Published in final edited form as:

Biomol NMR Assign. 2015 October ; 9(2): 235–238. doi:10.1007/s12104-014-9581-z.

Backbone assignments of mini-RecA intein with short native exteins and an active N-terminal catalytic cysteine

C. Seth Pearson¹, Georges Belfort¹, Marlene Belfort², and Alexander Shekhtman³

¹Howard P. Isermann Department of Chemical and Biological Engineering and The Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY 12180

²Department of Biological Sciences and The RNA Institute, University at Albany, Albany, NY 12222

³Department of Chemistry, University at Albany, Albany, NY 12222

Abstract

The backbone resonance assignments of an engineered splicing-inactive mini-RecA intein based on triple resonance experiments with [¹³C, ¹⁵N]-labeled protein are reported. The construct contains inactivating mutations specifically designed to retain most catalytic residues, especially those that are potentially metal-coordinating. The assignments are essential for protein structure determination of a precursor with an active N-terminal catalytic cysteine and for investigation of the atomic details of splicing.

Biological context

Inteins are naturally occurring protein elements that autocatalytically excise themselves from a non-functional precursor protein and ligate the flanking protein segments, called exteins, with a peptide bond, resulting in a functional protein. The study of inteins has been fueled by their applications in chemical biology (Muir 2003; Peck et al. 2011; Topilina and Mills 2014) and bioseparations (Wood et al. 1999; Wu et al. 2006), as the process of making and breaking of peptide bonds is of fundamental interest to these fields. In addition to these biotechnological applications, inteins have generated interest as potential antimicrobial drug targets. Inteins interrupt three critical genes of *Mycobacterium tuberculosis*: *recA*, *dnaB*, and *sufB*. Preventing splicing, and thus the maturation of non-functional precursor to functional spliced product presents a novel method of eliminating protein activity and suggests inteins as potential antimicrobial targets (Belfort 1998; Lew and Paulus 2002). Indeed, it has been shown that the potent *in vitro* intein inhibitor cisplatin kills *M. tuberculosis in vivo* (Zhang et al. 2011). This effect relied upon the N-terminal catalytic cysteine residue of the intein.

Although the overall stepwise mechanism of splicing has been characterized (Paulus 2000), and more recently simulation and mutational studies have elucidated the atomic rearrangements occurring at certain steps (Pereira et al. 2011; Liu et al. 2014), a detailed description of the entire process is lacking. Such mechanistic knowledge is essential for applications in biology and biotechnology, and for designing novel inhibitors.

Several early structures, using both crystallography (Klabunde et al. 1998) and solution NMR (Romanelli et al. 2004), have provided insight into the intein splicing mechanism. These include nucleophilic residues in the intein 1 and extein +1 positions, along with the conserved TXXH motif in the intein's B-block, a conserved penultimate histidine, and a conserved terminal asparagine. These residues are all within close proximity in three-dimensional space and form the core intein catalytic center. This general structure of the catalytic domain was found to be consistent across all subsequent structures (Moure et al. 2002; Sun et al. 2005; Johnson et al. 2007; Van Roey et al. 2007; Hiraga et al. 2009; Callahan et al. 2011; Du et al. 2011; Oeemig et al. 2012) with surprisingly little variation. In addition to the main catalytic center, an F-block aspartate was implicated as a catalytic residue (Van Roey et al. 2007). This finding was expanded using solution NMR, when it was observed that the abnormal pKa of the Cys1 was linked to this F-block aspartate, which itself displayed a similarly abnormal pKa (Du et al. 2011).

Solution NMR was used to study the peptide bond between the Cys1 of the GyrA intein and the preceding N-terminal extein residue (Romanelli et al. 2004). It was found that the amine was highly polarized and that the peptide bond was scissile and interactions with the B-block histidine were implicated as the cause of the effect. Later work showed a similarly strained bond in this location (Callahan et al. 2011), but implicated the nearby B-block threonine (Dearden et al. 2013).

With the residues in the catalytic center of the intein mostly defined, work turned to an examination of extein interactions. Solution NMR was used to study a splicing deficient mutant to obtain a structure with short extein chains (Johnson et al. 2007). This structure contained a mutated Cys+1 residue, but the position of the replacement Ser indicated that the Cys+1 sidechain likely extends into the catalytic site. The RadA intein of the hyperthermophilic *Pyrococcus horikoshii* was studied using solution NMR (Oeemig et al. 2012). It was demonstrated through mutational studies that splicing kinetics are dependent on the identity of the -1 residue of the N-extein and a solution NMR structure was used to correlate the favorability of extein-intein interactions with the modulation in splicing activity. This result is consistent with mutational (Wood et al. 2000; Amitai et al. 2009) and structural (Callahan et al. 2011) studies indicating modulation (acceleration, attenuation, or abolishment) of intein activity by neighboring extein residues. It was further demonstrated that native extein residues contribute to the sequential catalytic property of the Ssp DnaE intein (Sun et al. 2005).

The mechanism of Zn^{2+} inhibition of the Ssp DnaE intein was examined and it was found to be caused by Zn^{2+} coordination to the B-block His and F-block Asp (Sun, Ye et al. 2005), which interestingly differs from the Zn^{2+} coordination site observed for the PI-SceI intein (Moure et al. 2002). Many of the splicing site residues, such as cysteine, histidine, and aspartate, perform important catalytic functions while also providing potential coordination sites to metal ion inhibitors. It has additionally been shown that extein residues are modulators of intein activity and may also play a role in the action of inhibitors and their coordination. The participation of such a wide cast of residues in splicing and inhibition presents significant difficulty because it entails trapping precursor (exteins) while minimizing mutation of important catalytic residues. We therefore created a novel construct

based on the minimized RecA intein, whose backbone assignments were previously reported (Du et al. 2008), in which the B-block threonine is mutated to alanine. This mutation was shown to reduce strain between the first cysteine of the intein and the preceding N-extein residue with the effect of eliminating splicing and N-terminal cleavage (Dearden et al. 2013). Additionally, the terminal asparagine is mutated to alanine to eliminate C-terminal cleavage. These mutated residues allow the study of the atomic details of splicing and inhibition while simultaneously ensuring the presence of extein residues and the major catalytic residues.

As a first step toward elucidating the atomic details of protein splicing and addressing the complexities of intein inhibition, we have determined the backbone resonance assignments of the precursor-trapped RecA mini-intein with short native exteins and without mutations to potential metal-coordinating residues.

Methods and experiments

The construct I_{hh} -IM used for NMR assignment is the RecA mini-intein (Hiraga et al. 2005) containing the T70A and N139A mutations, as well as 5 N-extein and 5 C-extein residues, and a hexahistidine tag at the N-terminus linked by a TEV protease site for subsequent cleavage of the tag.

Uniformly labeled [U - ^{15}N] or [U - ^{13}C , ^{15}N] I_{hh} -IM was expressed in BL21(DE3) cells in M9 medium containing either [U - ^{15}N] ammonium chloride or [U - ^{15}N] ammonium chloride and [U - ^{13}C] dextrose as sole nitrogen and carbon sources and supplemented with 10% [U - ^{15}N] or [U - ^{13}C , ^{15}N] Isogro (Sigma). The labeled construct was purified by immobilized metal affinity chromatography using NiNTA resin columns (HisTrapHP, GE). This initial purification was followed by incubation with TEV protease overnight at 4°C to cleave the 6xHis affinity tag. The protease cleavage leaves a single non-native glycine as the N-terminal residue of the upstream extein. The intein was purified over a second NiNTA column to remove uncleaved I_{hh} -IM, cleaved His tag, and TEV protease. Cysteine-only labeled protein was also expressed in BL21(DE3) cells in M9 medium supplemented with [U - ^{13}C , ^{15}N] cysteine and 0.02% unlabeled casamino acids. Purification was performed in an identical manner. NMR samples (0.5 mM I_{hh} -IM) were prepared in 20 mM sodium phosphate, 100 mM sodium chloride, 1 mM TCEP in 90% H₂O/10% D₂O at pH 7.0. All NMR experiments were carried out at 25°C on a Bruker Avance 700 MHz or Avance II 500 MHz spectrometer equipped with a TXI cryoprobe. Spectra were processed with Bruker Topspin 2.1 software and analyzed using CARI (Keller 2004).

The sequence specific backbone $^1\text{H}^N$, $^{13}\text{C}^\alpha$, ^{15}N and sidechain $^{13}\text{C}^\beta$ assignments for I_{hh} -IM were obtained by standard triple resonance NMR methods, including HNCA, HNCACB, HNCOCACB, and ^{15}N NOESY experiments (Cavanagh et al, Protein NMR spectroscopy, 2nd edition, 2006).

Extents of assignments and data deposition

I_{hh} -IM contains 150 amino acids, including the N-terminal glycine left behind by protease cleavage. Nearly complete assignments of the backbone (>99%) $^1\text{H}^N$, ^{15}N , $^{13}\text{C}^\alpha$

and $^{13}\text{C}^\beta$ resonances were obtained with the exception of Ser+2, which precedes two proline residues, Cys1, which is assumed to be broadened due to chemical exchange, Gln51, Gly63, His128, Thr129, His138, and the ^{15}N resonances of the 7 proline residues. Compared to similar mini-RecA intein (BMRB entry 17414) (Du et al., 2011), all chemical shift differences were within 0.5 ppm with the exception of 8 residues. Four residues (Asp72, Lys74, Trp81, and Ala84) are very close spatially to the T70A mutation based on the crystal structure (Van Roey et al., 2007). The remaining four residues (Leu122, Glu123, Leu127, and His138) are very close spatially to the N139A mutation. The chemical shifts have been deposited in the BioMagResBank under accession number 25051.

Acknowledgments

This work was supported by NIH Biomolecular Science and Engineering training grant GM067545 (C.S.P.), NIH grant GM44844 (M.B.), and NIH grant GM085006 (A.S.).

References

- Amitai G, Callahan BP, Stanger MJ, Belfort G, Belfort M. Modulation of intein activity by its neighboring extein substrates. *Proc Natl Acad Sci U S A*. 2009; 106(27):11005–11010. [PubMed: 19541659]
- Belfort, M. Google Patents. 1998. Inteins as antimicrobial targets: genetic screens for intein function.
- 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(5):630–633. [PubMed: 21460844]
- Dearden AK, Callahan B, Roey PV, Li Z, Kumar U, Belfort M, Nayak SK. A conserved threonine spring-loads precursor for intein splicing. *Protein Sci*. 2013; 22(5):557–563. [PubMed: 23423655]
- 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(44):38638–38648. [PubMed: 21914805]
- Du Z, Liu Y, Zheng Y, McCallum S, Dansereau J, Derbyshire V, Belfort M, Belfort G, Van Roey P, Wang C. ^1H , ^{13}C , and ^{15}N NMR assignments of an engineered intein based on *Mycobacterium tuberculosis* RecA. *Biomol NMR Assign*. 2008; 2(2):111–113. [PubMed: 19636882]
- Du Z, Zheng Y, Patterson M, Liu Y, Wang C. pK(a) coupling at the intein active site: implications for the coordination mechanism of protein splicing with a conserved aspartate. *J Am Chem Soc*. 2011; 133(26):10275–10282. [PubMed: 21604815]
- Hiraga K, Derbyshire V, Dansereau JT, Van Roey P, Belfort M. Minimization and stabilization of the *Mycobacterium tuberculosis* recA intein. *J Mol Biol*. 2005; 354(4):916–926. [PubMed: 16288917]
- Hiraga K, Soga I, Dansereau JT, Pereira B, Derbyshire V, Du Z, Wang C, Van Roey P, Belfort G, Belfort M. Selection and structure of hyperactive inteins: peripheral changes relayed to the catalytic center. *J Mol Biol*. 2009; 393(5):1106–1117. [PubMed: 19744499]
- Johnson MA, Southworth MW, Herrmann T, Brace L, Perler FB, Wuthrich K. NMR structure of a KlbA intein precursor from *Methanococcus jannaschii*. *Protein Sci*. 2007; 16(7):1316–1328. [PubMed: 17586768]
- Keller, RLJ. *The Computer Aided Resonance Assignment Tutorial*. Switzerland: Cantina Verlag; 2004.
- Klabunde T, Sharma S, Telenti A, Jacobs WR Jr, Sacchettini JC. Crystal structure of GyrA intein from *Mycobacterium xenopi* reveals structural basis of protein splicing. *Nat Struct Biol*. 1998; 5(1):31–36. [PubMed: 9437427]
- Lew BM, Paulus H. An in vivo screening system against protein splicing useful for the isolation of non-splicing mutants or inhibitors of the RecA intein of *Mycobacterium tuberculosis*. *Gene*. 2002; 282(1–2):169–177. [PubMed: 11814689]

- Liu Z, Frutos S, Bick MJ, Vila-Perello M, Debelouchina GT, Darst SA, Muir TW. Structure of the branched intermediate in protein splicing. *Proc Natl Acad Sci U S A*. 2014; 111(23):8422–8427. [PubMed: 24778214]
- Moure CM, Gimble FS, Quioco FA. Crystal structure of the intein homing endonuclease PI-SceI bound to its recognition sequence. *Nat Struct Biol*. 2002; 9(10):764–770. [PubMed: 12219083]
- Muir TW. Semisynthesis of proteins by expressed protein ligation. *Annu Rev Biochem*. 2003; 72:249–289. [PubMed: 12626339]
- Oeemig JS, Zhou D, Kajander T, Wlodawer A, Iwai H. NMR and crystal structures of the *Pyrococcus horikoshii* RadA intein guide a strategy for engineering a highly efficient and promiscuous intein. *J Mol Biol*. 2012; 421(1):85–99. [PubMed: 22560994]
- Paulus H. Protein splicing and related forms of protein autoprocessing. *Annu Rev Biochem*. 2000; 69:447–496. [PubMed: 10966466]
- 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(5):619–630. [PubMed: 21609843]
- 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(3):430–442. [PubMed: 21185311]
- Romanelli A, Shekhtman A, Cowburn D, Muir TW. Semisynthesis of a segmental isotopically labeled protein splicing precursor: NMR evidence for an unusual peptide bond at the N-extein-intein junction. *Proc Natl Acad Sci U S A*. 2004; 101(17):6397–6402. [PubMed: 15087498]
- 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(5):1093–1105. [PubMed: 16219320]
- Topilina NI, Mills KV. Recent advances in in vivo applications of intein-mediated protein splicing. *Mob DNA*. 2014; 5(1):5. [PubMed: 24490831]
- 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(1):162–173. [PubMed: 17254599]
- 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(6):1055–1063. [PubMed: 11101334]
- Wood DW, Wu W, Belfort G, Derbyshire V, Belfort M. A genetic system yields self-cleaving inteins for bioseparations. *Nat Biotechnol*. 1999; 17(9):889–892. [PubMed: 10471931]
- Wu WY, Mee C, Califano F, Banki R, Wood DW. Recombinant protein purification by self-cleaving aggregation tag. *Nat Protoc*. 2006; 1(5):2257–2262. [PubMed: 17406465]
- 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(2):1277–1282. [PubMed: 21059649]

Figure 1.
 ^1H - ^{15}N HSQC spectrum of the engineered RecA intein with short native exteins. The interior crowded section is expanded in the upper left.