The success of genome sequencing has heightened the demand for new means to manipulate proteins. An especially desirable goal is the ability to modify a target protein at a specific site with a functional group of orthogonal reactivity. Here, we achieve that goal by exploiting the intrinsic electrophilicity of the thioester intermediate formed during intein-mediated protein splicing. Detailed kinetic analyses of the reaction of nitrogen nucleophiles with a chromogenic small-molecule thioester revealed that the α-hydrazino acetyl group was the optimal nucleophile for attacking a thioester at neutral pH to form a stable linkage. A bifunctional reagent bearing an α-hydrazino acetamido and azido group was synthesized in high overall yield. This reagent was used to attack the thioester linkage between a target protein and intein, and thereby append an azido group to the target protein in a single step. The azido protein retained full biological activity. Furthermore, its azido group was available for chemical modification by Huisgen 1,3-dipolar azide–alkyne cycloaddition. Thus, the mechanism of intein-mediated protein splicing provides the means to install a useful functional group at a specific site—the C terminus—of virtually any protein.

Introduction

Proteins are nucleophilic. Their side chains contain no electrophiles, other than the disulfide bonds of cystines or functional groups installed by post-translational modification. Accordingly, the chemical reactivity of proteins necessarily entails nucleophilic side chains, such as those of lysine[1] and cysteine.[2,3] The prevalence of these residues obviates control over the regiochemistry of reactions,[4] and produces heterogeneous reaction products—often at the expense of biological function.[5,6]

An intermediate that forms during the intein-mediated splicing of proteins contains an electrophile—a thioester (Scheme 1).[6] The orthogonal reactivity of this functional group can be exploited for the site-specific modification of a protein either by reaction with cysteine derivatives[7] or tandem reaction with a small-molecule thiol and amine.[8] Although thiols are potent nucleophiles for thioesters, the resultant thioesters are inherently unstable to hydrolysis;[9] this makes the simple transthioesterification of an intein-derived thioester unsuitable for the chemical modification of proteins. The powerful methods of native chemical ligation[10] and expressed protein ligation[11,12] offer an ingenious solution to this problem. After trans-thioesterification with a cysteine residue, S—N-acyl transfer regenerates the thiol and forms a stable amide linkage. This approach, which has been used for protein modification and immobilization,[12,13] introduces a residual thiol that can be the focal point for undesirable side reactions. For example, cysteine is by far the most reactive residue toward disulfide bonds, O2(g), and other common electrophiles.[14] In addition, the sulfhydryl group of cysteine can either suffer β elimination to generate dehydroalanine,[15] or disrupt self-assembled monolayers on gold or silver surfaces.[16] These detrimental attributes caused us to search for an alternative means to exploit the intein-derived thioester for the installation of an orthogonal functional group.

In contrast to sulfur nucleophiles, nitrogen nucleophiles can, in theory, react directly with the thioesters formed during intein-mediated protein splicing to form inert linkages. This reaction has been neither explored nor exploited previously. Moreover, we reasoned that an appropriate bifunctional nitrogen nucleophile could both attack an intein-derived thioester to form a stable linkage, and install an orthogonal (and thus useful) functional group.

The azido group can serve as an orthogonal functional group because it is absent from natural proteins, nucleic acids, and carbohydrates.[17] Moreover, chemical reactions of the azido group, such as the Cu I-catalyzed Huisgen 1,3-dipolar azide–alkyne cycloaddition[18] and Staudinger ligation,[19] can lead to site-specific modification or immobilization. Azido proteins have been produced previously. For example, Schultz and co-workers have developed a method for incorporating azido-lysine into proteins.[20] Their approach involves the production of an azidolysoine-charged suppressor tRNA that inserts the residue into a protein, as directed by an engineered gene. This method, although site-specific, is labor intensive and low yielding. Tirrell, Bertozzi, and co-workers have incorporated an azido group into a protein by using azidothymoalanne, which replaces methionine in proteins produced in methionine-depleted bacterial cultures.[21] This method is not site specific for proteins that contain more than one methionine residue.

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Here, we report a general strategy for producing proteins that are site-specifically labeled with an azido group. We produced these azido proteins by semisynthesis by using a variation of expressed protein ligation (Scheme 1).[11] Our strategy involves production of the protein of interest as a fusion protein with an intein molecule and a tag for affinity chromatography. On-resin cleavage of the intein-derived thioester was induced with a bifunctional reagent that carried a nucleophile for thioesters and an azido group. Kinetic analyses with a chromogenic thioester were used to identify the optimal nucleophile, and the desired bifunctional reagent was synthesized on a multigram scale. This strategy was used to produce an azido protein that maintained full biological activity and displayed an azido group at its C terminus that is available for chemoselective modification. Thus, exploiting the intrinsic and orthogonal reactivity of the thioester produced during intein-mediated protein splicing enabled site-specific chemical modification of a protein.

Results and Discussion
Identification of the optimal nucleophile for thioesters
To identify the optimal nitrogen nucleophile for a thioester, kinetic studies were performed on a model chromogenic thioester, AcGlySC₆H₄-p-NO₂ (Scheme 2). The rate of release of the thiophenolate anion was monitored by measuring the change in absorbance at 410 nm. Nitrogen nucleophiles with conjugate-acid pKₐ values ranging from 4.6 to 10.6 (Table 1) were used in the experiments. The logarithmic values of the second-order rate constants (k₂) of the unprotonated primary amines were plotted against the pKₐ values of their conjugate acids to yield the Brønsted plot shown in Figure 1A. The data were fitted to the equation:[24]

\[
\log k_2 = \log (AB) + (\beta + \beta') pK_a - \log (A10^{b_k} + B10^{b_k})
\]

In Equation (1), A and B are constants, \(\beta\) is the slope of the former part of the Brønsted plot, and \(\beta'\) is the slope of the latter part.

The Brønsted plot in Figure 1A is biphasic. The slope changes from 0.81 with nucleophiles of low pKₐ to 0.42 with nucleophiles of high pKₐ. This change is due to the known alteration in the rate-determining step from the formation of a tetrahedral-zwitterionic intermediate to the decomposition of the intermediate into products.[26–28] The value of the slope obtained (\(\beta' = 0.81\)) is in agreement with that for the aminolysis of oxygen esters.[29] As expected,[30] “α-effect” nucleophiles (alkoxy amines, α-hydrazino acetyl, and alkyl hydrazine) exhibited much greater nucleophilicity than that predicted from their pKₐ values. In water, the α effect could arise from the nucleophile being less solvated and hence more reactive because of the inductive withdrawal of electrons by the adjacent heteroatom.[31]
The Brønsted plot reports on the nucleophilicity of unprotonated (that is, neutral) amines. According to Figure 1A, the best nucleophile for thioesters in an environment in which all the amines are deprotonated, is ethyl amine. Indeed, other simple amines have been used in tandem with a small-molecule thiol to modify an intein-derived thioester.[8] At the high pH necessary to deprotonate an amine, however, thioesters are prone to hydrolysis.[9] Moreover, proteins are subject to unfolding and subsequent aggregation at high pH values.[32] Performing the reaction at pH 7.0 provides an acceptable trade-off between deprotonation of the nitrogen nucleophile and hydrolysis of the thioester.[36,37] The cleavage of thioesters by hydroxylamine, however, relies on the formation of an O-acylated hydroxylamine intermediate[36,37] that is inaccessible during the attack of an alkoxy amine on a thioester. Accordingly, an alkoxy amine is not an especially potent nucleophile for a thioester (Figure 1).

Synthesis of bifunctional azides

After identification of two optimal nucleophiles, we proceeded to synthesize two bifunctional reagents that carried those nucleophiles on one end and an azido group on the other. Azides 1 and 2 are both amides of 1-azido-2-aminoethane. Azide 1 has an α-hydrazino acetamido group, which is a more stable analogue of the α-hydrazino acetyl group of C₂H₅O(O)CCH₂NHNH₂ (Table 1; Figure 1); azide 2 has a γ-hydrazino acetamido group and is effectively an alkyl hydrazine.

Azide 1 was synthesized by the route shown in Scheme 3. Briefly, Boc-protected 1-azido-2-aminoethane was synthesized from Boc-protected 1-bromo-2-aminoethane. After Boc deprotection, the mixture was coupled to tri-Boc-protected α-hydrazino acetic acid. The Boc groups were removed, and azide 1 was isolated as a free base after cation-exchange chromatography with an overall yield of 72%.

Appending a Functional Group to a Protein
Azide 2 was synthesized by the route shown in Scheme 4. Briefly, 4-pentenoic acid was subjected to ozonolysis, and the resulting aldehyde was treated, in situ, with Boc-protected hydrazine. The azido group was installed by coupling 1-azido-2-hydrazino acetyl group (Figure 1). The intrinsic instability of the azido group at the C terminus of a model protein. As our protease, we inserted a glycine residue between the C terminus of RNase A and the intein. The resulting Met(−1)RNase A–Gly–mxe intein–chitin-binding domain fusion protein (Mw~36 kDa) was produced in E. coli, and the cell lysate was loaded onto chitin resin. Azides 1 and 2 were used to induce the on-resin cleavage of the fusion protein. As expected from the kinetic studies, azide 1 was found to be much more effective than azide 2 in cleaving the Met(−1)RNase A–Gly–mxe intein thioester. Its shorter and higher yielding synthesis, superior stability, and higher cleavage efficiency make azide 1 the optimal bifunctional reagent for the semisynthesis of proteins labeled with the azido group. The purity of the azido-RNase A (even upon elution from the chitin column) was apparent from SDS-PAGE analysis (Figure 2A); the integrity of the azido-RNase A was verified by using MALDI-TOF mass spectrometry (Figure 2B). This procedure produced an overall yield of ~1 mg of purified azido-RNase A per liter of E. coli culture.

Incubating a protein with a potent nucleophile, such as the α-hydrazino acetamido group of azide 1, could compromise the structure of the protein. For example, the target protein in this study has 142 amide bonds in its main chain and side chains that could be attacked by the α-hydrazino acetamido group, but it has only one thioester bond. In addition, the target protein has eleven amino groups that could serve as intramolecular nucleophiles for that thioester bond. Enzymatic catalysis provides an extremely sensitive measure of native-protein structure. This measure is especially useful for detecting the inadvertent modification of RNase A, as one of its eleven amino groups is both especially reactive and critical for enzymatic activity. Purified azido-RNase A had $k_{cat}/K_m = (3.2 \pm 1.0) \times 10^3 \text{ M}^{-1} \text{s}^{-1}$ for the cleavage of RNA. This value was in gratifying agreement with that of the wild-type enzyme, which had $k_{cat}/K_m = (5.2 \pm 0.4) \times 10^3 \text{ M}^{-1} \text{s}^{-1}$.

The α-hydrazino acetamido group was found to be the optimal nitrogen nucleophile for producing azido proteins. Still, we had to use a 450 mM solution of azide 1 to produce the desired hydrazide product. Lower concentrations led to the hydrolyzed protein (that is, the protein with a C-terminal carboxyl group) to be a dominant product. In contrast, a 50 mM solu-
tion of thiol typically suffices for transthioesterification during expressed protein ligation.\textsuperscript{11} The resulting thioester must then, however, react with a peptide (present in vast excess) that contains an N-terminal cysteine residue.\textsuperscript{12,13} The ability to obtain an azido protein in a single step by on-resin cleavage and the absence of the residual sulfhydryl group installed during expressed protein ligation are noteworthy advantages of our strategy (Scheme 1). These attributes are of particular importance for high-throughput procedures, such as the fabrication of protein microarrays.\textsuperscript{46}

Huisgen 1,3-dipolar cycloaddition to an azido protein

For our strategy to be useful, the azido group in azido-RNase A must be available for further reaction. We used a chemoselective reaction, Cu\textsuperscript{i}-catalyzed Huisgen 1,3-dipolar azide–alkyne cycloaddition,\textsuperscript{18,47,48} to probe for the availability of the azide functionality. To effect this “functional-group test”, alkynyl fluorescein \textsuperscript{10} was synthesized by the route shown in Scheme 5. Azido-RNase A was treated with \textsuperscript{10} in the presence of the Cu\textsuperscript{i} catalyst and its polytriazole ligand.\textsuperscript{48} The resulting protein had a molecular mass of \textit{m/z} 14449, which agreed well with that expected for the conjugate (C\textsubscript{586}H\textsubscript{936}N\textsubscript{180}O\textsubscript{201}S\textsubscript{13} = 14424). The protein was also subjected to SDS–PAGE and visualized by staining with Coomassie blue and fluorescence imaging. The azido-RNase A was found to be fluorescent as a result of the cycloaddition, whereas wild-type RNase A treated in the same manner was not fluorescent (Figure 3). Neither the mass spectrum nor the SDS–PAGE gel showed evidence of cleavage products, as have been observed in an azido protein exposed to reducing agents.\textsuperscript{49} Thus, an azido group was not only installed into a specific site on RNase A, but was also available for reaction. In ongoing work, the Staudinger ligation\textsuperscript{19} is being used for the site-specific immobilization of proteins produced by the novel route shown in Scheme 1.

Conclusion

We have exploited the mechanism of intein-mediated protein splicing to develop a general strategy for installing a functional group at the C terminus of a protein. The strategy is based on the capture of an intein-derived thioester with a nucleophile that was selected by using Brønsted analysis. We used this strategy to append an azido group to a model protein. The azido group did not affect the function of the protein and was...
available for Husgen 1,3-dipolar azide–alkyne cycloaddition of a fluorophore. We anticipate that such a C-terminal azido group could also be used for site-specific protein immobilization, and for modification by Staudinger ligation. Finally, we note that our strategy can be used to append other functional groups with orthogonal reactivity (such as an alkene, alkyne, or nitrile) to a target protein.

**Experimental Section**

The chromogenic thioester AcGlySC$_6$H$_4$-$\text{O}_2$N (a generous gift from B. L. Nilsson) was purified by recrystallization from methylene chloride and stored in a tightly sealed vial in a desiccator to prevent hydrolysis by moisture present in air. Fluorescein–NHS ester was a generous gift from L. D. Lavis. All other chemicals were commercial reagent grade or better, and were used as received except for benzyl azide, which was purified by flash chromatography before use. Anhydrous THF, DMF, and CH$_3$Cl, were obtained from a CYCLE-TAINER solvent delivery system (J. T. Baker, Phillipsburg, NJ, USA). Other anhydrous solvents were obtained in septum-sealed vials. Synthetic reactions were monitored by thin-layer chromatography (TLC) and visualized by using UV-light or stained with vanillin, ninhydrin, or I$_2$. In all reactions involving anhydrous solvents, glassware was flame-dried. Flash chromatography was performed with columns of silica gel 60, 230–400 mesh (Silicycle, Quebec City, Quebec, Canada).

**Instrumentation:** A Cary Model 3 UV/VIS spectrophotometer (Varian, Palo Alto, CA, USA) was used to perform kinetic assays and measure ultraviolet absorbance. NMR spectra were acquired with a Bruker AC+$\text{D}$ 300 spectrometer ($\text{H}$: 300 MHz; $\text{C}$: 75 MHz) at the Magnetic Resonance Facility in the Department of Chemistry, or (as indicated) on a Bruker DMX-400 Avance spectrometer ($\text{H}$: 400 MHz; $\text{C}$: 100 MHz) at the National Magnetic Resonance Facility at Madison (NMRFAM); $\text{C}$ spectra were proton-decoupled. Mass spectra of proteins were obtained with MALDI-TOF spectrometry on small organic molecules were obtained with ESI techniques. Mass spectra of proteins were obtained with MALDI-TOF mass spectrometry by using a Voyager-DE-PRO Biospectrometry workstation (Applied Biosystems, Foster City, CA, USA) and a 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) matrix (Aldrich). Fluorescence measurements were made with a QuantaMaster 1 workstation (Applied Biosystems, Foster City, CA, USA) and a 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) matrix (Aldrich). Mass spectra of proteins were obtained with MALDI-TOF spectrometry on small organic molecules were obtained with ESI techniques. Mass spectra of proteins were obtained with MALDI-TOF mass spectrometry by using a Voyager-DE-PRO Biospectrometry workstation (Applied Biosystems, Foster City, CA, USA) and a 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) matrix (Aldrich). Fluorescence measurements were made with a QuantaMaster 1 workstation (Applied Biosystems, Foster City, CA, USA) and a 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) matrix (Aldrich). Mass spectra of proteins were obtained with MALDI-TOF spectrometry on small organic molecules were obtained with ESI techniques. Mass spectra of proteins were obtained with MALDI-TOF mass spectrometry on small organic molecules were obtained with ESI techniques. Mass spectra of proteins were obtained with MALDI-TOF mass spectrometry on small organic molecules were obtained with ESI techniques. Mass spectra of proteins were obtained with MALDI-TOF mass spectrometry on small organic molecules were obtained with ESI techniques. Mass spectra of proteins were obtained with MALDI-TOF mass spectrometry on small organic molecules were obtained with ESI techniques.

**Kinetics of thioester cleavage**

**Synthesis of BocNHCH$_2$CH$_2$N$_3$ (3):** BocNHCH$_2$CH$_2$Br (10.00 g, 44.62 mmol) was dissolved in DMF (200 mL). NaN$_3$ (14.48 g, 223.1 mmol) was added, and the mixture was stirred at 110°C for 12 h. The solvent was removed under reduced pressure, and the residue was dissolved in water (200 mL). The resulting aqueous solution was extracted with ethyl acetate (2 x 200 mL). The organic layers were combined and dried over anhydrous MgSO$_4$. After filtration, the organic layer was concentrated under reduced pressure and the residue was dissolved in methylene chloride (10–20 mL) and purified by flash chromatography (silica gel, methylene chloride). BocNHCH$_2$CH$_2$N$_3$ (6.60 g, 80%) was isolated as a colorless oil. HRMS (ESI) [M+Na]$^+$ calculated for C$_9$H$_{14}$N$_4$O$_2$Na, 209.1014; found, 209.1010; $\text{H}$ NMR (400 MHz, CDCl$_3$) $\delta$ = 4.88 (brs, 1H), 3.42 (t, $J$ = 5.3 Hz, 2H), 3.34–3.26 (m, 2H), 1.45 (s, 9H); $\text{C}$ NMR (100 MHz, CDCl$_3$) $\delta$ = 155.8, 79.8, 51.4, 40.2, 28.5.

**Synthesis of (Boc$_2$)NN(Boc)CH$_2$CONHCH$_2$CH$_3$N$_3$ (5):** HCH$_2$NCH$_2$CH$_2$N$_3$ (4) was synthesized by dissolving azide 3 (2.11 g, 11.33 mmol) in HCl (4 N) in dioxane (100 mL). The mixture was then stirred at room temperature for 1 h. The solvent was removed under reduced pressure to give a dirty-white powder. Boc$_2$NN(Boc)CH$_2$CONHCH$_2$CH$_3$N$_3$ (5.9 g, 11.33 mmol) was then added, and the mixture was dissolved in methylene chloride (70 mL) and DMF (45 mL). The mixture was cooled to 0°C, and PyBOP (5.9 g, 11.33 mmol) and Et$_3$N (3.2 mL, 22.66 mmol) were added. The reaction mixture was allowed to warm to room temperature and stirred under Ar(g) for 21 h. The solvent was removed under reduced pressure, and the residue was purified by flash chromatography (silica gel, 50% (v/v) ethyl acetate in hexanes) to give (Boc$_2$)NN(Boc)CH$_2$CONHCH$_2$CH$_3$N$_3$ as a colorless viscous oil (4.9 g, 95%). HRMS (ESI) [M+Na]$^+$ calculated for C$_{26}$H$_{33}$N$_6$O$_2$Na, 481.2387; found,
Appendix A


481.2389; 1H NMR (400 MHz, CDCl3, 2 rotamers) δ = 8.51 and 8.27 (brs, 1H), 4.06 and 3.99 (s, 2H), 3.49–3.40 (m, 4H, 1.56–1.44 (m, 27H); 13C NMR (100 MHz, CDCl3, 2 rotamers) δ = 169.5, 169.2, 154.0, 153.5, 151.4, 151.2, 85.2, 85.1, 83.5, 82.8, 56.5, 54.8, 50.6, 38.9, 34.1, 29.9, 28.2, 28.1.

Synthesis of H2NNHCONHCH2CH2N3 (1): Azide 5 (4.66 g, 10.17 mmol) was dissolved in HCl (4 N) in dioxane (200 mL) and the solution was stirred at room temperature for 5 h. Solvent was removed under reduced pressure, the residue was dissolved in water (15 mL), and purified by cation-exchange chromatography (Dowex 50WX-800 ion-exchange resin, 1 M NaOH) to give H2NNHCONHCH2CH2N3 as a yellow oil (1.52 g, 95%). HRMS (ESI) [M+Na]+ calcd for C11H20N6O3Na, 307.1495; found, 307.1494; 1H NMR (300 MHz, [D6]DMSO) δ = 8.18 (brs, 1H), 8.09–7.90 (m, 1H), 4.34 (brs, 1H), 3.32 (t, J = 5.7 Hz, 2H), 3.26–3.18 (m, 2H), 2.66–2.53 (m, 2H), 2.22–2.07 (m, 2H), 1.64–1.48 (m, 2H), 1.46–1.22 (m, 9H); 13C NMR (100 MHz, [D6]DMSO, 2 rotamers) δ = 172.5, 156.4, 78.2, 56.1, 54.9, 50.4, 50.0, 38.2, 33.0, 28.2, 23.5, 22.9.

Synthesis of CF3CO2H·H2NNHCONHCH2CH2N3 (2): Compound 9 (197.8 mg, 0.69 mmol) was dissolved in methylene chloride (6.4 mL), and trifluoroacetic acid (6.4 mL) was added to the resulting solution. The reaction mixture was stirred at room temperature for 15 min. Solvent was removed under reduced pressure to afford CF3CO2H·H2NNHCONHCH2CH2N3 as a yellow oil (130 mg, 100%). HRMS (ESI) [M+H]+ calcd for C7H10N3O3F3, 218.0787; found, 218.0784; 1H NMR (300 MHz, [D6]DMSO) δ = 10.5 (s, 1H), 7.93 (t, J = 5.3 Hz, 1H), 7.28 (d, J = 5.7 Hz, 1H), 7.15 (d, J = 5.3 Hz, 1H), 7.09 (d, J = 7.0 Hz, 2H), 6.81 (d, J = 5.5 Hz, 1H), 6.69 (d, J = 2.3 Hz, 2H), 6.49 (d, J = 8.6 Hz, 2H), 6.54 (dd, J = 8.7, 2.3 Hz, 2H), 4.11 (dd, J = 5.3, 2.2 Hz, 2H), 3.17 (t, J = 5.3 Hz, 1H); 13C NMR (100 MHz, [D6]DMSO) δ = 171.9, 151.9, 148.0, 145.9, 137.5, 129.2, 126.7, 124.4, 112.9, 108.1, 102.3, 83.8, 81.0, 73.1, 28.8.

Synthesis of alkynyl fluorescein (10): Fluorescein–NHS ester (100 mg, 0.21 mmol) was dissolved in THF (10 mL) and propargylamine (23.27 mg, 0.42 mmol) was added. The reaction mixture was stirred at room temperature for 4 h. Solvent was removed under reduced pressure, and the residue was purified by flash chromatography (silica gel, 35% (v/v) hexanes in ethyl acetate containing 1% (v/v) AcOH) to give the desired product as a red solid (72.80 mg, 84%). HRMS (ESI) [M+Na]+ calcd for C11H18N2O3Na, 346.0797; found, 346.0777; 1H NMR (400 MHz, [D6]DMSO) δ = 8.08 (bs, 2H), 9.41 (t, J = 5.7 Hz, 1H), 7.95–5.5 (brs, 3H), 3.34 (t, J = 5.8, 2H), 3.28–3.20 (m, 2H), 2.89 (t, J = 7.5 Hz, 2H), 2.21–2.11 (m, 2H), 1.85–1.68 (m, 2H); 13C NMR (100 MHz, [D6]DMSO) δ = 171.9, 150.1, 50.0, 38.2, 32.2, 21.0.

Synthesis of polytriazole ligand: A polytriazole ligand for the CuI catalyst was synthesized essentially as described. Tripropargylamine (0.56 g, 4.28 mmol) was dissolved in acetonitrile (5.7 mL), and benzyl azide (2 g, 15.02 mmol) and 2,6-lutidine (0.46 g, 4.28 mmol) were added to the resulting solution. The reaction mixture was cooled to 0°C, and CuCl2·4H2O (81.25 mg, 0.22 mmol) was added. The reaction mixture was stirred to room temperature and stirred under Ar(g) for 2.5 days. The reaction mixture was filtered, and the white precipitate obtained was dried under high vacuum to yield the polytriazole ligand (0.34 g, 15%). HRMS (ESI) [M+Na]+ calcd for C31H47N13O4Na, 553.2553; found, 553.2570; 1H NMR (400 MHz, CDCl3) δ = 7.66 (s, 3H), 7.40–7.20 (m, 15H), 5.50 (s, 6H), 3.70 (s, 6H); 13C NMR (100 MHz, CDCl3) δ = 134.8, 129.2, 128.8, 128.1, 123.9, 54.2, 47.2.

Production of RNase A–intein–chitin-binding domain fusion protein: The plasmid that directs the expression of an RNase A–intein–chitin-binding domain fusion protein was a generous gift from U. Arnold. A glycine codon was introduced between RNase A and the intein genes by using the QuickChange site-directed mutagenesis kit from Stratagene (LaJolla, CA, USA). The resulting plasmid, pJK01, was transformed into E. coli BL21(DE3) cells, and the production of Met–RNase A–Gly–intein-chitin binding domain fusion protein was induced as described previously.

Production of azido–RNase A: Cells were resuspended in 3-(N-morpholino)propane sulfonic acid (MOPS; 20 mM)–NaOH buffer at
pH 6.8 containing NaCl (0.50 mM), ethylenediaminetetraacetic acid (EDTA; 0.10 mM), and Triton X-100 (0.1%, v/v). Cells were lysed with a French pressure cell, and the cell lysate was subjected to centrifugation at 15,000 g for 30 min. The supernatant was dialyzed to a final volume of 25 mL (per g of cells) and applied (flow rate: 0.75 mL min⁻¹) to a chitin column that had been equilibrated with the same buffer. The loaded resin was washed with two column volumes of buffer and then with four column volumes of MOPS–NaOH (0.5 mM) buffer at pH 7.0 containing NaCl (0.50 mM) and EDTA (0.10 mM). Azide 1 was dissolved in the latter buffer to a final concentration of 450 mM, and three column volumes of this solution were loaded onto the resin, out of which, two column volumes were allowed to flow through and one column volume was allowed to sit on top of the resin. This incubation was carried out for three days at room temperature in order to enable the reaction to proceed to completion. The hydrazide product was eluted with three column volumes of an aqueous solution of NaCl (2 M). Azido-RNase A was precipitated out of the eluate by adding an aqueous solution of sodium deoxycholate (NaDOC; to 0.72 mM) and tri-chloroacetic acid (TCA; to 260 mM). This precipitate was washed with acetone and dissolved in an aqueous solution of guanidine–HCl (4 M).

The solution of protein was added, with gentle stirring, in 20 μL aliquots into a refolding solution (50 mM) consisting of Tris–HCl (100 mM) buffer at pH 8.0 containing NaCl (100 mM), reduced glutathione (1 mM), and oxidized glutathione (0.2 mM). The final concentration of guanidine-HCl was 0.05 M. The refolding solution was incubated at room temperature for 24 h.

The refolding solution was dialyzed for 12 h against sodium acetate (50 mM) buffer at pH 5.0. The azido protein was purified by cation-exchange chromatography as described previously.[3]

Ribonuclease activity: Values of $k_{cat}/K_m$ for the enzymatic cleavage of a fluorogenic substrate, 6-carboxyfluorescein-dArU(dA)2-6-carboxyfluorescein–NHS ester, and U. Arnold for a plasmid that directs the expression of the RNase A–mxe intein–chitin-binding domain fusion protein. This work was supported by Grant GM44783 (NIH) and the Materials Research Science and Engineering Center at the University of Wisconsin–Madison (NSF-DMR 0079983). The Magnetic Resonance Facility was supported by Grants CHE-9208463 (NSF) and RR08389 (NIH). NMRFAM was supported by Grant P41RR02301 (NIH).

Keywords: bioconjugation · click chemistry · protein design · protein engineering · protein modifications


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