JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Communication

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Target-Activated Coumarin Phototriggers Specifically Switch on Fluorescence and Photocleavage upon Bonding to Thiol-Bearing Protein

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Supporting Information

ABSTRACT: A new concept in which only the molecular target, such as a thiol-bearing protein, can activate the phototrigger has been demonstrated. Such target-activatable phototriggers comprise three parts: a 7-aminocoumarin phototrigger, an electron acceptor (maleimide) that efficiently quenches the coumarin excited state, and a caged leaving group attached to the coumarin. In the absence of mercaptans, photoinduced electron transfer between coumarin and maleimide effectively blocks both the fluorescence and photocleavage pathways. Thiol-bearing molecules, however, readily annihilate the electron acceptor and thus restore the phototrigger for photorelease of the caged cargo (e.g., biotin). Unlike traditional phototriggers, functional-group-activated phototriggers allow easy handling under ambient light, report specific bonding to the target, and enable photocleavage capability selectively at the binding site in situ, thus effectively positioning the photoreleased cargo at the target. Meanwhile, the unique feature of thiol-specific activation of the fluorescence and photocleavage make our new phototrigger a universal tool that can be used to identify accurately protein cysteine S-nitrosylation, a physiologically important posttranslational modification.

Photocleavage of a specific bond enables biomolecule uncaging, protein purification, fluorescence activation, and drug release with spatial and temporal precision. Such photolabile protecting groups or phototriggers have recently been recognized as a powerful tool in biotechnologies and biomedical applications.[1,2] Typically, phototriggers are activated and armed but not yet fired.[3] Such phototriggers, however, cannot avoid undesirable firing when inadvertently exposed to ambient light. Ideally, a phototrigger should have an additional state in which the armed trigger is locked and cannot accidentally misfire. In this design, only the targeted molecule can unlock the phototrigger, thus activating the triggered downstream events. Specifically, the target molecule functions as a stimulating signal and triggers a cascade of events of fluorescence activation and subsequent photocleavage. Herein we report such target-activated phototriggers (TPTs) that are capable of receiving molecular signals from thiol- or *S*-nitroso (SNO)-bearing proteins. Because the armed yet locked state of TPTs exhibits superior photostability, not only can they be handled in ambient light, but also the specific target-unlocking characteristic controls the highly selective photocleavage with accurate positioning.

To date, none of the commonly used photolabile protecting groups (e.g., 2-nitrobenzyls, coumarins, benzoins, and phenacyls) have been reported to achieve successful target-activated photo-uncaging.[3] Thus, we employed the intrinsic photo-S_N1-dependent cleavage[4] of the coumarin trigger to construct a TPT molecule by borrowing designs from activatable fluorescent probes.[5] Our design incorporated an electron-transfer quencher to control the photocleavage by locking or unlocking the coumarin phototrigger. In the locked state, any photoexcitation of the coumarin dye relaxes via photoinduced electron transfer (PET) to the electron acceptor, thus switching off the coumarin fluorescence as well as the photocleavage. In the unlocked state, the coumarin phototrigger is reactivated to generate fluorescence and liberate the caged compounds under visible light.

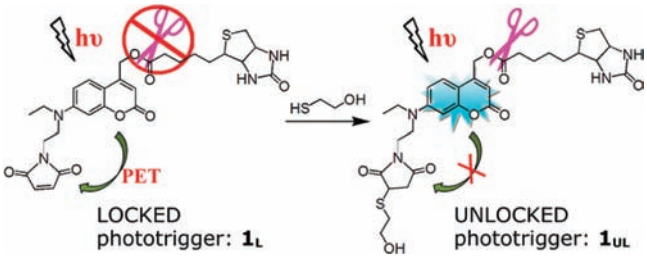
Considering that thiols play key roles in biological redox systems and protein post-translational modification,[6] we decided to use the mercapto group as the signal to unlock the phototrigger. Thus, a thiol-reacting maleimide that also functions as an electron acceptor was conjugated to the 7-aminocoumarin phototrigger.[5,7] The resultant compound can be activated only by a thiol group, and the unlocked state is unambiguously confirmed by coumarin fluorescence. After the trigger is unlocked, the caged cargo on the coumarin can be photoreleased (Scheme 1). Such target-activated uncaging uses visible light and thus avoids UV-caused phototoxicity to biological systems.

The phototrigger carrying a biotin cargo was synthesized in the locked state $\mathbf{1}_L$ [Figure S1 in the Supporting Information (SI) and Scheme 1]. Photolysis of the locked phototrigger $\mathbf{1}_L$ was attempted without success. HPLC revealed that compound $\mathbf{1}_L$ had no significant photolytic products after an extended (>20 min) visible-light irradiation (Figure 1A). Additionally, the locked phototrigger $\mathbf{1}_L$ was exposed to daylight for 2 days, and no photochemical products were observed. However, when mercaptans were used to convert the maleimide to a succinimide via Michael addition, the locked phototrigger $\mathbf{1}_L$ was converted to the unlocked state $\mathbf{1}_{UL}$, and the unlocked phototrigger underwent photolysis smoothly.

Received: January 15, 2012
Published: March 6, 2012

dx.doi.org/10.1021/ja300475k | *J. Am. Chem. Soc.* 2012, 134, 5052–5055

Scheme 1. Thiols Activate the Locked Phototrigger (1_L), Converting It into the Unlocked Phototrigger (1_{UL})



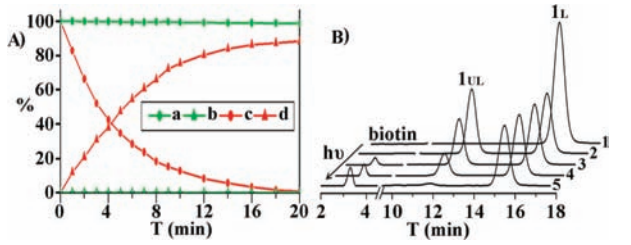


Figure 1. (A) Photolysis monitored by HPLC: (a) consumption curve of 1_L; (b) release curve of biotin from 1_L; (c) consumption curve of 1_{UL}; (d) release curve of biotin from 1_{UL}. (B) HPLC profiles of 1_L: (1) 1_L alone; (2) mixture of 1_L and 1_{UL} by partial thiol activation of 1_L; (3–5) photolysis of the mixture in (2) for (3) 3, (4) 8, and (5) 18 min.

To determine the thiol activation of the phototrigger, we added a desired amount of β-mercaptoethanol to the solution of 1_L [10^{-5} M in 6:4 MeOH/tris-HCl buffer (pH 7.4)]. Michael addition of the thiol to the maleimide rapidly converted some 1_L to 1_{UL}, thus unlocking a subpopulation of the phototrigger. The resulting mixture containing both 1_L and 1_{UL} in a cuvette was then irradiated with visible light. The photolysis profiles were monitored with HPLC and are displayed in Figure 1B. Even under the same irradiation, the locked phototrigger 1_L remained intact. Photolysis of the unlocked phototrigger 1_{UL}, however, was complete in 20 min ($\varphi_{chem} \approx 0.25$). Unlocking of the phototrigger enabled photorelease of the attached biotin (>87%), as evidenced by the HPLC (Figure 1) and UV–vis absorption and fluorescence emission (Figure S2).

According to the photo-S_N1 cleavage mechanism reported by Hagen and Bendig,[4] photochemical reaction and fluorescence represent the two main deactivation pathways of the S_1 state, provided that no other quenching routes are available. Hence, fluorescence measurements were conducted to determine the mechanism of the thiol-activated photocleavage. For the locked phototrigger 1_L, the fluorescence was negligible (Figure S3). However, the intrinsic fluorescence of coumarin was rapidly recovered once a sufficient amount of the thiol was introduced to convert 1_L to the unlocked 1_{UL}. Figure 2A displays the time-dependent response of 1_L upon addition of β-mercaptoethanol. The 480 nm fluorescence intensity exhibited a 43-fold enhancement within 150 s after the reaction with the thiol was complete. The fluorescence quantum yield was recovered from $\Phi_f = 0.01$ for 1_L to $\Phi_f = 0.43$ for 1_{UL} (Table S1 in the SI). These results confirm the previous report that the maleimide ring effectively quenches the S_1 excited state of coumarin dye by intermolecular PET.[5,7] Like thiol-activatable fluorescence probes, coumarin dyes are turned "off" by maleimides and "on" by thiol groups because Michael addition converts the

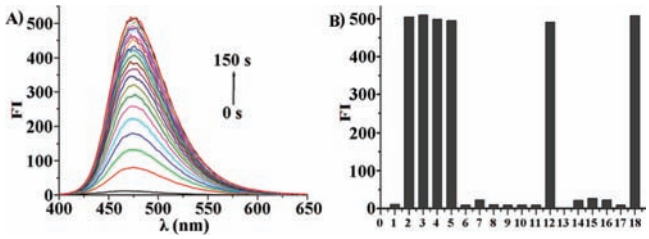


Figure 2. (A) The presence of 100 μM β-mercaptoethanol in the 10 μM 1_L solution of 6:4 MeOH/tris-HCl buffer (pH 7.4) gradually converted 1_L to 1_{UL}, thus enhancing the fluorescence intensity at 480 nm (λ_{ex} = 390 nm). (B) Responses of 10 μM 1_L upon exposure to different analytes (100 μM) in 6:4 solutions of MeOH or DMSO (for proteins) and Tris-HCl buffer (pH 7.4). The thiol-bearing analytes could unlock 1_L and thus produce strong luminescence, whereas solutions of those that could not unlock 1_L remained dark: 1, blank; 2, β-mercaptoethanol; 3, cysteine; 4, homocysteine; 5, glutathione; 6, glutamic acid, phenylanaline, alanine; 7, lysine; 8, glucose; 9, ascorbate; 10, H_2O_2; 11, Mg^{2+}, Fe^{3+}, Zn^{2+}, Ca^{2+}; 12, BSA; 13, BSA + NEM; 14, transferrin; 15, ovalbumin; 16, trypsin inhibitor; 17, lysozyme; 18, lysozyme + TCEP.

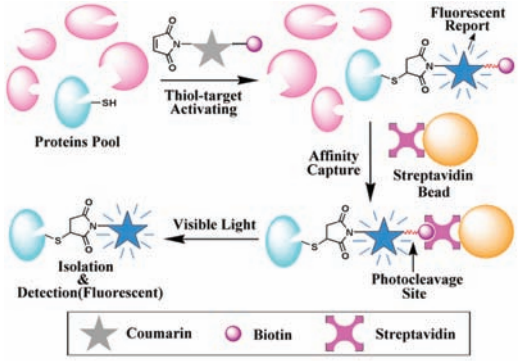
maleimide to a succinimide. Consequently, it was inferred that PET from coumarin to the maleimide effectively blocked the fluorescence and forbade the photocleavage. The key to open this forbidden pathway is mercaptans, which reactivate the fluorescence pathway along with photochemical reaction from the S_1 state. The dramatic contrast between highly photostable 1_L and rapidly photolytic 1_{UL} reveals that a new class of phototriggers, which can be only activated by their specific target molecules, have been successfully created.

To examine the selectivity of target activation, we treated the locked trigger 1_L with various biologically relevant compounds including thiol-containing amino acids (cysteine, homocysteine, glutathione), non-thiol-bearing amino acids (glutamic acid, phenylalanine, alanine, lysine), glucose, a reactive oxygen species (H_2O_2), a reducing agent (ascorbate), metal ions (Mg^{2+}, Fe^{3+}, Zn^{2+}, Ca^{2+}), non-thiol-bearing proteins (transferrin, ovalbumin, lysozyme, trypsin inhibitor), a thiol-bearing protein [bovine serum albumin (BSA)], a thiol-disabled protein [BSA + *N*-ethylmaleimide (NEM)], and finally, a protein containing reduced disulfide bonds [lysozyme + tris(2-carboxyethyl)phosphine hydrochloride (TCEP)]. The histogram in Figure 2B reveals that the locked phototrigger was highly specific for mercaptans. Both molecular and macromolecular thiols could unlock the phototrigger, whereas the other biologically relevant species could not. These results are in good agreement with the highly chemoselective nature of the thiol–maleimide Michael addition reaction.

To evaluate further the transduction of the activation signal from the thiol-bearing protein to the locked phototrigger 1_L, the experiments outlined in Scheme 2 were carried out. The thiol-bearing protein in the presence of other non-thiol-bearing proteins was used to test (1) the selectivity of the phototrigger for its target and (2) the activation by the target signal followed by subsequent transduction of the signal to the trigger to photocleave the cargo on the coumarin. Thus, the locked phototrigger 1_L carrying a caged biotin was incubated with a mixture of a thiol-bearing protein (BSA) and several non-thiol-bearing proteins (transferrin, ovalbumin, lysozyme, trypsin inhibitor) for 0.5 h. Next, streptavidin agarose beads were used for affinity capture of the biotinylated target protein in the mixture because of the strong biotin–streptavidin association

dx.doi.org/10.1021/ja300475k | J. Am. Chem. Soc. 2012, 134, 5052–5055

Scheme 2. Our Strategy Uses the High Biotin−Streptavidin Affinity for Specific Capture of the Thiol-Bearing Protein from the Mixture and Confirmation of Thiol−Protein-Activated Photocleavage of the Caged Biotin



(association constant $K_a \approx 10^{14}-10^{15}$ M^{-1}). After isolation, the agarose beads adsorbed a layer of the targeted protein carrying unlocked phototrigger ($\mathbf{1}_{UL}$). The selective capture of the target protein was signaled by the on-switching of the blue coumarin fluorescence (Figure 3B).

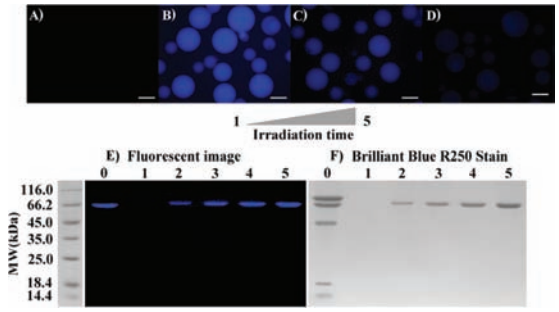


Figure 3. Photolysis of streptavidin agarose beads to release the captured biotinylated protein using visible-light irradiation (CHF-XM-500w lamp, >400 nm filter, 60 mW/cm^2). (top) fluorescence microscope images of photolyzed beads: (A) blank beads, (B−D) protein-loaded beads after 0, 2, and 8 min of light exposure. (bottom) SDS-PAGE of the supernatant viewed via (E) fluorescence or (F) Coomassie brilliant blue R250 stain: lane 0 contained the original protein mixtures incubated with $\mathbf{1}_L$ before affinity capture, whereas lanes 1−5 were loaded with the supernatant after different light exposure times (1, 0 min; 2, 1 min; 3, 2 min; 4, 4 min; 5, 8 min).

Next, these loaded beads were resuspended in a tris-HCl buffer and photolyzed with visible light (CHF-XM-500w lamp, >400 nm filter, 60 mW/cm^2). The supernatant was monitored by UV−vis absorption, fluorescence, and SDS-PAGE, whereas the beads were collected to monitor the disappearance of the fluorescence. Figure 3 and Figure S4 reveal that the photolysis was almost complete within 10 min, as evidenced by the absorption and fluorescence increase in the supernatant and the disappearance of fluorescence on the beads (Figure 3B−D). SDS-PAGE revealed a single fluorescence band of protein at ~66 kDa, corresponding to the thiol-bearing BSA. Lane 0 in Figure 3 confirms that the phototrigger specifically bonded to the thiol-bearing protein from a complex mixture of proteins. Lanes 1−5 in Figure 3 verify that the target-unlocked photolysis of the phototrigger was well-behaved and efficient. These results indicate again that only thiols can efficiently activate the phototrigger, which allows the capture of BSA protein from the mixture. Mild visible-light illumination releases the targeted protein from the beads in high purity and with efficient protein recovery (>70%), avoiding harsh UV conditions or aqueous SDS/heating that could cause denaturation of the biomacromolecules. More generally, the new target-unlocked phototriggers have the potential to become useful tools for the isolation and detection of targeted biomacromolecules. These results represent a significant step toward the next generation of phototriggers, capable of selective recognition, fluorescence detection, and signal transduction to activate photorelease at the specified location precisely.

To apply phototrigger $\mathbf{1}_L$ in a physiologically relevant sample, we chose to investigate post-translational modification of thiol-bearing proteins, in particular the formation of SNO-bearing proteins in human blood serum. S-Nitrosylation is the major mechanism for cellular nitric oxide (NO)-mediated signal transduction and plays pervasive roles in normal physiological and pathological modulation of protein functions.[8] Hence, using phototrigger $\mathbf{1}_L$ to identify *S*-nitrosoproteome as displayed in Figure 4A is significant. Here we utilized the

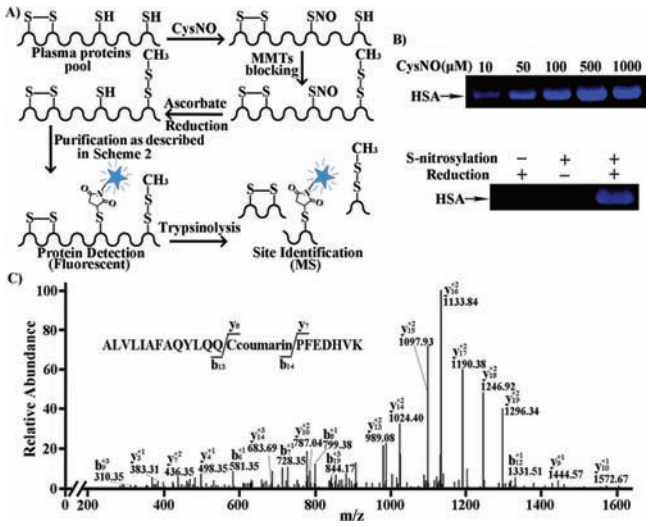


Figure 4. (A) Schematic of the protocol for identifying protein Cys−SNO modification using phototrigger $\mathbf{1}_L$ and $\mathbf{1}_{UL}$. (B) Top: Fluorescence imaging of the SDS-PAGE of human plasma proteins treated with various concentrations of CysNO (1, 0 μM; 2, 10 μM; 3, 50 μM; 4, 100 μM; 5, 500 μM; 6, 1000 μM) and purified by affinity capture followed by photolysis. Bottom: Fluorescence imaging of the SDS-PAGE of the S-nitrosylated plasma protein under different control conditions. (C) Tandem MS/MS spectra of the extracted ion on the $[M + H]^{3+}$ peak (*m*/*z* 926.1 in Figure S8) of peptides T21−41 in HSA with the unique coumarin adduct.

biotin-switch technique (BST),[9] in which free thiols on proteins in human plasma are blocked with an alkylation agent after exogenous treatment with the NO donor (CysNO). Subsequently, the S−NO bonds were specifically reduced with ascorbate (Figure S5),[8a] thereby recovering the thiol site from SNO−Cys in situ for labeling by and simultaneous activation of the phototrigger $\mathbf{1}_L$.

Figure 4A reveals that phototrigger $\mathbf{1}_L$ exhibits multi-functionality within a single molecule, functioning as a universal tool for thiol-proteomics research. The unique thiol-activated fluorescence of phototrigger $\mathbf{1}_{UL}$ enables the convenient semiquantitative determination of S-nitrosylation (Figure S6). Meanwhile, effective tracking of $\mathbf{1}_{UL}$ provides direct visual-

dx.doi.org/10.1021/ja300475k | *J. Am. Chem. Soc.* 2012, 134, 5052−5055

ization of the S-nitrosylated protein during protein enrichment and purification as well as SDS-PAGE in-gel analysis, as shown in Figure 4B. Again, mild photocleavage avoided the use of a harsh, high-concentration reductant such as dithiothreitol or mercaptoethanol as an exogenous elution agent, as in the previous isolation methodologies, which might induce fictitiously reduced disulfide bonds in the targeted protein during the elution. SDS-PAGE of the photolyzed supernatant (Figure 4B and Figure S7) indicated that only the human serum albumin (HSA, 66kD) of all blood proteins can be S-nitrosylated, confirming a previous report that SNO-albumin in plasma serves as a stable circulating storage form of NO.[10]

Importantly, avidin−biotin affinity capturing introduces a specific coumarin tag to the S-nitrosylation site after photocleavage. This tagging feature allows the direct determination of the residue that received SNO modification using mass spectrometric localization of the modified cysteine. Accordingly, nano-flow LC−MS/MS analysis of the peptides was carried out after in-gel trypsinolysis of the HSA band. In Figure S8, the triply charged precursor ion with four isotopic peaks corresponds to the peptide ALVLIAFAQYLQQCcoumarin PFEDHVK with the coumarin adduct ($\Delta m/z$ = 342.1), suggesting that the S-nitrosylation occurred on sequence T21−41 of HSA. As revealed by the tandem MS/MS of the extracted ion on the $[M + H]^{3+}$ peak (m/z 926.1) in Figure 4C, the $\Delta m/z$ range between the two informative fragment ions y_9^+ and y_7^{2+} corresponds to m/z 591.2 [glutamine (Q) + cysteine (C) + coumarin adduct − $2H_2O$], which unambiguously identifies the SNO site in HSA as the Cys-34 residue. These results demonstrate that our new phototrigger has the desired superiority in thiol-proteomics studies because it serves as an efficient fluorescent marker for visualization of the target proteins, provides photocleavage for affinity purification, and introduces an identification tag for determining the specific SNO site in the protein by mass analysis.

In summary, we have successfully constructed a new class of target-activated phototriggers. Target binding sends a signal to unlock the phototrigger, thus causing specific photocleavage. The use of maleimide enabled the phototrigger to target mercaptans selectively and efficiently in both small molecules and large biomacromolecules. The locking and unlocking of the phototrigger were used to demonstrate fluorescence detection of thiol-bearing proteins, selective capture, target-activated release, and tag conservation for mass identification. The new concept of target-activated phototriggers offers a powerful tool for significant applications in thiol-proteomics research, such as protein cysteine S-nitrosylation.

■ ASSOCIATED CONTENT

Supporting Information

Preparation and experimental details (Figures S1−S8 and Table S1) and complete ref 8b. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This research was supported by NSFC (21173078, 20903039), Shanghai Rising-Star Program (10QA1401600), Shanghai Science and Technology Commission (11JC1403300), and the Fundamental Research Funds for the Central Universities.

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