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Protein splicing of the three *Pyrococcus abyssi* ribonucleotide reductase inteins

Adam M. Kerrigan, Taryn L. Powers, Deirdre M. Dorval, Julie N. Reitter, Kenneth V. Mills *

Department of Chemistry, College of the Holy Cross, Worcester, MA 01610, USA

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ABSTRACT

An intein is a polypeptide that interrupts the functional domains of a protein, called the exteins. The intein can facilitate its own excision from the exteins, concomitant with the ligation of the exteins, in a process called protein splicing. The alpha subunit of the ribonucleotide reductase of the extreme thermophile *Pyrococcus abyssi* is interrupted by three inteins in separate insertion sites. Each intein can facilitate protein splicing when over-expressed in *Escherichia coli*, with affinity domains serving as the exteins. The influence of the N-terminal flanking residue on the efficiency of splicing is specific to each intein. Each intein has a different downstream nucleophilic residue, and cannot tolerate substitution to a residue of lesser or equal nucleophilicity. The influence of the conserved penultimate His also differs between the inteins.

Introduction

Protein splicing is the process by which an intervening polypeptide, called an intein, facilitates its excision from flanking sequences, the exteins, concomitant with the ligation of the exteins. This converts the precursor fusion protein (NIC) to linked exteins (NC) and excised intein (I).

The standard mechanism occurs in four steps [1]. The first is an amide to ester or thioester rearrangement of the peptide bond linking the N-terminal extein (N-extein) to the intein, which has an N-terminal Cys or Ser. The second step is a transesterification, whereby the N-extein is transferred to the side chain of the N-terminal residue of the C-terminal extein (C-extein), a Cys, Ser or Thr. This results in a branched ester intermediate. The third step is cleavage of the intein from the linked exteins via cyclization of the conserved C-terminal Asn of the intein. Finally, the ester linking the exteins is converted to a peptide bond, and the C-terminal aminosuccinimide of the intein may be hydrolyzed to Asn or iso-Asn.

If the order of the steps is disrupted, usually due to mutation of conserved residues, side reactions may occur [1]. If the third step is slowed or prevented, the linear ester from step one or the branched ester from step two may undergo hydrolysis or thiolysis. This process, N-terminal cleavage, results in release of the N-extein (N) from the linked intein and C-extein (IC) fragment. If the first or second step is slowed or prevented, the third step may become uncoupled from splicing. This process, called C-terminal cleavage, releases the C-extein (C) from the linked N-extein and intein (NI). For numbering, the first residue of the intein is designated residue 1, the first residue of the C-extein is C+1, and the final residue of the N-extein is N-1, with numbering proceeding upstream [2].

The alpha subunit of the ribonucleotide reductase (RIR) of the extreme thermophile *Pyrococcus abyssi* is interrupted by three separate inteins (Fig. 1) [3,4]. We show that each intein is capable of facilitating efficient protein splicing when over-expressed in *Escherichia coli* as a fusion protein between affinity domains serving as exteins. We study the flexibility of the insertion sites of the inteins by substituting residues at the splice junctions of each intein and studying the influence of these mutations on splicing efficiency.

Materials and methods

Plasmid preparation. To amplify the intein genes via PCR, *P. abyssi* was cultured and genomic DNA isolated as described [5]. Intein RIR1 was amplified with primers RIR1U (5′-TGAAAAGGCCTCACG ATGTTG) and RIR1L (5′-GGCTGCCCCATCGATTGTTC), intein RIR2 with RIR2U (5′-CATGCATGCAACAACTACGGG) and RIR2L (5′-CACTA ACGGCATCGATGAGG), and intein RIR3 with RIR3U (5′-GCAAAGGG AAGGCCTATAAG) and RIR3L (5′-ATGGCTTCCCGTTATCGATGT).

To express a fusion protein of *E. coli* maltose binding protein (MBP) with each intein and a poly-His tag, plasmids pMIHRIR1, pMIHRIR2, and pMIHRIR3 were created by digesting the PCR products above with StuI and ClaI (for RIR1 and RIR3) or SphI and ClaI (for RIR2), and ligating the product of the digestion into the same sites in plasmid pPabPol1His, described previously [5].

To increase the rate of cell growth during expression and the amount of expression, mutations were made to conserved homing

* Corresponding author. Fax: +1 508 793 3530.
E-mail address: kmills@holycross.eduw (K.V. Mills).

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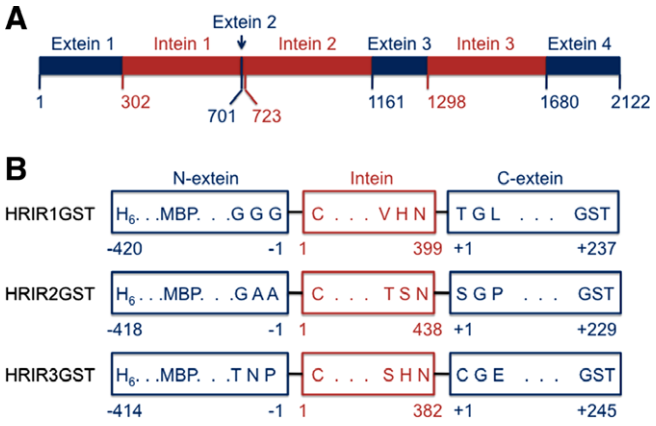

Fig. 1. Diagram of native precursor protein and constructs used in this communication. (A) Diagram, to scale, of sequence of exteins and inteins in the alpha subunit of ribonucleotide reductase from *P. abyssi*. Numbering indicates amino acid order. (B) Diagram of fusion proteins used in this work, not to scale. Amino acid numbering scheme as described in *Introduction*. H_6 is poly-His tag, MBP is *E. coli* maltose binding protein tag, and GST is glutathione-*S*-transferase tag. Relevant amino acids given by one-letter code.

endonuclease residues in the inteins, using appropriate primers and the Stratagene QuikChange mutagenesis kit (Invitrogen, Carlsbad, CA). For pMIHRIR1, Asp193 and Glu195 were changed to Ala. For pMIHRIR2, Asp254 and Asp256 were changed to Ala, and for pMIHRIR3, Ser247 and Asp249 were changed to Ala.

To code for an N-terminal poly-His tag and improve expression, pET28b-(+) (Novagen, Madison, WI) was altered by site directed mutagenesis to remove its ClaI site. For each intein, a vector was created by introducing a new ClaI site into the multi-cloning sequence of the altered pET28b-(+) by inserting an oligonucleotide pair between the BamHI/HindIII sites. Then, the intein from each pMIHRIR plasmid, with the homing endonuclease mutations, was inserted between the NdeI/ClaI sites of the modified pET28b-(+) vectors to create pHMRIR1, pHMRIR2, and pHMRIR3.

To express the inteins flanked by exteins of different sizes and detectable by commercial antibodies, the gene for glutathione-*S*-transferase (GST) was amplified from pET41b-(+) (Novagen) by PCR using primers GSTU (5′-GAAGGAGATATACATATCGATCCTAT) and GSTL (5′-TGACCGGTACCACTGCTAGCTCAATCCGATTTTGGA) and inserted between the ClaI and KpnI sites of pHMRIR1, pHMRIR2, and pHMRIR3 to create plasmids pHMRIR1GST, pHMRIR2GST, and pHMRIR3GST.

The DNA sequences of all plasmids were confirmed by Macrogen, Inc. (Seoul, Korea). The DNA sequence of the inteins was consistent with the NCBI database (Accession No. 000868).

Expression and protein purification. Plasmid-encoded proteins were over-expressed in *E. coli* BL21DE3 (Novagen). The cultures were grown at 37 °C with shaking to an A_{600} of 0.7, induced with isopropyl-1-thio-β-D-galactopyranoside (final concentration of 0.4 mM), and grown overnight at 20 °C. The cells were harvested by centrifugation and resuspended in 2.5 mL of buffer A (100 mM Bis–Tris Propane, pH 7.5, 500 mM NaCl), supplemented with 12 units/ml benzonase nuclease (Novagen), 100 μM phenylmethylsulfonyl fluoride and 20 μl of Protease Inhibitor Cocktail P8849 (Sigma–Aldrich, St. Louis, MO). The cells were lysed by passage through a French Pressure cell and pelleted by centrifugation at 15,000*g*. The supernatant was applied to 1 mL of amylose resin (New England Biolabs, Ipswich, MA) pre-equilibrated with buffer A. The resin was washed with 3 × 10 ml of buffer A and eluted in three 500-μl fractions of buffer A supplemented with 20 mM maltose. Protein concentrations were determined by the Bradford method [6].

To change the pH of the elution buffer, buffer was exchanged against Buffer 9.5 (200 mM CHES, pH 9.5, 500 mM NaCl) using a Millipore Ultrafree-0.5 centrifugal filter device.

Protein splicing analysis by SDS–PAGE, Western blot, N-terminal sequencing, and MALDI-TOF mass spectrometry. For SDS–PAGE, pre-cast 4–20% gradient Tris–glycine gels (Lonza, Rockland, ME) were used via the Laemmli method [7].

For Western blot, gels were blotted onto nitrocellulose. The membranes were incubated in 1% bovine serum albumin in Wash buffer (PBS and 0.1% Tween 20) and then with either 0.2 μg/ml anti-His monoclonal antibody (GenScript, Piscataway, NJ) or a 1:10,000 dilution of GST·Tag monoclonal antibody (Novagen). The blots were washed in Wash buffer, incubated with a 1:10,000 dilution of goat anti-mouse IgG alkaline phosphatase conjugate (Novagen), and washed again. The blots were developed with Western Blue stabilized substrate (Promega, Madison, WI).

For N-terminal sequencing, gels were blotted onto polyvinylidene fluoride membrane (Millipore, Billerica, MA). The blots were stained with 0.1% Ponceau S in 1% aqueous acetic acid, destained in 50% methanol/10% acetic acid, and washed with deionized water. The band of interest was excised and analyzed by the Tufts University Core Facility (Boston, MA).

For MALDI-TOF mass spectrometry, samples were analyzed by the University of Massachusetts Proteomic and Mass Spectrometry Core Facility (Worcester, MA).

Results

Protein expression and purification

We over-expressed and purified the protein products from three plasmids that separately express the inteins that interrupt the alpha subunit of the RIR from *P. abyssi* [3,4]. The insertion points of the three inteins in the *P. abyssi* RIR are indicated in Fig. 1a.

Each intein was expressed separately as a fusion protein between an N-terminal poly-His tag and MBP and C-terminal GST (Fig. 1b). In each fusion protein, native extein residues flank the inteins. For HRIR1GST, there are 12 native N-extein residues and 17 native C-extein residues. For HRIR2GST, there are six and 11 residues, respectively, and for HRIR3GST, there are eight and 24 residues, respectively.

Protein expression was improved by mutation of conserved homing endonuclease domain residues in the inteins, particularly for inteins two and three (data not shown). This is consistent with the results found in a study of the *in vitro* endonuclease activity of inteins two and three [8].

Splicing activity of intein one

Protein HRIR1GST exhibited protein splicing on over-expression in *E. coli*, as shown in Fig. 2. There is a faint band consistent with the size of the unspliced precursor, NIC (119 kDa), and a heavy band consistent in size with the spliced product, NC (73.5 kDa) (Fig. 2a). Both products react with the antibody against the C-terminal GST (Fig. 2b), and were purified using the N-terminal MBP. Mutation of the N-1 Gly to Ala significantly reduces splicing, and mutation to Pro leads to isolation of only unspliced precursor. Mutation of the penultimate His residue, His398, to Ala or Ser results in diminished splicing and the appearance of four major bands: the precursor NIC and spliced product NC, both of which reacted with the antibody against the C-terminal GST, the N-terminal product of N-terminal cleavage (N, 46.0 kDa), and the branched ester intermediate (BE). Mutation of the N-terminal Thr of the C-extein to Cys has little effect on splicing efficiency, but mutation to

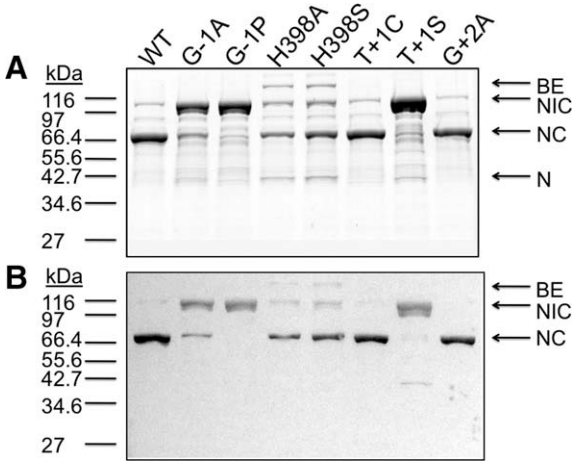

Fig. 2. Splicing and cleavage reactions of HRIR1GST and mutants. (A) SDS–PAGE analysis of about 1 μg of protein per lane. (B) Western blot using about 1.5 μg per lane, detected with primary antibody directed against GST.

Ser results in the accumulation of mostly precursor (NIC). Mutation of the Gly at position +2 of the C-extein to Ala does not appear to significantly change splicing efficiency.

Splicing activity of intein two

Protein HRIR2GST exhibits protein splicing on over-expression in *E. coli* (Fig. 3). The major band in SDS–PAGE corresponds in size to the spliced product, NC (72.4 kDa). There are also minor bands corresponding to unspliced precursor (NIC, 122.9 kDa), the N-terminal product of N-terminal cleavage (N, 45.7 kDa), and both products of C-terminal cleavage (NI, 96.2 kDa, and C, 26.7 kDa). This cleavage likely occurs after protein purification, as band C should not be retained by the MBP resin. The Western blot using anti-GST antibody identifies bands NIC, NC and C, with anomalous faint detection of band N. Mutation of residue N-1 from Ala to Gly, residue Ser437 to Ala or His, residue C+1 from Ser to Cys or C+2 from Gly to Ala results in proteins that exhibit similar splicing and side reaction patterns. Mutation of the C+1 Ser to Thr results in a reduced amount of splicing. Mutation of the N-1 Ala to Pro produces a band at about 30 kDa that reacts with the anti-GST antibody but is larger than the size predicted for C. Although it produces a strong band on the Western blot, the protein is a very low percentage of the protein mixture as observed by SDS–PAGE and thus we could not identify it via mass spectrometry or N-terminal sequencing.

Splicing activity of intein three

Protein HRIR3GST exhibits mostly protein splicing and some N-terminal cleavage (Fig. 4). SDS–PAGE reveals a major band corresponding in size to the product of splicing, NC (74.0 kDa), and bands corresponding in size to the unspliced precursor (NIC, 118 kDa), the product of C-terminal cleavage (NI, 89.5 kDa) and the product of N-terminal cleavage (N, 45.4 kDa). Western blot analysis using anti-GST antibody corroborates the assignments of NIC and NC. Mutation of the N-1 Pro to Gly reduces the extent of splicing and induces some post-purification C-terminal cleavage, as demonstrated by the appearance of band C in the Western blot. Similar behavior is seen if the C+1 Cys is changed to Ser or Thr. Mutation of His381 to Ser or Ala, or mutation of C+2 Gly to Ala, reduces the extent of splicing and leads to accumulation of the branched ester intermediate (BE). Mutation of the N-1 Pro to Ala results in a reduced extent of splicing and the production of two faint bands on SDS–PAGE that react with the anti-GST antibody but were of low concentration and were not identified.

Confirmation of spliced product by mass spectrometry

The identity of the spliced product was verified by MALDI-TOF mass spectrometry. Respectively, the m/z of the spliced product for HRIR1GST, HRIR2GST, and HRIR3GST were 73051 (73464.8 expected), 72038 (72414.5 expected) and 74635 (73952.3 expected).

The branched ester intermediate

The band of high molecular mass in the His398Ala and His398-Ser samples of HRIR1GST (Fig. 2) and H381A, H381S and G+2A lanes of HRIR3GST (Fig. 4) may correspond to the branched ester intermediate. We verified that these bands react with both anti-His tag (data not shown) and anti-GST antibodies. Exchange of the elution buffer with buffer 9.5 results in loss of the branched ester band, likely by alkaline hydrolysis, as analyzed by SDS–PAGE (data not shown). We submitted the band from HRIR1GST H398A

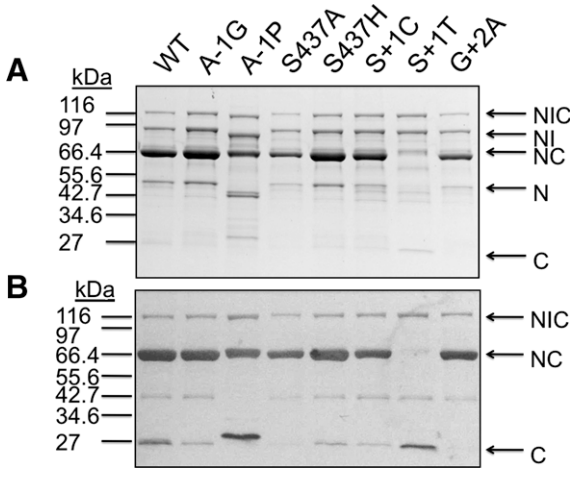

Fig. 3. Splicing and cleavage reactions of HRIR2GST and mutants. (A) SDS–PAGE analysis of about 1 μg of protein per lane. (B) Western blot using about 1.5 μg per lane, detected with primary antibody directed against GST.

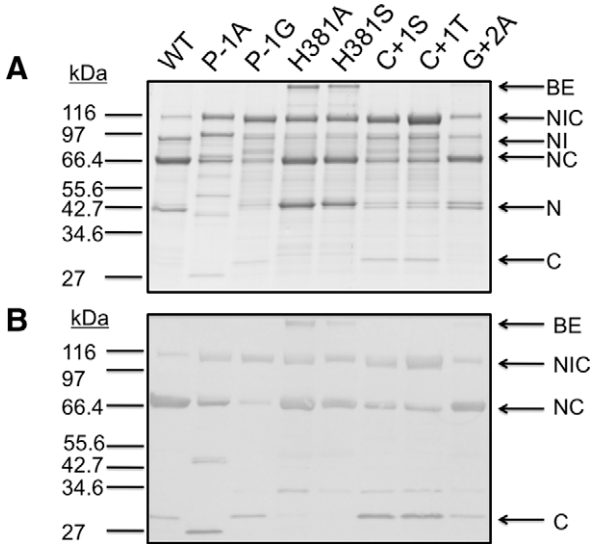

Fig. 4. Splicing and cleavage reactions of HRIR3GST and mutants. (A) SDS–PAGE analysis of about 1 μg of protein per lane. (B) Western blot using about 1.5 μg per lane, detected with primary antibody directed against GST.

and HRIR3GST H381A for N-terminal sequencing, and analysis for the first six residues resulted in overlapping sequences consistent with those expected of the branched intermediate: the N-termini of the N-exteins and inteins (save for the N-terminal Cys of the inteins, not detectable by this method).

Discussion

The overall splicing reaction

Each of the three *P. abyssi* RIR inteins facilitate protein splicing on over-expression in *E. coli* at 20 °C overnight. In contrast, the *P. abyssi* PolII intein requires *in vitro* incubation at temperatures above 30 °C [5]. Intein RIR1 has 78% amino acid sequence identity to the RIR1 intein from *Pyrococcus furiosus* and intein RIR3 has 84% identity to the RIR2 intein from *P. furiosus* [9], both of which splice in *E. coli* [10]. The three inteins have little sequence similarity to each other.

The first step of splicing

A different residue precedes each intein: Gly for RIR1, Ala for RIR2 and Pro for RIR3. Intein RIR2 is relatively flexible in its requirement for a particular amino acid at this position, whereas inteins RIR1 and RIR3 are sensitive to substitution (Figs. 2–4). For RIR1, the N-1 Gly likely allows conformational flexibility about the N-terminal scissile bond, whereas the N-1 Pro in RIR3 promotes structural rigidity. Therefore, for these two inteins, the local conformation of the peptide bond is important for promoting splicing, likely via an influence on the first step. Such role for the N-1 residue is widely supported in the literature, perhaps relating to the stabilization of a local conformation around the scissile bond that results in loss of amide resonance and a shift in the equilibrium toward the thioester. Two compelling examples link structural and biochemical evidence. The crystal structure of the *Mycobacterium xenopi* GyrA intein shows a cis peptide bond linking the N-extein and the intein [11], and NMR evidence of a loss of amide bond resonance could be due to scissile peptide bond rotation [12]. The crystal structure of the *Saccharomyces cerevisiae* VMA intein shows main chain distortion near the scissile bond [13]. Biochemical evidence suggests that the conformation of this bond is important in both inteins, as the N-1 residue influences the extent of cleavage at the splice junction, used as a measure of the efficiency of ester formation [14,15].

The second step of splicing

Each intein is followed downstream by a different nucleophile: Thr for RIR1, Ser for RIR2 and Cys for RIR3. For RIR3, substitution of the C+1 Cys for the less nucleophilic Ser or Thr significantly impairs splicing (Fig. 4). For RIR1 and RIR2, substitution of C+1 Thr or Ser for the more nucleophilic Cys does not decrease the efficiency of splicing (Figs. 2 and 3). However, substitution of C+1 Thr to Ser in RIR1 and C+1 Ser to Thr in RIR2 results in significant loss of splicing activity. The downstream nucleophiles in RIR1 and RIR2 may be activated by a general base mechanism. Substitution to Cys is tolerated, as it could be sufficiently nucleophilic to promote step two without activation, even with the thermodynamic disadvantage of forming a thioester in step two rather than an oxygen ester. The subtle structural difference between Ser and Thr must sufficiently influence the organization of the active site to prevent activation of the non-native nucleophilic residue for step two for inteins RIR1 and RIR2. For comparison, the relative nucleophilicity of the flanking nucleophiles was studied for the *Mycobacterium tuberculosis* RecA intein [16]. The downstream nucleophilic Cys has a measured pK_a of 5.8, whereas the upstream Cys has a pK_a of 8.2. Cleavage of the linear ester intermediate with DTT is more rapid with the *S. cerevisiae* VMA intein than with the *M. tuberculosis* RecA intein, suggesting that the VMA intein might have basic residues to activate the DTT (thus, the downstream nucleophile in step two) whereas the natively low pK_a of the downstream Cys in the RecA intein is sufficient to promote the nucleophilic attack.

The third step of splicing

The C+2 position for each intein is Gly. Mutation of this Gly to Ala had little effect on splicing for inteins one and two, but reduced splicing for intein three and led to the formation of a small amount of branched ester intermediate, suggesting that the Asn cyclization was slowed or the transition from step two to step three was affected.

The conserved penultimate His, which has been shown to influence the third step of splicing of many inteins, is replaced by Ser in intein RIR2. Substitution of Ser to His or Ala does not significantly affect the extent of protein splicing (Fig. 3), suggesting that this residue does not play a role in catalyzing the third step of splicing and its location in the active site does not interfere with activation of this step. However, substitution of the conserved His to Ala or Ser in inteins RIR1 and RIR3 reduces the extent of protein splicing and leads to accumulation of branched ester intermediate. This implies an effect on the rate of the third step of splicing, leading to the buildup of the product of step two. Computational analysis of a model intein based on the *Ssp* DnaB intein predicts that the imidazole of its penultimate His may be too distant to directly activate the side chain of the adjacent Asn [17], but assigns that role to an upstream His in intein block F, conserved in inteins RIR2 and RIR3 but not RIR1. Instead, the penultimate His may stabilize the tetrahedral intermediate of the cyclization and protonate the scissile peptide bond [17]. The crystal structure of the *P. furiosus* RIR1 intein [18] has the penultimate His 5.6 Å from the side chain amide of the C-terminal Asn, but only 3 Å from the backbone amide nitrogen. The conserved His in block B is close to hydrogen bonding distance to both the modeled Cys1 and the side chain of the C-terminal Asn, suggesting a role in coordinating the steps of splicing. Biochemically, evidence for the importance of the penultimate His is mixed. Branched ester intermediate was first shown to accumulate upon mutation of the penultimate His to Ala in the *Pyrococcus* GB-D Pol intein [19]. Protein splicing can be enhanced in inteins that lack a penultimate His by adding it via mutagenesis [20,21], but in certain inteins the His is not required for splicing [20,22,23] and in some cases replacing the penultimate residue with His impairs splicing [20,22].

Acknowledgments

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