Streamlined Expressed Protein Ligation Using Split Inteins
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ABSTRACT: Chemically modified proteins are invaluable tools for studying the molecular details of biological processes, and they also hold great potential as new therapeutic agents. Several methods have been developed for the site-specific modification of proteins, one of the most widely used being expressed protein ligation (EPL) in which a recombinant α-thioester is ligated to an N-terminal Cys-containing peptide. Despite the widespread use of EPL, the generation and isolation of the required recombinant protein α-thioesters remain challenging. We describe here a new method for the preparation and purification of recombinant protein α-thioesters using engineered versions of naturally split DnaE inteins. This family of autoprocessing enzymes is closely related to the inteins currently used for protein α-thioester generation, but they feature faster kinetics and are split into two inactive polypeptides that need to associate to become active. Taking advantage of the strong affinity between the two split intein fragments, we devised a streamlined procedure for the purification and generation of protein α-thioesters from cell lysates and applied this strategy for the semisynthesis of a variety of proteins including an acetylated histone and a site-specifically modified monoclonal antibody.

INTRODUCTION

The chemical modification of proteins is an established tool for studying the structure, function, and regulation of this class of biopolymer.1 Moreover, in recent years, a great deal of effort has been directed toward the modification of proteins for therapeutic applications.2 Traditionally, protein conjugation chemistries have exploited the reactivity of surface-exposed nucleophilic amino acids, such as cysteine or lysine, however, these methods typically result in heterogeneous mixtures of products, which can complicate biological studies or efficacious medicinal applications. To address this concern, several strategies have been developed for the site-specific modification of proteins, ranging from total chemical synthesis (usually via native chemical ligation, NCL)3 to the genetic incorporation of unnatural amino acids4 or bio-orthogonal functional groups.5 In between these two extremes lie a variety of semisynthetic approaches.5−7

The most widely used protein semisynthesis technique is an extension of NCL termed expressed protein ligation (EPL) in which a recombinant protein α-thioester building block is ligated to a synthetic molecule equipped with a 1,2-aminothiol moiety (most commonly an N-terminal Cys-containing peptide) through the formation of a native peptide bond8,9 (Scheme 1). Since its inception, EPL has been applied to a wide variety of proteins, including enzymes,10 ion channels,11 transcription factors,12 transmembrane receptors,13 and anti-

Scheme 1. Protein Splicing (A), trans-Splicing (B) and EPL (C)∗

*ExN and ExC represent N- and C-exteins, respectively. IntN and IntC represent N- and C-intein fragments, respectively.

bodies14 (for reviews see refs 15 and 16). One of the basic requirements of EPL is a thioester group at the C-terminus of a...
recombinant protein. This reactive handle is introduced by exploiting a process known as protein splicing (Scheme 1), which is mediated by an autoprocessing domain called an intein.\textsuperscript{16,17} Protein splicing typically takes place through the formation of one or more protein thioester intermediates, which ultimately resolve to form a native peptide bond between the sequences flanking the intein (referred to as N- and C-exteins). By using appropriate intein mutants, it is possible to intercept these intermediates with exogenous thiols, resulting in an N-extein of choice being cleaved from the mutant intein as a reactive α-thioester derivative suitable for chemical ligation.\textsuperscript{18}

Despite the many successes of EPL, the approach often suffers from low overall efficiency due to complications associated with the generation of protein α-thioesters. In particular, fusions to inteins are, to varying extents, susceptible to premature extein cleavage, both \textit{in vivo} and during initial purification from cell lysates, which reduces the isolated yield of the intein fusion needed for the subsequent thiolysis step.\textsuperscript{19} Importantly, the cleaved extein side-product is unreactive toward EPL, and its separation from the desired α-thioester or the ligation product is often difficult for large proteins, such as antibodies.\textsuperscript{19} To compound matters, the thiolysis reaction itself can be slow and inefficient, further strengthening the need to develop customized purification regimes, involving multiple chromatographic steps, to isolate the desired product from complex mixtures.\textsuperscript{20−22} Collectively, these technical issues mean that a considerable investment in time and resources is usually required before a semisynthetic protein is obtained in useful quantities.

To overcome the various drawbacks associated with the intein thiolysis process central to EPL, we envisioned an alternative strategy based on naturally occurring split inteins. Unlike inteins used in standard EPL, which are contiguous polypeptides that catalyze protein splicing in \textit{cis}, split inteins consist of two discrete polypeptides, herein termed Int\textsuperscript{N} and Int\textsuperscript{C}, which, upon association, catalyze protein splicing in \textit{trans} (protein \textit{trans}-splicing, PTS, Scheme 1).\textsuperscript{23} Split inteins have two important properties that make them attractive for an improved EPL strategy. First, cognate Int\textsuperscript{N} and Int\textsuperscript{C} pairs often bind tightly to one another; dissociation constants in the low nanomolar range, reflecting extremely fast on-rates, have been reported for members of the naturally split DnaE inteins from cyanobacteria.\textsuperscript{24,25} This ability of split inteins to self-associate has recently been exploited by Lu et al. as part of a traceless protein purification system, in this case using an artificially split intein pair.\textsuperscript{26} The potential utility of split inteins in EPL is further enhanced by the remarkable splicing efficiency of some members of the family.\textsuperscript{27,28} For instance, many of the split DnaE inteins have half-lives for the splicing reaction of less than a minute, as compared to several hours in the case of the \textit{cis}-splicing inteins commonly used in EPL.\textsuperscript{27,28} Moreover, recent mechanistic investigations indicate that these ”ultrafast” DnaE inteins have a highly activated N-terminal splice junction, making them superior reagents for protein α-thioester generation.\textsuperscript{28} Given the unique properties of split inteins, in particular, the ultrafast split DnaE inteins, we conceived the integrated protein modification system shown in Figure 1 in which the split intein association is employed both to purify the desired protein from complex biological mixtures and to trigger the generation of a desired protein α-thioester for EPL. In principle, this complementation system should address the major issues attendant to the standard EPL protocol, including premature cleavage of the intein, which cannot occur in the case of a split intein fragment absent its cognate partner.\textsuperscript{29,30}

\section*{RESULTS AND DISCUSSION}

\subsection*{Split Intein Mediated Thiolyis and EPL}

To implement our system, we designed a mutant of the ultrafast \textit{Nostoc punctiforme} (Npu) split DnaE intein suitable for efficient α-thioester generation. Specifically, we mutated the catalytic C-terminal residue in the Int\textsuperscript{C} fragment (Asn137) and the first residue in the C-extein (Cys+1) to Ala, to allow for efficient build up of the desired splicing intermediates upon exposure to an N-extein-Npu\textsuperscript{N} fusion (Figure 1). Preliminary studies showed that mixing N-extein-Npu\textsuperscript{N} fusions (where N-extein corresponded to various model proteins) with the mutant Npu\textsuperscript{C} (Npu\textsuperscript{AA}) led to highly efficient N-extein-α-thioester formation in a thiol-dependent manner (Figures S3 and S4). Importantly, only very low levels of intein cleavage (i.e., unwanted hydrolysis) were observed in the absence of thiols, thereby fulfilling a requirement of our integrated strategy.

Encouraged by these results, we adapted the system for the one-pot purification and generation of C-terminally modified proteins by taking full advantage of the strong and specific interaction between the split intein fragments. Accordingly, the Npu\textsuperscript{AA} peptide was immobilized on a solid support through a unique Cys residue engineered within its C-extein region (Figure S5). The resulting Npu\textsuperscript{AA} column (hereafter referred to as Int\textsuperscript{C} column) was then evaluated as an affinity-modification resin. Three test proteins maltose binding protein (MBP), ubiquitin (Ub), and protein histidine phosphatase type 1 (PHPT1) were genetically fused to Npu\textsuperscript{N} and expressed in \textit{Escherichia coli}. In each case, cells were lysed, and the soluble fraction was loaded onto the Int\textsuperscript{C} column to allow binding of the Npu\textsuperscript{N} tagged protein to the immobilized Npu\textsuperscript{CAA}. After a brief incubation (~ 5 min at rt) the column was extensively washed to remove contaminants, and thiolysis was triggered by

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure1.png}
\caption{Streamlined EPL using split inteins. (A) Schematic of the procedure for the isolation of an α-thioester derivative of a protein of interest (POI) using engineered split intein fragments (Int\textsuperscript{N} and Int\textsuperscript{C}). EPL can be performed in a one-pot fashion during thiolysis from the split intein or immediately after elution, without need of any further purification. (B) Sequences of Npu\textsuperscript{WT} (WT) and the Npu\textsuperscript{AA} mutant used on the split intein column. Catalytic residues mutated in Npu\textsuperscript{AA} are shown in bold, and the linker sequence added for immobilization onto the solid support is underlined. Sequences are numbered according to the intein sequence alignment shown in Figure S2.}
\end{figure}
addition of a buffer containing the thiol, mercaptoethansulfonate (MES). In all three cases, the desired α-thioester protein eluted from the IntC column with high recovery (75–95%) and high purity (~95% as determined by RP-HPLC and mass spectrometry) (Figure 2). Total isolated yields of purified protein α-thioesters varied from one protein to another and ranged from 2.5 mg (per L of bacterial culture) for Ub-MES to 40 mg for MBP-MES. The calculated loading capacity of the IntC column used in these experiments was 340 mg for MBP-MES. The calculated loading capacity of the IntC column used in these experiments was 3–6 mg of protein per mL of beads (0.12 μmol/mL), but higher or lower loadings could easily be achieved by modifying the amount of NpuC-AA immobilized on the solid support. Furthermore, we showed that the IntC column could be regenerated and reused at least 5 times with only minimal loss of capacity (Figures S11 and S12). The utility of the α-thioester derivatives of Ub, MBP, and PHPT1 obtained from the column was demonstrated by ligating each of them to an N-terminal Cys-containing fluorescent peptide (CGK(Fl)) to give the corresponding semisynthetic products in excellent yield (Figure S10). Importantly, one-pot thiolysis/ligation reactions could be carried out, which allowed us to obtain a site-specifically modified protein directly from cell lysates without isolating the intermediate thioester (Figure 3).

Dependence on the Identity of the C-Terminal Amino Acid. An attractive feature of EPL is that it can allow for the preparation of site-specifically modified proteins in a virtually traceless manner. This is contingent on the ability to efficiently generate recombinant protein α-thioesters when any of the 20 proteinogenic amino acids are present at the C-terminus of the protein. The activity of split inteins is known to be sensitive to the identity of the amino acids immediately flanking the splice junction. Thus, we were eager to test the generality of our strategy and asked whether we could generate α-thioesters of all 20 amino acids, using ubiquitin as the N-extein template. Twenty Ub-NpuN fusion proteins were individually expressed in E. coli and purified over the IntC column as before. Thiol-induced cleavage yields from the solid support were determined by SDS-PAGE analysis of the eluted and resin beads fractions, and levels of competing side reactions (mainly hydrolysis) were measured by RP-HPLC and ESI-TOF MS. The results clearly show that for most amino acids >60% of the bound proteins were recovered after MES treatment. Furthermore, 80–95% of the eluted material was the desired α-thioester product. The only exceptions were Pro and Glu, for which recovery were 49 and 50%, respectively (Figure 4). The Asn mutant displayed high levels of cleavage from the split intein, but almost no α-thioester could be isolated due to side-chain cyclization to form a C-terminal succinimide. A second problematic residue was Asp, for which we observed some premature cleavage during initial binding to the IntC resin. Moreover, RP-HPLC analysis of the eluted fractions upon thiolysis revealed two species with the molecular weight of the desired α-thioester. These results were not wholly unexpected, as Asp is known to cleave

![Figure 2](image-url)  
**Figure 2.** Purification of α-thioester proteins expressed in E. coli. (A) MBP, (B) PHPT1, and (C) Ub mercaptoethansulfonate (MES) α-thioesters were purified in one step from E. coli cell lysates using the IntC column. The purifications were monitored by coomassie stained SDS-PAGE analysis (top) (inp: input, FT: flow-through, W1-3: washes, E1-4: elutions, and bds: resin beads). RP-HPLC (detection at 214 nm) and ESI-TOF MS analysis of the eluted fractions (bottom left and right, respectively) confirmed the identity of all protein α-thioesters and indicated high purity.

![Figure 3](image-url)  
**Figure 3.** One-pot purification/ligation of ubiquitin to the H-CGK(Fluorescein)-NH2 peptide (CGK(Fl)). Ub-NpuN from E. coli cell lysates was bound to the IntC column, as shown in Figure 2, and after removal of contaminants through extensive washes, intein cleavage and ligation were triggered by addition of 200 mM MES and 1 mM CGK(Fl) peptide. Coomassie stained SDS-PAGE analysis and in gel fluorescence of the purification/ligation (left). RP-HPLC (detection at 214 and 440 nm) and ESI-TOF MS (right) of the eluted fractions confirm the desired ligated protein was obtained in one step directly from cell lysates with a ligation yield close to 95% (quantified by RP-HPLC).
The 20 mutants of the protein Ub-X-NpuN that were expressed in E. coli varying the identity of the C-terminal amino acid of Ub (X) from the WT Gly to all other proteinogenic amino acids. Thiolytic cleavage yields from the IntC column were calculated from the SDS-PAGE analysis of the eluted fractions and left over resin beads. Ratios of α-thioester vs side products were determined from RP-HPLC and ESI-TOF MS analysis of the eluted fractions. The major competing reaction for all amino acids was hydrolysis with the exception of Asn for which its succinimide form was isolated instead. See main text for a discussion on the problems associated with Asp. Error bars ± SD (n = 3).

Thioester Formation under Denaturing Conditions.
Next we investigated if our system was compatible with denaturing conditions. Protein semisynthesis frequently requires the preparation of protein fragments, which are often poorly behaved and need to be purified in the presence of strong chaotropic agents. We first confirmed that the model protein Ub-NpuN could bind the IntC column in the presence of 2 and 4 M urea and that the corresponding Ub α-thioester could be generated with similar yields as obtained under native conditions (Figure S14). This is consistent with a previous study that our streamlined EPL system is compatible with the majority of amino acids are present as the last residue in the protein of interest.

Site-Specific Modification of a Monoclonal Antibody.
Finally, we tested our streamlined EPL methodology for the modification of a monoclonal antibody. The site-specific modification of antibodies has become highly desirable in the area of biopharmaceuticals and diagnostics. Currently, most commercially utilized methods to conjugate cargo to antibodies are relatively nonspecific and result in polydisperse mixtures that may vary from batch-to-batch. Since this heterogeneity can adversely affect both efficacy and safety of the conjugate, attention has turned toward technologies that afford site-specifically modified antibodies. Indeed, protein semisynthesis via standard EPL and PTS has recently been used to generate monoclonal antibody conjugates with full activity. Given this, we were eager to see whether our streamlined EPL process could be used in the facile generation of antibody conjugates. As a model immunoglobulin (IgG) for our studies, we used an antibody against the DEC205 receptor, a C-type lectin found predominantly on dendritic cells. Accordingly, we designed a construct in which NpuN was fused to the C-terminus of the heavy chain of the antibody (αDEC205-NpuN). Initial expression tests of αDEC205-NpuN in 293T cells resulted in very low levels of the antibody being secreted (Figure 6A). We have observed previously that the identity of the IntC column could be directly used in EPL reactions without further purification. Accordingly, we successfully ligated the protein to a hH2B(117−125) peptide containing an acetylated Lys at position 120 to yield semisynthetic hH2B-K120Ac (Figure 5).

Data and materials available at: dx.doi.org/10.1021/ja309126m
of secreted αDEC20S-IntN by varying the identity of the intein N-fragment. Several new αDEC20S-IntN constructs were generated in which IntN corresponded to the N-fragment of a series of ultrafast split DnaE inteins, namely, Ava, Csp, Cra, Cwa, Mcht, Oli, and Ter. We also tested an NpuN mutant (C-S) where the noncatalytic cysteines, Cys28 and Cys59, were mutated to Ser, to determine whether these residues in fluorescence monitoring not only by MS analysis of deglycosylated and fully reduced HC after ligation, showing 75% of the HC is labeled. Expected mass for ligation product = 50221.2 Da. Free HC = 49575.0 Da.

Figure 6. Purification of a monoclonal antibody α-thioester using a split intein and its site-specific modification. (A) Test expression of αDEC20S genetically fused to the contiguous Mxe GyrA intein and different split DnaE inteins through the C-terminus of its heavy chain (HC). Western blot of 293T cell supernatants of several αDEC20S-Int fusions using an antibody against mouse IgG. A loading control is shown below. (B) Purification of αDEC20S-MES thioester using the split intein column. (C) Elution fractions containing were concentrated to 20 μM for 48 h at rt. (D) SEC-MALS analysis of the ligated protein showing that it retains its tetrameric structure after thiolysis and ligation (Mn = 151 kDa, Mcalc = 148 kDa). (E) ESI-TOF MS analysis of deglycosylated and fully reduced HC after ligation, showing 75% yield (Figure 6E). Importantly, we demonstrated that the semisynthetic αDEC-CGK(Fl) retains its ability to bind the DEC20S receptor to the same extent as a control αDEC20S, previously shown to be fully functional in vivo (Figure 7).

Figure 7. Binding of αDEC20S-CGK(Fl) to the DEC20S receptor. (A) Dose dependent binding of αDEC20S-CGK(Fl) to CHO cells expressing the mouse DEC20S receptor monitored by flow cytometry using a PE-labeled α-mouse IgG. Binding to control CHO/NEO cells, which do not express the receptor is shown in gray. (B) As in (A) but using a control α-DEC20S antibody.

Conclusion

We have shown that split DnaE inteins can be engineered for the efficient generation and isolation of protein α-thioesters. Furthermore, the strategy can be seamlessly integrated with EPL, and one pot purification/ligation can be performed without isolation of the α-thioester intermediates. We note, however, that the ligation step (i.e., NCL) still requires the use of high concentrations (high μM or above) of N-terminal cysteine peptide for efficient reactions. The strong and specific interaction between the two intein fragments facilitates the purification of protein α-thioesters under a variety of conditions, including strong denaturants, and the isolation of these products directly from cell lysates proceeds significantly faster than via many mainstream EPL strategies, which often take multiple days and several intermediate enrichment/purification steps. Additionally, while split intein fusions are usually associated with low levels of expression, we show here that certain DnaE IntN fragments express to the same levels as commonly used contiguous inteins when fused to a monomodal Ab. Moreover, the absence of premature cleavage events allowed us to generate semisynthetic proteins, such as H2B-K120Ac in far superior yields than those of previously established protocols. Importantly, we have shown the utility of chromatic (SEC) coupled to multiple angle light scattering (MALS) analysis confirmed that the antibody retained its tetrameric folded state after thiolysis and ligation (Figure 6D). We also performed MS analysis of the deglycosylated and fully reduced antibody, which confirmed the formation of a stable, nonreducible amide bond between the αDEC20S heavy chain and the fluorescent peptide with a 75% yield (Figure 6E). Importantly, we demonstrated that the semisynthetic αDEC-CGK(Fl) retains its ability to bind the DEC20S receptor to the same extent as a control αDEC20S, previously shown to be fully functional in vivo (Figure 7).
of this methodology for the modification of complex biomolecules such as an IgG. Thus, streamlined EPL via our split DnaE intein column should provide an efficient route to site-specifically modified proteins for basic biochemical research as well as translational applications.

**EXPERIMENTAL SECTION**

**Preparation of IntC Column.** Npu-CA–AA-Cys-OMe peptide (0.5 μmol per mL of resin) was dissolved in 2 column volumes (CV) of coupling buffer (50 mM Tris-HCl at pH 8.5, 250 μL for 15 CV of resin) and treated with 25 mM TCEP from a 1 M stock for 15 min. The peptide solution was then added to 1 CV of agarose SulfoLink resin (from Pierce, loading: 18.4 μmol iodoacetyl groups/mL of resin) in a small fritted column and incubated for 15 min on a nutator, followed by 30 min standing at rt. The column flow-through was collected, and the column was washed twice with 2 CV of coupling buffer. Unreacted iodoacetyl groups on the resin were blocked by a treatment with 50 mM H-Cys-OMe in coupling buffer for 15 min on a nutator, followed by 30 min standing at rt. Column was washed twice with 1 CV of coupling buffer, 2 CV of 1 M NaCl, and finally 2 CV of water. IntC columns were stored in storage buffer (100 mM NaCl, 150 mM MES, 1 mM EDTA, 1 mM TCEP, pH 7.2), and 300 μL of binding buffer (100 mM NaCl, 500 mM NaCl, 1 mM EDTA, 1 mM TCEP, pH 7.2) was caged and incubated with 75 μM CGK(Fl) peptide for 18 h. The column was capped and incubated with 75 μM CGK(Fl) peptide for 18 h. The column was washed three times with 3 CV of Ab elution buffer. Elutions containing dDEC205-MES were combined and concentrated down to 20 μL. Ligature was initiated by addition of 1 mM CGK(Fl) peptide and 1 mM TCEP and adjusting pH to 7.5–8.0. The reaction was incubated in the dark at rt for 48 h and monitored by SDS-PAGE imaging using a fluorescence scanner and coomassie staining. Once the reaction was completed, the ligated Ab was diluted to 200–500 μL and dialyzed into 100 mM phosphate, 150 mM NaCl, 1 mM EDTA, 1 mM TCEP, pH 7.2.

**ASSOCIATED CONTENT**

**Supporting Information**

Full experimental details including materials and methods, cloning, protein purification, and characterization, calculation of thiolysis and recovery yields, IntC column regeneration protocols, and antibody characterization. This material is available free of charge via the Internet at http://pubs.acs.org.

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**Notes**

The authors declare no competing financial interest.

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