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Photogenerated Quinone Methides as Protein Affinity Labeling Reagents

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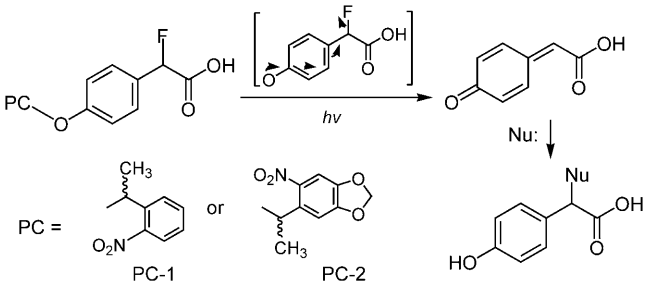
Photoaffinity labeling is a powerful approach for the study of protein–ligand and protein–protein interactions, especially for the characterization of low-affinity and transient binding.[1] In this technique, a molecule capable of generating a reactive intermediate upon UV light illumination is incorporated into a ligand or a protein, enabling it to form a covalent bond with its interacting partners. These stable complexes can then be analyzed by robust but harsh tools that usually disrupt noncovalent protein complexes—such as SDS-PAGE, HPLC, and MS—to identify target proteins and recognize binding interfaces. Therefore, novel phototriggered affinity labeling reagents with improved properties, including efficient photolysis at longer wavelengths, high reactivity of photogenerated intermediates, and inertness of intermediates to solvent water, should greatly facilitate the elucidation of protein–protein interaction networks. Here we report that photogenerated quinone methides (QMs) can be used as excellent photoinitiated affinity labeling reagents.

QMs are naturally occurring reactive metabolic intermediates in the biosynthesis of phenol-related biopolymers, such as melanin and lignin,[2] and have a variety of applications in organic synthesis[3] and biotechnology.[4] Their roles as DNA crosslinking and alkylating agents have been extensively investigated as well.[5] As a result, many parent molecules that release QMs upon UV illumination are already available.[6] However, most of them require short-wavelength UV light, which can damage biological macromolecules.

To overcome this problem, we synthesized two new QM precursors containing photocaging groups: PC-1 and PC-2 (Scheme 1). When a PC-1 or PC-2 group is released by UV light, a fluoride ion is spontaneously eliminated to generate a highly reactive QM, providing an electrophilic intermediate that can rapidly form stable Michael addition products with nucleophiles. We found that both PC-1 and PC-2 could be deprotected with 365 nm UV light, but that PC-2 was removed more rapidly than PC-1 at this wavelength (see the Supporting Information). Furthermore, visible light at 400 nm could be employed to remove PC-2, if the biomolecules in studies were extremely sensitive to UV light.



Scheme 1. Structures of two QM precursors and their phototriggered reactions. PC = photocaging group, PC-1 = α-methyl-l-(2-nitrophenyl)ethyl, PC-2 = (α-methyl-2-nitropiperonyl)methyl, Nu = nucleophile.

To use QMs as affinity labeling reagents, it is also important to know what amino acids are capable of reacting with them. Previous studies indicate that QMs crosslink with the side chains of Cys, Lys, and His, as well as the α-amine of each amino acid residue, although the thiol group in Cys is by far the best nucleophile and can readily outcompete reactions between QMs and other amino acids.[7] However, because most of those experiments were preformed in solutions in which free QMs and amino acids were simply mixed together,[8] the observed reactivities of amino acids with QMs might be different from what really occurs in an affinity labeling reaction in which an amino acid is within a short distance (several Å) of a nearby QM. To mimic this situation, we therefore designed a template molecule containing both a QM precursor and an amino acid under investigation, in which the QM precursor and the amino acid side chain were brought into proximity through a flexible backbone (Figure 1). A chromogenic group (R) with strong absorbance at 473 nm was also included to help the analysis of final products by HPLC. Because our initial data suggested that a thiol scavenger might significantly improve the specificity of crosslinking between QM-containing ligands and their targets, we tested the template molecules in the presence of excess amounts of β-mercaptoethanol (βMe). Once the QM is formed by UV light, it can go through three competing pathways: intramolecular addition with the nucleophile in the side chain of the amino acid, intermolecular addition with βMe, and reaction with water. We examined nine amino acids (Lys, Arg, Ser, Met, Trp, Tyr, His, Asp, Asn), representing all functional groups existing in the 20 naturally occurring proteinogenic amino acids except for the thiol of cysteine. Even though the concentration of βMe was much higher than those of the template molecules, the ε-amine of Lys, the imidazole of His, and the carboxylate of Asp/Glu were still able to form significant amounts of intramolecular cyclic adducts, implying that their reactivities with the nearby QMs were high enough to compete with a great excess of βMe in solution. The phenol of Tyr and the indole of Trp could also react with

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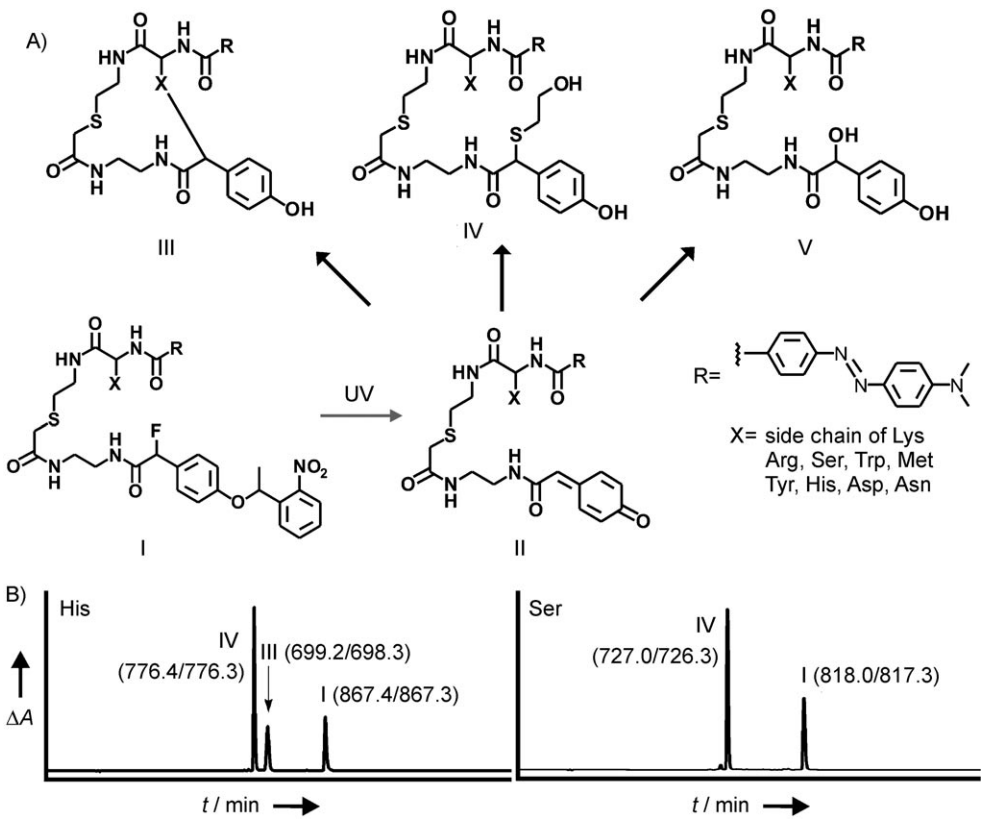


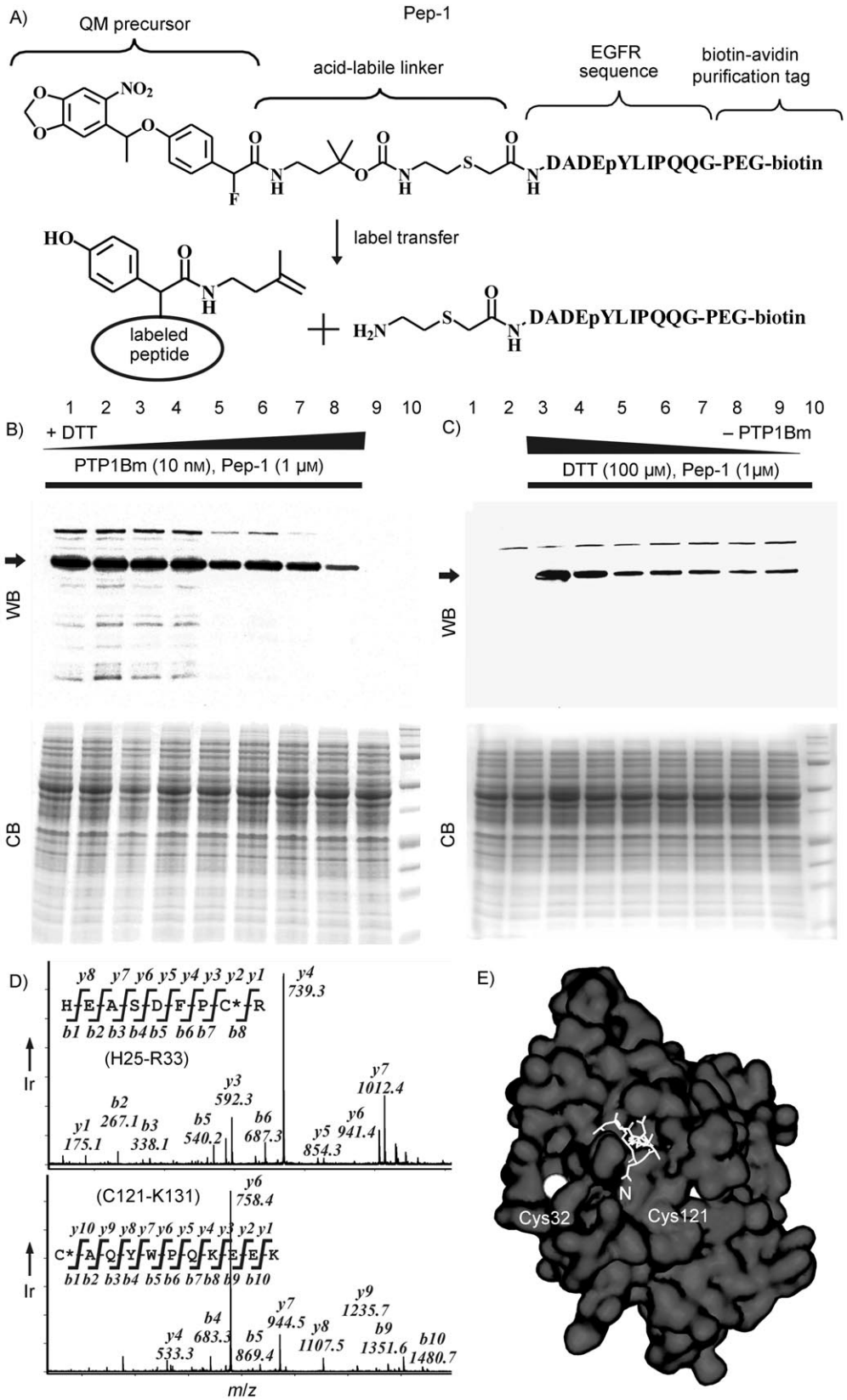
Figure 1. A) Structures of the template molecules (I) and three possible product types that might be obtained upon UV illumination, consisting of the intramolecular adducts (III), the βMe adducts (IV), and the water adducts (V). Structures II are the QM intermediates. R is a chromogenic group with maximum absorbance at 473 nm. X is the side chain of one of the nine amino acids tested. B) Representative HPLC chromatograms of final products resulting from two amino acids—His (reactive) and Ser (unreactive). In the presence of a thiol scavenger (βMe), the intramolecular adduct between the QM and the imidazole of His (in left chromatogram: peak III) is still observed, as well as the βMe adduct (in both chromatograms: peaks IV). No intramolecular adduct is formed between the QM and the hydroxyl of Ser. Peaks I (in both chromatograms) are the unreacted template molecules. Numbers in parentheses are the *m/z* (mass/charge) ratios of the corresponding peaks. The first values are those measured by MS and the second values are calculated.

this intramolecular QM if thiol was absent, but these reactions were completely suppressed by the presence of excess βMe. Other amino acids, including Arg, Met, Ser, Asn, and hydrophobic residues, could not react with this QM (Figure 1B and the Supporting Information). This result revealed that proximity could accelerate reactions between QMs and weak nucleophiles so that QMs should display broader reactivity profiles with amino acids on the surfaces of proteins during affinity labeling than with those in free solution.

To apply QMs for affinity labeling in a biological system, we prepared a peptide conjugate (pep-1) containing a photogenerated QM precursor, an acid labile linker, a short 11-mer peptide (DADEpYLIPQQG) adopted from the 988–998 residues of epidermal growth factor receptor (EGFR), and a biotin tag (Figure 2A). The EGFR (988–998) peptide was chosen from an autophosphorylation site (Y992) of EGFR, a receptor tyrosine kinase that plays critical roles in many aspects of cell development. Because the function of EGFR is regulated in vivo by protein tyrosine phosphatase 1B (PTP1B), which removes the phosphate group from Y992, the EGFR (988–998) peptide is an excellent substrate for PTP1B and is commonly used for the measurement of PTP1B activity. In addition, this peptide binds tightly with an inactive mutant of PTP1B (PTP1Bm, C215S).[9] Therefore, the pep-1/PTP1Bm system should form a crosslinked complex upon UV irradiation, serving as a good model for study of the properties of QMs during affinity labeling. In this design, the biotin tag allows us not only to detect labeled proteins by Western blot, but also to enrich them for further identification of crosslinked sites by standard LC-MS/MS analysis. Nevertheless, crosslinked peptides generated from trypsin digestion of labeled proteins are usually large and branched, so they tend to ionize poorly in MS, and their fragmentation often results in mass spectra that are too complicated to be interpreted, making it difficult to determine where crosslinking takes place.[10] The acid-labile linker allows us to achieve a label transfer in which the bulk of pep-1 is cleaved from the crosslinked peptides to leave a small phenol-like tag (+217 Da) on the crosslinked sites, greatly facilitating their identification.

To demonstrate the effect of thiol scavengers on the specificity of QM-based labeling reactions, we performed a dithiothreitol (DTT) titration experiment (Figure 2B) in which PTP1Bm and pep-1 were kept at 10 nM and 1 μM, respectively, in eight tubes of 100 μg protein extracts from *Escherichia coli*, but the concentrations of DTT in them varied from 0 to 5 mM. All samples were then irradiated with UV light under the same conditions, separated by SDS-PAGE, and blotted with streptavi-

Figure 2. A) Structure of pep-1, consisting of a QM precursor, an acid-labile linker, an EGFR (988–998) sequence, and a biotin. A small phenol-like tag (+217 Da) is left on a labeled peptide after a label transfer procedure as described in the text. B) DTT titration experiment. DTT concentration. Lane 1: 0 nM; lane 2: 0.1 μM; lane 3: 1 μM; lane 4: 10 μM; lane 5: 100 μM; lane 6: 500 μM; lane 7: 1 mM; lane 8: 5 mM; lane 9: control (0 nM, no UV irradiation); lane 10: protein ladder. All samples (1–9) contain protein extracts from *Escherichia coli* (100 μg), PTP1Bm (10 nM), and pep-1 (1 μM). C) PTP1Bm concentration dependence experiment. PTP1Bm concentration. Lane 1: control (0 nM, no UV irradiation); lane 2: 0 nM; lane 3: 1 μM; lane 4: 100 nM; lane 5: 25 nM; lane 6: 10 nM; lane 7: 5 nM; lane 8: 1 nM; lane 9: 0.1 nM; lane 10: protein ladder. All samples (1–9) contain protein extracts from *Escherichia coli* (100 μg), DTT (100 μM), and pep-1



(1 μM). WB: Western blot, CB: Coomassie blue staining. The PTP1Bm bands in WB are indicated with an arrow. D) MS/MS spectra of two peptides (H25–R33 and C121–K131) from PTP1Bm with the phenol-like modification on their cysteine residues (indicated by an asterisk). Identified *b*- and *y*-series ions are labeled. The second peptide (C121–K131) contains a missed trypsin cutting site (K128). Ir: relative intensity, *m/z*: mass/charge ratio. E) Crystal structure of the PTP1Bm bound to a truncated EGFR peptide (988–993) (PDB ID: 1PTU). The peptide is displayed in stick mode and its N terminus is marked. The surface of PTP1Bm is shown in gray. The sulfur atoms of two cysteines (C32 and C121) are displayed in CPK mode.

din-peroxidase conjugate to visualize labeled products. As a comparison, another gel loaded with the same set of samples was stained with Coomassie blue to show the total amount of proteins in them. When the concentration of DTT was low, multiple protein bands were observed; these might be caused by nonspecific labeling between pep-1 and some other proteins. Once the concentration of DTT was higher than 100 μM, however, only the strong PTP1Bm and a faint unidentified protein band were still visible, indicating that thiol could indeed dramatically improve the specificity of this light-activated affinity labeling reaction. Because reduced glutathione, a thiol-containing molecule, is present in various living cells in the 100 μM to 10 mM range,[11] QM-based affinity labeling would potentially be highly specific even inside cells.

Next, to test the sensitivity of this technique, we performed a PTP1Bm concentration dependence experiment (Figure 2C) in which most conditions were the same as in the DTT titration experiment. The concentration of DTT was kept at a constant 100 μM, whereas that of PTP1Bm was changed from 0.1 nM to 1 μM. Even at 0.1 nM, the PTP1Bm band was still clearly visible, indicating that minute amounts of PTP1Bm could be labeled with pep-1 even in the presence of a 10^6-fold excess of a thiol scavenger (100 μM vs. 0.1 nM).

We then tried to identify which residues of PTP1Bm reacted with pep-1. After the labeled PTP1Bm had been digested with trypsin, the crosslinked peptides were enriched by monoavidin-agarose and were then briefly incubated with acid before LC-MS/MS analysis. Two cysteine-containing peptides (H25-R33:HEASDFPC*R and C121–K131:C*AQYWPQKEEK) were found to contain the expected tag (+217 Da) on their cysteine residues (Figure 2D), indicating that they were the cleaved products of crosslinked peptides upon acid treatment. Even though PTP1Bm contains several cysteine residues, no other cysteine-bearing peptides were found to be crosslinked. By inspecting the crystal structure of PTP1Bm bound to a truncated EGFR peptide (988–993) (1PTU in the Protein Data Bank),[12] we found that these two cysteines lie near the N terminus of this short EGFR peptide (Figure 2E), implying that a suitable geometry is required for this affinity labeling reaction and suggesting that this QM-based labeling could be applied to mapping of interaction sites of proteins with unknown structures. Furthermore, we found no evidence of crosslinked products between pep-1 and other residues, confirming that QMs reacted preferably with cysteine, yet posing an interesting question of how efficient the labeling reaction would be if no cysteine residues on the target proteins were near to the QM.

To summarize, we have demonstrated that photogenerated QMs can be used as light-controllable protein affinity labeling reagents. The reactions between QMs and nucleophiles are less heterogeneous than those of other photogenerated active intermediates, such as carbenes and nitrenes, making it easier to identify labeled species.[13] In addition, the labeling reaction is highly specific between interacting protein–ligand and protein–protein pairs when a thiol scavenger is present. Finally, a wide choice of photocaging groups that are structurally similar to PC-1 and PC-2, but have distinct properties—including photolysis quantum yield, water solubility, and inertness of photolysis by-products—are available, offering great flexibility for different biological applications.

Keywords: crosslinked peptides · label transfer · mass spectrometry · photoaffinity labeling · quinone methides

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