Photoaffinity Reagents

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Isotope-Labeled Photoaffinity Reagents and Mass Spectrometry To Identify Protein–Ligand Interactions**

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The rate of drug discovery is greatly dependent on the development and improvement of rapid and reliable analytical methods for screening protein–ligand interactions. Photoaffinity labeling presents a valuable method for studying the interactions of biologically active, small molecules with their target proteins.[1] In photoaffinity labeling, a covalent linkage is created between a ligand and a protein upon irradiation by UV light. The requirements for the ideal photoaffinity label include its chemical stability prior to photoactivation, its easy photolysis at wavelengths that do not cause photochemical damage to the protein, and high reactivity of the intermediate product to C–H groups and to nucleophilic X atoms from X–H groups. Moreover, the reactions of the photoaffinity label with proteins should lead to stable and homogeneous products that can be isolated, purified, and analyzed subsequently by mass spectrometry. Reproducible high-efficiency labeling of target proteins is achieved with phenyl azides, diazirines, and benzophenone photophores.[2,3]

For in vivo studies of protein–protein interactions, diazirine groups have been incorporated into the amino acids methionine, leucine, and isoleucine.[4] If tri- or tetrafunctional photoaffinity labels containing a biotin group are used, the created products can be enriched by affinity purification on avidin beads. If a cleavage site is also incorporated, the biotin label can be released subsequently.[5]

A novel photoaffinity label **1** (Figure 1) has been presented recently by Lamos et al. which contains the following subunits:[6]

- A) a reactive site for coupling the biologically active ligand,
- B) a photoreactive site for reaction with the target protein,
- C) a biotin label, which allows purification of the protein–ligand com-

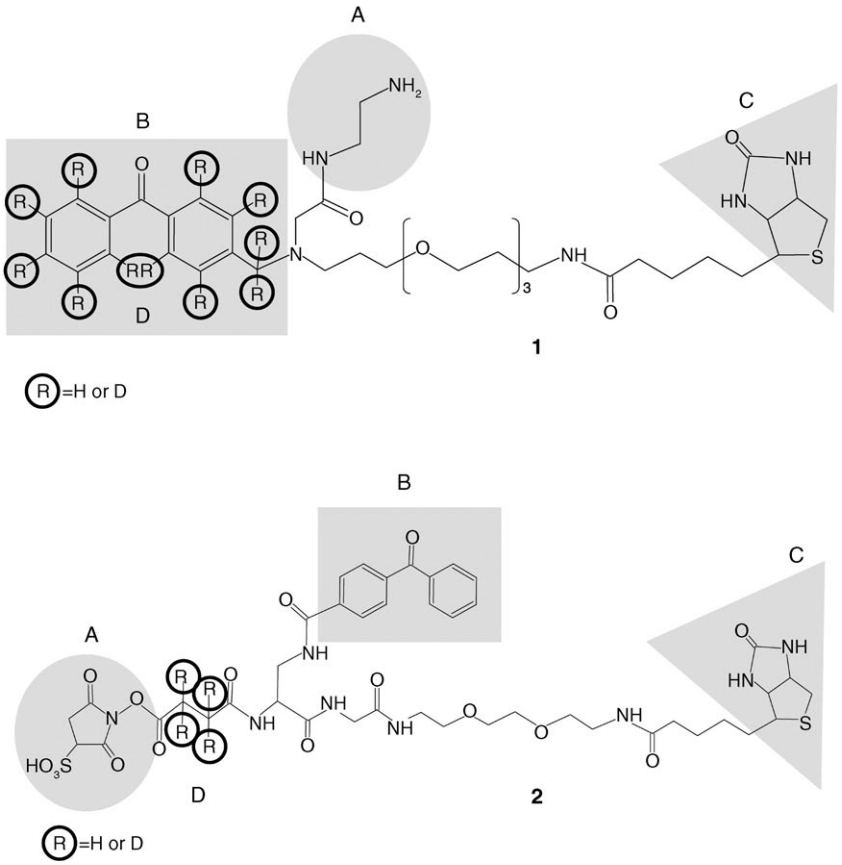

Figure 1. Isotope-labeled photoaffinity reagents **1**[6] and **2**.[7] The different structural elements are indicated as A) reactive site 1 (gray circle), B) photoreactive site (gray square), C) biotin label (gray triangle), and D) isotope label (open circles).

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plex by affinity chromatography on avidin beads,

- D) a stable isotope label to facilitate mass spectrometric identification of the protein–ligand complex.

Structural features A–D are also present in **2** (Figure 1), which has previously been developed by Trester-Zedlitz et al. for analyzing protein–protein interactions in chemical cross-linking studies.[7] However, the authors reported a low cross-linking efficiency for **2** in cross-linking studies of a heterodimeric protein complex and a great diversity of created cross-linked products.[7]

The newly developed photoaffinity probe **1**, which the authors termed "Target-Identification Probe (TIP)", has been successfully employed for identifying the interface region between the immunosuppressive drug cyclosporin A (CsA) with its target protein cyclophilin A (CypA) in the presence of the three nonbinding proteins ovalbumin, carbonic anhydrase, and FK binding protein (FKBP).[6] The general strategy is outlined in Figure 2 A. In the first step, the 1:1 mixture of non-deuterated and deuterated photoaffinity label is coupled to the bioactive ligand. After the coupling reaction, the conjugate is incubated with a protein mixture and the photoreaction is induced by irradiating the mixture with long-wavelength UV light. Only the target protein, which specifically interacts with the ligand, undergoes the photo-cross-linking reaction, whereas nonbinding proteins are not covalently attached to the ligand. The created protein–ligand complex is purified by affinity chromatography using avidin beads. The purified complex is enzymatically digested, for example, by trypsin, which cleaves proteins at the C-terminal site of lysine and arginine residues. Mass spectrometry (MS) using the "soft" ionization techniques electrospray ionization (ESI)[8] or matrix-assisted laser-desorption/ionization (MALDI)[9] is performed to analyze the created peptide mixtures. MS is the method of choice for the analysis of these complicated mixtures: its inherent high speed and sensitivity make it especially suited for high-throughput analysis of minute sample amounts. Tandem mass spectrometry (MS/MS) can be conducted to obtain sequence informa-

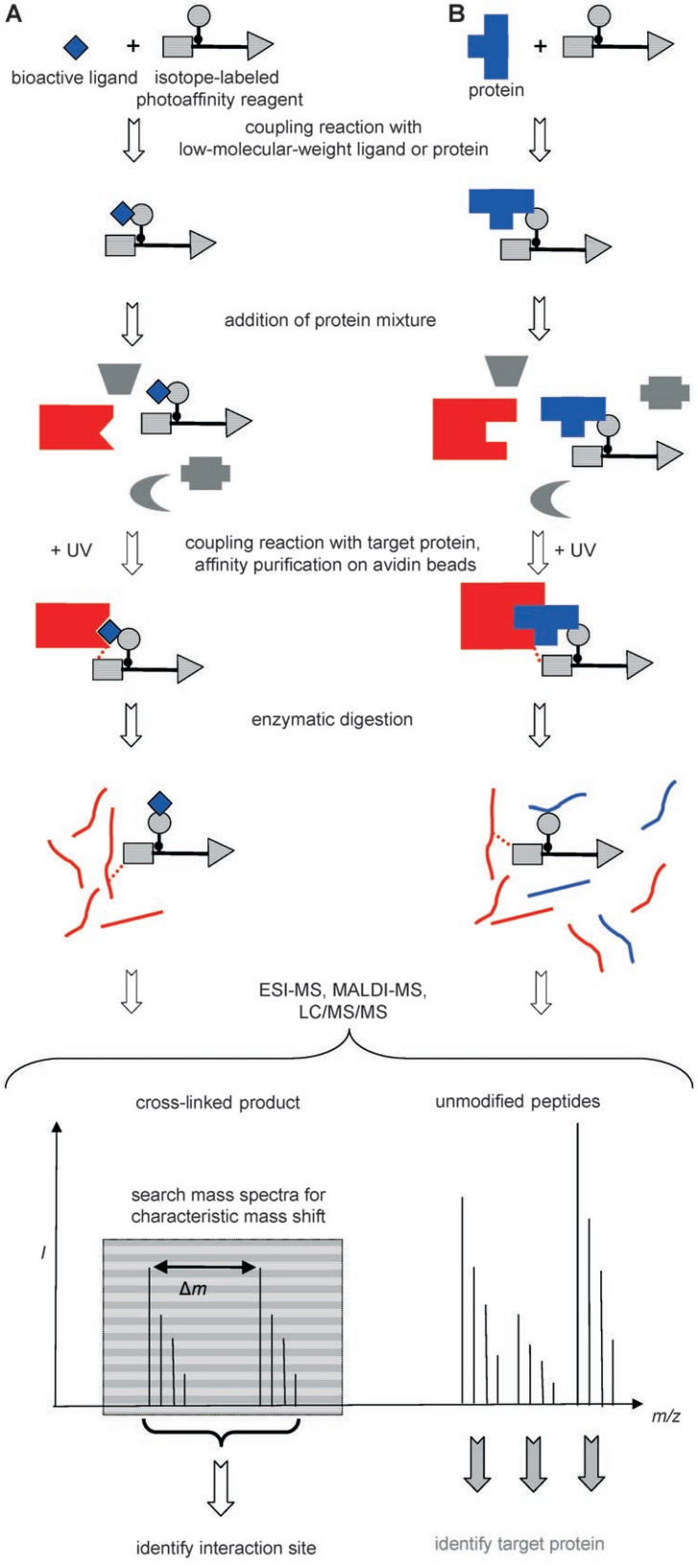

Figure 2. A) Strategy for analyzing protein–ligand interactions using **1** in a 1:1 mixture of [D_0] and [D_{11}] derivatives, as presented in reference [6] for studying the CypA–CsA interaction. B) Strategy concept for mapping protein–protein interactions using photoaffinity reagents, such as **1** or **2**. The structural components of the photoaffinity label are schematically depicted according to Figure 1.

tion for the proteolytic peptides of the target protein and to identify which amino acids have been modified by the photoaffinity-labeling procedure.[6]

Applying the photoaffinity label to a fixed ratio of non-deuterated and deuterated derivatives greatly facilitates MS identification of peptide–ligand adducts owing to the characteristic isotope patterns of the modified peptides. Signals exhibiting the characteristic mass shift caused by the heavy isotope label are attributed to adducts between peptides derived from the target protein and the ligand, thus, revealing information on the ligand binding site within the target protein. Unmodified peptides identify the target protein itself, which has been "fished out" from the protein mixture using the ligand as "bait" (Figure 2). It can be envisaged that this strategy can be employed to screen for protein binding partners of a target protein and to map their interaction sites (Figure 2B); however, this concept still awaits successful application.

When **1** was used for analyzing the interaction between CypA and CsA, the coupling efficiency of the photoaffinity label was rather low,[6] making it necessary to employ high-sensitivity analytical methods. Moreover, analysis of the reaction mixtures created by photoaffinity labeling can be hampered by the enormous complexity of the created mixtures. The application of stable isotope-labeled reagents (D, ^{18}O, ^{13}C), which are employed in a fixed ratio with their non-labeled counterparts, allows reaction products to be easily detected in the mass spectra by their distinctive isotopic patterns after enzymatic digestion of the created protein–ligand complexes.[6,7,10] One should be aware, however, that as a result of the incorporation of the isotope label, the MS signal intensity for a specific reaction product is reduced since a single signal is split into two signals. For the photoaffinity label **1**, the incorporation of eleven deuterium atoms introduces a large mass shift in the reaction product. Thus, the characteristic mass shift between non-deuterated and deuterated species is easily detected even in multiply charged ions that might be created by electrospray ionization.

One could envision employing photoaffinity reagents containing a large number of deuterium atoms for analyzing protein–ligand complexes in a "top-down" approach.[11] Here, the proteins under investigation are not enzymatically digested, but the intact protein–ligand complexes are fragmented inside the mass spectrometer. Fourier transform ion cyclotron resonance (FTICR)[11] or orbitrap mass spectrometers[12] have proven especially valuable for the "top-down" approach. One major drawback when deuterium atoms are incorporated as isotope labels is that the retention times of deuterated species in liquid chromatographic separation are slightly different from the retention times of their non-deuterated counterparts.[13] When the number of deuterium atoms is increased in order to enhance the mass difference between heavy- and light-isotope-labeled derivatives of the photoaffinity reagent, there is a corresponding increase in chromatographic resolution of the isotopic isoforms, which makes it more difficult to determine isotope ratios in the mass spectra. Therefore, the incorporation of ^{13}C or ^{18}O isotopes, which do not exhibit isotope effects during LC/MS analysis, seems to be advantageous for the future design of isotope-labeled reagents.[14]

The strategy of using isotope-labeled photoaffinity reagents in combination with MS presents a versatile method that allows screening for protein–ligand interactions from minute sample amounts within a short time. The development of novel and improved reagents can be foreseen.

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