Bioorthogonal Chemistry

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A Photoinducible 1,3-Dipolar Cycloaddition Reaction for Rapid, Selective Modification of Tetrazole-Containing Proteins**

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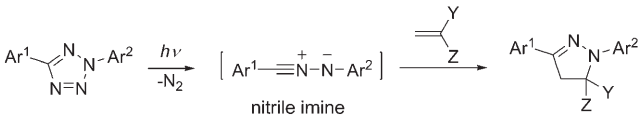
Bioorthogonal chemistry provides an exciting new strategy to visualize protein expression, track protein localization, measure protein activity, and identify protein interaction partners in living systems.[1] Two steps are typically involved in this approach: 1) the incorporation of a bioorthogonal group into a protein through either a biochemical pathway or semisynthesis; 2) a site-specific reaction between the protein that carries the bioorthogonal group and a cognate small-molecule probe. Although a plethora of methods have been developed to address the first step, such as non-sense suppression mutagenesis,[2] expressed protein ligation,[3] metabolic engineering,[4] and tagging-via-substrate,[5] only a small number of bioorthogonal reactions are known for the second step. These site-specific reactions include the acid-catalyzed nucleophilic addition of hydrazine to a ketone or aldehyde,[6] Staudinger ligation,[7] Cu^{I}-catalyzed azide–alkyne 1,3-dipolar cycloaddition (click chemistry),[8] strain-promoted azide–alkyne 1,3-dipolar cycloaddition,[9] and the oxidative coupling of aniline.[10] To fully realize the potential of bioorthogonal chemistry in probing protein function, there is an urgent need for the discovery of additional bioorthogonal reactions with robust reaction attributes. Herein, we report a bioorthogonal, photoinducible 1,3-dipolar cycloaddition reaction that allows rapid and highly selective modification of proteins carrying a diaryl tetrazole group in biological media.

Forty years ago, Huisgen and co-workers reported a photoactivated 1,3-dipolar cycloaddition reaction between 2,5-diphenyltetrazole and methyl crotonate.[11] A concerted reaction mechanism was proposed, whereby the diaryl tetrazole undergoes a facile cycloreversion reaction upon photoirradiation to release N_2 and generate in situ a nitrile imine dipole, which cyclizes spontaneously with an alkene dipolarophile to afford a pyrazoline cycloadduct (Scheme 1). The photolysis of diaryl tetrazoles was found to be extremely efficient upon UV irradiation at 290 nm, with quantum yields in the range 0.5–0.9.[12] Despite its robust mechanism, this photoactivated reaction has seen very few applications in the past four decades.[13]



Scheme 1. Photoactivated 1,3-dipolar cycloaddition reaction between a 2,5-diaryl tetrazole and a substituted alkene dipolarophile.

In our initial studies, we identified an extremely mild photoactivation procedure in the use of a hand-held UV lamp from UVP (UVM-57, 302 nm, 115 V, 0.16 amps). Under these mild conditions, the solvent compatibility, functional-group tolerance, regioselectivity, and yield of the photoactivated 1,3-dipolar cycloaddition reaction were excellent.[14] We then examined the reaction kinetics by incubating a tetrazole peptide with acrylamide in phosphate-buffered saline (PBS) at pH 7.5 under UV light (302 nm; see Figure S1 in the Supporting Information). We found that the photolysis of the tetrazole peptide to generate the nitrile imine intermediate was extremely rapid, with a first-order rate constant $k_1 = 0.14\ s^{-1}$; the subsequent cycloaddition with the dipolarophile acrylamide proceeded very efficiently, with a second-order rate constant $k_2 = 11.0\ M^{-1}s^{-1}$.[15] In the absence of a dipolarophile, however, slow formation of the nitrile imine–H_2O adduct was observed (see Figure S2 in the Supporting Information). Moreover, the pyrazoline cycloadducts showed strong fluorescence with variable emissions in the region of 487–538 nm, depending on the structure of the dipolarophile (see Figure S3 in the Supporting Information), and a high fluorescence quantum yield ($\Phi = 0.29$).

To examine the bioorthogonality of this reaction for residue-specific protein modification, we introduced the diphenyltetrazole moiety into the lysozyme by acylating the surface lysine residues of the lysozyme with the water-soluble tetrazole succinimide **1** (Figure 1a). A mixture of tetrazole-modified lysozymes (44% monoacylated, 5% bisacylated) was obtained, together with the unmodified lysozyme (51%). After the removal of small molecules by size-exclusion chromatography, the mixture was incubated with acrylamide (50 equiv) and irradiated with UV light (302 nm) for 2 min (Figure 1a). The pyrazoline product was analyzed along with the unmodified lysozyme and the tetrazole-modified lysozyme by LC–ESIMS. The expected intact masses of the lysozyme, the tetrazole-modified lysozyme (Lyso-Tet), and the pyrazoline product (Lyso-Pyr) were observed (Figure 1b–d). The conversion of Lyso-Tet into Lyso-Pyr was estimated to be 90% on the basis of LC–MS analysis (see Figure S5 in the Supporting Information). No obvious side products, such as

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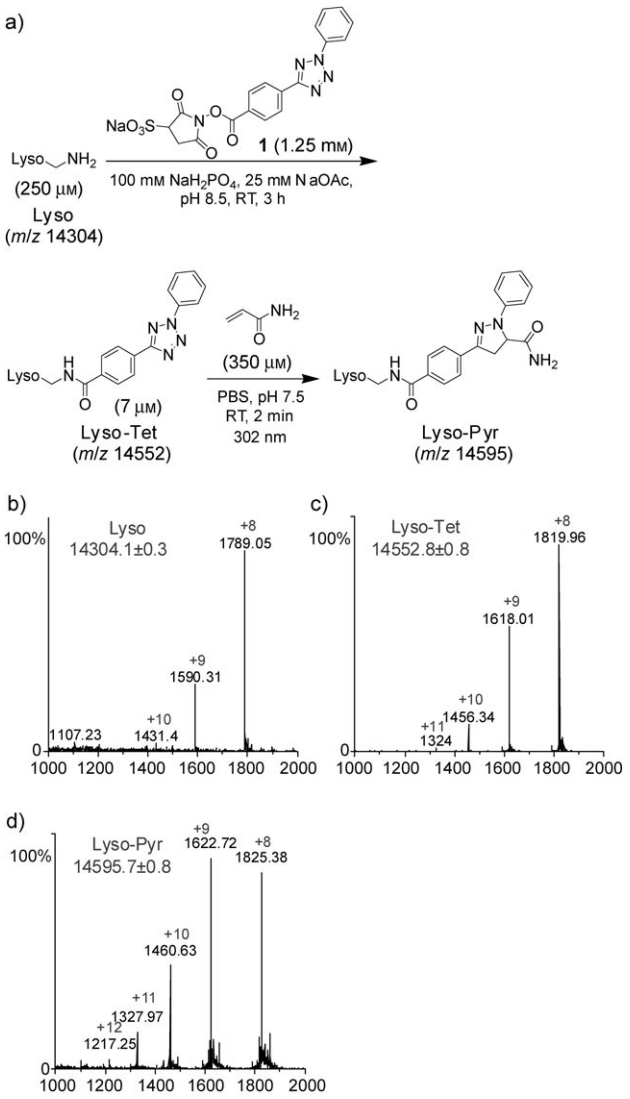

Figure 1. Fast and selective modification of the tetrazole-containing lysozyme by acrylamide. a) Introduction of 2,5-diphenyltetrazole into the lysozyme through acylation, and subsequent photoinduced, tetrazole-specific modification by acrylamide. ESIMS analysis of b) the lysozyme, c) the tetrazole-containing lysozyme, Lyso-Tet, and d) the photoinduced cycloaddition product, Lyso-Pyr.

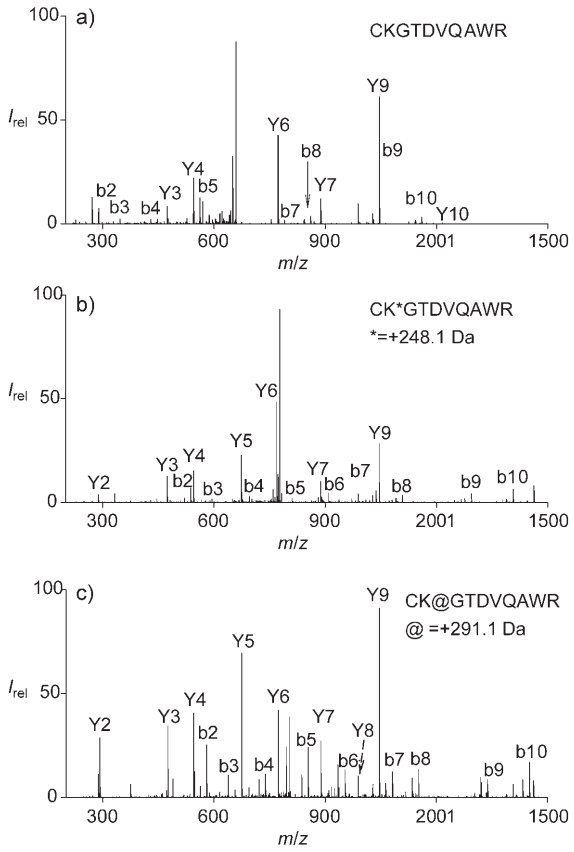

Figure 2. Sequence-informative MS2 fragment spectra of a representative tryptic peptide (CKGTDVQAWR) derived from chicken lysozyme: a) the fragment derived from the unmodified lysozyme; b) the fragment derived from Lyso-Tet (the addition of a mass corresponding to that of the tetrazole (248.1 Da) to K116 was observed); c) the fragment derived from Lyso-Pyr (the addition of a mass corresponding to that of the pyrazoline (291.1 Da) to K116 was observed).

lysozyme adducts derived from Michael addition to acrylamide, were observed in the product mixture.

To confirm that modification occurred on the tetrazole-modified Lys side chains, the unmodified lysozyme, Lyso-Tet, and Lyso-Pyr samples were digested with trypsin, and the resulting peptide fragments were analyzed by tandem mass spectrometry. Of six surface lysine residues in the lysozyme, four (K13, K33, K97, and K116) showed mass increases of 248.1 Da (tetrazole mass) in the digested Lyso-Tet sample, whereas five (K13, K33, K96, K97, and K116) showed mass increases of 291.1 Da (pyrazoline mass) in the digested Lyso-Pyr sample (Figure 2). Neither the 248.1-Da increases (Lyso-Tet starting materials) nor the 71.0-Da increases (Michael addition to acrylamide) were observed in the digested Lyso-Pyr sample, which indicates that the cycloaddition reaction was very specific and essentially quantitative. Furthermore, purified, homogeneous Lyso-Tet produced fluorescent adducts upon UV irradiation for 1 min in the presence of various dipolarophiles, including acrylamide (**2**), coumarine methacrylamide (**3**), and palmityl methacrylamide (**4**; lanes 3, 6, and 9 in Figure 3), whereas the unmodified lysozyme did not (lanes 1, 4, and 7). These results again confirm specific cycloaddition at the tetrazole sites.

To further evaluate the bioorthogonality of this cycloaddition reaction for site-specific protein modification, we prepared a tetrazole-containing enhanced green fluorescent protein, EGFP-Tet, by using the intein-based chemical ligation strategy (Figure 4a). When EGFP-Tet was incubated with the lipid dipolarophile **4** under UV irradiation at 302 nm for 1 min, a fluorescent band indicating the formation of the fluorescent EGFP-Pyr adduct was observed in the denaturing SDS-PAGE gel (lane 3 in Figure 4b). No fluorescent band was observed upon the irradiation of EGFP itself (lane 6, Figure 4b). Furthermore, fluorescence monitoring of the photoinduced cycloaddition reaction in solution revealed the appearance of an emission band for pyrazoline adducts at around 460 nm along with a concomitant roughly twofold increase in EGFP fluorescence intensity at 509 nm. These observations can be attributed to fluorescence resonance

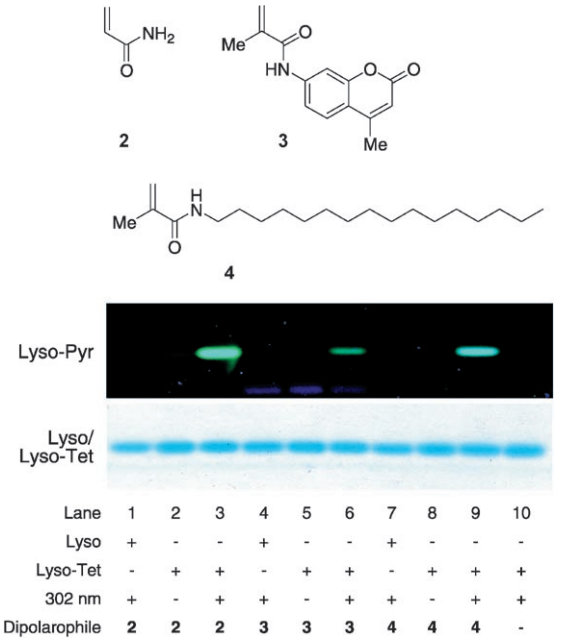

Figure 3. Photoinduced formation of fluorescent pyrazoline adducts with the tetrazole-containing lysozyme. The structures of the dipolarophiles are shown at the top. Fluorescence imaging (top panel, $\lambda_{ex} = 365$ nm) and Coomassie Blue staining (bottom panel) are shown for various lysozyme samples. Photoinduction was carried out for a duration of 1 min with UV irradiation at 302 nm. The blue fluorescence of coumarin is visible at the front edge of the gel in the fluorescence panel (lanes 4–6).

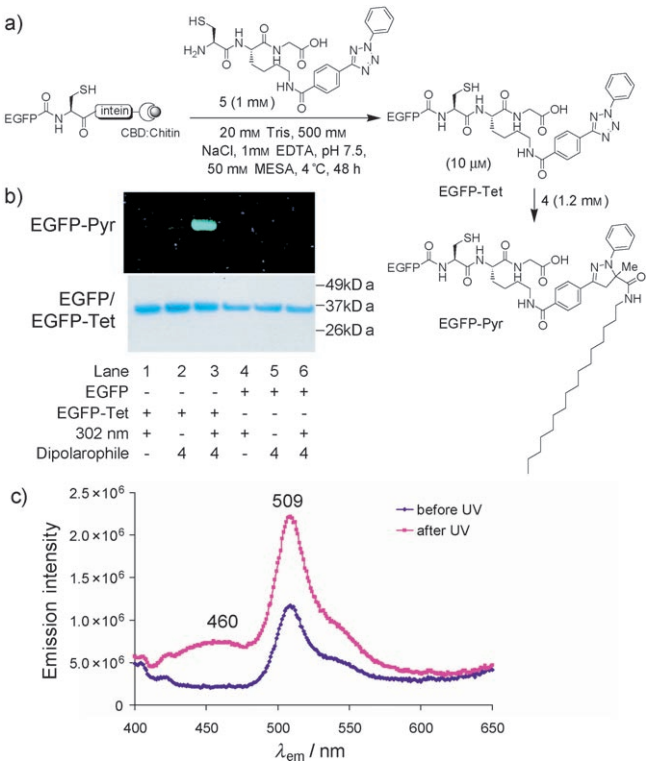

Figure 4. Photoinduced lipidation of EGFP with a tetrazole motif at its C terminus. a) Preparation of EGFP-Tet and subsequent photoinduced lipidation by the dipolarophile **4**. b) Fluorescence imaging (top panel, $\lambda_{ex} = 365$ nm) and Coomassie Blue staining (bottom panel) of EGFP and EGFP-Tet upon photoirradiation in the presence or absence of **4**. Photoinduction was carried out for a duration of 1 min with UV irradiation at 302 nm. c) Fluorescence spectra of EGFP-Tet (before UV irradiation) and EGFP-Pyr (after UV irradiation) with $\lambda_{ex} = 370$ nm. MESA = 2-mercaptoethanesulfonic acid.

energy transfer from the pyrazoline fluorophore to the EGFP fluorophore in the EGFP-Pyr adduct (Figure 4c). An increase of 280.5 Da in intact mass was also observed by ESIMS. This value matches well with the expected mass increase of 281.3 Da during the cycloaddition reaction (see Figures S6 and S7 in the Supporting Information). The conversion of EGFP-Tet into EGFP-Pyr was estimated to be 52% on the basis of LC–MS analysis (see Figure S7 in the Supporting Information). Furthermore, the photoinduced modification of EGFP-Tet by the dipolarophile **4** was found to proceed in the bacterial cell lysates with the same efficiency as in the biological medium (see Figure S8 in the Supporting Information).

In summary, we have developed a robust, photoinducible 1,3-dipolar cycloaddition reaction for selective protein modification in biological media. This reaction is extremely fast ($\leq$1 min) and tolerant of proteinaceous groups. Moreover, as fluorescent adducts are formed, the labeling of nonfluorescent proteins can be monitored in a facile manner. We attached the tetrazole group to the proteins by protein semisynthesis followed by treatment with a simple set of alkenes; however, the reverse procedure, that is, the attachment of alkenes (or alkynes[16]) to proteins, followed by treatment with diaryl tetrazoles, proceeds with similar efficiency (see Figures S9 and S10 in the Supporting Information). As several methods exist for the introduction of alkenes and alkynes into proteins in cellular systems,[8b,17] this bioorthogonal reaction should be useful for manipulating protein function in living systems. Efforts to optimize the photoreactivity of diaryl tetrazoles, for example, by tuning photoactivation to occur in the long-wavelength region, are under way. We are also investigating the site-specific incorporation of tetrazole-derived amino acids into proteins in mammalian cells and the application of this chemistry to intracellular protein regulation.

Experimental Section

Preparation of EGFP-Tet: The pTXB1–EGFP plasmid was transformed into the Rosetta competent cells (Novagen) by the heat-shock method and plated onto an Laria–Bertani (LB) agar plate containing ampicillin (50 μg mL^{-1}). The single colony picked from the plate was allowed to grow in LB medium (20 mL) supplemented with ampicillin (50 μg mL^{-1}) at 37 °C. The overnight culture was diluted into fresh LB medium (500 mL) containing ampicillin (50 μg mL^{-1}) and allowed to grow at 30 °C until the OD_{600} value reached 0.6. The induction of EGFP was initiated by adding isopropyl β-D-1-thiogalactopyranoside (IPTG; 0.3 mM), and the culture was allowed to grow for an additional 6 h. The cells were then harvested, and the EGFP–intein–CBD fusion protein (CBD = chitin-binding domain) in the supernatant was captured on a freshly prepared affinity column loaded with chitin beads (1.2 mL). The column was washed with a wash buffer (5 × 10 mL; Tris (20 mM), NaCl (500 mM), EDTA (1 mM), pH 7.5; Tris = tris(hydroxymethyl)aminomethane, EDTA = ethylenediaminetetraacetic acid), and the beads were then treated with a

cleavage buffer (2 mL; wash buffer supplemented with compound **5** (1 mM) 50 mM MESA) for 48 h at 4 °C. The filtrate was subjected to size-exclusion chromatography (superdex 75) to remove excess **5**, and the fractions were examined by SDS-PAGE. The pure fractions were pooled to give EGFP-Tet.

Lipidation of EGFP-Tet: The lipid dipolarophile **4** (16 mM in EtOH, 7.5 μL) was added to a 100-μL sample of either EGFP (8 μM) or EGFP-Tet (10 μM) in a wash buffer (Tris (20 mM), NaCl (500 mM), EDTA (1 mM), pH 7.5) in a quartz test tube. After irradiation with a hand-held 302-nm UV lamp for 1 min, 20-μL aliquots were withdrawn and analyzed by SDS-PAGE on a NuPAGE 4–12 % Bis-Tris gel. The EGFP-Pyr adducts in the gel were visualized by illuminating the gel with a hand-held 365-nm UV lamp and recorded with a digital camera. Afterwards, the gel was stained with Coomassie Blue to confirm the equal loading of proteins.

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