Kinetic Analysis of the Individual Steps of Protein Splicing for the Pyrococcus abyssi PolII Intein*

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Protein splicing involves the excision of an intervening polypeptide, the intein, from flanking polypeptides, the exteins, concomitant with the specific ligation of the exteins. The intein that interrupts the DNA polymerase II DP2 subunit in Pyrococcus abyssi can be over-expressed and purified as an unspliced precursor, which allows for a detailed in vitro kinetic analysis of the individual steps of protein splicing. The first order rate constant for splicing of this intein, which has a non-canonical Gln at its C terminus, is $9.3 \times 10^{-6}$ s$^{-1}$ at 60 °C. The rate constant for splicing increases 3-fold with substitution of Asn for the C-terminal Gln. The pseudo first order rate constant of dithiothreitol-dependent N-terminal cleavage is $1 \times 10^{-4}$ s$^{-1}$. The first order rate constant of C-terminal cleavage is $1.2 \times 10^{-7}$ s$^{-1}$ with Gln at the C-terminal position, $2.8 \times 10^{-4}$ s$^{-1}$ with Asn, and decreases significantly with mutation of the penultimate His of the intein to Ala. N-terminal cleavage is most efficient between pH 7 and 7.5 and decreases at both more acidic and alkaline pH values, whereas C-terminal cleavage and splicing are both efficient over a broader range of pH values.

Protein splicing involves the post-translational, self-directed excision of an intervening polypeptide, or intein, from flanking polypeptides, or exteins. The splicing process also involves the specific ligation of the extein segments by an amide bond, concomitant with the excision of the intein (1).

The canonical mechanism of protein splicing consists of four separate but coordinated chemical steps each directed by the intein without the assistance of other proteins or cofactors (1). The first step of splicing is an N–S or N–O acyl rearrangement of the amide bond linking the N-extein and intein (see Fig. 1, step 1). This is followed by a transesterification that transfers the N-extein to the side chain of the N-terminal Cys, Ser, or Thr of the C-extein (see Fig. 1, step 2). The third step of splicing couples the cyclization of the C-terminal Asn of the intein to peptide bond cleavage, and excises the intein from the newly ligated exteins (see Fig. 1, step 3). Finally, the ester bond linking the exteins is rearranged to an amide (see Fig. 1, step 4), and the C-terminal aminosuccinimide of the intein is presumably hydrolyzed to either Asn or iso-Asn (see Fig. 1, step 5).

Inteins that splice via variations of the canonical mechanism have previously been described. For instance, our group recently described the protein splicing of the intein that interrupts the DNA polymerase II DP2 subunit in Pyrococcus abyssi (Pab), which is able to facilitate protein splicing with a C-terminal Gln residue (2). These results are complementary to those of Pietrokovski and coworkers (3), who demonstrated that the intein that interrupts the ribonucleotide reductase (RNR) of the Chilo iridescent virus can facilitate protein splicing with a C-terminal Gln and that the intein interrupting the RNR of Carboxydothermus hydrogenoformans can facilitate protein splicing with a C-terminal Asp.

Our group also demonstrated that the penultimate His residue is required for efficient protein splicing of the Pab PolII intein and that N-terminal cleavage (see Fig. 1, step 7) occurs in place of splicing in its absence (2). The role of the penultimate His in facilitating Asn cyclization and protein splicing varies by intein. For instance, its replacement by mutation can inhibit protein splicing (4, 5) or lead to accumulation of branched ester intermediates (6). However, other inteins undergo efficient protein splicing with penultimate residues other than His, and the replacement of the these residues with His in these inteins can either reduce, enhance, or have no measurable effect on the extent of splicing (7–9).

The discovery that the Pab PolII intein can be isolated as an unspliced precursor and induced to splice by an increase in temperature allows for the measurement of rate constants for protein splicing and side reactions (2). This use of temperature-inducible splicing of inteins isolated from extreme thermophiles was also useful in the discovery of the chemical mechanism of splicing (6, 10–13). Previously, Evans and coworkers (9, 14, 15) have described the kinetics of the trans-splicing of the intein that interrupts the DNAe protein of Synchocystis sp. PCC6803 (Ssp). In this paper, we report a detailed kinetic analysis of the individual steps of protein splicing for the Pab PolII intein and compare the results from this cis-splicing intein to those of the trans-splicing Ssp DNAe intein.

**EXPERIMENTAL PROCEDURES**

**Plasmid Preparation, Protein Expression and Purification**—The preparation of plasmids pPabPolIIHis, pPabPolWT, pPabPolQN, pPabPolHA, and pPabPolQNH was previously described (2). Plasmid pPabPolWT encodes WT (defined as PolWT in Ref. 2), an in-frame fusion of Escherichia coli maltose-binding protein with the...
seven C-terminal residues of the *P. abyssi* PolII N-extein and the 185 residues of the intein, fused to the six N-terminal residues of the C-extein and a hexahistidine tag. To generate mutations of pPolWT, plasmid pPabPolIHis was digested with XmnI and ClaI and ligated with appropriate oligonucleotides to generate pPabPolQN and pPabPolQNHA, which encode proteins QN and QNHA, described previously as proteins PolQN and PolQNHA (2). The same procedure was used to generate plasmids pPabPolQACA and pPabPolQA, which encode proteins QACA and QA, respectively (Fig. 2).

To study C-terminal cleavage reactions, plasmid pPabC1AWT was prepared by the digestion of pPabPolWT with NdeI and XmaI, and ligation of the 6033-bp DNA fragment with the oligonucleotide pair PABNOEXU (5'-TATGAAAGCTTAAGGAGAAACGCGTTT-3') and PABNOEXL (5'-CCGGAACCCGGTTTCTCTTTAAAGCTTCA-3'). Plasmid pPabC1AWT encodes C1AWT, an in-frame fusion of the N-terminal sequence MKL with the four C-terminal residues of the N-extein, the 185 residues of the intein (with a C1A mutation), the six N-terminal residues of the C-extein, and a hexahistidine tag. This shortened N-extein is used to make the precursor and product of C-terminal cleavage easier to resolve via SDS-PAGE. Plasmids pPabPolQN, pPabPolHA, and pPabPolQNHA were used in the same manner to prepare plasmids pPabC1AQN, pPabC1AHA, and pPabC1AQNHA, respectively, which encode proteins C1AQN, C1AHA and C1AQNHA, respectively (see Fig. 2).

The DNA sequence of the newly created plasmids was verified by sequencing at Macrogen, Inc. (Seoul, South Korea). Plasmid-encoded proteins were overexpressed and purified as described previously (2).

**Reaction Conditions**—Except where indicated, N-terminal cleavage

**Fig. 1. Mechanism of protein splicing and cleavage reactions.** Most inteins with Cys residues at the N termini of the intein and C-extein splice via the following steps, 1) an N–S acyl shift resulting in a linear thioester, 2) a transesterification reaction involving the newly formed thioester and downstream Cys, generating a branched ester, and 3) cyclization of the intein C-terminal Asn coupled to peptide bond cleavage, resulting in ligation of the extein segments by a thioester bond and excision of the intein. The reaction is completed by 4) an S–N acyl shift of the thioester that links the exteins to an amide and 5) probable hydrolysis of the C-terminal aminosuccinimide of the intein. In the *Pab PolIII* intein, the C-terminal Asn of the intein is replaced by Gln. Mutations at the intein N terminus may result in Asn cyclization uncoupled from protein splicing and hence C-terminal cleavage (step 6). Mutations at the intein C terminus may result in thiolysis of the linear or branched thioester intermediates by exogenous thiol and therefore N-terminal cleavage of the intein (step 7).
results

Analysis of N-terminal Cleavage—We were able to isolate proteins consisting of an in-frame fusion of *E. coli* maltose-binding protein with the seven C-terminal residues of the *P. abyssi* PolII N-extein and the 185 residues of the intein, fused to the six N-terminal residues of the C-extein and a hexahistidine tag. The sequence at the N- and C-terminal splice junctions of these proteins is presented schematically in Fig. 2A. Proteins QA and QACA were purified at room temperature using immobilized metal affinity chromatography and analyzed by SDS-PAGE, and a single band corresponding to the predicted molecular mass of 66.6 kDa was observed for each protein (Fig. 3, lanes 1 and 3).

These fusion proteins were incubated for 18 h at 60 °C under the N-terminal splicing conditions described under “Experimental Procedures,” and the results were analyzed by SDS-PAGE (Fig. 3, lanes 2 and 4). In *vitro* N-terminal cleavage results in the predicted conversion of the 66.6-kDa precursor protein, MIH, to the cleaved N-terminal extein, M (43.7 kDa), and the intein-C-extein fusion, HI (22.9 kDa). The identity of these bands was verified by Western blot (data not shown). As expected, the protein bands corresponding to the precursor MIH and intein-C-extein fusion HI react with an anti-His-tag antibody, and the protein band corresponding to the N-extein, M, does not.

Time Course of N-terminal Cleavage—To determine the pseudo first order rate constants for DTT-dependent N-terminal cleavage of the fusion proteins QA, QACA, and QNHA, the proteins were incubated at 60 °C for 12 h under the conditions described under “Experimental Procedures,” and aliquots of the reaction were stopped by the addition of SDS-PAGE buffer at seven time points and analyzed by SDS-PAGE. The pseudo first order rate constants were calculated using data from two trials over the first 6 h of the reaction and are reported in Table II. One representative SDS-PAGE analysis is shown in Fig. 4.

To compare our pseudo first order rate constants for DTT-dependent N-terminal cleavage to those described previously (2), we incubated QNHA at 60 °C under the experimental conditions described above, except for including 25 mM DTT or excluding DTT. In the absence of DTT, the pseudo first order rate constant of N-terminal cleavage of QNHA was 1.6 × 10⁻⁵ s⁻¹, whereas in the presence of 25 mM DTT the pseudo first order rate constant calculated from the slopes of plots of ln(M1/M0) versus time in seconds. Rate constants of C-terminal cleavage assume a pseudo first order rate constant calculated from the slopes of plots of ln(M1/M0) versus time in seconds. Each data point for the percentage of N- or C-terminal cleavage or the calculation of rate constants is the average of two separate experiments.
order rate constant was $1.0 \times 10^{-4} \text{ s}^{-1}$. Similar rate constants for QNHA were obtained using both 50 and 100 mM DTT. Pseudo first order rate constants measured in the absence of DTT were $2.0 \times 10^{-5} \text{ s}^{-1}$ for QA and $2.4 \times 10^{-5} \text{ s}^{-1}$ for QACA.

**Dependence of N-terminal Cleavage on pH**—For QA, QACA, and QNHA, N-terminal cleavage is inefficient below a pH of 6.5 (Fig. 5). The maximum extent of N-terminal cleavage occurs around pH 7 and 7.5 for QA and QACA and around pH 8 for QNHA. The extent of N-terminal cleavage greatly decreases at pH values approaching pH 9.

**Analysis of C-terminal Cleavage**—We were able to isolate proteins consisting of an in-frame fusion of the N-terminal sequence MKL with the four C-terminal residues of the N-extein, the 185 residues of the intein (with a C1A mutation), the six N-terminal residues of the C-extein, and a hexahistidine tag. The sequence at the C-terminal splice junction of these proteins is presented schematically in Fig. 2B. Proteins C1AWT, C1AQN, C1AHA, and C1AQNHA were purified at room temperature using immobilized metal affinity chromatography and analyzed by SDS-PAGE, and a single band corresponding to the predicted molecular mass of 23.9 kDa was observed for each protein (Fig. 6, lanes 1, 3, 5, and 7).

These fusion proteins were incubated for 18 h at 60 °C under the C-terminal splicing conditions described under “Experimental Procedures,” and the results were analyzed by SDS-PAGE. In vitro C-terminal cleavage results in the predicted conversion of the 23.9-kDa precursor protein, NIH, to the cleaved N-extein-intein fusion, NI (22.4 kDa). The cleaved C-extein would be too small to observe on the gel.

The identity of these protein bands was verified by Western blot (data not shown). As expected, the protein band corresponding to the precursor NIH reacts with an anti-His-tag antibody; but the protein band corresponding to the N-terminal product of C-terminal cleavage, NI, does not react with the antibody.

MALDI-TOF mass spectrometry was also used to identify the products of C-terminal cleavage of C1AWT and C1AQN. MALDI-TOF mass spectrometry analysis of purified C1AWT and C1AQN resulted in peaks with $m/z$ of 23,859 and 23,839, respectively, which is consistent with the predicted molecular masses of 23,876 Da and 23,862 Da. An analysis of a C-terminal cleavage reaction mixture of C1AWT at 60 °C for 18 h revealed two major peaks with $m/z$ of 23,860 and 22,416, consistent with the predicted molecular masses of NIH (23,876 Da) and NI (22,418). Analysis of a C-terminal cleavage reaction mixture for C1AQN at 60 °C for 18 h revealed one major peak with $m/z$ of 22,393, consistent with the predicted molecular mass of NI (22,404).

C1AQN cleaves nearly to completion (93%) at 60 °C in 18 h, whereas C1AWT cleaves to ~59%. The cleavage of QNHA proceeds to ~15%, whereas cleavage of HA is barely detectable (~2%).

**Time Course of C-terminal Cleavage**—To determine the first order rate constants for C-terminal cleavage of the fusion proteins C1AWT, C1AQN, and C1AQNHA, the proteins were incubated at 60 °C under the conditions described under “Experimental Procedures,” and aliquots of the reaction were stopped by the addition of SDS-PAGE buffer at the appropriate time points and analyzed by SDS-PAGE. The first order rate constants were calculated using data from two trials over the first 60 min of the reaction for C1AQN, 18 h for C1AWT, and 24 h for C1AQNHA and are reported in Table III.

**Dependence of C-terminal Cleavage on pH**—For C1AWT, C1AQN, and C1AQNHA, C-terminal cleavage is optimal below pH 7.5, and the extent of cleavage decreases significantly at higher pH values (Fig. 7).

**DISCUSSION**

This paper expands on our previous characterization of the protein splicing of the Pol II intein (2). We discovered that the Pol II intein, which has a C-terminal Gln, can facilitate protein splicing with a rate of $9.3 \times 10^{-8} \text{ s}^{-1}$ at 60 °C and that this rate increases to $3.4 \times 10^{-5} \text{ s}^{-1}$ upon the substitution of Asn for the C-terminal Gln (2). Pietrokovski and coworkers (3) previously showed that the evolutionarily distinct Chilo iridescent virus RNR intein, which also has a C-terminal Gln, can facilitate splicing, although a substitution of the C-terminal Gln to Ala results in a 7-fold reduction in splicing yield. We also showed that substitution of the penultimate residue, a highly conserved His, of the Pol II intein with Ala results in N-terminal cleavage in place of protein splicing (Fig. 1, step 7 (2)). In this report, we took further advantage of the temperature-inducible activity of the Pol II intein to study the kinetics of protein splicing, the first such study of an intein that naturally splices in cis.

Mutation of the C-terminal Gln of the intein to Ala (Fig. 2, fusion protein QA) results in temperature-dependent N-terminal cleavage (Fig. 1, step 7) in place of splicing, as does a double mutation of both the C-terminal Gln of the intein and the N-terminal Cys of the C-extein to Ala (Fig. 2, fusion protein QACA) (Fig. 3). Similar N-terminal cleavage was previously shown for fusion protein QNHA (2). The rates of DTT-dependent N-terminal cleavage at 60 °C for each of these constructs are very similar (Table II). For QACA, thiol-induced N-terminal cleavage could be the

![Fig. 5](http://www.jbc.org/)

**Fig. 5.** pH dependence of N-terminal cleavage of QA, QACA, and QNHA. The effect of pH on N-terminal cleavage of QA (●), QACA (○), and QNHA (△) was examined as described under “Experimental Procedures.” The value for percent N-terminal cleavage is the average of two trials, and the pH values are as determined for the reaction mixture at 23 °C.

![Fig. 6](http://www.jbc.org/)

**Fig. 6.** SDS-PAGE analysis of C-terminal cleavage of C1AWT, C1AQN, C1AHA, and C1AQNHA. Lanes 1, 3, 5, and 7 consist of ~0.6 μg of C1AWT, C1AQN, C1AHA, and C1AQNHA, respectively. Lanes 2, 4, 6, and 8 consist of an 18-h incubation of C1AWT, C1AQN, C1AHA, and C1AQNHA, respectively, under the conditions described under “Experimental Procedures.”

![Fig. 7](http://www.jbc.org/)

**Fig. 7.** SDS-PAGE analysis of C-terminal cleavage of C1AWT, C1AQN, C1AHA, and C1AQNHA. Lanes 1, 3, 5, and 7 consist of ~0.6 μg of C1AWT, C1AQN, C1AHA, and C1AQNHA, respectively. Lanes 2, 4, 6, and 8 consist of an 18-h incubation of C1AWT, C1AQN, C1AHA, and C1AQNHA, respectively, under the conditions described under “Experimental Procedures.”
result of a nucleophilic attack on either the linear or branched ester. The fact that the rates of these cleavage reactions are so similar suggests either that the transesterification reaction is rapidly reversible with respect to formation of the linear ester, or that the cleavage of QA is primarily due to a nucleophilic attack on the linear ester. A definitive conclusion awaits the isolation of mutations that allow us to purify the branched intermediate and compare the rates of N-terminal cleavage of the linear and branched esters.

The rate of DTT-induced N-terminal cleavage is 3–10 times faster than that for the overall splicing reaction (Tables I and II), which is similar to the results observed with the trans-splicing Ssp DnaE intein, in which N-terminal trans-cleavage at 23 °C is 15–24 times faster than trans-splicing (9, 14). Our previously reported rate constants for N-terminal cleavage of fusion proteins QNHA, HA, and CA were for hydrolytic N-terminal cleavage and therefore a measurement of both ester formation and hydrolysis. The DTT-dependent rate constants reported here are relatively constant at 25, 50, and 100 mM concentrations of DTT and are a better gauge of the first order rate constant of thioester formation.

We were also able to measure the rate of C-terminal cleavage at 60 °C (Fig. 1, step 6) by substitution of the N-terminal Cys of the intein with Ala (Fig. 6). C-terminal cleavage with Gln at the intein C terminus (C1AWT) proceeds with a first order rate constant of 1.2 x 10^-5 s^-1. A substitution of Asn for Gln at the intein C terminus increases the first order rate constant for C-terminal cleavage to over 20-fold (Table III). This increase in rate is reflected in the greater than 3-fold increase in the first order rate constant of splicing of fusion protein QN with respect to fusion protein WT (Table I). Substitution of the penultimate His of the intein with Ala, along with the C-terminal Gln-Asn substitution (fusion protein C1AQNHA), results in an almost 100-fold decrease in the first order rate constant for C-terminal cleavage with respect to that of C1AQ (Table III). The substitution of penultimate His in a fusion protein that retains the C-terminal Gln results in C-terminal cleavage that is barely detectable after 18 h (Fig. 6).

These results imply that the intein may coordinate the overall splicing reaction by catalyzing the individual steps at appropriate relative rates. For the wild-type intein with C-terminal Gln, Gln cyclization as measured by C-terminal cleavage is ~10-fold slower than the combination of linear and branched ester formation, as measured by DTT-dependent N-terminal cleavage. Therefore, steps 1 and 2 of splicing would occur before step 3, preventing C-terminal cleavage from occurring prematurely and being uncoupled from splicing (Fig. 1, step 6). The substitution of the penultimate His for Ala results in a 100-fold reduction in the rate of side-chain cyclization, such that the rate of ester formation and hence thiolysis or hydrolysis, is far faster than side-chain cyclization. Therefore, it is not surprising that the penultimate His-Ala mutation would result in N-terminal cleavage uncoupled from splicing in fusion proteins HA and QNHA (Fig. 1, step 7) (2). In light of this hypothesis, the fact that the fusion protein QN splices efficiently and does not undergo significant C-terminal cleavage speaks to the flexibility of the control of the intein over relative reaction rates.

Our results also imply a role for the penultimate His residue. As the rates of cleavage with C-terminal Gln and Asn are both significantly decreased upon substitution of the penultimate His for Ala, it seems unlikely that the His serves to deprotonate the side-chain nucleophile. However, similar decreases in the rate of C-terminal cleavage could be explained by the His playing a role in increasing the electrophilicity of the peptide bond. Such a hypothesis is supported by structural studies. In the Mycobacterium xenopi GyrA intein structure, the penultimate His is in position to protonate the amide nitrogen of the scissile peptide bond upon Asn cyclization (16). The penultimate His (His-153) in the Ssp DnaB intein crystal structure could serve as part of a charge-relay system to stabilize the negative charge that forms on the amide oxygen of the scissile peptide bond in the tetrahedral intermediate of the cyclization of the C-terminal Asn (17). In addition, in the Ssp DnaB intein structure, the upstream His-143 is in a position to increase the nucleophilicity of the side chain of the C-terminal Asn via an
interaction through a water molecule (17). It will be interesting to determine whether substitution of the equivalent His in the Pab PolII intein (His-173) will effect the rate of splicing and/or C-terminal cleavage.

The first order rate constant of C-terminal cleavage of the Ssp DnaE trans-splicing intein is ∼5–8 times that of N-terminal trans-cleavage (14), so the control of the order of the steps of splicing by optimization of their relative rates may be a general phenomena. However, in the Ssp DnaE intein, N-terminal cleavage must precede C-terminal cleavage (9, 14). C-terminal cleavage is also significantly less efficient upon substitution of the upstream Ser in the Pyrococcus sp. GB-D Pol intein (6), and requires thiol-induced cleavage of the N-terminal scissile bond in the Saccharomyces cerevisiae VMA intein (18). Therefore, for these inteins, the order of the steps of the reaction may also be controlled by a sequential conformational change in the intein subsequent to steps 1 or 2 of splicing that optimally configures the C-terminal splice junction for cleavage.

The Pab PolII intein does not require prior N-terminal cleavage for the C-terminal cleavage to occur, so a catalytic mechanism involving a conformational change seems unlikely. Likewise, other inteins, including the ones that interrupt the ribonucleotide reductase of Methanobacterium thermoautotrophicus (19), DnaB of Ssp (20), and GyrA of M. xenopi (21), do not require N-terminal cleavage prior to C-terminal cleavage. This could be explained by the Ssp DnaB intein crystal structure, which suggests two separate active sites that may be separately responsible for the N- and C-terminal reactions (17). The separation of the active sites may preclude the need for a conformational change. The very different pH profiles for N-terminal cleavage (Fig. 5) and C-terminal cleavage (Fig. 7) suggests that the active sites for these reaction steps could be separate in the Pab PolII intein as well.

The pH profile of C-terminal cleavage (Fig. 7) is more similar to that of the pH profile of splicing (2) than is the pH profile for N-terminal cleavage, which lends further support to the hypothesis that the reactions at the intein C terminus control the rate of splicing.

The mechanism of the C-terminal cleavage reaction in the Pab PolII intein is still unclear. In the most likely scenario, Asn cyclization coupled to peptide bond cleavage proceeds via nucleophilic attack of the nitrogen of the side-chain amide on the scissile bond, resulting in bond cleavage coupled to formation of an aminosuccinimide as the C-terminal intein residue (Fig. 8, path 1). In support of this mechanism, groups using the Pyrococcus sp. GB-D Pol intein or S. cerevisiae VMA intein have purified and identified excised inteins with C-terminal aminosuccinimide residues (12, 13, 22). However, for the Pab PolII intein, we were unable to purify excised inteins with C-terminal cyclized residues both with WT, which has C-terminal Gln, and QN, which has C-terminal Asn. This may be explained by the increase in stability of C-terminal aminosuccinimides in model peptides at elevated temperatures (13), and the instability to alkaline hydrolysis of glutarimides relative to succinimides (23–25). However, the failure of Pietrokovski and co-workers (3) to isolate an excised intein with a cyclized C-terminal residue resulting from the splicing of the Chilo iridescent virus RNR intein, and in particular with its C-terminal Gln/Asn mutant, led them to suggest that the splicing of this non-canonical intein may proceed via a different mechanism at the intein C terminus. They proposed that the C-terminal residue may cyclize via a nucleophilic attack of the amide oxygen of the side chain on the scissile peptide bond, which would result in the excision of an intein with a C-terminal glutaranoxydride residue; that is, cleavage would occur via formation of an acylimidate (Fig. 8, path 2) (3). This mechanism was first proposed by Clarke (26) and was based on the observation that the amide oxygen of asparagine serves as a nucleophile in the reaction of 3-ketosteroid Δ⁵-isomerase from Pseudomonas testosteroni with an acetylenic mechanism-based inhibitor (27, 28). This would result in a mixed imido anhy-
dride, which should be less stable to hydrolysis than the imide and could explain their inability to isolate a cyclized intermediate. Further support for this mechanism rests with the fact that the C. hydrogenoformans RNR intein can splice with a C-terminal Asp, likely via a similar succinanyldride intermediate. The C. hydrogenoformans RNR intein also can facilitate C-terminal cleavage in a mutant intein in which the C-terminal Asp is replaced by Ala. To explain this observation, Pietrokovski and coworkers (3) invoke a mechanism first proposed by Nussbaum (29) in which the upstream nucleophile liberated by the transesterification reaction serves as the nucleophile that cleaves the downstream scissile bond, liberating a cyclized intein intermediate (Fig. 8, path 3). For the Pab PolII intein, our results preclude path 3 as a mechanism for C-terminal cleavage, as C-terminal cleavage can occur in our C1A mutants (Fig. 6). However, we cannot distinguish between path 1 or 2 and are initiating model peptide studies to examine this question.

We can purify unspliced and uncleaved precursor fusion proteins involving the Pab PolII intein expressed at 20 °C and can selectively induce the N- or C-terminal cleavage of inteins modulated by mutation via an increase in temperature. This suggests that this intein may be particularly useful in protein purification protocols (30), particularly if N- and C-terminal cleavage can be shown to be independent of the specific identity of the flanking sequences.

It is interesting to ponder why an intein would evolve to splice in an inefficient manner. What evolutionary forces resulted in the Pab PolII intein using Gln as its C-terminal residue, when the highly conserved Asn at that position results in splicing that is more than three times faster? Although there is no direct evidence for this proposal, the selection of the intein excision rate might imply that the intein plays or played some role in controlling the functional expression of the interrupted gene. Along these lines, the fact that the intein that interrupts the Poi of Thermococcus fumicolans also selectively splices at high temperatures (31) suggests that thermophilic inteins may have evolved to correlate proper growth temperature with the functional expression of proteins involved in DNA replication. Such possibilities are far more satisfying that the view of the intein as a molecular parasite and follows the speculation that the naturally split Ssp DnaE intein could serve a regulatory role in DnaE expression (32).

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