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Specific Modulation of Protein Activity by Using a Bioorthogonal Reaction

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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 mutagenesis to insert an unnatural amino acid containing an azide group (Z) into the target protein at positions such that a "click" reaction with an alkyne modulator (X) will alter the function of the protein. This bioorthogonally 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 *Escherichia coli* enzyme aminoacyl transferase by both active-site occlusion and allosteric mechanisms. We have termed this a "clickable magic bullet" strategy, and it should be generally applicable to studying the effects of protein inhibition, within the limits of unnatural amino acid mutagenesis.

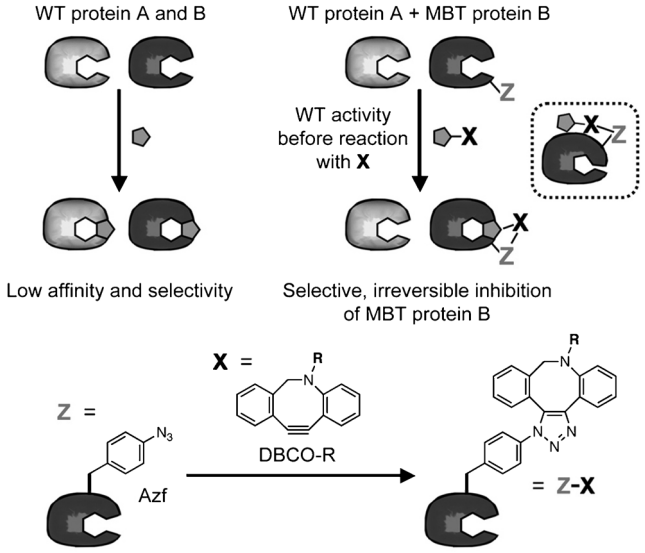

Figure 1. Clickable "magic bullet" strategy. Top: Replacing WT protein B with MBT protein B enables selective inhibition by reaction of Z with X. Inset: Allosteric inhibition through ZX reaction. Bottom: Cycloaddition of azidophenylalanine (Azf = Z) with the dibenzocyclooctyne derivative (DBCO-R = X).

Most pharmaceutical agents are designed to function as molecular entities acting on specific biological targets, what Erlich referred to as "magic bullets" (MBs).[1] A key early event in the development of any drug is target-validation: confirmation that modulation of a particular gene or protein will improve a disease phenotype.[2] 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.[3] However, there are limitations to each of these techniques, and in many cases a greater understanding of the biological pathways can be attained by using small molecules with high target specificity, even if they do not ultimately become drugs.[4] The field of chemical genetics encompasses an array of strategies to link genotypes and phenotypes using small molecules in model systems.[5] Here, we present a method designed to attain small-molecule specificity rapidly and simply using a combination of genetic manipulation and molecular design.

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, thus taking advantage of the strain-promoted cycloaddition of Azf and DBCO to rapidly and specifically attach X to the MBT protein.[6] We note that our general strategy is equally applicable to other bioorthogonal "click" chemistry reactive pairs such as cycloalkene/tetrazine.[7] Protein modulation should be specific, provided that the reactions are truly chemoselective and only the MBT protein contains the Z-group target. Different positions of the Z-group-targeting moiety can be evaluated to identify positions for which the Z moiety is not inherently destabilizing, that is, positions for which the MBT protein has near wild-type (WT) activity prior to reaction with an X molecule. The identified MBT mutants will be taken forward to studies in which they are reacted with X molecules and the phenotype of the modified protein will be evaluated.

The two previous chemical genetics experiments that are most relevant to our work are the "bump-and-hole" strategy employed by Schreiber, Shokat, and others,[8b,c] and the tethering approach of Erlanson et al.[8a] 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.[9] To be well implemented, this strategy generally requires prior structural knowledge and a deep binding pocket like an active site. Tethering takes place by reaction of an exogenous thiol group of a Cys residue to covalently attach small molecules to the surface of pro-

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teins.[8a,10] These molecules can act as allosteric modulators, so they do not need to reside in active sites.[11] Moreover, the Cys thiol group can be scanned across the surface of a protein in a screening protocol, so that careful, structure-based design is not necessary. However, as many proteins contain Cys residues, specificity derived from tethering is not easily retained in cell lysates or in vivo. Our method shares many features with these two strategies, but the ability to substitute any of the amino acids in a protein sequence with the Z group should make it much more broadly applicable than the bump-and-hole strategy, and the selectivity of click-chemistry 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.[12] Finally, it should be noted that unnatural amino acids, including those with bioorthogonal reactive handles, have been used extensively to probe protein structure and function, particularly in membrane proteins by Sakmar, Wang, and Dougherty.[13] However, none of these previous studies made use of the reactive handle as a general tool for perturbing protein function as we do here.

To incorporate Azf into the MBT proteins site-specifically, we can employ unnatural amino acid mutagenesis, developed by Wang and Schultz.[14] This method allows an unnatural amino acid to be inserted by using a 21st aminoacyl tRNA synthetase (aaRS) which charges a $tRNA_{CUA}$ that recognizes the UAG stop codon. Two plasmids were transformed into *Escherichia coli*: pAzfRS (previously referred to as $pDULE2_{Azf}$), encoding Azf aaRS and $tRNA_{CUA}$, and a plasmid encoding the MBT protein with a UAG mutation at the desired site of incorporation.[15] Following growth in the presence of Azf, the MBT protein is either purified for in vitro studies or reacted with DBCO reagents in vivo.

As a proof-of-concept, we have used the MB strategy to inhibit the *E. coli* aminoacyl transferase (AaT) protein.[16] 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.[17] 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.[18]

Using existing crystal structures of AaT,[19] we chose six locations for incorporation of Azf. These included three positions inside 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 predicted that replacement of these amino acids with Azf would minimize the perturbance to protein function prior to reaction with Azf. We expressed and purified His-tagged versions of each AaT mutant and assessed the ability of each mutant to react with X using DBCO rhodamine derivatives ($DBCO_{545}$ or $DBCO_{TMR}$, Figure S1 in the Supporting Information). Data from MALDI MS and PAGE analysis showed that for those positions taken forward for additional study, the labeling reaction was quantitative within the limits of MS detection (Table S1 and Figure S5). Next, we tested the activity of these Azf mutants prior to reaction with $DBCO_{545}$. Five mutants—Z47, Z59, Z68, Z81, and Z135—were found to have activities of at least 25% that of WT. The positions of these amino acids are shown in Figure 2. All five of these mutants were taken forward to inhibition assays. While it is surprising that Azf replacement of the 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.[19a] The reduction in activity on mutation of Y42 is less surprising as this residue forms a hydrogen bond with the Lys or Arg of the peptide substrate.[19b]

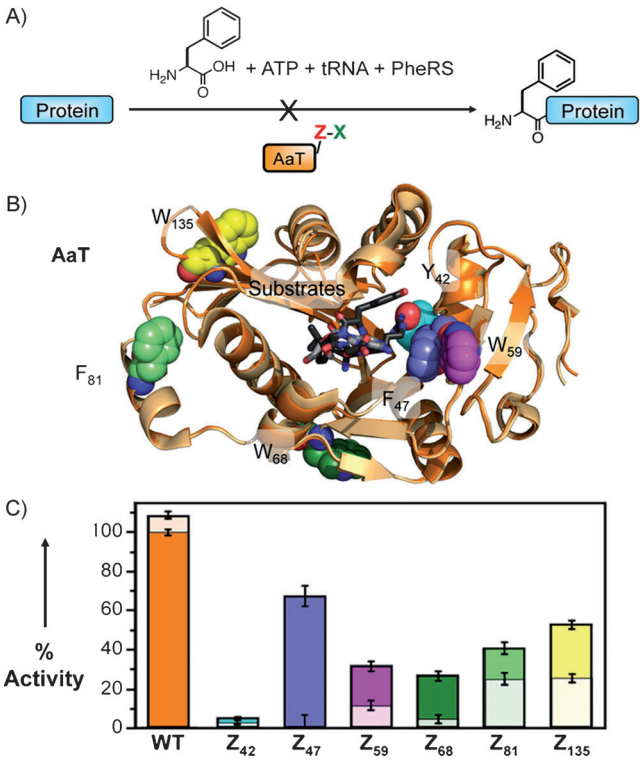

Figure 2. Analysis of AaT inhibition by reaction with $DBCO_{545}$. A) Transferase activity assay uses a tRNA that is aminoacylated in situ with Phe (for detection by HPLC). AaT activity is evaluated before and after the reaction of the Azf mutants with $DBCO_{545}$. B) Structures of seven AaT Azf mutants showing the position of the adenosine donor (gray), peptide product (black), and Azf substitution sites. Structures with aminoacyl adenosine (PDB ID: 2Z3K, orange) or peptide product (PDB ID: 2Z3N, cream) are overlaid.[19b] C) Transferase activity before (dark colors) and after (light colors) reaction with $DBCO_{545}$; evaluated based on conversion of LysAlaAcm in an HPLC assay (see Supporting Information).

All five mutants were inhibited by $DBCO_{545}$ treatment. The levels of inhibition that we observed varied from 52% for Z135 (relative to unmodified protein) to 98% for Z47. It is perhaps not surprising that Z47 was most inhibited, because this mutation occurs in the active site. Inhibition presumably occurs by simply occluding access to the active site. The Z59 modification would also block the active site. Inhibition of activity by Z68, Z81, or Z135 mutants, 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[19b] (Figure S9). The $DBCO_{545}$ and $DBCO_{TMR}$ adducts should not be able to reach the active site in either state of the enzyme. In WT AaT, W135 occurs at a crystallographic dimer interface, and some weak dimerization has been observed by gel electrophoresis analysis of purified AaT. However, our analysis of WT AaT indicates that the activity varies linearly with concentration (Table S2). Therefore, we believe that AaT is monomeric in the concentration range used herein, and that Z68, Z81, and Z135 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 six Cys residues. Blocking with iodoacetamide can be used to eliminate this reaction (Figure 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 had to carry out the reaction under conditions where the DBCO inhibitor is present at much higher concentrations than the enzyme so that a pseudo-first-order kinetic analysis could be applied.[20] Time-points must also be selected so that the irreversible reaction has not been allowed to reach completion. Under the conditions of our assay (1 μM AaT, 30 min), $DBCO_{545}$ has an apparent IC_{50} value of 8.5 μM for the Z47 mutant (Figure S8). Although a more rigorous analysis would be required to determine a true IC_{50} value, this is sufficient to show that inhibition is dose-dependent, provided that the assay time is comparable to the half-life for the $DBCO_{545}$ cycloaddition (i.e. hours).

Initially, we used $DBCO_{545}$ as an inhibitor because we could assess the levels of protein modification using the chromophore. However, modeling studies implied that for Z47, the rhodamine moiety was unnecessary because the DBCO moiety occupied the binding pocket and rhodamine extended into solution (Figure S9). Therefore, we also tested inhibition of the DBCO amine ($DBCO_A$) alone and saw 99% inhibition, similar to that obtained with $DBCO_{545}$. For the Z68 and Z135 mutants, $DBCO_A$ actually gave higher levels of inhibition than $DBCO_{545}$, 87 and 94%, respectively (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 the reaction of DBCO with AaT in cell lysates and in cells.

Next, we wanted 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 α-synuclein (αS) in lysates of TS351G *E. coli* cells, which lack endogenous AaT activity.[17b] Here, we determined the activity of AaT based on its ability to transfer Azf to the reporter protein from Azf-acylated tRNAs 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 crossreactivity of the click reagents is expected because of the order of addition. To make appropriate calculations of the 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. Because no Azf should be transferred in the −ATP control reactions, this allowed us to determine that the level of non-specific fluorescein binding was very low. Therefore, we calculated the degree of inhibition by comparing the levels of fluorescence in $DBCO_{545}$-treated lanes to those treated with DMSO. We chose the Z47, Z68, and Z135 mutants for further study. Z47 showed almost complete inhibition by active-site occlusion, and Z68 and Z135 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 Z47, 66% for Z68, and 73% for Z135. (Supporting Information, Figure S12) This shows that we can effectively inhibit AaT in the presence of the other proteins in the TS351G lysates by using 200 μM $DBCO_{545}$.

Finally, in order to demonstrate inhibition of AaT mutants in living cells, we reacted $DBCO_{TMR}$ (which we expected to be more cell permeable than $DBCO_{545}$ or $DBCO_A$) with AaT in intact *E. coli* cells, and then analyzed activity in crude lysates as described above. Our method 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

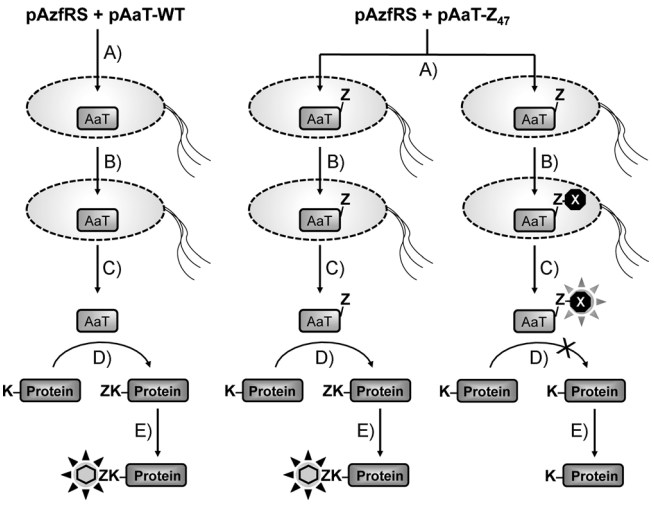

Figure 3. In vivo assay. Cells are transformed with an AzfRS plasmid and a WT AaT plasmid (left) or a mutant AaT (e.g. Z47) plasmid (right) and subjected to the following conditions: A) AaT production is induced with the addition of Azf and IPTG; B) Cells are pelleted, washed, and reacted with $DBCO_{TMR}$ 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 are determined by using a Cu-catalyzed reaction with a fluorescein alkyne probe. WT control experiments determined the level of transfer activity that should be expected from uninhibited AaT as well as the levels of non-specific binding of the $DBCO_{TMR}$ reagent. Vehicle control experiments determined 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 $DBCO_{TMR}$ reaction was determined by comparing the levels of labeling in the final step to the levels seen in the vehicle control.

IPTG, the cells were pelleted, washed repeatedly to remove unincorporated Azf, and then treated with a $DBCO_{TMR}$ 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 the steps, no cross-reactions are expected. Also, note that BL21(DE3) cells were used for this experiment because we observed little endogenous AaT activity in these cells as compared to overexpressed AaT (data not shown) and we only obtained low yields of protein containing the unnatural amino acid in TS351G cells.

We successfully inhibited all three Azf-tagged AaT proteins by using in vivo treatment with $DBCO_{TMR}$ (Figure 4). Because

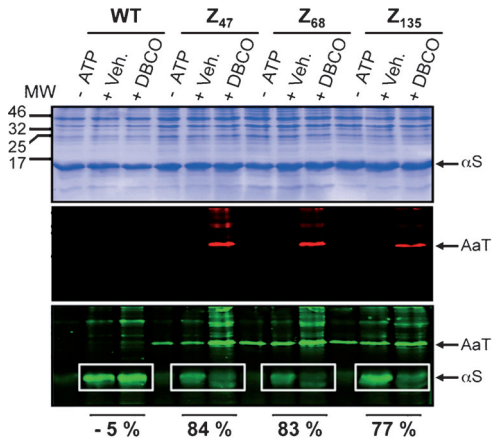

Figure 4. AaT inhibition in cells. BL21(DE3) cells expressing AaT WT and mutants were subjected to one of three conditions, +DBCO = AaT-expressing cells treated with $DBCO_{TMR}$ in DMSO; lysates of these cells were used to transfer Azf to the αS reporter protein; +Veh. = AaT-expressing cells treated with DMSO only; lysates of these cells were used to transfer Azf to the αS reporter protein; −ATP = AaT-expressing cells treated with DMSO only; 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 by using a fluorescein-alkyne. Transfer yields were calculated from the fluorescence intensity of the αS bands and were used to determine the 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 with $\lambda_{ex} = 532$ (middle) or $\lambda_{ex} = 473$ nm (bottom). MW = molecular weight markers (in kDa). The methods for the +DBCO and +Veh. experiments are shown in Figure 3.

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 $DBCO_{TMR}$ 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 labeling of other proteins (possibly endogenous AaT substrates) with the fluorescein alkyne probe, analysis of the αS bands allowed us to assay for AaT activity.

By comparing the +DBCO and +Veh. lanes (Figure 4), significant inhibition of AaT activity was observed in all cases: 84% for Z47, 83% for Z68, and 77% for Z135. This shows that AaT was effectively inhibited by dosing $DBCO_{TMR}$ in intact *E. coli* cells. Moreover, we can track the selectivity of the DBCO reagent by analyzing the red fluorescence channel. Relatively low levels of nonspecific binding were seen 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. Notably, the green bands at the bottom of the gel correspond to fluorescein labeling of unreacted Azf in AaT mutants that had not been treated with $DBCO_{TMR}$. This shows that Azf was present in the active site of these AaT samples (+DBCO lanes appear to have higher background fluorescence owing to incomplete filtering of TMR emission, excited with 473 nm light). Thus, we saw specific, DBCO-dependent inhibition of Azf mutants of AaT at levels comparable to or greater than those seen for the purified enzyme. However, it must be noted that this method still needs to be optimized to make it a truly in vivo technology. While the DBCO reaction was carried out in cells, the assay was carried out in cell lysates, as is common for AaT; this is because AaT inhibition does not produce a clear phenotype (even the TS351G AaT-deletion cell line does not exhibit an obvious phenotype).[13,17b,21]

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-based techniques for target validation. While it is more complex to implement than RNAi, it offers validation in the context of a dosable small molecule. Because many small interfering RNAs (siRNAs) act catalytically, controlling the dosage can be challenging.[22] A few additional advantages of our method versus an RNAi-based method are worth noting. First, RNAi prevents translation of the protein of interest, which may lead to phenotypes resulting from the absence of multiple, independent protein interactions rather than inhibition of one specific activity. Such changes would be hard to replicate with a small-molecule or protein therapeutic, which typically bind to only one face or pocket of the target protein. Second, although we have only found examples of inhibitors 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, thus activators could not be found.

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 siRNAs in the appropriate cell lines. Careful usage of codons can prevent the miRNA from targeting the MBT as well as the WT protein.[23] 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 to coincide with expression of the replacement MBT protein.

3) The UAG stop codon, though rare (~5% in *E. coli*), is present in many natural open reading frames (~300).[24] 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 $tRNA_{CUA}$ does not efficiently deliver the unnatural amino acid at the UAG stop codon, high levels of truncated protein may be synthesized, which would result 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 might not be optimal for this application. Although the fact that we are able to achieve nearly 100% inactivation of the Z47, Z68, and Z135 mutants implies that the cycloaddition goes to completion with respect to the protein, this will not be true for many residues in many proteins.[25] 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.[7a]
6) The ultimate goal of connecting the genotype to a 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.

Despite these potential complications, the ability to rapidly generate a protein that can be selectively targeted 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.[26] Although we used an enzyme with a known crystal structure in this proof-of-principle experiment, this is not required. The protein sequence could be scanned by the unnatural amino acid, analogous to an Ala scan, and then the activity of the protein simply assayed to determine which positions are non-perturbed prior to reaction with the small molecule. Herein, our method has been limited to a single-celled model organism, *E. coli*, however, unnatural amino acid mutagenesis has also been demonstrated in mammalian cells as well as multicellular organisms such as *Caenorhabditis elegans* and *Drosophila melanogaster*.[27] Moreover, alternate coding strategies have been employed leading to efficient incorporation at four base codons, which might address issues 3) and 4) listed above.[27a,28] Schultz, Church, Isaacs, and co-workers have also generated a strain of *E. coli* with the TAG stop codons replaced by TAA codons.[29] We will explore applying these techniques to continue to improve our method. We have begun to apply our method to other systems including membrane transport proteins implicated in drug resistance and aggregating proteins like α-synuclein. These types of gain-of-function systems may be more amenable to our technique because 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_{10}-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.[17b] Briefly, *E. coli* cells were grown in a primary culture of LB (4 mL) at 37 °C until they achieved an OD_{600} of 0.5. This culture was then diluted into a secondary culture of LB (500 mL) and grown to an OD_{600} of 0.6–0.8. AaT expression was induced using isopropyl β-D-thiogalactoside (IPTG; 0.1 mM) and cells were grown at 25 °C for 16 h. Cells were pelleted at 6000 rpm using a centrifuge (Sorvall RC-5, GS3 rotor). Cell pellets were resuspended in Ni-NTA binding buffer (50 mM Tris, 10 mM imidazole, 300 mM KCl, pH 8.0) that included a protease inhibitor cocktail, phenylmethylsulfonyl fluoride (PMSF, 1 mM), and DNAse1-Grade II (10 units/mL). Following resuspension, the cells were lysed by using sonication. Soluble proteins were collected by centrifugation at 14227 *g* for 15 min. Collected soluble protein was gently shaken for 1 h on ice with Ni-NTA Superflow resin (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, 300 mM KCl, pH 8.0). Fractions containing *E. coli* AaT were dialyzed overnight in transferase buffer (50 mM HEPES, 30% glycerol, 120 mM $(NH_4)_2SO_4$, 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_{XX}). The sequences and mutagenic primers are shown in Figure S3. BL21-Gold (DE3) cells were transformed with the pAaT-Z_{XX} plasmid and a pAzfRS (previously referred to as $pDULE2_{Azf}$) plasmid containing genes for an *M. jannaschii* mutant tRNA synthetase and $tRNA_{CUA}$ pair. Cells were selected using ampicillin (100 mg L^{-1}) and streptomycin (100 mg L^{-1}). Azf was incorporated into AaT as described above with the addition of Azf (1.0 mM, solubilized by dropwise addition of 1 M NaOH) to the growth media. When working with Azf, cultures and purified proteins were kept shielded from the light.

AaT cyclooctyne labeling: Ni-NTA-purified AaT and AaT-Z_{XX} mutants (0.20 mg mL^{-1}) were incubated with $DBCO_A$, $DBCO_{545}$, or $DBCO_{TMR}$ (200 μM final concentration) from a DMSO stock for 2 h at 37 °C. As a vehicle control, AaT and AaT-Zxx mutants were also treated with an equal volume DMSO. Excess DBCO reagent was removed by dialyzing twice in transferase buffer (50 mM Tris, 30% glycerol, 120 mM $(NH_4)_2SO_4$, 1 μM β-ME, pH 8.0). Labeled proteins

were then analyzed by using PAGE or trypsin digest followed by MALDI-MS (as described in the Supporting Information) and stored at −80 °C.

Activity of AaT using LysAlaAcm ligation assay: The activity of DMSO- and $DBCO_{TMR}$-treated purified AaT and AaT-Z_{XX} mutants were assayed by transfer of Phe to the N terminus of a LysAlaAcm peptide (Bachem; Torrance, CA, USA) as previously described.[18b] Each ligation reaction (125 μL total volume) contained the following reagents: Phe (1 mM), *yeast* Phe tRNA (12.5 μg), adenosine-5′-triphosphate (ATP, 1.25 mM), *yeast* Phe tRNA aminoacyl synthetase (yPheRS, 2.5 μg), AaT (1 μM), and LysAlaAcm (100 μM) in the AaT ligation buffer (50 mm HEPES, 50 mM Tris, pH 8.0, 150 mM KCl, 10 mM $MgCl_2$, 30% glycerol; 120 mM ammonium sulfate). The reaction mixtures were incubated at 37 °C for 4 h and quenched with 1% acetic acid. Proteins were removed from the peptides by washing with four volumes of acetone and cooled at −20 °C for 1 h. The reactions were centrifuged at 14227 *g* at 4 °C for 20 min to separate the peptides from the precipitated proteins. The supernatant was transferred to a centrifuge tube (1.5 mL) 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 min. The resulting reaction volume was dissolved in a total volume of 1 mL in acetonitrile/water (1:9, 0.1% TFA) and analyzed by HPLC (see Supporting Information for details) to determine the ligation efficiency by integrating the peak intensities for PheLysAlaAcm and LysAlaAcm (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_{XX} plasmids using ampicillin and streptomycin. A primary culture (4 mL) was used to inoculate a secondary culture (100 mL). Secondary cultures were grown to an OD_{600} of 0.6 and then protein expression was induced with Azf (1.0 mM) and IPTG (0.1 mM) for 16 h at 25 °C. Cell pellets were harvested at 6000 rpm for 10 min using a centrifuge (Sorvall RC-5, GS3 rotor). Cell pellets were washed three times with wash buffer (50 mM Tris, 300 mM KCl, pH 8.0) to remove excess Azf. Washed cell pellets were resuspended in wash buffer (2 mL) and incubated with either DMSO or $DBCO_{TMR}$ (500 μM) at 37 °C for 2 h with 250 rpm shaking. Cells were then washed three times with wash buffer containing DMSO (1%) and then washed once with wash buffer. Cell pellets were then resuspended in wash buffer (1 mL) and sonicated (one minute on then one minute off) three times. Lysed cells were stored on ice until further use. $\alpha S_{2-140}K_2$ protein (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 with a modified AaT buffer (50 mm HEPES, pH 8.0, 150 mM KCl, 10 mM MgCl2) and AaT (1.25 μg). ATP was omitted and replaced with an equivalent volume of MilliQ water for the negative controls. The reaction mixtures were incubated at 37 °C for 2 h. The crude reaction mixture (12.5 μL) was then labeled with fluorescein-alkyne (33 μM) using tris(3-hydroxypropyltriazolylmethyl)amine (THPTA, 3.546 mM), $CuSO_4$ (681 μM), and sodium ascorbate (5.4 mM) at 37 °C for 1 h. The reaction mixture was then heated in gel loading dye LDS (5 μL, Pierce) for ten minutes at 95 °C and analyzed by SDS-PAGE. Fluorescence images were obtained by using a Typhoon FLA 7000 scanner. Fluorescein was imaged by using an excitation wavelength (λ_{ex}) of 473 nm and a Y520 filter. TMR was imaged by using λ_{ex} = 532 nm and an O580 filter. Images were collected by using a 100 μM pixel size. Total protein content was visualized with Coomassie Brilliant Blue dye.

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