

Precise Probing of Residue Roles by Post-Translational β,γ -C,N Aza-Michael Mutagenesis in Enzyme Active Sites

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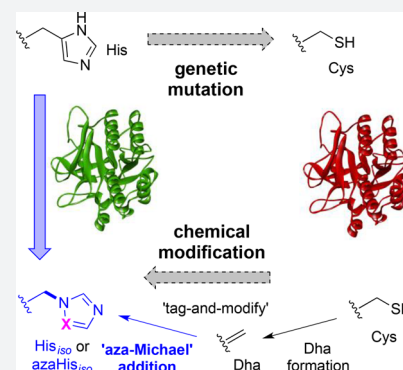
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Supporting Information

ABSTRACT: Biomimicry valuably allows the understanding of the essential chemical components required to recapitulate biological function, yet direct strategies for evaluating the roles of amino acids in proteins can be limited by access to suitable, subtly-altered unnatural variants. Here we describe a strategy for dissecting the role of histidine residues in enzyme active sites using unprecedented, chemical, post-translational side-chain- β,γ C–N bond formation. Installation of dehydroalanine (as a “tag”) allowed the testing of nitrogen conjugate nucleophiles in “aza-Michael”-1,4-additions (to “modify”). This allowed the creation of a regioisomer of His (iso-His, His_{iso}) linked instead through its pros-N π atom rather than naturally linked via C4, as well as an aza-altered variant aza-His_{iso}. The site-selective generation of these unnatural amino acids was successfully applied to probe the contributing roles (e.g., size, H-bonding) of His residues toward activity in the model enzymes subtilisin protease from *Bacillus lentus* and *Mycobacterium tuberculosis* pantothenate synthetase.



INTRODUCTION

Covalent protein chemistry provides well-appreciated opportunities in protein labeling^{1–3} and the attachment of cargoes.⁴ It also has great potential in allowing the creation of subtle changes and in the creation of precise alterations and functional mimics in proteins, beyond the limits of traditional biology, that in turn can allow deeper understanding of protein mechanism.^{5,6} Among one of the most intriguing strategies is a notion of alteration to test recapitulation of (e.g., enzymatic) activity through the replacement of a “lost” functional group with a variant (sometimes known as chemical “rescue”).^{7,8} This has been applied in various ways including, for example, the rescue by a noncovalently associated prosthetic^{9,10} (that may have relevance to more general activation by small molecules^{11,12}); or by the “uncaging” of encoded “caged” unnatural amino acids.^{13–15} As a form of “covalent chemical rescue”, chemical modifications of amino acid residues in enzyme active sites have primarily explored cysteine (Cys) aminoethylation to mimic lysine (Lys) (e.g., ribulosebiphosphate carboxylase/oxygenase,¹⁶ aspartate aminotransferase,¹⁷ leader peptidase,¹⁸ aldolase,¹⁹ topoisomerase,²⁰ sugar lyase,²¹ and pantothenate synthetase²²) with other, more rare, examples of attempted mimicry of arginine (Arg),²³ glutamate (Glu),²⁴ and even histidine (His).^{25–27} However, most of these mimics differ in critical parameters such as size, constitution, and/or side-chain length. To our knowledge, no strategies for mimicking amino acids as their direct regioisomers have been possible to date;

these would have the potential to act as precisely different isosteres allowing careful dissection of contributing functions. Here, we create and test the effectiveness of the first such regioisomer of His in proteins, using a variant that is linked regioisomerically through its pros-N π atom (iso-His, His_{iso}) rather than naturally linked via C4 (Figure 1a) and its aza-analogue (aza-His_{iso}).

RESULTS

We chose to explore the effect of chemical mutagenesis on enzyme activity through conversion via cysteine (Cys) to the His-mimic residue iso-histidine (His_{iso}, Figure 1). His can be a sensitive catalytic residue in several enzymes,²⁸ and Cys would provide a dramatically different side chain in a logical mutational analysis pathway: e.g., His → Cys → His_{iso} (Figure 1a). We reasoned that His_{iso} (and variants) could in principle be introduced site-selectively using a “tag-and-modify” approach²⁹ (Figure 1b) based on the installation of dehydroalanine (Dha) (potentially from cysteine (Cys)³⁰) followed by conjugate Michael-type addition. Until now, addition of sulfur nucleophiles to Dha (“thio-Michael-type” additions)³¹ has been the dominant use of this unnatural amino acid residue in proteins, including use in “chemical rescue”.²¹ However, other potential nucleophiles, such as amines, could

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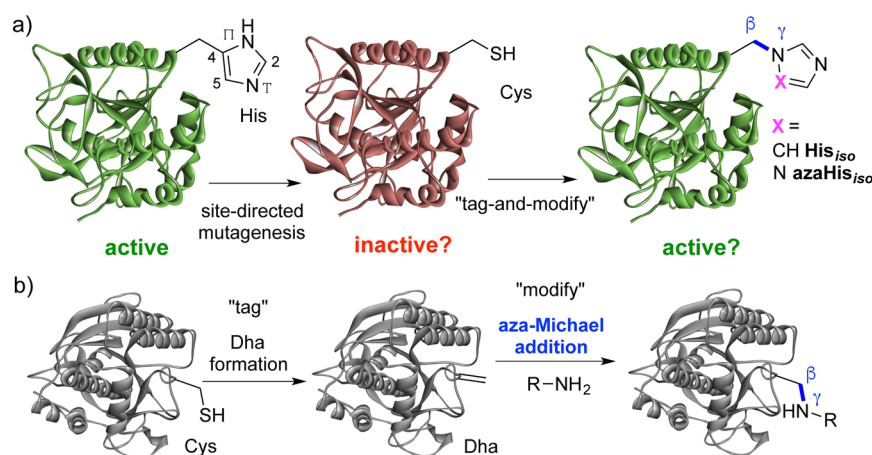


Figure 1. (a) Concept of protein activity probing via post-translational mutagenesis to regioisomeric His_{iso} or aza-His_{iso} in a protein (e.g., enzyme active site). (b) Proposed “tag-and-modify” approach to the installation of His_{iso} or aza-His_{iso} based on dehydroalanine (Dha) formation followed by novel β,γ,C,N aza-Michael addition.

be considered (“aza-Michael-type” additions, Figure 1b), thereby opening the door to this new form of protein modification.³² We report here a validation of this approach on intact proteins through the first examples of β,γ,C,N bond formation.^{33–35} Prior examples of aza-Michael additions, attempted only on extended acrylamido/vinylsulfonamido motifs, are rare³⁶ and would not allow the precise mutagenesis via β,γ,C,N bond formation intended here. While aza-Michael reactions have typically required essentially protein-incompatible conditions (harsh catalysis³⁷ and/or the use of organic solvents^{38,39}), the noted beneficial effect of aqueous media upon rate⁴⁰ and the success of vinylsulfonamides³⁶ suggested some potential.

We tested the proposed β,γ,C,N “aza-Michael” addition of imidazole to Dha on intact proteins using the subtilisin from *Bacillus lentus*⁴¹ (SBL, EC 3.4.21.62). SBL is a suitably robust model protein for this approach; it has no native Cys allowing the positioning of Dha via the chemical conversion of a Cys residue introduced through mutagenesis. Thus, the single cysteine mutant SBL-S156C (Ser156 → Cys156) was used to generate SBL-Dha156 using 2,5-dibromohexanediamide (DBHDA).³⁰ Site 156 sits at the base of the S₁ (Schechter–Berger nomenclature⁴²) pocket of the active site. Gratifyingly, full conversion to the desired SBL-S156His_{iso}, demonstrating the first examples of β,γ,C,N bond formation on proteins, was observed after incubation of SBL-S156Dha with imidazole for 5 h at 37 °C (Figure S1). Importantly, determination of the activity of SBL-His_{iso}156 revealed that the conditions of installation were compatible with maintaining protein function. Prior studies have allowed chemical installation of bulky charged groups⁴³ or classical mutagenesis to His at this site,^{44,45} through the determination of kinetic parameters (SBL-His_{iso}156, $k_{\text{cat}} = 122 \pm 6 \text{ s}^{-1}$; $K_{\text{M}} = 0.71 \pm 0.06 \text{ mM}^{-1}$; SBL-wt, $k_{\text{cat}} = 153 \pm 4 \text{ s}^{-1}$; $K_{\text{M}} = 0.73 \pm 0.05 \text{ mM}^{-1}$), it was confirmed that this site, where His_{iso} is essentially isosteric to His without H-bond donation, has a minimal role in activity determination, consistent with an outward facing disposition.⁴⁶

Next, *M. tuberculosis* pantothenate synthetase (PanC; EC 6.3.2.1)⁴⁷ was selected as a model protein to probe the proposed His-to-His_{iso} activity “modulation” (Figure 1a). PanC catalyzes the ATP-dependent condensation of D-pantoate and β -alanine forming pantothenate (Figure 2a).⁴⁷ Biosynthesis of pantothenate (vitamin B5) is essential for the growth of

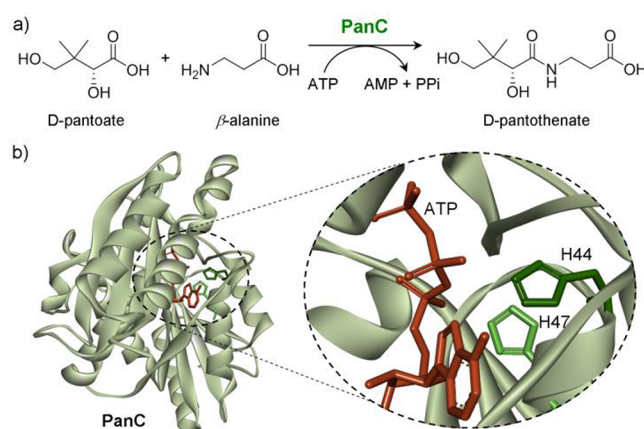


Figure 2. (a) The condensation of D-pantoate with β -alanine catalyzed by pantothenate synthetase (PanC). (b) PanC active site image highlighting His47 and His44 and their proposed interaction with the α and β phosphates of ATP, respectively, during catalysis [derived from PDB 1N2E (ref 48)].

bacteria, yeast, and plants and, in particular, for virulence of *M. tuberculosis*; it is thus a potential therapeutic target.⁴⁸ In the catalytic site of PanC, histidines His44 and His47 were identified as having a proposed⁴⁹ critical role in ATP binding (Figure 2b); prior mutation of His → Ala in either site led to decrease in activity.²² These therefore presented potentially sensitive sites at which to test regioisomeric His_{iso} mutation. Notably, both sites are buried (cf. the more surface-exposed SBL-156 site) and would provide a more stringent test of the His_{iso} formation strategy.

Dha was introduced to position 44 or 47 of PanC protein by site-directed mutagenesis first to Cys44 or Cys47 to give PanC-H44C or PanC-H47C. We then explored chemical conversion to Dha through bis-alkylation/elimination reagent. Wild type PanC-wt has no native cysteine, which, as for SBL, we envisaged would enable full control of the site of Dha formation. Consistent with literature reports,²² PanC proved a challenging substrate protein, strikingly susceptible to unfolding and precipitation: this necessitated a novel strategy for Dha generation. Typical Dha-forming reaction with DBHDA³⁰ (Figure 3, R₁ = R₂ = CONH₂) or the use of temperatures >25 °C over prolonged periods with any reagents

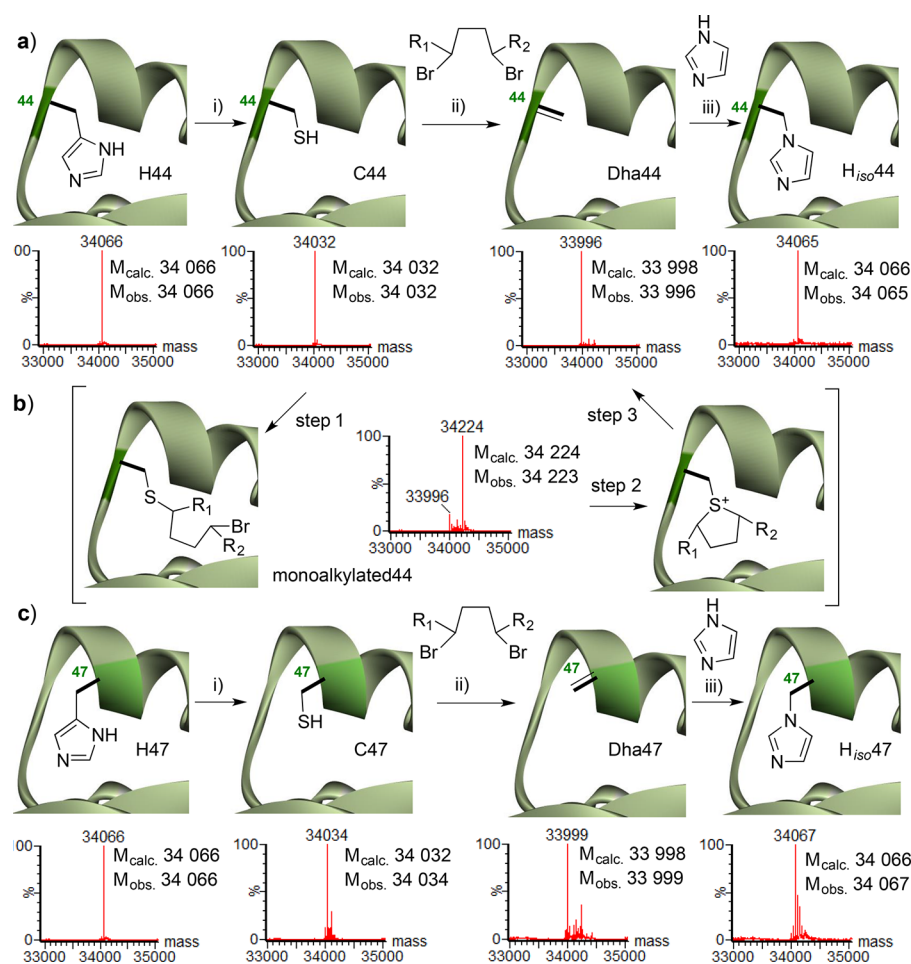


Figure 3. Creation of His_{iso} in PanC at sites (a) 44 and (c) 47 using a novel “interrupted” elimination strategy to generate Dha: step 1 monoalkylation and purification in this interrupted state prevented loss of this sensitive protein; then steps 2 and 3 were simply performed by incubation to give Dha. (b). Conditions: (i) site-directed mutagenesis; (ii) 50 equiv of dibromide, 25 °C, aqueous buffer (NaPi 50 mM, pH 8.0); DBHDA, R₁ = R₂ = CONH₂; MDBP, R₁ = COOMe, R₂ = H, then gel filtration, then incubation 25 °C; (iii) imidazole (aqueous), pH 9–10.

proved detrimental to PanC (as judged by CD spectroscopy (Figure S5)). It was therefore not possible to directly chemically introduce Dha into this sensitive protein using standard methods.

Methyl 2,5-dibromopentanoate (MDBP)⁵⁰ (Figure 3, R₁ = COOMe, R₂ = H), however, proved both sufficiently reactive with PanC and advantageously soluble in aqueous buffer so as to allow rapid monoalkylation. This allowed us to consider the exploitation of a rate-limiting second alkylation step to enable isolation of intermediates not typically accessible with DBHDA. This, in turn, allowed us to develop a novel, “interrupted” alkylation–elimination method (Figure 3b) as a solution to the problems of accessing Dha: first, rapid monoalkylation (prior to purification away from conditions detrimental to protein stability), followed by, second, simple prolonged incubation to allow sulfonium formation and elimination to Dha. Thus, with only 50 equiv of MDBP complete monoalkylation (step 1, Figure 3b) of PanC-H44C was observed after 45 min at 25 °C (Figure S6). Removal of excess MDBP by gel filtration at this monoalkylated state successfully ablated protein precipitation in later steps and then allowed incubation at 25 °C for 16 h with concomitant full conversion to PanC-H44Dha via intramolecular alkylation–elimination (steps 2 and 3, Figure 3b, and Figure S7). In this way, even these highly sensitive proteins proved tractable substrates. Moreover, an essentially identical

“interrupted” approach also allowed the site variant PanC-H47Dha, in which site 47 is part of a helix and thus more buried, to be generated (Figure 3c). Thus, PanC-H47C was fully monoalkylated after 16 h incubation with MDBP (50 equiv), purified, then incubated, and hence, gratifyingly, fully converted after excess reagent removal and incubation (48 h at 25 °C, Figure 3c and Figure S8) to PanC-H47Dha.

Next, the reactivities of these relatively buried sites (Dha44 and Dha47) were tested. First, “thio-Michael-type” addition of β -mercaptoethanol to PanC-H44Dha allowed complete conversion to the desired adduct (Figure S9), thereby confirming both accessibility and electrophilic reactivity of Dha44 in PanC. Then, β,γ -C,N “aza-Michael” addition of imidazole to PanC-H44Dha and PanC-H47Dha was initially tested at 25 °C due to the noted instability (Figure S5). Pleasingly, initial examples of aza-Michael reaction at pH 9–10 were observed to PanC-H44H_{iso} and PanC-H47H_{iso}; however these required high excesses of imidazole and long reaction times (3 days) and typically resulted in only partial or variable conversions (50 to >95%, Figure S10). Despite instability over long periods, shorter reaction times at 37 °C proved successful (Figure 3a,b). It also proved possible to install an aza-analogue to create PanC-H44azaH_{iso}, simply through variation of the nucleophile to 1,2,4-triazole (>80% conversion, Figure S14).⁵¹ The precise site locations of installation of His_{iso} and aza-His_{iso} in PanC-

Table 1. Steady-State Kinetic Parameters of Wild-Type and “Chemical Mutants” of *M. tuberculosis* PanC^a

entry	PanC	k_{cat} [s^{-1}]	D-pantoate		β -alanine	
			K_{M} [mM]	$k_{\text{cat}}/K_{\text{M}}$ [$\text{s}^{-1}\cdot\text{M}^{-1}$]	K_{M} [mM]	$k_{\text{cat}}/K_{\text{M}}$ [$\text{s}^{-1}\cdot\text{M}^{-1}$]
1	wild-type	1.6 ± 0.1	1.3 ± 0.1	1219	0.61 ± 0.01	2598
2	H44C	0.011 ± 0.001	0.5 ± 0.1	22	0.03 ± 0.02	368
3	H44Dha	0.020 ± 0.003	1.1 ± 0.3	19	0.106 ± 0.008	193
4	H44H _{iso}	0.08 ± 0.01	nd ^b	nd ^b	0.16 ± 0.09	500
5	H44azaH _{iso}	0.0123 ± 0.002	nd ^b	nd ^b	0.12 ± 0.01	102

^a25 °C, 100 mM Hepes (pH 7.8), 10 mM MgCl₂, 10 mM ATP, [D-pantoate] or [β -alanine] is 0.01–5 mM while the other is saturated and constant at 5 mM. Coupled assay with 1 mM phosphoenolpyruvate, 200 μM NADH, myokinase, pyruvate kinase, and lactate dehydrogenase (18 U/mL each). ^bnd = not determined due to an inability to determine a K_{M} using concentration range <5 mM, and the associated β -alanine. K_{M} is thus a K_{Mapp} ; see Supporting Information.

H44H_{iso}, PanC-H44azaH_{iso}, and PanC-H47H_{iso} (as well as stability to enzymatic digestion) were fully confirmed by peptide mapping using proteolytic digest followed by LC–MS/MS analysis (Figures S11, S12, and S14). Notably, attempted use of a variety of substituted imidazoles under essentially identical reaction conditions (Figure S13) failed, suggesting that only something isosteric to His (such as His_{iso} or aza-His_{iso}) could be accommodated at either site and highlighting the highly hindered nature of these sites. Interestingly, lower conversions were observed (Figure S15) for unoptimized test reactions with other *N*-heterocycles, suggesting that electronics (with potential influence upon nucleophilicity and/or basicity) may also play an important role in such reactions.

Having successfully installed His_{iso}, even into these buried sites, we next tested its effect upon the enzyme activity of PanC. Kinetic parameters for PanC variants were determined by coupled spectrophotometric assay (Table 1 and Figure S16–18).⁵² Regioisomeric His_{iso}, which lacks hydrogen bond donor character, allows examination of hydrogen bonding; His44's primary role has been suggested as such a hydrogen bond donor. The measurement of these steady state parameters in the Bi-Uni-Uni-Bi Ping-Pong mechanism suggested for PanC²² requires some caution with direct mechanistic interpretation, but k_{cat} reports directly on ADP formation allowing interrogation of the first half-reaction, the reversible formation of adenylyl-pantoate·ADP. Mutation of His44 to Cys44 or Dha44 reduced this activity to 1–2%, as judged by k_{cat} to levels consistent with prior analyses for Ala44.²² Through the creation of PanC-H44H_{iso} we were able to probe the effect of altered function through this regioisomeric, “chemical mutation”. Thus, while His_{iso} is a poor substitute for His in PanC, its insertion apparently creates some activity (as judged by k_{cat}), ~5% of that of wt. Notably, insertion of the aza variant in PanC-H44azaH_{iso} gives only the lower levels of activity seen for Ala, Cys, or Dha.

To probe the potential structural implications of the intermediate activity that was observed only for His_{iso} further, we conducted a series of molecular dynamics simulations (Figure 4 and Figures S21–25), based on the available X-ray structure.⁴⁸ Three cases were considered: the wt enzyme and the His44His_{iso} and His44Cys mutants (Figure 4). Both neutral and protonated forms were considered for His44His_{iso}. Stable Michaelis complexes were readily obtained for each complex after 40 ns; no substantial changes were seen upon increasing the simulation time. The simulations of the wt enzyme show that the side chain of His44 forms a *tele*-N–H···O–ATP hydrogen bond with the β -phosphate (Figure 4, top left). This interaction is still present in the His44His_{iso} mutant if His_{iso}44 is protonated (Figure 4, top right). However, only a small population of the protonated form would be expected to be

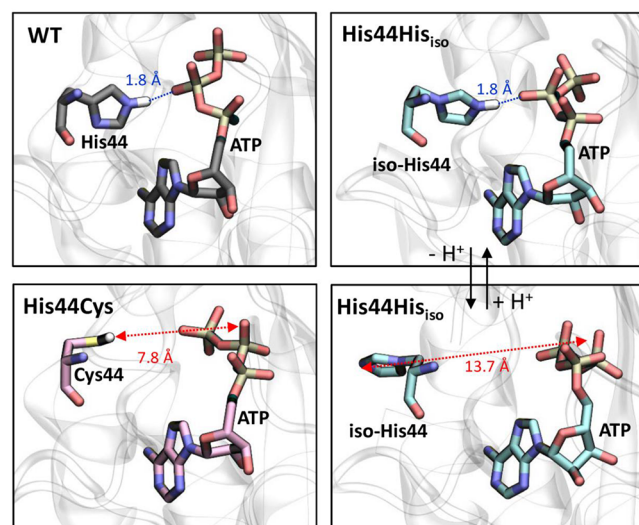


Figure 4. Representative structures obtained from molecular dynamics simulations (40 ns) of the wt enzyme (top left) and the mutants of the His44 residue to Cys (bottom left), neutral iso-His (bottom right), and protonated iso-His (top right).

present at the working pH, and the predominant form (neutral His_{iso}) preferentially adopts a rotated conformation that displays no interaction with the β -phosphate. In this case His_{iso} moves out toward the solvent (Figure 4, bottom right). Together these observations are consistent with the intermediate k_{cat} of PanC-His_{iso}44:⁵³ the retained 5% activity might be attributed to a small population of protonated His_{iso} and/or the facilitation of ADP release caused by the conformational rearrangement that His_{iso}44 undergoes upon deprotonation. This population would be expected to be altered in the aza variant aza-His_{iso}44, a mutant that showed only lower activity. Mutation of His44 to Cys (Figure 4, bottom left) also suppresses the hydrogen bond, since Cys is further away (almost 8 Å) and prefers to interact with other residues, consistent with the lowest k_{cat} . Notably, suppression of the hydrogen bond in all cases changes the orientation of the β -phosphate of ATP, moving it away from the histidine that aids its activation (Figure S26), suggesting that this conformational change could be a structural determinant in the lower activities of the His_{iso} and Cys mutants.

DISCUSSION

We demonstrate for the first time the creation of a regioisomeric pro-*N*-linked imidazole ring in the chemical mutant His_{iso} as a mimic histidine for the C4-linked imidazole

in His in two enzymes, as well as a corresponding aza variant (aza-His_{iso}). This ability to perform aza-Michael-type chemical, post-translational mutation not only allows mechanistic hypothesis testing, as we have shown here, but may have broader utility in exploring other His-mediated modulation of activities such as nucleophilicity, basicity, metal-binding, and even the pH range in which a protein might operate. As such, iso-His highlights the broader value of regioisomeric mutants and might now be considered a new, conservative mutation that may be installed even into active sites in sensitive proteins, as we show here. As such, both this post-translational strategy and the new His_{iso} and aza-His_{iso} mutations disclosed here complement other methods for exploring the role of histidine in proteins.^{54–56} For example, prior cotranslational biosynthetic methods using residue-specific replacement⁵⁴ or amber-codon suppression^{55,56} have allowed incorporation of certain non-isomeric analogues but all can struggle with other analogues, dependent on the method and associated translational and biosynthetic tolerances. More generally, post-translational, aza-Michael-type chemistry at Dha may provide a usefully general mode of protein conjugation for the attachment of other cargoes, probes, or labels.³²

■ ASSOCIATED CONTENT

■ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acscentsci.7b00341](https://doi.org/10.1021/acscentsci.7b00341).

Figures S1–26 and details of protein expression and purification, LC–MS and MS/MS analyses, enzyme activity assays, chemical mutations, structural analyses, sequences, and computational methods (PDF)

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Notes

The authors declare no competing financial interest.

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