Mechanistic insights into cyclic peptide generation by DnaE split-inteins through quantitative and structural investigation

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Abstract: Inteins carry out protein-splicing reactions, which are utilised in protein chemistry, protein engineering and biotechnological applications. Rearrangement of the order of the domains in split-inteins results in a head-to-tail cyclisation of the target sequence, which can be used to genetically encode and express libraries of cyclic peptides. The efficiency of the splicing reaction depends on the target sequence. Here we used mass spectrometry to assess the cyclic peptide formation of different, hexameric target sequences *in vivo* by the DnaE split-inteins from *Synechocystis* sp. and *Nostoc punctiforme*, revealing a strong impact by the target sequence and intein on the intracellular peptide concentration. Furthermore, we determined the crystal structures of their pre-splicing complexes, which allowed us to identify F-Block Asp17 as crucial for the DnaE-mediated splicing reaction.

Inteins (*internal protein*), which are found in all domains of life, are single-turnover catalysts of posttranslational protein splice reactions. These intervening protein sequences self-excise themselves in a multistep autoprocessing event, followed by the ligation of the exons (*external protein*).^[1] Moreover, the two intein halves and their respect exons can also be encoded in different genomic locations, termed split-inteins. Here protein-splicing and ligation is carried out *in trans* between the two separate precursors.^[2] In the last 20 years, methods for utilisation of inteins for protein engineering, chemical biology as well as pharmaceutical applications, such as expressed protein ligation and the semi-synthetic generation of modified proteins, have been developed.^[3] Furthermore, split-inteins can be used to genetically encode and express libraries of cyclic peptides (CPs), a method that was termed split-intein mediated circular ligation of peptides and proteins (SICLOPPS).^[4] Here the order of the two split-intein-domains is inverted and encoded as a single polypeptide, resulting in the head-to-tail cyclisation of the sequence between the IntC and IntN domains (Supporting Fig.1). Due to their cyclised backbone structure, CPs are extremely stable against proteolytic degradation and the entropic cost upon binding is reduced.^[5] Thus CPs provide useful scaffolds for biotechnological and pharmaceutical applications.^[6]

Two naturally occurring, homologous split-inteins have been used for SICLOPPS in bacteria, yeast and human cells: the

and *Synechocystis* sp. PCC6803 (*Ssp*).^[4b, 7] They share an overall sequence identity of 67% (N-domain) and 53% (C-domain), respectively (Supporting Fig. 2). Despite the highly conserved molecular architecture and mechanism of action of inteins, they exhibit different splicing efficiencies and extein sequence preferences. The *Ssp* DnaE intein was shown to possess a relatively slow splicing velocity at the optimal reaction temperature of 30 °C.^[8] In contrast, *Npu* DnaE has a 33-170 fold higher splicing rate and an overall yield ranging from 80-90%.^[9] Its temperature optimum of 37 °C is ideal for CP generation in *Escherichia coli* or human cell culture and was reported to be less influenced by the extein sequence adjacent to the essential Cys residue.^[9a, 9c, 10] Although engineered and other natural inteins were shown to possess higher splicing efficiency and to be partly less influenced by the extein sequences,^[9a, 11] so far only the naturally occurring *Npu* and *Ssp* DnaE inteins have been used to generate expressed cyclic peptide libraries in screening campaigns. For such CP libraries, one needs to consider that the resulting intracellular CP concentration could impact on the outcome of an *in vivo* screening assay.

To gain mechanistic insights into the *in vivo* CP generation by split-inteins, we quantitatively and structurally investigated the sequence bias of the *Npu* and *Ssp* DnaE inteins. By using synthetic ¹³C-labeled reference peptides and liquid chromatography mass spectrometry (LC/MS), we determined the amount of four different cyclic hexameric target sequences produced by the two homologous inteins in *E. coli*. Three of these sequences were chosen for their diversity in sequence (CFAHPQ, CGWIKK, CLNSLS), while the fourth peptide (CAFHPQ) was used to investigate the previously reported increased splicing efficiency with a Phe at the extein⁺² position in *Ssp* DnaE.^[12] An equivalent Phe is also present in *Npu*, but it

Figure 1. Hexameric cyclic peptide generation in *E. coli* and splicing efficiency of *Npu* und *Ssp* DnaE split-inteins. A) Quantification of four target CP sequences by LC-ESI-MS/MS in *E. coli* cell extracts. Measurements were repeated four times per extracts and average values with the respect standard deviations correspond to four biological replicates. B) Analysis of splicing efficiency of the purified DnaE inteins with four target sequences. C) Splicing efficiency of the purified DnaE-CFAHPQ wild type, CAsp¹⁷Ala and CAsp¹⁷Asn mutant inteins. Mutation of the F-Block Asp to Ala strongly impedes DnaE splicing. The *Npu* DnaE^{D17N} mutant shows slightly higher, residual activity. *Ssp* DnaE is more sensitive to CAsp¹⁷-mutations. Highlighted by an arrow are the products of the premature cleavage at the IntC-splice junction.

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was shown that *Npu* DnaE splices even faster when the Phe is substituted with Trp or Met.^[13] Our quantification revealed that the overall yield of produced CP ranges from 0.1–3.7 nmol/g cells, depending on the used target sequence (Fig. 1A and Supporting Table 1). In addition, the previously reported higher splicing efficiency of the *Npu* DnaE results in a higher yield of CP for two of the four hexameric target sequences. However, we used here the *Ssp* DnaE^{A35H}-intein variant shown to enhance the splicing velocity as published for the SICLOPPS method.^[14a]

The amounts of produced CP can be correlated with the ratios between spliced and pre-spliced proteins for the *Ssp* DnaE intein: While almost complete processing can be seen for the CFAHPQ-target sequence, only about one third of the protein has undergone splicing with the CAFHPQ peptide, albeit the yields for the CFAHPQ and CAFHPQ sequences are similar with 3.3 and 2.7 nmol/g cells (Fig. 1A and Supporting Table 1). The CFAHPQ and CAFHPQ target peptide for both inteins is encoded by the same DNA sequence without any rare codons (Supporting Table 2). For the *Npu* DnaE intein apparently no direct link between the splicing ratios and produced CPs can be observed. However, analysis by immunoblotting revealed that *Npu* DnaE-CLNSLS might be marginally higher expressed than the CAFHPQ and CFAHPQ constructs (Fig. 1B and Supporting Fig. 3). In addition, premature cleavage at the C-terminal splice junction can be seen for the three *Npu* DnaE target sequence constructs with the lowest peptide yields (CGWIKK, CFAHPQ, CAFHPQ), but not for the CLNSLS peptide or in the *Ssp* DnaE intein (Fig. 1B). Early, off-pathway C-terminal cleavage, which is caused by the cyclisation of the inteins C-terminal Asn prior to branch intermediate formation, arises when coordination of the reaction steps is either perturbed by mutation or by expression between foreign exteins.^[14] Furthermore, it was recently shown that *Npu* DnaE expression can be toxic for *E. coli* cells.^[15b] Taken together the overall intracellular concentration of the CP product depends on the expression level, premature cleavage at the C-terminal splice junction, possible toxic effects and the sequence-dependent splicing efficiencies. Moreover, the pre-organisation imposed by the short hexapeptide target sequence, due to steric restraints, possibly influences the splicing reaction. With short target peptides the impact of the extein sequence is likely to be higher compared to longer, more flexible target sequences.^[9b, 15]

To gain insights into the molecular mechanism of the splicing reactions, we determined the crystal structures of the catalytically incompetent pre-splice complexes of both split-inteins *Npu* DnaE^{C1A/N36A} and *Ssp* DnaE^{C1A/A35H/N36A}, with the target sequences CAFHPQ and CFAHPQ. To our best knowledge, these are the first fully resolved structures of intein-extein complexes, showing the organisation of the splice junctions prior to the splicing reaction. All DnaE inteins investigated here adopt the typical horse-shoe like folding topology, with the hexameric target sequence protruding from the catalytic side (Fig. 2A and Supporting Fig. 4). Furthermore, a second conformation is observed in the crystal of *Ssp* DnaE^{C1A/A35H/N36A}-CAFHPQ (Fig. 2B) trapped by an intermolecular disulphide-bridge between the Cys⁺ of two proteins at the C-terminal splice junction (Supporting Fig. 4). For *Npu* DnaE a "capture and collapse"-intein-folding mechanism was postulated, where the second IntN-lobe attacks the first IntC-part, forming a native-like intermediate, which is followed by a

collapse of this intermediate onto the first IntN-lobe.^[16] In our trapped structure the IntC-IntN subdomain needs to rotate 89° around the bending residues 52–54 (IntN; ^Nβ5) and 10–13 (IntC, ^Cβ1), in order to form the fully assembled intein (Fig. 2B). This could have resemblance to the predicted native-like folding intermediate of intein-folding model-mechanism "capture and collapse",^[16] albeit we cannot prove that the here observed conformation is directly on the folding pathway.

In all here reported structures, the hexameric target peptides are engaged in intermolecular crystal contacts (Supporting Fig. 6–8), which likely impact on torsion angles. However, our structures of the *Npu* and *Ssp* DnaE-pre-splice complexes provide further insights into the interconnected and complex hydrogen bonding network at the splice junctions that is responsible for the DnaE-mediated protein-splicing reaction.^[3d] The orientation of ^CAla³⁶ and ^NAla¹ (Cβ-atoms), which correspond to ^CAsn³⁶ and ^NC¹ in the active inteins, are equivalent orientated as found in other available wild type intein structures. Thus, we modelled the sidechain atoms of ^CAsn³⁶ and ^NC¹ stereo chemically into our mutant intein structures (Supporting Fig. 9+10). The distances between the S_Y (^NC¹) and the C-terminal splice junction range from ~8–10 Å. Such distances observed in the structures of other inteins were argued to correspond to an "open conformation" of the splice junctions^[17] (Supporting Fig. 11+12, Supporting Table 3).

Figure 2 Structure of DnaE-pre-splicing complex. A) Schematic representation of the intein pre-splicing complex, in which important sequence motifs are highlighted. Below: X-ray crystal structure of the *Ssp* DnaE^{C1A/A35H/N36A}-CAFHPQ pre-splicing complex. The split-intein is drawn as ribbon (IntN: grey; IntC: black), the hexameric target cyclic peptide sequence (blue), ^CAla³⁶ (pink), C⁺ and ^NAla¹ (golden) are highlighted as sticks. The F_o-DF_c simulated annealing difference electron density map, contoured at 2.5σ, is depicted as blue mesh. B) The two conformation observed in the crystal structures of the *Ssp* DnaE^{C1A/A35H/N36A}-CAFHPQ pre-splicing complex, representing the fully assembled intein (grey) and a second conformation (red), possibly resembling the intermediate of the "capture and collapse"-mechanism of intein folding. Highlighted in green are the bending residues (IntN 52–54, IntC 10–13). Please note: crystallisation experiments were set up in the presence of reducing agent, with monomeric and monodispersed protein (Supporting Fig. 5).

Figure 3 Role of amino acid residues in the different steps of the protein splicing reaction based on the X-ray crystal structure of the Ssp DnaE^{C1A/A35H/N36A}-pre-splicing complex. Residues involved in S-N-acyl shift (left), transthioesterification (middle) and Asn-cyclisation (right) are highlighted. The carbon atoms of the cyclic target sequence are coloured in light blue, the IntN domain in dark grey, the IntC domain light grey, ^CAsn³⁶ in pink, ^NC¹ and Cys⁺¹ in golden. Please note that the side chain atoms for ^CAsn³⁶ and ^NC¹ are not present in our expression construct, and thus were modelled stereo chemically, based on the comparison with other available intein structures. For clarity, we have removed the oxygen from the Cys⁺¹, which is oxidised in our structure.

The first step of the protein-splice reaction is the energetically unfavoured N-S-acyl shift at the N-terminal splice junction. The nucleophile (^NCys¹) needs to be deprotonated by a base and the ground state is destabilised.^[3d] Here, a direct interaction of the peptide bond at the splice-junction with the amid-nitrogen and O^γ of ^NThr⁶⁹ mediates the ground state destabilisation and positions the splice junction (Fig. 3, left). It was previously shown for DnaE, that ^NThr⁶⁹ likely enhances the rate of the N-S acyl shift through this interaction.^[18]

In the *Mycobacterium tuberculosis* (*Mtu*) RecA intein His⁷³ and Asp⁴²² (in DnaE corresponding to ^NHis⁷² and ^CAsp¹⁷; Supporting Fig. 11+12) undergo large pK_a shifts during protein splicing and are essential for the required acid-base catalysis.^[19] Mutation of ^CAsp¹⁷ to Ala impedes splicing in both inteins, whereas the substitution of ^CAsp¹⁷ by Asn retained some splicing activity in *Npu* DnaE, but not in *Ssp* DnaE (Fig. 1C). While for *Ssp* DnaE such a pK_a shift appears to be necessary, for *Npu* DnaE replacement of the charged Asp with a polar Asn is sufficient to maintain some protein splicing activity during the 16 h expression time. The Asn could here fulfil a structural or charge-stabilising role. In addition, for both *Npu* DnaE mutants the same extend of premature cleavage at the C-terminal junction can be observed. Asn cyclisation resulting in premature C-terminal cleavage was previously reported for the otherwise splicing deficient *Npu* DnaE ^NCys¹ to Ala mutant.^[9b] In contrast, C-terminal cleavage in the *Ssp* DnaE mutant only occurs in the ^CAsp¹⁷ to Asn, but not the ^CAsp¹⁷ to Ala mutant (Fig. 1C). Furthermore, it was shown for both inteins that mutation of residues outside of the active side can positively and negatively modulate the splicing efficiency, most likely due to the perturbation of amino acid networks.^[11, 20]

Our data show ^CAsp¹⁷ is important for DnaE-mediated protein splicing in CP generation. In our structures, ^NHis⁷² is located below the splice junction, too far away from the ^NCys¹-side chain for its deprotonation. Nevertheless, deprotonation could be carried out by the action of ^CAsp¹⁷. ^NHis⁷² would then be ideally positioned to support the destabilisation of the ground state^[21] and the protonation of the tetrahedral intermediate. In addition, ^CAsp¹⁷ could also activate Cys⁺¹ for the following transthioesterification step by deprotonation, and act as an acid

to mediate bond-cleavage, resulting in the branched (or lariat) intermediate (Fig. 3, middle). Such a role was postulated for Asp⁴²² in *Mtu* RecA.^[19b, 22] In the *Ssp* DnaB mini-intein the deprotonation of Asn is mediated by an Asp, followed by an attack on the C-scissile peptide bond and subsequent cyclisation.^[23] Our structure reveals that ^CAsp¹⁷ is indeed positioned, that is could deprotonate ^CAsn³⁶ (Fig. 3, right). Ground state destabilisation of the C-terminal splice junction for the Asn cyclisation possibly occurs through the conserved ^CHis²⁴ and the action of the penultimate ^CHis/Ser³⁵, shown to be important for efficient splicing of DnaE split-inteins.^[24] Furthermore, ^CHis/Ser³⁵ are likely not only to stabilise the tetrahedral intermediate, but also to protonate the amide nitrogen of the scissile peptide bond, turning it a good leaving group.^[25] An Ala at this position, analogue to the native *Ssp* DnaE intein, is not able to fulfil this function. In addition, ^NArg⁵⁰ was shown to act positively on splicing.^[26] According to our structure ^NArg⁵⁰ could influence the orientation of ^NCys¹, ^CAsp¹⁷ and/or the exteins peptide backbone (Fig. 3 and Supporting Fig. 6). The last step in protein splicing is the uncatalysed S-N-acyl shift to rearrange to the more stable amide bond, which does not need support by surrounding residues.

In summary, our results show that intracellular concentrations of genetically encoded CP produced by *Ssp* and *Npu* DnaE inteins largely vary, depending on the extein sequence. Nevertheless, both inteins were successfully used to generate CP libraries in screening campaigns. Furthermore, we provide mechanistic insight into the expressed CP generation by these two inteins, identifying the F-Block ^CAsp¹⁷ is crucial for efficient splicing of linear peptides in *Npu* and *Ssp* DnaE intein.

Experimental Section

Chemical synthesis of unlabelled and ¹³C-labeled reference cyclic peptides was done by Fmoc-solid phase peptide synthesis, followed by cyclisation in solution.^[27] The DnaE split-Inteins from *Nostoc punctiforme* PCC73102 and *Synechocystis* sp. PCC6803 were expressed in *Escherichia coli*. Cyclic peptides were extracted using butanol, the cell extracts spiked with the respect ¹³C-labeled reference peptides and

analysed by HPLC–ESI-MS/MS (Supporting Fig. 13–18). For crystallisation catalytically inactive variants *Ssp* DnaE^{C1A/A35H/N36A} and *Npu* DnaE^{C1A/N36A} were expressed, purified and crystallised. Structures were solved by molecular replacement. The structure factors and models have been deposited in the PDB (accession codes: 5OL1, 5OL5, 5OL6, 5OL7) For detailed materials and methods see the Supporting Information online.

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Keywords: split-intein • protein splicing • cyclic peptide • intein structure

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COMMUNICATION

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Inteins carry out protein-splicing reactions and can be used to genetically encode and express cyclic peptides. We show that the target sequence and DnaE-intein strongly impact on the intracellular peptide concentration. The crystal structures of the intein pre-splicing complexes provide further molecular details on DnaE-mediated protein splicing.