Published in final edited form as:
Biochemistry. 2013 August 27; 52(34): 5920–5927. doi:10.1021/bi400736c.

An internal disulfide bond acts as a switch for intein activity

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Abstract

Inteins are intervening polypeptides that catalyze their own removal from flanking exteins, concomitant to the ligation of the exteins. The intein that interrupts the DP2 (large) subunit of DNA Polymerase II from *Methanoculleus marisnigri (Mma)* can promote protein splicing. However, protein splicing can be prevented or reduced by over-expression under non-reducing conditions, due to the formation of a disulfide bond between two internal intein Cys residues. This redox sensitivity leads to differential activity in different strains of *E. coli* as well as in different cell compartments. The redox-dependent control of *in vivo* protein splicing in an intein derived from an anaerobe that can occupy multiple environments hints at a possible physiological role for protein splicing.

Protein splicing is a self-catalyzed process facilitated by an intein. The intein interrupts two flanking polypeptides, called the N- and C-exteins, and promotes both its own excision as well as the ligation of these exteins [(1, 2)].

Protein splicing usually follows a four-step mechanism (Fig. 1A). First, the peptide bond linking the N-extein and intein is converted to a linear ester or thioester by nucleophilic attack by the intein's N-terminal Ser or Cys. Next, the N-extein is transferred from the side chain of the first residue of the intein to the side chain of the first residue of the C-extein (Ser, Thr or Cys) via transesterification. In step three, cyclization of the conserved C-terminal Asn residue of the intein, coupled to peptide bond cleavage and aminosuccinimide formation, separates the intein from the exteins. Concomitantly, an ester bond links the exteins. Finally, the aminosuccinimide may be hydrolyzed, and the exteins' ester linkage is rapidly converted to a peptide bond [(1, 2)]. If the steps of splicing are poorly coordinated, side reactions can occur. In N-terminal cleavage, the linear or branched ester formed in steps one and two can be cleaved, uncoupled from splicing. In addition, Asn cyclization uncoupled from splicing can result in C-terminal cleavage.

Protein splicing can be essential for the activity of the flanking proteins [(3, 4)]. It has been suggested that inteins, particularly in association with intervening homing endonuclease domains, are parasitic genetic elements. As such, inteins persist in their host sequence because they have little effect on fitness, are present in highly conserved sites, or are capable of using their associated homing endonuclease to invade intein-less alleles [(5–8)]. If, instead,

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Supporting Information

A table with the MS/MS matching data from Figure 4D is given as Table S1. Supplemental figures S1–S4 are also provided, as described in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

inteins are present in host proteins because they play a beneficial role, perhaps by regulating the activity of their flanking exteins, splicing would need to be responsive to a trigger.

Some native inteins, and others modified by molecular engineering, can promote conditional protein splicing. Inteins from thermophilic organisms can be purified as unspliced precursors from over-expression in *E. coli* and induced to splice at high temperature [9–11]. Engineered inteins have been designed to facilitate splicing (or cleavage side reactions) in response to changes in light or protease activity [12, 13], small molecules [14–16], pH [17, 18] or temperature [10, 19–22]. Protein splicing in *trans*, in which the intein is split into two separate fragments, depends on re-association of these fragments. Split-intein re-association has been triggered in engineered systems by light or the addition of a small molecule, both *in vitro* and *in vivo* [13, 23–28].

Conditional protein splicing also can be triggered by reduction of a disulfide bond that prevents splicing of the intein. Such disulfide bonds have been engineered between intein and extein Cys residues to control splicing activity [29–33]. Native disulfide bonds that influence activity, either between Cys1 and Cys+1 [34, 35] or between Cys1 and a Cys in the N-extein [30], have also been described. Autoprocessing by hedgehog domains, which are similar in structure and sequence to inteins, depends on reduction of a disulfide bond between Cys1 of the processing domain and a conserved downstream Cys [36].

We report an intein with *in vivo* splicing activity that is controlled by the oxidation state of a disulfide bond. Unlike inteins with disulfide bonds between extein and intein Cys residues, this intein has a disulfide bond between Cys residues within the intein. We show that the intein that interrupts the DP2 subunit of DNA Polymerase II from *Methanoculleus marisnigri* (the *Mma* PolII intein) [37] can promote protein splicing when over-expressed in *E. coli* BL21(DE3). However, when over-expressed in an *E. coli* strain that promotes disulfide bond formation, we isolate unspliced precursor. This precursor can be induced to undergo N-terminal cleavage upon *in vitro* incubation with 1,4-dithiothreitol (DTT). Unusual migration on SDS-PAGE, resolvable by reduction, indicates the presence of a disulfide bond in the unspliced precursor. We also show that splicing is dependent on cellular oxidation state, with differential activity in different *E. coli* strains as well as between the cytoplasm and periplasm of the same strain.

Experimental Procedures

Plasmid preparation

We created an *E. coli* expression vector for a fusion protein of the *E. coli* maltose binding protein (MBP) to the 10 C-terminal residues of the N-extein, the 165-residue intein, the six N-terminal residues of the C-extein, and a poly His-tag. Short linkers connect the MBP and His-tag to the extein segments. To create this vector, pMIHMma, we amplified the *Mma* PolII gene by PCR from genomic DNA (ATCC, 35101D) with primers MmaU (5′-GGCTACGGCAGGCCTTTCTTCCACG) and MmaL (5′-GAGGAGCGAATTCCAGTCCTCGTCGCCGTC). The PCR product was inserted between the *Stu*I and *Eco*RI sites of plasmid pPabPol1His [11]. To facilitate periplasmic expression, we digested pMIHMma with *Stu*I and *Hin*dIII and transferred the intein-containing sequence to pMal-p2x (New England Biolabs) to make pMIHMma-p2x. We created site-directed mutants of the intein with appropriate primers as described [38]. The sequence of the intein was verified by DNA sequencing and was consistent with the NCBI database (accession number ABN58047).

Protein expression and purification

To facilitate overexpression under reducing or oxidizing conditions, plasmids were transformed into *E. coli* BL21(DE3) or Origami 2(DE3), respectively (Novagen). We induced expression at mid-log phase with 1 mM isopropyl-β-D-1-thiogalactopyranoside and incubated the cells with shaking at 20°C for 16 h.

Overexpressed fusion proteins were purified through their C-terminal His tags. *E. coli* were pelleted by centrifugation at 3000 × g, and pellets were suspended in buffer A (20 mM HEPES, pH 7.5, 500 mM NaCl) supplemented with BugBuster Extraction buffer (Novagen), 12 units/ml benzonase nuclease, 100 μM phenylmethylsulfonyl fluoride and Protease Inhibitor Cocktail P8849 (Sigma-Aldrich). The cellular extract was either analyzed by Western blot or further purified using Talon metal affinity resin (Clontech), prewashed with buffer A. After loading the sample, the column was washed with 3 × 10 ml of buffer A supplemented with 10 mM imidazole and 0.1% Tween-20 and eluted with 3 × 500 μl of buffer A supplemented with 200 mM imidazole. Protein concentration was determined by the Bradford method [(39)]. Proteins were exchanged against buffer A using a Millipore Ultracel-0.5 centrifugal filter, 3000 MWCO.

Protein analysis

To analyze protein splicing by SDS-PAGE, we used precast 4–20% gradient Tris-glycine gels via the Laemmli method [(40)]. Sample buffer was supplemented with DTT to 50 mM for reducing SDS-PAGE, with about 2 μg of protein per lane. Western blot analysis was performed by blotting onto PVDF, blocking with 1% BSA in Buffer W (PBS and 0.1% Tween-20) and incubating with a 1:4000 dilution of His-Detector Nickel-AP conjugate. We washed the blots in buffer W and developed them with Western Blue stabilized substrate.

LC/FT-MS and LC/MS-MS were performed on tryptic digests as described [(34)] to determine which Cys residues participated in a disulfide bond. For MALDI-TOF analysis, protein samples were desalted using a C18 ZipTip and spotted with 3,5-dimethoxy-4-hydroxycinnamic acid. We analyzed samples with an UltraFLEX III TOF mass spectrometer in linear mode.

Results and Discussion

Protein splicing activity

We are able to over-express a fusion of the *Mma* PolII intein with an N-terminal MBP and a C-terminal His tag. The intein was selected for study for two reasons: i) a non-canonical C-terminal Gln replaces the highly conserved Asn, and ii) the intein has Cys residues in intein blocks F and G that we suspected might be near the active site and promote disulfide-bond formation (Fig. 1B).

On over-expression in *E. coli* and purification utilizing the C-terminal His tag, we observed that the wild-type intein promotes mostly N-terminal cleavage with limited splicing, whereas substitution of the C-terminal Gln165 for the highly conserved Asn results in a considerable increase in splicing efficiency (Fig. 2).

The identity of the bands MIH, MH and IH were confirmed by Western blot confirming the presence of a His-tag (data not shown) and by MALDI-TOF/MS. For MIHMma, we observed a peak for MALDI-TOF/MS consistent with IH (expected m/z 20735, experimental 20739). For MIHMma Q165N expressed in *E. coli* BL21(DE3), we observed MALDI-TOF/MS data consistent with IH (m/z expected 20721, observed 20712), MH (m/z expected 46458, observed 46528), and I (m/z expected 18404, observed 18393). We observed a MALDI-TOF/MS peak consistent with the precursor MIH in the MIHMma

Q165N sample expressed from *E. coli* Origami 2(DE3) (m/z expected 64844, observed 65026).

Influence of potentially catalytic residues on splicing efficiency

It is curious that the intein with native C-terminal Gln splices poorly, given that ligation of the flanking native exteins is likely essential. Cyclization with Gln is slow and results in cleavage of the thioester from steps one or two uncoupled from splicing, or perhaps the poor splicing in the wild-type context is due to expression in the context of modified exteins and a non-native host organism. Regardless, we show that the control of intein activity by disulfide bond formation described below holds true for both wild type intein and a Q165N mutant, so this redox effect is likely physiologically relevant.

In the *Pyrococcus abyssi* PolII (*Pab* PolII) intein, both splicing and C-terminal cleavage via side-chain cyclization are faster with the conserved Asn than with native Gln [41]. The highly conserved penultimate His has been shown to be important in promoting the third step of splicing in some inteins [42–44]. However, in other cases replacement of non-conserved residues at this position with His has either no effect or is deleterious to splicing [42, 45, 46]. We hypothesized that replacing the native penultimate Gly residue (Gly164) with the conserved His might increase the extent of splicing with the native Gln. However, substitution of Gly164 to His does not increase the efficiency of splicing in the wild-type *Mma* PolII intein, and disrupts splicing and N-terminal cleavage in the Q165N mutant (Fig. 2), suggesting that the conformational flexibility of the penultimate Gly might be important for coordinating and promoting the steps of splicing.

Besides Cys1 and Cys+1, the *Mma* PolII intein has Cys residues at two important positions. The first is at position four of block F (residue F:4). In other inteins, this residue is the Cys responsible for initiating splicing in class three inteins [47–49] or Asp and Ser residues that may coordinate the steps of splicing [18, 50–53]. Although Asp is the most common F:4 residue in canonical inteins, Cys is among the next most conserved options. The second Cys residue is in block G, three residues removed from the active site Cys+1 (Fig. 1b). Cys is an unusual residue for this position [54].

Mutation of conserved Cys residues disrupt splicing (Fig. 2). Mutation of Cys1 to Ala, which should prevent step one, results in precursor accumulation rather than C-terminal cleavage, suggesting that step one or two of splicing is required to promote step three. Mutation of both Cys+1 and Q165 to Ala, which blocks steps two and three, results in accumulation of precursor and some N-terminal cleavage, likely from cleavage of the linear thioester formed in step one.

The MIHMma triple mutant of E152R/Q165N/S+9R was prepared for tryptic digests/LC-MS described below, in order to separate the block F and G Cys residues on different tryptic fragments. The mutation allows for splicing, albeit reduced in extent. We note aberrant migration of the bands IH and I with SDS-PAGE under reducing conditions. However, MALDI-TOF/MS confirmed the identity of these bands as IH (m/z 20831 observed versus 20817 expected) and I (m/z 18443 observed versus 18431 expected), as does reactivity of IH with the reagent directed against the C-terminal His-tag (Fig. S1).

Mutation of the block F Cys147 or block G Cys163 also interrupts splicing in MIHMma Q165N (Fig. 2). (Although MIHMma promotes mostly N-terminal cleavage, there is a modest effect due to these mutations (Fig. S2).) We hypothesized that one of these Cys residues, both likely to be near the active site, might form a disulfide bond that would prevent or regulate splicing. There is precedence for redox-controlled protein splicing; however, this would be the first example of a native *internal* intein disulfide bond regulating

splicing. Although a disulfide bond formed between an internal Cys residue could be inhibitory, it is not surprising that mutation of the Cys residue could interfere with splicing under reducing conditions, as the reduced Cys may play a role in properly coordinating the active site.

Protein splicing controlled by a disulfide bond – SDS-PAGE evidence

For the *Mma* PolII intein, the first evidence of a disulfide bond came from a comparison of protein splicing activity when proteins were overexpressed in *E. coli* BL21(DE3) versus *E. coli* Origami 2(DE3), which has deletions of two oxidoreductases, trxB and gorA, that results in cytoplasmic disulfide bond formation [55]. We noted an increase in unreacted precursor, as well as a decrease in intensity of bands due to splicing or N-terminal cleavage, when proteins are expressed in the oxidizing *E. coli* Origami 2(DE3) cells rather than the reducing *E. coli* BL21(DE3) cells (Fig. 3A).

In addition, we incubated unspliced precursor isolated from *E. coli* Origami 2(DE3) with increasing amounts of DTT. When SDS-PAGE is performed in the absence of additional DTT, the band representing the unspliced precursor has an altered migration that is restored to the expected migration on reduction, suggesting a change in molecular shape due to the presence or absence of a disulfide bond (Fig. 3B).

We made separate mutations to each Cys residue in the context of the Q165N mutant to determine which Cys residues might be responsible for making a disulfide bond. We then analyzed the change in migration by SDS-PAGE for the unspliced precursor under reducing or non-reducing conditions (Fig. 3C). For Q165N, we observe a change in migration of the precursor band in the absence of reductant, which we attribute to a disulfide bond. However, the shift in migration is lost with mutation of Cys1 to Ala or Cys147 to Ser, suggesting that these residues are involved in the disulfide bond. We observe a mixed phenotype when Cys +1 is converted to Ala or Cys163 is converted to Thr, suggesting that neither residue is absolutely required for disulfide bond formation (Fig. 3C). Mutation of Cys163 to Thr also affects the extent of splicing (Fig. 2), so it may play some structural role in the active site. We observe similar gel shifts with wild-type MIHMma (Fig. S3).

Protein splicing controlled by a disulfide bond – Mass spectrometry evidence

To verify the Cys residues involved in the disulfide bond, we performed further analysis by mass spectrometry. We purified MIHMma E152R/Q165N/S+9R from *E. coli* Origami 2(DE3) and analyzed the sample by SDS-PAGE both with and without the addition of DTT. The unspliced precursor bands boxed in Fig. 4A were excised from the gel and digested by trypsin. We analyzed the digests by LC/FT-MS and MS/MS. We identified a LC peak present in non-reduced sample, but not present in the reduced sample, with a retention time of 29.34 min (Fig. 4B). FT-MS analysis of this fraction gave a z=3 peak of 836.0650, compared to a theoretical value of 836.0618 for a peptide linking the tryptic fragments containing Cys1 and Cys147 (Fig. 4C). MS/MS analysis of this fraction was also consistent with this peptide (Fig. 4D). We observed over 90% coverage of tryptic peptides for both the reduced and oxidized samples.

In vitro activity of unspliced precursor

We wanted to determine if we could purify redox-trapped precursor and induce splicing or cleavage *in vitro.* We purified MIHMma Q165N and incubated the protein at 37°C at pH 7.5 in the presence of 50 mM DTT, 150 mM DTT, 4 mM TCEP or no reductant. We observed that DTT induces N-terminal cleavage in a concentration and time-dependent manner (Fig. 5). Incubation with TCEP results in cleavage as well, although less efficiently. This may result from DTT both reducing the disulfide bond and serving as the nucleophile to induce

thiolysis of the linear or branched ester. Similar results are seen with the wild type MIHMma. After N-terminal cleavage, a band appears that migrates consistently with the size of the excised intein. (This band does not react with the anti-His-tag antibody, further suggesting that it is I and not IH with aberrant migration. Bands attributed to IH and I were also confirmed with MALDI-TOF/MS.) Although there may be a small amount of splicing generating the I band, it is more likely that the IH formed on N-terminal cleavage then undergoes uncoupled C-terminal cleavage, supported by the decrease of the IH band and increase of I band in Fig. 5. This suggests that the intein undergoes a mechanism-linked conformational change after step one or two. Therefore, the resulting cleavage of the thioester is required to permit step three, consistent with precursor accumulation when step one of splicing is blocked (Fig. 2). Although we isolate less precursor on expression in *E. coli* BL21(DE3) (Fig. 3A), treatment of the remaining precursor with DTT or TCEP gives similar results (Fig. S4), suggesting that the precursor is catalytically competent to facilitate the steps of splicing, even if they are not properly coordinated. In attempts to induce splicing rather than cleavage, we varied pH, temperature, time of incubation, and the means by which we disrupted the cells. We also added solubilizing reagents and attempted to refold the protein under reducing or non-reducing conditions both by rapid dilution and slow dialysis, and consistently observed stable precursor with some uncoupled cleavage reactions rather than *in vitro* splicing (data not shown). However, our *in vivo* results show that the intein splices under reducing conditions and does not splice under non-reducing conditions, suggesting potential physiological relevance (see below). As we are able to induce N-terminal cleavage efficiently *in vitro* from an inactive precursor, the intein might serve use in biotechnology applications such as intein-mediated protein purification.

Potential regulatory role for an intein disulfide bond

We also wanted to determine if this disulfide bond could control protein splicing in different cell compartments. Either MIHMma or MIHMma Q165N was expressed to the cytoplasm or periplasm. Total cellular extracts were analyzed for splicing activity by reducing SDS-PAGE followed by Western blot (Fig. 3A). In the BL21(DE3) strain, splicing and N-terminal cleavage were significantly reduced on expression in the periplasm, which is a more oxidizing environment than the cytoplasm. Similar reductions in activity were observed in Origami 2(DE3) cells. This suggests that this redox trigger allows splicing to be sensitive to the different redox states in different cellular compartments. Given that the native *Mma* host is an obligate anaerobe that can colonize diverse anaerobic environments [(56)], it is exciting to speculate that intein-mediated redox control of the activity of the replicative polymerase might have physiological relevance.

Relative strengths of intein disulfide bonds

It appears that the disulfide bond between residues 1 and 147 is strong enough to be fully oxidized in *E. coli* Origami 2(DE3), particularly when expressed to the periplasm. When expressed in BL21(DE3), the fraction of the precursor that is not oxidized appears to either splice or undergo cleavage. This led us to question whether the disulfide bond in the *Mma* PolII intein is more resistant to reduction than the disulfide we previously observed in the *Pab* PolII intein [(34)].

We previously reported that the *Pab* PolII intein could be isolated as a disulfide-inactivated precursor on over-expression in *E. coli* BL21(DE3) [(34)]. We also showed that the disulfide bond was sensitive to temperature and flanking sequence. To compare these results with the *Mma* PolII intein, we examined the *Pab* PolII intein in the same MIH context, as previously described [(11)]. We purified both MIHMma and MIHPab from over-expression in *E. coli* Origami 2(DE3). In Fig. 6A, we show that although over 10% of our purified MIHPab precursor was in the reduced state, the MIHMma precursor was more easily reduced by DTT

on incubation at 20°C for 30 min with 1 mM EDTA. This could be due to the relative strength of the disulfide bonds and/or to the rigidity of the thermophilic *Pab* intein at low temperature. However, the *Pab* intein was more sensitive to reduction by glutathione under the same conditions (Fig. 6B), suggesting that the disulfide bond between Cys1 and Cys+1 of the *Pab* intein might be more assessable to the bulkier reducing agent than the internal Cys1-Cys147 disulfide in the *Mma* intein. The *Mma* intein was also more sensitive to DTT-induced reduction in the presence of 1 mM EDTA (Fig. 6C), which we did not observe with *Pab* intein. This might indicate metal ion involvement with disulfide bond formation or in the active site. Divalent cations have been shown to inhibit protein splicing with other inteins [57–60].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

B. Funding Source Statement: This material is based upon work supported by the National Science Foundation under grants MCB-0950245 and MCB-1244089 (KVM), by the National Institutes of Health grants GM38060 (RJL) and GM81408 (CW), and by a Henry Dreyfus Teacher-Scholar Award (KVM).

Abbreviations and Textual Footnotes

MBP	*E. coli* maltose binding protein
MIH	a fusion protein of MBP, an intein, and a poly His-tag
MIHMma	MIH with the *Mma* PolII intein
MIHPab	MIH with the *Pab* PolII intein
Mma	*Methanoculleus marisnigri*
Pab	*Pyrococcus abyssi*

References

1. Paulus H. Protein splicing and related forms of protein autoprocessing. Annu Rev Biochem. 2000; 69:447–496. [PubMed: 10966466]
2. Volkmann G, Mootz HD. Recent progress in intein research: from mechanism to directed evolution and applications. Cell Mol Life Sci. 2013; 70:1185–1206. [PubMed: 22926412]
3. Davis EO, Jenner PJ, Brooks PC, Colston MJ, Sedgwick SG. Protein splicing in the maturation of M. tuberculosis recA protein: a mechanism for tolerating a novel class of intervening sequence. Cell. 1992; 71:201–210. [PubMed: 1423588]
4. Kawasaki M, Nogami S, Satow Y, Ohya Y, Anraku Y. Identification of three core regions essential for protein splicing of the yeast Vma1 protozyme. A random mutagenesis study of the entire Vma1-derived endonuclease sequence. J Biol Chem. 1997; 272:15668–15674. [PubMed: 9188457]
5. Gogarten JP, Hilario E. Inteins, introns, and homing endonucleases: recent revelations about the life cycle of parasitic genetic elements. BMC Evol Biol. 2006; 6:94. [PubMed: 17101053]
6. Gogarten JP, Senejani AG, Zhaxybayeva O, Olendzenski L, Hilario E. Inteins: structure, function, and evolution. Annu Rev Microbiol. 2002; 56:263–287. [PubMed: 12142479]
7. Pietrokovski S. Intein spread and extinction in evolution. Trends Genet. 2001; 17:465–472. [PubMed: 11485819]
8. Swithers KS, Senejani AG, Fournier GP, Gogarten JP. Conservation of intron and intein insertion sites: implications for life histories of parasitic genetic elements. BMC Evol Biol. 2009; 9:303. [PubMed: 20043855]

9. Cambon-Bonavita MA, Schmitt P, Zieger M, Flaman JM, Lesongeur F, Raguenes G, Bindel D, Frisch N, Lakkis Z, Dupret D, Barbier G, Querellou J. Cloning, expression, and characterization of DNA polymerase I from the hyperthermophilic archaea Thermococcus fumicolans. Extremophiles. 2000; 4:215–225. [PubMed: 10972190]
10. Choi JJ, Nam KH, Min B, Kim SJ, Soll D, Kwon ST. Protein trans-splicing and characterization of a split family B-type DNA polymerase from the hyperthermophilic archaeal parasite Nanoarchaeum equitans. J Mol Biol. 2006; 356:1093–1106. [PubMed: 16412462]
11. Mills KV, Manning JS, Garcia AM, Wuerdeman LA. Protein splicing of a Pyrococcus abyssi intein with a C-terminal glutamine. J Biol Chem. 2004; 279:20685–20691. [PubMed: 15024006]
12. Berrade L, Kwon Y, Camarero JA. Photomodulation of protein trans-splicing through backbone photocaging of the DnaE split intein. Chembiochem. 2010; 11:1368–1372. [PubMed: 20512791]
13. Vila-Perello M, Hori Y, Ribo M, Muir TW. Activation of protein splicing by protease- or light-triggered O to N acyl migration. Angew Chem Int Ed Engl. 2008; 47:7764–7767. [PubMed: 18767096]
14. Peck SH, Chen I, Liu DR. Directed evolution of a small-molecule-triggered intein with improved splicing properties in mammalian cells. Chem Biol. 2011; 18:619–630. [PubMed: 21609843]
15. Skretas G, Wood DW. Regulation of protein activity with small-molecule-controlled inteins. Protein Sci. 2005; 14:523–532. [PubMed: 15632292]
16. Zhang L, Zheng Y, Callahan B, Belfort M, Liu Y. Cisplatin inhibits protein splicing, suggesting inteins as therapeutic targets in mycobacteria. J Biol Chem. 2011; 286:1277–1282. [PubMed: 21059649]
17. Wood DW, Derbyshire V, Wu W, Chartrain M, Belfort M, Belfort G. Optimized single-step affinity purification with a self-cleaving intein applied to human acidic fibroblast growth factor. Biotechnol Prog. 2000; 16:1055–1063. [PubMed: 11101334]
18. Wood DW, Wu W, Belfort G, Derbyshire V, Belfort M. A genetic system yields self-cleaving inteins for bioseparations. Nat Biotechnol. 1999; 17:889–892. [PubMed: 10471931]
19. Adam E, Perler FB. Development of a positive genetic selection system for inhibition of protein splicing using mycobacterial inteins in Escherichia coli DNA gyrase subunit A. J Mol Microbiol Biotechnol. 2002; 4:479–487. [PubMed: 12432958]
20. Cann IK, Amaya KR, Southworth MW, Perler FB. Bacteriophage-based genetic system for selection of nonsplicing inteins. Appl Environ Microbiol. 2004; 70:3158–3162. [PubMed: 15128583]
21. Tan G, Chen M, Foote C, Tan C. Temperature-sensitive mutations made easy: generating conditional mutations by using temperature-sensitive inteins that function within different temperature ranges. Genetics. 2009; 183:13–22. [PubMed: 19596904]
22. Zeidler MP, Tan C, Bellaiche Y, Cherry S, Hader S, Gayko U, Perrimon N. Temperature-sensitive control of protein activity by conditionally splicing inteins. Nat Biotechnol. 2004; 22:871–876. [PubMed: 15184905]
23. Binschik J, Zettler J, Mootz HD. Photocontrol of protein activity mediated by the cleavage reaction of a split intein. Angew Chem Int Ed Engl. 2011; 50:3249–3252. [PubMed: 21384476]
24. Buskirk AR, Ong YC, Gartner ZJ, Liu DR. Directed evolution of ligand dependence: small-molecule-activated protein splicing. Proc Natl Acad Sci U S A. 2004; 101:10505–10510. [PubMed: 15247421]
25. Mootz HD, Blum ES, Tyszkiewicz AB, Muir TW. Conditional protein splicing: a new tool to control protein structure and function in vitro and in vivo. J Am Chem Soc. 2003; 125:10561–10569. [PubMed: 12940738]
26. Mootz HD, Muir TW. Protein splicing triggered by a small molecule. J Am Chem Soc. 2002; 124:9044–9045. [PubMed: 12148996]
27. Tyszkiewicz AB, Muir TW. Activation of protein splicing with light in yeast. Nat Methods. 2008; 5:303–305. [PubMed: 18272963]
28. Yuen CM, Rodda SJ, Vokes SA, McMahon AP, Liu DR. Control of transcription factor activity and osteoblast differentiation in mammalian cells using an evolved small-molecule-dependent intein. J Am Chem Soc. 2006; 128:8939–8946. [PubMed: 16819890]

29. Callahan BP, Stanger M, Belfort M. A redox trap to augment the intein toolbox. Biotechnol Bioeng. 2013; 110:1565–1573. [PubMed: 23280506]
30. Callahan BP, Topilina NI, Stanger MJ, Van Roey P, Belfort M. Structure of catalytically competent intein caught in a redox trap with functional and evolutionary implications. Nat Struct Mol Biol. 2011; 18:630–633. [PubMed: 21460844]
31. Cui C, Zhao W, Chen J, Wang J, Li Q. Elimination of in vivo cleavage between target protein and intein in the intein-mediated protein purification systems. Protein Expr Purif. 2006; 50:74–81. [PubMed: 16884922]
32. Saleh L, Southworth MW, Considine N, O'Neill C, Benner J, Bollinger JM Jr, Perler FB. Branched intermediate formation is the slowest step in the protein splicing reaction of the Ala1 KlbA intein from Methanococcus jannaschii. Biochemistry. 2011; 50:10576–10589. [PubMed: 22026921]
33. Shi J, Muir TW. Development of a tandem protein trans-splicing system based on native and engineered split inteins. J Am Chem Soc. 2005; 127:6198–6206. [PubMed: 15853324]
34. Chen W, Li L, Du Z, Liu J, Reitter JN, Mills KV, Linhardt RJ, Wang C. Intramolecular disulfide bond between catalytic cysteines in an intein precursor. J Am Chem Soc. 2012; 134:2500–2503. [PubMed: 22280304]
35. Mills KV, Lew BM, Jiang S, Paulus H. Protein splicing in trans by purified N-and C-terminal fragments of the Mycobacterium tuberculosis RecA intein. Proc Natl Acad Sci U S A. 1998; 95:3543–3548. [PubMed: 9520402]
36. Chen X, Tukachinsky H, Huang CH, Jao C, Chu YR, Tang HY, Mueller B, Schulman S, Rapoport TA, Salic A. Processing and turnover of the Hedgehog protein in the endoplasmic reticulum. J Cell Biol. 2011; 192:825–838. [PubMed: 21357747]
37. Perler FB. InBase, the Intein Database. Nucleic Acids Res. 2000; 28:344–345. [PubMed: 10592269]
38. O'Brien KM, Schufreider AK, McGill MA, Reitter JN, Mills KV. Mechanism of protein splicing of the Pyrococcus abyssi lon protease intein. Biochem Biophys Res Commun. 2010; 403:457–461. [PubMed: 21094142]
39. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976; 72:248–254. [PubMed: 942051]
40. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970; 227:680–685. [PubMed: 5432063]
41. Mills KV, Dorval DM, Lewandowski KT. Kinetic analysis of the individual steps of protein splicing for the Pyrococcus abyssi PolII intein. J Biol Chem. 2005; 280:2714–2720. [PubMed: 15557319]
42. Chen L, Benner J, Perler FB. Protein splicing in the absence of an intein penultimate histidine. J Biol Chem. 2000; 275:20431–20435. [PubMed: 10770923]
43. Mizutani R, Nogami S, Kawasaki M, Ohya Y, Anraku Y, Satow Y. Protein-splicing reaction via a thiazolidine intermediate: crystal structure of the VMA1-derived endonuclease bearing the N and C-terminal propeptides. J Mol Biol. 2002; 316:919–929. [PubMed: 11884132]
44. Wang S, Liu XQ. Identification of an unusual intein in chloroplast ClpP protease of Chlamydomonas eugametos. J Biol Chem. 1997; 272:11869–11873. [PubMed: 9115246]
45. Southworth MW, Perler FB. Protein splicing of the Deinococcus radiodurans strain R1 Snf2 intein. J Bacteriol. 2002; 184:6387–6388. [PubMed: 12399510]
46. Wu H, Hu Z, Liu XQ. Protein trans-splicing by a split intein encoded in a split DnaE gene of Synechocystis sp. PCC6803. Proc Natl Acad Sci U S A. 1998; 95:9226–9231. [PubMed: 9689062]
47. Brace LE, Southworth MW, Tori K, Cushing ML, Perler F. The Deinococcus radiodurans Snf2 intein caught in the act: detection of the Class 3 intein signature Block F branched intermediate. Protein Sci. 2010; 19:1525–1533. [PubMed: 20521254]
48. Tori K, Dassa B, Johnson MA, Southworth MW, Brace LE, Ishino Y, Pietrokovski S, Perler FB. Splicing of the mycobacteriophage Bethlehem DnaB intein: identification of a new mechanistic class of inteins that contain an obligate block F nucleophile. J Biol Chem. 2010; 285:2515–2526. [PubMed: 19940146]

49. Tori K, Perler FB. Expanding the definition of class 3 inteins and their proposed phage origin. J Bacteriol. 2011; 193:2035–2041. [PubMed: 21317331]
50. Du Z, Liu J, Albracht CD, Hsu A, Chen W, Marieni MD, Colelli KM, Williams JE, Reitter JN, Mills KV, Wang C. Structural and mutational studies of a hyperthermophilic intein from DNA polymerase II of Pyrococcus abyssi. J Biol Chem. 2011; 286:38638–38648. [PubMed: 21914805]
51. Frutos S, Goger M, Giovani B, Cowburn D, Muir TW. Branched intermediate formation stimulates peptide bond cleavage in protein splicing. Nat Chem Biol. 2010; 6:527–533. [PubMed: 20495572]
52. Pereira B, Shemella PT, Amitai G, Belfort G, Nayak SK, Belfort M. Spontaneous proton transfer to a conserved intein residue determines on-pathway protein splicing. J Mol Biol. 2011; 406:430–442. [PubMed: 21185311]
53. Van Roey P, Pereira B, Li Z, Hiraga K, Belfort M, Derbyshire V. Crystallographic and mutational studies of Mycobacterium tuberculosis recA mini-inteins suggest a pivotal role for a highly conserved aspartate residue. J Mol Biol. 2007; 367:162–173. [PubMed: 17254599]
54. Perler FB. InBase: the Intein Database. Nucleic Acids Res. 2002; 30:383–384. [PubMed: 11752343]
55. Bessette PH, Aslund F, Beckwith J, Georgiou G. Efficient folding of proteins with multiple disulfide bonds in the Escherichia coli cytoplasm. Proc Natl Acad Sci U S A. 1999; 96:13703–13708. [PubMed: 10570136]
56. Romesser JA, Wolfe RS, Nayer F, Speiss E, Walther-Mauruschat A. *Methanogenus*, a new genus of marin methanogenic bacteria, and characterization of *Methanogenium cariaci* sp. nov. and *Methanogenium marisnigri* sp. nov. Archives of Microbiology. 1979; 121:147–153.
57. Ghosh I, Sun L, Xu MQ. Zinc inhibition of protein trans-splicing and identification of regions essential for splicing and association of a split intein*. J Biol Chem. 2001; 276:24051–24058. [PubMed: 11331276]
58. Mills KV, Paulus H. Reversible inhibition of protein splicing by zinc ion. J Biol Chem. 2001; 276:10832–10838. [PubMed: 11152694]
59. Nichols NM, Benner JS, Martin DD, Evans TC Jr. Zinc ion effects on individual Ssp DnaE intein splicing steps: regulating pathway progression. Biochemistry. 2003; 42:5301–5311. [PubMed: 12731871]
60. Sun P, Ye S, Ferrandon S, Evans TC, Xu MQ, Rao Z. Crystal structures of an intein from the split dnaE gene of Synechocystis sp. PCC6803 reveal the catalytic model without the penultimate histidine and the mechanism of zinc ion inhibition of protein splicing. J Mol Biol. 2005; 353:1093–1105. [PubMed: 16219320]
61. Abramoff MD, Magalhaes PJ, Ram SJ. Image Processing with ImageJ. Biophotonics International. 2004; 11:36–42.

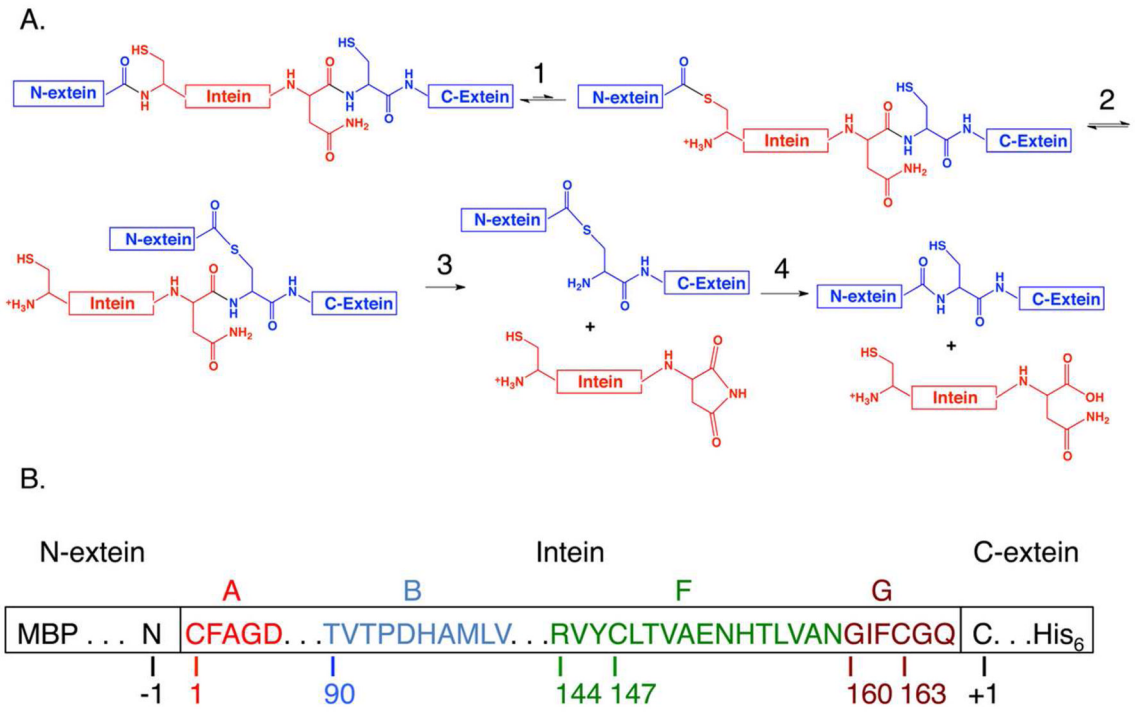

Figure 1. Mechanism of protein splicing and schematic of the *Mma* PolII intein

A) Overview of canonical mechanism for protein splicing. B) Schematic of the fusion protein, not to scale. The letters above the boxes indicate the conserved intein sequence blocks, the color-coded residues indicate the sequence, and the numbers below the boxes indicate the numbering scheme. For numbering the intein and C-extein, residue numbers increase downstream with residue 1 and +1 the first residues, respectively. For the N-extein, the final residue is −1, with values proceeding upstream.

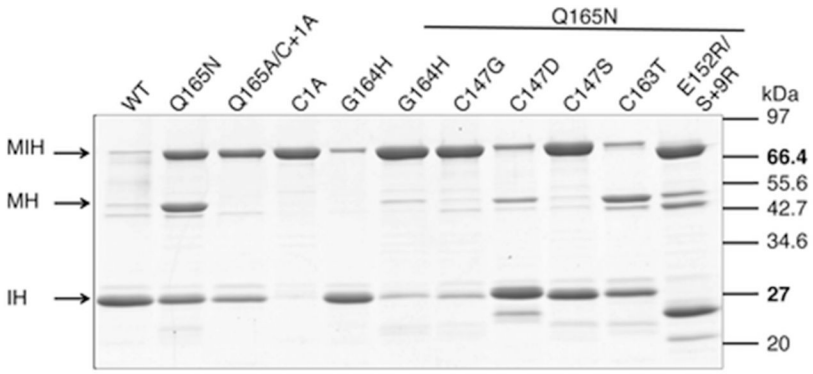

Figure 2. Activity of the *Mma* PolII intein and mutants in *E. coli* BL21(DE3)

Reducing SDS-PAGE analysis of proteins after metal-affinity purification. For protein splicing, the precursor MIH (64.8 kDa) is converted to MH (46.0 kDa) and I (18.4 kDa, not detected due to lack of the His-tag). For N-terminal cleavage, MIH is converted to IH (20.7 kDa) and M (44.1 kDa). Mutations to MIHMma are given above the lanes; the final six lanes each have the Q165N mutation.

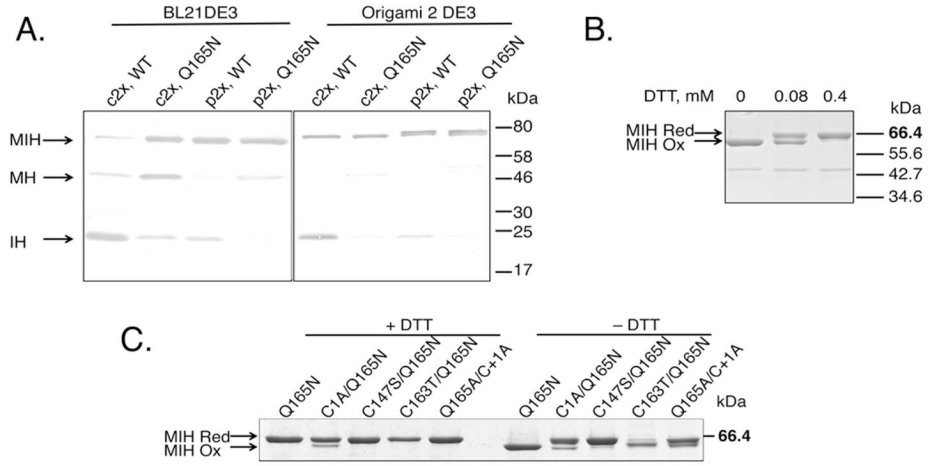

Figure 3. Disulfide bond formation and *in vivo* splicing or cleavage activity

A) Western blot analysis of total cellular extracts, produced from two separate SDS-PAGE experiments under reducing conditions. Bands indicate the detection of the C-terminal His tag. Lane label of c2x indicates cytoplasmic expression, p2x indicates periplasmic expression. Cell strain indicated at top. MIHMma indicated by WT, MIHMma Q165N represented by Q165N. B) Non-reducing SDS-PAGE analysis of MIHMma Q165N purified by metal-affinity chromatography from *E. coli* Origami2(DE3), incubated with DTT at the given concentrations for 15 min at 50°C. C) SDS-PAGE analysis of purified proteins to examine the influence of mutations to intein Cys residues on disulfide bond formation, with or without 50 mM DTT in the gel-loading buffer.

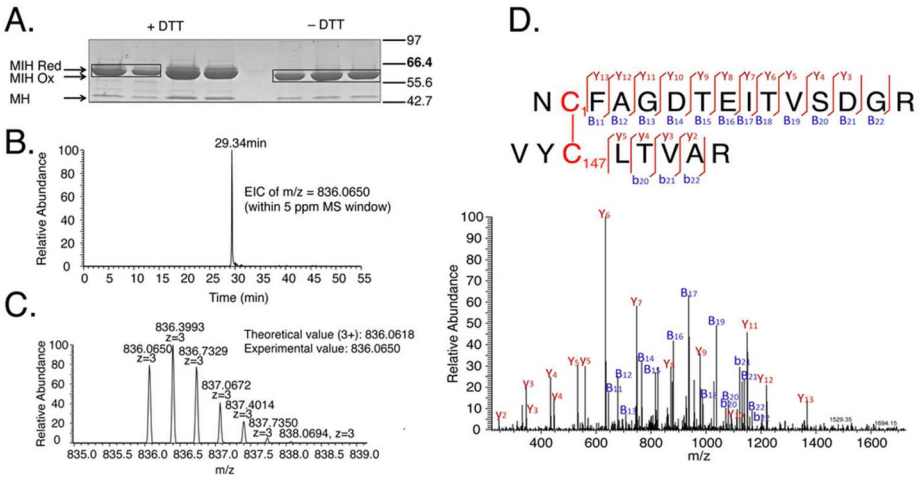

Figure 4. Identification of intramolecular disulfide bond using LC-FT/MS and MS/MS after in-gel tryptic digest

A) SDS-PAGE analysis of samples of protein MIHMma Q165N/E152R/S+9R used for tryptic digest and subsequent LC-FT/MS. The first two boxed lanes were used for the "reduced" sample and the last three boxed lanes were used in subsequent analysis as the "oxidized" sample. B.) Extracted ion chromatography analysis of disulfide peptide (m/z=836.0650) using accurate MS (within 5 ppm mass accuracy window). The peptide eluted at 29.34 min on a C18 HPLC column in the tryptic digestion of the oxidized sample from SDS-PAGE in A. C.) Experimental FT/MS data with isotope distribution for the identified intramolecular disulfide bond peptide. Mono isotopic mass matched well with theoretical data (m/z=836.0618, M=2505.162). D.) MS/MS matching of the intramolecular disulfide bond sequence. Matching data shown as Table S1.

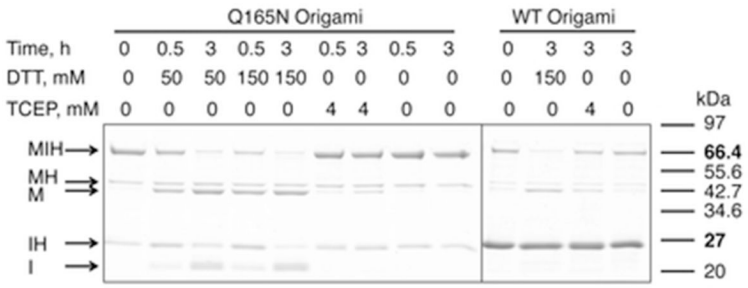

Figure 5. *In vitro* incubation of disulfide-linked precursor with reducing agents results in mostly N- and C-terminal cleavage

SDS-PAGE analysis of incubation of MIHMma and MIHMma Q165N purified from *E. coli* Origami 2(DE3). Proteins were purified as described and exchanged into buffer A supplemented with reducing agents to the final concentration indicated above the lanes, in addition to 4 mM EDTA. Samples were incubated at 25°C for time indicated.

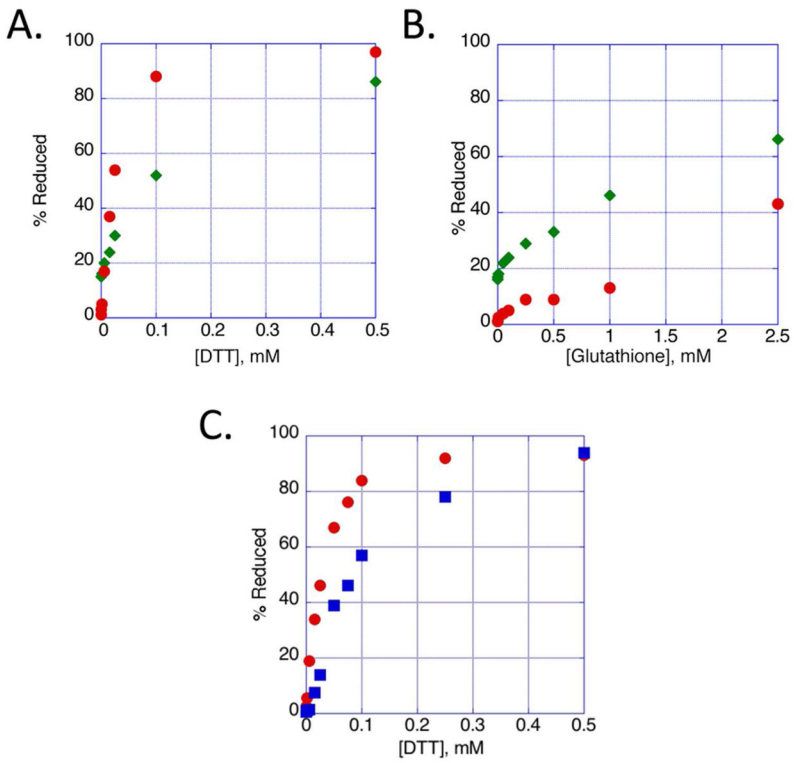

Figure 6. Comparison of reduction of disulfide-linked precursors

We calculated the percent reduction of the unspliced precursor by estimating the relative amount of protein in the oxidized or reduced precursor band by densitometry using ImageJ [(61)], such that % reduction = 100 * (Reduced)/[(Reduced) + (Oxidized)]. In each case, reduction was for 30 min at 20°C. A.) Reduction with DTT of *Pab* (◆) versus *Mma* (●) precursor. B.) Reduction with reduced glutathione of *Pab* (◆) versus *Mma* (●) precursor. C.) Reduction of *Mma* precursor by DTT with (●) or without (■) 1 mM EDTA.