Protein Splicing

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# Activation of Protein Splicing by Protease- or Light-Triggered O to N Acyl Migration**

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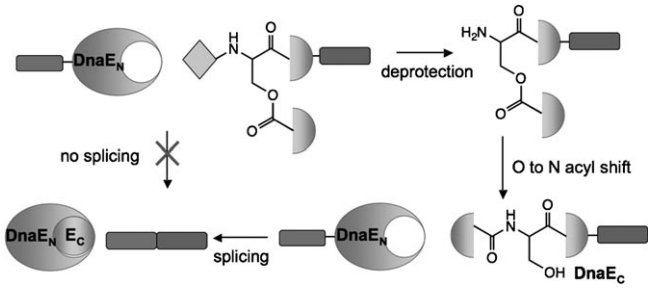
Intramolecular O to N acyl migrations are well known reactions in organic chemistry.[1–3] This type of rearrangement has been the object of renewed interest in chemical biology as it provides opportunities for the spatial and temporal control of peptide conformation and function.[4,5] Incorporation of an *O*-acyl isomer of a peptide bond at a serine or threonine residue in a peptide sequence (herein referred to as an *O*-acyl linkage) introduces a kink into the main chain of the biopolymer and interrupts the normal backbone hydrogen-bonding characteristics, both of which can affect structure and function.[5–7] In the case where the Ser/Thr α-amino group is transiently protected, recovery of the normal amide backbone and, as a consequence native structure and function, can be achieved by removal of this protecting group, thereby triggering the spontaneous O to N acyl shift. Accordingly, a variety of triggers of O to N acyl migrations have been developed that allow the restoration of native structure following the administration of light,[8] changes in pH value,[9] or enzymatic activity.[10]

Despite the successful use of O to N acyl migrations to control peptide conformation and function,[11–13] the strategy has yet to be applied to more complex processes, such as the regulation of protein catalysis. Of particular interest to us was whether a reversible *O*-acyl modification could be used to control protein splicing, which is a naturally occurring autocatalytic process in which an internal protein domain (intein) removes itself from a polypeptide sequence with concomitant ligation of its flanking regions (exteins).[14,15] Engineered inteins have found widespread use in biotechnology and chemical biology.[16] Of relevance to the current study are the conditional protein *trans*-splicing (CPS) systems which permit the activity of a given protein to be controlled by triggering the assembly of an artificially split intein.[17–19] In principle, use of the O to N acyl migration strategy would allow other types of chemical and biochemical triggers to be incorporated into these CPS systems. Moreover, it might be possible to generate conditional inteins based on the naturally split DnaE inteins,[20] which are incompatible with current CPS strategies owing to their self-assembling nature.

We hypothesized that the *trans*-splicing activity of the split *Ssp* DnaE intein could be controlled by the judicious introduction of an *O*-acyl linkage (Scheme 1). Analysis of




***Scheme 1.*** Conditional protein splicing based on initiation of an O to N acyl migration. An *O*-acyl linkage judiciously placed within the sequence of DnaE$_C$ blocks protein *trans*-splicing activity, but upon rearrangement to an amide after deprotection, the intein regains normal activity.

the crystal structure of *Ssp* DnaE suggested two potential sites for the modification (Figure 1 A). The first site, Ser9, is the only native Ser/Thr residue in the C-terminal half of the split intein (referred to as DnaE$_C$). This residue is located in the

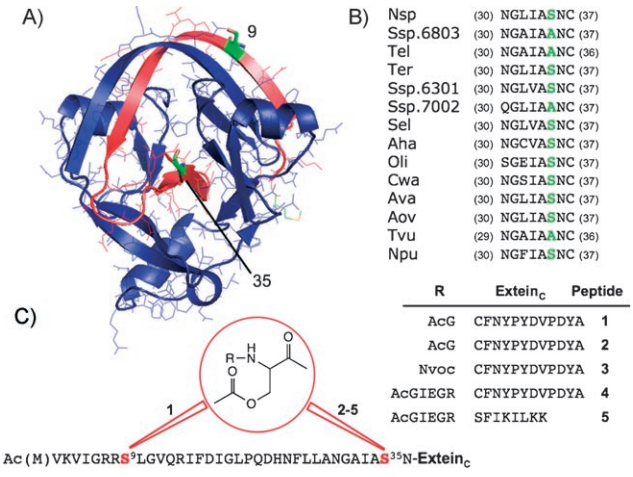



***Figure 1.*** A) Crystal structure of *Ssp* DnaE intein.[21] DnaE$_N$ is shown in blue and DnaE$_C$ in red, with residues 9 and 35 highlighted in green. B) Sequence alignment of the conserved block G of the DnaE intein family, showing that both Ala and Ser are found at position 35. C) Structures of *O*-acyl peptide analogues of DnaE$_C$ used in this study. An *O*-acyl linkage was introduced at position 9 for peptide **1** and at position 35 for analogues **2**–**5**.

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middle of a long β strand that forms an antiparallel β sheet with the $DnaE_N$ fragment in the intein complex.[21] Introduction of a kink in this β strand would be expected to disrupt this key element of secondary structure, destabilize the complex, and prevent splicing. The second site was Ala35, which is serine in many other inteins of the DnaE family and is in the vicinity of the intein active site (Figure 1 A,B). Introduction of a main-chain kink at this position would be expected to alter the position of catalytic residues, which could lead to a loss of splicing activity. Importantly, both these sites are within the short $DnaE_C$ fragment, which is accessible to chemical synthesis.[22,23] Accordingly, we synthesized two $DnaE_C$ analogues containing an *O*-acyl linkage at the β-hydroxy group of either Ser9 (peptide **1**) or 35 (peptide **2**) and their α-amino groups irreversibly blocked by acetylglycine (Figure 1 C; see also the Supporting Information).

To evaluate the effect of each *O*-acyl linkage on *trans*-splicing activity, we compared splicing reactions between $DnaE_N$ and either wild-type $DnaE_C$, an Ala35Ser mutant, *O*-acyl analogue **1**, or *O*-acyl analogue **2**. As model extein sequences, we fused maltose binding protein linked to ubiquitin (MBP-Ub) and a hemagglutinin (HA) peptide tag to the N terminus of $DnaE_N$ and the C terminus of $DnaE_C$, respectively (Figure 2 A). The reactions were followed by western blot using an anti-HA antibody to detect formation of the MBP-Ub-HA splicing product (Figure 2). As predicted by the sequence analysis, the $DnaE_C$A35S mutant had identical activity to the wild-type $DnaE_C$. More surprising was the splicing activity of *O*-acyl analogue **1**, which was also

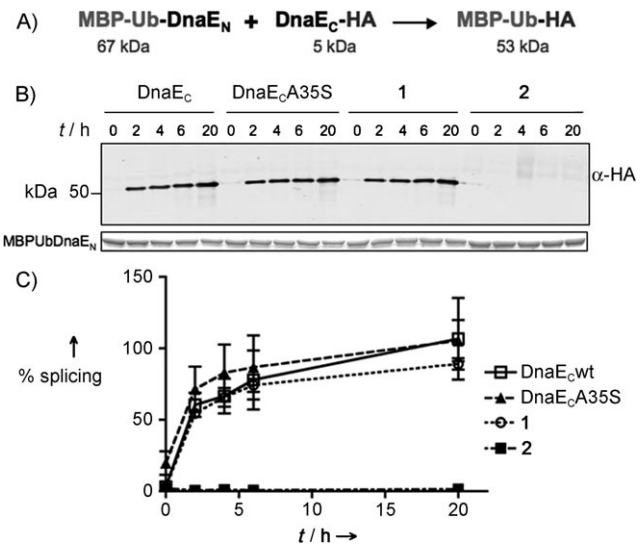


***Figure 2.*** Effect of *O*-acyl linkages on DnaE-mediated *trans* splicing. A) Scheme of the *trans*-splicing reaction with the indicated model exteins. B) Western blot analysis with an anti-HA antibody of representative splicing reactions between $DnaE_N$ and $DnaE_C$ constructs at 20 and 12.5 μM, respectively, in splicing buffer (100 mM phosphate, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, pH 7.5). Lower box: Coomassie-stained loading control, showing MBP-Ub-$DnaE_N$. The splicing product (MBP-Ub-HA) can be detected as a band at just above 50 kDa in the western blot. C) Plot of the fraction splice product formed at different timepoints relative to the maximum level obtained for the wild-type (wt) intein (wt reaction went to about 50% completion; see Supporting Information). Errors = s.e.m. ($n = 3$).

indistinguishable from that of the wild-type $DnaE_C$. This indicates that the split intein can tolerate local disruption to the interfacial β sheet caused by the *O*-acyl linkage. In contrast, no splicing product was observed for the reaction between *O*-acyl analogue **2** and $DnaE_N$. Moreover, HPLC and MS analysis of this reaction indicated that known splicing side reactions, such as N terminal cleavage,[24] also did not occur at detectable levels (see Supporting Information). Thus, introduction of the *O*-acyl linkage at Ser35 blocks all *trans*-splicing activity of $DnaE_C$. Steady-state and stopped-flow fluorescence experiments showed that *O*-acyl analogue **2** associates with $DnaE_N$ with similar affinity and kinetics to wild-type $DnaE_C$, and circular dichroism spectroscopy indicated the secondary and tertiary structure of the $DnaE_N$/*O*-acyl analogue **2** complex was not globally perturbed from that of the all-amide structure (see Supporting Information). We therefore conclude that the lack of *trans*-splicing activity associated with *O*-acyl analogue **2** is due to local perturbations in the active site of the complex rather than an inability of the fragment to associate with $DnaE_N$ and adopt the canonical intein fold.

We investigated whether *O*-acyl analogue **2** could be used as the basis of a CPS system. Triggering the O to N acyl shift at this site would generate the $DnaE_C$A35S mutant, which we have already shown to support *trans*-splicing (Figure 2). Thus, incorporation of a transient protecting group on the Ser35 α-amino group should lead to a conditional DnaE intein. Many α-amino protecting groups are known to be viable triggers for this kind of rearrangement.[8,10,13] We were especially attracted to protecting groups that are removed by proteases or light. Given that proteases are implicated in the pathology of many diseases,[25] the ability to activate splicing in response to their activities could lead to conditional inteins which sense the presence of these disease markers or generate polypeptide therapeutics (e.g. a toxin) in response to them. Photoremovable protecting groups have been widely used in the development of caged versions of bioactive molecules, including inteins.[26] Accordingly, we synthesized two protected versions of *O*-acyl peptide **2**, one containing a photocleavable Nvoc group on the α-amino group of Ser35 (*O*-acyl analogue **3**) and one containing a Factor Xa protease recognition sequence at the same position (*O*-acyl analogue **4**; Figure 3 A). Initial studies showed that peptides **3** and **4** could be deprotected by treatment with 325 nm laser light and Factor Xa protease, respectively, and that spontaneous O to N acyl migration then ensues (see Supporting Information). As expected, in their protected forms, both **3** and **4** were inactive in our *trans*-splicing assay with $DnaE_N$ (Figure 3). However, exposure of the splicing reactions to the appropriate stimuli, either UV light or the protease, led to dramatic increases in the amount of splice product formed. The kinetics of the splicing reaction between **3** and $DnaE_N$ were indistinguishable from the wild-type reaction, whereas we observed a slight decrease in both the rate and extent of the reaction of **4** with $DnaE_N$ compared to the controls. This difference in behavior most likely reflects the significantly slower kinetics associated with the enzymatic deprotection compared to the photolysis reaction, and also the fact that a small amount of nonspecific proteolysis of the intein occurs with prolonged incubations with Factor Xa (see

  

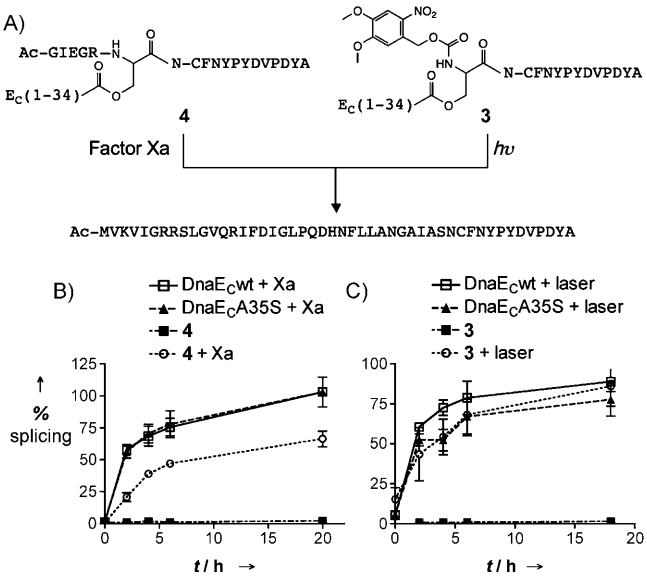


***Figure 3.*** Light- and protease-triggered O to N acyl migration to activate protein *trans* splicing. A) Protease- and photo-mediated activation of *O*-acyl DnaE$_C$ analogues. B) Protease-activated protein *trans* splicing: MBP-Ub-DnaE$_N$ and indicated peptides were incubated at 20 and 12.5 mM, respectively, in buffer (20 mM Tris, 100 mM NaCl, 2 mM $CaCl_2$, 1 mM DTT, pH 8.0) with or without Factor Xa (0.1 U/50 μg) protease. Errors = s.e.m. ($n = 3$). C) Light-activated protein *trans* splicing: MBP-Ub-DnaE$_N$ and indicated peptides were incubated at 20 and 12.5 μM, respectively, in splicing buffer with or without 325 nm laser irradiation. The reactions were monitored as in Figure 2. Errors = s.e.m. ($n = 3$).

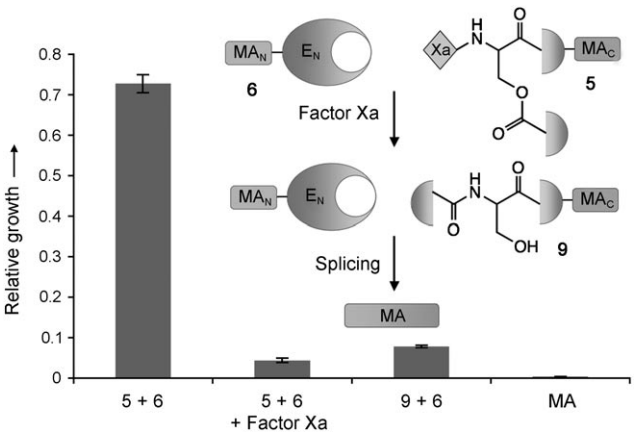


***Figure 4.*** Protease activation of intein-mediated antimicrobial activity. Growth of *E. coli* incubated with constructs **5** and **6** at 30 μM in the presence and absence of Factor Xa protease. Magainin (MA) and the constitutively active wild-type intein (**9** + **6**) at 30 μM are also shown as positive controls. Bacterial growth ($OD_{650}$ after 18 h incubation) of each sample relative to a culture control is shown. The inset shows the DnaE-mediated splicing of magainin.

Supporting Information). Nonetheless, the key result is that we were able to trigger the DnaE *trans*-splicing reaction using either a photochemical or a biochemical stimulus.

Finally, to illustrate the generality and potential applications of our CPS system, we asked whether the approach could be used to proteolytically trigger the synthesis of a toxin. Among the many extein options possible, we focused on the antimicrobial peptides,[27] as the vast structure–activity relationship literature available on these molecules helped with the design of functionally silent mutations that would allow the use of DnaE *trans* splicing for the assembly of the peptide. Accordingly, we designed an analogue of magainin[28] that had native-like activity and could be split and regenerated upon DnaE-mediated *trans* splicing leading to an antimicrobial response (Figure 4; see also the Supporting Information). We then fused the C terminus of this magainin peptide to *O*-acyl analogue **4**, which contains the Factor Xa trigger, to give peptide **5**. Consistent with the initial model studies, we could not detect any *trans* splicing between **5** and the corresponding DnaE$_N$ fused to the N terminus of magainin (protein **6**) in the absence of the protease. Treatment of **5** with Factor Xa in the presence **6** resulted in the formation of magainin and the recovery of antimicrobial activity against both *E. coli* and *S. aureus* strains, thus demonstrating the potential of the approach (Figure 4; see also the Supporting Information). By exporting this system to more active toxins under the control of pathogenesis related proteases, potent and specific antimicrobial agents could be developed.

Our results demonstrate that an *O*-acyl linkage conveniently placed at the Ser35 side chain of the C-terminal fragment of the DnaE intein abolishes protein splicing. The modification does not have a significant effect on either the association of N- and C-terminal intein fragments or the global conformation of the complex between them, which indicates that inhibition is accomplished by a localized perturbation of the intein active site. More importantly, intein activity can be recovered by an O to N acyl migration triggered by proteolytic or photochemical removal of specific protecting groups at the switch site. This result is, to the best of our knowledge, the first application of the O to N acyl shift method for the control of protein activity. In principle, this type of reversible modification could be used to control the activity of other proteins accessible to chemical synthesis.

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