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Construction of a Photoactivatable Profluorescent Enzyme Via Propinquity Labeling

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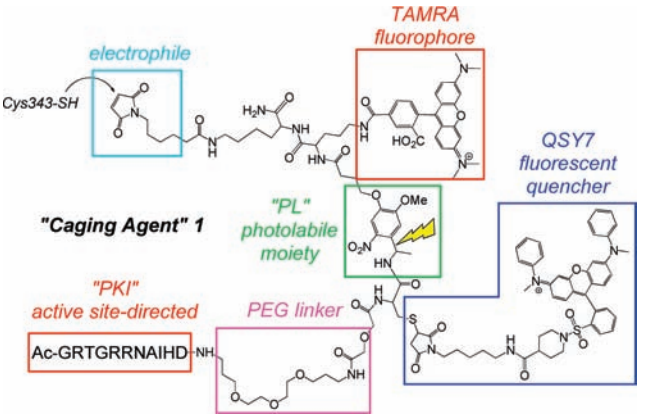
Supporting Information

ABSTRACT: A strategy for the construction of a profluorescent caged enzyme is described. An active site-directed peptide-based affinity label was designed, synthesized, and employed to covalently label a nonactive site residue in the cAMP-dependent protein kinase. The modified kinase displays minimal catalytic activity and low fluorescence. Photolysis results in partial cleavage of the enzyme-bound affinity label, restoration of enzymatic activity (60−80%) and a strong fluorescent response (10−20 fold). The caged kinase displays analogous behavior in living cells, inducing a light-dependent loss of stress fibers that is characteristic of cAMP action. This strategy furnishes molecularly engineered enzymes that can be remotely controlled in time, space, and total activity.

Elucidation of protein structure/function relationships has a long and storied history. However, a crucial turning point proved to be Pauling's conjecture of the relationship between a hemoglobin mutation and sickle cell anemia, which established the field of molecular medicine.[1] The subsequent identification of proto-oncogenes and oncogenes firmly demonstrated the link between protein structure and function, cell behavior, and disease.[2] Indeed, deciphering relationships between intracellular protein activity and cellular behavior represents, in large part, the *raison d'etre* of modern cell biology. However, for a wide variety of reasons, these relationships can be difficult to discern. For example, there is often a pronounced spatiotemporal component associated with protein action and the attendant biological consequences. In this regard, one particularly noteworthy protein is the cAMP-dependent protein kinase (PKA), which is associated with an array of organelles and supramolecular complexes, yet the ramifications of its action at these sites are distinct.[3] Light-activatable ("caged") proteins represent a potentially powerful means to address the relationship between protein activity and cellular behavior as a function of timing and location.[4] Unfortunately, confirmation that activation has occurred must often await a biological readout. Furthermore, the spatially localized persistence of the activated protein as well as how much has been generated is critical for verifying cause and effect. In 2004, Muir and his colleagues established a means to link photoactivation with a fluorescent response, using an engineered protein prepared via the expressed protein ligation method.[5] We report herein a strategy that utilizes active site recognition for propinquity labeling of a nonactive site residue for the preparation of a caged profluorescent protein.[6]

The catalytic subunit (C) of PKA contains two readily modifiable Cys residues, one at the base of the active site (Cys-199) and the other approximately 20 Å removed (Cys-343) (Figure 1a). Covalent modification of Cys-199 destroys catalytic activity and thus represents a poor choice for tagging the enzyme with a fluorophore label.[7] Consequently, we focused our attention on the nonactive site Cys-343 moiety for this purpose. The C subunit catalyzes the phosphorylation of Ser and Thr residues ensconced within targeted sequences (-Arg-Arg-Xaa-Ser-) of protein and peptide substrates.[8] In addition, peptide-based inhibitors have been constructed by replacing the phosphorylatable Ser moiety with a nonphosphorylatable Ala residue.[9] **Caging Agent 1** contains a (i) validated active site-directed



inhibitory peptide sequence, GRTGRRNAIHD ("PKI"),[9] (ii) a polyethylene glycol (PEG) spacer to optimally position an appended electrophilic maleimide near Cys-343, and (iii) a tripeptide cassette composed of a photolabile (PL) moiety inserted between a fluorophore and a fluorescent quencher. The cassette employed in this study [Cys(QSY7)-PL-Lys-(TAMRA)], shown explicitly in **Caging Agent 1** and schematically in panels b and c of Figure 1, was derived from a library of fluorescently quenched cassettes.[10] The latter were screened to identify an arrangement of fluorophore, quencher, and photolabile moiety that optimizes the fluorescent response upon photocleavage. In short, photolysis of the covalently modified, and therefore inactive, protein kinase (Figure 1c) is designed to both restore

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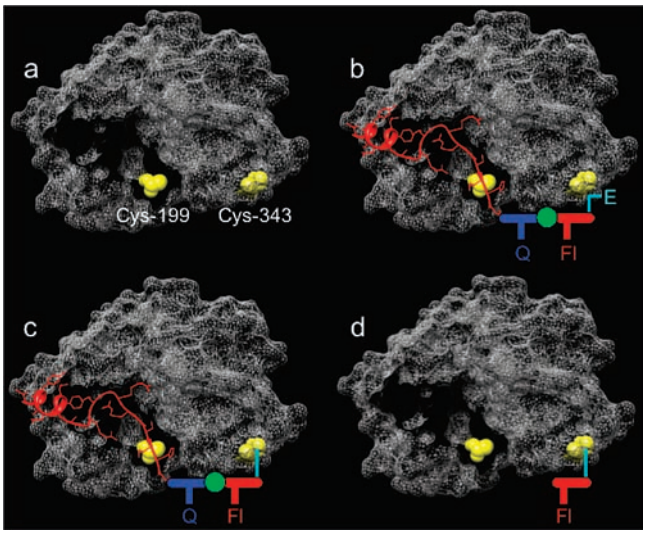
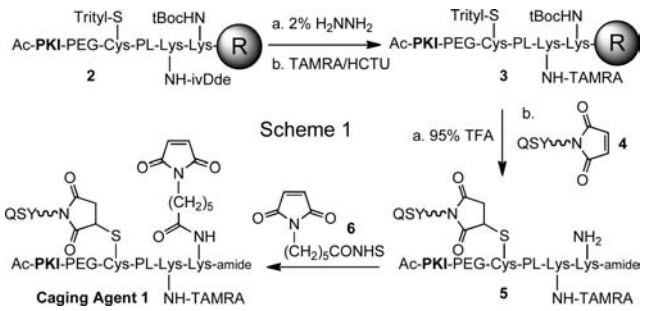


Figure 1. (a) Native C subunit highlighting the active site Cys-199 and its nonactive site Cys-343 counterpart. (b) Active site-directed alignment of the maleimide (E) of **Caging Agent 1** near Cys-343. (c) Caged C subunit. (d) Photolyzed C subunit. Q = Quencher; F = Fluorophore; ● (green) = PL.

Scheme 1



enzymatic activity and simultaneously generate a fluorescent protein via release of the active site-directed PKI fragment and the appended quencher moiety (Figure 1d).

Although **Caging Agent 1** is "information dense", it is readily prepared in a stepwise fashion via solid-phase synthesis and three subsequent side-chain modification steps (Scheme 1). The peptide backbone was synthesized on the TGR resin with the side chains of the C-terminal Lys dyad orthogonally protected to ensure site-selective attachment of the fluorophore (TAMRA) and electrophilic (maleimide) components. The hydrazine sensitive ivDde protecting group was removed first, allowing the TAMRA fluorophore to be positioned proximal to the PL moiety (**3**). Subsequent global deprotection and cleavage of the peptide from the resin (95% CF_3CO_2H) exposed both the Cys and remaining Lys side-chain functionalities. The former was modified with the QSY−maleimide conjugate **4** (explicit structure highlighted in blue in **Caging Agent 1**) to furnish **5**, which was then conjugated with the activated *N*-hydroxysuccinimide (NHS) ester of 4-maleimido-butyric acid **6**, which provided the desired **Caging Agent 1**.

The caged enzyme (<3% residual activity) was acquired by exposing the native C subunit to **Caging Agent 1** (2 equiv, see

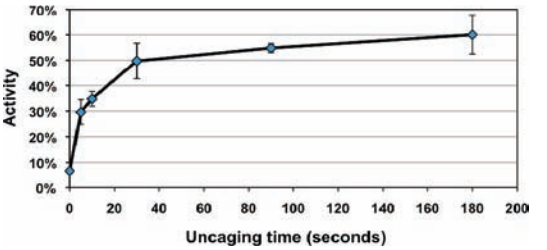


Figure 2. Percent protein kinase activity as a function of irradiation time.

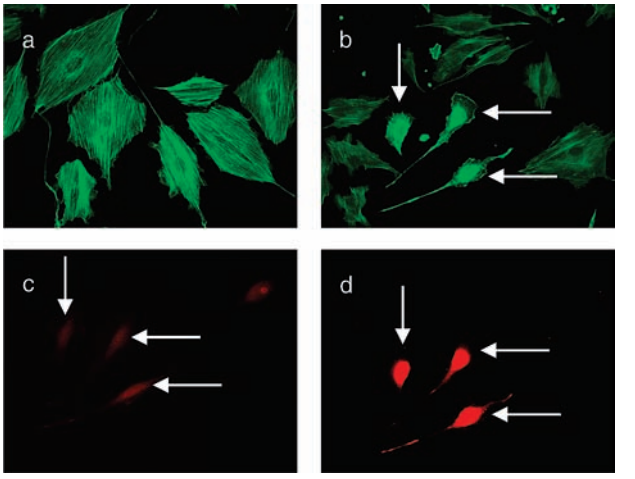


Figure 3. (a) Photolyzed nonmicroinjected REF52 cells. (b) Only microinjected cells (arrows) display stress fiber loss and morphological changes upon global illumination. Rhodamine fluorescence (c) prior to and (d) following photolysis. The PKA construct is freely diffusible in live cells (c and d), and thus the associated rhodamine fluorescence is strongest in the nuclear region (thickest path length). Stress fiber stained cells are fixed (a and b). Panels b−d are of the same field.

Supporting Information for experimental details). Subsequent photolysis provided the catalytically active (vide infra) C subunit. The gel migratory and photophysical behavior of the caged and photolyzed enzymes are consistent with light-induced loss of the PKI-PEG-Cys(QSY7) fragment (Figures S-2, S-3, S-4, S-5 in Supporting Information). Specifically, the light-exposed C subunit displays enhanced migratory aptitude relative to its higher molecular weight caged counterpart. In addition, the photolyzed enzyme displays enhanced fluorescence relative to the nonphotolyzed species (Figure S-6 in Supporting Information). In the presence of Prionex (porcine collagen with protein stabilizing properties) maximal restoration of catalytic activity is 80% and the fluorescence enhancement is 10-fold. In the absence of Prionex, 60% of native catalytic activity is recovered (Figure 2), but a 20-fold fluorescence yield is achieved (Figure S-6 in Supporting Information).

Stress fibers are bundles of actin that interface with focal adhesions and thereby link the cytoskeleton of the cell with the extracellular matrix. cAMP, as well as PKA activity, drives the disassembly of stress fibers along with attendant morphological changes.[11] We examined the ability of caged PKA to promote stress fiber disassembly in a light-dependent fashion in a rat embryonic fibroblast cell line (REF52). Microinjection of caged PKA, in the absence of photolysis, has no effect on REF52 stress fiber integrity (Figure S-10 in Supporting Information). By contrast, cells

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microinjected with PKA, upon subsequent photolysis, exhibit both the characteristic loss of stress fibers as well as a 6.2 ± 2.1-fold enhancement in fluorescence (panels b and d of Figure 3).

Many, but certainly not all, protein kinases possess a nonactive site Cys residue positioned at the Cys-343 site in PKA. However, any modifiable Cys moiety that lies outside, but within the vicinity, of the active site could potentially serve as a covalent anchoring residue. Alternatively, it is relatively straightforward to incorporate a reactive Cys at an appropriate position via site-directed mutagenesis. Finally, we do note that, although microinjection is a common biological tool, it is inconvenient and labor intensive, and only a relatively small number of cells (~100) can be treated in this fashion at any given time. By contrast, cell-permeable reagents offer a more robust strategy applicable to large cell populations. Cell permeable analogues of **Caging Agent 1** could prove useful in this regard since they can potentially modify the target protein in situ.

■ ASSOCIATED CONTENT

Supporting Information. Figures S-1−S-10 and procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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