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Active/Inactive Dual-Probe System for Selective Photoaffinity Labeling of Small Molecule-Binding Proteins

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Selective detection is a critical first step in the identification of small molecule-binding proteins from a complex mixture of cellular proteins.[1,2] Photoaffinity labeling (PAL) offers a potentially ideal strategy for isolating direct binding proteins from the proteome, especially when the structural details or chemical reactivities of the proteins at the binding sites are completely unknown.[3,4] PAL involves the use of bioactive small-molecule ligands derivatized with a photoreactive group and a reporter group.[3e–f] Upon photoactivation, a highly reactive intermediate generated from a probe can react with a variety of amino acid residues of a protein in a distant-dependent manner. The unique reactivity provides the basis for affinity-selective crosslinking of the small molecule–protein complexes. Once crosslinked, the chemically stable complexes become amenable to various biochemical analyses such as proteolytic digestion followed by identification of the binding proteins or the binding sites within the binding proteins by mass spectrometry (MS).[3d–f] However, the general utility of PAL in the discovery of binding proteins has often been limited in reality largely due to low selectivity and low crosslinking yields.[3,5] These problems are particularly pronounced in cases where the binding affinity is modest ($K_d > \mu M$) or the binding proteins are not abundant in the cell.[6] The development of PAL methods to date has been aimed primarily toward increasing the *reactivity* rather than the *selectivity* of photoactivatable functionalities.[3,7] Herein, we report a novel strategy to control the selectivity of PAL by simultaneously using a bioactive PAL probe and its inactive analog. We believe that it represents a step toward expanding the scope of PAL especially in the search of small molecule-binding proteins with modest affinity, which has traditionally been difficult.

In a conventional PAL reaction with a mixture of cellular proteins, a probe displaying a bioactive ligand can form equilibrium complexes with its specific binding proteins as well as various other nonspecific proteins, both of which are then photocrosslinked through a fast intramolecular reaction (Figure 1, upper path). The formation of nonspecific complexes brings about two undesirable effects in PAL reactions. First, it leads to multiple reaction products, complicating subsequent product analysis. Second, sequestering of the probe by nonspecific proteins decreases the effective concentration of the probe. We hypothesized that the probe could be tuned to selectively react with its specific binding proteins if the formation of undesired nonspecific complexes is disfavored by an inhibitor prior to the PAL reaction step. Small molecules are known to nonspecifically bind to nonpolar patches of protein surfaces through hydrophobic interaction.[8] The strength of hydrophobic interaction is considered roughly proportional to the buried surface area of the bound complex. The overall magnitude of the nonspecific small molecule–protein interaction typically follows a simple absorption isotherm as there are numerous nonspecific binding sites on proteins.[8a] Our approach therefore is to introduce a biologically inactive structural analog, which would mimic the active PAL probe in the nonspecific protein binding property (Figure 1, lower path). Excess amounts of an inactive PAL probe would predominantly form complexes and subsequently react with nonspecific proteins as a scavenger,[9] while the free active probe would be fully available to complex with its specific binding protein.

To explore this new PAL approach, we employed benzenesulfonamide as a model bioactive small-molecule ligand and studied the reactivity of its derivatives as PAL probes. Benzenesulfonamide is a potent inhibitor of human carbonic anhydrase II (hCAII; $IC_{50} = 0.27$ nM[10a]), a ubiquitous cytosolic protein, which catalyzes the reversible hydration of CO_2. The protein–ligand interaction has been well characterized biochemically and crystallographically.[10,11] To detect the binding protein of benzenesulfonamide by PAL, we designed a trifunctional probe **1** based on an L-lysine scaffold that contains a benzenesulfonamide moiety as a protein-binding ligand, benzophenone as a photoactivatable group, and biotin as a reporter group,[12] which allows the detection of protein–photoadducts (Figure 2). Compound **3** possessing the ligand group but lacking the biotin reporter group was designed as a positive control. As a biologically inactive analog of the benzenesulfonamide group, the 4-methoxybenzoyl group was chosen on the basis of its similar shape and surface area. Compounds **2** and **4** represent inactive analogs

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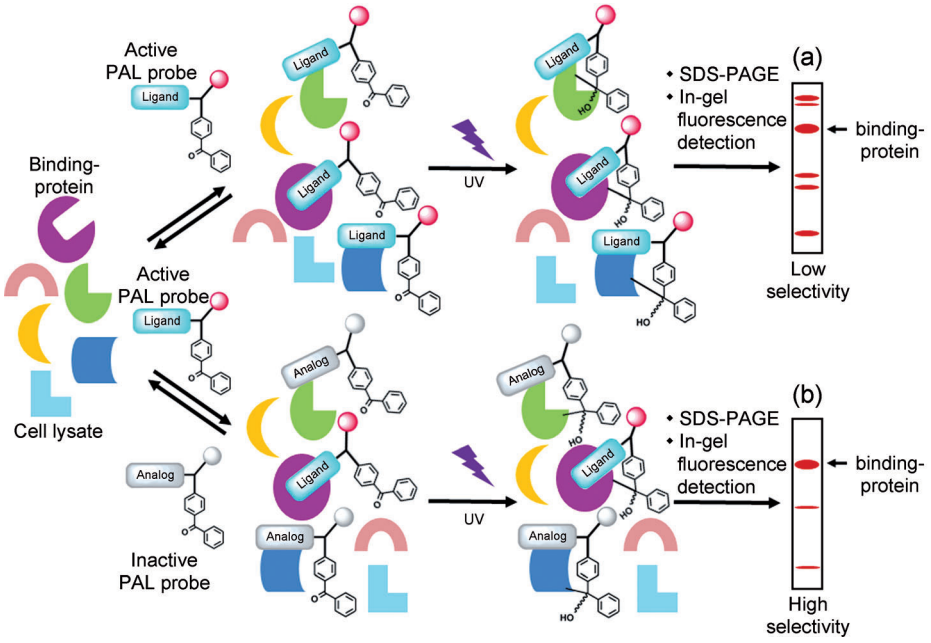


Figure 1. PAL reaction in a cell lysate with a) traditional PAL probe and b) dual-probe PAL system. The binding protein of a ligand is shown in magenta. Magenta circle, reporter group; gray circle: non-reporter group.

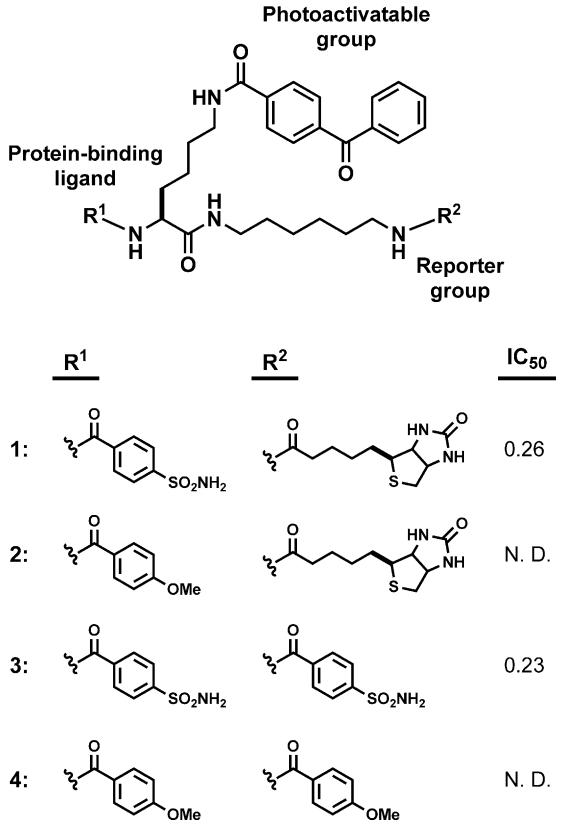
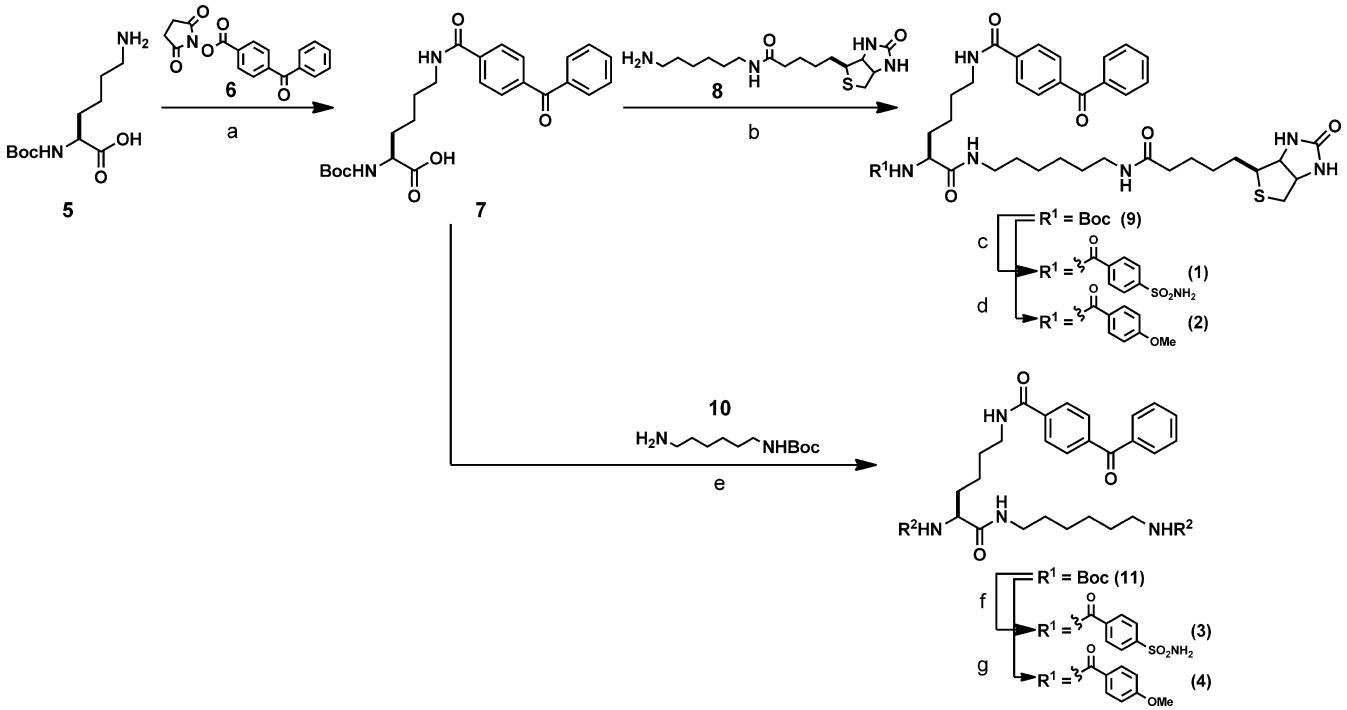


Figure 2. Structures of the active PAL probe **1** and the inactive probes **2**–**4** and their inhibitory activity against hCAII (μM). N. D., no activity detected.

with and without biotin reporter group and are expected to bind proteins nonspecifically in a manner similar to **1**. Probe **1** was synthesized from *N*-Boc-L-lysine in four steps with an overall yield of 38% (Scheme 1). Analogs **2**–**4** were obtained in two steps from the intermediate **7**. We evaluated the protein-binding activity of compounds **1**–**4** by performing an enzyme activity inhibition assay for hCAII.[8a] Whereas **2** and **4** showed no activity ($IC_{50}>1$ mM) as expected, the PAL probes **1** and **3**, which were designed to bind hCAII, displayed IC_{50} values of 0.26 μM and 0.23 μM, respectively. Thus, the incoporation of the photoactivatable group or the biotin reporter group into the probe did not interfere with the small molecule–protein interaction.

PAL reactions were conducted to first evaluate the reactivity of compounds **1** and **2** toward proteins. Typically, reactions were initiated by UV irradiation ($\lambda=365$ nm) of the reactants on ice; subsequently, the reaction mixture was separated by SDS-PAGE. The photoadducts derived from **1** and **2** as biotin-labeled proteins were detected by using fluorophore-conjugated Streptavidin and analyzed by using a fluorescence imager. On the other hand, the photoadducts of **3** or **4** without the biotin group did not give rise to fluorescent signals. The efficiency of each reaction was evaluated by the determining the intensity of fluorescence signals corresponding to the quantity of the protein photoadducts in the gels. When **1** was reacted with its known binding protein, hCAII, a photoadduct (**12**) of a single fluorescent band was generated in a dose-dependent manner for **1**, with $EC_{50}=0.19$ μM (Figure 3a).[13] While the active analog **3** inhibited the generation of the photoadduct **12** with an IC_{50} of 13 μM (Figure 3b), the inactive analog **4** displayed no inhibitory activity, even at a concentration of 1 mM. These results confirmed that the PAL reaction between **1** and hCAII is based on specific binding. To assess if **1** reacts with other proteins in a nonspecific fashion, it was also reacted with bovine serum albumin (BSA), which is not known to bind benzenesulfonamide but known to nonspecifically bind various hydrophobic small molecules.[14] The reaction generated the photoadduct **13**; with an increase in the concentration of **1**, increasing amounts of **13** were formed with no saturation observed up to 1 mM of **1** (extrapolated to $EC_{50}=2.9$ mM; Figure 3c), which is characteristic for a phenomenon driven by nonspecific binding.[8a,15] Compound **2** also showed a dose-dependent reactivity with hCAII and BSA, with EC_{50} values of respectively 1.7 μM and 0.84 μM (Figure S1 in the Supporting Information), thus indicating that the apparent binding affinities of **2** for both proteins are relatively high. To test the hypothesis that **1** and its inactive probe **4** compete for similar nonspecific binding



Scheme 1. Synthesis of the active PAL probe **1** and the inactive probes **2**–**4**. Reagents and conditions: a) **6**, 20% Na_2CO_3 aq., DMF, 77%; b) **8**, DMT-MM, MeOH, 86%; c) (i) 4 M HCl-dioxane, CH_2Cl_2, (ii) 4-sulfamoylbenzoate *N*-hydroxysuccinimide ester, CH_2Cl_2, DIPEA, DMF, 55%; d) (i) TFA/CH_2Cl_2 = 1:2, (ii) 4-methoxybenzoyl chloride, DIPEA, DMF, 47%; e) **10**, HATU, DIPEA, DMF, 84%; f) (i) 4 M HCl-dioxane, CH_2Cl_2, (ii) 4-sulfamoylbenzoic acid, HATU, DIPEA, DMF, 17%; g) (i) 4 M HCl-dioxane, (ii) 4-methoxybenzoic acid, HATU, DIPEA, DMF, 60%.

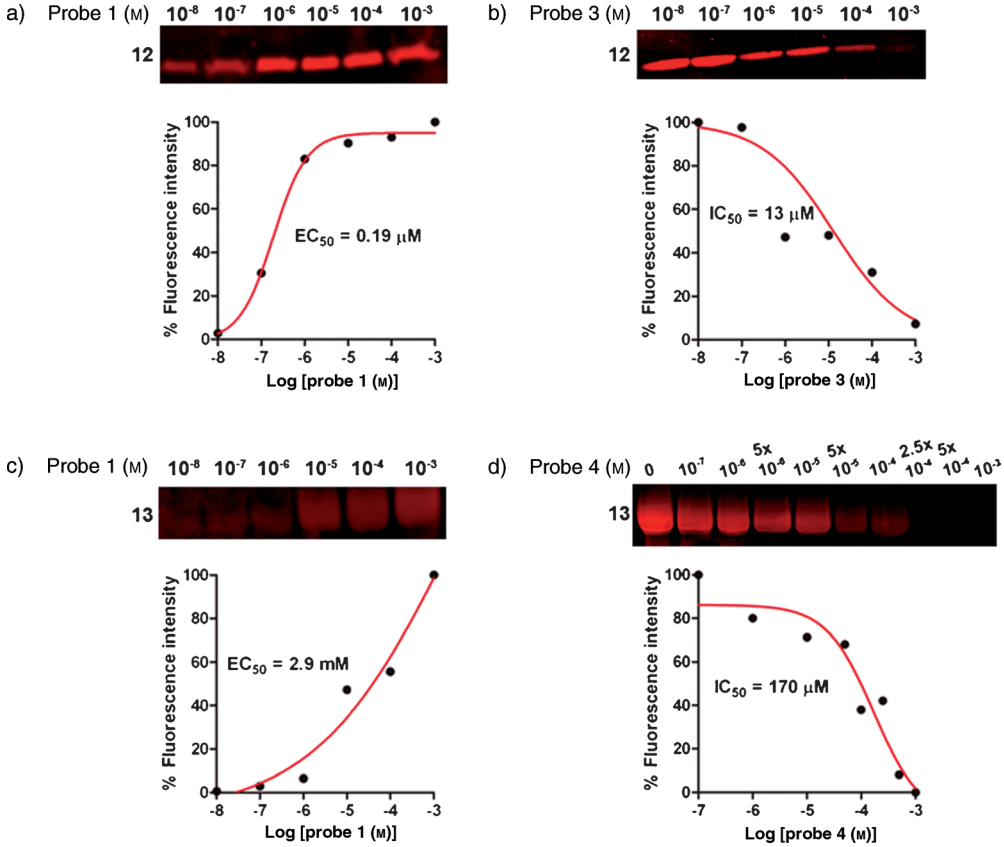


Figure 3. a) PAL reactions of **1** at various concentrations with hCAII (1 μg, 1.7 μM). b) Effects of **3** on the PAL reaction of **1** (1 μM) with hCAII (1 μg, 1.7 μM). c) PAL reactions of **1** at various concentrations with BSA (5 μg, 3.8 μM); d) Effects of the inactive probe **4** on the PAL reaction of **1** (1 μM) with BSA (5 μg, 3.7 μM). Fluorescence data were normalized to the largest value for each experiment.

sites on a protein, we reacted **1** with BSA in the presence of varying amounts of **4**. Significantly, we observed a decrease in the formation of photoadduct **13** at increasing concentrations of **4** ($IC_{50}=170\ \mu M$; Figure 3d). This result indicated that BSA preferentially bound and reacted with the excess reactant **4** over **1**. Therefore, it raised the possibility that a high selectivity of the active PAL probe could be achieved even in a protein mixture by simply disfavoring the formation of undesired complexes through addition of an inactive PAL probe.

We next performed the PAL reaction of **1** in mixtures of hCAII and BSA. As indicators of effective PAL reactions, we employed the selectivity for the outcome of each reaction, which is calculated from the following equation: selectivity (%) = (fluorescent intensity of **12**/total fluorescent intensity) × 100. When the initial ratio of hCAII to BSA was decreased from 10:1 to 1:100, the PAL probe **1** generated **12** with a decreasing selectivity (from 90% to 37%, see Figure 4a). Thus, probe **1** reacted with abundant BSA more efficiently than with its specific binding protein hCAII, consistent with the typical outcome of PAL experiments under conventional conditions. Under the same reaction conditions, **2** gave a photoadduct with BSA (**17**) as the major product reflecting the initial ratio of the two proteins (Figure S1c in the Supporting Information). When **1** was reacted with a mixture of hCAII and BSA at the ratio of 1:100 in the presence of various amounts of **4**, we found that the highest selectivity of 65% was achieved with 1000-fold excess of **4** relative to **1**. Therefore, the inactive probe **4** selectively inhibited the formation of **13** in a dose-dependent manner. Due to the limited solubility of **4** in aqueous solution, we were unable to explore its ability at much higher concentrations to improve the selectivity for the active probe **1**.

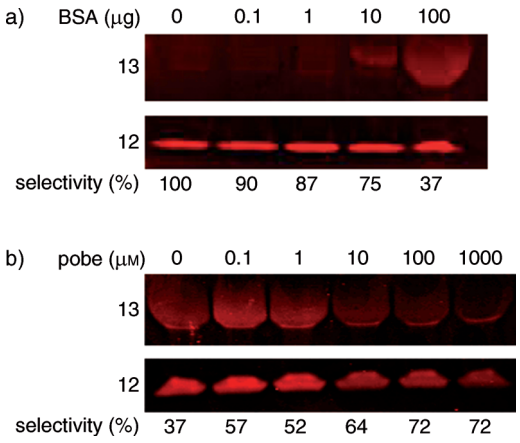
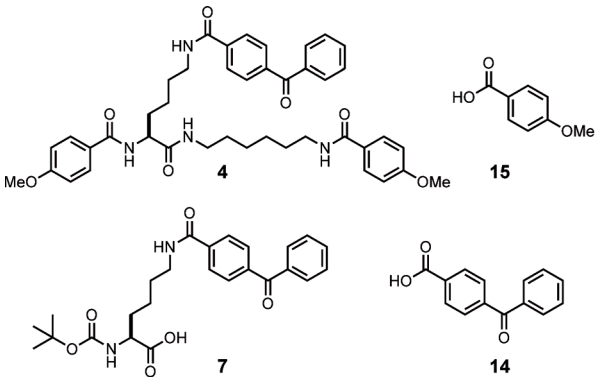


Figure 4. a) Effects of BSA on the PAL reactions of **1** (1 μM) with hCAII (1 μg). b) Effects of the inactive probe **4** on the PAL reactions of **1** (1 μM) with hCAII (1 μg) and BSA (100 μg).

To gain insight into the structural basis of the nonspecific PAL reaction, we also evaluated the effect of substructures of the inactive probe (Figure 5, **4**, **7**, and **14**) in the PAL reaction of **1** with a 1:100 mixture of hCAII and BSA. As shown in Figure 5, compound **7** also exhibited an inhibitory effect on the nonspecific reactivity of **1** with BSA; however, its efficiency was weaker than that of **4**. Lack of competitive inhibition by **14** confirmed that the nonspecific PAL reactions by **1** involve nonspecific protein binding events and do not occur via simple intermolecular reactions. From these data, all parts of the inactive probe **4** were found necessary to maximally compete with **1**. Therefore, our data indicate that designing an appropriate analog is important for achieving PAL detection of specific binding proteins with a high selectivity. Nevertheless, when a structurally close inactive ligand is not readily available, using a simple scaffold structure such as **7** may sufficiently improve the selectivity of PAL reactions.

An ideal PAL probe should react selectively with specific binding proteins in the presence of a complex array of cellular proteins in a reaction solution. With the conventional PAL method, when **1** was reacted with hCAII in the presence of increasing amounts of proteins from HeLa cell lysates, an increasing amount of photoadducts with various proteins was also formed apart from the desired photoadduct **12** (Figure 6a). Due to the overlap between fluorescent bands for **12** and other photoadducts in the SDS-PAGE gel, the selectivity of product formation was not assessed quantitatively in these cases. When the inactive probe **4** was added at 1000-fold excess over the active probe **1** to the reaction mixture of hCAII and lysate proteins (ratio of 1:100), the formation of undesired photoadducts was visibly inhibited, as observed in the SDS-PAGE gel (Figure 6b). This showed that the inactive probe **4** served effectively as a mimic of



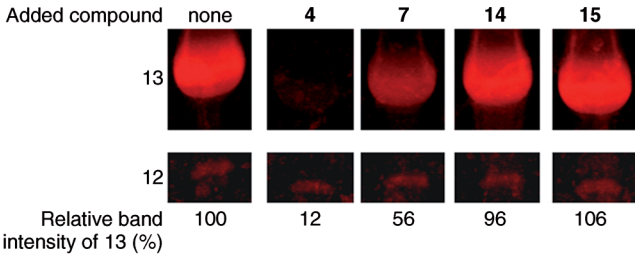


Figure 5. Effects of inactive analogs **4**, **7**, **14**, or **15** (1 mM) on the PAL reactions of **1** (1 μM) with hCAII (1 μg) and BSA (100 μg).

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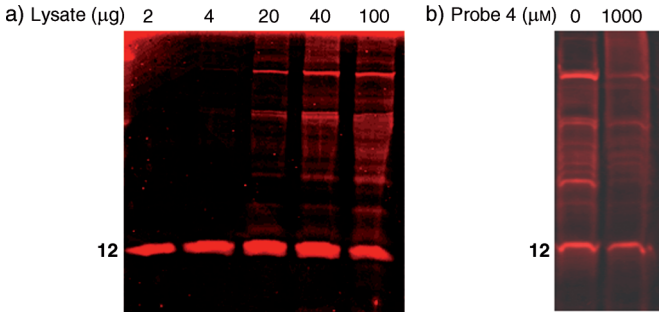

Figure 6. a) In-gel western blot analysis of PAL reactions of **1** (1 μM) with hCAII (1 μg) mixed with various amounts of HeLa cell lysate. b) In-gel western blot analysis of PAL reactions of **1** with hCAII (1 μg) mixed with HeLa cell lysate (100 μg) in the absence (lane 1) or presence (lane 2) of **4** (1 mM).

probe **1** in its nonspecific interaction with various cellular proteins but not in the specific interaction with hCAII. These results demonstrate that it is possible to tune the selectivity of an active PAL probe in a complex protein mixture by simultaneously employing an inactive probe as a scavenger of nonspecific proteins.

In conclusion, we have described an active/inactive dual-probe approach to control the selectivity of PAL reactions toward detecting specific small molecule-binding proteins. We demonstrated that our new PAL system is applicable to levels of a binding protein in the cell lysate as low as 1% (w/w). In addition, we found that a simple inactive analog representing the scaffold moiety of the PAL probe can also improve the labeling selectivity, which should be useful in cases where inactive analog is not easily obtained. With judicious choice of the inactive PAL probe, our method should be generally applicable to a wide range of bioactive small molecules for straightforward detection of their binding proteins. It should be particularly useful for exploring yet unknown small molecule–protein interactions with modest affinity, which have been previously difficult to detect.

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Keywords: analog · ligand–protein interaction · nonspecific binding · photoaffinity labeling · proteins

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[15] We excluded the possibility of intermolecular PAL reactions because no quenching was observed when radical quenchers such as phenol (0.1 M) were used as an additive and because the concentrations of the reactants are beyond the regime for simple bimolecular reactions.

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Photoaffinity Labeling

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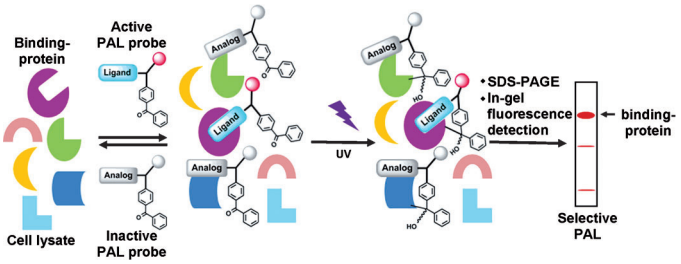

Two are better than one: A new approach to selective photoaffinity labeling is described in which a bioactive probe is used in combination with its inactive analog as a scavenger of nonspecific proteins.

