SpyTag/SpyCatcher Cyclization Confers Resilience to Boiling on a Mesophilic Enzyme**

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Abstract: SpyTag is a peptide that spontaneously forms an amide bond with its protein partner SpyCatcher. SpyTag was fused at the N terminus of β-lactamase and SpyCatcher at the C terminus so that the partners could react to lock together the termini of the enzyme. The wild-type enzyme aggregates above 37°C, with irreversible loss of activity. Cyclized β-lactamase was soluble even after heating at 100°C; after cooling, the catalytic activity was restored. SpyTag/SpyCatcher cyclization led to a much larger increase in stability than that achieved through point mutation or alternative approaches to cyclization. Cyclized dihydrofolate reductase was similarly resilient. Analyzing unfolding through calorimetry indicated that cyclization did not increase the unfolding temperature but rather facilitated refolding after thermal stress. SpyTag/SpyCatcher sandwiching represents a simple and efficient route to enzyme cyclization, with potential to greatly enhance the robustness of biocatalysts.

Biological catalysts frequently show superlative regioselectivity and stereoselectivity compared to chemical catalysts but they suffer from instability. The stabilization of enzymes can be achieved without the use of chemical modifications by looking for homologues in thermophiles, inferring a consensus or ancestral sequence, or structure-based design. Proteins obtained from thermophiles usually achieve their optimum catalytic efficiency at a high temperature, a costly trait for application to biotransformations. Therefore, it is desirable to obtain a protein with a high catalytic efficiency at ambient temperature, whilst maintaining the thermal tolerance of thermophile-derived proteins.

Directed evolution and rational design of enzymes have led to important successes, but these approaches require time-consuming individual optimization and the gains are often marginal. For example, after more than 700 mutations on T4 lysozyme, the best thermostabilization from combining individual stabilizing point mutations was 8°C, at a cost of losing most of the catalytic activity. Therefore, generic approaches that require neither extensive structural knowledge nor library selection are needed to provide a faster and more broadly applicable route to enzyme stabilization.

To overcome the limited stability of peptide interactions, we previously developed a peptide tag that effects spontaneous intermolecular amide bond formation. This tag was developed through the dissection and modification of a protein domain from Gram-positive bacteria. We engineered SpyTag, a 13 amino acid peptide, to rapidly form an irreversible amide bond to the 15 kDa protein SpyCatcher. The system is genetically encodable and the tag and protein are functional on either terminus. Since the termini are one of the most flexible regions of proteins and fluctuations here may initiate unfolding, we hypothesized that connecting the N and C termini of a protein through SpyTag/SpyCatcher cyclization could enhance stability (Figure 1A).

Protein cyclization has been previously achieved through the use of carbodiimide cross-linking, sortase, or (most often) inteins. TEM-1 β-lactamase (BLA) is an important model system for enzyme evolution, as well as having clinical relevance from the emergence of bacteria with broad spectrum antibiotic resistance. Previous cyclization of BLA with a split intein achieved a 5°C increase in thermal tolerance. We genetically fused SpyTag to the N terminus of BLA and SpyCatcher to the C terminus (Figure 1A). This SpyTag-BLA-SpyCatcher construct expressed efficiently in Escherichia coli. To test whether we had successfully cyclized BLA, we generated negative controls unable to react in both SpyTag and SpyCatcher: we mutated the reactive Asp in SpyTag to Ala (SpyTagDA) or the catalytic Glu residue in SpyCatcher to Gln (SpyCatcherE). When we analyzed the linear mutants alongside the cyclized construct by SDS-PAGE, we found that the cyclized form had a lower mobility, a result consistent with efficient cyclization (75%; Figure 1B and Figure S1a in the Supporting Information).

To further confirm that we had successfully cyclized BLA, we introduced a TEV protease cleavage site between the BLA and SpyCatcher. Following TEV protease cleavage, the cyclized SpyTag-BLA-TEV-SpyCatcher migrated to the same apparent molecular weight as the linear construct (Figure 1B). We also witnessed the disappearance of polymeric forms (generated from a low level of intermolecular reaction of SpyTag-BLA-SpyCatcher) and the appearance of new bands from polymer cleavage (Figure 1B).

Furthermore, we verified the molecular weight of SpyTag-BLA-SpyCatcher, as well as of the DA and EQ controls, by...
using electrospray ionization mass spectrometry (calculated $M_w$ of SpyTag-BLA-SpyCatcher before reaction 45,786.4 Da; calculated $M_w$ of SpyTag-BLA-SpyCatcher after loss of H$_2$O from spontaneous amide bond formation 45,768.4 Da; observed $M_w$ 45,768 Da; Figure 1C and Figure S1 in the Supporting Information).

To test the effect of cyclization on protein thermal stability, we incubated SpyTag-BLA-SpyCatcher and unmodified BLA in phosphate-buffered saline over a wide range of temperatures. Surprisingly, we found that SpyTag-BLA-SpyCatcher was not lost from solution at any of the temperatures tested, even up to 100°C. On the other hand, BLA started aggregating at 37°C and had completely aggregated at 55°C (Figure 2A,B). Therefore, SpyTag/SpyCatcher cyclization conferred an increase in aggregation temperature of more than 60°C. It was conceivable that the different stability resulted from different protease contamination of the preparations. However, after mixing BLA and SpyTag-BLA-SpyCatcher, there was still a much greater loss of BLA than SpyTag-BLA-SpyCatcher upon heating (Figure S2).

To test whether SpyTag-BLA-SpyCatcher retained activity as well as solubility, we measured enzymatic activity with the colorimetric substrate nitrocefin after incubation at 25 or 100°C. Nearly all BLA activity was lost after high-temperature incubation, whereas nearly all the activity of SpyTag-BLA-SpyCatcher was retained after 100°C exposure (Figure 2C).

To understand to what extent the fused components conferred resistance to aggregation, we tested the noncyclized SpyTagDA-BLA-SpyCatcher in equivalent assays. Interestingly, SpyTagDA-BLA-SpyCatcher did show enhanced resistance to aggregation compared to BLA. However, covalent cyclization was important, because the recovery of activity following heating of SpyTagDA-BLA-SpyCatcher was inferior to SpyTag-BLA-SpyCatcher (Figure S3). SpyTag-BLA-SpyCatcherEQ (with the E77Q mutation blocking the SpyCatcher reaction) showed low resistance to aggregation and weak recovery of catalytic activity (Figure S4). Fusing the whole CnaB2 domain (to give BLA-CnaB2, which undergoes isopeptide formation within the CnaB2 domain but without connecting the termini of BLA) gave excellent resistance to aggregation but only moderate recovery of catalytic activity (Figure S4).

To see whether the effect of SpyTag/SpyCatcher could be extended to another class of enzyme, we tested our approach on dihydrofolate reductase (DHFR). SpyTag-DHFR-SpyCatcher cyclized in high yield, as shown by SDS-PAGE and mass spectrometry (Figure S5). SpyTag-DHFR-SpyCatcher, like the SpyTagDA-DHFR-SpyCatcher control, resisted aggregation at 100°C (Figure S5). However, SpyTag-DHFR-SpyCatcher retained its catalytic activity following the 100°C heating, unlike SpyTagDA-DHFR-SpyCatcher (Figure S5).
To dissect the mechanism of SpyTag/SpyCatcher stabilization, we used differential scanning calorimetry (DSC) to test the thermal unfolding profiles of SpyTag-BLA-SpyCatcher, SpyTagDA-BLA-SpyCatcher, and BLA. SpyTag-BLA-SpyCatcher and SpyTagDA-BLA-SpyCatcher each generated two peaks (Figure 3A). The common peak for all constructs, corresponding to BLA domain unfolding, showed a melting temperature (T_m) of 39.6 °C for SpyTag-BLA-SpyCatcher, 39.6 °C for SpyTagDA-BLA-SpyCatcher, and 41.3 °C for BLA (Figure 3A). The second peak for SpyTag-BLA-SpyCatcher, with a T_m value of 85.4 °C, likely corresponds to unfolding of the reconstituted SpyTag/SpyCatcher domain. The second peak for SpyTagDA-BLA-SpyCatcher, related to the noncovalent SpyTagDA/SpyCatcher complex, showed a T_m value of 54.0 °C (Figure 3A).

To test the capability of the BLA domain to refold, we used DSC to scan from 20 to 60 °C (where the signature from BLA unfolding is observed), allowed the sample to cool, and then scanned over this temperature range again. BLA gave no peak on the second scan, a result consistent with an inability to refold (Figure 3B). By contrast, SpyTag-BLA-SpyCatcher produced a signal of similar shape but reduced intensity on the second scan (Figure 3C), thus suggesting that the BLA domain is able to refold successfully in this cyclized context. SpyTagDA-BLA-SpyCatcher gave no peak in the range 40–45 °C, thus indicating an absence of BLA refolding; the peak from 45–60 °C corresponds to the SpyTagDA/SpyCatcher moiety (Figure 3D). Therefore, cyclization through SpyTag-SpyCatcher chemistry did not appear to increase resistance to unfolding, but instead increased the ability to refold.

In conclusion, we have developed a method to dramatically increase the ability of a protein to recover from denaturation by using cyclization through spontaneous isopeptide bond formation. SpyTag/SpyCatcher cyclization conferred resistance to aggregation and enabled the recovery of catalytic activity following heating. DSC showed that the T_m value for the BLA domain was largely unaffected by cyclization, thus the enhanced resilience is likely due to an increase in the ability of the cyclized protein to refold. The greatly enhanced stability to aggregation conferred by SpyTag/SpyCatcher cyclization compared to other cyclization strategies may relate to the shielding of interprotein associations by the presence of a stable domain,[26] but intriguingly, DSC indicates that the stabilization effect extends beyond temperatures at which the SpyCatcher/SpyTag complex can unfold.

Disulfide bonds may also be engineered to lock protein termini but can interfere with existing disulfides, will not form in reducing environments, can break through β-elimination at elevated temperatures,[21] and often only give modest stability enhancements.[22] While this work was in progress, elastin-like peptides containing terminal SpyTag and SpyCatcher were shown to cyclize, but no functional effect of cyclization was established.[23] For some protein architectures, it will be hard to achieve SpyTag/SpyCatcher cyclization, but approximately 50% of single-domain proteins in the PDB have N- and C-terminal structural elements within 5 Å,[29] and future work may be able to harness the reactivity of SpyTag and SpyCatcher in internal regions of proteins.[14,28] Since protein stability can confer tolerance to mutations advantageous for function but deleterious for folding to the native structure,[19] it will also be interesting to explore whether SpyTag/SpyCatcher stabilization could facilitate library-based evolution of novel protein function.


Supporting Methods

Cloning

PCR was performed with KOD Hot Start DNA polymerase (Roche). All DNA was transformed into chemically competent *Escherichia coli* DH5α (Invitrogen). β-lactamase (BLA) constructs were N-terminally His6-tagged in pET28a (Novagen) and are missing the signal sequence that localizes BLA to the periplasm. TEM-1 BLA, the antibiotic resistance gene present in the pDEST14 plasmid (Life Technologies), was amplified using primers 5'-GGTTCAAGGGGTTCCGGTCAACCAAGAAGCTGGAAGTAAAGATG and 5'-TCCGCTGCCACCACTCCCCAATGCTTAATCAGTGAGGACCACCTATTCAG.

To generate pET28a SpyTag-BLA-SpyCatcher (GenBank KJ645919, Addgene ID 70943) and pET28a SpyTag DA-BLA-SpyCatcher (reactive Asp of SpyTag converted to Ala), we used Overlap Extension PCR.[1] We required a fragment encoding SpyTag-BLA or SpyTag DA-BLA in combination with a fragment encoding SpyCatcher (with each fragment joined via a GSGGSG linker).[2] In order to add SpyTag to BLA via a GSGGSG spacer, the PCR product was amplified using primers 5'-AGGACATATGGGAGCCACATCGTGATGGTGACGCCTACAAAGCGAGAAGGGGTTCCAGGGTGTCCCGGT and 5'-TCCGCTGCCACCACTCCCCAATGCTTAATCAGTGAGGACCACCTATTCAG. In order to add SpyTag DA, the PCR product was amplified using primers 5'-AGGACATATGGGAGCCACATCGTGATGGTGACGCCTACAAAGCGAGAAGGGGTTCCAGGGTGTCCCGGT and 5'-TCCGCTGCCACCACTCCCCAATGCTTAATCAGTGAGGACCACCTATTCAG. SpyCatcher was amplified from pDEST14-SpyCatcher[2] using 5'-GGGAGTGGTGGCAGCGGAGGCGCCATGGTTGACCTCTTTCAG and 5'-TTTAAAGCTTTCATTAAATATGAGCGTCACCTTTAGTGGCCTTGC. The final PCR product for SpyTag-BLA or SpyTag DA-BLA PCR fragment was mixed at an equimolar ratio with the SpyCatcher PCR product. The final PCR product was amplified with primers 5'-AGGACATATGGGAGCCACATCGTGATGGTGACGCCTACAAAGCGAGAAGGGGTTCCAGGGTGTCCCGGT and 5'-TTTAAAGCTTTCATTAAATATGAGCGTCACCTTTAGTGGCCTTGGCC. The final PCR product for SpyTag DA-BLA-SpyCatcher was amplified with primers 5'-AGGACATATGGGAGCCACATCGTGATGGTGACGCCTACAAAGCGAGAAGGGGTTCCAGGGTGTCCCGGT and 5'-TTTAAAGCTTTCATTAAATATGAGCGTCACCTTTAGTGGCCTTGGCC. The amplified PCR products were digested with Ndel (NEB) and HindIII (NEB) and ligated into pET28a.

pET28a-BLA was generated using pET28a SpyTag-BLA as a template, with inverse PCR using primers 5'-CACCAGAAACGCTGGAAGAG and 5'-TCCCATATGGCTGCGGCGC. We digested with DpnI (NEB) and re-ligated using T4 Polynucleotide Kinase (NEB) and T4 DNA ligase (NEB).

To generate pET28a SpyTag-BLA-TEV-SpyCatcher and pET28a SpyTag DA-BLA-TEV-SpyCatcher, a Tobacco Etch Virus (TEV) protease cleavage site was inserted between BLA and
SpyCatcher using the pET28a SpyTag-BLA-SpyCatcher and pET28a SpyTag DA-BLA-SpyCatcher plasmids via Site-directed, Ligase-Independent Mutagenesis (SLIM)\(^3\) using 5'-GGCGCCATGGGTATTGCAGTACCTATCAG, 5'-GAAAACCTGTATTTTCTCCCCCGGACCAGTACCTATCAG, 5'-TCCGCTGCCACCACCTCCCC and 5'-GCCCTGAAAATACAGGTTCCTCGGTGCCCACCACCTCCCC.

To generate pET28a SpyTag-BLA-SpyCatcher EQ (Glu77 promoting isopeptide bond formation converted to Gln), pET28a SpyTag-BLA-SpyCatcher was used as a template employing the QuikChange (Stratagene) protocol with 5'-GGAAAATATACATTTGTCAGACACCCCGCACCAGCG and 5'-CGTCTGGGTGTAGCCAGATACATTTTCTCCCGGTGCCCACCACCTCCCC. The same primers were used to generate pET28a SpyTag-DHFR-SpyCatcher EQ, with pET28a SpyTag-DHFR-SpyCatcher as a template.

pET28a BLA-CnaB2 was cloned by SLIM, adding the remainder of SpyTag back onto the C-terminus of BLA-SpyCatcher, thereby recreating the intact CnaB2 domain with the "YKPTK" motif from SpyTag at the C-terminus. BLA-SpyCatcher was used as a template with the primers 5'-TAATGGAAGCTTGCCGACGTAC, 5'-GTGATGTTGATGCGTACAA GCCGACGAAGTAATGAAAGCCGACGTAC, 5'-AATATGAGCGTCACCTTTAGTGGCTTGC and 5'-CTTCGTCGGCTTTGATGCATCCACCAAAATGATGGAAGTAATGAAAGCCGACGTAC.

pET9d encoding His\(_6\)-tagged TEV protease was kindly supplied by Kim Nasmyth (University of Oxford)\(^4\).

**Circular polymerase extension cloning**

Circular polymerase extension cloning (CPEC)\(^5\) was used to insert DHFR into the SpyTag-SpyCatcher or SpyTag DA-SpyCatcher scaffold in pET28a, to generate pET28a SpyTag-DHFR-SpyCatcher and pET28a SpyTag DA-DHFR-SpyCatcher. In CPEC, vector and insert are each separately amplified by PCR, using primers such that the insert and vector products will contain regions overlapping each other. Vector and insert are then connected together by a PCR reaction.

To amplify the vector, we used primers 5'-GGGAGTGGTGTCGACGAGGAG (codes for GSGGSG) and 5'-ACCAGAACCACCTGACAGAGGAGG (codes for KGSGGSG) with pET28a-SpyTag (DA)-BLA-SpyCatcher as a template with 25 cycles of PCR. The product was digested with DpnI and PCR-purified.

To amplify the insert, we added adaptor sequences to each end of the insert (matching the GSGGSG linkers). The adaptor sequence on the 5’ was CCGACGAAGGGTTCAGGGGGTGCCGGT which codes for PTKGSGGSG (PTK being the three amino acids of the C-terminus of SpyTag\(^2\)) and the adaptor sequence on the 3’ was GGGAGTGGTGTCGACGAGGAGGCG which codes for GSGGSG. The DHFR insert (FolA) was amplified from the genome of *E. coli* BL21 DE3 RIPL (Stratagene) using primers 5’-
CCGACGAAGGGTTACGGGGTTCCGGTATGATCAGTCTGATTGCGGC and 5'-GCCTCCGCTCCACCACCTCCCCCGCCTCCAGAATCTCAAAGC with 20 cycles of PCR and then PCR-purified. 500 ng vector was mixed with equimolar insert and joined by PCR with KOD DNA polymerase. Cycling conditions were 95 °C for 3 min followed by 10 cycles of 95 °C for 30 s, 62 °C for 30 s and 68 °C for 4 min. The presence of the reaction product was confirmed on a 0.7% agarose gel and then the reaction mixture was transformed into chemically competent E. coli DH5α. All constructs were verified by Sanger sequencing.

SDS-PAGE

SDS-PAGE was performed on 10 or 14 % polyacrylamide gels, using an XCell SureLock (Life Technologies) at 200 V. Dithiothreitol (DTT, Sigma) was added to a final concentration of 100 mM. Samples were then mixed with 6x SDS-PAGE loading buffer (0.23 M Tris-HCl, 0.24 % glycerol, 6.7 % SDS and 12 mM bromophenol blue). Samples were heated at 95 °C for 7 min in a Bio-Rad C1000 Thermal Cycler, before loading onto the gel. Gels were stained with InstantBlue Coomassie (Expedeon), imaged using a Bio-Rad ChemiDoc XRS+, and analyzed using Image Lab 3.0 software (Bio-Rad). The apparent mobility of the cyclized proteins depended on the acrylamide percentage of the gel as well as the sequence between SpyTag and SpyCatcher. For SpyTag-BLA-SpyCatcher cyclization decreased mobility, whereas for SpyTag-DHFR-SpyCatcher cyclization increased mobility.

Recombinant protein expression and purification

Proteins were expressed using E. coli BL21 DE3 RIPL for BLA and DHFR constructs or B834 DE3 cells (Novagen) for TEV protease, grown in LB with 0.8 % glucose and either with 0.1 mg/mL ampicillin or 0.5 mg/mL kanamycin, depending on the plasmid. Overnight cultures were diluted 100-fold, grown at 37 °C to an OD600 of 0.5, and induced with 0.4 mM IPTG. In the case of TEV protease, the culture was grown at 22 °C overnight and in the case of BLA and DHFR constructs the culture was grown at 18 °C overnight. For mass spectrometry to avoid glyconylation,[6] SpyTag-BLA-SpyCatcher, SpyTag DA-BLA-SpyCatcher and SpyTag-BLA-SpyCatcher EQ were grown in B834 in LB without glucose and induced at 30 °C for 3 h. His6-tagged proteins were purified using Ni-NTA (Qiagen) using standard methods and dialyzed in PBS. In the case of TEV protease, no dialysis was performed because TEV protease precipitated when dialyzed in PBS. To improve desalting ahead of mass spectrometry, SpyTag DA-BLA-SpyCatcher and SpyTag-BLA-SpyCatcher EQ were dialyzed into 0.1 M ammonium acetate. BLA construct concentration was determined from OD280 on a Nanodrop ND-1000 (Thermo Scientific) and calculated using the molar extinction coefficient from the ProtParam tool.[7] DHFR construct concentrations were determined using the Microplate bicinchoninic acid (BCA) Protein Assay Kit (Thermo Scientific). Protein purity was analyzed by SDS-PAGE.

Temperature-dependent solubility assay

20 µl 25 µM BLA construct or 20 µl 20 µM DHFR construct in PBS pH 7.4 containing 100 mM DTT was incubated for 10 min at 25, 37, 55, 75, 90 or 100 °C and then cooled down to 10 °C in a Bio-Rad C1000 Thermal Cycler at 3 °C/s. For the mixing assay, 25 µM BLA was mixed with 25 µM SpyTag-BLA-SpyCatcher and incubated as above. The samples were spun at 17,000 g
at 4 °C for 30 min and the supernatant was removed. 6x SDS loading buffer was added to the supernatant and the samples were heated at 95 °C for 7 min in a Bio-Rad C1000 Thermal Cycler, before 10 % SDS-PAGE. Each gel contained a triplicate control sample of protein that had been left on ice during the experiment, which was used to standardize concentrations between different gels. Recovery was calculated from the band intensity relative to the control sample on that gel, defined as 100 % recovery.

**β-lactamase enzymatic assay**

A 96 well polystyrene plate (Greiner) was blocked by incubating the wells with 350 µl PBS pH 7.4 containing 3 % bovine serum albumin (BSA, ≥ 98 %, Sigma) for 2 h at 37 °C. 20 µl 25 µM BLA construct in PBS pH 7.4 containing 100 mM DTT was incubated for 10 min at 25, 37, 55, 75, 90 or 100 °C and then cooled down to 10 °C in a Bio-Rad C1000 Thermal Cycler at 3 °C/s. The samples were diluted 1 in 400 using 0.1 M NaH₂PO₄ pH 7.0 containing 1 mM EDTA and 1 % BSA. 3 nM of BLA construct was allowed to react with 100 µM Nitrocefin (Merck) in 0.1 M NaH₂PO₄ pH 7.0 containing 1 mM EDTA at 25 °C in a 96 well plate. The reaction was quenched at different time points by adding a final concentration of 250 µM potassium clavulanate (Sigma). The absorbance at 486 nm was measured using a Spectramax M3 microplate reader (Molecular Devices) shortly after the addition of the clavulanate. The absorbance was corrected for the dilution caused by the clavulanate and blanked by a control lacking enzyme.

**DHFR enzymatic assay**

A 96 well polystyrene plate was blocked by incubating the wells with 350 µl phosphate buffered saline (PBS) pH 7.4 containing 3 % BSA for 2 h at 37 °C. 20 µl 20 µM SpyTag (DA)-DHFR-SpyCatcher in PBS pH 7.4 containing 100 mM DTT was incubated for 10 min at 25 or 100 °C and then cooled down to 10 °C in a Bio-Rad C1000 Thermal Cycler at 3 °C/s. The samples were diluted 1 in 20 using PBS pH 7.4. 100 nM SpyTag (DA)-DHFR-SpyCatcher was incubated at 25 °C with 100 µM β-nicotinamide adenine dinucleotide phosphate reduced (NADPH, Sigma) and 100 µM dihydrofolate acid (DHF, Sigma) in 1x assay buffer (Dihydrofolate Reductase Assay Kit, Sigma). OD₃₄₀ was measured using a Spectramax M3 microplate reader shortly after the addition of the substrates to the enzyme. Absorbance was blanked using 1x assay buffer containing 100 nM SpyTag-DHFR-SpyCatcher. DHFR activity was plotted as the decrease in OD₃₄₀ following mixing the enzyme with substrates.

**Mass spectrometry**

Salt was removed from a 10 µM sample of SpyTag-DHFR-SpyCatcher using a C₄ resin ZipTip (Merck). The sample was then analyzed using a Micromass LCT time-of-flight electrospray ionization mass spectrometer (Micromass UK). The other protein samples were desalted using a Chromolith RP-18e column (Merck) and these samples in acetonitrile with water + 0.1% formic acid were introduced by electrospray ionisation (ESI) into a Micromass LCT Premier XE orthogonal acceleration reflecting TOF mass spectrometer in positive ion mode. MassLynx V 4.00.00 (Waters Corporation) was used to convert the m/z spectrum to a mass spectrum using a Maximum Entropy algorithm. Predicted masses were obtained using the ProtParam tool.\[^{[7]}\]
The second prominent peak for DHFR proteins expressed in *E. coli* RIPL is likely to correspond to the $M_W$ increase from non-enzymatic gluconylation (178 Da).\[^6\]

**DSC**

DSC profiles of SpyTag-BLA-SpyCatcher, SpyTag DA- BLA-SpyCatcher and BLA were measured on the VP Cap DSC (GE Healthcare). The blank (PBS pH 7.4) signal was subtracted from the sample, the values were corrected for concentration and volume, and the baseline was subtracted using the MicroCal DSC Origin Pro 7.0 software (GE Healthcare). Deconvolution of the DSC trace was achieved by MicroCal DSC Origin Pro 7.0 software. A two-state transition model was applied to SpyTag DA- BLA-SpyCatcher and the peak at 85.4 °C for SpyTag- BLA-SpyCatcher. A non-two-state transition model was applied to the peak at 39.6 °C for SpyTag- BLA-SpyCatcher and BLA.\[^8\] The DSC conditions to determine the $T_m$ were 20 µM protein in PBS pH 7.4 from 20 to 110 °C at a scan rate of 1 °C/min at a pressure of 3 atm. To determine reversibility of unfolding, conditions were 20 µM protein in PBS pH 7.4 from 20 to 60 °C with a scan rate of 2 °C/min at a pressure of 3 atm. The protein was allowed to cool to 20 °C and then reheated at the same rate to 60 °C.

**TEV protease cleavage**

10 µM TEV protease was incubated with 10 µM SpyTag (DA)-BLA-TEV-SpyCatcher at 34 °C for 2 h in 50 mM Tris-HCl pH 8.0 containing 0.5 mM EDTA and 1 mM DTT. To stop the reaction, DTT was added to a final concentration of 100 mM and 6x SDS loading buffer was added. Samples were heated at 95 °C for 7 min in a Bio-Rad C1000 Thermal Cycler.

**Molecular visualization**

Protein structures were rendered in PyMOL (DeLano Scientific), based on Protein Data Bank files 2X5P\[^9\] and 1BTL\[^10\].

**Effect of mutation in SpyTag or SpyCatcher on gel mobility and MS.**

a) SpyTag-BLA-SpyCatcher, SpyTag DA-BLA-SpyCatcher and SpyTag-BLA-SpyCatcher EQ were boiled in SDS-loading buffer and analyzed by SDS-PAGE with Coomassie staining. M: molecular weight markers.
b) MS of SpyTag DA-BLA-SpyCatcher.
c) MS of SpyTag-BLA-SpyCatcher EQ.
SpyTag-BLA-SpyCatcher stability was retained after mixing with BLA. a) SpyTag-BLA-SpyCatcher was mixed with BLA, heated at the indicated temperature for 10 min, centrifuged and the supernatant analyzed by SDS-PAGE with Coomassie staining. M: molecular weight markers. Triplicate samples are shown. The control was without any incubation. b) Quantification of soluble fractions from a) (mean of triplicate ± 1 s.d.). Some error bars are too small to be visible.
SpyTag-BLA-SpyCatcher thermal tolerance compared to DA mutant. 

a) SpyTag-BLA-SpyCatcher or SpyTag DA-BLA-SpyCatcher was heated at the indicated temperature for 10 min, centrifuged and the supernatant analyzed by SDS-PAGE with Coomassie staining. C is control without any incubation.

b) Quantification of soluble fractions from a) (mean of triplicate ± 1 s.d.).

c) Nitrocefin assay of enzyme activity after 10 min incubation at 25 or 100 °C (mean of triplicate ± 1 s.d.). Some error bars are too small to be visible.
Figure S4

A) BLA-CnaB2 or SpyTag-BLA-SpyCatcher EQ was heated at the indicated temperature for 10 min, centrifuged and the supernatant analyzed by SDS-PAGE with Coomassie staining. C is control without any incubation.

B) Quantification of soluble fractions from A) (mean of triplicate ± 1 s.d.).

C) Nitrocefin assay of enzyme activity for SpyTag-BLA-SpyCatcher EQ, BLA-CnaB2 and SpyTag-BLA-SpyCatcher after 10 min incubation at the indicated temperature (mean of triplicate ± 1 s.d.). Some error bars are too small to be visible.

**Thermal tolerance of CnaB2 and SpyCatcher EQ cyclization controls.** a) BLA-CnaB2 or SpyTag-BLA-SpyCatcher EQ was heated at the indicated temperature for 10 min, centrifuged and the supernatant analyzed by SDS-PAGE with Coomassie staining. C is control without any incubation. b) Quantification of soluble fractions from a) (mean of triplicate ± 1 s.d.). c-e) Nitrocefin assay of enzyme activity for SpyTag-BLA-SpyCatcher EQ, BLA-CnaB2 and SpyTag-BLA-SpyCatcher after 10 min incubation at the indicated temperature (mean of triplicate ± 1 s.d.). Some error bars are too small to be visible.
SpyTag/SpyCatcher cyclization rendered DHFR activity stable to boiling. a) SpyTag (DA)-DHFR-SpyCatcher (EQ) was boiled in SDS-loading buffer and analyzed by SDS-PAGE with Coomassie staining. b) SpyTag (DA)-DHFR-SpyCatcher was heated at the indicated temp. for 10 min, centrifuged, and protein recovered in the supernatant analyzed by SDS-PAGE with Coomassie staining (mean of triplicate ± 1 s.d.). c) MS of SpyTag-DHFR-SpyCatcher and linear variants. d) Catalytic activity, after 10 min incubation at 25 or 100 °C (mean of triplicate ± 1 s.d.). Some error bars are too small to be visible.