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Intramolecular Disulfide Bond between Catalytic Cysteines in an Intein Precursor

Wen Chen[†], **Lingyun Li**[‡], **Zhenming Du**[†], **Jiajing Liu**[†], **Julie N. Reitter**, **Kenneth V. Mills**[§], **Robert J. Linhardt**[‡], and **Chunyu Wang**[*,†]

[†]Department of Biology, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, New York 12180, United States

[‡]Department of Chemical and Biological Engineering, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, New York 12180, United States

[§]Department of Chemistry, College of the Holy Cross, Worcester, Massachusetts 01610, United States

Abstract

Protein splicing is a self-catalyzed and spontaneous post-translational process in which inteins excise themselves out of precursor proteins while the exteins are ligated together. We report the first discovery of an intramolecular disulfide bond between the two active site cysteines, Cys1 and Cys+1, in an intein precursor composed of the hyperthermophilic *P. abyssi* PolII intein and extein. The existence of this intramolecular disulfide bond is demonstrated by the effect of reducing agent on the precursor, mutagenesis, and liquid chromatography–mass spectrometry (LC-MS) with tandem MS (MS/MS) of the tryptic peptide containing the intramolecular disulfide bond. The disulfide bond inhibits protein splicing, and splicing can be induced by reducing agents such as tris (2-carboxyethyl) phosphine (TCEP). The stability of the intramolecular disulfide bond is enhanced by electrostatic interactions between the N- and C-exteins but is reduced by elevated temperature. The presence of this intramolecular disulfide bond may contribute to the redox control of splicing activity in hypoxia and at low temperature and point to the intriguing possibility that inteins may act as switches to control extein function.

Keywords

intein; protein splicing; intramolecular disulfide bond; extein; catalytic cysteine; MS

Protein splicing is a self-catalyzed post-translational process in which an intervening protein, called an intein, is excised from a precursor protein, together with the ligation of the two flanking sequences immediately N- and C-terminal to the intein, termed N- and C-exteins, respectively[1–3] (supporting information Fig. S1). Protein splicing is strictly intramolecular[4], requiring no external co-factor or energy input[4–5]. Inteins have been found in all domains of life[6], but exist only in unicellular organisms[7]. Hedgehog (Hh) proteins, crucial for the embryonic patterning of higher eukaryotes, undergo similar auto-processing in the cholesterolylation of the Hh signaling domain by the Hh processing domain[8–10]. The Hh processing domain and inteins share many conserved sequence motifs and a common Hh-intein (HINT) fold[11].

Corresponding Author wangc5@rpi.edu.

Supporting Information. Detailed materials and methods are provided along with supporting figures showing the four steps of protein splicing and additional LC-MS and MS/MS data. This information is available free of charge via the Internet at http://pubs.acs.org.

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There is usually a residue with a side chain nucleophile (e.g. Cys, Ser, and Thr) at the junction between the N-extein and intein (N-terminal splice junction) and between C-extein and intein (C-terminal splice junction). These two conserved residues, Cys1 (the first residue of intein) and Cys+1 (the first residue of C-extein) for many inteins, serve as the nucleophiles for first two steps of splicing, N-X acyl shift and trans-esterification, respectively (supporting information Fig. S1). Therefore, differential reactivity of Cys residues can control the steps of protein splicing[12–13]. Recently, an intramolecular disulfide bond has been engineered in *Ssp* DnaE intein precursor with a CPGC motif[14], resulting in a stable intein precursor that can be reduced to induce splicing. Likewise, intein-mediated protein ligation was used to create a non-native disulfide bond to trap an intein precursor of the *M. jannaschii* KlbA intein[15]. Salic *et al.* have shown that there is an intramolecular disulfide bond in the Hh processing domain[16], which needs to be reduced before cholesterolylation can proceed. However, within native intein precursor sequences, intramolecular disulfide bonds have not been observed between the two catalytic cysteines. For Cys1 and Cys+1 to catalyze protein splicing efficiently, the N- and C- terminal splice junctions need to be close in space. However, crystal structures of inteins show variable distances between the two junctions, from 3~4 Å up to 8~9 Å[17–20].

Recently, we solved the NMR structure of a hyperthermophilic intein interrupting the *Pyrococcus abyssi* DNA polymerase II (*Pab* PolII)[21–22]. The *Pab* PolII intein only splices at high temperature, offering the opportunity to study a stable precursor containing native intein and extein sequences at room temperature. We overexpressed in *E. coli* BL21(DE3) a 25 kDa precursor composed of the *Pab* PolII intein, a short N-extein and a short His-tagged C-extein, which contains Cys1 and Cys+1, the only two cysteines in the sequence (see supporting information). The *Pab* PolII intein precursor was purified by nickel-NTA affinity chromatography. In the SDS PAGE in Fig. 1A, instead of the expected 25 kDa band, a strong 20 kDa band was observed along with a very weak 25 kDa band. We then treated the *Pab* PolII precursor protein with increasing amounts of reducing agent (tris (2-carboxyethyl) phosphine (TCEP) or β-mercaptoethanol) at room temperature, below the optimal temperature for protein splicing of this intein. The 25 kDa band became progressively stronger with the concomitant decrease of the 20 kDa band. We suspect that an intramolecular disulfide bond was formed between the two catalytic cysteine residues, resulting in a circle-like protein and increasing the rate of migration in non-reducing SDS PAGE[23]. Therefore, the 20 kDa protein band is likely the disulfide-linked form of the intein precursor (oxidized precursor), which is then converted to the expected 25 kDa band (reduced precursor) upon reduction at a temperature that does not permit splicing. Additional bands observed by SDS-PAGE between 40–50 kDa are likely intein dimers, as they disappear with the addition of reducing agent. There are two cysteines in each intein monomer, and therefore several different types of intermolecular disulfide bonds can form, giving rise to multiple bands.

The identification of the 20 kDa band as an intein with an intramolecular disulfide bond is further supported by the comparison with a Cys1Gly mutant, which cannot form an intramolecular disulfide bond. As expected, the 20 kDa band was absent for this mutant (Fig. 1B). The WT precursor protein migrating at 20 kDa was then excised from the SDS-PAGE, digested by trypsin and analyzed by LC-MS/MS. All predicted tryptic peptides were identified except for the ones containing a single cysteine (supporting information Fig. S2). Instead, a tryptic peptide containing covalently linked Cys1 and Cys+1 was detected and identified by accurate MS and MS/MS, conclusively demonstrating the presence of the intramolecular disulfide bond between the two active site cysteines (Fig. 1C). This disulfide in *Pab* PolII intein precursor is particularly strong, as it forms on over-expression in *E. coli* BL21 (DE3) rather than requiring a *trxB*- and *gor*-strain.

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Next, we tested if the presence of the intramolecular disulfide affects the splicing activity. Very little ligated extein was detected after overnight incubation at 60°C in the absence of reducing agent, indicating the intramolecular disulfide bond inhibits splicing. TCEP was then introduced to break the disulfide bond to initiate splicing. Ligated exteins, detected by LC/MS and confirmed by MS/MS, were generated in the presence of 0.25 mM TCEP (supporting information Fig. S3 and S4). Spliced exteins accumulated with TCEP concentration up to 2 mM (Fig. 2A and 2B).

Next, we explored the influence of extein residues on the formation of the disulfide bond. In this precursor, three of the four C-terminal residues of the N-extein are positively charged and three of the four N-terminal residues of the C-extein are negatively charged (see supporting information). This might provide attractive forces that favor disulfide bond formation. Mutation of Lys(-4), Arg(-3) and Arg(-2) in the N-extein to Glu, Asp, and Asp, respectively, results in a significant decrease of oxidized species with the concurrent increase of the reduced species in SDS-PAGE (Fig. 2C). This suggests that electrostatic interactions between the N- and C-exteins play an important role in the formation of the intramolecular disulfide bond and in coordinating the N- and C-terminal splice junction. Electrostatic interactions were also observed to assist in the reassociation of the fragments of the split *Npu* DnaE intein[24].

We tested if temperature can change the equilibrium between reduced and oxidized precursor. Purified *Pab* PolII intein precursor protein was incubated at 35°C, 45°C, 55°C, 65°C, and 75°C overnight at pH 6.5 (Fig. 2D). The protein aggregates when the temperature is above 80°C (data not shown). With increased temperature, there is an increased amount of reduced precursor and a decreased amount of oxidized precursor, indicating that the disulfide bond is weakened by high temperature, which may help account for the temperature dependence of the splicing activity.

This is the first time that an intramolecular disulfide bond between the two active site cysteines, Cys1 and Cys+1, has been discovered in a native intein precursor protein. The two cysteines are separated by 185 residues of primary sequence, in contrast to engineered intein disulfide bonds separated by just two residues, such as that formed by the CPGC motif.[14] In the Hh processing domain, the Cys1 forms a disulfide bridge with another cysteine that is *not* homologous to Cys+1, which must be reduced by a protein disulfide isomerase before cholesterolylation[16]. Salic et al. proposed that the disulfide may be important for the folding of the Hh processing domain, but this is not the case for the *Pab* PolII intein, which has a well folded structure without the intramolecular disulfide bond[22].

The *Pab* PolII intein interrupts a crucial extein, the DNA polymerase II DP2 subunit, which is essential for DNA replication in *Pab*[25–26]. *Pab* is anaerobic, and the presence of oxygen may impose oxidative stress and result in a more oxidizing cellular environment. Under these conditions, disulfide formation between C1 and C+1 may be promoted, inhibiting splicing and the formation of the fused active exteins, the DNA PolII DP2 subunit. This may arrest DNA replication to preserve the integrity of the genome during oxidative stress. Callahan et al. have proposed a similar redox-switch for the intein that interrupts the *Pab* MoaA[14]. The molybdopterin cofactor produced by active MoaA is used in a variety of redox-dependent enzymes. Although disulfide bonds are unusual in intracellular proteins, genomic studies suggest that they are more common in hyperthermophilic archaebacteria, including *Pab*, suggesting that the intramolecular disulfide bond might be relevant *in vivo*[27–28].

The temperature-dependence of the *Pab* PolII intein may also be a function of the redox switch, and possibly play a role in regulation of extein activity. Below its optimal growth

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temperature of ~100°C, *Pab* needs to shut down DNA replication. As shown in Fig. 2D, more intramolecular disulfide bond is present at lower temperatures for the *Pab* PolII precursor, inhibiting splicing. With native exteins, this would prevent the formation of active DNA polymerase DP2. The redox sensitivity of this intramolecular disulfide bond may contribute to the mechanism how *Pab* stops replication, even at relatively moderate temperature, such as 37°C.

Interestingly, the intein interrupting another essential DNA replication protein in *Pab*, replication factor 2[29], also has the conserved residues C1 and C+1. Although the intein has yet to be studied, it may further contribute to redox regulation in the physiological response to hyperoxia and low temperature in *Pab* using redox chemistry of the intramolecular disulfide bond. It is intriguing that inteins may not just be a parasitic element in protein or DNA sequence[7]; instead, inteins may act as switches and regulate the function of crucial exteins and cellular physiology through redox sensitivity conferred by intramolecular disulfide bonds.

In summary, for the first time, we have conclusively established the existence of an intramolecular disulfide bond between two active site residues, Cys1 and Cys+1, in an intein precursor composed of native *Pab* PolII intein and exteins. Our findings suggest that redox chemistry of intramolecular disulfide bond may regulate protein splicing and extein function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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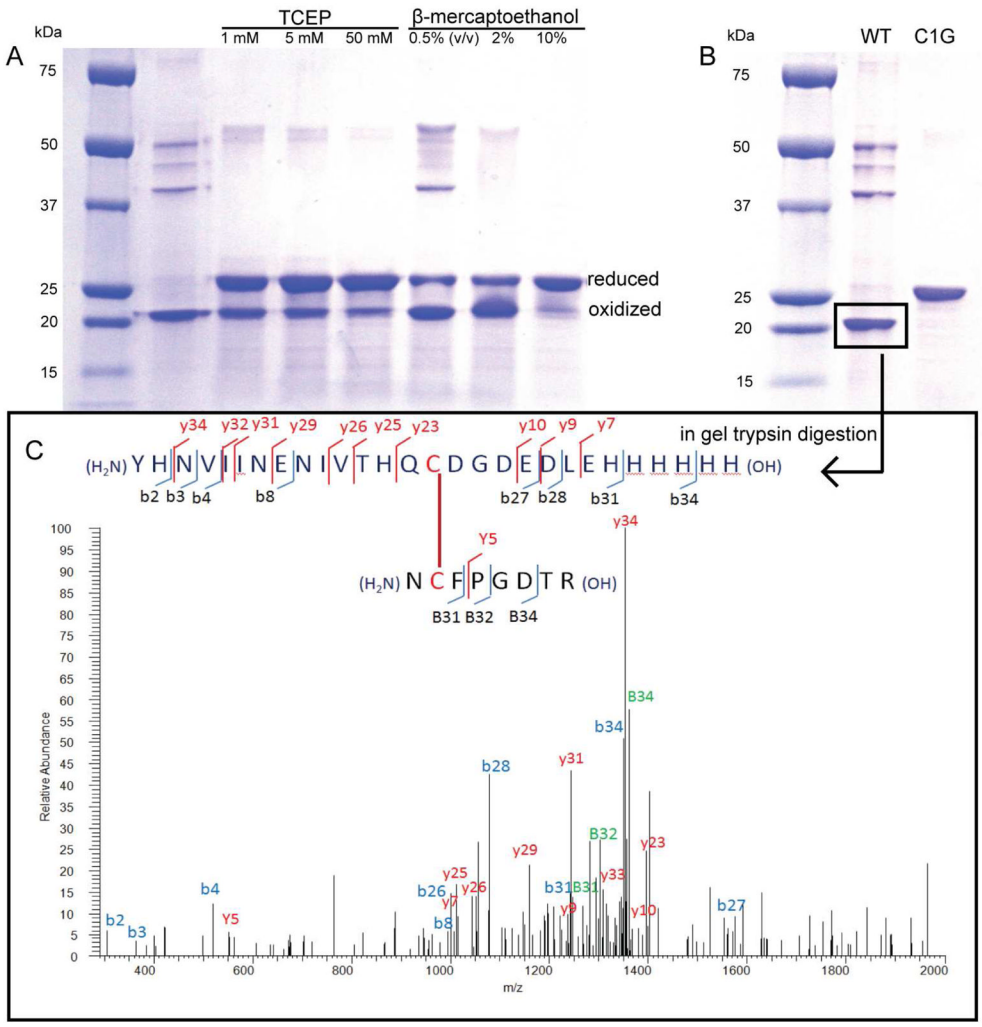

Figure 1.

Intramolecular disulfide bond between the two active site cysteines, Cys1 and Cys+1. (A) Effect of reducing agents. *Pab* PolII precursor was treated with 1 mM, 5 mM, or 50 mM TCEP, or with 0.5% (v/v), 2%, and 10% β-mercaptoethanol. Increasing amount of reducing agent decreased the intensity of the 20 kDa band while increasing the intensity of the 25 kDa band. (B) Effect of the Cys1Gly mutation, which prevents the formation of an intramolecular disulfide bond. Only the 25 kDa band (reduced precursor) is present in the SDS-PAGE of the Cys1Gly *Pab* PolII intein precursor. (C) MS/MS identification of the intramolecular disulfide bond. The band in panel B was excised and digested by trypsin and analyzed by LC-MS/MS. Full sequence coverage was achieved (Supporting information). A peptide containing the intramolecular disulfide bond was detected and identified by accurate MS and MS/MS in the digestion mixture, demonstrating that the *Pab* PolII precursor forms an intramolecular disulfide bond.

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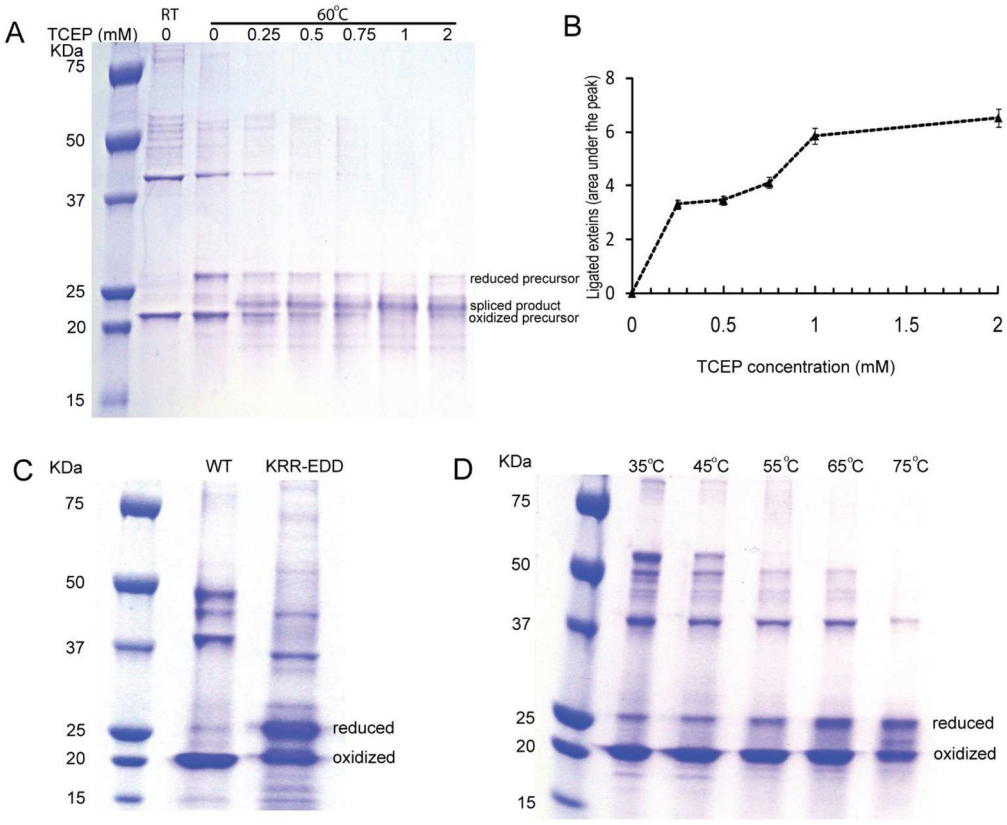

Figure 2.

(A) Precursor splicing with and without TCEP. The precursor protein was treated with 0.25 mM, 0.5 mM, 0.75 mM, 1 mM and 2 mM TCEP at 60°C overnight for splicing. (B) Greater amount of spliced extein was detected by MS/MS with increased amount of TCEP. (C) Comparison between WT and KRR-EDD mutant shows the effect of charge-charge interaction to disulfide bond formation. (D) Temperature dependence of the intramolecular disulfide bond, with higher temperature favoring the reduced species. *Pab* PolII intein precursor was incubated at 35°C, 45°C, 55°C, 65°C, and 75°C for overnight.