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Regulating Enzymatic Activity with a Photoswitchable Affinity Label

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Affinity labeling is a powerful method for exploring the active site of proteins. This technique involves the noncovalent binding of a ligand, which then guides a reactive functional group to form a covalent bond with amino acid residues in the immediate vicinity or at some distance to the binding site (endo- vs. exo-affinity labeling). These reactive groups can be various electrophiles, such as epoxides, haloketones, and dehydroazepines.[1]

The impact of this technique would be even greater if affinity-guided covalent attachment could be used to reversibly control the function of a target protein. This could be achieved by inserting a molecular switch between the ligand and the reactive functional group. Optical switches would be especially advantageous since they can be actuated with excellent spatial and temporal control. In addition, the chemistry of optical switches, in particular azobenzenes, has been developed to a high degree of sophistication and their compatibility with complex biological systems has been demonstrated.[2]

The resulting photoswitchable affinity label (PAL) consists of a ligand, a tether containing the photoswitch, and a reactive group (Figure 1). Noncovalent binding of the molecule positions the electrophile close to nucleophiles within reach of the photoswitch in the extended (*trans*) form. The molecule will therefore only react with a limited set—ideally one—of the nucleophiles that surround the binding site, which can be an active site or an allosteric site. Covalent conjugation then anchors the ligand, which results in persistent activation or inhibition of the protein. This effect can be reversed by switching the tether to the short (*cis*) form, which retracts the ligand from the binding site. Thus, as one switches between different wavelengths of light and changes the effective concentration of the ligand, the activity of the protein can be influenced.

We now report the application of this concept to control the activity of carbonic anhydrase with covalently tethered, photo-switchable inhibitors. Carbonic anhydrase catalyzes the reversible hydration of carbon dioxide and is involved in maintaining physiological pH. Human carbonic anhydrase II (HCAII), which plays a role in a variety of diseases, including glaucoma, epilepsy, and cancer, has been the subject of numerous pharmacological[3] and biophysical

investigations.[4] Importantly, recent studies have shown that this enzyme can undergo effective exo-affinity labeling with sulfonamide inhibitors tethered to reactive moieties.[5]

To probe the PAL concept with carbonic anhydrase, we synthesized several arylsulfonamides tethered to an electrophilic epoxide moiety through an azobenzene photoswitch (Scheme 1). Azobenzenes undergo a large geometrical change upon switching between their *trans* and *cis* states. Our compounds are based on sulfanilamide (**1**), an archetypical inhibitor of HCAII, which coordinates to the zinc ion in the active site. Analogues of increasing length (compounds **2–5**) were designed to react with nucleophiles positioned at various distances from the active site after noncovalent binding. Compound **6**, which lacks the epoxide and could not attach itself covalently to the surface of the protein, was prepared as a control.

For practical purposes, we decided to carry out our study with commercially available bovine carbonic anhydrase II (BCAII), which is highly homologous to HCAII.[6] Since carbonic anhydrase also functions as an esterase, its activity can be spectroscopically assayed by hydrolysis of *p*-nitrophenyl acetate (PNPA).[5b]

Incubation of BCAII with PAL **3** in the dark, followed by purification, led to a significant reduction in enzyme activity, as monitored by PNPA hydrolysis (Figure 2). The extent of covalent labeling of the enzyme with compound **3** was estimated by LC–MS analysis to be in the 65–85% range. MALDI-TOF MS/MS analysis showed that the sites of labeling were histidines 2 or 3, which is in accordance with observations made by Hamachi et al.[5b] on HCAII (see the Supporting Information).

Upon irradiation of the sample with 380 nm ultraviolet light, the rate of PNPA hydrolysis increased twofold from $(6.974 \times 10^{-8} \pm 3.327 \times 10^{-9}) \text{ M s}^{-1}$ to $(1.301 \times 10^{-7} \pm 3.815 \times 10^{-10}) \text{ M s}^{-1}$ (Figure 2). This result is consistent with partial isomerization of the azobenzene photoswitch to the *cis* state, and concurrent retraction of the sulfonamide inhibitor from the catalytically active zinc.

When the wavelength of the incident light beam was 460 nm, the rate of PNPA hydrolysis remained at levels found in the dark-adapted state. Notably, the rate of background hydrolysis was not influenced by irradiation alone.

The photostationary *cis/trans* ratio of **3** in DMSO solution was investigated by NMR spectroscopy and was found to be 25:75 at 460 nm, >98:2 at 380 nm, and <2:98 in the dark. These data indicate that the photostationary states of the azobenzene switch change significantly upon bioconjugation.

The control compound **6** was found to be a relatively weak and reversible inhibitor of BCAII with a K_i of $(1.5 \pm 0.09) \mu\text{M}$. Interestingly, irradiation with different wavelengths had no effect on inhibition with this compound. The shorter azobenzene derivative **2** gave less efficient labeling of BCAII, whereas the more extended compounds **4** and **5** failed to covalently modify the enzyme.

The enzyme activity could be changed repeatedly by switching between different wavelengths; this demonstrates the robustness of the azobenzene photoswitch. As shown in Figure 3, alternation of irradiation between 460 and 380 nm was matched by different rates of PNPA hydrolysis.

The relatively modest change in activity observed upon photoswitching (about twofold) could be explained by the nature of the two histidines involved in the bioconjugation. These nucleophiles are placed at the flexible N terminus of the enzyme, which according to a recent X-ray structure[6] of BCAII, reaches over the catalytically active site. It is conceivable that

conjugation to a single residue in a stiffer region of the enzyme would lead to a more pronounced effect.

In summary, we have demonstrated that enzymatic activity can be tuned with an inhibitor that functions as a photoswitchable affinity label (PAL). Photoresponsive versions of enzymes, such as horseradish peroxidase,[7] ribonuclease S,[8] or papain[9] have been previously designed through site-specific incorporation of azobenzene amino acids or unspecific labeling of lysine residues.[10] What distinguishes our work is the application of the PAL concept to the optical control of protein function. In principle, PALs allow for the photoregulation of native proteins, and avoid the site-specific introduction of highly reactive residues (e.g., cysteines) as points of attachment. This could have advantages in cases where the genetic engineering of mutant proteins is not desirable. The extension of this idea to other enzymes and receptors, for example, ion channels, is currently under investigation in our laboratories.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
The PAL concept.

Figure 2. Photoregulation of enzymatic activity. Hydrolysis of PNPA catalyzed by unlabeled BCAII (X), labeled BCAII in the dark (▲), labeled BCAII at 460 nm (□), and labeled BCAII at 380 nm (●).

Figure 3.

Reversibility of the photoregulation. Hydrolysis of PNPA with BCAII labeled with **3** under alternating irradiation at 460 nm (white boxes) and 380 nm (black boxes). The rates for hydrolysis were found to be (in M s^{-1}): $5.384 \times 10^{-8} \pm 1.951 \times 10^{-9}$ (0–120 s, 460 nm), $1.240 \times 10^{-7} \pm 8.000 \times 10^{-9}$ (120–240 s; 380 nm), $8.589 \times 10^{-8} \pm 5.813 \times 10^{-9}$ (240–360, 460 nm), $1.270 \times 10^{-7} \pm 7.367 \times 10^{-9}$ (360–480 s; 380 nm), $8.101 \times 10^{-8} \pm 4.210 \times 10^{-9}$ (480–600 s, 460 nm), $1.196 \times 10^{-7} \pm 9.872 \times 10^{-9}$ (600–720 s; 380 nm), and $7.719 \times 10^{-8} \pm 3.057 \times 10^{-9}$ (720–840 s, 460 nm).

Scheme 1.
PALs for carbonic anhydrase.