Enhancing protein stability with extended disulfide bonds

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Disulfide bonds play an important role in protein folding and stability. However, the cross-linking of sites within proteins by cysteine disulfides has significant distance and dihedral angle constraints. Here we report the genetic encoding of noncanonical amino acids containing long side-chain thiols that are readily incorporated into both bacterial and mammalian proteins in good yields and with excellent fidelity. These amino acids can pair with cysteines to afford extended disulfide bonds and allow cross-linking of more distant sites and distinct domains of proteins. To demonstrate this notion, we preformed growth-based selection experiments at nonpermissive temperatures using a library of random β-lactamase mutants containing these noncanonical amino acids. A mutant enzyme that is cross-linked by one such extended disulfide bond and is stabilized by ∼9 °C was identified. This result indicates that an expanded set of building blocks beyond the canonical 20 amino acids can lead to proteins with improved properties by unique mechanisms, distinct from those possible through conventional mutagenesis schemes.

noncanonical amino acids | extended disulfide bonds | β-lactamase | thermostability | evolutionary advantage

We are developing strategies that begin to address the question of whether an expanded genetic code provides an evolutionary advantage to an organism. For example, it has been recently shown that a unique noncanonical amino acid mutation in TEM-1 β-lactamase significantly increases the enzyme's catalytic activity for the substrate cephalexin, a result that cannot be recapitulated by substitution of canonical amino acids at this site (1). This same enzyme has been reengineered to be dependent on a noncanonical active site residue for activity, a dependency that was maintained for hundreds of generations without escape (2). Furthermore, addition of noncanonical amino acid building blocks to an unbiased library of ribosomally synthesized cyclic peptides provided a selective advantage in the evolution of inhibitors of cytotoxic intracellular proteases (3). Here we begin to explore whether noncanonical amino acids can provide *Escherichia coli* a selective growth advantage by increasing the thermal stability of essential proteins.

Cysteine is unique among the 20 canonical amino acids in that it can form reversible covalent cross-links in proteins. Disulfide bonds can stabilize monomeric and multisubunit proteins (4), play a role in catalysis (5, 6), and regulate protein activity (7); because of these unique properties, disulfide bonds are highly conserved in protein evolution (8, 9). There has been considerable success in the use of structure-based design to engineer disulfide bonds into proteins for both biopharmaceutical and industrial applications (10, 11). However, the sites in proteins that can be cross-linked by a cystine disulfide are typically constrained to a distance between the two β-carbons of ∼5.5 Å (10) and a near 90° dihedral angle for the disulfide bond (12). Thus, the relatively long distances that might be required to bridge distinct protein domains or subunits, or steric constraints at specific sites may preclude the introduction of a natural disulfide bond. These challenges led us to explore whether we could overcome the geometrical constraints of the cysteine disulfide by genetically encoding noncanonical amino acids (NCAAs) with longer thiol-containing side chains.

To this end, we designed a series of tyrosine derivatives (SetY, SprY, and SbuY) with *para*-substituted aliphatic thiols of various lengths (Fig. 1*A*). The calculated length between the two β-carbons of the SbuY-Cys disulfide bond is 14 Å when fully extended, which is significantly longer than a natural cystine cross-link. We genetically encoded these NCAAs in bacterial and mammalian cells by suppressing the nonsense codon TAG with an orthogonal, amber suppressor aminoacyl-tRNA synthetase (RS)/tRNA pair (13). Because statistical analyses of proteins have suggested that stabilizing disulfide mutations are most often found in regions of higher mobility near the protein surface and associated with longer loop lengths (>25 residues) (10, 14), we reasoned that these more flexible, extended disulfides might facilitate the introduction of stabilizing disulfide bonds into proteins. Using a N-terminal truncated β-lactamase as a model system (15), we carried out a growth-based selection under nonpermissive temperatures with a library of mutants in which the thiol-containing NCAAs were randomly incorporated, and identified a mutant enzyme cross-linked by an extended disulfide bond that is stabilized by ∼9 °C.

Results

Genetic Incorporation of NCAAs Containing Long-Chain Thiols. The development of orthogonal aminoacyl-tRNA synthetase/tRNA pairs has led to the genetic incorporation of various structurally and functionally diverse NCAAs into recombinant proteins (16, 17). Recently, a series of tyrosine derivatives containing *para*-substituted long-chain haloalkanes were genetically incorporated into proteins by Y.W. and coworkers using an engineered,

Significance

This work describes a facile system for incorporating noncanonical amino acids containing long side-chain thiols using an expanded genetic code. These amino acids begin to overcome the distance and geometric constraints of the cysteine disulfide and can pair with cysteines to cross-link more remote sites in proteins. To demonstrate this notion, we constructed a library of random β-lactamase mutants containing these noncanonical amino acids and grew them at nonpermissive temperatures. We identified a mutant enzyme that is cross-linked by one such extended disulfide bond that has significantly enhanced thermal stability. This study suggests that an expanded set of amino acid building blocks can provide novel solutions to evolutionary challenges.

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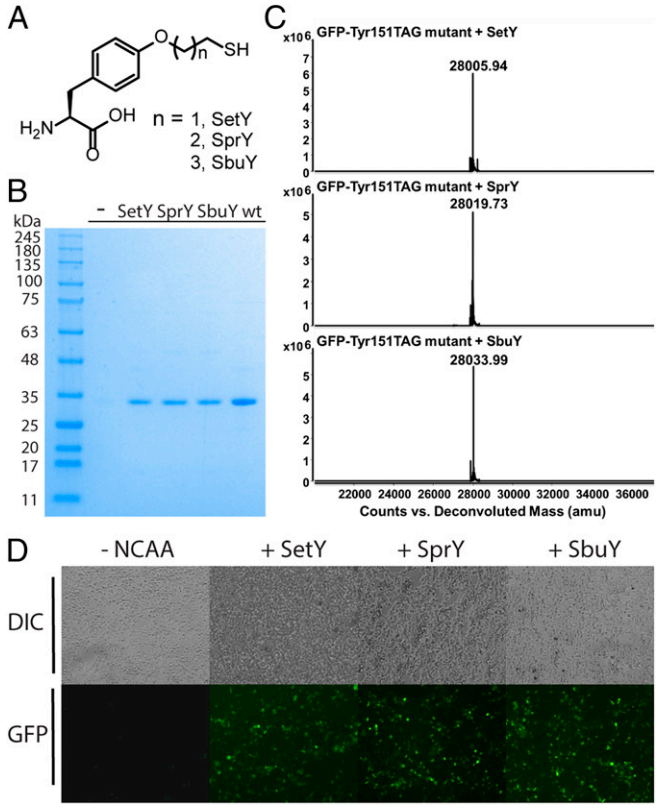

Fig. 1. Genetic incorporation of NCAAs containing long-side-chain thiols. (*A*) Structure of *O*-(2-mercaptoethyl)-L-tyrosine (SetY), *O*-(3-mercaptopropyl)-L-tyrosine (SprY), and *O*-(4-mercaptobutyl)-L-tyrosine (SbuY). (*B*) SDS/PAGE analysis of purified GFP (134TAG) expressed in *E. coli* DH10B using the *Mb*XYRS/$tRNA^{Pyl}$ pair in the presence or absence of 1 mM NCAAs. GFP mutants were expressed in LB medium and purified by standard Ni-NTA affinity chromatography. (*C*) Mass spectral analysis of GFP mutants containing the corresponding NCAAs. The calculated masses for the mutant GFPs containing SetY, SprY, and SbuY are 28,005 Da, 28,019 Da, and 28,033 Da, respectively. (*D*) Fluorescence microscopy (10×) of 293T cells expressing an EGFP mutant (Tyr39TAG) in the presence or absence of NCAAs. 293T cells were transiently cotransfected with pCMV-XYRS and pEGFP-Tyr39TAG and were grown in DMEM supplemented with 10 FBS in the presence or absence of 250 μM NCAAs.

polyspecific pyrrolysyl-tRNA synthetase termed "XYRS" (13). Based on this work, we designed and synthesized a series of the thiol-containing amino acids SetY, SprY, and SbuY as substrates for XYRS (Fig. 1*A*). The efficiency of incorporation of these NCAAs into proteins by XYRS was initially assessed using a GFP variant bearing an amber codon (TAG) at the permissive site, Asp134 (18). *E. coli* DH10B cells containing the reporter plasmid pLeiG-GFP-Asp134TAG (19) were transformed with a pULTRA plasmid containing the *E. coli* codon optimized amber suppressor $tRNA^{Pyl}$/XYRS pair. A quantitative fluorescence assay was carried out in the presence and absence of 1 mM NCAA. A significant increase in fluorescence was observed in the presence of all of the NCAAs (*SI Appendix*, Fig. S1) relative to controls lacking the NCAA.

To more precisely quantify the amount of protein produced, the GFP mutants bearing a C-terminal histag were expressed in LB medium in the presence or absence of 1 mM NCAAs, purified by standard Ni-NTA affinity chromatography, and analyzed by SDS/PAGE. As shown in Fig. 1*B*, full-length GFP mutants were expressed only in the presence of NCAAs. The final yields of all three purified GFP mutants were similar (~10 mg/L). Site-specific incorporation was further confirmed by high-resolution mass spectrometry [quadrupole-time-of-flight/MS (QTOF/MS)]. The calculated masses for the mutant GFPs containing SetY, SprY, and SbuY are 28,005 Da, 28,019 Da, and 28,033 Da, respectively, which agree with the observed masses (Fig. 1*C*).

Because the pyrrolysyl-tRNA synthetase/$tRNA^{Pyl}$ pair has been shown to be orthogonal in mammalian cells (18, 19), the utility of this system was also evaluated in mammalian cell culture using 293T cells. Incorporation of the NCAAs was evaluated by cotransfection of the reporter plasmid pEGFP-Tyr39TAG and the pCMV plasmid containing the XYRS/$tRNA^{Pyl}$ pair driven by CMV and U6 promoters, respectively. At 250 μM NCAAs, inspection of transfected cells by fluorescence microscopy revealed bright green fluorescence, suggesting the production of full-length EGFP by amber suppression (Fig. 1*D*).

A Growth-Based Assay for β-Lactamase Variants with Enhanced Thermostability. With a functional expression system in place, we next explored the potential impact of these long, thiol-containing

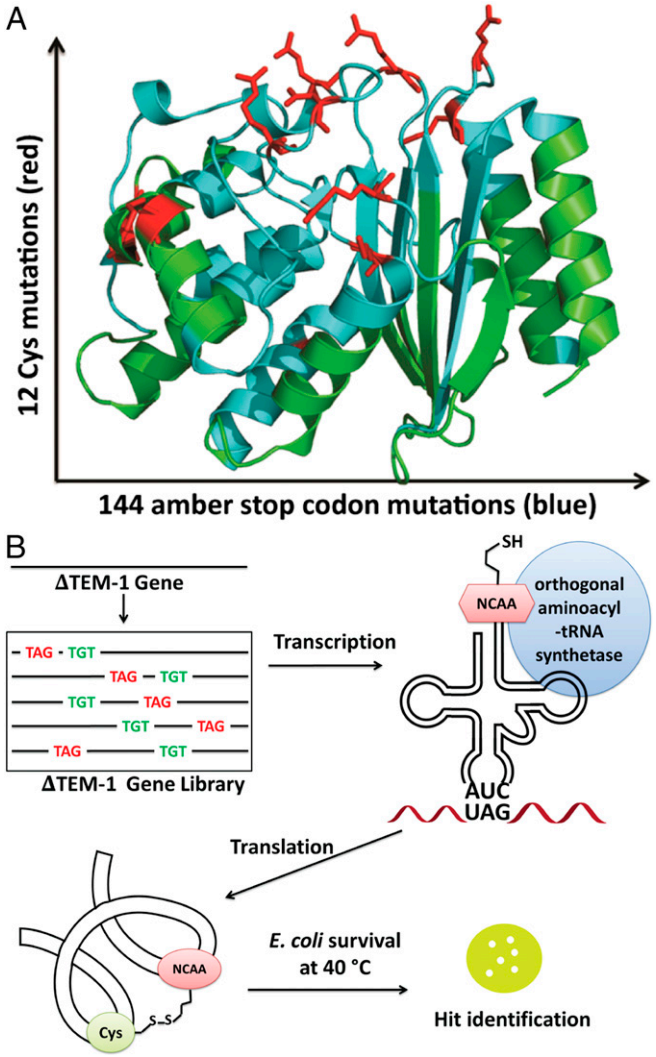

Fig. 2. Library design and selection scheme. (*A*) X-ray crystal structure of TEM-1 β-lactamase (PDB ID code 1FQG) showing the design of the library. Each library member contains 1 of the 12 Cys mutations (shown in red) and 1 of the 144 amber stop codon mutations (shown in blue). (*B*) Selection scheme for ΔTEM-1 protein mutant. Mutagenesis was used to insert Cys residues into a TAG-scanned library of ΔTEM-1, and the resulting library containing double mutants was subjected to a growth-based selection at 40 °C in the presence of the thiol NCAAs; the mutations in surviving clones were determined by sequence analysis.

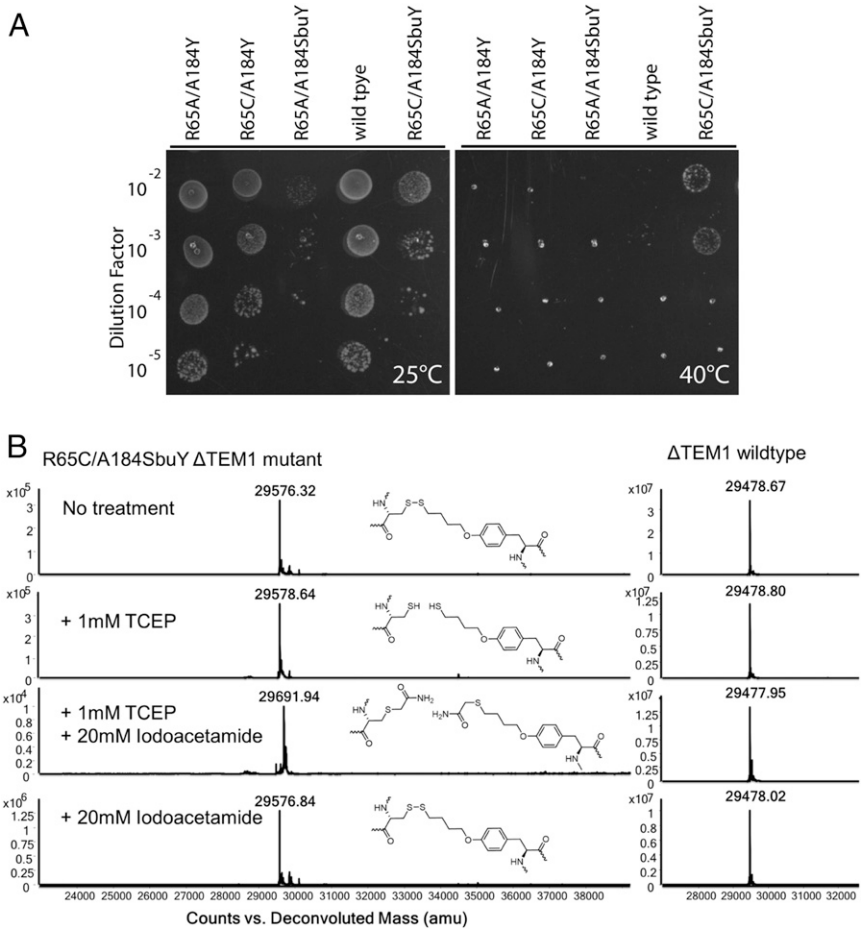

Fig. 3. Formation of an unnatural disulfide bond. (*A*) Characterization of the R65C/A184SbuY mutant. Serial dilutions of overnight *E. coli* cultures were grown on LB plates containing 10 μg/mL Amp and 1 mM SbuY at the indicated temperatures indicated on the graphs. (*B*) Mass spectral analysis of purified R65C/A184SbuY protein under four different treatment conditions.

NCAAs on protein thermostability. We recently demonstrated that screening a library of mutant TEM-1 β-lactamases containing randomly incorporated noncanonical amino acids with unique structures afforded an enzyme with significantly increased catalytic activity toward a β-lactam antibiotic (1). Using a similar system, we asked whether the random introduction of the thiol-containing NCAAs SetY, SprY, or SbuY into β-lactamase could increase its thermostability. The selection is based on a system reported by Hecky and Müller (15) that uses a structurally perturbed, destabilized TEM-1 β-lactamase lacking the first five N-terminal residues (ΔN5, here referred to as ΔTEM-1). Because TEM-1 contains no free cysteine residues (one disulfide bond), we constructed a disulfide-pairing ΔN5 TEM-1 library, in which each member contains two mutations: one cysteine (TGT) at a defined site and one thiol NCAA (TAG) at a random site. Hence, the library contains a combination of 12 different individual TGT point mutations and 144 TAG individual point mutations for each gene (Fig. 2*A*) (i.e., one TGT and one TAG mutation per library member; theoretical diversity = 1,728). The sites of amber mutations were based on our previous published work (1). Cysteine mutations (R65C, D115C, T140C, K146C, E171C, N175C, R178C, M182C, D209C, R240C, Q267C, and M270C) were introduced into regions of higher mobility near the protein surface. The DNA library was then introduced into *E. coli* DH10B cells containing the amber suppressor XYRS/tRNA^{Pyl} pair (Fig. 2*B*), and the transformed cells were grown at a nonpermissive temperature (40 °C) in the presence of 10 μg/mL ampicillin and 1 mM of SetY, SprY, or SbuY. After 24 h of growth, a single NCAA-dependent hit was identified on a SbuY-containing selection plate. This transformant contained the mutations Arg65 to Cys and Ala184 to SbuY (R65C/A184SbuY).

Formation of a SbuY-Cys Disulfide Bond in the β-Lactamase Mutant. An inspection of the wild-type crystal structure (*SI Appendix*, Fig. S2*A*) revealed that the distance between the two β-carbons of residues 65 and 184 is 11.1 Å, which is within the distance constraints of the two β-carbons of a Cys-SbuY disulfide bond that can be as long as 14.2 Å when fully extended (calculated with Chembio3D Ultra). In addition, modeling of the mutant (R65C/A184Y) using PyMOL suggested that the distance between the sulfur atom of the Cys65 side chain and the oxygen atom of the Tyr184 side chain is 7.7 Å (*SI Appendix*, Fig. S2*B*). This value is consistent with the calculated distance between these two atoms in an extended Cys-SbuY disulfide bond of ∼7.9 Å, raising the possibility of a cysteine-SbuY disulfide bond.

To test this hypothesis, three mutant clones (R65A/A184Y, R65C/A184Y, and R65A/A184SbuY) were generated by site-directed mutagenesis. *E. coli* DH10B cells transformed with these mutants, as well as ΔN5 TEM-1 and the R65C/A184SbuY putative cross-linked mutant, were then grown in the presence of 1 mM SbuY and antibiotic. As shown in Fig. 3*A*, all clones grew at 25 °C, but only the R65C/A184SbuY mutant survived at 40 °C. Intriguingly, at 40 °C, no colony was observed for the R65C/A184TAG clone in the presence of 1 mM SetY or SprY, suggesting that the enhanced thermostability for this mutant enzyme is

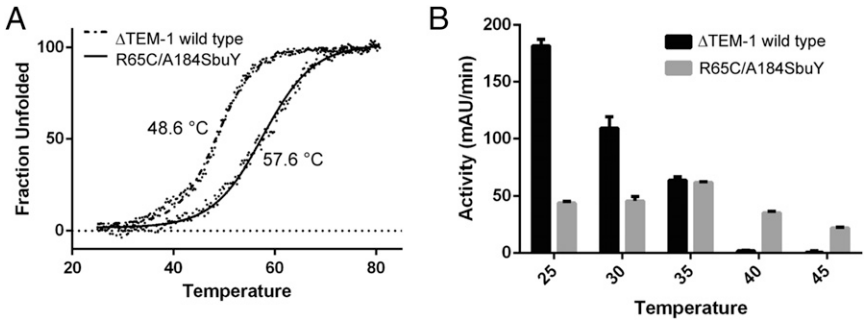

Fig. 4. Thermostability of the mutant β-lactamase. (*A*) Thermal denaturation curves of wild-type and mutant ΔTEM-1. For each protein, the CD signal was recorded at 223 nm as a function of temperature. (*B*) Temperature-dependent activity profiles of wild-type and mutant ΔTEM-1. Purified enzymes were preincubated at the each temperature for 30 min in a temperature-controlled plate reader followed by the addition of nitrocefin. The apparent activity was recorded by monitoring absorbance at 482 nm.

side-chain-length–dependent. Indeed, the calculated distances between the Cys sulfur atom and the aryl *para*-oxygen atom are around 6.7 Å and 5.4 Å for the extended Cys-SprY and Cys-SetY disulfide bonds, respectively (*SI Appendix*, Fig. S2*C*). These distances are shorter than the 7.7-Å distance obtained from modeling of the R65C/A184SbuY mutant.

To confirm formation of an unnatural disulfide, the R65C/A184SbuY ΔTEM-1 mutant was inserted into a pET26b vector following a PelB signal peptide, and protein expression (15) was carried out in the presence of 1 mM SbuY using *E. coli* BL21(DE3). Genetic incorporation of SbuY at position 184 in the ΔTEM-1 mutant was confirmed by trypsin digestion under standard reduction-alkylation condition followed by liquid chromatography (LC)-MS/MS analysis, which identified the peptide DTTMP(SbuY)AMATTLR containing SbuY at the expected site (*SI Appendix*, Fig. S3). Next, both the purified ΔTEM-1 and mutant protein were subjected to QTOF/MS analysis. The theoretical molecular mass, assuming all thiols form internal disulfides, is calculated to be 29,478.6 Da for ΔTEM-1 containing one conserved disulfide bond and 29,575.6 Da for the corresponding R65C/A184SbuY mutant containing two disulfide bonds (including the additional one from the noncanonical pair), respectively. As shown in Fig. 3*B*, the observed masses for the wild type and mutant enzymes are 29,478.7 Da and 29,576.3 Da, respectively, which is in agreement with the masses calculated based on proteins containing no free thiols.

The unnatural disulfide bond was engineered at the surface of the protein, whereas the natural disulfide bond is buried in the protein interior. It has been demonstrated that the bulky reducing reagent tris(2-carboxyethyl)phosphine (TCEP) preferentially reduces exposed versus buried disulfide bonds (20). Thus, we reasoned that under mild TCEP-reducing conditions, only the unnatural disulfide will be reduced. Indeed, treatment with 1 mM TCEP had no effect on the natural cysteine disulfide, as demonstrated by no mass change for the wild-type protein, but resulted in an observed mass change of "+2" for the R65C/A184SbuY mutant, consistent with reduction of the extended disulfide bond. Further treatment with 2-iodoacetamide following this limited reduction resulted in an observed mass of 29,691.9 Da for the R65C/A184SbuY mutant, which matched the calculated mass of a protein adduct containing two additional carbamidomethyl groups (29,691.6 Da). Finally, in a control experiment, treatment with 2-iodoacetamide in the absence of reducing reagent had no effect on the mass of either the wild-type or the mutant protein, indicating the absence of free thiol residues. These results collectively indicate the formation of an additional surface-exposed disulfide bond in the R65C/A184SbuY mutant.

Thermostability of the Mutant β-Lactamase. Next, we examined if survival of *E. coli* containing the R65C/A184SbuY ΔTEM-1 at 40 °C is indeed due to an enhanced thermostability of the mutant enzyme. Thermal denaturation curves were obtained using circular dichroism (CD) spectroscopy by measuring α-helix ellipticity while increasing the temperature (21). The melting temperatures (Tm) of the ΔTEM-1 and mutant enzymes were determined to be 48.6 °C and 57.6 °C, respectively (Fig. 4*A*). This result confirms that the R65C/A184SbuY mutation significantly improves the thermostability of the ΔN5 TEM-1 enzyme. Interestingly, an inspection of the structure revealed that the mutations are located across the hinge region between the two half-domains (22), which has been previously suggested as a hotspot for stabilizing substitutions for this enzyme (21). We also attempted to introduce this unnatural disulfide bond into full-length wild-type TEM-1 β-lactamase. Unfortunately, the protein expresses extremely poorly, with the majority of the protein forming insoluble aggregates, which precluded the further characterization of this protein. It is possible that the wild-type full-length protein folds to form mixed disulfides or disulfide oligomers.

Finally, a temperature-dependent activity profile was obtained for both ΔTEM-1 and mutant enzymes by preincubating the purified proteins at a given temperature for 30 min followed by the addition of the chromogenic substrate nitrocefin. The initial velocity was recorded with a temperature-controlled plate reader, and the apparent activity was plotted as a function of temperature. As shown in Fig. 4*B*, the activity of the mutant enzyme is somewhat reduced to that of ΔTEM-1 at 25 °C. However, the activity increases as the temperature is increased from 25 °C to a maximum activity at 35 °C and then decreases again to a low level of detectable activity at 45 °C. At 40 °C, the activity of the mutant is comparable to that at 25 °C, consistent with the in vivo survival results. For comparison, the activity of the wild-type enzyme decreases as temperature increases, and no enzymatic activity is detectable at temperatures above 40 °C, consistent with previously published results (15).

Discussion

Nature uses the disulfide linkage as one strategy to stabilize protein structure. However, natural disulfide bonds between two cysteine residues are limited by geometrical constraints. We have begun to address this limitation by genetically encoding NCAAs that are capable of forming disulfide bonds that bridge longer distances. These longer and more flexible linkages should make it much easier to cross-link sites within proteins. Indeed, it has recently been shown that electrophilic amino acids with extended structures can selectively stabilize proteins and protein complexes by intra- and intermolecular cross-links, respectively (13, 23–25). However, the use of electrophilic NCAAs to form noncanonical

cross-links in a bacterial selection system can be complicated by cellular toxicity, irreversible reactions, reaction rates, and scavenging by intracellular nucleophiles. The use of well-precedented disulfide bond formation obviates these concerns, although it does require in general the use of periplasmically secreted proteins.

With these constraints in mind, we chose a truncated β-lactamase as a model system for identification of mutant proteins with enhanced stability. One mutant, R65C/A184SbuY, contains a length-dependent unnatural disulfide bond and exhibits significantly enhanced thermostability both in vitro and in vivo. Inspection of the crystal structure of wild-type β-lactamase suggests that the unnatural disulfide bond extends over an 11-Å distance across a hinge region, linking the α-domain and the α/β-domain of TEM-1 β-lactamase. Previous studies on TEM-1 β-lactamase–stabilizing variants have found that many mutations are located at this hinge region, suggesting a potential hotspot for stabilizing substitutions (21). For example, structural studies on the clinically isolated stabilizing mutant M182T β-lactamase revealed a hydrogen bond from the backbone of Ala185 through a water molecule to the side chain of Glu63 (26) that stabilizes the protein by 6.5 °C (21). Here we show that this same region can be cross-linked with a long disulfide bond, which results in a 9 °C increase in thermal stability. A survey of the literature reveals that most engineered cysteine disulfides give a Tm increase around 5 °C, although there are a few cases where a more than 10 °C increase in Tm is observed (27–36). Interestingly, in work related to that described here, Xiang et al. (13) demonstrated that halogen-containing NCAAs with relatively long side chains are capable of forming a covalent linkages with a Cys residue and showed that they improved the thermal stability of an affibody by 13 °C in vitro.

We are currently screening our library of mutants for additional stabilizing extended disulfides, as well as introducing electrophilic amino acids that form reversible covalent adducts with nucleophilic Cys and Lys residues. We are also exploring the use of computational methods to engineer additional extended disulfide bonds into this and other protein scaffolds to further explore the degree to which cross-linking more distal sites in proteins enhances thermal stability. Finally, this study suggests that an expanded set of amino-acid–building blocks can provide novel solutions to evolutionary challenges.

Materials and Methods

Synthesis of NCAAs Containing Long Side-Chain Thiols. Chemical synthesis of NCAAs is described in *SI Appendix, Supplementary Methods* and Scheme S1.

Expression and Purification of Mutant GFPs. *E. coli* DH10B cells, cotransformed with pLeiG-GFP-Asp134TAG and pULTRA-MbXYRS [bearing mutations derived from MmXYRS (13)] plasmids, were grown in LB media supplemented with chloramphenicol (50 μg/mL) and spectinomycin (50 μg/mL) at 37 °C. When OD_{600} reached 0.3, 1 mM of NCAAs was supplemented to the media. After 40 min, protein expression was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM, and cells were grown for an additional 20 h at 30 °C. Cells were harvested by centrifugation at 4,750 × *g* for 10 min. The cell pellets were suspended in BugBuster protein extraction reagent and lysed at 30 °C. The resulting cell lysate was clarified by centrifugation at 14,000 × *g* for 30 min, and protein was purified on Ni-NTA resin (Qiagen) following the manufacturer's instructions.

Expression of mutant EGFP in mammalian cells is described in *SI Appendix, Supplementary Methods*.

Construction of Disulfide Pairing ΔN5 TEM-1 Library. A ΔN5 TAG-scanned TEM-1 gene library cassette was first constructed and inserted into the pBK screening vector (described in *SI Appendix, Supplementary Methods*, in detail). The plasmid containing random TAG mutations was then mutagenized to introduce Cys mutations using the Quik Change Site Directed mutagenesis kit (Agilent Technologies) and the primers listed in *SI Appendix*, Table S1, and verified by DNA sequencing (Genewiz). DNA plasmids prepared from each sublibrary were combined in a pool to yield a TAG-TGT combination library.

Screening for Enhanced Thermal Stability. The DNA library was cotransformed with the pULTRA-XYRS plasmid bearing the amber suppressor MbXYRS/$tRNA^{pyl}$ into *E. coli* DH10B cells. Transformants were plated on LB agar plates containing 10 μg/mL ampicillin, 100 μg/mL spectinomycin, 1 mM IPTG, and 1 mM of SetY, SprY, or SbuY. After incubation at 40 °C for 24 h, single colonies on the plates were picked, and their NCAA dependence was confirmed by plating in the presence or absence of NCAAs. Plasmids from NCAA-dependent colonies were isolated and subjected to DNA sequencing.

Expression and Purification of TEM-1 Mutants. TEM-1 mutants were inserted into pET-26b expression vector and expressed in *E. coli* BL21(DE3), similarly as described for GFP mutants. Details of expression and purification are included in *SI Appendix, Supplementary Methods*.

Mass Spectrometry Analysis. High-resolution mass spectrometry was carried out on an Agilent 6520 accurate-mass QTOF instrument. Protein mass was deconvoluted by extraction of the total ion count across the entire area of protein elution using Agilent Qualitative Analysis software. For GFP mutants, samples in PBS buffer were injected (10 μL) at a concentration of 0.1–0.5 mg/mL. For TEM-1 β-lactamase mutants, samples in sodium bicarbonate buffer (pH 8.5) were treated under four different conditions before injection: (*i*) no treatment; (*ii*) 1 mM TCEP (Thermo Scientific) for 30 min; (*iii*) 1 mM TCEP for 30 min followed by 20 mM iodoacetamide (Sigma-Aldrich) for an additional 30 min; and (*iv*) 20 mM iodo-acetamide for 30 min. For identification of the SbuY incorporation site, proteins were subjected to in-solution reduction, alkylation, and overnight digestion by trypsin (Promega) before LC-MS/MS analysis at Scripps Center for Metabolomics and Mass Spectrometry.

Thermal Stability Determination. Protein (0.15 mg/mL) in 200 mM sodium phosphate buffer (pH 7.0) was subjected to heat-induced denaturation. Signals were monitored at 223 nm on a 202SF CD spectrometer (AVIV), equipped with a Peltier temperature controller. The temperature was raised in increments of 0.25 °C from 25 °C to 80 °C at a rate of 2 °C/min. The fraction unfolded was calculated from observed ellipticity and plotted against temperature. The temperature midpoint of the unfolding curve was determined by data fitting to the Boltzmann model using GraphPad Prism 6.

To assess the effect of temperature on activity, a clear-bottom 96-well plate (Corning) containing 5.5 nM of the purified enzymes in 90 μL of 50 mM sodium phosphate buffer (pH 7.0) was preincubated at each given temperature (25 °C, 30 °C, 35 °C, 40 °C, and 45 °C) for 30 min. After incubation, 10 μL of preheated nitrocefin (BioVision) in the same buffer was added to a final concentration of 100 μM. The absorbance was recorded at 482 nm with a temperature-controlled plate reader (Spectramax 250). The initial reaction rate was determined by monitoring the increase in absorbance between 10 and 40 s.

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1. Xiao H, et al. (2015) Exploring the potential impact of an expanded genetic code on protein function. *Proc Natl Acad Sci USA* 112(22):6961–6966.
2. Tack DS, et al. (2016) Addicting diverse bacteria to a noncanonical amino acid. *Nat Chem Biol* 12(3):138–140.
3. Young TS, et al. (2011) Evolution of cyclic peptide protease inhibitors. *Proc Natl Acad Sci USA* 108(27):11052–11056.
4. Fass D (2012) Disulfide bonding in protein biophysics. *Annu Rev Biophys* 41:63–79.
5. Ellgaard L, Ruddock LW (2005) The human protein disulphide isomerase family: Substrate interactions and functional properties. *EMBO Rep* 6(1):28–32.
6. Gleiter S, Bardwell JC (2008) Disulfide bond isomerization in prokaryotes. *Biochim Biophys Acta* 1783(4):530–534.
7. Hogg PJ (2003) Disulfide bonds as switches for protein function. *Trends Biochem Sci* 28(4):210–214.
8. Thornton JM (1981) Disulphide bridges in globular proteins. *J Mol Biol* 151(2): 261–287.
9. Wong JW, Ho SY, Hogg PJ (2011) Disulfide bond acquisition through eukaryotic protein evolution. *Mol Biol Evol* 28(1):327–334.
10. Dombkowski AA, Sultana KZ, Craig DB (2014) Protein disulfide engineering. *FEBS Lett* 588(2):206–212.
11. Craig DB, Dombkowski AA (2013) Disulfide by Design 2.0: A web-based tool for disulfide engineering in proteins. *BMC Bioinformatics* 14:346.
12. Van Wart HE, Lewis A, Scheraga HA, Saeva FD (1973) Disulfide bond dihedral angles from Raman spectroscopy. *Proc Natl Acad Sci USA* 70(9):2619–2623.
13. Xiang Z, et al. (2014) Proximity-enabled protein crosslinking through genetically encoding haloalkane unnatural amino acids. *Angew Chem Int Ed Engl* 53(8): 2190–2193.

14. Dani VS, Ramakrishnan C, Varadarajan R (2003) MODIP revisited: Re-evaluation and refinement of an automated procedure for modeling of disulfide bonds in proteins. *Protein Eng* 16(3):187–193.
15. Hecky J, Müller KM (2005) Structural perturbation and compensation by directed evolution at physiological temperature leads to thermostabilization of beta-lactamase. *Biochemistry* 44(38):12640–12654.
16. Liu CC, Schultz PG (2010) Adding new chemistries to the genetic code. *Annu Rev Biochem* 79:413–444.
17. Dumas A, Lercher L, Spicer CD, Davis BG (2015) Designing logical codon reassignment: Expanding the chemistry in biology. *Chem Sci* 6(1):50–69.
18. Xiao H, Xuan W, Shao S, Liu T, Schultz PG (2015) Genetic incorporation of ε-N-2-hydroxyisobutyryl-lysine into recombinant histones. *ACS Chem Biol* 10(7):1599–1603.
19. Kim CH, Kang M, Kim HJ, Chatterjee A, Schultz PG (2012) Site-specific incorporation of ε-N-crotonyllysine into histones. *Angew Chem Int Ed Engl* 51(29):7246–7249.
20. Cline DJ, et al. (2004) New water-soluble phosphines as reductants of peptide and protein disulfide bonds: Reactivity and membrane permeability. *Biochemistry* 43(48): 15195–15203.
21. Brown NG, Pennington JM, Huang W, Ayvaz T, Palzkill T (2010) Multiple global suppressors of protein stability defects facilitate the evolution of extended-spectrum TEM β-lactamases. *J Mol Biol* 404(5):832–846.
22. Fonzé E, et al. (1995) TEM1 beta-lactamase structure solved by molecular replacement and refined structure of the S235A mutant. *Acta Crystallogr D Biol Crystallogr* 51(Pt 5):682–694.
23. Xiang Z, et al. (2013) Adding an unnatural covalent bond to proteins through proximity-enhanced bioreactivity. *Nat Methods* 10(9):885–888.
24. Chen XH, et al. (2014) Genetically encoding an electrophilic amino acid for protein stapling and covalent binding to native receptors. *ACS Chem Biol* 9(9):1956–1961.
25. Furman JL, et al. (2014) A genetically encoded aza-Michael acceptor for covalent cross-linking of protein-receptor complexes. *J Am Chem Soc* 136(23):8411–8417.
26. Wang X, Minasov G, Shoichet BK (2002) Evolution of an antibiotic resistance enzyme constrained by stability and activity trade-offs. *J Mol Biol* 320(1):85–95.
27. Matsumura M, Becktel WJ, Levitt M, Matthews BW (1989) Stabilization of phage T4 lysozyme by engineered disulfide bonds. *Proc Natl Acad Sci USA* 86(17):6562–6566.
28. Jeong MY, Kim S, Yun CW, Choi YJ, Cho SG (2007) Engineering a de novo internal disulfide bridge to improve the thermal stability of xylanase from *Bacillus stearothermophilus* no. 236. *J Biotechnol* 127(2):300–309.
29. Davoodi J, Wakarchuk WW, Carey PR, Surewicz WK (2007) Mechanism of stabilization of *Bacillus circulans* xylanase upon the introduction of disulfide bonds. *Biophys Chem* 125(2-3):453–461.
30. Han ZL, Han SY, Zheng SP, Lin Y (2009) Enhancing thermostability of a *Rhizomucor miehei* lipase by engineering a disulfide bond and displaying on the yeast cell surface. *Appl Microbiol Biotechnol* 85(1):117–126.
31. Chen XZ, et al. (2010) Site-directed mutagenesis of an *Aspergillus niger* xylanase B and its expression, purification and enzymatic characterization in *Pichia pastoris*. *Process Biochem* 45(1):75–80.
32. Le QA, Joo JC, Yoo YJ, Kim YH (2012) Development of thermostable *Candida antarctica* lipase B through novel in silico design of disulfide bridge. *Biotechnol Bioeng* 109(4):867–876.
33. Tan Z, Li J, Wu M, Wang J (2014) Enhancing the thermostability of a cold-active lipase from *Penicillium cyclopium* by in silico design of a disulfide bridge. *Appl Biochem Biotechnol* 173(7):1752–1764.
34. Zhang S, et al. (2014) Improving *Trichoderma reesei* Cel7B thermostability by targeting the weak spots. *J Chem Inf Model* 54(10):2826–2833.
35. Yin X, et al. (2015) Contribution of disulfide bridges to the thermostability of a type A feruloyl esterase from *Aspergillus usamii*. *PLoS One* 10(5):e0126864.
36. Yu XW, Tan NJ, Xiao R, Xu Y (2012) Engineering a disulfide bond in the lid hinge region of *Rhizopus chinensis* lipase: Increased thermostability and altered acyl chain length specificity. *PLoS One* 7(10):e46388.