Characterization of a Naturally Occurring Trans-Splicing Intein from *Synechocystis* sp. PCC6803

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ABSTRACT: A naturally occurring trans-splicing intein from the *dnaE* gene of *Synechocystis* sp. PCC6803 (*Ssp* DnaE intein) was used to characterize the intein-catalyzed splicing reaction. Trans-splicing/cleavage reactions were initiated by combining the N-terminal splicing domain of the *Ssp* DnaE intein containing five native N-extein residues and maltose binding protein as the N-extein with the C-terminal *Ssp* DnaE intein splicing domain (E_C) with or without thioredoxin fused in-frame to its carboxy terminus. Observed rate constants (k_{obs}) for dithiothreitol-induced N-terminal cleavage, C-terminal cleavage, and trans-splicing were $(1.0 \pm 0.5) \times 10^{-3}$, $(1.9 \pm 0.9) \times 10^{-4}$, and $(6.6 \pm 1.3) \times 10^{-5}$ s^{-1}, respectively. Preincubation of the intein fragments showed no change in k_{obs}, indicating association of the two splicing domains is rapid relative to the subsequent steps. Interestingly, when E_C concentrations were substoichiometric with respect to the N-terminal splicing domain, the levels of N-terminal cleavage were equivalent to the amount of E_C, even over a 24 h period. Activation energies for N-terminal cleavage and trans-splicing were determined by Arrhenius plots to be 12.5 and 8.9 kcal/mol, respectively. Trans-splicing occurred maximally at pH 7.0, while a slight increase in the extent of N-terminal cleavage was observed at higher pH values. This work describes an in-depth kinetic analysis of the splicing and cleavage activity of an intein, and provides insight for the use of the split intein as an affinity domain.

Protein splicing is a post-translational processing event in which the intein (*1*) and the first C-extein residue catalyze its excision from a precursor protein, followed by the formation of a peptide bond between the adjacent protein sequences, which are termed exteins (reviewed in refs *2*−*5*). Approximately 100 inteins have been identified in organisms from the eukaryotic, archaeal, and eubacterial kingdoms (*6*). Naturally occurring inteins can be grouped into three categories: inteins that contain a homing endonuclease between the splicing domains, inteins that lack a homing endonuclease region (mini-inteins), and inteins in which the splicing domain is split, and are capable of splicing in trans.

The mechanism of protein splicing has been well studied (*7*, *8*). In the majority of inteins, splicing involves four highly coupled nucleophilic displacement reactions that take place at the N- and C-terminal splice junctions, three of which are catalyzed by the intein: (i) (thio)ester bond formation, resulting from an N−S or N−O acyl shift at the N-terminal residue (cysteine or serine); (ii) a transesterification reaction involving the newly formed (thio)ester bond and the C-terminal extein nucleophile (cysteine, serine, or threonine), resulting in a branched (thio)ester intermediate; (iii) cyclization of the C-terminal asparagine, releasing the intein; and (iv) a spontaneous S−N or O−N acyl rearrangement to form a peptide bond between the newly ligated extein sequences. In trans-splicing, these steps are preceded by the association of the N- and C-terminal intein fragments.

Prior to the discovery of the naturally occurring trans-splicing intein in the *Synechocystis* sp. *dnaE* gene (*Ssp* DnaE intein)[1] (*9*), protein trans-splicing was performed with artificially split inteins (*10*−*13*). Various inteins have been engineered to act as trans-splicing elements: the *Mycobacterium tuberculosis* RecA intein, the *Pyrococcus* sp. GB-D pol-1 intein, and the PI-*pfu*I intein from *Pyrococcus furiosu*s. However, the requirement for urea treatment with these inteins hindered general utility. The *Ssp* DnaE intein does not require the urea treatment step (*14*). This technology provides many useful applications, including segmentally labeling proteins for NMR analysis (*13*), ordered three-fragment ligation (*15*), and cyclization of proteins and peptides (*14*, *16*).

The *dnaE* gene encodes the catalytic subunit of the bacterial DNA polymerase III, and in *Synechocystis* sp., it is separated into two coding regions. The first split gene encodes the 774 N-terminal amino acid residues of the DnaE protein fused to the 123 N-terminal amino acid residues of the *Ssp* DnaE intein sequence, while the second fragment

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[1] Abbreviations: *Ssp* DnaE intein, *Synechocystis* sp. PCC6803 DnaE intein; E_N, N-terminal 123-amino acid fragment of the *Ssp* DnaE intein; E_C, C-terminal 36-amino acid fragment of the *Ssp* DnaE intein; E_C-CF, E_C with two native C-extein residues; E_C-CFNK, E_C with four native C-extein residues; MBP, *E. coli* maltose binding protein; CBD, chitin binding domain from *Bacillus circulans*; Trx, *E. coli* thioredoxin; ME_NB, fusion protein of MBP, E_N with five native N-terminal extein residues, and the CBD; E_CT, fusion protein of E_C with four native C-terminal extein residues and Trx; E_CGFP_{uv}, fusion protein of E_C with four native C-terminal extein residues and the UV variant of the green fluorescent protein; DTT, dithiothreitol; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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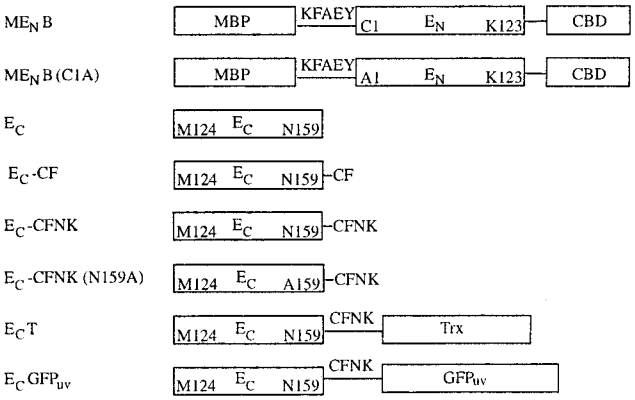

FIGURE 1: Fusion proteins and peptides used in the *Ssp* DnaE intein trans-splicing and N-terminal cleavage study. The fusion proteins ME_NB and ME_NB(C1A) both contain the 123-amino acid N-terminal splicing domain of the *Ssp* DnaE intein. The synthetic peptides E_C, E_C-CF, E_C-CFNK, and E_C-CFNK(N159A) and the fusion proteins E_CT and E_CGFP_{uv} contain the 36-amino acid C-terminal splicing domain of the *Ssp* DnaE intein. Amino acid substitutions indicated in parentheses are for the intein described, and are numbered according to that of the full-length 159-amino acid intein. Native extein residues are indicated by their single-letter amino acid code. Abbreviations for the fusion proteins are as follows: MBP, maltose binding protein; CBD, chitin binding domain; Trx, thioredoxin; and GFP_{uv}, UV variant of the green fluorescent protein.

consists of the 36 C-terminal amino acid residues of the *Ssp* DnaE intein followed by the 423 C-terminal amino acids of the DnaE polymerase. These two split genes are located 745 kilobases apart on the genome, and are found on opposite strands of the DNA (*9*). When the two intein halves are coexpressed in *Escherichia coli*, they are capable of trans-splicing to produce intact DNA polymerase III (*9*). In addition, these two fragments can trans-splice in vivo and in vitro when expressed in a foreign protein context (*14*, *16*).

Although the mechanism of protein splicing is well understood, the kinetic properties of splicing are not. This paper focuses on the investigation of the kinetic properties of trans-splicing and trans-cleavage of the *Ssp* DnaE intein. Unlike full-length inteins, the split *Ssp* DnaE intein serves as an ideal model system because the splicing or cleavage reaction is initiated only after combining the two trans-splicing domains. In this work, examination of the overall rate of reaction for both cleavage and splicing, elucidation of the rate-limiting steps, examination of the effect of temperature and pH on activity, and the affinity of the two intein fragments is addressed.

EXPERIMENTAL PROCEDURES

Construction of Plasmids. The construction of pMEB2 has been described previously (*14*). This plasmid contains an IPTG-inducible T7 promoter and ampicillin resistance and directs the expression of a fusion protein (ME_NB, Figure 1) consisting of the *E. coli* maltose binding protein (MBP), and the 123-residue N-terminal fragment of the *Ssp* DnaE intein (E_N) along with its five native N-extein residues adjacent to the N-terminus, followed by the chitin binding domain (CBD) from *Bacillus circulans* (*17*). Plasmid pMETXB1 encodes a fusion protein composed of the 36-residue C-terminal fragment of the *Ssp* DnaE intein (E_C), followed by its four native C-extein residues, *E. coli* thioredoxin (Trx), the *Mycobacterium xenopi* GyrA intein with asparagine[198] changed to alanine (*Mxe* GyrA intein), and the CBD. pMETXB1 contains an IPTG inducible T7 promoter and ampicillin resistance marker. Thioredoxin was obtained by PCR amplification from the plasmid pBSTXB1 (*18*) using the following primers: 5′-GTGGAAGCTCTTCAAATTGTGGTGGTATGAGCGATAAAATTATTCACCT-3′ and 5′-GTGGAAGCTCTTCAGCACATACGCATCGCCAGGTTAGCGTCGAG-3′. These primers incorporate *Sap*I sites at both ends of thioredoxin, as well as the amino acid sequences CGG before and MRM after the thioredoxin sequence. Sequencing results indicated that thioredoxin contained an in-frame deletion of the codon for Ser[89]. The amplified thioredoxin gene was inserted into the *Sap*I sites of pMECXB1 (5′-AATGGAAGAGCGCATATGGCGGCCGCGAATTCCTCGAGGGCTCTTCC-3′). The plasmid pMEB2A expresses the same fusion protein as pMEB2, but contains the C1A mutation in E_N. The amino acid substitution was made by linker insertion between the *Xho*I and *Kpn*I sites of pMEB2. The linker was comprised of the following oligomers: 5′-TCGAAAAATTTGCTGAATATGCTCTGTCTTTTGGTAC-3′ and 5′-CAAAAGACAGAGCATATTCAGCAAATTTT-3′.

Protein Expression and Purification. Plasmids pMEB2, pMEB2A, and pMETXB1 were transformed into ER2566 cells (New England Biolabs, Inc.), and the resulting transformants were grown at 37 °C until an OD_{600} of approximately 0.5 was reached. Protein expression was induced by the addition of IPTG to a final concentration of 0.5 mM, and cultures were grown overnight at 15 °C with shaking (250 rpm). Cells were harvested by centrifugation at 4 °C and lysed by sonication (over ice). Cellular debris was removed by centrifugation at 23000*g* for 30 min. The fusion proteins ME_NB and ME_NB(C1A) were purified at 4 °C using amylose resin as described previously (*19*). Briefly, the crude cell extract was applied to 15 mL of amylose resin (NEB) equilibrated in buffer A [20 mM Tris-HCl (pH 7.0) and 500 mM NaCl]. After application of the supernatant, the resin was washed with approximately 10−15 column volumes of buffer A. The desired protein was eluted with 3 column volumes of buffer B [20 mM Tris-HCl (pH 7.0), 500 mM NaCl, and 10 mM maltose]. The fusion protein E_CT, the product of protein expression from pMETXB1, was purified from a chitin resin at 4 °C using previously described methods (*19*,*20*). The crude protein extract was applied to 15 mL of chitin resin equilibrated in buffer C [20 mM Tris-HCl (pH 8.0) and 500 mM NaCl]. The column was washed with 10−15 column volumes of buffer C to remove the unbound material. Cleavage was induced by quickly washing the column with 3 column volumes of buffer D [20 mM Tris-HCl (pH 8.0), 500 mM NaCl, 30 mM dithiothreitol (DTT) (American Bioanalytical, Natick, MA), and 1 mM hydroxylamine (HA) (Sigma Chemical Co., St. Louis, MO)] and incubating overnight at 4 °C. Fractions were collected after overnight incubation, and those containing protein were dialyzed against 1 L of buffer C to remove residual DTT and HA. The dialyzed protein was concentrated using a Centriprep 10 apparatus (Amicon, Inc., Beverly, MA). Protein concentrations were determined using the Bio-Rad protein assay.

In Vitro Trans-Cleavage and/or Trans-Splicing. To examine trans-cleavage and trans-splicing, the protein ME_NB was used along with E_CT or several synthetic peptides.

Peptide synthesis was conducted as described previously (*19*), and included the following: a 36-amino acid peptide mimicking the 36 C-terminal amino acid residues of the *Ssp* DnaE intein (E_C), a 38-amino acid peptide corresponding to E_C with two native C-extein residues (E_C-CF), and two 40-amino acid peptides corresponding to E_C with four native C-extein residues (E_C-CFNK) with either Asn or Ala at residue 159. The standard assay contained ME_NB (24 μM) with or without a C1A mutation, 100 mM Tris-HCl (pH 7.0), and 500 mM NaCl. To monitor N-terminal cleavage, E_C (3−108 μM final concentration) and 50 mM DTT were added to the reaction mixture. To examine C-terminal cleavage, 12−50 μM E_CT was used in place of E_C, with 50 mM DTT. Trans-splicing reactions consisted of mixing ME_NB (24 μM) and E_CT (5−100 μM) in the absence of DTT. For kinetic analysis, aliquots were removed at specific time intervals, and the reaction was stopped by the addition of 3× SDS−PAGE sample buffer with 0.125 M DTT followed by boiling. Unless specified, all reactions were carried out at 23 °C. Cleavage reaction mixtures were visualized by electrophoresis using a 10 to 20% Tricine gel (Novex, San Diego, CA), while 10 to 20% Tricine and 8% Tris-glycine gels (Novex) were used to follow the splicing reactions. Gels were stained with Coomassie Brilliant Blue (Sigma) and scanned, and the density of the bands was quantified using NIH Image 1.59.

Analysis of the Interaction between Intein Fragments. To examine the interaction between the two intein fragments, the standard assay containing ME_NB with the C1A mutation (24 μM) and 12−96 μM E_C or E_CGFP_{uv} (Clontech, Palo Alto, CA, Lot 6079-1) were analyzed with nondenaturing 12% Tris-glycine gels (Novex). Resulting gels were either stained with Coomassie Brilliant Blue (Sigma) or analyzed by Western blot with antibodies against E_N and E_C as described previously (*21*).

Confirmation of the Thioester Intermediate. To identify the presence of a thioester intermediate, reaction mixtures containing 24 μM ME_NB and 24 μM E_C were incubated with and without 50 mM 2-mercaptoethanesulfonic acid (MESNA) and a 30-residue peptide (1 mM). The sequence of the 30mer is as follows: NH_2-CAYKTTGANKHIIVACEGNPYVPVHFDASV-COOH. Reaction mixtures were incubated overnight at 23 °C, resolved on a 10 to 20% Tricine gel (Novex), and visualized with Coomassie Brilliant Blue (Sigma).

Kinetic Analysis of Trans-Cleavage (N- and C-Terminal) and/or Splicing. Experimental evidence indicates that association of the two intein splicing domains is fast relative to the subsequent steps so that the reaction involving N-terminal cleavage of the linear thioester by DTT can be described by reaction I:

$$\mathrm{AB} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{AB^*} \xrightarrow[\mathrm{DTT}]{k_3} \mathrm{E + F} \qquad \text{(I)}$$

where AB=associated ME_NB and E_CT
AB*=activated thioester state
E=associated intein splicing domains with Trx fused to C-fragment
F=N-terminal cleavage product (MBP)

k_2 is the N−S acyl rearrangement rate constant, k_{-2} is the S−N acyl shift rate constant, and k_3 is the rate of cleavage by DTT. It is necessary to analyze the trans-cleavage reaction using pre-steady-state assumptions as described by Guan et al. (*22*). Since the reaction follows pseudo-first-order kinetics, and it is assumed that there is no accumulation of the thioester intermediate (i.e., $d[AB^*]/dt = 0$), then the rate of loss of AB is equivalent to the rate of formation of F. Using this information, when DTT is present in excess, the rate of formation of F can be described as follows:

$$d[F]/dt = k_3k_2/(k_{-2} - k_3)[AB] \qquad (1)$$

and the overall rate constant (k_{obs}) is equal to $k_3k_2/(k_{-2} - k_3)$.

Since N-terminal cleavage stimulates C-terminal cleavage (see Results), the C-terminal cleavage reaction can be represented by the following:

$$\mathrm{A + B} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{AB} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{AB^*} \xrightarrow[\mathrm{DTT}]{k_3} \mathrm{E + F} \xrightarrow{k_4} \mathrm{I + G} \qquad \text{(II)}$$

(k_{obs1} spans AB → E + F)

where A= ME_NB,
B=E_CT
AB=associated ME_NB and E_CT
AB*=activated thioester state
E=associated intein splicing domains with Trx fused to C-fragment
F=N-terminal cleavage product (MBP)
G=C-terminal cleavage product (Trx)
I=associated intein splicing domains

The k_{obs} for cleavage is a representation of all of the steps up until and including DTT-induced N-terminal cleavage. Since the association of the intein fragments is rapid and N-terminal cleavage is an irreversible process, reaction II can be simplified to the following:

$$\mathrm{AB} \xrightarrow[\mathrm{DTT}]{k_{obs1}} \mathrm{E + F} \xrightarrow{k_4} \mathrm{I + G} \qquad \text{(III)}$$

where k_{obs1} is the observed rate for N-terminal cleavage and k_4 is the rate of C-terminal cleavage. If k_4 is assumed to be first-order, this can be treated as an irreversible consecutive reaction (*23*). C-Terminal cleavage can be monitored; k_{obs1} is determined experimentally so that k_4 can be calculated using the following:

$$[G] = AB_o\left[1 + \frac{1}{k_{obs1} - k_4}(k_4e^{-k_{obs1}t} - k_{obs1}e^{-k_4t})\right] \qquad (2)$$

where G is the C-terminal cleavage product and AB_o is the associated state (determined by the concentration of the limiting fragment).

The trans-splicing process can be described by the following reaction scheme:

$$\mathrm{A + B} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{AB} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{AB^*} \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} \mathrm{AB^{**}} \xrightarrow{k_6} \mathrm{I + L^*} \xrightarrow{k_7} \mathrm{L} \qquad \text{(IV)}$$

(k_{obs2} spans A + B → I + L*; AB** → C (hydrolysis))

where A= ME_NB,
B=E_CT
AB=associated ME_NB and E_CT
AB*=activated thioester state
AB**=branched intermediate
C=hydrolysis product (MBP)
I=associated intein splicing domains
L*=spliced product prior to S-N shift
L=spliced product (MBP-Trx)

Like the cleavage reaction, the splicing reaction is assumed to be pseudo-first-order, and the rate of association of the two intein fragments is assumed to be very fast. Also, the

last step (S−N shift) is extremely fast and not considered to be catalyzed by the intein (*24*). For the splicing reaction, one cannot assume that there is a lack of accumulation of the branched intermediate. Therefore, the contribution of each step to the overall rate constant cannot be derived.

In this work, an average k_{obs} for both N-terminal cleavage and trans-splicing was calculated from data at various intein C-fragment concentrations. Reported k_{obs} values were obtained from at least three trials, with standard deviations reported. These reactions were treated as irreversible, pre-steady-state first-order processes; k_{obs} was obtained by fitting the data to the equation $P = P_o(1 - e^{-kt})$, where P is the percentage of cleaved or spliced product at time t, P_o is the maximum percentage of cleavage or spliced product obtained, and k is the observed rate (*25*). This method was used previously to analyze the kinetic data for the trans-acting δ ribozyme (*26*).

RESULTS

We have previously shown in vitro trans-splicing and/or trans-cleavage activity of the *Ssp* DnaE intein (*14*). The current work focuses on the further biochemical characterization of the *Ssp* DnaE intein. This unique splicing element allows a known starting point for kinetic studies so that the relative rates of the splicing events can be tested.

N-Terminal Cleavage. To determine whether the presence of C-terminal extein residues was necessary for the activity of the *Ssp* DnaE intein, three different peptides consisting of the 36-amino acid splicing domain with no (E_C), two (E_C-CF), or four (E_C-CFNK) native extein residues were used in the trans-cleavage reaction (for a summary of the constructs that were used, see Figure 1). Incubation of the ME_NB precursor protein with any of these three peptides resulted in processing of the fusion protein, suggesting that the C-extein sequence was not necessary for activity. E_C was chosen for the study of N-terminal cleavage because it lacks the C-extein residue normally involved in the second step of protein splicing, trans-thioesterification. Therefore, the mechanism of ME_NB processing is through N-terminal cleavage of the linear thioester intermediate. This reduces the steps in the trans-cleavage reaction to the first two steps of the splicing reaction (association and N−S acyl rearrangement). The mechanism of cleavage of the *Ssp* DnaE intein is illustrated in Figure 2. Several nucleophiles were tested for cleavage activity, including DTT, hydroxylamine, and MESNA. DTT was chosen because it cleaved with the fastest rate and to the greatest extent (data not shown). The concentration of DTT in the assay was varied from 50 to 250 mM, and did not have an effect on k_{obs1}. N-Terminal cleavage occurred with time after mixing the appropriate intein domains (Figure 3A,B). The k_{obs1} for DTT-induced N-terminal cleavage was determined to be $(1.0 \pm 0.5) \times 10^{-3}$ s^{-1} (Table 1) using equations described in Experimental Procedures (Figure 3C). In addition, the reaction behaved in a pseudo-first-order fashion when the E_C concentrations were varied at conditions below saturation (data not shown).

Preincubation of the Cleavage Reaction Mixture. To determine if the association of the two intein fragments contributes to the overall rate, the reaction mixtures were incubated for various periods of time prior to the addition of DTT. Reaction mixtures containing 24 μM ME_NB and E_C (12, 24, and 48 μM) were incubated for either 5 or 90 min at room temperature prior to the addition of DTT (50 mM). Similar rates were obtained whether the reaction mixtures were allowed to incubate for 0, 5, or 90 min (Table 1). These data suggest that the association step is very fast relative to the subsequent cleavage steps under these conditions and does not contribute to the overall rate of reaction.

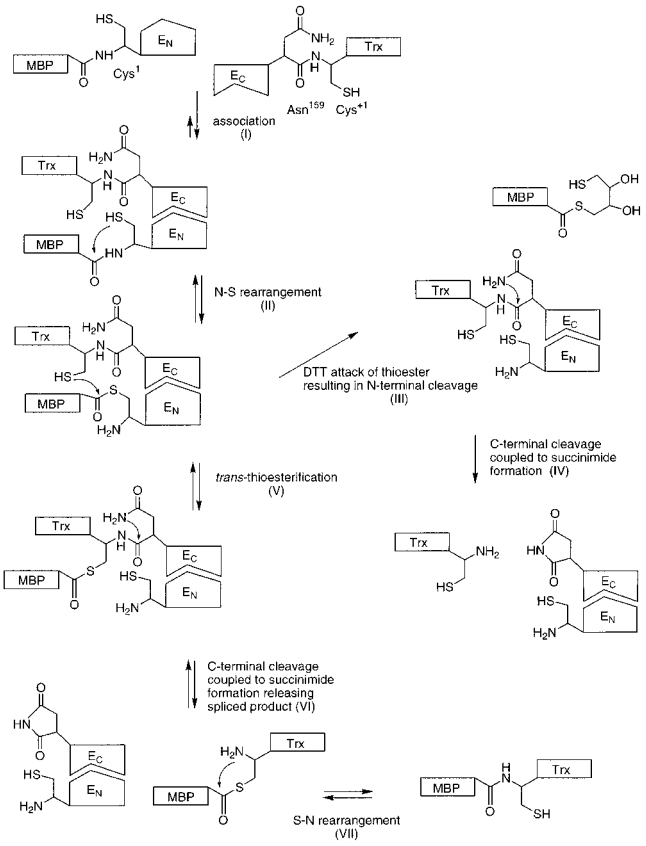

FIGURE 2: Mechanism of trans-cleavage and trans-splicing of the *Ssp* DnaE intein. Association (I) of the two intein fragments aligns the splice junctions so that splicing or cleavage can occur. Following the N−S acyl rearrangement (II), one of two pathways is possible. The activated complex can undergo N-terminal cleavage by a nucleophile (III), followed by C-terminal cleavage via succinimide formation (IV), or trans-thioesterification leading to branched intermediate formation (V) can occur. Succinimide formation (VI) and an S−N acyl rearrangement (VII) yield the spliced product. During the process of trans-splicing, the complex can also undergo spontaneous hydrolysis after thioester formation or trans-thioesterification (not shown).

Intein Fragment Turnover. The ability of the C-terminal fragment of the *Ssp* DnaE intein to dissociate once bound to an N-terminal domain and then bind to another available precursor was addressed by examining N-terminal cleavage reactions at longer time intervals and differing reactant ratios. Following overnight incubation, the amount of N-terminal cleavage product formed approximated the concentration of E_C, consistent with the C-fragment acting only once (Figure 3A,B,D). Whether E_C was added at concentrations below (6 μM) or above (60 μM) the 1:1 ratio of ME_NB to E_C, the results showed that by 90 min maximal cleavage had occurred, with no significant cleavage occurring thereafter (Figure 3D). To further examine the interaction between the two splicing domains, reaction mixtures containing 24 μM ME_NB with the C1A mutation and either E_C or E_CGFP_{uv} (12−96 μM) were analyzed with nondenaturing 12% Tris-glycine gels. The C1A mutation prevents cleavage or splicing

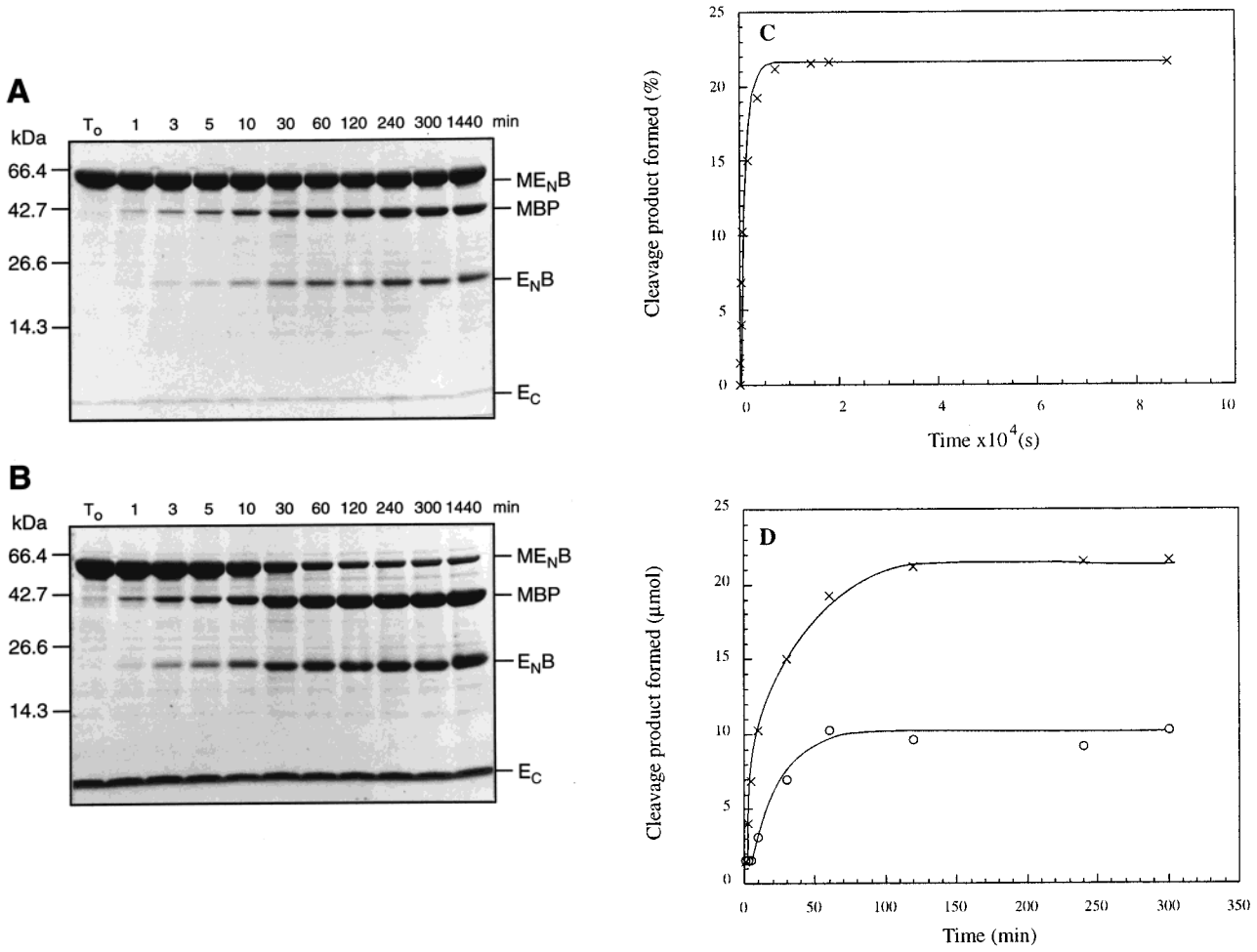

FIGURE 3: Analysis of N-terminal cleavage of the *Ssp* DnaE Intein. Assay of the reaction mixture containing 24 μM ME_NB, 50 mM DTT, and either 6 (A) or 60 μM E_C (B). Reaction mixtures were incubated at 23 °C and visualized on a 10 to 20% Tricine gel stained with Coomassie Brilliant Blue. Time intervals at which the aliquots were taken are indicated above the appropriate gel lane. Precursors (ME_NB and E_C) as well as N-terminal cleavage products (MBP and E_NB) are also labeled. The k_{obs} for N-terminal cleavage was determined by fitting a plot of percent cleavage as a function of time using the program KaleidaGraph (Synergy Software, Reading, PA) and the equation $P = P_o(1 - e^{-kt})$ as described in Experimental Procedures (C). A plot of micromoles of cleavage product vs time (D) summarizes the data for the reaction mixtures containing either 6 (○) or 60 μM E_C (×) and 24 μM ME_NB. Results show that after 90 min maximal cleavage has occurred. No additional cleavage is detected after 24 h (data not shown). The data represent an average of two trials.

Table 1: Rate Data Obtained for N-Terminal Cleavage, C-Terminal Cleavage, and Trans-Splicing Reactions of the *Ssp* DnaE Intein[a]

N-terminal cleavage reaction with DTT	k_{obs} (s^{-1})
$ME_NB + E_C$ (0 min preincubation)[b]	$(1.0 \pm 0.5) \times 10^{-3}$
$ME_NB + E_C$ (5 min preincubation)[b]	$(7.5 \pm 0.7) \times 10^{-4}$
$ME_NB + E_C$ (90 min preincubation)[b]	$(1.1 \pm 0.7) \times 10^{-3}$
$ME_NB + E_CT$ (0 min preincubation)[b]	$(1.7 \pm 0.3) \times 10^{-3}$
$ME_NB + E_CT$ (0 min preincubation)[c]	$(1.8 \pm 0.1) \times 10^{-3}$
C-terminal cleavage reaction	**k_4 (s^{-1})**
$ME_NB + E_CT$	$(1.9 \pm 0.9) \times 10^{-4}$
trans-splicing reaction	**k_{obs} (s^{-1})**
$ME_NB + E_CT$	$(6.6 \pm 1.3) \times 10^{-5}$

[a] See Experimental Procedures for details. Errors represent the standard deviation from at least three trials. [b] With 24 μM ME_NB in the reaction mixture. [c] With 48 μM ME_NB in the reaction mixture.

from occurring. E_C fused to thioredoxin was not used because it accumulated near the top of the native gel due to its very basic pI value, complicating interpretation. When ME_NB was incubated with E_CGFP_{uv}, a shift in the ME_NB band was observed by native polyacrylamide gel electrophoresis, suggestive of a noncovalent interaction (Figure 4). The comigration of the N- and C-terminal splicing domains was verified by Western blot analysis using an antibody raised against E_N and E_C (data not shown).

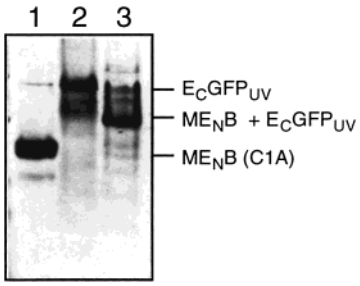

FIGURE 4: Analysis of splicing domain association by native PAGE. The interaction of proteins containing the N- and C-terminal *Ssp* DnaE intein splicing domains was investigated by mixing ME_NB(C1A) (24 μM) and E_CGFP_{uv} (48 μM) and incubating overnight at 23 °C. The reaction mixture was visualized on a nondenaturing 12% Tris-glycine gel that was stained with Coomassie Brilliant Blue: lane 1, ME_NB(C1A); lane 2, E_CGFP_{uv}; and lane 3, ME_NB(C1A) and E_CGFP_{uv}. The disappearance of the ME_NB band, which is the limiting reactant, and the appearance of a new band are suggestive of complex formation.

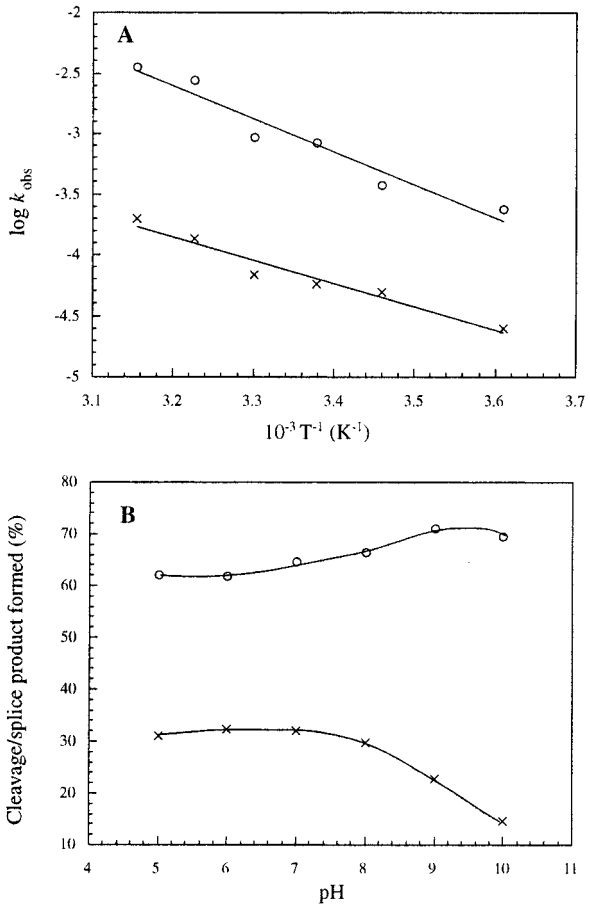

FIGURE 5: Effect of temperature and pH on trans-splicing and N-terminal cleavage activity. (A) An Arrhenius plot was constructed for N-terminal cleavage reaction mixtures (○) that consisted of 24 μM ME_NB, 24 or 48 μM E_C, and 50 mM DTT and trans-splicing reaction mixtures (×) that contained 24 μM ME_NB and 24 or 48 μM E_CT. Reaction mixtures were incubated at various temperatures from 4 to 44 °C. The k_{obs} for each temperature was determined as described in Experimental Procedures. The slope of the line yielded activation energies of 12.5 and 8.9 kcal for the cleavage and splicing reactions, respectively. The data represent an average of two trials. (B) The effect of pH on the amount of N-terminal cleavage product (○) that formed was determined for a reaction mixture containing 24 μM ME_NB, 100 μM E_C, and 50 mM hydroxylamine, while the effect on the spliced product (×) was determined for a reaction mixture containing 24 μM ME_NB and 100 μM E_CT. Reaction mixtures were incubated overnight at 23 °C.

Sensitivity of Trans-Cleavage to Temperature and pH. To examine the temperature sensitivity of N-terminal cleavage, reaction mixtures containing 24 or 48 μM E_C were incubated at temperatures between 4 and 44 °C. From these data, k_{obs1} was calculated at each temperature and an Arrhenius plot was constructed (Figure 5A). The k_{obs1} values that were obtained were linear for the temperature range that was examined. The activation energy of cleavage was determined to be 12.5 kcal/mol. To determine if extein sequence has an effect on E_a, reaction mixtures containing 24 μM ME_NB and 24 μM E_CT were incubated at 16 and 44 °C. k_{obs1} values similar to that of E_C were observed, indicating that the lack of extein sequence does not dramatically affect E_a (data not shown). The effect of pH on the N-terminal cleavage reaction was examined over the pH range of 5−10, with hydroxylamine as the nucleophile. Hydroxylamine was present in large excess (50 mM), and the pH of each reaction mixture was confirmed after the addition of all components. The results indicated activity over the entire pH range that was examined, with a slight increase at the higher pH values (Figure 5B).

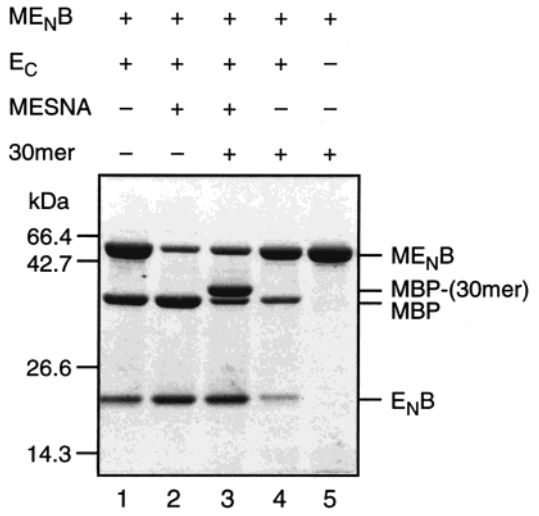

FIGURE 6: Confirmation of thioester intermediate formation. All reaction mixtures contained 24 μM ME_NB and 24 μM E_C and were incubated at 23 °C overnight: lane 1, ME_NB and E_C; lane 2, ME_NB, E_C, and 50 mM MESNA; lane 3, ME_NB, E_C, MESNA, and 1 mM 30mer; lane 4, ME_NB, E_C, and 30mer (no MESNA); and lane 5, ME_NB and 30mer (no E_C). ME_NB, cleavage product (MBP), E_NB, and ligated product (MBP−30mer) are visible on a 10 to 20% Tricine gel stained with Coomassie Brilliant Blue.

Effect of C-Extein Sequence on k_{obs1}. To determine if the presence of C-extein residues affected the overall rate of N-terminal cleavage, reaction mixtures were incubated at 23 °C with differing concentrations of E_CT (15−50 μM). These data yielded a k_{obs1} of $(1.7 \pm 0.3) \times 10^{-3}$ s^{-1} (Table 1). This value falls within the range obtained for the reaction mixture containing E_C.

Confirmation of Thioester Formation. To confirm that the splicing reaction of the *Ssp* DnaE intein proceeds through a thioester intermediate, as do those of other inteins, a 30-amino acid peptide was added to a reaction mixture containing ME_NB, E_C, and MESNA. The 30mer possesses an N-terminal cysteine, and is added to the reaction mixture for ligation with the thioester generated by N-terminal cleavage. In the presence of the 30mer, there was a shift in the molecular mass of the N-terminal cleavage product, MBP, indicating it had undergone ligation (Figure 6). For this to occur, MESNA must have attacked the thioester intermediate of the intein to generate a new reactive thioester at the C-terminus of the cleavage product. Similar results were observed when the extein sequence was present on the C-terminal splicing domain (data not shown).

C-Terminal Cleavage. To study C-terminal cleavage, E_CT (5−50 μM) was used in the reaction in place of E_C. The C-terminal cleavage product (Trx) could be detected, but only after N-terminal cleavage had occurred (Figure 7A). When DTT was eliminated from the reaction mixture, significantly less C-terminal cleavage product could be detected (data not shown). The small amount of C-terminal cleavage product formed in the absence of DTT may have been triggered by hydrolysis at the N-terminal splice junction. Using eq 2 and k_{obs1} for N-terminal cleavage, the rate of C-terminal cleavage was determined to be $(1.9 \pm 0.9) \times 10^{-4}$ s^{-1} (Table 1). When E_CT was present in excess, it appeared that the extent of

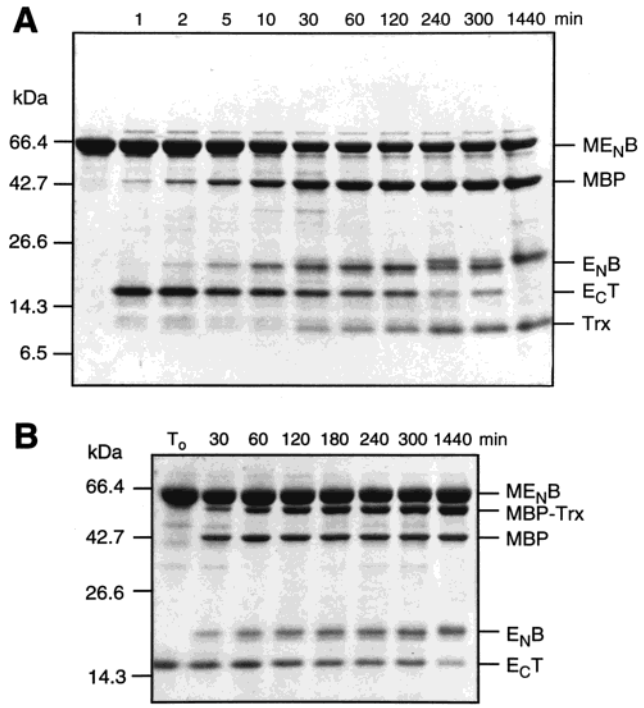

FIGURE 7: Analysis of the C-terminal cleavage and trans-splicing reactions of the *Ssp* DnaE intein. The assay for monitoring C-terminal cleavage (A) contained 24 μM ME_NB, 15 μM E_CT, and 50 mM DTT, while the assay for monitoring trans-splicing (B) contained 24 μM ME_NB and 24 μM E_CT. All reaction mixtures were incubated at 23 °C and visualized by SDS−PAGE, using a 10 to 20% Tricine gel stained with Coomassie Brilliant Blue. Time intervals at which the aliquots were taken are indicated at the top of the gels. Precursors (ME_NB and E_CT), N-terminal cleavage or hydrolysis products (MBP and E_NB), the C-terminal cleavage product (Trx), and the spliced product (MBP-Trx) are labeled.

C-terminal cleavage was limited by the ME_NB concentration, supporting the hypothesis that C-terminal cleavage follows association and N-terminal cleavage. To determine if C-terminal cleavage could occur independently of N-terminal cleavage or splicing, ME_NB with a C1A mutation was used in the reaction. When N-terminal cleavage or splicing was blocked, no accumulation of C-terminal cleavage product was detected (data not shown).

Kinetic Study of Splicing. The trans-splicing mechanism involves several steps in which no relationship between rate constants could be derived with confidence. However, it is reasonable to suggest that all of the steps following association contribute to the k_{obs2}. To examine the splicing reaction, aliquots were taken from reaction mixtures and analyzed over the course of several hours (Figure 7B). Formation of the spliced product (MBP−Trx) as well as the hydrolysis product (MBP) was quantitated, and the k_{obs2} for splicing was determined to be $(6.6 \pm 1.3) \times 10^{-5}$ s^{-1} (Table 1) as described in Experimental Procedures. Once again, evaluation of initial velocities suggested the reaction behaved in a pseudo-first-order fashion below saturation (data not shown).

Sensitivity of Trans-Splicing to Temperature and pH. The sensitivity of trans-splicing to temperature was examined by incubating reaction mixtures containing 25 or 50 μM E_CT at various temperatures from 4 to 44 °C. The k_{obs} at each temperature was calculated, and an Arrhenius plot was constructed (Figure 5A). On the basis of the slope obtained from the Arrhenius plot, the activation energy was determined to be 8.9 kcal/mol. Although k_{obs2} continues to increase over the temperature range that was examined, the overall extent of splicing begins to decrease with increasing temperature above 30 °C (data not shown). In contrast, it appears that cleavage by spontaneous hydrolysis is driven by an increase in temperature. Splicing was also examined over the pH range of 5−10. A reaction mixture consisting of 24 μM ME_NB, 100 μM E_CT, and Tris-HCl buffer at the appropriate pH was allowed to incubate overnight at room temperature. Maximal splicing activity occurred at pH 7 (Figure 5B).

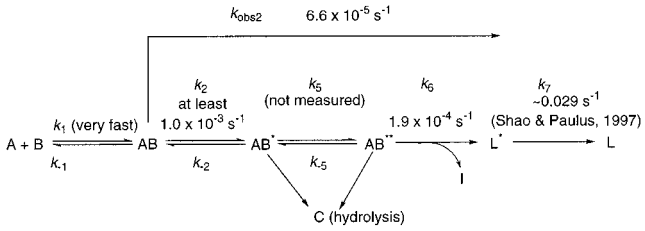

FIGURE 8: Overall trans-splicing mechanism with determined rate constants. Values for the N−S acyl shift, C-terminal cleavage, and k_{obs} for splicing were determined experimentally. The S−N shift rate data were determined previously. A represents ME_NB. B represents E_CT. AB represents the associated state. AB* represents the activated thioester state. AB** represents the branched intermediate. C represents the hydrolysis product (MBP). I represents associated intein splicing domains. L* represents the spliced product prior to the S−N shift. L represents the spliced product with a peptide bond.

DISCUSSION

Past attempts to examine the kinetics of protein splicing have been hindered by the difficulty of determining the start of the reaction. Upon isolation of a full-length intein, its progression along the splicing pathway is not known, thereby affecting interpretation of the data. With the *Ssp* DnaE intein, the reaction cannot proceed until both intein fragments are present. Other advantages of the *Ssp* DnaE intein are (i) it is the first documented naturally occurring split intein, where in previous work inteins were artificially split (*9*, *10*, *12*, *13*), (ii) it splices without a denaturation/renaturation step, and (iii) it is highly soluble. Some of these advantages may be the result of the intein evolving to become more proficient in the cell. For these reasons, the *Ssp* DnaE intein is a potentially powerful means of in vivo and in vitro protein manipulation.

With the exception of trans-thioesterification, each step in the splicing process has been examined in this work in some detail (Figure 8). The first two steps of trans-splicing are identical to the first two steps of N-terminal cleavage: association and an N−S acyl rearrangement. No detectable change in the k_{obs} for N-terminal cleavage whether a preincubation was conducted or not demonstrated that association of the intein splicing domains is very rapid compared to subsequent splicing steps (Table 1). Also, it was determined that the rate of the N−S acyl shift is at least 15 times faster than the overall k_{obs} for trans-splicing. The C-terminal extein residues were not required for N-terminal cleavage to occur, nor did they appear to affect the rate of cleavage. The next stage in the splicing pathway is trans-thioesterification leading to formation of the branched intermediate, but unfortunately, there was no direct method

of examining this step. The fourth step, C-terminal cleavage coupled to succinimide formation, appeared to be about 2−3 times faster than the overall k_{obs} for trans-splicing. The last step in the splicing mechanism is an S−N acyl rearrangement that is not catalyzed by the intein. Previous studies with model peptides have shown that the S−N acyl rearrangement is too fast to measure at pH 7 and has a low activation energy (*24*). Examination of the steps in the splicing mechanism, as well as previous work studying the rate of cleavage next to asparagine in model peptides (*27*), and the S−N acyl rearrangement (*24*) suggests that trans-thioesterification and/or succinimide formation are the slow steps in the *Ssp* DnaE intein trans-splicing mechanism.

This work is the most in-depth study on the rates of the steps in the splicing reaction to date. However, previous work permits a general comparison of the rates of splicing or N-terminal cleavage of other inteins to the *Ssp* DnaE intein. Study of the *Psp* GB-D pol-1 intein in a foreign protein context (*28*) indicated that splicing was complete in 4 h. Similarly, the *Ssp* DnaE intein in vitro splicing reaction was also complete in 3−4 h, suggesting that the two values may be comparable. However, a more quantitative comparison requires a study to determine the actual k_{obs} of splicing or cleavage for the *Psp* pol-1 intein. It is interesting that the trans-splicing activity of the *Ssp* DnaE intein appears to be a relatively slow process. It has been suggested that a slow splicing step may be a method of controlling production of the DnaE protein in *Synechocystis* sp. (*9*). Even though the splicing reaction generates a protein necessary for DNA replication, the cell only needs a few copies of this protein for viability (*9*, *21*).

Previous studies on N-terminal cleavage by Southworth et al. measured percent cleavage as a function of time, and determined the rate of product formation for the full-length *Mxe* GyrA intein to be 0.036 s^{-1} at 23 °C (*29*); however, no kinetic rate constants were reported. For the *Sce* VMA intein, Chong et al. measured half-times of N-terminal cleavage at pH 6.0 and 8.0 (*30*). Using these half-times and assuming a first-order reaction, k_{obs} values of 1.9×10^{-3} s^{-1} at pH 8.0 and 4.0×10^{-5} s^{-1} at pH 6.0 were calculated. The rate at pH 8.0 is strikingly similar to the data obtained for the *Ssp* DnaE intein, indicating that the study presented here may be applicable to other inteins.

It is interesting that C-terminal cleavage was not detected until DTT-induced N-terminal cleavage had occurred. In both the N-terminal cleavage and splicing pathways, the scissile peptide bond at the N-terminus of the intein is broken. The *Ssp* DnaE intein may coordinate the protein-splicing steps by linking cleavage of this scissile peptide bond (step V of Figure 2) to a conformational change that permits C-terminal cleavage to occur (step VI of Figure 2). This behavior was similar to that reported for the H453Q/C455A mutant of the *Sce* VMA intein (*30*). However, an N-terminal cleavage-induced conformational change may not be a universal control mechanism as other inteins do not couple C-terminal cleavage to N-terminal cleavage (*29*−*34*). Thus, there may be a heterogeneity in the internal signals inteins use to tightly regulate the splicing reaction.

In this study, the unreacted and cleaved precursor protein was observed in the splicing reaction mixtures. This could have been due to the intein being in a foreign protein context or due to the lack of chaperonins found in its native environment. Both of these cases may cause some misfolding of the precursor, thereby affecting the overall extent of the reaction. Protein misfolding may account for slight variations in the extent of reaction observed from different protein preparations; however, the overall rate constants between preparations were reproducible.

It is also possible that the native extein sequence plays a role in the extent of protein splicing, even though it is not necessary for trans-splicing or trans-cleavage to occur. We have previously shown that the *Ssp* DnaE intein is capable of cis-splicing with two proximal N-extein or three proximal C-extein residues (*14*), but did not examine the minimum requirement for cleavage. The constructs used for trans-splicing in the work presented here contained five native N-extein and four native C-extein residues. Also, it was speculated previously that complex formation is at least partially and perhaps entirely dominated by the intein fragments and not by the extein residues (*14*). It is possible that the extein residues are more important in later steps, perhaps by optimizing the spatial orientation of amino acid residues so that splicing can occur. Interestingly, these same extein residues do not appear to be as crucial for the first two steps of protein splicing (association and the N−S acyl shift) as they do for the later steps, since the extent of the N-terminal cleavage reaction was typically >80%, compared to a splicing extent of ca. 30%. Furthermore, the rate of N-terminal cleavage was the same, within experimental error, whether there was zero or four native C-extein residues (Table 1). When Wu et al. examined trans-splicing of the split *dna*E gene product in *E. coli* by Western blot analysis, they reported that 80% of the precursor was converted to the spliced product (*9*). The fact that the extent of trans-splicing in this study was lower suggests that native context may play a role in proficiency. Further work is needed to determine the role extended extein sequences play in intein folding and whether this impacts only on the extent of splicing or also the rate of the splicing reaction.

Preincubation of the reaction mixture prior to the addition of DTT indicated that association is fast relative to the subsequent reaction steps. Unfortunately, the rate of association could not be measured using the techniques described in this work. When the E_C fragment was limiting in the reaction mixture, there was no additional turnover over a 24 h period, suggesting that the N- and C-terminal *Ssp* DnaE intein splicing domains interact with a high affinity. This interaction was confirmed by native gel and Western blot analysis. This tight binding may be biologically important for this intein by providing a mechanism of controlling interference from end products of the splicing reaction. If there were a rapid turnover of the two splicing domains, then a small amount of the released C-terminal splicing domain would be able to produce a large amount of unwanted cleavage of the N-terminal precursor protein. As a comparison, the trans-acting δ ribozyme was able to turn over up to four times (*26*).

There are other possible methods of preventing product interference of the splicing reaction. The first method would be to have the intein domains without extein sequence be unable to participate in trans-cleavage reactions. This is not likely to be the case in vivo because the in vitro rates of N-terminal cleavage are similar whether or not the C-terminal splicing domain contains C-extein residues. A second method

would be to have the two splicing domains undergo a conformational change to create an even higher affinity interaction between the two fragments so that they do not dissociate over the relevant lifetime of the cell. Elucidation of the binding constants for the two splicing domains is necessary to determine whether the second possibility is occurring. Studies on the artificially split *Psp* GB-D Pol-1 intein showed that after reconstitution, endonuclease activity could be restored (*10*). Although this work does show interaction between the intein fragments, it does not address the issue of turnover. In addition, affinity could be attributed to the endonuclease domain, not only to the splicing domains.

Both trans-splicing and trans-cleavage activities occurred over a wide temperature range (4−44 °C). These temperatures are similar to the growth conditions of *Synechocystis* sp. PCC6803 which is viable up to 39 °C (*35*). The activation energies of 12.5 and 8.9 kcal/mol for trans-cleavage and trans-splicing, respectively, are moderate compared to the activation energies of other processes. For example, the activation energy of the S−N acyl rearrangement, which is not catalyzed by the intein, is 4−5 kcal/mol (*24*). Alternatively, the cis to trans proline isomerization of prothrombin, whose kinetics are generally very slow, has an E_a of ∼24 kcal/mol (*36*). It is interesting that the activation energy for cleavage is higher than that for splicing, since cleavage occurs at a faster rate than splicing under these conditions. The higher E_a may be due to the fact that cleavage is a bimolecular process (i.e., requires intein and separate nucleophile), and additional energy is required for the nucleophile to enter the active site. Even though the initial rates for both cleavage and splicing increased linearly over the temperature range that was examined, the extent of splicing began to decrease at higher temperatures. Perhaps this is due to a slow denaturation occurring at the higher temperature.

A pH titration indicated that the trans-splicing activity was maximal at pH 7.0. Activity dropped off at higher pH, consistent with data reported for other inteins (*28*, *37*). It has been suggested that inhibition at higher pH occurs because the acyl rearrangement (N−O or N−S) favors the amide bond at higher pH, while the ester bond is favored at lower pH (*24*, *28*, *30*). N-Terminal cleavage activity was detected over the entire pH range that was examined, and even slightly higher at the upper pH limits. This indicates that the intein may misfold slightly at the higher pH so that splicing is inhibited but not N-terminal cleavage. This again shows that conditions are much less stringent for N-terminal cleavage than they are for trans-splicing.

The study presented here is the first detailed kinetic examination of the steps in the splicing reaction and further characterizes the properties of the naturally occurring split *Ssp* DnaE intein. Additionally, the high affinities of the N- and C-terminal domains make it an attractive tool for protein−protein interaction methodology. The results in this work are consistent with previous mechanistic observations of other inteins, suggesting that the *Ssp* DnaE intein provides a useful model system for understanding the biochemical processes of other protein splicing elements. Future work will focus on gaining a better understanding of the trans-thioesterification step, as well as determining the association and dissociation rates of the intein splicing domains.

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