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Mechanistic investigation and further optimization of the aqueous Glaser-Hay bioconjugation

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Abstract

The Glaser-Hay bioconjugation has recently emerged as an efficient and attractive method to generate stable, useful bioconjugates with numerous applications, specifically in the field of therapeutics. Herein, we investigate the mechanism of the aqueous Glaser-Hay coupling to better understand optimization strategies. In doing so, it was identified that catalase is able to minimize protein oxidation and improve coupling efficiency, suggesting that hydrogen peroxide is produced during the aqueous Glaser-Hay bioconjugation. Further, several new ligands were investigated to minimize protein oxidation and maximize coupling efficiency. Finally, two novel strategies to streamline the Glaser-Hay bioconjugation and eliminate the need for secondary purification have been developed.

Graphical Abstract

Introduction

Protein bioconjugates, in which a protein is conjugated to another molecule, represent a critical area of research with widespread applications, most notably in the development of strategies for site-specific drug delivery and improved cellular imaging.^{1–5} Unnatural amino acid (UAA) technology introduces novel chemical moieties into proteins and allows for the

Conflicts of interest

There are no conflicts to declare.

preparation of well-defined, homogenous protein bioconjugates, which display therapeutic advantages over the heterogenous bioconjugate mixtures afforded through reactivity of native amino acid residues.^{6–11}

The Glaser-Hay coupling represents an attractive reaction to develop as a bioconjugation, given its many chemical advantages, among them the formation of a highly stable carbon-carbon bond in the form of a well-defined linear 1,3-diyne. Moreover, the reaction has been demonstrated to be tolerant of a wide range of functional groups.¹² Our previous work reported the first successful biological Glaser-Hay coupling in a full-length protein under mild, aqueous reaction conditions.^{13–15} The Glaser-Hay bioconjugation relies on the incorporation of an UAA containing a terminal alkyne: *p*-propargyloxyphenylalanine (*p*PrF, 1) (Figure 1). The diyne product of the Glaser-Hay reaction has many useful downstream applications, including its potential to obtain bioconjugates with diverse biological, photochemical, and optoelectronic properties.^{16–20}

While the biological Glaser-Hay coupling is effective, protein oxidation has been observed after around 6 hours of reaction time.¹³ Our previous work reported two optimized conditions for the Glaser-Hay bioconjugation: the use of the traditional TMEDA ligand in a pH 6.0 reaction to afford faster coupling, and the use of a carboxylated biphenyl ligand in a pH 8.0 reaction to minimize protein degradation.¹⁵ This bipyridyl ligand approach was also elucidated by others as a superior ligand system.²¹ Despite the utility of the Glaser-Hay bioconjugation and this previous work, we hypothesized there was a potential for further optimization, which could be facilitated via an enhanced understanding of the reaction mechanism in aqueous solution.

Results and Discussion

Investigating the aqueous mechanism of the Glaser-Hay coupling

The mechanism of the Glaser-Hay coupling in organic solvents has been studied in detail. In 1964, Bohlmann et al. reported the formation of a dicopper(II)-diacetylide complex as the rate-limiting step in the observed second-order kinetics.²² Later work suggested that the mechanism progresses through a dicopper(III) intermediate (Scheme 1A; bottom pathway).²³

Most recently, Vilhelmsen et al. reported the Glaser-Hay reaction as involving a Cu^{2+} intermediate, leading to the generation of hydroxyl radicals (Scheme 1A; top pathway).²⁴ This study utilized UV/Vis spectroscopy as well as ^{13}C NMR to study reaction progression under various conditions. They reported that increased copper(I) and/or increased TMEDA resulted in an increased reaction rate. However, they also notably reported that the reaction rate slowed after some time, due to the build-up of water in the reaction through its absorption from the air. Given the detrimental effects of water on the rate of the Glaser-Hay coupling in an organic solvent, we sought to investigate the kinetics of the aqueous Glaser-Hay coupling, as the reaction pathway in water may differ from the pathways previously observed for the reaction in organic solvents.

For this study, we employed UV/Vis spectroscopy as well as both ^{13}C and ^1H NMR to monitor the Glaser-Hay coupling of the dimerization of propargyl alcohol in an aqueous solution. For NMR experiments, deuterated water (D_2O) was employed as the solvent to allow for an NMR lock and for clarity of NMR spectra. Notably, reaction progress was tracked via relative integration of ^{13}C product and reactant peaks. Although such a method for quantitative kinetics studies is abnormal, it has been proven effective in quantitative analyses, specifically in kinetic studies of *in vivo* and *in vitro* processes.^{25–30} Further, Vilhlemsen et al. previously utilized integration of ^{13}C NMR to successfully track the kinetics Glaser-Hay coupling in organic solvent.²⁴ While poor signal to noise ratio and the effects of the nuclear Overhauser effect are cited as pitfalls of integrating ^{13}C NMR spectra, the strength of the 400 MHz NMR instrument coupled with the deuterated solvent used appears to be sufficient to overcome these limitations.²⁴

The Glaser-Hay dimerization of propargyl alcohol was prepared and air was bubbled through the solution for 10 minutes (see Supporting Information). The vial was then sealed and allowed to stir at 80°C . At specific timepoints, the reaction was monitored by NMR. The relatively low equivalencies of copper(I) iodide (2.4 mol%) and TMEDA (4.0 mol%) were chosen as the baseline to reflect the low concentrations of catalyst and ligand when the reaction is conducted on a biological system. Given these starting conditions, our results are translatable to the previously developed aqueous Glaser-Hay bioconjugation despite the higher concentrations of alkyne and lack of protein partner.

In order to probe the reaction kinetics of the aqueous Glaser-Hay coupling, the concentrations of reaction components were systematically adjusted. Each trial was then compared as a function of time for the rate of formation of a methylene product peak observed at 50 ppm in the ^{13}C NMR relative to the corresponding starting material peak at approximately 49.3 ppm for each set of reaction conditions (Figure 2A). The 50 ppm peak corresponds to the methylene peak in the diyne-containing product. The peak seen at approximately 49.3 ppm corresponds to the methylene peak in the alkyne starting material.

Under all reaction conditions tested, we observed the instantaneous disappearance of the terminal alkyne peak in the ^1H NMR (see Supporting Information), suggesting the rapid conversion to a copper acetylide intermediate during the aqueous Glaser-Hay coupling. This was corroborated by the presence of a triplet in the ^{13}C spectra due to the coupling of the carbon to the copper.

Varying the amount of copper(I) iodide in the reaction yielded surprising results. We observed that increases in the level of copper(I) in the reaction resulted in decreased reaction rates and less conversion of starting material to product, even at ten times (24 mol%) the original copper (I) iodide amount (Figure 2B). This finding is significant, as it suggests that the aqueous Glaser-Hay coupling may not progress through a dicopper acetylide intermediate, as had been reported as the mechanism of the reaction in organic solution. This is logical given the high coordination of water as the solvent; its ability to act as a good ligand to bind copper may make the dicopper complex less likely to form. Instead, these results suggest that copper coordinates to just a single alkyne at a time to generate a copper acetylide, which has been reported to be fairly stable.³¹ At this stage, the copper acetylide

could coordinate a free alkyne or a second copper acetylide; however, water solvation of the complex may inhibit diacetylide formation (Scheme 1B). Conversely, if a mono-acetylide complex reacting with a free alkyne is employed, increased copper in the aqueous reaction could result in most of the alkyne being converted to its copper acetylide form, leaving little free terminal alkyne starting material to react to form the dimer product. Further, the addition of more copper may lead to the formation of larger copper acetylide clusters, possibly limiting the reactivity of the catalyst in an aqueous solution.³²

Varying the amount of TMEDA in the reaction demonstrated kinetics similar to those found in organic solution. Increases in the amount of TMEDA led to increased reaction rates, suggesting increases in nitrogenous ligand concentration may help facilitate quicker reaction and more efficient coupling (See Supporting Information). This could be due to the fact that with more TMEDA in the system, more of the copper is coordinated to TMEDA and forming a reactive copper complex, rather than being solvated by just water.

UV/Vis spectroscopy indicated a shift in absorbance immediately after the addition of propargyl alcohol to the catalyst mixture in aqueous solution. When only copper(I) iodide and TMEDA were present in an aqueous solution, the solution was blue and had a maximal absorbance at a wavelength just above 600 nm (See Supporting Information). When the propargyl alcohol was added to the reaction, the color immediately converted to a green-yellow, as the absorbance shifted to a wavelength of just above 400 nm. This shift confirms the rapid formation of a copper acetylide complex, which have been reported to be yellow in color.³¹ The shift in color also indicates the changing oxidation state of copper in the reaction, as the blue color of the copper complex is lost upon coordination to propargyl alcohol. Taking specific timepoints after the addition of propargyl alcohol showed that the maximum wavelength of absorption did not change over the course of the reaction.

Optimizing the biological Glaser-Hay coupling

With our newly improved understanding of the potential mechanism of the aqueous Glaser-Hay coupling, we sought to further optimize the aqueous Glaser-Hay bioconjugation to reduce protein oxidation and improve coupling efficiency. We previously reported that the addition of the radical scavengers ascorbic acid, cysteine, and oleic acid did not afford better coupling additions and that ascorbic acid actually increased protein oxidation and inhibited coupling.¹⁵

Taking these results along with aforementioned observations of the instantaneous formation of the copper acetylide and the potential lack of formation of a dicopper intermediate, we hypothesized that the oxidative damage to protein in the aqueous Glaser-Hay bioconjugation is more likely due to damage from hydrogen peroxide (H₂O₂) generation rather than free radicals.

This hypothesis is also supported by our previous report that ascorbic acid results in increased protein oxidation and inhibits coupling. Previous studies have reported that ascorbic acid can be cytotoxic due to its production of the reactive oxygen species (ROS) hydrogen peroxide, which damages proteins and other cellular components.^{33, 34} However, it has also been demonstrated that the presence of the enzyme catalase in cells can mitigate the

cytotoxicity of ascorbic acid through the breakdown of hydrogen peroxide. Given these results, we sought to investigate whether catalase could decrease protein oxidation and improve coupling efficiency in the Glaser-Hay bioconjugation.

In order to probe the effects of catalase, we performed a 250 mL expression of GFP containing *pPrF* at residue 151. Following purification, the protein was buffer exchanged into PBS and concentrated to a standard concentration of 1.0 mg/mL to remove variability in initial protein concentration. With the GFP-151-*pPrF* in hand, a series of reactions to test the effectiveness of catalase in a coupling reaction between the mutant protein and a Fluor-488 alkyne dye were prepared.

The addition of catalase to the Glaser-Hay bioconjugation resulted in significantly less protein degradation. Better coupling efficiency was also observed when catalase was added to the reaction (Figure 3). These results suggest that the previously observed protein oxidation in the Glaser-Hay bioconjugation is likely due to the production of hydrogen peroxide, which can be broken down by catalase.

It has also been reported that hydroxyl radicals are generated through the interaction of Cu^{2+} ions and hydrogen peroxide in phosphate-buffered solution.³² Additionally, it has been suggested that the level of radical formation is at least partially dependent on the ligand chelating the Cu^{2+} ion in solution. Given this as well as our previous report of improved coupling with a carboxylated biphenyl ligand, we sought to investigate whether other ligands could be employed to minimize protein degradation in the Glaser-Hay bioconjugation.¹⁵

For the ligand, the traditionally used **2** as well as the recently reported **3** were investigated along with biquinoline (**4**), a dimethylated bipyridyl (**5**) and terpyridine (**6**) to examine the electronic contributions of the ligand to the progress of the reaction (Figure 4). We hypothesized that altering the electronics of the coordinating ligand could impact the ability of copper to facilitate the aqueous Glaser-Hay bioconjugation. The electron donating/withdrawing capability of each ligand may alter the ability of the Cu to coordinate alkyne and participate in the oxidative addition. Further, we hypothesized the various substituents on these bipyridines could afford extra rigidity, aiding in chelation and activation of the copper center to improve the reaction. Finally, the commercial availability and affordable cost of these ligands proffer practicality, improving the potential utilization of these ligands that do not require additional synthetic steps to access. Due to varied solubilities, a stock solution of ligand **2** was prepared as a 500 mM solution in H_2O . For ligand **3**, a 500 mM solution was prepared in 1 M NaOH. For ligands **4**, **5**, and **6**, 500 mM stock solutions were prepared in DMSO. To test these conditions, the reactions were again conducted between the same mutant protein and alkyne-containing dye. When conducting Glaser-Hay reactions with **2**, GFP-151-*pPrF* at pH = 6.0 was employed for 4 hours, based on previously reported optimized conditions.¹⁵ Similarly, when conducting reactions with **3**, the protein at pH = 8.0 was employed for 8 hours, in accordance with previously optimized conditions. For reactions with **4**, **5**, and **6**, the same reaction conditions as **3** were employed due to similarities in ligand structure.

A total of ten reactions were prepared to test the effectiveness of each ligand with and without the addition of catalase. Gratifyingly, the addition of catalase improved coupling and reduced protein oxidation for each of the ligands examined (Figure 5). However, differences in protein oxidation were observed between the different ligands. Reactions with ligands **2**, **3**, and **4** demonstrated little protein oxidation in general, especially in the presence of catalase. However, the reaction with ligand **5** demonstrated significantly more protein oxidation, especially in the absence of catalase. These results indicate that ligand selection has a significant impact on protein degradation during the aqueous Glaser-Hay bioconjugation. Further, we identified 2,2'-biquinoline (**4**) as a promising ligand choice.

Overall, we demonstrate that the addition of catalase as well as appropriate ligand selection can greatly reduce protein degradation and improve coupling in the Glaser-Hay bioconjugation.

Streamlining the biological Glaser-Hay coupling

Bioconjugation reactions such as the Glaser-Hay on proteins containing UAAs can be time-consuming and involve multiple purification steps. Therefore, we sought to streamline the process by attempting the Glaser-Hay bioconjugation reaction directly on the lysate from purification as well as during the protein purification process (Figure 6). Each of these reaction timepoints was analyzed with ligands **2**, **3**, and **4** in Glaser-Hay bioconjugations with a Fluor-488 alkyne dye. Preparation of ligand solutions and reaction duration remained the same as the aforementioned conditions.

To examine the Glaser-Hay bioconjugation during the protein purification process, protein purification was performed using the terminal 6XHis tag and a Ni-NTA resin. Purification was performed using a Qiagen Ni-NTA Quik Spin Kit according to manufacturer's protocol up until the elution step. At this point, the GFP-151-*pPrF* remained bound to the Ni-NTA resin, and Glaser-Hay reactants were added in the hope of performing the Glaser-Hay bioconjugation with the protein bound to the purification matrix. To test the Glaser-Hay bioconjugation on cell lysate, protein expressions were pelleted, resuspended and lysed using commercially available BugBuster for 20 minutes. After centrifugation, the lysate was used directly in the Glaser-Hay bioconjugation.

Both reaction preparation strategies were successful, as indicated by the presence of fluorescence bands on SDS-PAGE (Figure 7). Binding the mutant protein to the nickel purification resin prior to conducting the Glaser-Hay bioconjugation afforded better coupling than conducting the reaction on the lysate. Nevertheless, each of these approaches allows for increased applicability of the Glaser-Hay bioconjugation by streamlining the reaction process and eliminating additional purification steps.

Experimental

Materials and Methods

General.—Reactions were conducted under ambient atmosphere with non-distilled solvents. NMR data was acquired on a Varian Gemini 400 MHz. All GFP proteins were

purified according to manufacturer's protocols using a Qiagen Ni-NTA Quik Spin Kit. pEvol plasmids were obtained from the laboratory of Dr. Peter Schultz.

Dimerization of propargyl alcohol.—The following was used as a standard to monitor the aqueous Glaser-Hay coupling. Amounts of Cu(I), TMEDA, and propargyl alcohol were varied to analyze the effect of each on the reaction kinetics. To a flame-dried vial, 8 mg of copper(I) iodide (2.4 mol %) was added to 2 mL D₂O, along with 10 μ L of TMEDA (4.0 mol %). Next, 100 μ L of propargyl alcohol (1.7 mmol, 1 eq) was added, and air was bubbled through the reaction for 10 minutes. The reaction vial was sealed and stirred at 80°C for 12 hours. Timepoints were taken at 1 hour, 3 hours, 6 hours, 8 hours, 10 hours, and 12 hours. At each timepoint, a sample was removed from the vial and directly added to an NMR tube for ¹H and ¹³C NMR analysis. Following data acquisition, these samples were returned to their respective reactions.

Synthesis of *p*-dipropargylaminophenylalanine (pPrF).—Boc-Tyrosine-OMe (114 mg, 2 eq, 0.385 mmol) was added to a flame-dried vial. Cesium carbonate (254 mg, 3 eq, 0.58 mmol) was then added, followed by dry DMF (3 mL). This mixture was stirred at 100°C for 20 mins. 5-Bromo-1-pentyne (20 μ L, 1 eq, 0.19 mmol) was then added to the mixture, as well as a catalytic potassium iodide. The reaction was stirred overnight at 100°C, then cooled to room temperature and washed with brine (10 mL) and diethyl ether (10 mL). The ether layer was then washed with brine (10 mL x 3). The brine layer was then back-extracted with ether (10 mL). The organic layers were combined, dried with magnesium sulfate, filtered, and excess solvent was removed *in vacuo*. Column chromatography (silica gel, 5:1 hexanes/ethyl acetate) was performed to yield the protected product. The product was then dissolved in 1,4-dioxane (2 mL). Then, 1 M lithium hydroxide (2 mL) was added and the reaction was stirred at room temperature for 2 hours. 1,4-dioxane was then removed *in vacuo* and the resulting water solution was acidified through the dropwise addition of 6 M HCl. The reaction was then extracted into ethyl acetate and the organic layer dried with magnesium sulfate and filtered. Excess solvent was removed *in vacuo* to yield a colorless oil. This oil was dissolved in dichloromethane (DCM, 1.5 mL). Trifluoroacetic acid (TFA, 0.5 mL) was added and the reaction was stirred at room temperature for 1 hour. Excess solvent was removed *in vacuo* to yield pPrF as a white solid (22 mg, 0.061 mmol, 31.6% yield). ¹H NMR (400 MHz, CDCl₃): δ 7.02 (d, J = 12 Hz, 2 H), 6.82 (d, J = 12 Hz, 2 H), 4.95 (d, J = 8 Hz, 1 H), 4.53 (d, J = 8 Hz, 1 H), 4.03 (t, J = 4 Hz, 2 H), 3.71 (s, 3 H), 3.02 (m, J = 8 Hz, 1 H), 2.39 (t, J = 4 Hz, 2 H), 1.97 (m, J = 8 Hz, 2 H), 1.55 (s, 1 H), 1.41 (s, 9 H). ¹³C NMR (400 MHz, CDCl₃): δ 172.4, 157.9, 130.3, 127.9, 114.5, 83.5, 79.9, 68.8, 66.0, 54.5, 52.2, 37.4, 28.3, 28.2, 21.1, 15.1.

Expression of pPrF-containing GFP-151.—*Escherichia coli* BL21(DE3) cells were co-transformed with a pET-GFP-TAG-151 plasmid (2.0 μ L) and a pEvol-pCNF plasmid (2.0 μ L) using an Eppendorf electroporator. Cells were then plated on LB-agar plates supplemented with ampicillin (50 mg/mL) and chloramphenicol (34 mg/mL) and grown at 37°C. After 16 hours, a single colony was used to inoculate LB media (10 mL) supplemented with ampicillin and chloramphenicol. The culture was grown to confluence at 37°C over 16 hours. This culture was then used to begin an expression culture in LB media

(250 mL) at $OD_{600} = 0.1$, then incubated at 37°C until it reached an OD_{600} of between 0.7 and 0.8. At this point, mutant protein expression was induced through the addition of 1 M ITPG (250 μ L) and 20% arabinose (250 μ L), as well as 100 mM *p*PrF (2.5 mL). Induced cells were grown for an additional 16 hours at 30°C, then harvested via centrifugation (10 mins, 5000 rpm). The media was decanted, and the cell pellet was stored in a –80°C freezer for 20 minutes. Mutant GFP was then purified using commercially available Ni-NTA spin columns according to the manufacturer's protocol. Protein yield and purity was assessed via SDS-PAGE and spectrophotometrically via a Nanodrop spectrophotometer. Protein was then transferred into phosphate buffered saline solution (PBS) using 10k MWCO spin columns prior to use in bioconjugation reactions.

Protocol for Glaser-Hay bioconjugation.—To a sterile 1.5 mL Eppendorf tube, the following were added: 5 μ L of a vigorously shaken solution of CuI (500 mM in H₂O) and 5 μ L of nitrogenous ligand (500 mM). The two solutions were thoroughly mixed by pipetting and allowed to incubate for ten minutes. 30 μ L of GFP containing a terminal alkyne UAA (GFP/ *p*PrF; pH = 8.0, 1.04 ± 0.03 mg/mL) and 20 μ L of Fluor-488 Alkyne (1 mM in DMSO) were then added to the tube. Finally, 5 μ L of catalase (10 mg/mL in H₂O) was added to the tube. For control reactions, 5 μ L of PBS at the appropriate pH was added in place of catalase. The reaction was incubated at room temperature (22°C) for the appropriate reaction duration. Excess reactants were then removed by buffer exchange using 10k MWCO concentrator columns. The reaction was washed with PBS (8×200 μ L) to a final volume of 50 μ L. The reaction was analyzed by SDS-PAGE and imaged immediately to analyze fluorescence. Fluorescence intensity indicated the effective coupling reaction, as the GFP had been denatured and thus was no longer fluorescent, while the coupling to the fluorophore re-establishes a fluorescent signal. The gel was then stained for 3 hours using Coomassie Brilliant Blue, then destained overnight using a methanol solution (60% deionized H₂O, 30% MeOH, 10% acetic acid). The gel was then analyzed again on the gel imager to indicate protein presence and relative degradation.

Protocol for Glaser-Hay bioconjugation during protein purification.—A 250 mL expression of GFP/*p*PrF was spun down and cells were lysed using commercially available BugBuster. 250 μ L of lysate was added to 100 μ L of Ni-NTA resin and allowed to bind before being washed with an imidazole-containing wash buffer according to manufacturer's protocol. Next, the resin was washed with PBS (5×200 μ L) and then dried. 75 μ L of PBS was then added to wet the resin. Next, 10 μ L of a premixed 1:1 solution of CuI (500 mM in H₂O) and nitrogenous ligand (500 mM) was added. Finally, 40 μ L of Fluor-488 Alkyne (1 mM in DMSO) was added. The reaction was incubated at room temperature (22 °C) for the appropriate reaction duration. The nickel resin was then washed with PBS (8×200 μ L) and imidazole-containing wash buffer (3×200 μ L); protein was eluted following manufacturer's protocol. The reaction was analyzed by SDS-PAGE and imaged immediately to analyze fluorescence. Fluorescence intensity indicated the effective coupling reaction, as previously explained. The gel was then stained for 3 hours using Coomassie Brilliant Blue, then destained overnight using a methanol solution (60% deionized H₂O, 30% MeOH, 10% acetic acid). The gel was then analyzed again on the gel imager to indicate protein presence and relative degradation.

Protocol for Glaser-Hay bioconjugation on cell lysate.—Expression of GFP/pPrF was spun down and cells were lysed using commercially available BugBuster. The lysed cells were centrifuged again and the lysate was decanted. 20 μ L of a premixed 1:1 solution of CuI (500 mM in H₂O) and nitrogenous ligand (500 mM) was added to 250 μ L of lysate. Next, 40 μ L of Fluor-488 Alkyne (1 mM in DMSO) was added. The reaction was incubated at room temperature (22°C) for the appropriate reaction duration. The lysate was then bound to Ni-NTA resin and protein was purified according to manufacturer's protocol. The reaction was analyzed by SDS-PAGE and imaged immediately to analyze fluorescence. Fluorescence intensity indicated the effective coupling reaction, as previously explained. The gel was stained for 3 hours using Coomassie Brilliant Blue, then destained overnight using a methanol solution (60% deionized H₂O, 30% MeOH, 10% acetic acid). The gel was then analyzed again on the gel imager to indicate protein presence and relative degradation.

Conclusions

Overall, a better understanding of the aqueous Glaser-Hay reaction has been obtained, which facilitates the further optimization of the reaction to broaden its utility. Our results suggest that the aqueous Glaser-Hay reaction may not progress through a dicopper intermediate, which would make it mechanistically different from the organic solvent based Glaser-Hay reaction. These mechanistic insights also led to a better understanding of hydrogen peroxide as the source of the previously observed protein degradation. Furthermore, we successfully demonstrated that the addition of catalase to the Glaser-Hay bioconjugation improves coupling and reduces protein degradation. We further investigated the impact of various nitrogenous ligands on the Glaser-Hay bioconjugation, identifying 2,2'-biquinoline (**4**) as a promising ligand for future use. Finally, we illustrated the feasibility and applicability of a streamlined approach to conducting the Glaser-Hay bioconjugation through carrying out the reaction on the cell lysate or during protein purification, eliminating secondary purification required via conducting the reaction post-expression purification. Overall, these improvements further the utility of the Glaser-Hay bioconjugation reaction to facilitate a covalently-linked, stable, and linear bioconjugate.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A) Structure of alkynyl UAA, *pPrF*, which is incorporated into protein for utilization in Glaser-Hay bioconjugations. (B) Generalized Glaser-Hay bioconjugation between a protein and a soluble alkyne. The blue star can represent a fluorophore, protein, surface, or resin.

Figure 2.

Effect of varying copper (I) iodide on the rate of the aqueous Glaser-Hay reaction. (A) ¹³C NMR timecourse studies monitoring the methylene carbon conversion between starting material and product. (B) Normalized ¹³C integration data with increasing copper concentrations (1.2, 2.4, 4.8, and 24 mol% respectively) demonstrating decreasing rates of reaction.

Lane	L	1	2
Catalase		+	-

Figure 3. Standard Glaser-Hay bioconjugation of GFP-151-*pPrF* with Fluor-488 alkyne using the CuI/TMEDA system. The reaction was conducted in the presence (Lane 1) and absence (Lane 2) of catalase. Both reactions afforded fluorophore coupling to the protein (top gel); however, less protein degradation was observed when catalase is employed (Coomassie stained gel, bottom).

Tetramethylethylenediamine, **2**

2,2'-Bipyridine-4,4'-dicarboxylic acid, **3**

2,2'-Biquinoline, **4**

6,6'-Dimethyl-2,2'-dipyridyl, **5**

Terpyridine, **6**

Figure 4.
Nitrogenous ligands employed in the Glaser-Hay optimization.

Figure 5.

Investigation of the effects of catalase and various nitrogenous ligands on the biological Glaser-Hay reaction. Protein degradation was assessed in the presence (blue) and absence (red) of catalase via SDS-PAGE normalization of protein concentrations for each reaction. Moreover, coupling efficiency (grey) of each reaction was determined by ratios of fluorescence resulting from conjugation of the fluorescent dye to the total amount of protein in the lane. These ratios were normalized to the previously reported conditions (ligand 2).

Figure 6. Streamlining of the Glaser-Hay bioconjugation. Following protein expression with the *pPrF* UAA, the Glaser-Hay reaction can be conducted at various stages of the purification process. This reaction can occur directly from the lysate (first column), from the Ni-NTA immobilized protein (second column), or following elution from the Ni-NTA resin (third column).

Figure 7. SDS-PAGE results from investigations of the Glaser-Hay bioconjugation. The best coupling was observed from conducting the reaction on the completely purified protein (Lane 1). Coupling was observed with both the Glaser-Hay reaction being performed on the Ni-NTA resin (Lane 2), and directly on the lysate (Lane 3). No coupling is observed in the absence of the CuI/TMEDA system as observed by the presence of protein but lack of fluorescence in Lane 4.

Scheme 1.

Potential Glaser-Hay mechanisms. (A) Previously proposed Glaser-Hay mechanisms in organic solution.^{23, 24} (B) Potential Glaser-Hay mechanism in aqueous solution utilizing a single Cu atom.