging proteins bearing N-terminal Lys or Arg with Leu, Phe, or Met using aminoacyl tRNA as a substrate.<sup>[17]</sup> Addition of the hydrophobic amino acid constitutes a recognition motif for ClpS, which targets the tagged protein for degradation by ClpAP. We and others have previously shown that AaT can accept a variety of hydrophobic unnatural amino acids, which can be used to report on AaT activity.<sup>[18]</sup>

Using existing AaT crystal structures, we chose six locations for incorporation of Azf.<sup>[19]</sup> These included three positions right in the active site, and three positions near the exterior of AaT that were not expected to directly alter activity. In all cases, we mutated aromatic amino acids because we expected replacement of these amino acids with Azf to be minimally perturbing to protein function prior to reaction with Azf. We expressed and purified His-tagged versions of each AaT mutant and assessed the ability to react them with X molecules using DBCO rhodamine derivatives (DBCO<sub>545</sub> or DBCO<sub>TMR</sub>, Supporting Information, Fig. S1). MALDI MS and PAGE gel data showed that for those positions taken forward to additional study, labeling was quantitative within the limits of MS detection. (Supporting Information, Table S1 and Fig. S5) Next, we tested the activity of these Azf mutants prior to reaction with DBCO<sub>545</sub>. Five mutants – Z<sub>47</sub>, Z<sub>59</sub>, Z<sub>68</sub>, Z<sub>81</sub>, and Z<sub>135</sub> – were found to have activities within 4-fold of WT. The positions of these amino acids are shown in Figure 2. All five mutants were taken forward to inhibition assays. While it is surprising that Azf replacement of nearly isosteric Phe at position 81 should cause such a large change in activity, this is consistent with previous reports of the sensitivity of AaT to mutation.<sup>[19a]</sup> The reduction in activity on mutation of Y<sub>42</sub> is less surprising as this makes a hydrogen bond with the Lys or Arg of the peptide substrate.<sup>[19b]</sup>

All five mutants were inhibited by DBCO<sub>545</sub> treatment. The levels of inhibition that we observed varied from 52% for Z<sub>135</sub> (relative to unmodified protein) to 98% for Z<sub>47</sub>. It is perhaps not surprising that Z<sub>47</sub> was most inhibited, since the mutation occurred in the active site. Inhibition presumably occurs by simply occluding access to the active site. Z<sub>59</sub> modification would also block the active site. Inhibition of Z<sub>68</sub>, Z<sub>81</sub>, or Z<sub>135</sub>, on the other hand, probably occurs through an allosteric mechanism. Using crystal structures of either the acyl nucleotide-bound or peptide product-bound states of AaT, we made simple computational models of the DBCO adducts.<sup>[19b]</sup> (Supporting Information, Fig. S9) The DBCO<sub>545</sub> and DBCO<sub>TMR</sub> adducts should not be able to reach the active site in either state of the enzyme. In WT AaT, W<sub>135</sub> occurs at a crystallographic dimer interface, and some weak dimerization has been observed in gel analysis of purified AaT. However, our analysis of WT AaT indicates that activity varies linearly with concentration. (Supporting Information, Table S2) Therefore, we believe that AaT is monomeric in the concentration range used, and that Z<sub>68</sub>, Z<sub>81</sub>, and Z<sub>135</sub> inhibition occurs through allostery. It should be noted that we observed some reactivity between WT AaT and the DBCO reagent, presumably at one or more of the 6 Cys residues. Blocking with iodoacetamide can be used to eliminate this reaction (Supporting Information, Fig. S6), but this strategy is not viable in cells. Moreover, the differences in inhibition seen for the Azf mutants give us confidence

that high levels of inhibition are only achieved upon DBCO reaction with a precisely placed Azf residue.

We also wished to determine whether inhibition was in fact dose-dependent. The cycloaddition is essentially irreversible, therefore we must carry out the reaction under conditions where the DBCO inhibitor is present at much higher concentrations than the enzyme so that pseudo-first-order kinetic analysis can be applied.<sup>[20]</sup> Timepoints must also be selected so that the irreversible reaction has not been allowed to reach completion. Under the conditions of our assay (1  $\mu$ M AaT, 30 min), DBCO<sub>545</sub> has an apparent IC<sub>50</sub> of 8.5  $\mu$ M for the Z<sub>47</sub> mutant. (Supporting Information, Fig. S8) While a more rigorous analysis would be required to determine a true IC<sub>50</sub>, this is sufficient to show that inhibition is dose-dependent, provided that the assay time is comparable to the half-life for the DBCO<sub>545</sub> cycloaddition (hours).

Initially, we used DBCO<sub>545</sub> as an inhibitor because we could assess the levels of protein modification using the chromophore. However, modeling studies implied that for Z<sub>47</sub>, the rhodamine moiety was unnecessary since the DBCO moiety occupied the binding pocket and rhodamine extended into solution (Supporting Information, Fig. S9). Therefore, we also tested inhibition of the DBCO amine (DBCO<sub>A</sub>) alone and saw 99% inhibition, similar to that obtained with DBCO<sub>545</sub>. For the Z<sub>68</sub> and Z<sub>135</sub> mutants, DBCO<sub>A</sub> actually gave higher levels of inhibition than DBCO<sub>545</sub>, 87% and 94%, respectively (Supporting Information, Table S3). If necessary, the DBCO moiety can also be functionalized with other groups (R in Figure 1), so that a variety of inhibitors can be made. In the cell studies below, we used a fluorescent R group in order to confirm reaction with AaT in lysates and in cells.

Next, we wished to demonstrate that our method could be used to inhibit AaT selectively in complex mixtures. Therefore, we carried out labeling reactions using a reporter protein derived from  $\alpha$ -synuclein ( $\alpha$ S) in lysates of TS351G *E. coli* cells, which lack endogenous AaT activity.<sup>[17b]</sup> Here, we determined the activity of AaT based on its ability to transfer Azf to the reporter protein from Azf-acylated tRNA generated *in situ* by a mutant aaRS. Transfer of Azf was then imaged using a copper-catalyzed “click” reaction to append a fluorescein derivative. Note: Although azide-alkyne chemistry is being used for both inhibition and the AaT assay, no cross-reactivity of the click reagents is expected because of the order of addition. To make appropriate calculations of inhibition levels, we carried out control experiments in which we either withheld ATP (necessary for acylation of the tRNA with Azf) from the transfer reaction or added DMSO vehicle only. Since no Azf should be transferred in the “-ATP” controls, this control allowed us to determine that the level of non-specific fluorescein binding was very low. Therefore, we calculated inhibition by comparing the levels of fluorescence in DBCO<sub>545</sub> treated lanes to those treated with DMSO. We chose three mutants for further study: Z<sub>47</sub>, Z<sub>68</sub>, Z<sub>135</sub>. Z<sub>47</sub> showed almost complete inhibition by active site occlusion, and Z<sub>68</sub> and Z<sub>135</sub> showed the highest levels of presumed allosteric modulation. In all cases, we observed inhibition levels comparable to those observed with the small reporter peptide in purified reactions: > 99% for Z<sub>47</sub>, 66% for Z<sub>68</sub>, and 73% for Z<sub>135</sub>. (Supporting Information, Fig. S12) This shows that we can effectively inhibit AaT in the presence of the other proteins in the TS351G lysates using 200  $\mu$ M DBCO<sub>545</sub>.

Finally, in order to demonstrate that we can inhibit AaT mutants in living cells, we reacted DBCO<sub>TMR</sub> (which we expected to be more cell permeable than DBCO<sub>545</sub> or DBCO<sub>A</sub>) with AaT in intact *E. coli* cells, and then analyzed activity in crude lysates as described above. Our protocol is outlined in Figure 3. We transformed BL21(DE3) cells with pAzfRS and plasmids encoding the AaT mutants. After growth in the presence of Azf and IPTG, the cells were pelleted, washed repeatedly to remove unincorporated Azf, and then treated with a DBCO<sub>TMR</sub> solution or DMSO vehicle. The cells were washed, resuspended, and lysed. The levels of AaT activity were analyzed by Azf transfer and reaction with fluorescein alkyne as described above. Note that Azf is used both to introduce the reactive handle for inhibition *in vivo* and then to determine AaT activity. However, because of the order of our steps, no crosstalk is expected. Note also that BL21(DE3) cells were used here since we observed little endogenous AaT activity in these cells as compared to overexpressed AaT (data not shown) and we obtained low yields of unnatural amino acid protein in TS351G cells.

As one can see in Figure 4, we successfully inhibited all three Azf-tagged AaT proteins through *in vivo* treatment with DBCO<sub>TMR</sub>. Since we used a tetramethylrhodamine label on our inhibitor and a fluorescein probe for AaT activity, the red channel reports on reaction of the Azf residue in AaT with the DBCO<sub>TMR</sub> inhibitor, while the green channel reports on the levels of Azf reporter transferred in the activity assay. Once again, trials in which ATP was withheld from the transfer reaction served as controls for non-specific fluorescein binding. Although we observed labelling of other proteins (possibly endogenous AaT substrates) with the fluorescein alkyne probe, analysis of the  $\alpha$ S bands allowed us to assay AaT activity.

As one can see by comparing the “+ DBCO” and “+ Veh.” lanes, significant inhibition of AaT activity was observed in all cases: 84 % for Z<sub>47</sub>, 83 % for Z<sub>68</sub>, and 77 % for Z<sub>135</sub>. This shows that we can effectively inhibit AaT by dosing DBCO<sub>TMR</sub> in intact *E. coli* cells. Moreover, we can track the selectivity of the DBCO reagent by analyzing the red fluorescence channel. We see relatively low levels of non-specific binding for WT AaT, indicating that the bands seen for the Azf mutants may be due to off-target incorporation of Azf or association of AaT with other proteins. One feature of note is the green bands observed in the bottom gel, corresponding to fluorescein labeling of unreacted Azf in AaT mutants that had not been treated with DBCO<sub>TMR</sub>. This shows that Azf was present in the active site of these AaT samples (“+ DBCO” lanes appear to have higher background due to emission from TMR excited with 473 nm light). Thus, we see specific, DBCO-dependent inhibition of Azf mutants of AaT at levels that are comparable to or greater than those seen for the purified enzyme. However, it must be noted that there are still steps to be taken to make this a truly *in vivo* technology. While the DBCO reaction was carried out in cells, the assay was carried out in lysates, as is common for AaT, which lacks a clear activity-dependent phenotype (even the TS351G AaT-deletion cell line does not exhibit an obvious phenotype).<sup>[13, 17b, 21]</sup> A discussion of potential improvements to this method is given below.

We have shown, for the first time, that unnatural amino acids and bioorthogonal chemical reactions can be used as part of a general strategy to connect genotype to phenotype. We believe that our method could be a valuable complement to RNAi for target validation. While it is more complex to implement than RNAi, it offers validation in the context of a

dosable small molecule. Since many microRNAs (miRNAs) act catalytically, controlling dosage can be hard.<sup>[22]</sup> A few additional advantages of our method are worth noting. Firstly, RNAi prevents translation of the protein of interest, which may lead to phenotypes resulting from the absence of all of its protein interactions rather than inhibition of one specific activity. These changes would be hard to replicate with a small molecule or protein therapeutic, which typically bind to one face of the target protein. Second, although we have only found examples of inhibition in this initial study of AaT, the method can conceivably also be used to find activators. RNAi can only reduce the activity of its protein target, not enhance it.

While we are excited about the many possible applications of this method, several considerations are worth noting:

1. Washout of unincorporated Azf is essential. Azf can act as a buffer, consuming DBCO-R and preventing modification of Azf in the MBT protein.
2. The ability to remove the endogenous copy of the protein, at least from cell lysates, is essential. This can be done with a chromosomal deletion as in this work, or using miRNA in appropriate cell lines. Judicious codon usage can prevent the miRNA from targeting the MBT as well as the WT protein.<sup>[23]</sup> However, deleting the endogenous copy must either have a very mild phenotype (as AaT deletion does here), or the knockdown of the endogenous gene must be carefully timed with expression of the replacement MBT protein.
3. The UAG stop codon, though rare (~ 5 %), is present in many natural proteins (~ 300).<sup>[24]</sup> The presence of AzfRS could lead many of these codons to be read through, yielding proteins with aberrant activity and/or susceptibility to inhibition by reaction with DBCO reagents. These proteins may be misfolded and degraded, but they may also contribute to the observed phenotype.
4. If the tRNA<sub>CUA</sub> does not efficiently deliver the unnatural amino acid at the UAG stop codon, high levels of truncated protein may be synthesized, resulting in a dominant negative phenotype that would mask the effect of any DBCO-based modulation of the MBT protein.
5. Click chemistry is imperfect and the reactions used here may not be optimal for this application. Although the fact that we are able to achieve nearly 100 % inactivation of the Z<sub>47</sub>, Z<sub>68</sub>, and Z<sub>135</sub> mutants implies that the cycloaddition goes to completion with respect to the protein, this will not be true for many residues in many proteins.<sup>[21c]</sup> Also, the cyclooctyne probes used here are relatively hydrophobic and may be prone to non-specific binding. Newer, faster bioorthogonal reactions are continually being developed, and a great variety of R groups and X/Z reactive partners are possible, so there are many options, including more polar reactive groups or meta-stable linkages (e.g. oxime) to make reversible inhibitors.<sup>[7a]</sup>
6. The ultimate goal of connecting genotype to phenotype requires that the phenotype be either intrinsically-discernable or that a reporter construct be used to assess the phenotype. In the latter case, this could mean transformation of a third plasmid and maintenance of the cell line in the presence of three antibiotics, which could

introduce intolerable levels of stress to the cells (as we observed in attempts to perform an *in vivo* assay of AaT activity with a GFP reporter). Chromosomal insertion of the synthetase and tRNA or the use of multi-gene vectors like pET-DUET would reduce this burden and may be necessary for phenotypic characterization.

In spite of these potential complications, the ability to rapidly generate a protein that is selectively-targetable by simple small molecules could have tremendous value in the drug discovery process. The fact that we can do this through a single amino acid substitution is a significant advantage relative to other similar technologies that require a greater perturbation to confer selectivity.<sup>[25]</sup> Although we use an enzyme with a crystal structure in this proof-of-principle experiment, this is not required. One could scan the unnatural mutation through a protein sequence as one does in an Ala scan and simply rely on an activity assay to determine which positions are non-perturbing prior to reaction with the small molecule. The demonstration of our method in this manuscript is limited to a single-celled model organism, *E. coli*. However, unnatural amino acid mutagenesis has been demonstrated in mammalian cells as well as multicellular organisms such as *C. elegans* and *Drosophila melanogaster*.<sup>[26]</sup> Moreover, alternate coding strategies have been employed leading to efficient incorporation at four base codons, which may address issues 3 and 4 above.<sup>[26a, 27]</sup> Schultz, Church, Isaacs, and coworkers have also generated a strain of *E. coli* with the TAG stop codons replaced by TAA codons.<sup>[28]</sup> We will explore these applications and continue to improve our method. We have begun to apply our method to other systems including membrane transporters implicated in drug resistance and aggregating proteins like  $\alpha$ -synuclein. These types of gain-of-function systems may be more amenable to our methods, as deletion of the endogenous copy of the protein may be less important and there are clear phenotypes. The generality of our approach should make it equally applicable to the discovery of competitive inhibitors, allosteric modulators, or modulators of protein interactions with DNA or other proteins.

## Experimental Section

### Wild type AaT expression and purification

His<sub>10</sub>-tagged *E. coli* AaT was expressed from the pAaT-WT plasmid (previously referred to as pEG6) in *E. coli* BL21-Gold (DE3) cells using a procedure modified from Graciet *et al.*<sup>[17b]</sup> Briefly, *E. coli* were grown in a primary culture of 4 mL LB at 37 °C to OD<sub>600</sub> of 0.5 which was diluted into a secondary culture of 500 mL LB and grown to OD<sub>600</sub> of 0.6 – 0.8. AaT expression was induced using 0.1 mM isopropyl  $\beta$ -D-thiogalactoside (IPTG) and cells were grown at 25 °C for 16 hours. Cells were pelleted at 6,000 rpm using a GS3 rotor and Sorvall RC-5 centrifuge. Cell pellets were resuspended in the Ni-NTA binding buffer (50 mM Tris, 10 mM imidazole, and 300 mM KCl, pH 8.0) that included protease inhibitor cocktail, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 10 units/mL DNase I–Grade II. Following resuspension, the cells were lysed using sonication. Soluble proteins were collected *via* centrifugation at 13,200 rpm for 15 minutes. Collected soluble protein was gently shaken for 1 hour on ice with Ni-NTA Superflow (Qiagen). Protein was purified by rinsing with Ni-NTA binding buffer and then washing with Ni-NTA wash buffer (50 mM Tris, 50 mM imidazole, and 300 mM KCl, pH 8.0). The proteins were eluted with elution

buffer (50 mM Tris, 250 mM imidazole, and 300 mM KCl, pH 8.0). Fractions containing *E. coli* AaT were dialyzed overnight in transferase buffer consisting of 50 mM HEPES, 30 % glycerol, 120 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1 μM β-mercaptoethanol (β-ME), pH 8.0. The dialyzed enzymes were stored at -80 °C.

### Unnatural amino acid incorporation into AaT

Site-directed mutagenesis was used to insert TAG stop codons into the pAaT-WT plasmid in order to introduce an unnatural amino acid at position XX (pAaT-Z<sub>XX</sub>). The sequences and mutagenic primers are shown in Figure S3 (Supporting Information). BL21-Gold (DE3) cells were transformed with the pAaT-Z<sub>XX</sub> plasmid and a pAzfRS (previously referred to as pDULE2<sub>Azf</sub>) plasmid containing an *M. jannaschii* mutant tRNA synthetase and tRNA<sub>CUA</sub> pair. Cells were selected using ampicillin (100 mg/L) and streptomycin (100 mg/L). Azf was incorporated into AaT as described above with the addition of 1.0 mM Azf (solubilized by dropwise addition of 1 M NaOH) to the growth media. When working with Azf, cultures and purified proteins were kept shielded from light.

### AaT cyclooctyne labeling

Ni-NTA-purified AaT and AaT-Z<sub>XX</sub> mutants (0.20 mg/mL) were incubated with 200 μM (final concentration) DBCO<sub>A</sub>, DBCO<sub>545</sub>, or DBCO<sub>TMR</sub> from a DMSO stock for 2 hours at 37 °C. As a vehicle control, AaT and AaT-Z<sub>XX</sub> mutants were also treated with an equal volume dimethyl sulfoxide. Excess DBCO reagent was removed by dialysis twice in transferase buffer (50 mM Tris, 30 % glycerol, 120 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1 μM β-ME, pH 8.0). Labeled proteins were then analyzed by PAGE gel or trypsin digest and MALDI MS as described above and stored at -80 °C.

### Activity of AaT using LysAlaAcm ligation assay

The activity of DMSO- and DBCO<sub>TMR</sub>-treated purified AaT and AaT-Z<sub>XX</sub> mutants were assayed by transfer of Phe to the N-terminus of a LysAlaAcm peptide (Bachem; Torrance, CA, USA) as previously described.<sup>[18b]</sup> Each ligation reaction, 125 μL total volume, contained the following reagents: Phe (1 mM), *yeast* Phe tRNA (12.5 μg), Adenosine-5'-triphosphate (1.25 mM, ATP), *yeast* Phe tRNA aminoacyl synthetase (yPheRS, 2.5 μg), AaT (1 μM), and LysAlaAcm (100 μM) in the AaT Ligation Buffer (50 mM HEPES pH 8.0, 150 mM KCl, 10 mM MgCl<sub>2</sub>, 50 mM Tris, pH 8.0; 30 % glycerol; 120 mM ammonium sulfate). The reaction mixtures were incubated at 37 °C for 4 hours and quenched with 1 % acetic acid. Proteins were removed from the peptide using four volumes of acetone and cooled at -20 °C for 1 hour. The reactions were centrifuged at 13, 200 rpm at 4 °C for 20 minutes to separate the peptide from precipitated protein. The supernatant was transferred to a 1.5 mL centrifuge tube and the acetone was allowed to evaporate overnight at room temperature. Residual acetone was removed by drying in a Speedvac (Savant, Thermo Scientific, Fisher Inc.) for 30 minutes. The resulting reaction volume was dissolved in a total volume of 1 mL of 1:9 0.1 % TFA in acetonitrile:water and analyzed by HPLC (see Supporting Information for details) to determine ligation efficiency by integration of separated PheLysAlaAcm and LysAlaAcm peak intensities monitored at 325 nm.



### **In vivo AaT labeling activity assay**

*E. coli* BL21(DE3) cells were grown with pAzfRS and either pAaT-WT or pAaT-Z<sub>XX</sub> plasmids using ampicillin and streptomycin. A 4 mL primary culture was used to inoculate a 100 mL secondary culture. Secondary cultures were grown to an OD<sub>600</sub> of 0.6 and induced with 1.0 mM Azf and 0.1 mM IPTG. Protein expression was induced for 16 hours at 25 °C. Cell pellets were harvested at 6,000 rpm for 10 min using a GS3 rotor and Sorvall RC-5 centrifuge. Cell pellets were washed three times with wash buffer (50 mM Tris and 300 mM KCl, pH 8.0) to remove excess Azf. Washed cell pellets were resuspended in 2 mL wash buffer and incubated with either DMSO or 500 μM DBCO<sub>TMR</sub> at 37 °C for 2 h with 250 rpm shaking. Cells were then washed three times with wash buffer containing 1% DMSO and then washed once with wash buffer. Cell pellets were then resuspended in 1 mL wash buffer and sonicated (one minute on then one minute off) three times. Lysed cells were stored on ice until further use. αS<sub>2-140</sub>K<sub>2</sub> (31.3 μg) was modified with Azf (0.85 mM) in the *E. coli* lysate using *E. coli* total tRNA (125 μg), ATP (2.1 mM), and *E. coli* AzfRS (2.5 μg) in a reaction volume of 73.2 μL in modified AaT buffer (50 mM HEPES pH 8.0, 150 mM KCl, 10 mM MgCl<sub>2</sub>) and AaT (1.25 μg). ATP was omitted and replaced with an equivalent amount of MilliQ water for negative controls. The reaction mixture was incubated at 37 °C for 2 hours. The crude reaction (12.5 μL) was then labeled with fluorescein-alkyne (33 μM) using THPTA (3.546 mM), CuSO<sub>4</sub> (681 μM), and sodium ascorbate (5.4 mM) at 37 °C for 1 hour. The reaction was boiled with gel loading dye LDS (5 μL, Pierce) for ten minutes at 95 °C and analyzed by SDS-PAGE. Fluorescence images were obtained with a Typhoon FLA 7000. Fluorescein was imaged using 473 nm excitation and an Y520 filter. TMR was imaged using 532 nm excitation and an O580 filter. Images were collected using a 100 μM pixel size. Total protein content was visualized using Coomassie Brilliant Blue.

### **Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

### **Acknowledgments**

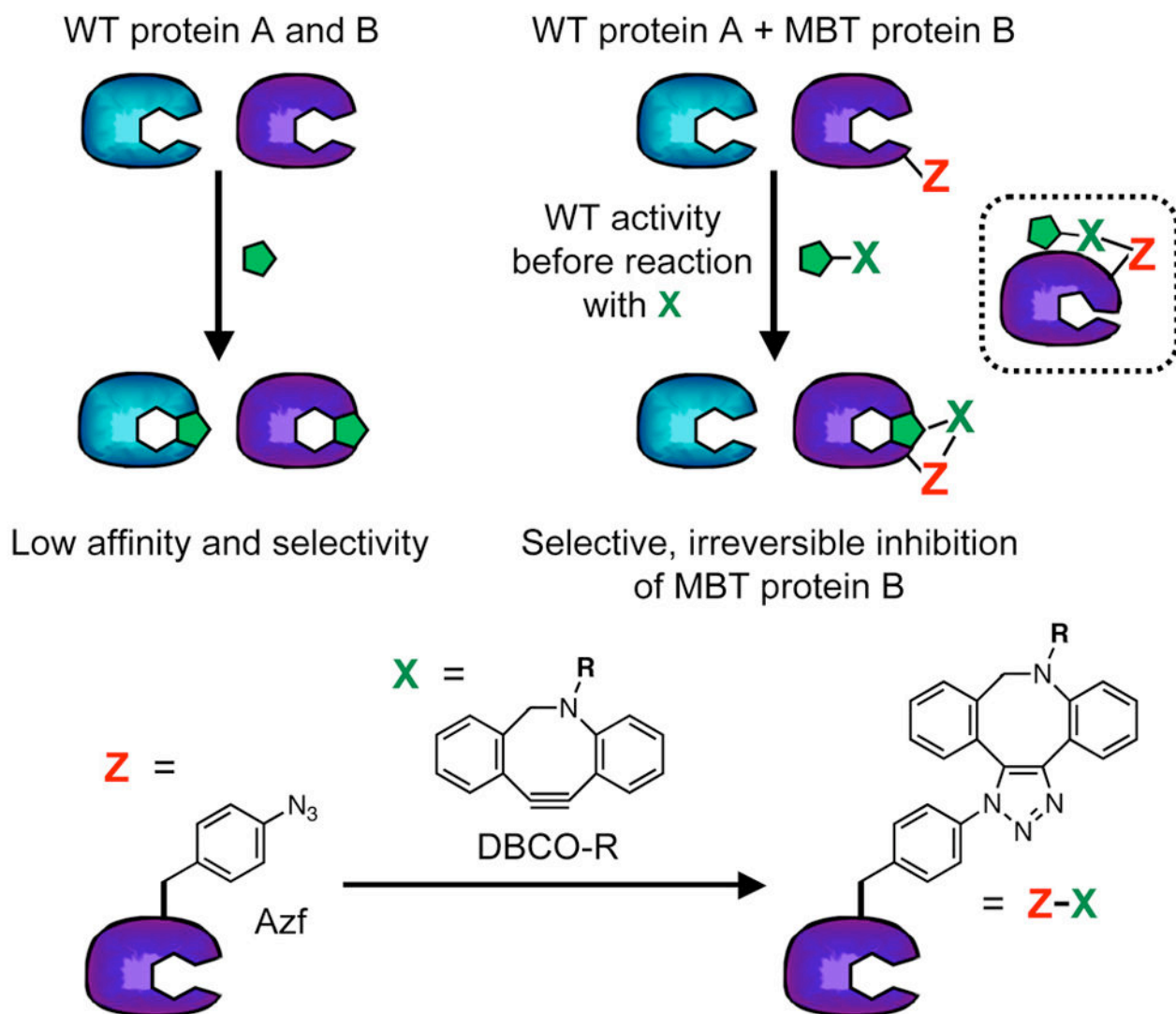
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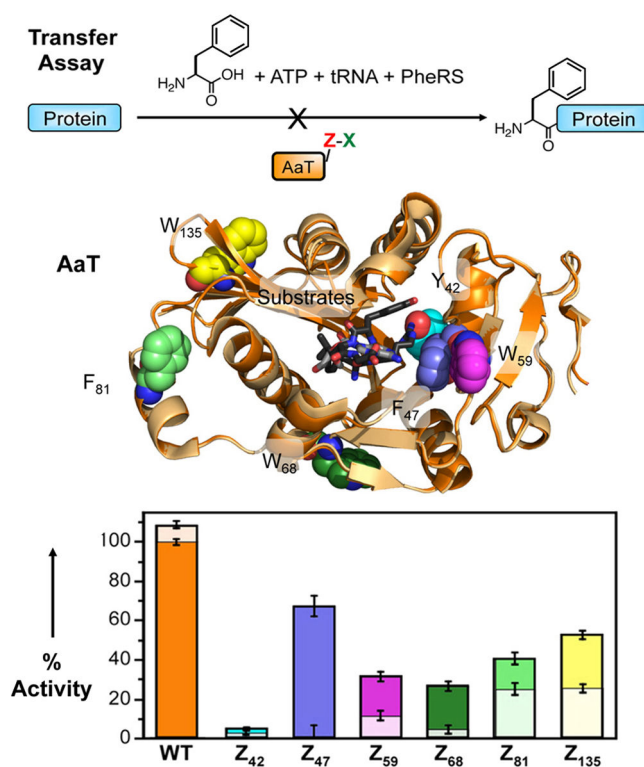
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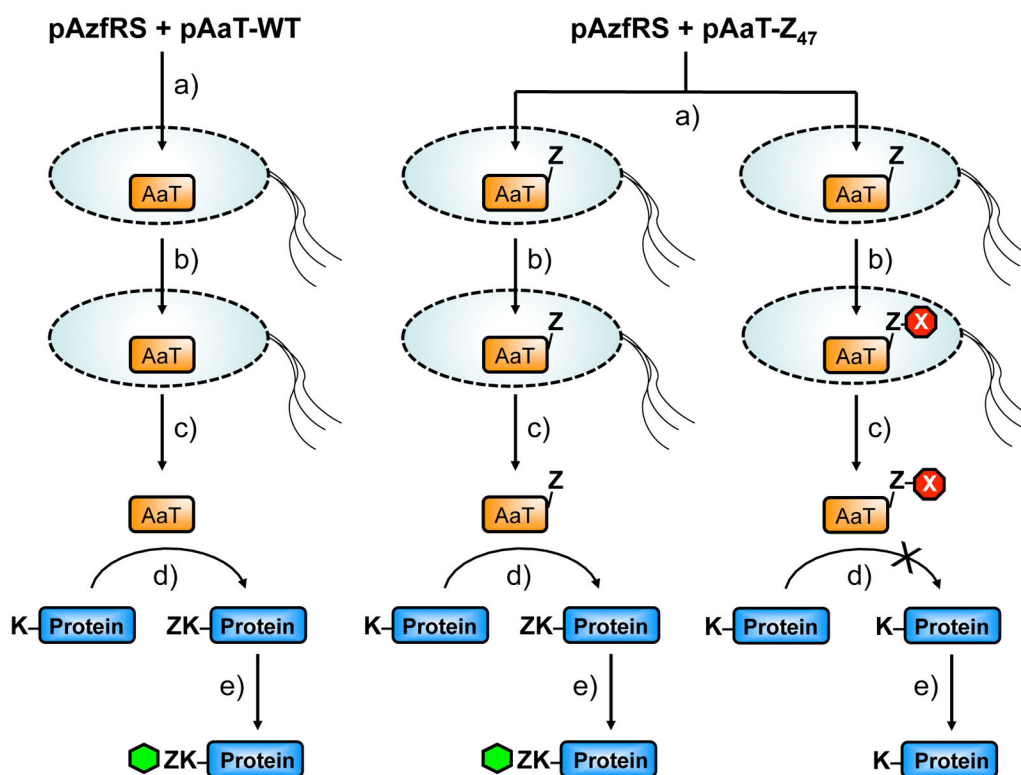
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**Figure 1.** Clickable Magic Bullet Strategy. Top: Replacing WT protein B with MBT protein B enables selective inhibition by reaction of Z with X. Inset: Allosteric inhibition through ZX reaction. Bottom: Cycloaddition of azidophenylalanine (Azf, Z) with dibenzocyclooctyne derivative (DBCO-R, X).

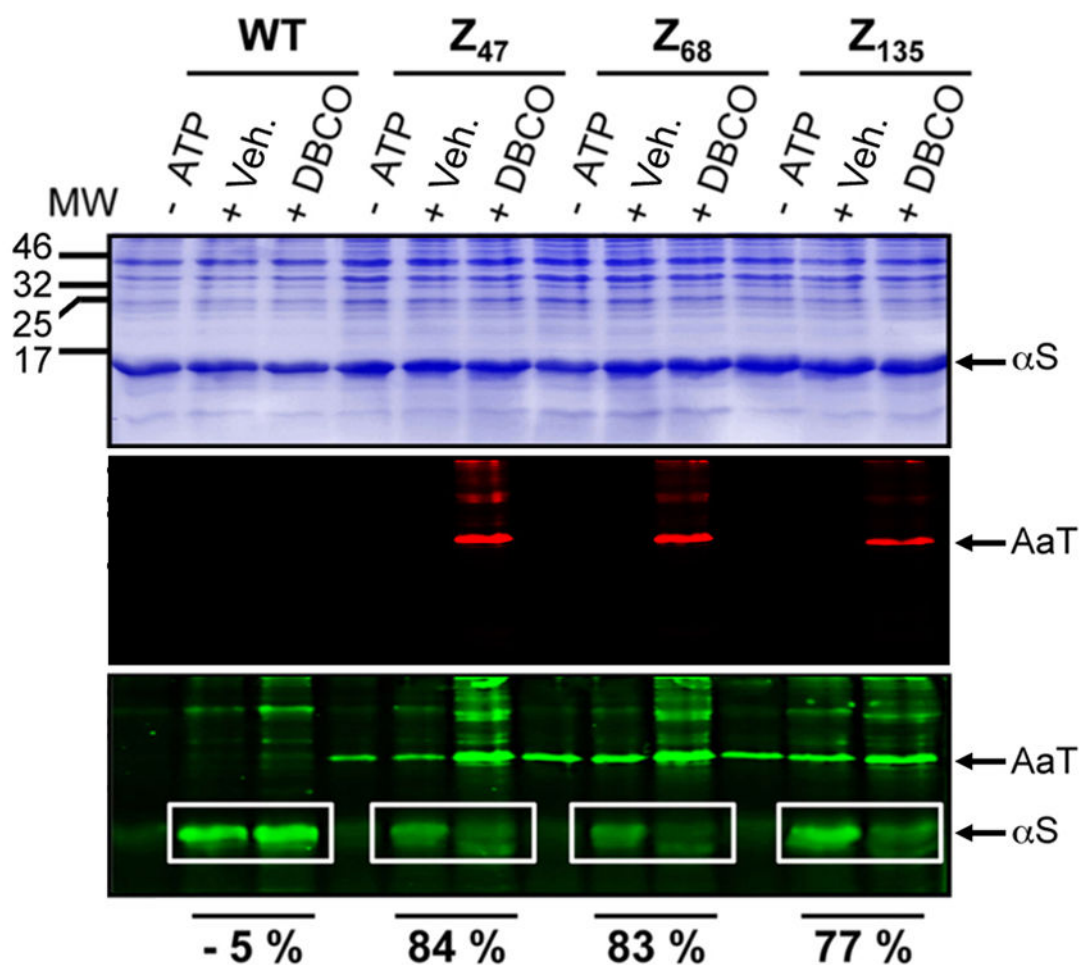


**Figure 2.** Analysis of AaT Inhibition by DBCO<sub>545</sub> Reaction. Top: Transferase activity assay uses tRNA aminoacylated *in situ* with Phe (for detection by HPLC). AaT activity is evaluated before and after reaction of AaT mutants with DBCO<sub>545</sub>. Middle: Structures of seven AaT AaT mutants showing the position of adenosine donor (gray), peptide product (black), and AaT substitution sites. Structures with aminoacyl adenosine (PDB ID: 2Z3K, orange) or peptide product (PDB ID: 2Z3N, cream) are overlaid.<sup>[19b]</sup> Bottom: Activity before (dark colors) and after (light colors) reaction with DBCO<sub>545</sub> evaluated based on conversion of LysAlaAcm in an HPLC assay (see Supporting Information).



**Figure 3.**

*In Vivo* Assay Protocol. Cells are transformed with an AzfRS plasmid and a WT AaT plasmid or a mutant AaT (e.g. Z<sub>47</sub>) plasmid and subjected to the following conditions: a) AaT production is induced with the addition of Azf and IPTG; b) Cells are pelleted, washed, and reacted with DBCO<sub>TMR</sub> or DMSO vehicle; c) Cells are grown again after resuspension; d) Cells are lysed and the lysates subjected to an Azf transfer assay using the αS reporter protein, *E. coli* AzfRS, and AaT; e) Azf transfer levels determined by Cu-catalyzed reaction with a fluorescein alkyne probe. WT control experiments determine the level of transfer activity that should be expected from uninhibited AaT as well as the levels of non-specific binding of the DBCO<sub>TMR</sub> reagent. Vehicle control experiments determine the level of transfer activity that should be expected from uninhibited AaT mutants (i.e., What is the effect of the Azf mutation alone?). Inhibition by the DBCO<sub>TMR</sub> reaction is determined by comparing the levels of labeling in the final step to the levels seen in the vehicle control.



**Figure 4.**

**AaT Inhibition in Cells.** BL21(DE3) cells expressing AaT WT and mutants were subjected to one of three conditions, + DBCO: AaT-expressing cells were treated with DBCO<sub>TMR</sub> in DMSO, then lysates of these cells were used to transfer Azf to the  $\alpha$ S reporter protein; + Veh.: AaT-expressing cells were treated with DMSO only, then lysates of these cells were used to transfer Azf to the  $\alpha$ S reporter protein; - ATP: AaT-expressing cells were treated with DMSO only, then lysates of these cells were used in a mock transfer assay in which ATP was withheld to prevent Azf transfer. In all cases, Azf transfer was detected with a fluorescein alkyne. Transfer yields calculated from the fluorescence intensity of  $\alpha$ S bands were used to determine percent inhibition (relative to the corresponding + Veh. lane in the white box). All three images depict the same gel, stained with Coomassie dye (top), imaged using 532 nm excitation (middle), or using 473 nm excitation (bottom). MW: Molecular weight markers (kDa). The protocols for the + DBCO and + Veh. experiments are shown in Figure 3.