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Protein Engineering **Hot Paper**

Enzymatic C-to-C Protein Ligation

Fabian B. H. Rehm[+], Tristan J. Tyler[+], Simon J. de Veer, David J. Craik,* and Thomas Durek**

Abstract: *Transpeptidase-catalyzed protein and peptide modifications have been widely utilized for generating conjugates of interest for biological investigation or therapeutic applications. However, all known transpeptidases are constrained to ligating in the N-to-C orientation, limiting the scope of attainable products. Here, we report that an engineered asparaginyl ligase accepts diverse incoming nucleophile substrate mimetics, particularly when a means of selectively quenching the reactivity of byproducts released from the recognition sequence is employed. In addition to directly catalyzing formation of L-/D- or α-/β-amino acid junctions, we find C-terminal Leu-ethylenediamine (Leu-Eda) motifs to be bona fide mimetics of native N-terminal Gly-Leu sequences. Appending a C-terminal Leu-Eda to synthetic peptides or, via an intein-splicing approach, to recombinant proteins enables direct transpeptidase-catalyzed C-to-C ligations. This work significantly expands the synthetic scope of enzyme-catalyzed protein transpeptidation reactions.*

Introduction

The generation of defined, site-specifically modified proteins is a crucial aspect of modern chemical biology, enabling new ways of studying protein function and the manufacture of therapeutic or diagnostic bioconjugates.[1] Although several regio-and chemoselective chemical conjugation strategies have been developed, these approaches often require case-by-case optimization and the synthesis of specialized reagents.[2] By contrast, enzymatic approaches readily achieve exquisite specificity following genetic incorporation of a recognition motif into the protein of interest.[3] In particular, recombinantly-accessible transpeptidases that operate on natural amino acid substrates have seen widespread use, with the best studied example being the bacterial enzyme sortase A.[4] Recently, asparaginyl endopeptidases (AEPs) that preferentially catalyze transpeptidation reactions (asparaginyl ligases) have emerged as next-generation enzymes due to their short tripeptide recognition motifs and high catalytic efficiencies.[5] Asparaginyl ligases are remarkably versatile enzymes—they have been employed for peptide and protein head-to-tail cyclization,[6] N- or C-terminal protein labeling with small synthetic peptides,[7] the generation of N-to-C protein-protein fusions, including polyproteins,[8] the promiscuous modification of protein N-termini on red blood cell and extracellular vesicle surfaces,[9] and for C-terminal protein labeling with structurally diverse, non-peptidyl amines.[20] However, like all known transpeptidases, asparaginyl ligases have, until now, been thought to be constrained to operating on α-amino acid substrates and in the N-to-C orientation. Here we expand the synthetic scope of asparaginyl ligase-catalyzed reactions via substrate mimetics, enabling intermolecular ligation at L-/D- and α-/β-amino acid ligation junctions and C-to-C protein fusions.

Results and Discussion

[C247A]*Oa*AEP1 is a catalytically improved asparaginyl ligase that is accessible via recombinant production in *E. coli*.[10] It acts on Asn-Gly-Leu (P1-P1′-P2′) motifs and, via a thioester-linked acyl enzyme intermediate, joins the P1 Asn to a protein or peptide bearing a compatible N-terminal dipeptide (e.g. Gly-Leu, P1″-P2″) (Figure 1A). As the initial P1′-P2′ leaving group is released from the recognition motif over the course of the reaction, this byproduct competes with the desired incoming nucleophile N-termini, culminating in a product-limiting equilibrium. By simply extending the Asn-Gly-Leu motif by a single His, the nucleophilicity of released Gly-Leu-His byproducts can be quenched via metal complex formation upon addition of Ni^{2+} to the reaction (Figure 1A) thereby suppressing the reverse reaction.[8a] We hypothesized that this quenching approach could enable the efficient incorporation of suboptimal incoming N-termini (P1″-P2″) which would otherwise be readily outcompeted by the more efficiently incorporated Gly-Leu (P1′-P2′) byproduct. We synthesized a series of N-terminal Gly-Leu substrate mimetics and monitored their conjugation to a model peptide substrate (Ac-RWRGWR**NGL**H, recognition sequence in bold). Reactions contained 100 μM peptide-**NGL**H, 500 μM substrate mimetic, 100 nM [C247A]*Oa*AEP1, and 2 mM $NiSO_4$ in 100 mM Tris-HCl, pH 7.5, and were run until equilibrium was reached (1 h,

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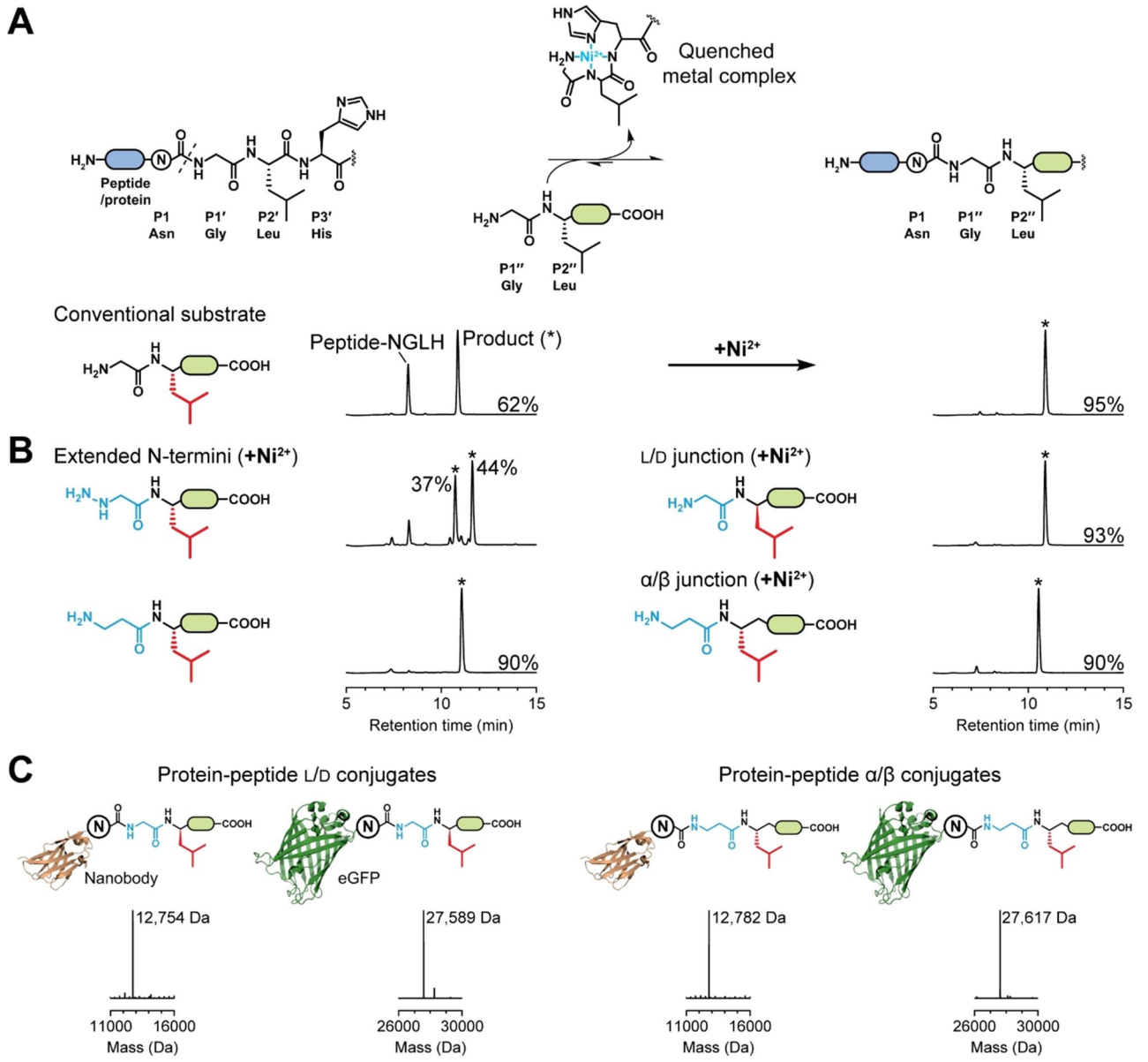

Figure 1. [C247A]*Oa*AEP1-catalyzed formation of non-natural ligation junctions. A) Scheme illustrating the Ni^{2+}-mediated nucleophile quenching strategy. Below the scheme are RP-HPLC chromatograms (280 nm) of the ligation of a model NGLH-containing peptide substrate (Ac-RWRGWR**NGL**H, 100 μM) to the conventional substrate (GLRL, at 2 equiv) with/without $NiSO_4$ addition (as indicated, 2 mM) as catalyzed by 100 nM [C247A]*Oa*AEP1 in 100 mM Tris-HCl, pH 7.5, for 1 h. B) Ligation of substrate mimetics to the model **NGL**H-containing peptide as in (A), except all shown reactions were supplemented with $NiSO_4$ and the mimetic peptides were provided at 5 equiv Figures S1 and S2 show RP-HPLC chromatograms for all Ni^{2+}-free reactions and MALDI-TOF MS traces for all reactions, respectively. Table S1 lists observed and calculated peptide conjugate masses. C) Ligation of the D- and β-amino acid substrate mimetics (500 μM) to **NGL**H-modified protein substrates (a nanobody, VHH_{6e}, and eGFP, as indicated; 50 μM) as catalyzed by 400 nM [C247A]*Oa*AEP1 in 100 mM HEPES pH 7, 25 °C for 2 h. Shown are reconstructed ESI-MS spectra with the observed product masses. Table S2 lists observed and calculated protein conjugate masses.

25 °C), then analyzed by RP-HPLC (Figure 1B, Figure S1) and MALDI-TOF MS (Figure S2). We found that replacement of the P1″ Gly with N-terminally extended residues was well tolerated, with both an N-terminal β-Ala and a hydrazino Gly analogue being efficiently incorporated, although the latter produced two putative products on RP-HPLC but a single major mass on MALDI-TOF MS of the crude reaction mixture, potentially suggesting ligation through either the β- or α-nitrogen atoms (Figure 1B, Figures S1 and S2). A similar product mixture was observed in a study that examined ribosomal incorporation of hydrazino Phe.[11] Substitution of the critical P2″ Leu residue with D-Leu was also compatible, enabling intermolecular cross-chiral ligation. Similarly, α-/β-amino acid ligation junctions could be readily formed with an N-terminal β-Ala-β-homoLeu sequence when Ni^{2+} was supplemented in the reaction (90 % conversion to product with Ni^{2+}, 48 % without; Figure S1). To demonstrate these non-natural L-/D- and

α-/β-amino acid ligation junctions in the context of C-terminal protein modification, we prepared enhanced green fluorescent protein (eGFP) and a single-domain antibody (nanobody) with C-terminal extended **NGL**H motifs. Defined protein-peptide conjugates containing D- or β-amino acids at the ligation site could then be generated using 10 mol eq. of the substrate mimetics (Figure 1C).

Given that [C247A]*Oa*AEP1 could readily produce diverse, non-natural N-to-C ligation junctions intermolecularly, we took inspiration from retro (reversed sequence) peptidomimetics,[12] to examine whether C-terminal mimetics of the N-terminal Gly-Leu sequence could also serve as substrates. As the P2″ Leu residue is a key driver of transpeptidation, we hypothesized that a C-terminal Leu modified with a suitable diamine at the carboxy group could potentially mimic the N-terminal Gly-Leu sequence. Initially, we prepared an N-acetylated peptide bearing a C-terminal Leu-hydrazide motif (Ac-GLRL-hydrazide) and examined its C-to-C ligation to our model **NGL**H-containing peptide under identical reaction conditions to the earlier substrate mimetics. However, we found incorporation to be substantially more challenging, reaching only 2 % and 20 % conversion to product in the absence and presence of Ni^{2+}, respectively (Figure 2A and Figure S1). To assess whether a diamine that distances the nucleophilic amine from the Leu sidechain comparably to Gly-Leu would be more appropriate, we prepared a peptide carrying a C-terminal Leu-ethylenediamine motif (Leu-Eda). This peptide could be readily synthesized using standard Fmoc solid-phase peptide chemistry by treating 2-chlorotrityl chloride resin with 10 % (v/v) Eda in DMF containing DIPEA prior to coupling of the first Leu (Figure S3). We found that [C247A]*Oa*AEP1 could readily ligate this retro substrate mimetic to the model **NGL**H-containing peptide at levels comparable to the N-terminal substrate mimetics, presenting the first example of direct transpeptidase-catalyzed ligation in the C-to-C orientation (Figure 2A). To verify that the Leu-Eda motif is a legitimate substrate mimetic, i.e. that it contains enzyme recognition elements beyond the amine, we synthesized a range of variants where the P2″ Leu was substituted (Figure 2B). In all cases, replacement of Leu resulted in substantially reduced ligation efficiency, further indicating that a C-terminal Leu-Eda genuinely mimics native N-terminal Gly-Leu substrates (Figure 2B). Next, we prepared defined protein-peptide C-to-C conjugates using the **NGL**H-containing nanobody and eGFP substrates and 10 mol eq. of the Leu-Eda peptide (Figure 2C). Protein-peptide C-to-C conjugates have previously been generated in multiple steps, relying on transpeptidase-mediated attachment of a click handle and a subsequent click reaction for peptide attachment.[13] Here we do so in a single step, obviating the need for specialized click handle building blocks and intermediate purification steps.

Having established the C-terminal Leu-Eda motif as an efficient retro substrate mimetic, we turned our attention to short bifunctional linker peptides that could be ligated

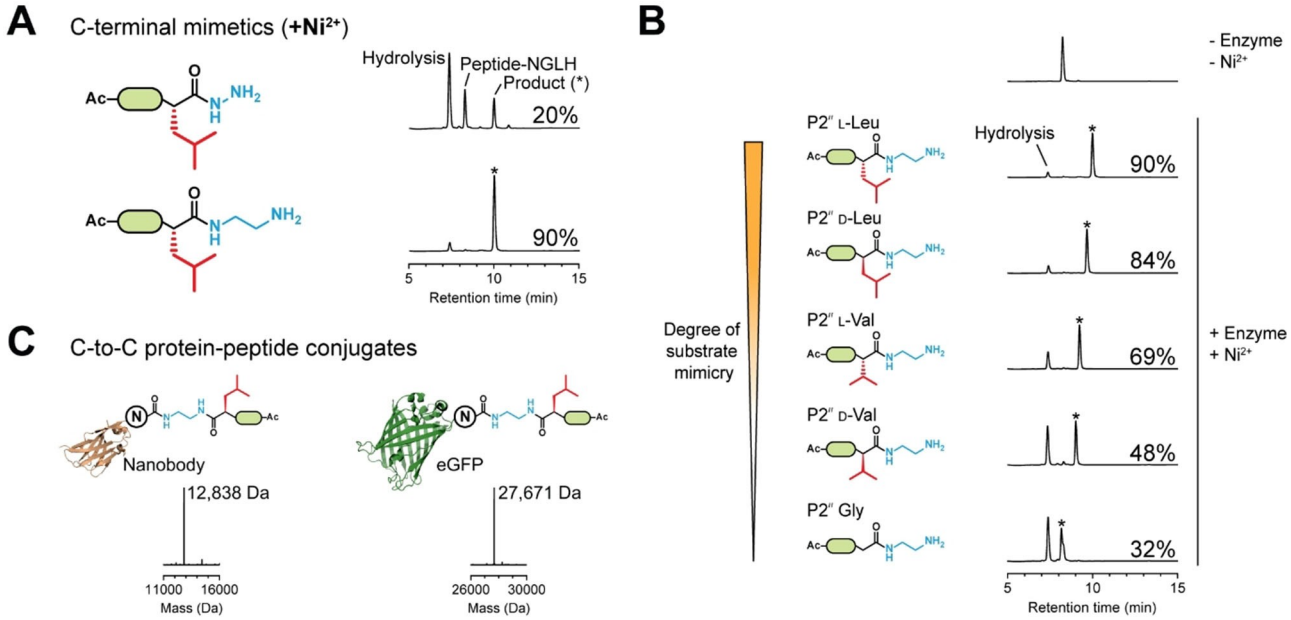

Figure 2. [C247A]*Oa*AEP1-catalyzed C-to-C ligation. A) Ligation of C-terminal substrate mimetics (Ac-GLRL-hydrazide or Ac-GLRL-Eda) to the model substrate Ac-RWRGWR**NGL**H. Reactions were conducted as in Figure 1A. Shown are RP-HPLC chromatograms (280 nm) of the crude reaction mixtures and % conversion to product (*) is indicated. B) Assessing the degree of substrate mimicry via by monitoring the ligation of a series of Xaa-Eda (Ac-GLRX-Eda) peptides to Ac-RWRGWR**NGL**H as in (A). Figures S4 and S5 show RP-HPLC chromatograms for all Ni^{2+}-free reactions and MALDI-TOF MS traces for all reactions, respectively. The chromatogram for the P2″ Leu-Eda peptide is the same as shown in Figure 2A. Table S1 lists observed and calculated peptide conjugate masses. C) Ligation of the Leu-Eda substrate mimetics to **NGL**H-modified protein substrates as in Figure 1C. Shown are reconstructed ESI-MS spectra with the observed product masses. Table S2 lists observed and calculated protein conjugate masses.

through both termini. We anticipated that a native N-terminal Gly-Leu sequence would readily outcompete a C-terminal Leu-Eda motif. Thus, with the aim of controlling the orientation of peptide conjugation through addition of Ni^{2+}, we designed a peptide with an extended Gly-Leu-His N-terminal sequence and a C-terminal Leu-Eda (GLHRL-Eda) (Figure 3A). Ni^{2+} addition would thus simultaneously function to control peptide orientation (by deactivating the N-terminal GLH) and to improve Leu-Eda ligation efficiency by quenching byproducts from **NGL**H-containing substrates. Conjugation of the GLH-linker-L-Eda to our model **NGL**H-containing peptide indeed revealed that ligation in the absence of Ni^{2+} predominantly proceeded through the N-terminus of the bifunctional peptide. In contrast, Ni^{2+} addition (10 mol eq. relative to [bifunctional peptide]) resulted in almost exclusive ligation through the C-terminus as evidenced by retention time shifts of product peaks of identical mass as assessed by RP-HPLC and MALDI-TOF MS analysis (Figure 3A and Figure S6A). In further support of this Ni^{2+}-mediated orientation control is the finding that ligating excess (4 eq.) of our model **NGL**H peptide to the bifunctional linker peptide yielded peptide-linker-peptide C-to-C ligation products only when Ni^{2+} was not added and, as a result, the GLH motif was not quenched (Figure S6B). Furthermore, we found trypsin digests of peptide-linker conjugates to contain C-to-C or N-to-C peptides spanning the ligation site only in the presence or absence of Ni^{2+}, respectively (Figure S6C, D).

We then sought to apply this bifunctional linker peptide for the generation of protein-linker-protein C-to-C fusions. Following initial attachment of the peptide to a protein bearing a C-terminal **NGL**H sequence, a second protein-

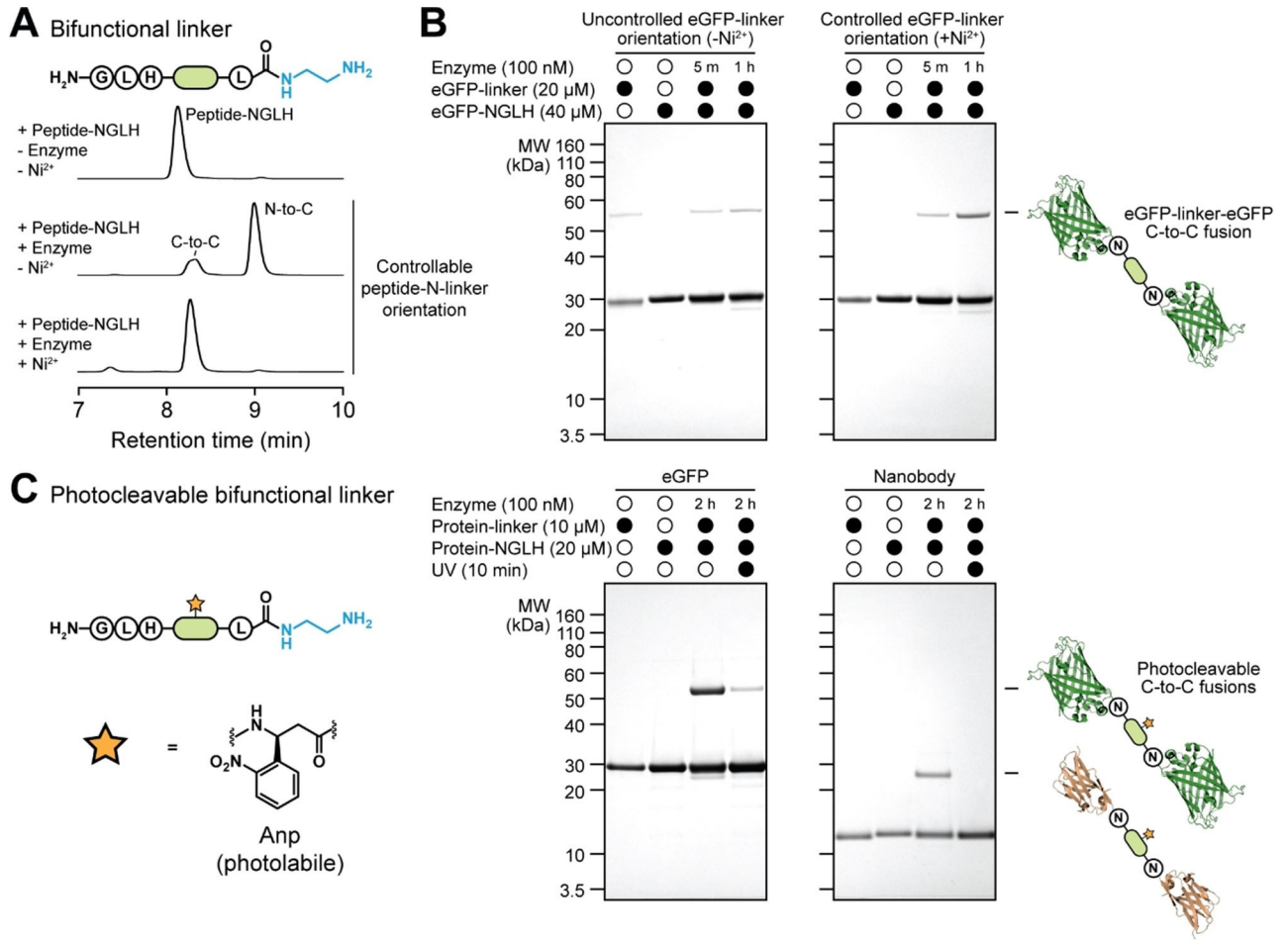

Figure 3. Generation of C-to-C protein fusions using orientation-controllable bifunctional linker peptides. A) Ligation of the bifunctional linker peptide (GLHRL-Eda) to the model substrate Ac-RWRGWR**NGL**H with/without Ni^{2+}, as indicated. Reactions were conducted as in Figure 1A except that the bifunctional linker was provided at 1 mM (10 mol equiv), enzyme at 200 nM, and $NiSO_4$ at 5 mM. Reactions containing Ni^{2+} were incubated for 15 min prior to enzyme addtion. Shown are RP-HPLC chromatograms (280 nm) of the crude reaction mixtures. Figure S6 shows MALDI-TOF MS traces of the same reactions. Table S1 lists observed and calculated peptide conjugate masses. B) SDS-PAGE analysis of eGFP-linker-eGFP C-to-C fusion formation when the initial attachment of the linker to eGFP-**NGL**H is controlled via Ni^{2+} addition or uncontrolled. $NiSO_4$ was removed prior to protein-linker to protein-**NGL**H ligations. C) SDS-PAGE analysis of the formation of photocleavable eGFP and nanobody (VHH_{6e}) protein-linker-protein C-to-C fusions and their cleavage via UV irradiation. Initial linker (GLHG-Anp-L-Eda) attachment was conducted in the presence of Ni^{2+} to control ligation orientation. Reconstructed ESI-MS spectra of the protein-linker conjugates are shown in Figure S7. Table S2 lists observed and calculated protein conjugate masses. Anp = 3-amino-3-(2-nitro-phenyl)propionic acid.

protein ligation would need to be conducted to generate such a fusion construct. We hypothesized that ligation through the more efficient N-terminal Gly-Leu sequence in the second [C247A]*Oa*AEP1-catalyzed reaction would result in enhanced conversion to product. To test this hypothesis, we examined the generation of eGFP-linker-eGFP fusion proteins following attachment of the bifunctional linker peptide to eGFP-**NGL**H in the presence or absence of Ni^{2+}—when the linker orientation is controlled to ligate through Leu-Eda or uncontrolled, respectively (Figure 3B, Figure S7). As anticipated, controlled attachment of the linker, with intermediate removal of Ni^{2+}, yielded more product in the subsequent reaction (Figure 3B, Figure S8). In fact, when linker attachment was uncontrolled (-Ni^{2+}) we also observed unwanted, premature formation of protein-linker-protein conjugates during the attachment step itself, further supporting a need for Ni^{2+} inclusion during this initial conjugation.

Next, to demonstrate the utility of a synthetic linker dividing protein-protein conjugates, we generated photocleavable C-to-C protein fusions via incorporation of photolabile 3-amino-3-(2-nitro-phenyl)propionic acid (Anp) into the synthetic linker (GLHG-Anp-L-Eda) (Figure 3C). Following Ni^{2+}-controlled attachment of the linker and subsequent generation of the protein-linker-protein fusions via consecutive [C247A]*Oa*AEP1-catalyzed reactions with intermediate removal of excess linker peptide, the C-to-C nanobody and eGFP fusions could be cleaved via exposure to UV irradiation (Figure 3C). Such photocleavable nanobody-nanobody C-to-C fusions, where both N-terminal antigen-binding faces are unperturbed, could be employed as catch-and-release delivery systems.

Although we could readily generate synthetic peptides bearing C-terminal Leu-Eda motifs by standard solid-phase peptide chemistry, we next investigated alternative approaches for the preparation of proteins bearing this motif. Given the well-established methodologies for generating recombinant protein-thioesters through intein splicing,[14] we hypothesized that direct aminolysis of protein-Leu-thioesters with Eda could yield recombinant Leu-Eda-functionalized proteins (Figure 4A). Indeed, we found that we could readily convert SUMO-Leu-2-mercaptoethanesulfonate (MES) thioesters, generated via standard *Mycobacterium xenopi* GyrA intein splicing, to SUMO-Leu-Eda by simply buffer exchanging (PD10 column) the thiolysed chitin column eluate into pH 7.5 20 mM HEPES/50 mM NaOAc buffer containing 100 mM NaCl, 20 mM MESNa, and 0.5 M Eda and incubating at 25 °C for 6–8 h (Figure 4A and

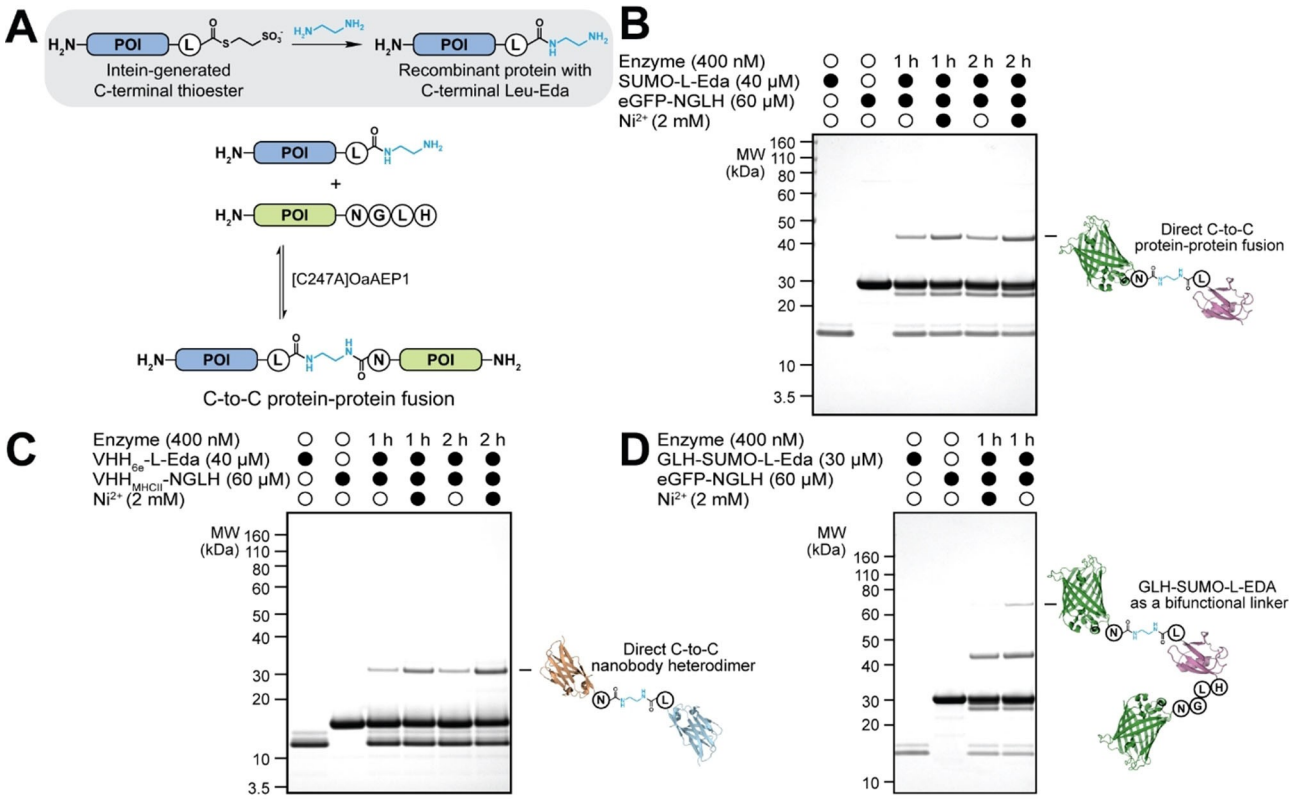

Figure 4. Direct [C247A]*Oa*AEP1-catalyzed C-to-C ligation of recombinant proteins. A) Scheme detailing the intein-mediated strategy for generating recombinant proteins bearing a C-terminal Leu-Eda motif. B) SDS-PAGE analysis of the direct C-to-C ligation of SUMO-L-Eda to eGFP-**NGL**H with/without $NiSO_4$, as indicated. C) Generation of a nanobody C-to-C heterodimer with reactions and analysis as in (B). D) SDS-PAGE analysis of the ligation of bifunctional GLH-SUMO-L-Eda to eGFP-**NGL**H with/without $NiSO_4$, as indicated. The addition of Ni^{2+} suppresses formation of the trimeric protein conjugate (lane 3). Reconstructed ESI-MS spectra of the C-terminal Leu-Eda-modified proteins are shown in Figure S9. POI = protein of interest.

Figure S9). Like all protein substrates prepared in this study, this model SUMO substrate had an N-terminal Pro to ensure that any observed ligated product could be attributed to ligation through its C-terminus. Combining SUMO-Leu-Eda, eGFP-**NGL**H and [C247A]*Oa*AEP1, all recombinantly produced proteins, resulted in the direct formation of genetically inaccessible SUMO-eGFP C-to-C fusions (Figure 4B). Supplementing these reactions with $NiSO_4$ facilitated enhanced product formation. Similarly, we could prepare nanobody C-to-C heterodimers by ligating a nanobody bearing a C-terminal Leu-Eda to another with a C-terminal **NGL**H sequence (Figure 4C). In this example, we ligated VHH_{6e} (also known as VHH05), which binds a 14 amino acid tag (QADQEAKELARQIS, 6e-tag),[15] to VHH_{MHCII} (also known as VHH07), which recognizes major histocompatibility complex class II (MHCII) molecules.[16] VHH_{MHCII} has been used to deliver antigenic peptides or proteins to MHCII-positive cells, which include professional antigen-presenting cells (such as dendritic cells, B cells, and macrophages), to develop self-antigen tolerance for the treatment of autoimmune disorders[17] or to elicit immune responses to a foreign antigen for the development of vaccines, as was recently demonstrated for the SARS-CoV-2 Spike receptor-binding domain.[18] Thus, our C-to-C VHH_{6e}-VHH_{MHCII} conjugates could be useful for the rapid screening of the delivery of diverse 6e-tagged antigens to MHCII for the development of similar biomedical applications. Finally, akin to the bifunctional peptides introduced above, we prepared bifunctional GLH-SUMO-Leu-Eda and monitored its ligation to eGFP-**NGL**H. This reaction enabled transpeptidase-catalyzed formation of trimeric protein fusions from two protein substrates in the absence of uncontrolled oligomerization (Figure 4D).

Conclusion

Transpeptidases are valuable tools for protein engineering. Here, we demonstrate that the asparaginyl ligase [C247A]*Oa*AEP1 can break free of standard transpeptidation reaction constraints via the design and characterization of peptides that mimic the native N-terminal Gly-Leu substrates. We report the first examples of intermolecular L-/D- and α-/β-amino acid ligation junction formation at the C-termini of both peptides and proteins. However, like all previously reported transpeptidase-catalyzed ligations, these reactions operate exclusively in the N-to-C orientation. This fundamental orientation restriction could be overcome using C-terminal Leu-Eda retro (reversed sequence) substrate mimetics, enabling ligation in the C-to-C orientation. To our knowledge, the Leu-Eda motif represents the first described C-terminal retro mimetic of an N-terminal peptide substrate. We apply synthetic peptides bearing this C-terminal motif for the generation of protein-peptide and, via short, orientation-controllable bifunctional linkers, protein-linker-protein C-to-C fusions. Finally, we demonstrate that synthetic peptide linkers can be entirely circumvented by preparing recombinant proteins with C-terminal Leu-Eda via aminolysis of intein-generated protein-thioesters, enabling direct formation of unnatural C-to-C fusion proteins using only recombinant protein substrates and enzyme. New routes to C-to-C fusion proteins, which are genetically inaccessible, are sought after for the preparation of protein fusions where key functional features are located at the protein N-termini, as is the case for nanobodies.[13c,19] Finally, a bifunctional protein (SUMO), modified with an N-terminal GLH sequence and a C-terminal Leu-Eda, enabled the controlled formation of trimeric protein conjugates with an intermediate C-to-C ligation junction. Thus, this study greatly expands the scope of transpeptidase-catalyzed reactions.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the Supporting Information of this article.

Keywords: Biorthogonal · Enzyme Bioconjugation · Enzyme Catalysis · Protein Engineering · Site-Specificity

[1] O. Boutureira, G. J. Bernardes, *Chem. Rev.* **2015**, *115*, 2174–2195.
[2] M. Kumar, N. C. Reddy, V. Rai, *Chem. Commun.* **2021**, *57*, 7083–7095.
[3] Y. Zhang, K. Y. Park, K. F. Suazo, M. D. Distefano, *Chem. Soc. Rev.* **2018**, *47*, 9106–9136.
[4] a) J. M. Antos, G. L. Chew, C. P. Guimaraes, N. C. Yoder, G. M. Grotenbreg, M. W. Popp, H. L. Ploegh, *J. Am. Chem. Soc.* **2009**, *131*, 10800–10801; b) J. M. Antos, M. W. Popp, R. Ernst, G. L. Chew, E. Spooner, H. L. Ploegh, *J. Biol. Chem.* **2009**, *284*, 16028–16036; c) M. W. Popp, J. M. Antos, G. M. Grotenbreg, E. Spooner, H. L. Ploegh, *Nat. Chem. Biol.* **2007**, *3*, 707–708; d) J. M. Antos, G. M. Miller, G. M. Grotenbreg, H. L. Ploegh, *J. Am. Chem. Soc.* **2008**, *130*, 16338–16343.
[5] a) K. S. Harris, T. Durek, Q. Kaas, A. G. Poth, E. K. Gilding, B. F. Conlan, I. Saska, N. L. Daly, N. L. van der Weerden, D. J. Craik, M. A. Anderson, *Nat. Commun.* **2015**, *6*, 10199; b) G. K. T. Nguyen, S. J. Wang, Y. B. Qiu, X. Hemu, Y. L. Lian, J. P. Tam, *Nat. Chem. Biol.* **2014**, *10*, 732–738; c) F. B. H. Rehm, T. J. Tyler, J. Xie, K. Yap, T. Durek, D. J. Craik, *ChemBioChem* **2021**, *22*, 2079–2086.

[6] a) J. Du, K. Yap, L. Y. Chan, F. B. H. Rehm, F. Y. Looi, A. G. Poth, E. K. Gilding, Q. Kaas, T. Durek, D. J. Craik, *Nat. Commun.* **2020**, *11*, 1575; b) X. Hemu, Y. Qiu, G. K. Nguyen, J. P. Tam, *J. Am. Chem. Soc.* **2016**, *138*, 6968–6971; c) K. H. Ngo, R. Yang, P. Das, G. K. T. Nguyen, K. W. Lim, J. P. Tam, B. Wu, A. T. Phan, *Chem. Commun.* **2020**, *56*, 1082–1084; d) G. K. Nguyen, A. Kam, S. Loo, A. E. Jansson, L. X. Pan, J. P. Tam, *J. Am. Chem. Soc.* **2015**, *137*, 15398–15401; e) N. Pi, M. Gao, X. Cheng, H. Liu, Z. Kuang, Z. Yang, J. Yang, B. Zhang, Y. Chen, S. Liu, Y. Huang, Z. Su, *Biochemistry* **2019**, *58*, 3005–3015; f) F. B. H. Rehm, M. A. Jackson, E. De Geyter, K. Yap, E. K. Gilding, T. Durek, D. J. Craik, *Proc. Natl. Acad. Sci. USA* **2019**, *116*, 7831–7836; g) I. Saska, A. D. Gillon, N. Hatsugai, R. G. Dietzgen, I. Hara-Nishimura, M. A. Anderson, D. J. Craik, *J. Biol. Chem.* **2007**, *282*, 29721–29728; h) K. Yap, J. Du, F. B. H. Rehm, S. R. Tang, Y. Zhou, J. Xie, C. K. Wang, S. J. de Veer, L. H. L. Lua, T. Durek, D. J. Craik, *Nat. Protoc.* **2021**, *16*, 1740–1760; i) K. Yap, J. Q. Du, F. Y. Looi, S. R. Tang, S. J. de Veer, A. R. Bony, F. B. H. Rehm, J. Xie, L. Y. Chan, C. K. Wang, D. J. Adams, L. H. L. Lua, T. Durek, D. J. Craik, *Green Chem.* **2020**, *22*, 5002–5016.

[7] a) G. K. Nguyen, Y. Cao, W. Wang, C. F. Liu, J. P. Tam, *Angew. Chem. Int. Ed.* **2015**, *54*, 15694–15698; *Angew. Chem.* **2015**, *127*, 15920–15924; b) F. B. H. Rehm, T. J. Harmand, K. Yap, T. Durek, D. J. Craik, H. L. Ploegh, *J. Am. Chem. Soc.* **2019**, *141*, 17388–17393; c) T. J. Harmand, D. Bousbaine, A. Chan, X. Zhang, D. R. Liu, J. P. Tam, H. L. Ploegh, *Bioconjugate Chem.* **2018**, *29*, 3245–3249; d) D. Zhang, Z. Wang, S. Hu, S. Balamkundu, J. To, X. Zhang, J. Lescar, J. P. Tam, C. F. Liu, *J. Am. Chem. Soc.* **2021**, *143*, 8704–8712.

[8] a) F. B. H. Rehm, T. J. Tyler, K. Yap, T. Durek, D. J. Craik, *Angew. Chem. Int. Ed.* **2021**, *60*, 4004–4008; *Angew. Chem.* **2021**, *133*, 4050–4054; b) Y. Deng, T. Wu, M. Wang, S. Shi, G. Yuan, X. Li, H. Chong, B. Wu, P. Zheng, *Nat. Commun.* **2019**, *10*, 2775.

[9] a) T. J. Harmand, N. Pishesha, F. B. H. Rehm, W. Ma, W. B. Pinney, Y. J. Xie, H. L. Ploegh, *ACS Chem. Biol.* **2021**, *16*, 1201–1207; b) T. C. Pham, M. K. Jayasinghe, T. T. Pham, Y. Yang, L. Wei, W. M. Usman, H. Chen, M. Pirisinu, J. Gong, S. Kim, B. Peng, W. Wang, C. Chan, V. Ma, N. T. H. Nguyen, D. Kappei, X. H. Nguyen, W. C. Cho, J. Shi, M. T. N. Le, *J. Extracell Vesicles* **2021**, *10*, e12057.

[10] R. Yang, Y. H. Wong, G. K. T. Nguyen, J. P. Tam, J. Lescar, B. Wu, *J. Am. Chem. Soc.* **2017**, *139*, 5351–5358.

[11] J. A. Killian, M. D. Van Cleve, Y. F. Shayo, S. M. Hecht, *J. Am. Chem. Soc.* **1998**, *120*, 3032–3042.

[12] J. Rai, *Chem. Biol. Drug Des.* **2019**, *93*, 724–736.

[13] a) R. W. Cheloha, F. A. Fischer, A. W. Woodham, E. Daley, N. Suminski, T. J. Gardella, H. L. Ploegh, *Nat. Commun.* **2020**, *11*, 2087; b) R. W. Cheloha, F. A. Fischer, T. J. Gardella, H. L. Ploegh, *RSC Chem. Biol.* **2021**, *2*, 1692–1700; c) M. D. Witte, J. J. Cragnolini, S. K. Dougan, N. C. Yoder, M. W. Popp, H. L. Ploegh, *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 11993–11998.

[14] A. C. Conibear, E. E. Watson, R. J. Payne, C. F. W. Becker, *Chem. Soc. Rev.* **2018**, *47*, 9046–9068.

[15] J. Ling, R. W. Cheloha, N. McCaul, Z. J. Sun, G. Wagner, H. L. Ploegh, *Mol. Immunol.* **2019**, *114*, 513–523.

[16] J. N. Duarte, J. J. Cragnolini, L. K. Swee, A. M. Bilate, J. Bader, J. R. Ingram, A. Rashidfarrokhi, T. Fang, A. Schiepers, L. Hanke, H. L. Ploegh, *J. Immunol.* **2016**, *197*, 4838–4847.

[17] N. Pishesha, T. Harmand, L. Y. Smeding, W. Ma, L. S. Ludwig, R. Janssen, A. Islam, Y. J. Xie, T. Fang, N. McCaul, W. Pinney III, H. R. Sugito, M. A. Rossotti, G. Gonzalez-Sapienza, H. L. Ploegh, *Nat. Biomed. Eng.* **2021**, *5*, 1389–1401.

[18] N. Pishesha, T. J. Harmand, P. W. Rothlauf, P. Praest, R. K. Alexander, R. van den Doel, M. J. Liebeskind, M. A. Vakaki, N. McCaul, C. Wijne, E. Verhaar, W. Pinney III, H. Heston, L. M. Bloyet, M. C. Pontelli, M. X. G. Ilagan, R. Jan Lebbink, W. J. Buchser, E. Wiertz, S. P. J. Whelan, H. L. Ploegh, *Proc. Natl. Acad. Sci. USA* **2021**, *118*, e2116147118.

[19] K. Wagner, M. J. Kwakkenbos, Y. B. Claassen, K. Maijoor, M. Bohne, K. F. van der Sluijs, M. D. Witte, D. J. van Zoelen, L. A. Cornelissen, T. Beaumont, A. Q. Bakker, H. L. Ploegh, H. Spits, *Proc. Natl. Acad. Sci. USA* **2014**, *111*, 16820–16825.

[20] F. B. H. Rehm, T. J. Tyler, K. Yap, S. J. de Veer, D. J. Craik, T. Durek, *J Am Chem Soc* **2021**, *143*, 19498–19504.

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