Mutational Analysis of Protein Splicing, Cleavage, and Self-Association Reactions Mediated by the Naturally Split *Ssp* DnaE Intein

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ABSTRACT: The ability to separately purify the naturally split *Synechocystis* sp. PCC6803 (*Ssp*) DnaE intein domains has allowed detailed examination of both universal and *Ssp* DnaE intein-specific steps in the protein splicing pathway. By engineering substitutions at both the +1 and penultimate intein positions, we have further characterized intein reaction kinetics in this system. Replacement of the crucial +1Cys with serine decreased N-terminal cleavage and trans-splicing rates; however, this substitution did not prevent splicing or the ability of $ZnCl_2$ to inhibit it. Substitution of the penultimate intein residue (alanine) with a typically conserved histidine did not increase the rate or extent of trans-splicing or cleavage under typical assay conditions. Despite the observation that this histidine aids in asparagine cyclization for other inteins, it did not encourage C-terminal cleavage for the *Ssp* DnaE intein or uncouple it from N-terminal cleavage. Both the +1Ser and Ala to His mutants were insensitive to $ZnCl_2$ during trans-cleavage experiments, uncoupling a previously linked inhibition in asparagine cyclization from an inhibition in trans-thioesterification detected for the wild-type intein.

Intein turnover has never previously been detected, presumably due to a high affinity between the two domains. Characterization of the N-terminal cleavage rates of the wild-type and mutant C-domain proteins allowed us to describe the first example of intein domain dissociation and rebinding via exploitation of an inactive N-domain competitor. A significant affinity was demonstrated between the wild-type C-domain and the competitor, and amino acid substitution, either at the +1 residue or at the penultimate intein position, reduced this affinity.

Inteins posttranslationally initiate their own excision from the surrounding protein sequence (*1*, *2*). This process results in ligation of the previously interrupted sequence into a single continuous polypeptide and the release of what was the *in*ternal pro*tein*, or *intein* (*3*). In addition to protein splicing, inteins can initiate peptide bond cleavage at both their N- and C-termini in the absence of a ligation event. To date, more than 170 inteins have been identified from all three branches of life (data compiled in InBase, the Intein Database, at New England Biolabs, Inc. (NEB), http://www.neb.com/neb/inteins.html) (*4*). A homing endonuclease domain can be found in the central region of many inteins and is thought to mediate lateral gene transfer (*5*, *6*). Removal of this domain does not appear to affect protein splicing activity (*7*), and a number of naturally occurring mini-inteins lacking this domain have been described.

From *Synechocystis* sp. PCC6803 (*Ssp*), a DNA coding for a mini-intein was found flanking a naturally split gene (located 745 kb apart in the genome) which encodes two domains of the DNA polymerase III subunit (extein) (*8*, *9*). Although direct evidence for intein splicing in *Synechocystis* sp. is lacking, no other coding sequence for the polymerase has been located in this genome. It has therefore been implied that a relatively high affinity between the two *Ssp* DnaE intein domains facilitates the assembly of the two extein domains and, upon splicing, generates the full-length polymerase posttranslationally.

Evidence suggesting a high affinity between the two *Ssp* DnaE intein domains can be found throughout intein literature. Both trans- and cis-splicing (fusion of the two domains to create one polypeptide sequence) have been demonstrated in vitro as well as in vivo (*Escherichia coli*) (*8*, *10*, *11*), suggesting that the added step of assembly in trans-splicing has a negligible effect on splicing activity. Immobilization of one domain of the intein via an affinity tag allows the other domain to be purified from an *E. coli* lysate (*12*). The *Ssp* DnaE intein domains have also been used as an affinity system to bring together two domains of a single reporter protein (*12*, *13*). Further, both a high affinity and slow dissociation rate relative to those of either cleavage or splicing reactions have been proposed to explain the lack of detectable turnover during intein reactions (*14*). A tight association between the two domains may also explain why they are resistant to denaturation during SDS−PAGE and can be detected as a single protein band with two N-termini (*15*). Despite the wealth of indirect evidence supporting a high affinity between the two domains, attempts to quantitate this association have not previously been reported.

Structures of various inteins have demonstrated that the two termini of an intein fold into close proximity, forming a single active site (*16*−*18*). Residues required for splicing activity are conserved and map mainly to this active site, at the junction of the intein/extein sequence. In addition, at the first C-extein position (the +1 residue), a nucleophilic residue (Ser/Thr/Cys) is required for splicing activity (*19*−*24*).

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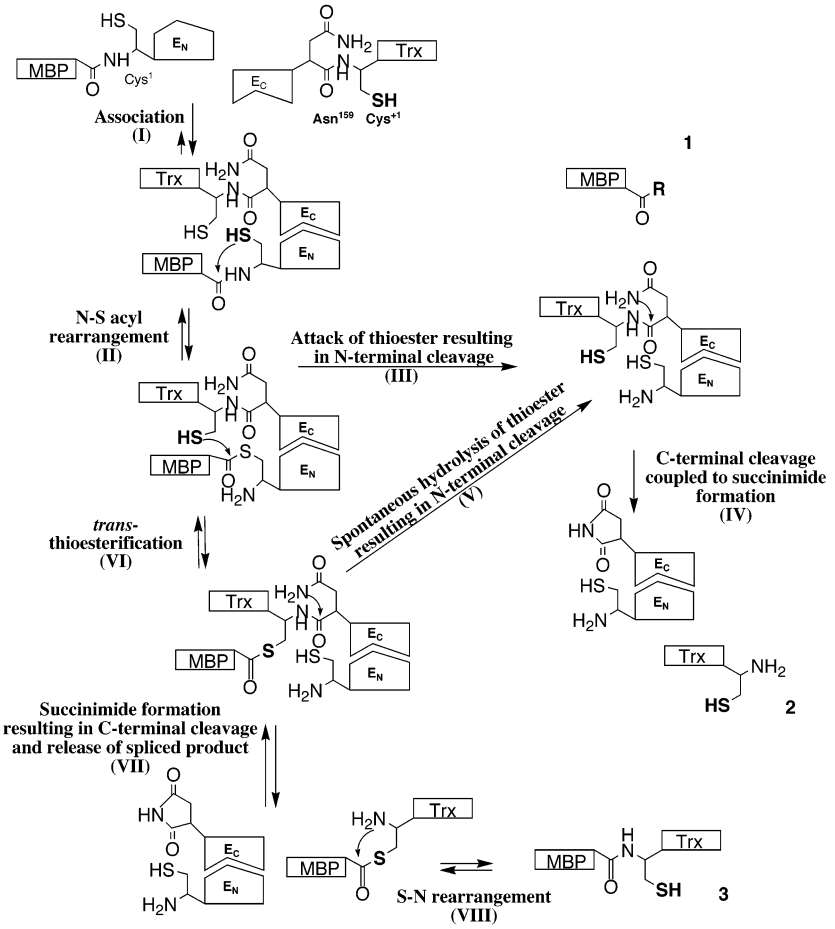


FIGURE 1: Mechanism of the naturally split *Ssp* DnaE intein trans-splicing and trans-cleavage reactions. Association of the two intein domains (I) has been demonstrated not to contribute to the overall rate of splicing and cleavage reactions. Attack by Cys1 results in a rearrangement of the N-terminal scissile bond (II), yielding a reactive thioester that can then be attacked by nucleophilic reagents. This leads to N-terminal cleavage (III), typically monitored by the appearance of species **1**. R is either $C_4H_9SO_2$ or OH depending on whether DTT or H_2O induces cleavage, respectively. Cyclization of the C-terminal asparagine residue (N159) results in C-terminal cleavage (IV), monitored by the appearance of species **2**. Alternatively, spontaneous trans-splicing occurs in vitro in the absence of an exogenous nucleophile. Attack of the N-terminal thioester by the +1 cysteine residue yields a branched thioester intermediate (VI). Cyclization of N159 liberates the associated extein domains from the intein halves (VII). In a step uncatalyzed by the intein, the ligated exteins undergo a final S−N rearrangement to yield a single polypeptide chain (VIII). Note that trans-cleavage does not involve extein ligation. Although spontaneous N-terminal cleavage can occur via hydrolysis of either the linear or branched thioester intermediates, only the major pathway (V) is shown.

Although a histidine residue, demonstrated to aid in asparagine cyclization (Figure 1, steps IV and VII; *23*, *25*), can be found as the penultimate residue of most inteins, this position is occupied by an alanine in the *Ssp* DnaE intein. Previous studies have suggested that a "reversion" to histidine can either increase or restore absent splicing activity in certain histidine-lacking inteins (*26*, *27*). However, previous work with this mutation in the *Ssp* DnaE intein has resulted in seemingly contradicting reports of both enhanced activity (*11*) and no effect upon substitution (*28*).

The many mutational studies of inteins have provided considerable insight into the mechanism of intein splicing and have identified the residues most critical for this activity. However, typical intein splicing and cleavage reactions are spontaneous processes both in vivo and in vitro, normally precluding the systematic study of intein reaction kinetics. Detailed kinetics studies (*14*, *15*) have been made possible by the ability not only to separately purify the two domains of the naturally split mini-intein but to subsequently reconstitute activity simply by mixing (*10*). Recently, another group has exploited a thermostable intein whose activity was suppressed during growth at low temperatures and subsequently triggered in vitro to detail its reaction kinetics (*29*). The usefulness of the naturally split *Ssp* DnaE intein system lies partially in the ability to quantitate the kinetics of intein reactions, providing, in a number of cases, an additional layer of insight into previously described qualitative observations. This investigation quantitatively examines the effects of altering two historically important amino acid positions in the *Ssp* DnaE intein system. In addition, we employ these mutants and the information gained from their analysis in a novel intein assay system to obtain information on the relative affinities of the N- and C-domains of both wild-type and mutant intein sequences. Previous work that employed the use of Zn^{2+} as an intein inhibitor (*15*, *28*, *30*) is also expanded to further our understanding of intein kinetics.

MATERIALS AND METHODS

Strains and Constructs. Plasmids pMEB2 and pMETXB1 have been described previously (*10*, *14*). pMEB2 encodes a three-part fusion protein consisting of the *E. coli* maltose binding protein (MBP, or M), the N-terminal domain of the *Ssp* DnaE intein (E_n) preceded by five native N-terminal extein residues (KFAEY), and the *Bacillus circulans* chitin

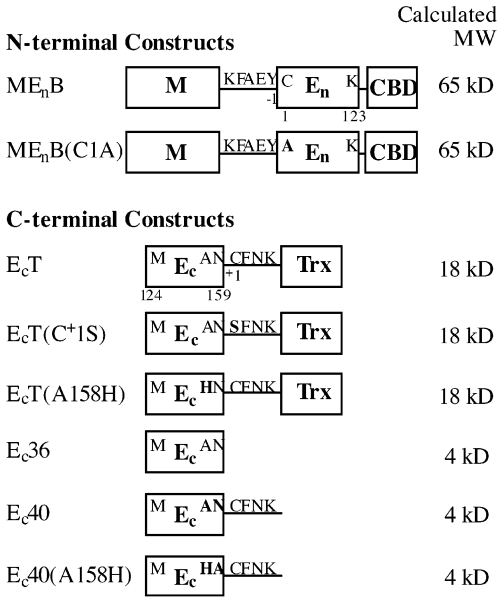


FIGURE 2: Diagram of designed fusion proteins and synthetic peptides. The N- and C-terminal domains of the *Ssp* DnaE intein (E_n and E_c) were purified separately as fusion proteins [ME_nB, ME_nB(C1A), E_cT, E_cT(C+1S), and E_cT(A158H)] or synthesized as peptides [E_c36, E_c40, and E_c40(A158H)] for use in experiments described herein. Thioredoxin (Trx) and the maltose-binding protein (MBP) of *E. coli* and the *B. circulans* chitin binding domain (CBD) flanked the intein N- or C-terminal domain. Native extein residues KFAEY and CFNK were included at the intein N- and C-termini, as indicated. Synthetic peptides were designed either to match the sequence of E_c with, or without, the four native C-extein residues or to incorporate both an Ala158His and an Asn159Ala mutation (the latter to block C-terminal cleavage). Amino acids in bold denote mutations from the native sequence.

binding domain (CBD, or B) all under the control of an IPTG[1]-inducible tac promoter. Cassette mutagenesis was used to mutate the first residue of the intein from cysteine to alanine, generating plasmid pME_nB*2A, which was verified by DNA sequencing (NEB). The notation B* signifies the presence of an elutable version of the chitin binding domain (*31*) which was added for studies outside the scope of this work. The plasmid pMETXB1 encodes a four-part fusion protein consisting of the *Ssp* DnaE intein C-terminal domain (E_c), four native extein residues (CFNK), *E. coli* thioredoxin (Trx, or T), the *Mycobacterium xenopi* GyrA (*Mxe* GyrA) intein containing an asparagine 198 to alanine substitution, and the CBD, all under the control of an IPTG-inducible T7 promoter. The codons for the penultimate residue of the intein (Ala158) or the first C-extein residue (+1) were mutated to encode histidine or serine by cassette mutagenesis to generate single-point mutants E_cT(A158H) and E_cT(C+1S), respectively. The resulting plasmids, pMETXB1PH and pMETXB1S, were verified by DNA sequencing (NEB). *E. coli* strains ER1992 and ER2566 (*15*) were used for plasmid and protein purifications, respectively.

Protein Purification. The fusion protein ME_nB (Figure 2) was expressed and purified from the pMEB2 plasmid as reported previously (*14*) by elution from an amylose column equilibrated in buffer A (20 mM Tris, pH 7.0, 500 mM NaCl) by addition of buffer B (20 mM Tris, pH 7.0, 500 mM NaCl, 10 mM maltose). The inactive mutant ME_nB(C1A) was purified similarly. The E_cT fusion protein (Figure 2) was purified as published previously from the pMETXB1 plasmid (*14*) by elution from a chitin column equilibrated in buffer C (20 mM Tris, pH 8.0, 500 mM NaCl) by addition of buffer D (20 mM Tris, pH 8.0, 500 mM NaCl, 30 mM DTT), which caused the *Mxe* GyrA intein to cleave at its N-terminus, releasing the two-part fusion E_cT. After purification, E_cT was dialyzed against buffer C to remove DTT and concentrated using an Amicon Centriprep 10 (Millipore Corp.). Purification of E_cT mutant proteins [E_cT(C+1S) and E_cT(A158H)] was conducted similarly. A Bradford assay using BSA as a standard was employed to determine protein concentrations (*32*). Purified samples were aliquoted, stored at −80 °C, and used immediately after being thawed at 4 °C. All buffers and stock solutions used for experiments described herein were prepared with ultrapure MilliQ water.

Peptides representing the wild-type and mutant C-terminal domains of the *Ssp* DnaE intein (Figure 2) were synthesized (NEB) as described previously (*33*) as 36-mers without the extein sequence (E_c36) or as 40-mers containing the four native extein residues, CFNK [E_c40 and E_c40(A158H)]. Stocks of 10 mg/mL peptide were aliquoted, frozen at −20 °C, and used immediately after being thawed at room temperature.

N- and C-Terminal Trans-Cleavage Reactions. Trans-cleavage reactions were conducted on the basis of a method described previously (*14*, *15*). Specifically, fusion proteins ME_nB and E_cT (wild-type or mutant sequences) were incubated in buffer A at room temperature at a 1:3 ratio with excess E_cT protein or E_c peptide (36 μM). Nucleophilic attack (Figure 1, step III) was typically encouraged by the addition of 50 mM DTT. In some experiments, DTT was replaced by 0.5 M HA (pH 7.0) to verify that potential Zn^{2+}−DTT interactions did not affect analysis. Addition of nucleophile to the reaction mixture served as the start of the trans-cleavage time courses. Aliquots of the reaction mixture were removed at intervals up to 24 h later and quenched by dilution into 1/3 volume of 3X SDS sample buffer containing DTT (NEB). Quenched samples were incubated for 30 min at room temperature before being transferred to 4 °C to await electrophoresis. Where included, $ZnCl_2$ was added to the reactions immediately prior to nucleophile addition, unless noted otherwise.

Trans-cleavage experiments were also conducted with substoichiometric amounts of C-terminal peptide (9 μM) to N-terminal protein (24 μM) for 48 h at room temperature or 37 °C in buffer A with 50 mM DTT to investigate the potential for the peptide to induce N-terminal cleavage from more than one N-domain, i.e., reaction turnover. Additionally, the C-terminal peptides, E_c36, E_c40, and E_c40(A158H), were incubated with the wild-type N-domain, ME_nB, for 24 h. The reaction mixture was divided into two halves, and additional ME_nB (18 μM) was added to one of the halves while the inactive N-domain, ME_nB(C1A) (18 μM), was added to the second half. The mixtures were then allowed to incubate for an additional overnight period. Similar experiments were conducted in which the E_c36 peptide was incubated initially with the inactive N-domain for 24 h before the experiment was split and continued as above. Samples taken during these time courses were treated as for typical cleavage experiments prior to electrophoresis.

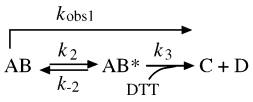
[1] Abbreviations: DTT, 1,4-dithio-DL-threitol; IPTG, isopropyl β-D-thiogalactopyranoside; LB, Luria−Burtani broth; PVDF, poly(vinylidene fluoride); HA, hydroxylamine hydrochloride.

Relative Affinity/Replacement Assays. To assess the relative affinities of the various C-terminal proteins for the N-terminal domain of the intein, competition assays were conducted. C-terminal protein E_cT, E_cT(A158H), or E_cT(C+1S) (1.2 nmol) was incubated for either 2 or 4 h with the inactive N-terminal mutant ME_nB(C1A) (1.5 nmol) prior to addition of 2.2 nmol of ME_nB protein. To substantially minimize the effects of the various E_cT (wild-type or mutant)/ME_nB(C1A) ratios on rates, excess ME_nB(C1A) was used and equilibrium between the two species was established. Where applicable, 0.5 or 1.0 mM $ZnCl_2$ was added either at the beginning or at the end of the incubation. Time points were taken at the beginning and at the end of the incubation and then up to 24 h after the addition of ME_nB.

In Vitro Trans-Splicing Reactions. Reactions designed to study trans-splicing were conducted by incubation of wild-type or mutant ME_nB and E_cT fusion proteins at room temperature in buffer A at a 1:3 molar protein ratio (12:36 μM) in the absence of DTT or other exogenous nucleophiles. Addition of the C-terminal intein domain to the reaction mixture containing the N-domain signaled the start of the time course. Trans-splicing was typically monitored for 24 h. Samples were quenched and treated as described for trans-cleavage experiments.

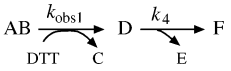
Data Acquisition. Samples from trans-splicing or trans-cleavage reactions were equilibrated to room temperature before electrophoresis on Novex 10−20% tricine gels (Invitrogen). Coomassie Blue R250 staining was used for protein band visualization. Wild-type or mutant ME_nB and E_cT starting materials migrated with an apparent mass of 65 and 18 kDa, respectively, and could be easily distinguished from both trans-cleavage and trans-splicing products. N- and C-terminal trans-cleavage products, M and T, migrated with mobilities of approximately 43 and 12 kDa, respectively. The trans-splicing product, MT, was visible at ~57 kDa. Both protein sequencing and Western blot analysis were used to verify band identities. Quantitation of protein bands was achieved by digitization of the gels on a flatbed scanner (Microtek) followed by densitometric analysis conducted with NIH Image software (http://rsb.info.nih.gov/nih-image/).

Kinetic Analysis of Trans-Cleavage and Trans-Splicing. The mechanism of intein splicing and cleavage has been described elsewhere (*2*, *23*, *24*, *34*) and occurs via two related pathways (Figure 1). For the *Ssp* DnaE intein, the first step in either pathway involves association of the two halves, a step that has been demonstrated previously to be rapid with respect to the overall reaction (*14*, *15*). Procedures designed for the analysis of the trans-cleavage and -splicing reactions have been described in detail elsewhere (*14*, *15*). The rate of N-terminal cleavage (k_{obs1}) was measured by the formation of free maltose binding protein as described by eq 1.



(1)

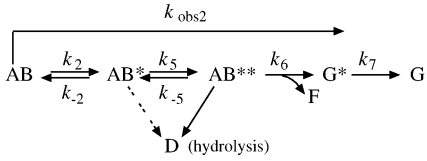
AB is the associated intein fusion complex (ME_nB:E_cT), AB* is the linear thioester intermediate, C is the N-extein, maltose-binding protein (M), and D is the associated complex of E_nB:E_cT, as identified in Figure 1. For the *Ssp* DnaE intein, C-terminal cleavage has never been reported in the absence of N-terminal cleavage, although this phenomenon has been described for other inteins (*23*, *24*). Therefore, the dependence of C-terminal cleavage on upstream reactions was described in terms of the observed rate of N-terminal cleavage, k_{obs1}, plus the additional step of asparagine cyclization (Figure 1, step IV, and eq 2) as described previously (*14*, *15*).



(2)

E is the C-extein (T), and F is the associated intein complex (E_nB:E_c). The rate of trans-splicing, k_{obs2}, was determined by the appearance of the spliced product MT (eq 3).

AB** is the branched thioester intermediate, and G* and G represent the spliced product MT before and after S−N rearrangement, respectively, as identified in Figure 1.



(3)

The processes of both N-terminal cleavage and trans-splicing can be treated as first-order, irreversible, pre-steady-state reactions and therefore fit to a simple exponential model (Igor Pro, Wavemetrics) described as $P = P_0(1 - e^{-kt})$, where P is the fraction of cleaved or spliced product (M or MT, respectively), P_0 is the maximum product formed, k is the observed rate (s^{-1}), and t is time (s) (*14*, *15*). P_0 was allowed to float during fitting routines. For individual N-terminal trans-cleavage experiments, the fraction of cleaved product, P, was derived from the expression $M_n - M_0$ for each given time point, n, where M_0 represents the quantity of M present at the beginning of the reaction. The extent of trans-cleavage was calculated relative to the amount of starting material ME_nB as M_f/ME_nB_0. For trans-splicing experiments the fraction of spliced product (MT) was calculated by the expression $MT_n - MT_0$. The extent of trans-splicing was calculated relative to the amount of starting material ME_nB as MT_f/ME_nB_0.

The amount of C-terminal cleavage product T was derived from the expression $T_n - T_0$. The extent of C-terminal cleavage was calculated relative to the amount of starting material E_cT, as T_f/E_cT_0. Data from trans-splicing or -cleavage experiments (number of replicates, $n \geq 3$) were fitted and normalized on the basis of the value for P_0 determined by the fitting routine for each individual experiment and averaged to yield the average fraction of product formed, R, for each experimental condition.

N-Terminal Sequencing. For N-terminal sequencing, protein bands electrophoresed on Novex 10−20% tricine gels were subsequently transferred to a PVDF membrane (Problott, Applied Biosystems Inc.) according to the procedure of Matsudaira (*35*), with previously described modifications (*36*). Desired bands were excised from the surrounding membrane after staining with Coomassie Blue R250, and subjected to sequential degradation on a Procise 494 protein/peptide sequencer (Applied Biosystems) (*36*).

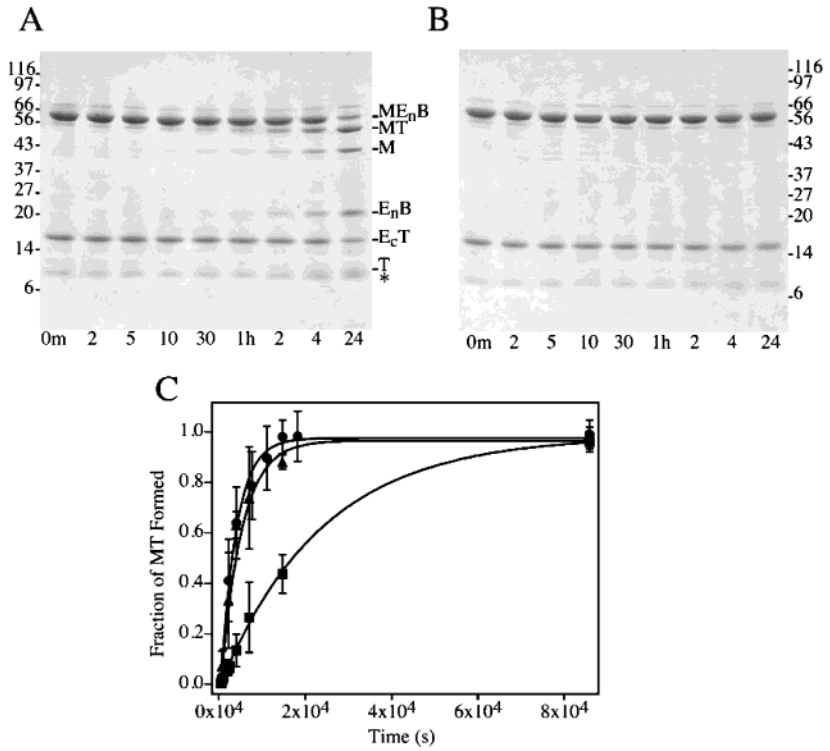


FIGURE 3: Effect of C-terminal substitutions on trans-splicing activity. Trans-splicing experiments were conducted with 12 μM ME_nB and 36 μM E_cT(C+1S) proteins in buffer A in the absence (A) or presence (B) of 1 mM $ZnCl_2$. Samples taken at the times indicated on the bottom of each lane were quenched and electrophoresed on a 10−20% tricine gel. Coomassie Blue staining was used for visualization. Marker molar masses are expressed in kilodaltons. Abbreviations note the starting materials (ME_nB and E_cT), spliced product (MT), and trans-cleavage products (M, E_nB, and T). The asterisk denotes a protein contaminant. (C) Protein gels from wild-type (●), A158H (▲), and +1Ser (■) containing trans-splicing experiments [including those pictured in (A) and (B)] were quantitated (NIH Image), and the fraction of MT formed was plotted vs time. Data ($n \geq 3$) were fitted (solid lines, Igor Pro), and vertical bars represent the error in each data point.

RESULTS

Effects of Serine Substitution at the +1 Extein Residue on Trans-Splicing and -Cleavage. For the *Ssp* DnaE intein, a cysteine residue at the first C-extein position is responsible for nucleophilic attack on the linear thioester to form the branched intermediate (Figure 1, step VI) (*19*, *21*−*24*, *37*, *38*). For a number of inteins, it is a +1 serine that serves as the nucleophile for this step of the reaction (*4*). Serine substitution for the native +1Cys of the *Ssp* DnaE intein results in a 7-fold reduction in the rate of trans-splicing (Figure 3) but in an identical maximum extent of spliced product formed compared to those of the native domain (Table 1). Trans-splicing and spontaneous hydrolysis with the serine mutant are abolished by the addition of 1 mM $ZnCl_2$ (Figure 3B), consistent with the hypothesis that Zn^{2+} inhibition requires the presence of an amino acid capable of metal chelation at the +1 position (*15*, *30*).

The rate of N-terminal trans-cleavage of the C+1S mutant was decreased relative to that detected from native reactions (Figure 4). The magnitude of the rate decrease is similar to that reported previously for a much less conservative +1Val mutation (*15*) as well as for a +1Thr substitution (data not shown), suggesting that a universal complexity such as proper folding or active site assembly may be at the root of this effect. In contrast to C+1S splicing, the N-terminal cleavage rate was unaffected by the addition of $ZnCl_2$ (Figure 4B). C-terminal cleavage rates in the absence and presence of $ZnCl_2$ were also similar [$k_{obs3} = (0.10 \pm 0.03) \times 10^{-3}$ s^{-1}, extent 50 ± 17%; $k_{obs3,Zn^{2+}} = (0.12 \pm 0.02) \times 10^{-3}$ s^{-1}, extent 39 ± 11%], unlike those measured for the wild-type cysteine, which demonstrated a dramatic inhibition by the metal ion (*15*).

Table 1: Observed Rates and Extents of Reaction for N-Terminal Trans-Cleavage and Trans-Splicing of the *Ssp* DnaE Intein[a]

N-Terminal Trans-Cleavage[b]

protein domains	k_{obs} (10^{-3} s^{-1})	extent (%)
ME_nB/E_cT	1.6 ± 0.10	78 ± 11
ME_nB/E_cT (+ 1 mM $ZnCl_2$)	0.54 ± 0.09	69 ± 14
ME_nB/E_cT(C+1S)	0.19 ± 0.07	69 ± 10
ME_nB/E_cT(C+1S) (+ 1 mM $ZnCl_2$)	0.19 ± 0.06	75 ± 0.6
ME_nB/E_cT(A158H)	0.58 ± 0.06	78 ± 2.1
ME_nB/E_cT(A158H) (+ 1 mM $ZnCl_2$)	0.08 ± 0.04	53 ± 18

Trans-Splicing[c]

protein domains	k_{obs} (10^{-4} s^{-1})	extent (%)
ME_nB/E_cT	3.0 ± 0.4	54 ± 3.6
ME_nB/E_cT(C+1S)	0.42 ± 0.16	54 ± 8.3
ME_nB/E_cT(A158H)	2.2 ± 1.1	42 ± 16

[a] Observed rates and extents of reaction were calculated as described in the Materials and Methods. [b] N-terminal cleavage time courses commenced upon the addition of 50 mM DTT to mixtures containing 12 μM N-domain and 36 μM C-domain. Where indicated, $ZnCl_2$ was added immediately prior to DTT addition. [c] Trans-splicing time courses commenced upon the addition of the C-domain (12 μM) to the N-domain (36 μM).

Effect of Penultimate Residue Substitution from Alanine to Histidine on Intein Activity. Although a histidine residue at the penultimate intein position is one of the few highly conserved intein residues, the naturally split *Ssp* DnaE intein

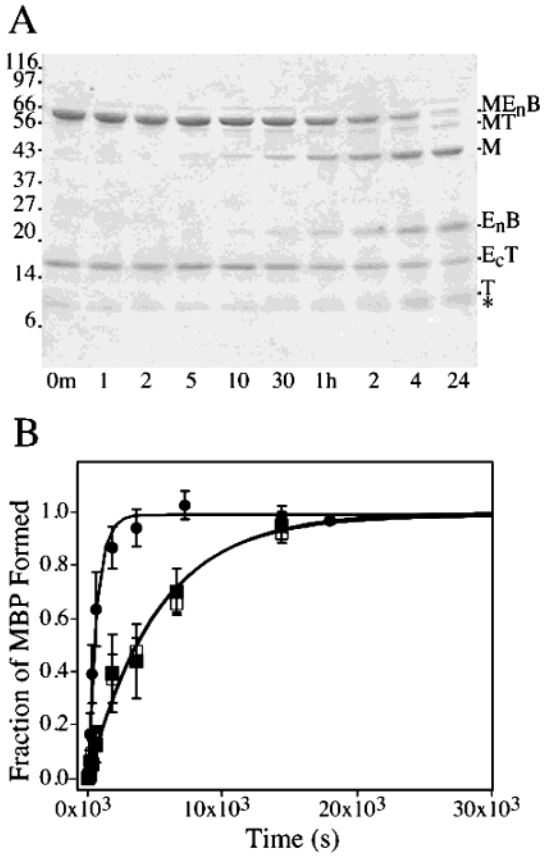


FIGURE 4: Effect of +1 substitution on trans-cleavage activity. Trans-cleavage experiments were conducted with 12 μM ME_nB and 36 μM E_cT(C+1S) proteins in buffer A supplemented with 50 mM DTT. (A) Samples from experiments lacking $ZnCl_2$ were quenched at the times indicated on the bottom of each lane and electrophoresed on a 10−20% tricine gel prior to Coomassie Blue staining. Standard molar masses are expressed in kilodaltons, and abbreviations are as in Figure 3. (B) Protein gels from +1Ser experiments with (□) and without (■) $ZnCl_2$ [including the one pictured in (A)] were quantitated, and N-terminal cleavage was expressed as the fraction of MBP formed vs time. Wild-type trans-cleavage data (●) are shown for comparison. Data ($n \geq 3$) were fitted (solid lines), and vertical bars represent the error in each given data point.

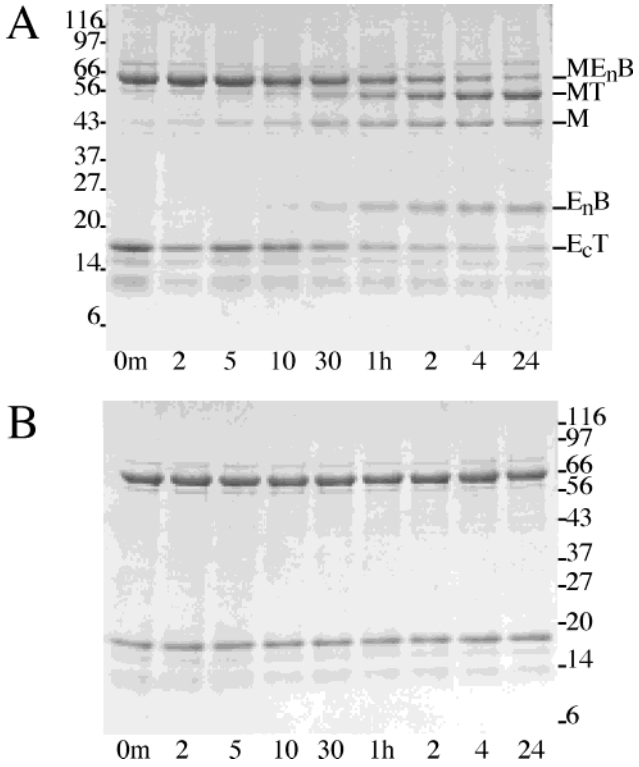


FIGURE 5: Trans-splicing reactions conducted with the A158H mutant C-terminal domain. Trans-splicing experiments were conducted with 12 μM ME_nB and 36 μM E_cT(A158H) proteins in buffer A in the absence (A) or presence (B) of 1 mM $ZnCl_2$. Samples taken from representative time courses were quenched and electrophoresed on a 10−20% tricine gel prior to Coomassie Blue staining. Standard molar masses are expressed in kilodaltons, and abbreviations are as in Figure 3. Small molecular mass bands are due to a contaminant in the E_cT(A158H) protein preparation.

has an alanine at this position (Ala158). Despite this fact, the wild-type intein splices moderately well in the MBP/Trx extein system (*14*, *15*). Substitution of the penultimate alanine with a histidine does not appear to enhance this activity under these assay conditions (Figures 3C and 5, Table 1). The addition of $ZnCl_2$ to the A158H splicing reactions resulted in total inhibition of both splicing and spontaneous hydrolysis, similar to reactions with the wild-type E_cT/ME_nB proteins. Modulation of buffer pH during trans-splicing experiments illuminated minor differences between the trans-splicing and spontaneous hydrolysis reactions of the wild-type and the A158H mutant domains. Whereas trans-splicing rates were similar for both proteins (Figure 6A), rates of spontaneous hydrolysis were faster for the wild-type domains at pH ≥ 8 (Figure 6B). The maximum extent of E_cT(A158H) splicing occurs at pH 6, whereas the wild-type domains experience a maximum at pH 7 (Figure 6C). The extent of hydrolysis appears not to vary as a function of pH for the wild-type domains but is decreased at both high and low pH for the mutant (Figure 6D). It is important to note, particularly when using inteins as purification or engineering tools, that optimal conditions for yielding the greatest extent of a given product do not always coincide with those conditions that display the fastest reaction rates.

N-terminal cleavage rates for the A158H mutant were decreased compared to those of the wild-type protein reactions only by approximately 2-fold (Table 1, Figure 7). The addition of $ZnCl_2$ resulted in a 7-fold reduction in the rate of N-terminal cleavage, greater than the 3-fold inhibition reported for the wild-type domains (Figure 7B) (*15*). Although difficult to rigorously quantitate due to the presence of small molecular mass contaminants, C-terminal cleavage rates of the A158H mutant appeared unaffected by the presence of the metal inhibitor (data not shown).

Trans-cleavage experiments were conducted with the E_cT(A158H) mutant and an N-terminal domain incapable of undergoing an N−S acyl shift [ME_nB(C1A)] (*20*, *23*) to examine whether "reversion" to a typically conserved penultimate histidine would allow the *Ssp* DnaE intein to undergo C-terminal cleavage in the absence of N-terminal cleavage as has been detected for other inteins (*23*, *25*). Although no C-terminal cleavage was observed, the appearance of a large molecular mass protein band (~92 kDa apparent molar mass) was detected at the later points of the experiment (data not shown). N-terminal protein sequencing identified this band as containing a sequence matching the N-termini of both ME_nB(C1A) and E_cT(A158H) proteins. During previous experiments with the ME_nB/E_cT domains, we were unable to routinely detect this full-length complex, presum-

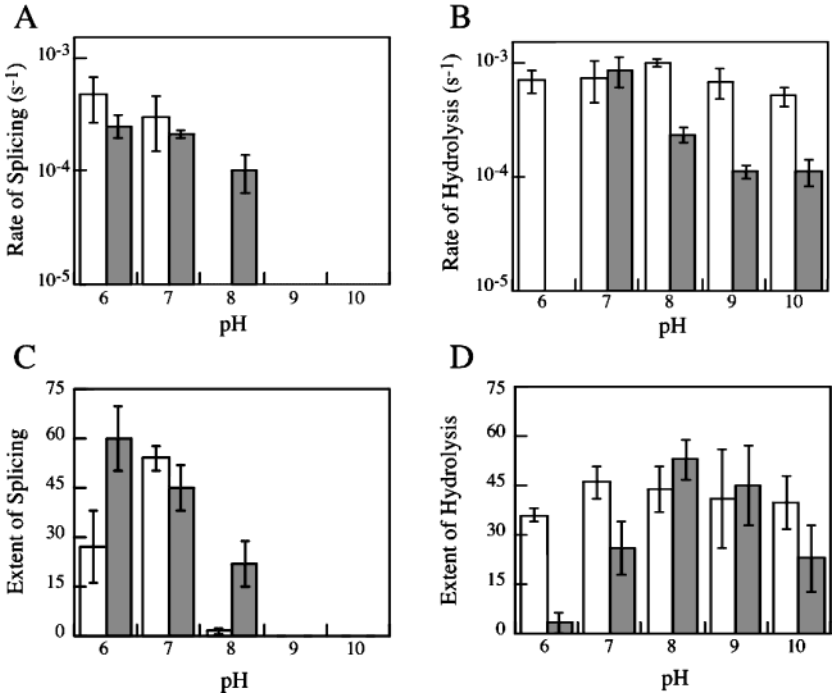


FIGURE 6: Rates and extents of splicing and spontaneous hydrolysis reactions. Trans-splicing experiments were conducted with 12 μM ME_nB and 36 μM E_cT (empty bars) or E_cT(A158H) (filled bars) proteins as a function of pH. Data were compiled from multiple experiments ($n \geq 3$) and expressed to describe the rates of splicing (A) and hydrolysis (B) as described in the Materials and Methods. The extent of product formed was expressed as the amount of spliced or cleaved product, MT or M, as a fraction of starting material ME_nB for trans-splicing (C) and spontaneous hydrolysis (D), respectively. The wild-type trans-splicing rate at pH 8 and the A158H rate of hydrolysis at pH 6 were not calculable due to the extremely low amount of product formed. Elsewhere, the lack of a bar in a given column indicates a value of 0, and error bars represent 1 standard deviation.

ably due to the lack of complex buildup when the splicing/cleavage pathways are allowed to progress (*15*). Current experiments employing the wild-type C-domain with the inactive N-domain indeed revealed this protein band on SDS−PAGE, consistent with this hypothesis (data not shown).

Competition of ME_nB(C1A) with ME_nB. Although often regarded as enzymes, inteins differ from classical enzymes in a number of ways, including the lack of detectable turnover. The regeneration of starting material or catalyst also appears impossible due to the nature of intein function (*39*). For the *Ssp* DnaE intein, the lack of detectable turnover has been suggested to arise from a relatively high affinity between the two domains, allowing little or no dissociation during a time frame relevant to the splicing and cleavage reactions. Various experiments designed to detect whether peptide E_c36 could induce N-terminal cleavage from more than one ME_nB molecule, including those conducted at increased temperature, provided no evidence of turnover (data not shown), similar to reports published previously (*14*). Additionally, no turnover was detected during experiments with either 40-mer peptide [E_c40 or E_c40(A158H)]. We therefore examined whether it was possible to detect intein dissociation via the use of the inactive N-terminal mutant ME_nB(C1A). Substoichiometric amounts of the C-domain peptide E_c were allowed to interact with ME_nB(C1A) for 24 h in the presence of DTT before the active N-domain ME_nB was added to the mixture (Figure 8A). After an additional overnight incubation, the presence of a protein band representing the N-terminal cleavage product MBP was visible, demonstrating the ability of ME_nB to compete with ME_nB(C1A) for the E_c peptide. The MBP band was detected only in the late time points of the reaction, supporting a slow off rate and/or high affinity of the E_c:ME_nB(C1A) complex. Adding more ME_nB(C1A) to the E_c/ME_nB(C1A) reaction mixture did not result in any detectable change, as expected (data not shown). The complementary experiment, in which substoichiometric amounts of E_c peptide were incubated with ME_nB for 24 h prior to dividing the reaction in half and adding either additional ME_nB or the inactive fragment ME_nB(C1A), verified that, once a small amount of N-terminal cleavage occurred (approximately equal to the concentration of the limiting fragment), no additional cleavage was detected (Figure 8B). These observations demonstrate the ability of the C-fragment to bind the inactive N-domain, dissociate, and rebind a second N-domain but do not provide evidence to support reaction turnover in the classical sense.

Examination of Relative Affinities via Replacement Reactions. Given the ability of wild-type ME_nB to compete with the inactive N-fragment ME_nB(C1A) for the C-terminal peptide and our understanding of N-terminal cleavage kinetics for wild-type and two C-domain mutants, we expanded the previous experiments to examine how C-terminal substitutions affect the affinity of the intein domains. Limiting amounts of C-domain [E_cT, E_cT(A158H), or E_cT(C+1S)] were allowed to interact with the inactive N-terminal domain ME_nB(C1A) in the presence of DTT for 2 h prior to the addition of excess wild-type N-domain, ME_nB. Samples were taken at the start of the experiment, and upon ME_nB addition, a time course was started (Figure 8C). The rate of MBP accumulation was monitored via SDS−PAGE as for typical trans-cleavage experiments. The rate of MBP appearance in the presence of the competitive inhibitor, ME_nB(C1A),

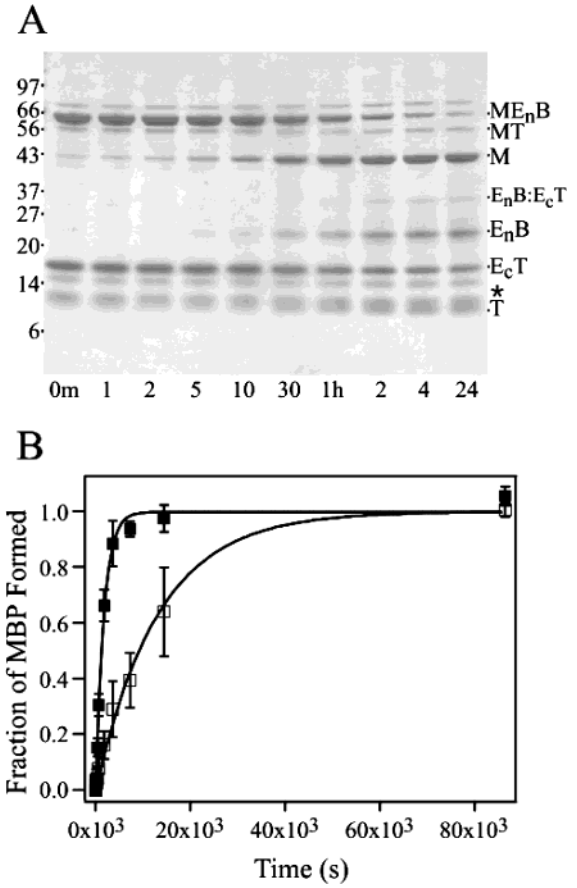


FIGURE 7: Effect of the A158H mutation on trans-cleavage. Trans-cleavage experiments were conducted with ME_nB and E_cT(A158H) proteins in buffer A in the presence of 50 mM DTT. (A) Samples were taken at the times indicated below each lane and quenched prior to electrophoresis on a 10−20% tricine gel. Coomassie Blue staining was used for visualization. Standard molar masses are expressed in kilodaltons, and abbreviations are as in Figure 3, with E_nB:E_cT representing the associated two-domain intein complex. Small molecular mass bands just above and below the asterisk represent the presence of a protein contaminant. (B) Data from A158H time course replicates [including that pictured in (A)] conducted in the presence (□) or absence (■) of 1 mM $ZnCl_2$ were compiled and expressed as the fraction of MBP formed vs time for N-terminal cleavage. Data ($n \geq 3$) were fitted (solid lines), and vertical bars represent the error in each given data point.

encompasses within it the affinity of the E_cT:ME_nB(C1A) complex as well as the rate of N-terminal cleavage. The previous determination of N-terminal cleavage rates for reactions with the wild-type and C-domain mutant proteins (Table 1) allowed a comparison to be made between the rates of MBP formation from experiments with and without the inhibitor. Because inherent effects of each mutation on the N-terminal cleavage activity can be taken into account in the comparison, these data report on the relative affinities of the C-terminal domains to the mutant N-terminal domain. For E_cT replacement experiments, a 30-fold reduction was seen in the rate of observed N-terminal cleavage compared to that of experiments conducted in the absence of ME_nB(C1A) (Figure 8D). The decreases in the rate of MBP formation for both the E_cT(A158H) and E_cT(C+1S) mutants were 10- and 9-fold, [E_cT(A158H) $k_{obs1,C} = (5.7 \pm 0.9) \times 10^{-5}$ s^{-1}; E_cT(C+1S) $k_{obs1,C} = (2.1 \pm 0.4) \times 10^{-5}$ s^{-1}], respectively, when compared to those of experiments lacking the competitor (compare to Table 1). These results suggest that both mutants display a decreased affinity for the mutant N-terminal domain compared to the native sequence.

As affinity and activity could now be separately examined, we employed the use of $ZnCl_2$ to examine whether Zn^{2+} inhibition occurred in part by reducing the affinity of the two intein domains for each other or solely through inhibition of the cleavage reaction steps downstream of association. An additional 3.5-fold decrease in the rate of MBP formation [beyond that already noted above; $k_{obs1,Zn^{2+}} = (1.7 \pm 0.6) \times 10^{-5}$ s^{-1}] was observed only for experiments employing the E_cT(A158H) mutant C-domain (for a total 34-fold inhibition over Zn^{2+}-free nonreplacement reactions, Figure 8D). It is interesting to note that, although $ZnCl_2$ was able to inhibit N-terminal cleavage in wild-type ME_nB/E_cT reactions (Table 1), the metal had no apparent inhibitory effect in the corresponding replacement reactions (Figure 8D). Experiments were also conducted in which Zn^{2+} was added at the end of the equilibrium incubation (concurrent with ME_nB addition, instead of at the beginning of the assay). Differences between experiments for which Zn^{2+} was added at the beginning of the reaction and at the end of the incubation period were seen only for assays conducted with the A158H C-domain. In this case, an additional 5-fold reduction in MBP formation was detected for the latter condition (data not shown). Reactions to which $ZnCl_2$ was added at 0.5 mM did not differ from those conducted at 1 mM $ZnCl_2$ for any protein (data not shown). Incubations up to 4 h (with or without $ZnCl_2$) were conducted to ensure that equilibrium had been reached prior to ME_nB addition. These studies yielded results identical to those of experiments employing a 2 h equilibrium (data not shown).

DISCUSSION

This work has expanded upon previous quantitative characterizations of the naturally split *Ssp* DnaE intein by examining substitutions at both the penultimate intein residue and the first C-extein position (+1 residue). The specific characterization of trans-cleavage rates has allowed subsequent investigations into the relative affinities of mutant and wild-type C-domains for the N-terminal intein domain. A cysteine to serine substitution at the +1 residue, which is responsible for forming the branched intermediate (*37*), had a moderate effect on the splicing kinetics of the naturally split intein but did not prevent splicing. A serine occurs naturally at the +1 position of a number of functional inteins, yet its substitution for a wild-type cysteine resulted in a significant decrease in the amount of spliced product formed for the *Sce* VMA intein (*21*, *24*, *40*). Although *Ssp* DnaE intein splicing was slowed by the substitution, the extent of spliced product achieved was the same, demonstrating how a classical examination of overnight splicing extents or on-column splicing (*40*) would not have been sufficient to quantitate the effect on this activity.

Previously, substitution of the *Ssp* DnaE intein +1Cys to an amino acid unable to chelate Zn^{2+} resulted in the abrogation of Zn^{2+}-mediated inhibition, consistent with the concept that the *Ssp* DnaE intein +1Cys participates in Zn^{2+} chelation (*15*, *30*). Because substitution of the +1Cys to Ser provides a side chain capable of binding Zn^{2+} and this protein cannot undergo splicing in the presence of the metal ion, Zn^{2+}-mediated inhibition does not depend solely on the

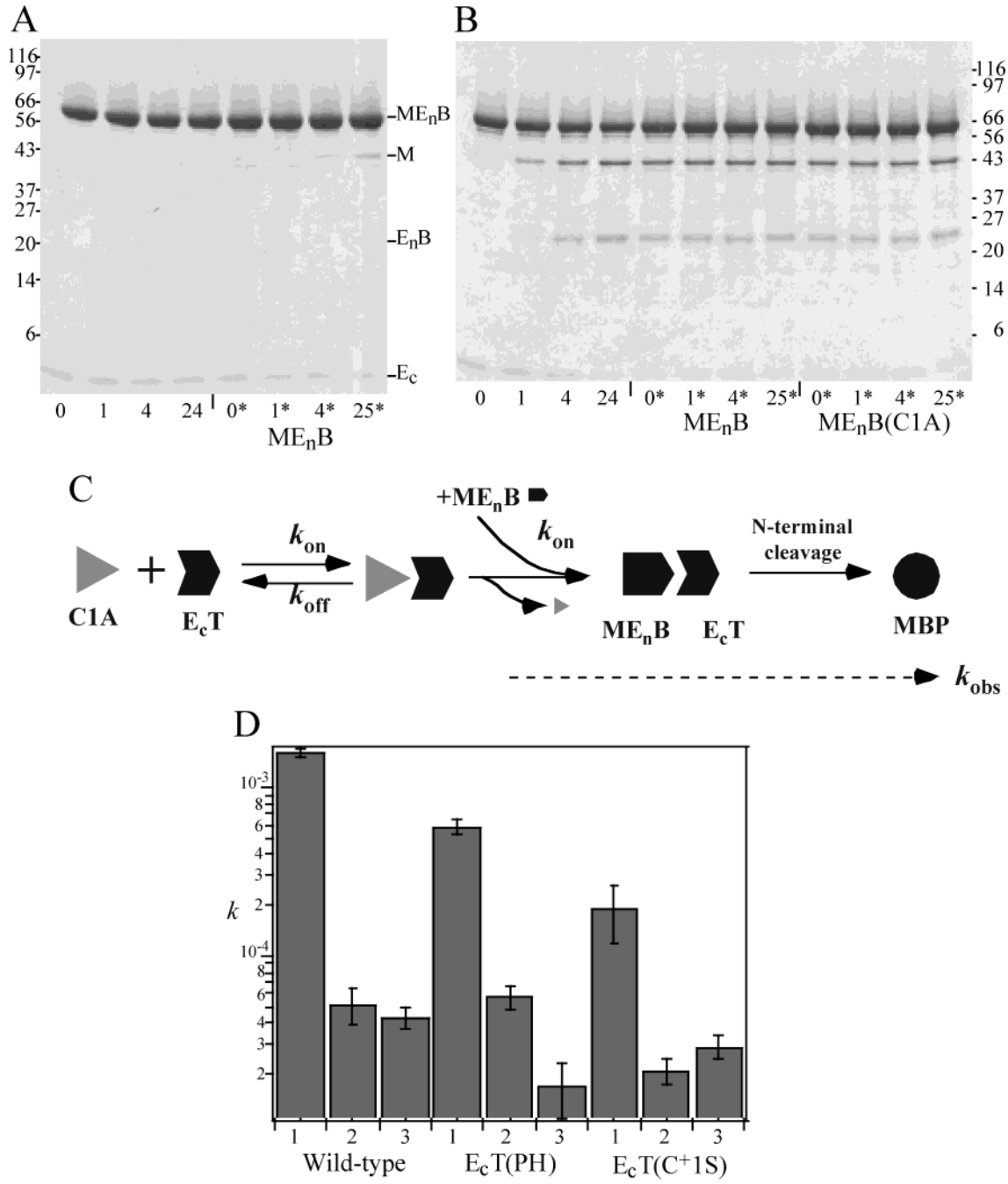


FIGURE 8: Turnover and replacement/competition reactions. E_c peptide was incubated initially either with the inactive N-fragment $ME_nB(C1A)$ (A) or with the wild-type fragment (B) for 24 h in the presence of DTT. The reaction mixtures were split into two halves, to which either ME_nB or $ME_nB(C1A)$ was added for another overnight incubation. Samples were taken at the times indicated at the bottom of each lane to monitor N-terminal cleavage. An asterisk indicates time (h) passed after the initial 24 h incubation. (C) Cartoon of replacement experiments. The inactive N-fragment [$ME_nB(C1A)$] was preincubated with a C-domain protein, and the mixture was allowed to come to equilibrium prior to the addition of ME_nB. The rate of MBP appearance takes into account the on rate for the C-domain:ME_nB complex. (D) Replacement experiments were conducted by preincubation of 1.5 nmol of $ME_nB(C1A)$ and 1.2 nmol of E_cT wild-type or mutant proteins in the presence of DTT for 2 h in the absence (lanes labeled 2) or presence (lanes labeled 3) of 1 mM $ZnCl_2$ prior to the addition of 2.2 nmol of ME_nB. Samples were then taken from the reaction mixture, and the results were quantitated to express the observed rate of MBP formation (k, s^{-1}) as for typical cleavage experiments. Lanes labeled 1 represent the rate of N-terminal cleavage for ME_nB and the C-domain listed below measured from typical trans-cleavage experiments [conducted in the absence of competitor $ME_nB(C1A)$] for comparison.

presence of a sulfhydryl at the first C-extein position. The split intein active site appears to be able to accommodate the substitution and maintain the interactions required for splicing inhibition. The +1Ser-substituted protein, however, is not subject to the Zn^{2+}-mediated inhibition of DTT-induced N-terminal cleavage detected for the +1Cys protein. This inhibition of the wild-type intein is not dramatic (3-fold) and may be relieved in the Cys+1Ser mutant by a decreased ability of serine to chelate the metal ion. In either the presence or absence of Zn^{2+}, the rate of N-terminal cleavage with the +1Ser is reduced by 10-fold compared to that of the wild-type domains. This effect may result from a general phenomenon upon +1 substitution as both a valine and threonine mutant behave similarly (*15*).

X-ray crystallographic studies of the *Sce* VMA intein documented that a Zn^{2+} molecule was bound to the +1Cys, the penultimate residue (histidine), a glutamate from the N-terminal intein domain (Glu80), and a water molecule (*17*). In the *Mtu* RecA intein (which also contains a His at its penultimate position), Zn^{2+}-mediated cleavage inhibition was reported despite substitution of the +1Cys to Ala (*28*), suggesting that multivalent chelation could be a general hallmark of intein−Zn^{2+} interactions. However, substitution of the +1Cys to a nonchelating residue such as Val or Thr abolishes the Zn^{2+} effect in the *Ssp* DnaE intein. The naturally split intein lacks a His at the penultimate position and contains an Arg at the position comparable to Glu80; therefore, other residues may serve to accommodate the metal ion in this intein. Examination of the chelating role of the histidine by substitution of Ala158 to His did result in an altered Zn^{2+} inhibition profile for the split intein. Interestingly, instead of a simple increase in inhibition upon the addition of a potential chelator, both an increase and a decrease could be detected, depending on the reaction step being monitored. Although A158H splicing was still entirely inhibited by Zn^{2+}, C-terminal cleavage inhibition appeared to be relieved. In addition, the magnitude of N-terminal cleavage inhibition was increased compared to that of the wild-type domains. A similar profile was also detected for the C+1S mutant, with Zn^{2+}-mediated inhibition of trans-splicing but not of C-terminal cleavage. The ability of Zn^{2+} to inhibit splicing but not C-terminal cleavage for these two mutants suggests that both substitutions alleviate inhibition at asparagine cyclization, but not at trans-thioesterification. On the basis of experiments with the wild-type domains, we hypothesized that both reaction steps could be inhibited by Zn^{2+} (*15*), but the specific inhibition on trans-thioesterification was difficult to demonstrate as the two effects could not previously be uncoupled in the splicing pathway. The ability of Zn^{2+} to inhibit trans-splicing despite the absence of asparagine cyclization inhibition supports the previous hypothesis (*15*) that the metal ion is able to specifically inhibit trans-thioesterification.

A histidine at the penultimate intein position has been shown to aid in the step of asparagine cyclization (*23*, *25*). Given the absence of a native His at position 158 and that C-terminal cleavage (a step dependent upon asparagine cyclization) occurs only after N-terminal cleavage for the wild-type *Ssp* DnaE intein, we also examined whether the A158H mutation would allow independent C-terminal cleavage in this intein. Despite the substitution, we did not observe spontaneous C-terminal cleavage under any set of conditions investigated, nor was there an increased extent of C-terminal cleavage seen. Therefore, the wild-type *Ssp* DnaE intein appears to have either optimized asparagine cyclization and C-terminal cleavage or to have developed a separate mechanism that is unaffected by the "replacement" of a histidine at the penultimate position, as has been suggested for other inteins (*27*).

Substitution of Ala158 to His did not result in faster splicing under typical assay conditions. In addition, we observed no increase in the extent of spliced product generated, similar to previous findings (*28*). A thorough examination of the effect of pH on both splicing and spontaneous hydrolysis revealed conditions that promote A158H splicing over the wild-type process (measured by the amount of spliced product formed). Previous work suggesting an enhancement in *Ssp* DnaE intein splicing activity upon substitution of a histidine for the native alanine (*11*) appears to be specific to given reaction conditions rather than a universal effect. It should be noted that the condition at which the fastest splicing is observed does not always coincide with the condition at which the greatest extent of splicing occurs. These data are consistent with the nature of a bifurcated pathway in which both hydrolysis and splicing can occur upon formation of a single precursor, in this case the branched intermediate.

Despite numerous efforts, the quantitation of an absolute intein domain affinity has not been possible to date. Instead, the discovery of ME_nB to compete with an inactive N-domain for E_cT in vitro has allowed us to make the first preliminary reports on relative affinities between the domains of the *Ssp* DnaE intein and its mutants. The on rate for the two active domains has been shown to be rapid relative to the overall rate of N-terminal cleavage (*14*, *15*), and the on and off rates of the C-domain:inhibitor complex do not contribute individually to the assay since the incubation time allows for equilibrium to occur between the two species. Therefore, the rate of MBP formation measured during the competition assay reflects the equilibrium dissociation of the C-domain and the rate of N-terminal cleavage. Because the rate of N-terminal cleavage can be measured during experiments without the inhibitor, decreases detected during the competition assays report on the relative affinity of the complex.

The significant rate reduction detected during wild-type C-domain experiments supports a high affinity and low turnover for the wild-type ME_nB:E_cT complex. The absence of any detectable turnover during ME_nB/E_c reactions suggests that the ME_nB/E_cT affinity is greater than the $ME_nB(C1A)/E_cT$ affinity. Because turnover is presumably prevented by a tight association after N-terminal cleavage, it is the E_nB/E_c interaction that is more accurately reflected as tighter than the $ME_nB(C1A)/E_cT$ interaction. Whether this reflects an enhanced affinity that occurs during/after N-terminal cleavage or is due to the reduced affinity of the inactive $ME_nB(C1A)$ N-domain for any C-domain is currently unclear. The concept that the N−S acyl shift increases the affinity between the two intein domains has been suggested previously (*14*, *15*, *39*) and may account for the lack of repeatable detection of the ME_nB:E_cT complex during SDS−PAGE [whereas the E_nB:E_cT complex could be routinely detected (*15*)]. Upon mutation at the C-terminus, either by replacement of the intein penultimate residue with histidine or by a conservative substitution of the +1 cysteine with serine, the affinity of the two intein domains appears to drop significantly. No mutation tested thus far has appeared to result in a higher N- and C-domain complex affinity than that observed for the wild-type domains.

The addition of Zn^{2+} to the replacement assays had an unexpected effect, that is, a reduction of the rate of the A158H-containing experiments but not of those employing the wild-type C-domain, despite the metal ion's ability to inhibit N-terminal cleavage for both proteins during typical trans-cleavage assays. Either Zn^{2+} interferes with the on rate of the wild-type C-domain:inactive N-domain complex and this effect is not detected during replacement reactions because equilibrium is reached prior to addition of ME_nB (a conclusion contradicted by previous and current incubation

experiments) or the minor effect of Zn^{2+} inhibition is efficiently swamped in the wild-type assays due to the larger magnitude of retardation brought about by the presence of the inactive N-domain. This latter hypothesis, while satisfying for the wild-type C-domain assays is less so for the E_cT(A158H) assays. For replacement experiments containing the histidine mutant, the presence of Zn^{2+} results in differing magnitudes of inhibition depending on the timing of addition (pre- or postincubation, with increased inhibition occurring upon postincubation addition). If Zn^{2+} retards the on rate of the E_cT(A158H):N-domain complex, this effect would be masked when the metal is added during preincubation and the complex is allowed to come to equilibrium. This hypothesis is consistent with the reduced magnitude of Zn^{2+} inhibition detected in the A158H competition assays (3-fold) compared to that detected in the assays conducted in the absence of ME_nB(C1A) (7-fold). However, further experimentation is required to verify this theory.

To date Zn^{2+} has been found to bind or inhibit three different inteins (*15*, *17*, *28*, *30*). We have demonstrated in this work that the Zn^{2+} effect can be more confidently attributed to an inhibition in the trans-thioesterification step of the intein splicing mechanism. Our studies have focused not only on the utility of the naturally split *Ssp* DnaE intein system to discern quantitative effects of specific amino acid substitutions on the activities of trans-splicing and -cleavage but also on the first step of split intein reactions, association. The use of an inactive N-domain as a competitor in replacement reactions allowed the first demonstration of intein domain dissociation and rebinding and permitted the determination of relative affinities of wild-type and mutant intein domains. A high-affinity interaction between the N- and C-terminal splicing domains may be a common intein feature as a number of artificially split inteins are capable of trans-splicing in vitro (*41*−*43*). Therefore, the ability to quantitate an affinity constant for the naturally split *Ssp* DnaE intein may have widespread application as inteins are increasingly used as protein engineering and interaction tools. These studies provide a baseline for future widespread scanning efforts designed to illuminate the requirements for intein complex affinity.

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