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Cysteine as a Monothiol Reducing Agent to Prevent Copper-Mediated Oxidation of Interferon Beta During PEGylation by CuAAC

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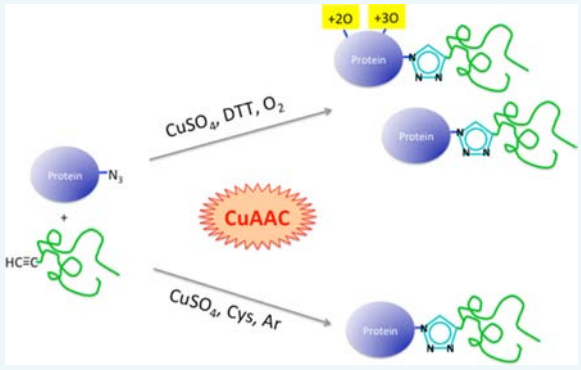
Supporting Information

ABSTRACT: Bioconjugation by copper-catalyzed azide−alkyne cycloaddition (CuAAC) provides a powerful means to produce site-specifically modified proteins. However, the use of a copper catalyst brings about the possible generation of reactive oxygen species that could cause degradation of vulnerable amino acid residues. We investigated whether PEGylation by CuAAC caused any modifications to the therapeutic protein interferon beta-1b, which was produced via global amino acid substitution with azidohomoalanine at the N-terminus and contains no methionine residues. Using previously reported reaction conditions, LC-MS peptide mapping detected +32 Da and +48 Da oxidation modifications of tryptic peptides 28−33 (LEYCLK) and 137−147 (EYSHCAWTIVR) in the protein post-PEGylation. The oxidative degradation increased with reaction time, whereas reducing the copper concentration slowed the PEGylation rate as well as the oxidation rate. Replacing dithiothreitol (DTT) with any of five different monothiol reducing agents in anaerobic conditions allowed efficient PEGylation in 2−4 h and abrogated oxidative degradation. Free cysteine provided reproducible reaction results as a reducing agent in this system and has been successfully applied to other protein conjugations. Monothiol reducing agents, such as cysteine, may be useful tools as protective reducing agents for CuAAC in some bioconjugation systems.



INTRODUCTION

Copper-catalyzed azide−alkyne cycloaddition (CuAAC) has emerged as a versatile addition to the bioconjugation chemist's toolbox.[1−3] In the presence of a Cu(I) catalyst, an alkyne and an azide group are joined to form a stable triazole linkage between the reactants (Scheme 1). The reaction is highly selective, leaving untouched the myriad functional groups present in the 20 canonical amino acids, and conveniently proceeds efficiently in aqueous and aerobic systems. This allows site-selective stable conjugation between many interesting biomolecules. A recent comparison of bioorthogonal conjugation reactions confirmed CuAAC as a chemoselective reaction producing a stable product at reasonable rates.[4] However, the necessary presence of the Cu(I) catalyst brings about the possibility of protein degradation resulting from the generation of reactive oxygen species (ROS) in the reaction system.[5]

Scheme 1. CuAAC Reaction Scheme

In order to employ CuAAC for clinical manufacture of a therapeutic bioconjugate, the possibility of Cu-induced protein degradation must be addressed. ROS such as hydroxyl radical, hydrogen peroxide, and superoxide can be generated when combining transition metals, reducing agents, and oxygen in aqueous systems.[6,7] These are capable of inducing oxidative modification to amino acid residues, particularly Cys and Met, but also Trp, His, and Tyr.[7,8] Ligands such as TBTA (Scheme 2) are employed during CuAAC to stabilize the Cu(I) ion and to provide protection from ROS.[9,10] Strain-promoted azide−alkyne cycloaddition (SPAAC) has been developed as a metal-free alternative to CuAAC, but it is not always an appropriate choice because it introduces large fused ring systems onto the protein that can add significant hydrophobic character and promote aggregation.[11,12] Consequently, the work herein was conducted to determine any degradants that form and to design reaction conditions to prevent degradation during CuAAC conjugation of a specific protein.

Using CuAAC, we have developed a long-lasting form of interferon beta-1b (IFNb) by site-specifically PEGylating an azide-containing nonnatural amino acid that is incorporated recombinantly into the protein sequence during expression.[13] IFNb is a 20 kDa cytokine containing a 4-α-helix bundle.[14−16]

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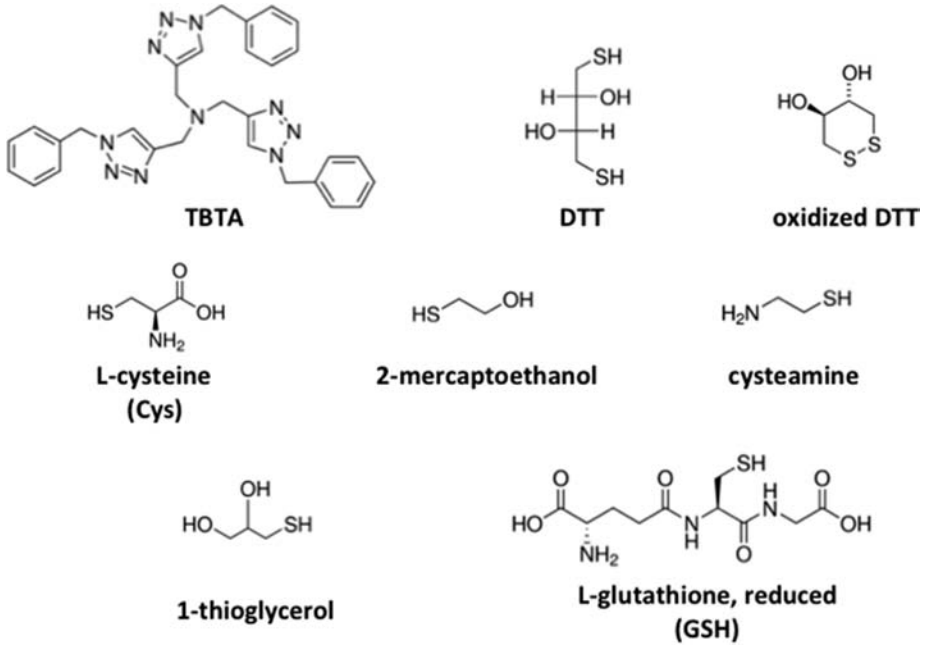
Scheme 2. Compound Structures



Scheme 3. PEGylation of IFNb by CuAAC

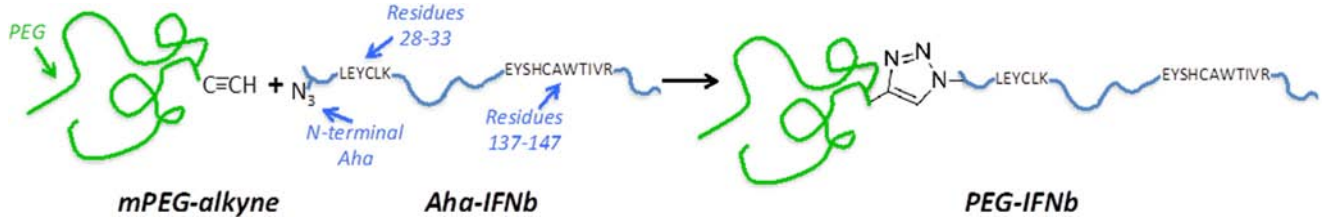


Table 1. Relative Amounts of Oxidative Degradation in Various Batches of IFNb and PEGylated IFNb[a]

species	batch	% +32 or +48 peptide 28−33	% +32 or +48 peptide 137−147	total % +32 or +48 modifications to peptides 28−33 and 137−147
IFNb	A	0.2%	1.6%	1.8%
IFNb	B	0.1%	0.5%	0.6%
IFNb	C	0.1%	0.5%	0.6%
IFNb	D	0.9%	1.8%	2.7%
IFNb	E	0.4%	0.6%	1.0%
IFNb	F	0.0%	0.0%	0.0%
IFNb	G	0.5%	0.2%	0.7%
IFNb average ± s.d.	**A−G**	**0.3% ± 0.3%**	**0.7 ± 0.7%**	**1.1% ± 0.9%**
PEG-IFNb	1	1.5%	3.0%	4.5%
PEG-IFNb	2	3.9%	4.5%	8.4%
PEG-IFNb	3	1.6%	4.5%	6.1%
PEG-IFNb	4	3.3%	4.8%	8.1%
PEG-IFNb average ± s.d.	**1−4**	**2.6% ± 1.2%**	**4.2% ± 0.8%**	**6.8% ± 1.8%**

[a]Total % +32 or +48 modifications to peptides 28−33 and 137−147 is the sum of % +32 or +48 peptide 28−33 and % +32 or +48 peptide 137−147. Overnight PEGylation in air with 2 mM DTT, 1.5 mM $CuSO_4$.

The native form contains one disulfide bond and a free cysteine. The clinically approved IFNb products have the free cysteine removed to reduce the formation of disulfide-linked aggregates of this highly hydrophobic aglycosylated recombinant protein produced in *E. coli*.[14−16] The intramolecular disulfide bond between Cys31 and Cys141, however, is essential to the potency of the molecule.[14] In our PEGylated IFNb, Cys31 and Cys141 are retained, but all Met residues in the protein have been replaced with other permissive natural amino acids in order to allow site-specific azidohomoalanine (Aha) incorporation at position 1 by global amino acid substitution (sequence in Supporting Information).[17−19] IFNb was purified from *E. coli* using standard methods[15] and maintained under reducing conditions to prevent the formation of disulfide-linked aggregates prior to refolding. PEGylation by CuAAC was performed on the reduced protein, allowing

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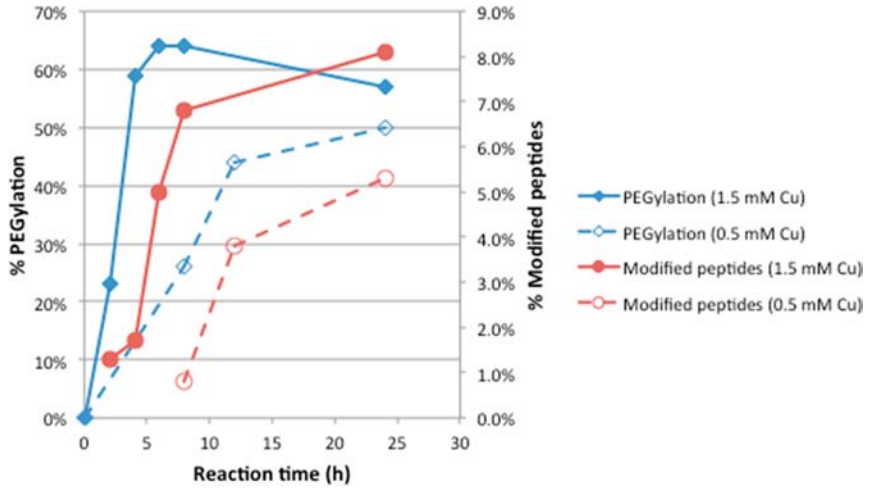

Figure 1. % PEGylation and % modification of peptides 28−33 and 137−147 in IFNb versus reaction time. % PEGylation represents the portion of IFNb that was converted to PEG-IFNb. Modified peptides represents the total of +32 and +48 modifications of the peptides 28−33 and 137−147. Results are shown for reactions conducted using either 1.5 mM or 0.5 mM Cu as catalyst.

refolding and disulfide bond formation following reaction cleanup (Scheme 3).

The purity and identity of IFNb species were characterized by LC-MS peptide mapping before and after the CuAAC PEGylation reaction. The effects of reaction conditions, including Cu concentration, reaction time, and reaction components, on both the identified oxidative degradation and the PEGylation efficiency were investigated. Monothiol reducing agents were identified as a class that protected the IFNb residues during the reaction and allowed for efficient PEGylation.

RESULTS

IFNb and PEGylated IFNb were subjected to peptide mapping with LC-MS analysis. All expected tryptic peptides longer than two amino acids were found in the chromatograms, with the exception of the N-terminal peptide, which is covalently linked to the PEG (Supporting Information). LC-MS/MS was used to verify the identity of peptide peaks. The peptides 28−33 (sequence LEYCLK) and 137−147 (sequence EYSHCAW-TIVR) were found to have varying levels of modification to +32 Da and +48 Da species (hereafter referred to as +32 and +48), which may correspond to addition of 2 or 3 oxygen atoms. The level of oxidative modification increased significantly after PEGylation by CuAAC. These peptides contain both of the Cys residues in the protein, as well as Tyr30, Tyr138, His140, and Trp143, all of which may be subject to oxidation. If any Cys residues were modified, they may not be available for subsequent disulfide bond formation during the refolding step later in the production process. The Asn residue in peptide 20−27 (and to a minor extent that in 153−159) underwent some deamidation, which was demonstrated to occur mainly during the tryptic digestion procedure (data not shown). Some oxidation of the Trp residue in peptide 20−27 was found, but the levels were always well below 1%.

The relative amounts of +32 and +48 oxidation events on tryptic peptides 28−33 and 137−147 were measured by LC-MS in multiple batches of IFNb starting material and following PEGylation (Table 1) by comparing the extracted ion peak areas of all variants of both peptides. The results are reported for each peptide, and also as the summed total of oxygen-modified versions of these peptides (% +32 or +48 peptide 28−33 plus % +32 or +48 peptide 137−147). If the oxidation were to occur on one Cys residue per molecule, this would represent the maximum percentage of IFNb molecules that could be blocked from forming disulfide bonds. Prior to PEGylation, IFNb had an average total of 1.1% 28−33 and 137−147 peptides with +32 and +48 modifications, presumably due to minor oxidation events during the prior production and isolation steps. After CuAAC (2 mM DTT, 3 mM TBTA, 1.5 mM $CuSO_4$, pH 7.6, overnight in air), PEG-IFNb had an average total of 6.8% 28−33 and 137−147 peptides with +32 and +48 modifications, confirming a significant increase in oxidative modifications during the CuAAC step. Given that disulfide bond formation is necessary for IFNb potency and that any other oxidative modifications are also disadvantageous, we sought to develop reaction conditions that protect IFNb from oxidation during CuAAC while providing for efficient PEGylation.

The reaction time and Cu concentration were found to affect both the PEGylation rate and the degree of IFNb oxidative degradation (Figure 1). With 1.5 mM Cu, PEGylation reached 23% at 2 h with a total of 1.3% 28−33 and 137−147 peptides with +32 and +48 modifications. PEGylation appeared complete by 4−6 h, with a plateau at 57−64%, but the amount of oxidative degradation increased from 1.7% at 4 h to 5.0% at 6 h and 8.1% at 24 h. The level of Aha incorporation at position 1 varies with upstream conditions, as described previously; MALDI-TOF analysis has shown a plateau of PEGylation yield indicates nearly quantitative PEGylation of reactive Aha residues.[13] Thus, PEGylation proceeded efficiently and oxidative degradation of IFNb began in significance only toward the end of the reaction and continued to increase with time. With 0.5 mM Cu, PEGylation reached only 26% by 8 h and proceeded to 50% at 24 h, at which point the oxidative degradation was 5.3%. Hence lowering the Cu concentration served to slow the rate of PEGylation and oxidative degradation in concert.

The TBTA ligand is thought to protect other species from Cu-induced degradation as well as to accelerate the reaction at optimal ratios.[9,10] Increasing the TBTA:Cu ratio to 6:1 from our standard condition of 2:1 did not have a significant effect at 24 h. The PEGylation yields were 73% and 80% and the 28−33 and 137−147 peptides with +32 and +48 modifications totaled

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Table 2. Levels of PEGylation and Peptide Modification Using Various Reducing Agents, Under Air or Argon Atmosphere

reaction	mM $CuSO_4$	time (h)	reducing agent	atmosphere	% PEGylation	Total % +32 or +48 modifications to peptides 28−33 and 137−147
1	1.5	4	2-mercaptoethanol	Argon	71%	0.4%
2	1.5	4	Cys	Argon	70%	0.5%
3	1.5	4	GSH	Argon	69%	1.0%
4	1.5	4	Cysteamine	Argon	73%	0.7%
5	1.5	4	1-thioglycerol	Argon	72%	1.0%
6	1.5	4	2-mercaptoethanol	Air	69%	2.0%
7	1.5	4	Cys	Air	61%	2.6%
8	1.5	4	GSH	Air	59%	5.1%
9	1.5	4	Cysteamine	Air	68%	0.5%
10	1.5	4	1-thioglycerol	Air	71%	2.2%
11	1.5	4	DTT	Air	46%	2.4%

Table 3. Kinetics and Reproducibility of the PEGylation Reaction, With Cysteine As a Reducing Agent under an Argon Atmosphere[a]

reaction	reaction time (h)	% PEGylation	total % +32 or +48 modifications to peptides 28−33 and 137−147
1	2	72%	1.0%
1	3	72%	1.2%
1	4	73%	1.3%
2	2	68%	2.0%
2	4	70%	1.7%
3	4	70%	0.5%
4	4	75%	3.3%
5	4	74%	1.3%
Average ± s.d. (at 4 h)	4	72% ± 2.3%	1.6% ± 1.0%

[a]Reactions 1 and 2 were assayed at multiple time points to verify kinetics; the rest were stopped at 4 h.

8.1% and 8.4%, respectively, with the two ratios (6:1 and 2:1). The TBTA ligand was essential for reaction, as only 8% PEGylation occurred when TBTA was omitted. Screens with the water-soluble THPTA ligand produced lower PEGylation yields than with TBTA in this system.

Monothiol reducing agents were investigated as alternatives to dithiol-containing DTT, whose cyclic oxidation product contains no free thiols (Scheme 2, Table 2). With the monothiol reducing agents, the PEGylation proceeded well in both aerobic and anaerobic environments by 4 h. In air, PEGylation varied slightly from 59% to 71% and oxidative degradation ranged from 0.5 to 5.1% with the monothiol agents. In argon, the PEGylation yield was 69−73% amongst all five agents tested. Moreover, oxidative degradation, 0.4−1.0%, was significantly limited in anaerobic conditions. The control reaction with DTT in air produced 46% PEGylation and 2.4% oxidative degradation, indicating that this reaction is not robustly complete at 4 h (the reaction does not proceed when using DTT in the absence of air as discussed previously[13]).

Cysteine, a monothiol reducing agent with broad precedent in protein biopharmaceutical production, was chosen for the optimized reaction conditions. Kinetics and reproducibility of this system were tested. PEGylation appeared complete at 2 h and oxidative degradation remained low at 4 h (Table 3). This indicated that 4 h is a robust reaction time for PEGylation completion and minimal degradation. A total of five reactions run with the new conditions for 4 h averaged 1.6% oxidative peptide modification with 70−75% PEGylation. MALDI-TOF analysis has indicated that this represents complete PEGylation of Aha-containing IFNb, where the remaining unreacted protein does not contain a reactive Aha due to incorporation efficiency during manufacture. This level of oxidative degradation is comparable to the average level of 1.1% for the protein prior to PEGylation (Table 1, $P < 0.36$) and is significantly reduced from the value of 6.8% observed for the original PEGylation conditions ($P < 0.006$).

DISCUSSION

This study provides an investigation of the effect of copper exposure on IFNb during CuAAC. The exposure results in +32 and +48 Da modifications to tryptic peptides 28−33 and 137−147. It is possible that the oxidation to peptides 28−33 and 137−147 corresponds to addition of 2 or 3 oxygen atoms to cysteine residues and formation of cysteinesulfinic acid and cysteic acid, respectively. Thus, the observed oxidative degradation contains the risks of both native protein modification as well as the risk of blocking the protein from containing the disulfide bond essential for potency. The lack of observed +16 oxidation may be due to the lower stability of cysteine sulfenic acid versus higher oxidative forms.[20] The observed +32 and +48 species could also represent other modifications to these peptides. It should be noted that because this IFNb sequence contains no Met residues, the effect of the chemistry on Met residues was not investigated.

Regardless of the Cu concentration, when using DTT the oxidative degradation appears to accelerate after an induction time. Our previous work with DTT in aerobic conditions showed that the reaction mixture began as highly reducing and then inflected to a more oxidizing environment, after which the PEGylation reaction began.[13] In anaerobic conditions with DTT, the reaction did not proceed. A possible explanation is a competitive complexation of Cu by DTT that prevents productive access of Cu to the PEGylation reaction center. After oxidation, DTT forms a cyclic product, which may release Cu to TBTA and the reaction complex and also to amino acids in the protein that are vulnerable to oxidation. Due to the

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induction time, which can vary between reactions presumably due to variable oxygen incorporation with different scales and mixing, the reaction with DTT is not robustly complete by 4 h and was shown here to rapidly cause more oxidative degradation to IFNb after 4 h.

The use of a monothiol reducing agent in the reaction protected the residues in IFNb from modification while still providing for efficient PEGylation by 2−4 h. Any complexation of Cu by the free thiols of the reducing agent was not prohibitive to the reaction. Oxidation during the reaction may cause the formation of mixed disulfides between the monothiol reducing agents and the IFNb Cys residues, which may provide protection for the Cys residues in the protein sequence. The formation of mixed disulfides has been shown to protect Cys residues from oxidative degradation.[21] The free monothiol may also serve directly as a sacrificial oxidant for reaction with generated ROS. Exclusion of air can be expected to reduce the rate of ROS formation, and has been found previously to reduce protein oxidation during CuAAC.[22] We have scaled these conditions up for GMP manufacture of PEG-IFNb with similar results, and have also translated these conditions to CuAAC bioconjugation with other proteins.[23] Given the similar results with 5 different agents with varying polarities and redox potentials, the monothiol agents may be generalizable as protective reducing agents for CuAAC in similar aqueous systems, though the effect on Met residues is unknown. Further optimization for a given protein, such as concentrations of reducing agent and copper and use of other ligands, is possible. Finn and colleagues have developed an ascorbic acid/aminoguanidine reducing system, providing protection from potential protein degradation from resultant reactive dehydroascorbate, that has been used successfully in many bioconjugation reactions.[10] Both systems are available for protective CuAAC for protein conjugates, where differing proteins and reaction requirements may determine the best conditions for an application.

In conclusion, the use of CuAAC for PEGylation of a nonnatural amino acid-containing IFNb protein was found to cause +32 and +48 oxidation of tryptic peptides 28−33 and 137−147. The oxidation increased with time during the reaction, and reducing the concentration of Cu or increasing the TBTA ligand content did not ameliorate the effect. Use of five different monothiol reducing agents under anaerobic conditions protected the IFNb residues from oxidation. Cysteine, as a monothiol reducing agent, provided a robust protective system for bioconjugation by CuAAC that can be amenable to large-scale production of modified proteins for clinical use.

EXPERIMENTAL PROCEDURES

Reagents. Branched 40 kDa mPEG-alkyne and linear 30 kDa PEG diol were obtained from NOF, Japan. Aha hydrochloride was from SAFC and Iris Biotech GmbH Germany. TBTA was from SAFC. $CuSO_4$ pentahydrate (99.999% pure), all reducing agents, and modified proteomics grade trypsin were from Sigma-Aldrich.

IFNb Preparation. IFNb was expressed and purified as described previously.[13] The protein sequence is provided in the Supporting Information. Briefly, the protein was expressed in a Met auxotrophic *E. coli* expression host with Aha supplemented in the medium. Inclusion bodies were isolated from cells lysed by microfluidization in PBS and then washed first in 50 mM tris, 1% Triton X-100, 5 mM EDTA, 150 mM NaCl, pH 8.0 and then in PBS. The inclusion bodies were solubilized in 160 mM sodium phosphate, 100 mM DTT, 2% SDS, pH 7.4 by microfluidization. IFNb was extracted into an equal volume of 2-butanol and then precipitated by dripping this phase into 160 mM sodium phosphate, 0.1% SDS, with the pH lowered to 5.0 with acetic acid. Precipitated IFNb was isolated and stored at −80 °C until use.

PEGylation. PEGylation was performed as described previously.[13] Briefly, IFNb was solubilized at 4 mg/mL in 100 mM phosphate, 2% SDS, pH 7.6. This was combined with stocks of each component to result in the following final concentrations unless otherwise noted: 1 mg/mL IFNb, 2% SDS, 2 mM reducing agent, 1.4 wt % branched 40 kDa mPEG-alkyne, 0.6 wt % 30 kDa PEG diol, 3 mM TBTA, and 1.5 mM $CuSO_4$. The reaction with 0.5 mM $CuSO_4$ contained 1 mM TBTA to maintain the ligand:Cu ratio. The reactions were prepared in a vessel with a headspace at least equal to the reaction volume and allowed to react overnight or for the time listed. Reactions performed under argon had air removed by 3 cycles of vacuum purge/argon fill in a round-bottom flask. To quench reactions, they were filtered (to remove solid TBTA) directly into tubes containing a final concentration of 100 mM DTT and analyzed immediately or frozen at −80 °C (equivalent results were obtained with both methods). PEGylation efficiency was determined by densitometry of reducing Coomassie-stained SDS-PAGE gels, with integration of the free IFNb and PEGylated IFNb bands.

LC-MS Peptide Mapping. The crude PEGylation reaction mixtures were purified by reducing SDS-PAGE prior to peptide mapping LC-MS analysis. 400 μg of total protein was loaded onto a 4−20% tris-glycine gel (Invitrogen) and run under reducing conditions per the manufacturer's instructions. The gel was negatively stained with 4 M sodium acetate and the portion of the gel containing IFNb or PEGylated IFNb was excised. This sample was washed with water four times to remove SDS, macerated to increase surface area, and then placed in a solution of 100 mM tris pH 7.7 plus 0.05% Zwittergent 3−14. The sample was incubated at 4 °C in this buffer overnight, the supernatant was collected, and then another aliquot of buffer was added, followed by another round of incubation and supernatant collection. The supernatants were combined and concentrated in 10 000 MWCO filtration devices (Amicon). The concentrated protein was reduced by DTT, blocked with iodoacetic acid, and digested by trypsin at 37 °C overnight. Samples were then spiked with neat formic acid to 1% and analyzed by LC-MS. The equivalent of 6 μg of nondigested protein was injected onto an octadecylsilyl reversed-phase HPLC column (PROTO 200, Higgins Analytical, Inc.) and separated with a water/acetonitrile +0.1% formic acid gradient using a Waters Alliance 2695 HPLC instrument, and analyzed by electrospray time-of-flight MS using a MicroMass Q-TOF mass spectrometer (Waters). MS data were collected in full-scan mode over the range of 360−2000 m/z, and MS/MS fragment spectra were generated from the top MS ions. The resulting spectra were analyzed using MassLynx software (Waters). Predicted trypsin-generated peptides of IFNb were identified by MS matching to the theoretically predicted peptides, and the sequence was confirmed by MS/MS sequence matching. An extracted-ion chromatogram (XIC) was generated for the singly charged version of each peptide of interest to relatively quantify the peptides. The percentages of the signals for the 28−33 and 137−147 tryptic peptides and related modified versions were

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calculated by dividing the XIC peak area for each species by the sum of the peak areas for all versions of the corresponding peptide. Although the ionizing efficiency of peptides may vary, it is anticipated that the variations in the various versions of the same peptide will not have a large effect on the ionization efficiency of the same peptide family.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.5b00320.

IFNb sequence, Coomassie-stained SDS-PAGE gel of reaction mixture, LC-MS chromatogram of PEG-IFNb, mass spectra of oxidized peptides (PDF)

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Notes

The authors declare the following competing financial interest(s): All authors are or have been employed by Allozyne.

■ ABBREVIATIONS:

Aha, azidohomoalanine; CuAAC, copper-catalyzed azide–alkyne cycloaddition; DTT, dithiothreitol; IFNb, interferon beta-1b; ROS, reactive oxygen species

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