Caged Catalytic Subunit of cAMP-Dependent Protein Kinase

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The light-mediated release of active biological molecules from "caged" reagents has been widely applied to biological problems.[1] Caged reagents are especially useful as the extent, timing, and location of photorelease in cells can be controlled. Caged *small* molecules include nucleotides (ATP, GTP &c.), neurotransmitters, second messengers (cAMP, Ca^{2+}, IP_3, nitric oxide &c.), and, recently, peptides.[2] Light-mediated regulation of protein activity is also highly desirable. Two major approaches have been taken; one is reversible control (switching) of the activity of proteins modified with photoisomerizable molecules.[3] In this case, all-or-none switching has not yet been achieved, possibly because random rather than targeted modification of functional groups on proteins has generally been applied. The second approach is irreversible photoactivation (triggering) of inactivated proteins, i.e., the preparation of caged proteins. The latter have included proteinases,[4] T4 lysozyme,[5] an immunotoxin,[6] G-actin,[7] bovine serum albumin,[8] α-hemolysin,[9] heavy meromyosin,[10] β-galactosidase,[11] and antibodies.[12] The chemical modifications used to cage proteins with light-sensitive protecting groups have also often been random, and the lack of control in this approach is a serious drawback. Targeted modification is more desirable and we advanced this idea by using modification at single cysteine residues introduced by mutagenesis.[9,13]

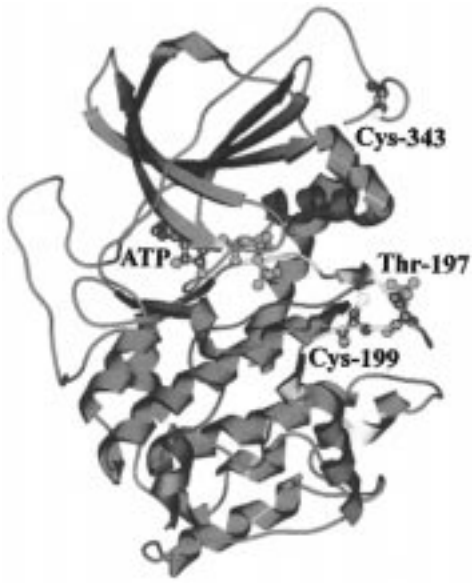

Figure 1. Ribbon diagram of the "closed" form of the wild-type C subunit complexed with a protein kinase inhibitor peptide (red) and ATP. The side chains of Thr-197, Cys-199, and Cys-343 are shown. In this study, a mutant with Cys-343 replaced by Ser was used. Thr-197 and Cys-199 are in the "activation loop" of the kinase (yellow). Thr-197 is phosphorylated (as shown) in the active form of the enzyme.[18] The figure was produced with the program Spock[19] by using coordinates for a recombinant mouse Cα subunit (1atp.pdb).

The caging of proteins involved in cell signaling such as protein kinases, G proteins, and transcription factors is an important challenge. The catalytic subunit of cAMP-dependent protein kinase (PKA) is a crucial participant in a wide range of signaling events including those involved in hormone action,[14] development,[15] and neuronal plasticity.[16] The tetrameric PKA holoenzyme, which contains two regulatory (R) and two catalytic (C) subunits, is inactive until cAMP binds to R. The holoenzyme then dissociates to form an R_2 dimer and two monomeric, active C subunits. The free C subunit can phosphorylate cytoplasmic or membrane protein substrates or diffuse into the nucleus and alter gene expression by acting on transcription factors.[17] Here, we describe a C subunit of PKA caged at Cys-199 at the mouth of the active site (Figure 1). The enzyme undergoes a 20- to 30-fold increase in activity upon photoactivation, and it is therefore suitable as a reagent for microinjection into living cells.

The C subunit, which contains two cysteine residues, Cys-199 and Cys-343 (Figure 1), is largely inactivated by a variety of sulfhydryl-specific reagents. The loss of activity is due to derivatization of Cys-199 as C subunit modified exclusively at Cys-343 is still active and unblocking of Cys-199 modified with 5,5′-dithiobis(2-nitrobenzoic acid) with dithiothreitol restores enzymatic activity.[20] Therefore, we chose to cage the C subunit by attaching a 2-nitrobenzyl group at Cys-199, which is in the

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(1) (a) *Bioorganic photochemistry Vol. 2: Biological applications of photochemical switches*; Morrison, H., Ed.; Wiley-Interscience: New York, 1993. (b) Adams, S. R.; Tsien, R. Y. *Annu. Rev. Physiol.* **1993**, *55*, 755. (c) Nerbonne, J. M. *Curr. Opinion Neurobiol.* **1996**, *6*, 379.
(2) (a) Pan, P.; Bayley, H. *FEBS Lett.* **1997**, *405*, 81. (b) Walker, J. W.; Gilbert, S. H.; Drummond, R. M.; Yamada, M.; Sreekumar, R.; Carraway, R. E.; Ikebe, M.; Fay, F. S. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 1568.
(3) Willner, I.; Shai, R. *Angew. Chem., Int. Ed. Engl.* **1996**, *35*, 367.
(4) (a) Turner, A. D.; Pizzo, S. V.; Rozakis, G.; Porter, N. A. *J. Am. Chem. Soc.* **1988**, *110*, 244. (b) Stoddard, B. L.; Koenigs, P.; Porter, N.; Petratos, K.; Petsko, G. A.; Ringe, D. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 5503.
(5) Mendel, D.; Ellman, J. A.; Schultz, P. G. *J. Am. Chem. Soc.* **1991**, *113*, 2758.
(6) Goldmacher, V. S.; Senter, P. D.; Lambert, J. M.; Blättler, W. A. *Bioconjugate Chem.* **1992**, *3*, 104.
(7) (a) Marriott, G.; Miyata, H.; Kinosita, K. *Biochem. Int.* **1992**, *26*, 943. (b) Marriott, G. *Biochemistry* **1994**, *33*, 9092. (c) Ottl, J.; Gabriel, D.; Marriott, G. *Bioconjugate Chem.* **1998**, *9*, 143.
(8) Thompson, S.; Spoors, J. A.; Fawcett, M.-C.; Self, C. H. *Biochem. Biophys. Res. Commun.* **1994**, *201*, 1213.
(9) Chang, C.-Y.; Niblack, B.; Walker, B.; Bayley, H. *Chem. Biol.* **1995**, *2*, 39.
(10) Marriott, G.; Heidecker, M. *Biochemistry* **1996**, *35*, 3170.
(11) Golan, R.; Zehavi, U.; Naim, M.; Patchornik, A.; Smirnoff, P. *Biochim. Biophys. Acta* **1996**, *1293*, 238.
(12) Self, C. H.; Thompson, S. *Nature Medicine* **1996**, *2*, 817.
(13) In vitro translation has also been used to place a photosensitive amino acid at a key site in a polypeptide chain by using a chemically aminoacylated tRNA [ref 5 and Cook, S. N.; Jack, W. E.; Xiong, X.; Danley, L. E.; Ellman, J. A.; Schultz, P. G.; Noren, C. J. *Angew. Chem., Int. Ed. Engl.* **1995**, *34*, 1629], but our approach is simpler and more conducive to scale-up. Modification with active-site directed reagents[4] is not as generally applicable as our approach.
(14) Daaka, Y.; Luttrell, L. M.; Lefkowitz, R. J. *Nature* **1997**, *390*, 88.
(15) Hammerschmidt, M.; Brook, A.; McMahon, A. P. *Trends in Genetics* **1997**, *13*, 14.
(16) Abel, T.; Martin, K. C.; Bartsch, D.; Kandel, E. R. *Science* **1998**, *279*, 338.
(17) Montminy, M. *Annu. Rev. Biochem.* **1997**, *66*, 807.
(18) Johnson, L. N.; Noble, M. E. M.; Owen, D. J. *Cell* **1996**, *85*, 149.

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Table 1. Activity of the Caged C Subunits Before and After Photoactivation[a]

caging reagent	BNPA	DMNBB	NBB
incubation[b] conditions	20 mM, 2.5 h	2 mM, 2.0 h	2 mM, 0.75 h
residual activity	8−12%	14%[c]	5−8%
residual activity after subsequent NEM treatment	5−7%	nd	3−5%
specific activity after photolysis[d]	15−20%	25−30%	80−100%
fold increase in specific activity after photolysis	2−3×	2×[e]	20−30×

[a] Based on three separate experiments for each reagent. The kinase activity assay used the peptide kemptide (LRRASLG) as substrate.[23] [b] All with 4.3 μM C343S at 25 °C in 100 mM Tris·HCl, pH 8.5, 1 mM EDTA. The reaction with DMNBB contained 8% DMF and that with NBB 4% ethanol. [c] Appeared to be completely modified from gel shift experiments. [d] Expressed as a percentage of the specific activity of the unmodified recombinant C subunit. Photolysis was performed at pH 6.0 for 20 min with a 30-W UV lamp (2200 μW cm^{-2}, peak emission 312 nm, Cole-Parmer E-09815-22). A glass filter, providing a cutoff at 285 nm (Oriel #51220) was used in all experiments. [e] No NEM treatment.

"activation loop" of the enzyme close to the critical Thr-197.[18,21] To be useful in cell biology experiments, a caged signaling protein must show at least a 10-fold increase in activity upon photolysis. Two reagents, 2-bromo-2-(2-nitrophenyl)acetic acid (BNPA) and 4,5-dimethoxy-2-nitrobenzyl bromide (DMNBB), were ruled out by this criterion (Table 1).[22] Therefore, we resorted to modification with 2-nitrobenzyl bromide (NBB). Because of the lability of the C subunit, considerable effort was required to find suitable conditions for this reaction, which occurred within a narrow window of pH and temperature in the presence of a small fraction of organic solvent (2 mM NBB in 100 mM Tris·HCl, pH 8.5, with 4% ethanol at 25 °C). A second modification with *N*-ethylmaleimide (NEM) after the NBB reaction lowered the residual activity of C343S still further to 3−5% (Table 1). To confirm that the residual activity is a property of the modified protein rather than residual unmodified protein, affinity chromatography with PKIP (protein kinase inhibitor peptide)-Affigel[23b] was carried out. Unmodified C subunit binds to the affinity matrix, while the chemically modified subunit does not (data not shown). The activity of the NBB-treated C subunit (C343S-NB) was similar before and after the PKIP-Affigel treatment suggesting that the residual activity belongs to the modified protein.

Not only did NBB modification give the lowest residual activity but also the modified C subunit was best activated by photodeprotection (Table 1). The efficiency of photolysis at 312 nm was higher at pH 6.0 than at pH 8.5, as determined from the time-course analyzed by SDS-polyacrylamide gel electrophoresis and by the rate of appearance of kinase activity.[24] By 20 min at pH 6.0, 100% of the protein was photodeprotected, whereas at pH 8.5, about 40% of the protein remained caged. In both cases, the specific catalytic activity of the released protein after 5 min irradiation was close to that determined before modification (pH 6.0, 104 ± 7%, $n = 3$), an increase of 20- to 30-fold upon uncaging. The specific activity of the released protein then slowly decreased with further irradiation (e.g., 20 min at pH 6.0, 88 ± 11%, $n = 3$). To measure the product quantum yield (Q_P) of C343S-NB, we used a 2-nitrobenzyl compound, 1-(2-nitrophenyl)-ethyl phosphate, with known $Q_P = 0.54$ as a reference.[25] At pH 6.0, the Q_P for uncaging C343S-NB was 0.84. At pH 8.5, the Q_P was 0.14.

In summary, we have produced a caged C subunit of PKA by targeted alkylation of a labile enzyme under carefully controlled conditions of temperature and pH. A significant lesson of this work is that a variety of photoremovable protecting groups must be tested for a given protein and that a reagent that works well with one protein may not with another. As alkylation can usually be pushed to completion, the critical factors appear to be different levels of residual activity after complete alkylation and different rates and yields of photoactivation. We believe that inactivation of the C subunit is a consequence of disruption of the activation loop (Figure 1),[18] and it is interesting that the least bulky of the three reagents is most effective. In addition to the intrinsic photochemistry of the caging group,[1] photoactivation may be affected by the microenvironment on a protein, which could promote a conformation of the caging group that is unfavorable for photolysis or cause quenching of the excited state. In these cases, the rates of side reactions may become significant and reduce the yield of photoactivated protein.

Most notably, we have produced an important new reagent for investigating cell signaling. The approach we have taken should be generalizable to other protein kinases. Because they have related primary sequences[26] and three-dimensional structures,[18,26] it seems likely that modification of natural or replacement cysteines at the position corresponding to Cys-199 in the C subunit of PKA should yield caged kinases with other members of the superfamily.[27]

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(19) Christopher, J. A. *SPOCK: the structural properties observation and calculation kit (program manual)*; Center for Macromolecular Design: Texas A&M University, College Station, TX, 1998.

(20) First, E. A.; Taylor, S. S. *Biochemistry* **1989**, *28*, 3598.

(21) To avoid potential complications, we chose to work with the single-cysteine mutant of the mouse Cα subunit C343S. The specific catalytic activity of recombinant C343S (16−17 μmol/min/mg) was close to that of recombinant wild-type C subunit (17−18 μmol/min/mg; literature 19 μmol/min/mg [Slice, L. W.; Taylor, S. S. *J. Biol. Chem.* **1989**, *264*, 20940]).

(22) Residual activity as well as a low efficiency of photolysis can adversely affect the extent (fold) of activation. The water-soluble sulfhydryl-specific caging reagent, BNPA, developed previously for caging the pore-forming R104C mutant of α-hemolysin,[9] reacted with C343S to yield C subunit with a residual activity of 8−12% (Table 1). Subsequent modification with *N*-ethylmaleimide (NEM) reduced the activity to 5−7%, rather than the 1−2% found by direct modification with NEM. Therefore, a large fraction of the residual activity after BNPA modification came from the modified protein. DMNBB was investigated because its derivatives can be photolyzed at longer wavelengths than unsubstituted 2-nitrobenzyl molecules. After C343S had been treated with DMNBB the residual catalytic activity was 14%. Upon irradiation at 312 nm, a disappointing 2-fold increase in activity was obtained (Table 1). Photoactivation with a 365 nm light source was even less at 1.5-fold.

(23) (a) Roskoski, R. *Methods Enzymol.* **1983**, *99*, 3. (b) Cheley, S.; Bayley, H. *Biochemistry* **1991**, *30*, 10246.

(24) While C343S-NB migrates faster than unmodified protein in 12% SDS-polyacrylamide gels, for analytical purposes, the difference in mobility was accentuated by treating samples with 4-acetamido-4′-((iodoacetyl)amino)-stilbene-2,2′-disulfonate (IASD), a sulfhydryl-directed disulfonate reagent [Krishnasastry, M.; Walker, B.; Braha, O.; Bayley, H. *FEBS Lett.* **1994**, *356*, 66]. C343S-NB is unable to react with IASD, whereas IASD-modified C343S migrates more slowly than the unmodified protein. Before photolysis, the modified C subunit was separated from unreacted NBB and other small molecules by passage through a Sephadex G-25 column in 20 mM Tris·HCl pH 8.5, 200 mM KCl. Dithiothreitol was then added to 8.5 mM.

(25) (a) Kaplan, J. H.; Forbush, B.; Hoffman, J. F. *Biochemistry* **1978**, *17*, 1929. (b) Walker, J. W.; Reid, G. P.; McCray, J. A.; Trentham, D. R. *J. Am. Chem. Soc.* **1988**, *110*, 7170.

(26) www.sdsc.edu/kinases.

(27) Another potentially powerful approach to caged signaling molecules is the attachment of photoremovable groups to phosphoproteins at sites of thiophosphorylation.[2a]