Protein Engineering Hot Paper

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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.[1] The stabilization of enzymes can be achieved without the use of chemical modifications by looking for homologues in thermophiles,[2] inferring a consensus or ancestral sequence,[3–6] selection from libraries,[7,8] or structure-based design.[9,10] Proteins obtained from thermophiles usually achieve their optimum catalytic efficiency at a high temperature,[11] 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.[12] 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.[13–16] We engineered SpyTag, a 13 amino acid peptide, to rapidly form an irreversible amide bond to the 15 kDa protein SpyCatcher.[14] The system is genetically encodable and the tag and protein are functional on either terminus.[14] Since the termini are one of the most flexible regions of proteins and fluctuations here may initiate unfolding,[17] 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,[18] sortase,[19] or (most often) inteins.[20–23] 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.[6,24] Previous cyclization of BLA with a split intein achieved a 5°C increase in thermal tolerance.[21] 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 (SpyCatcherEQ).[14] 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

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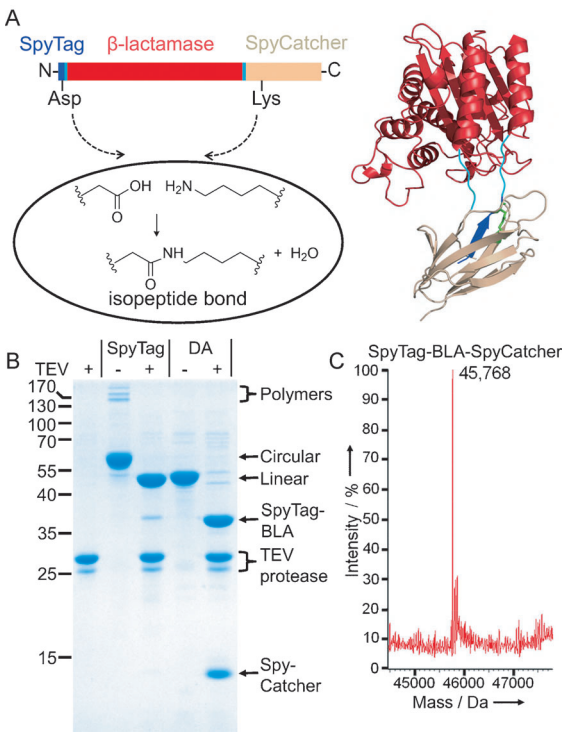

Figure 1. SpyTag/SpyCatcher-mediated cyclization of β-lactamase. A) A cartoon of the cyclization of BLA. B) SDS-PAGE with Coomassie staining for TEV protease or SpyTag-BLA-TEV-SpyCatcher and SpyTagDA-BLA-TEV-SpyCatcher with or without TEV protease. C) Mass spectrometry of SpyTag-BLA-SpyCatcher.

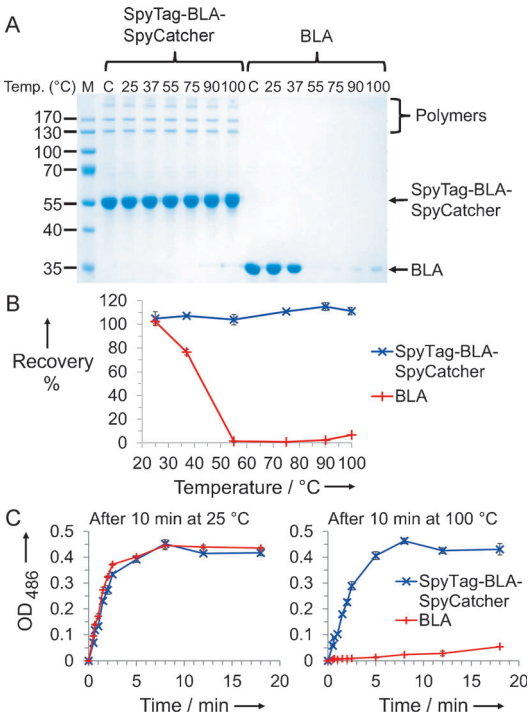

Figure 2. Increased thermal tolerance of the cyclized enzyme. A) Cyclized or original enzyme was heated at the indicated temperature for 10 min, centrifuged, and the supernatant analyzed by SDS-PAGE with Coomassie staining. M = molecular weight markers. C = control without any incubation. B) Quantification of soluble fractions from (A). The data points show the mean of triplicate samples ± 1 standard deviation (SD). C) Nitrocefin assay of enzyme activity after 10 min incubation at 25 or 100 °C (mean of triplicate ± 1 SD). Some error bars are too small to be seen. OD_{486} = absorbance at 486 nm.

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_2O 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)[14] 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)[14] 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).[25] 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

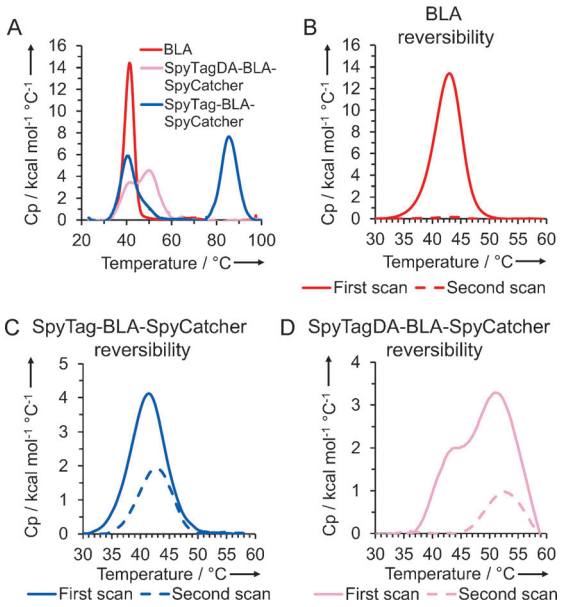

Figure 3. Thermal unfolding transitions of BLA, SpyTag-BLA-SpyCatcher, and SpyTagDA-BLA-SpyCatcher. A) DSC giving the specific heat capacity (Cp) of each enzyme construct scanned from 20–110 °C at 1 °C min^{-1}. B) DSC of BLA scanned from 20–60 °C at 2 °C min^{-1} before cooling and then repeating the scan. C) DSC as in (B) for SpyTag-BLA-SpyCatcher. D) DSC as in (B) for SpyTagDA-BLA-SpyCatcher.

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,[27] and often only give modest stability enhancements.[12] 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.[28] 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,[30] it will also be interesting to explore whether SpyTag/SpyCatcher stabilization could facilitate library-based evolution of novel protein function.

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