J|A|C|S
ARTICLES
Published on Web 06/17/2002

Catalytic Subunit of Protein Kinase A Caged at the Activating Phosphothreonine

Keyong Zou,[†] Stephen Cheley,[†] Richard S. Givens,[‡] and Hagan Bayley*[,†,§]

Contribution from the Department of Medical Biochemistry and Genetics, The Texas A&M University System Health Science Center, College Station, Texas 77843-1114, Department of Chemistry, University of Kansas, Lawrence, Kansas 66045-7582, and Department of Chemistry, Texas A&M University, College Station, Texas 77843-3255

Received March 19, 2002

Abstract: Caged reagents are photoactivatable molecules with applications in biological research. While a great deal of work has been carried out on small caged molecules, less has been done on caged macromolecules, such as proteins. Caged proteins would be especially useful in signal transduction research. Since most proteins involved in cell signaling are regulated by phosphorylation, a means to cage phosphorylated proteins would be generally applicable. Here we show that the catalytic subunit of protein kinase A can be activated by thiophosphorylation at Thr-197. The modified protein can then be caged with 4-hydroxyphenacyl bromide to yield a derivative with a specific catalytic activity that is reduced by ~17-fold. Upon photolysis at near UV wavelengths, an ~15-fold increase in activity is observed, representing an ~85−90% yield of uncaged product with a quantum yield $\phi_P = 0.21$. Because protein kinases belong to a superfamily with structurally related catalytic domains, the protein chemistry demonstrated here should be applicable to a wide range of signaling proteins.

Introduction

A caged reagent[1] is a molecule in which the activity has been blocked by chemical modification with a photolabile reagent. Photolysis removes the protecting group and restores activity. Because the photorelease of an effector can be temporally and spatially controlled, caged molecules, such as caged ATP, are widely applied in biological research.[2,3] While a great deal of work has been carried out on small caged molecules, less has been done on caged macromolecules.[4,5] In the experiments described here, a catalytic subunit (C) of cAMP-dependent protein kinase (PKA) caged on an activating phosphate was prepared with the recently developed 4-hydroxyphenacyl reagent.[6]

The idea of gaining photochemical control of protein activity is not new[7−9] and approaches other than direct caging have been taken including the use of proteins derivatized with photoisomerizable reagents[10−18] and the application of caged[19] or photoisomerizable[20,21] inhibitors and activators. In general, it has been difficult to achieve all-or-none effects with photoisomerizable reagents, although the prospect of reversible activation is intriguing. While the use of caged inhibitors and activators is a promising strategy, we focus here on the direct caging of proteins for which several approaches have been explored. The random introduction of photocleavable protecting groups through chemical modification of reactive amino acid side chains has proved surprisingly successful.[22−27] However,

* Corresponding author. E-mail: bayley@tamu.edu. Telephone: (979) 845-7047. Fax: (979) 862-2416.
[†] The Texas A&M University System Health Science Center.
[‡] University of Kansas.
[§] Department of Chemistry, Texas A&M University.

(1) Kaplan, J. H.; Forbush, B.; Hoffman, J. F. *Biochemistry* **1978**, *17*, 1929−1935.
(2) Adams, S. R.; Tsien, R. Y. *Annu. Rev. Physiol.* **1993**, *55*, 755−784.
(3) Dorman, G.; Prestwich, G. D. *Trends Biotechnol.* **2000**, *18*, 64−77.
(4) Bayley, H.; Chang, C.-Y.; Miller, W. T.; Niblack, B.; Pan, P. *Methods Enzymol.* **1998**, *291*, 117−135.
(5) Curley, K.; Lawrence, D. S. *Curr. Opin. Chem. Biol.* **1999**, *3*, 84−88.
(6) Givens, R. S.; Park, C.-H. *Tetrahedron Lett.* **1996**, *37*, 6259−6262.
(7) Singh, A. E. R.; Thornton, E. R.; Westheimer, F. H. *J. Biol. Chem.* **1962**, *237*, PC3006−PC3008.
(8) Erlanger, B. F. *Annu. Rev. Biochem.* **1976**, *45*, 267−283.
(9) Hug, D. H. *Photochem. Photobiol. Rev.* **1978**, *3*, 1−33.
(10) Montagnoli, G.; Monti, S.; Nannicini, L.; Giovannitti, M. P.; Ristori, M. G. *Photochem. Photobiol.* **1978**, *27*, 43−49.
(11) Willner, I.; Rubin, S.; Riklin, A. *J. Am. Chem. Soc.* **1991**, *113*, 3321−3325.
(12) Willner, I.; Rubin, S.; Zor, T. *J. Am. Chem. Soc.* **1991**, *113*, 4013−4014.
(13) Ueda, T.; Murayama, K.; Yamamoto, T.; Kimura, S.; Imanishi, Y. *J. Chem. Soc., Perkin Trans.1* **1994**, 225−230.
(14) Willner, I.; Shai, R. *Angew. Chem., Int. Ed. Engl.* **1996**, *35*, 367−385.
(15) Lien, L.; Jaikaran, D. C. J.; Zhang, Z. H.; Woolley, G. A. *J. Am. Chem. Soc.* **1996**, *118*, 12222−12223.
(16) Liu, D.; Karanicolas, J.; Yu, C.; Zhang, Z. H.; Woolley, G. A. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 2677−2680.
(17) Kumita, J. R.; Smart, O. S.; Woolley, G. A. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 3803−3808.
(18) Pieroni, O.; Fissi, A.; Angelini, N.; Lenci, F. *Acc. Chem. Res.* **2001**, *34*, 9−17.
(19) Wood, J. S.; Koszelak, M.; Liu, J.; Lawrence, D. S. *J. Am. Chem. Soc.* **1998**, *120*, 7145−7146.
(20) Bartels, E.; Wassermann, N. H.; Erlanger, B. F. *Proc. Natl. Acad. Sci. U.S.A.* **1971**, *68*, 1820−1823.
(21) Lester, H. A.; Krouse, M. E.; Nass, M. M.; Nerbonne, J. M.; Wassermann, N. H.; Erlanger, B. F. *J. Gen. Physiol.* **1980**, *75*, 207−232.
(22) Thompson, S.; Spoors, J. A.; Fawcett, M.-C.; Self, C. H. *Biochem. Biophys. Res. Commun.* **1994**, *201*, 1213−1219.
(23) Marriott, G.; Miyata, H.; Kinosita, K. *Biochem. Int.* **1992**, *26*, 943−951.
(24) Marriott, G. *Biochemistry* **1994**, *33*, 9092−9097.
(25) Golan, R.; Zehavi, U.; Naim, M.; Patchornik, A.; Smirnoff, P. *Biochim. Biophys. Acta* **1996**, *1293*, 238−242.
(26) Self, C. H.; Thompson, S. *Nat. Med.* **1996**, *2*, 817−820.
(27) Cambridge, S. B.; Davis, R. L.; Minden, J. S. *Science* **1997**, *277*, 825−828.

10.1021/ja020405e CCC: $22.00

because of its predictability and reproducibility, targeted modification is likely to be more useful and essential in cases of proteins with multiple functional domains. In a relatively sophisticated form of targeted modification, caged proteins have been obtained by the incorporation of unnatural amino acids at specific sites during translation in vitro[28−31] or in *Xenopus* oocytes.[32−35] Photoactivatable unnatural amino acids incorporated during translation or by synthesis, or semisynthesis, have also been used to initiate protein folding,[36,37] protein splicing,[38] and protein cleavage.[33] A second rational approach to the photoactivation of proteins involves the use of photolabile active-site directed reagents. For example, various proteases can be caged as *o*-hydroxycinnamoyl acyl enzymes.[39−42]

A third approach, which we have adopted, is targeted chemical modification of single cysteine residues,[43,44] which can be introduced by genetic engineering.[43] This strategy has been applied to a pore-forming toxin,[43] heavy meromyosin,[44] the catalytic subunit of protein kinase A,[45] and a protein tyrosine phosphatase.[46] Caged cysteine residues have also been introduced during translation in vitro.[35]

Most of the work with caged proteins has been done with 2-nitrobenzyl reagents that were first introduced for caging small molecules.[1,47] The 2-nitrobenzyl reagents have disadvantages. The protecting group is released as a 2-nitrosobenzaldehyde or 2-nitrosoacetophenone. These molecules react with biological molecules,[48] for example those containing sulfhydryl groups.[49,50] Because caged proteins are generally used at low concentrations, this may be less of a problem than it would be in other circumstances, such as the use of millimolar concentrations of a caged small molecule. In addition, the process of deprotection after the absorption of a photon is relatively slow ($k \approx 1-10^3$ s^{-1}) for 2-nitrobenzyl derivatives.[51] Hence, several new reagents have been introduced to replace the 2-nitrobenzyl compounds. Notable among them is the 4-hydroxyphenacyl group and its derivatives, which have been used to cage small molecules including ATP, phosphate, glutamic acid, GABA and various peptides.[6,52−58] A brief report of a caged protein tyrosine phosphatase obtained by selective modification at the active-site cysteine by various α-haloacetophenones, including 4-hydroxyphenacyl bromide, has also appeared.[46] Advantages of the 4-hydroxyphenacyl group include the formation of a spiroketone upon photolysis, which is hydrolyzed to the unreactive 4-hydroxyphenylacetic acid.[55,59] This photoproduct does not compete for incident light because of the blue-shift in absorbance. In addition, photodeprotection is rapid after the absorption of a photon ($k \approx 10^7-10^8$ s^{-1}).[53]

In this work, we describe a general approach for caging cell signaling proteins, with properties that are modulated by protein phosphorylation. Because almost all signaling proteins are subject to phosphorylation, the approach will be useful for virtually any signaling pathway, including, for example, those involved in cell division and oncogenesis.[60−62] The covalent attachment of a phosphate group to a critical residue, usually serine, threonine, or tyrosine, can dramatically alter the function of a protein. For example, kinase activity can be turned on or shut off, or protein−protein interactions can be reinforced. We first explored the caging of phosphoryl residues with thiophosphoserine-containing peptides and 2-nitrobenzyl reagents.[63] Recently, the idea of caging at thiophosphate was extended to tyrosine-containing peptides, using both 2-nitrobenzyl reagents and the 4-hydroxyphenacyl group.[58] In both cases, the thiophosphoryl peptides were successfully caged and subsequently deprotected in high yields. In the present work the approach is applied to a protein, the catalytic subunit of protein kinase A, a key enzyme in cell signaling and a model for other signaling proteins,[64,65] especially its structural homologues in the large kinase superfamily.[66]

PKA is the best studied of all kinases and serves as an archetype for understanding the entire kinase family. The inactive holoenzyme of PKA contains two catalytic subunits (C) and two regulatory subunits (R). Many cells respond to stimuli with increased levels of cAMP generated through the action of adenylate cyclase. Two molecules of cAMP bind to each regulatory subunit of PKA. Once occupied, the regulatory subunit undergoes a conformational change, and active mono-

(28) Mendel, D.; Ellman, J. A.; Schultz, P. G. *J. Am. Chem. Soc.* **1991**, *113*, 2758−2760.
(29) Cornish, V. W.; Mendel, D.; Schultz, P. G. *Angew. Chem., Int. Ed. Engl.* **1995**, *34*, 621−633.
(30) Pollitt, S. K.; Schultz, P. G. *Angew. Chem., Int. Ed.* **1998**, *37*, 2104−2107.
(31) Short, G. F.; Lodder, M.; Laikhter, A. L.; Arslan, T.; Hecht, S. M. *J. Am. Chem. Soc.* **1999**, *121*, 1.
(32) Nowak, M. W.; Kearney, P. C.; Sampson, J. R.; Saks, M. E.; Labarca, C. G.; Silverman, S. K.; Zhong, W.; Thorson, J.; Abelson, J. N.; Davidson, N.; Schultz, P. G.; Dougherty, D. A.; Lester, H. A. *Science* **1995**, *268*, 439−442.
(33) England, P. M.; Lester, H. A.; Davidson, N.; Dougherty, D. A. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 11025−11030.
(34) Miller, J. C.; Silverman, S. K.; England, P. M.; Dougherty, D. A.; Lester, H. A. *Neuron* **1998**, *20*, 619−624.
(35) Philipson, K. D.; Gallivan, J. P.; Brandt, G. S.; Dougherty, D. A.; Lester, H. A. *Am. J. Physiol. Cell Physiol.* **2001**, *281*, 195−206.
(36) Lu, H. S. M.; Volk, M.; Kholodenko, Y.; Gooding, E.; Hochstrasser, R. M.; DeGrado, W. F. *J. Am. Chem. Soc.* **1997**, *119*, 7173−7180.
(37) Hansen, K. C.; Rock, R. S.; Larsen, R. W.; Chan, S. I. *J. Am. Chem. Soc.* **2000**, *122*, 11567−11568.
(38) 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*, 1629−1630.
(39) Stoddard, B. L.; Bruhnke, J.; Porter, N.; Ringe, D.; Petsko, G. A. *Biochemistry* **1990**, *29*, 4871−4879.
(40) Stoddard, B. L.; Bruhnke, J.; Koenigs, P.; Porter, N.; Ringe, D.; Petsko, G. A. *Biochemistry* **1990**, *29*, 8042−8051.
(41) Jones, P. B.; Porter, N. A. *J. Am. Chem. Soc.* **1999**, *121*, 2753−2761.
(42) Porter, N. A.; Thuring, J. W.; Li, H. *J. Am. Chem. Soc.* **1999**, *121*, 7716−7717.
(43) Chang, C.-Y.; Niblack, B.; Walker, B.; Bayley, H. *Chem. Biol.* **1995**, *2*, 391−400.
(44) Marriott, G.; Heidecker, M. *Biochemistry* **1996**, *35*, 3170−3174.
(45) Chang, C.-Y.; Fernandez, T.; Panchal, R.; Bayley, H. *J. Am. Chem. Soc.* **1998**, *120*, 7661−7662.
(46) Arabaci, G.; Guo, X.-C.; Beebe, K. D.; Coggeshall, K. M.; Pei, D. *J. Am. Chem. Soc.* **1999**, *121*, 5085−5086.
(47) Kaplan, J. H.; Hollis, R. J. *Nature* **1980**, *288*, 587−589.
(48) Du, X.; Frei, H.; Kim, S. H. *Biopolymers* **2001**, *62*, 147−149.
(49) Zuman, P.; Shah, B. *Chem. Rev.* **1994**, *94*, 1621−1641.
(50) Barth, A.; Corrie, J. E. T.; Gradwell, M. J.; Maeda, Y.; Mantele, W.; Meier, T.; Trentham, D. R. *J. Am. Chem. Soc.* **1997**, *119*, 4149−4159.
(51) Corrie, J. E. T.; Trentham, D. R. Caged Nucleotides and Neurotransmitters. In *Biological Applications of Photochemical Switches*; Morrison, H., Ed.; John Wiley & Sons: New York, 1993; pp 243−299.
(52) Givens, R. S.; Jung, A.; Park, C.-H.; Weber, J.; Bartlett, W. *J. Am. Chem. Soc.* **1997**, *119*, 8369−8370.
(53) Park, C.-H.; Givens, R. S. *J. Am. Chem. Soc.* **1997**, *119*, 2453−2463.
(54) Conrad, P. G.; Givens, R. S.; Hellrung, B.; Rajesh, C. S.; Ramseier, M.; Wirz, J. *J. Am. Chem. Soc.* **2000**, *122*, 9346−9347.
(55) Conrad, P. G.; Givens, R. S.; Weber, J. F. W.; Kandler, K. *Org. Lett.* **2000**, *2*, 1545−1547.
(56) Givens, R. S.; Weber, J. F. W.; Conrad, P. G.; Orosz, G.; Donahue, S. L.; Thayer, S. A. *J. Am. Chem. Soc.* **2000**, *122*, 2687−2697.
(57) Du, X.; Frei, H.; Kim, S. H. *J. Biol. Chem.* **2000**, *275*, 8492−8500.
(58) Zou, K.; Miller, W. T.; Givens, R. S.; Bayley, H. *Angew. Chem., Int. Ed.* **2001**, *40*, 3049−3051.
(59) Anderson, J. C.; Reese, C. B. *Tetrahedron Lett.* **1962**.
(60) McDonald, E. R. *Ann. Med.* **2001**, *33*, 113−122.
(61) Sampath, D.; Plunkett, W. *Curr. Opin. Oncol.* **2001**, *13*, 484−490.
(62) O'Farrell, P. H. *Trends Cell. Biol.* **2001**, *11*, 512−519.
(63) Pan, P.; Bayley, H. *FEBS Lett.* **1997**, *405*, 81−85.
(64) Smith, C. M.; Radzio-Andzelm, E.; Madhusudan; Akamine, P.; Taylor, S. S. *Prog. Biophys. Mol. Biol.* **1999**, *71*, 313−341.
(65) Taylor, S. S.; Radzio-Andzelm, E.; Madhusudan; Cheng, X.; Ten Eyck, L.; Narayana, N. *Pharmacol. Ther.* **1999**, *82*, 133−141.
(66) Hanks, S. K.; Hunter, T. *FASEB J.* **1995**, *9*, 576−596.

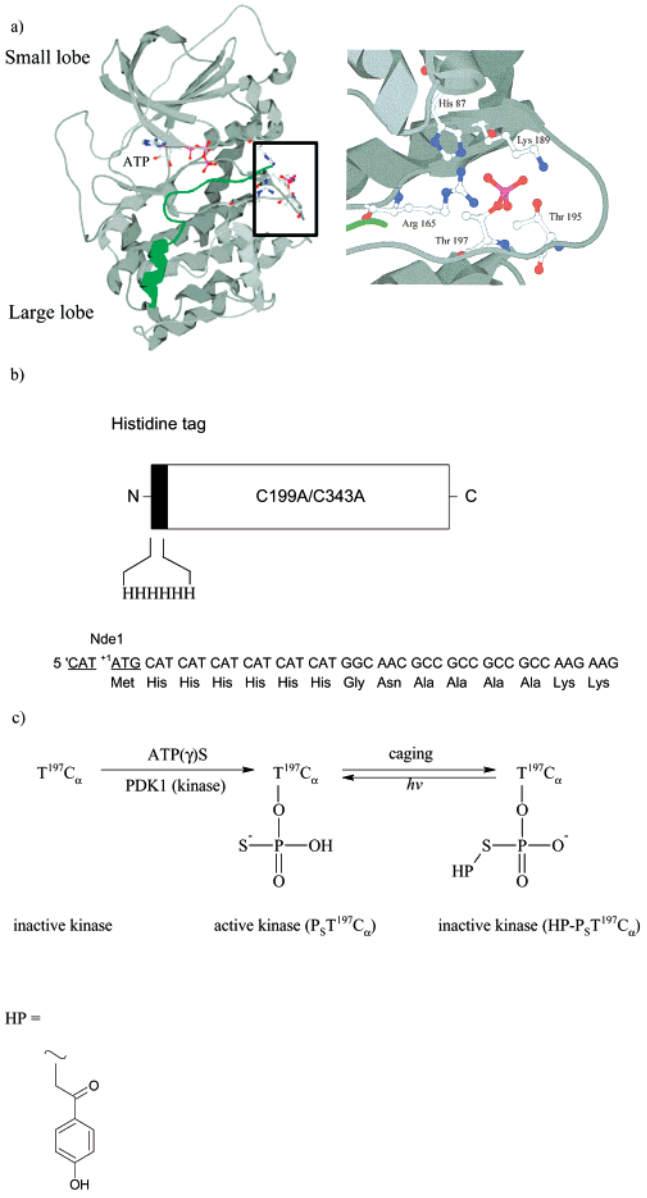

Figure 1. Caging the C subunit of PKA at Thr-197. (A) Structure of the C subunit, showing the small and large lobes, and the location of ATP and a peptide inhibitor (green) in the active site. The expanded view shows key residues, including Thr-197. (B) Linear structure of the C subunit mutant used in this work: H_6-(T^{197})C199A/C343A, $T^{197}C_\alpha$ for short. The sequence of the N-terminal His-tag is shown. (C) Caging and uncaging of the thiophosphorylated C subunit, $P_ST^{197}C_\alpha$. The caging reagent is 4-hydroxyphenacyl (HP) bromide.

meric C subunit is released. The C subunit consists of two lobes (Figure 1a).[65,67,68] The small lobe comprising the N-terminal sequences is associated with ATP binding, while the large lobe comprising the C-terminal sequences provides residues for catalysis and substrate binding. The activity of the C subunit is regulated by phosphorylation at Thr-197.[69−71] The nonphosphorylated catalytic subunit can be phosphorylated and activated by autophosphorylation,[69] but this is an inefficient process, and it was recently found that 3-phosphoinositide-dependent kinase 1 (PDK1) phosphorylates C exclusively at Thr-197 with high efficiency and is likely to be the physiological activator of the C subunit.[70,71] The phosphate group on Thr-197 interacts with several residues from both lobes, including Arg-165 and Lys-189 from the large lobe, and His-87 from the small lobe. These contacts are likely to contribute to the stabilization of the enzyme in an active conformation.[68,72] Therefore, we felt that the methodology developed for caged thiophosphoryl peptides might be applied to position 197 of the C subunit; a bulky photolabile protecting group would prevent the enzyme from assuming a fully active conformation.

The C subunit of PKA has previously been caged in a variety of ways. In one approach Cys-199, in an otherwise cysteine-free enzyme, was derivatized with 2-nitrobenzyl bromide.[45] The same residue in the natural C subunit, which also contains a cysteine at position 343, was modified with an active-site-directed peptide that had been coupled to the carboxyl group of α-bromo-2-nitrophenylacetic acid.[73] Lawrence and colleagues have also used a caged inhibitor of the C subunit to turn off kinase activity.[19] Here we describe the expression of an unphosphorylated C subunit in *Escherichia coli*, thiophosphorylation at Thr-197 with PDK-1, derivatization and inactivation with 4-hydroxyphenacyl bromide and subsequent uncaging and activation with near UV radiation (Figure 1c). Caging at Thr-197 offers the potential advantage of requiring no mutagenesis and the likelihood that the methodology will be generally applicable to members of the kinase superfamily, and perhaps to other signaling proteins. While no protein kinase has previously been caged directly on phosphate, the Lawrence group has recently shown that modification at cysteine with α-bromo-2-nitrophenylacetic acid can mimic phosphorylation.[74] In the case of LIM kinase, the enzyme can be inactivated in this way and subsequently reactivated by photolysis demonstrating an alternative to the present approach.[74]

Experimental Section

Materials. 4-Hydroxyphenacyl bromide was prepared as described.[53] *N*-[2-(*p*-Bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H89) was purchased from Alexis. ATP, kemptide (LRRASLG), benzamidine, and phenylmethylsulfonyl fluoride (PMSF) were from Sigma. Adenosine 5′-*O*-(3-thiotriphosphate) [(ATP(γ)S, #1-162-306)], and bovine serum albumin (#238-031) were from Roche. Prionex (10%) (a BSA substitute, Pentapharm product) was from Centerchem. [^{35}S]ATP(γ)S (600 Ci/mmol, #55000) was purchased from ICN, and [γ-^{32}P]ATP was from Amersham. (4-Acetamido-4′-[(iodoacetyl)amino]stilbene-2,2′-disulfonate (IASD, #A-484) was from Molecular Probes. 3-Phosphoinositide-dependent kinase 1 (PDK1, #14-280) was from Upstate Biotechnology. A monoclonal antibody against the thiophosphate-epitope (PhosphFind, #2001) was from Boston Biologicals. An antibody specific for peptides and proteins containing phosphothreonine (P-Thr-Polyclonal, #9381), HRP-conjugated anti-mouse IgG (secondary antibody, #7072-1), HRP-conjugated anti-rabbit IgG (secondary antibody, #7071-1), and LumiGLO for Western blot detection (#7003) were purchased from Cell Signaling Technology. Nitrocellulose membrane (pore size 0.45 μm, #21640) was from Schleicher & Schuell. Bio-Gel P-6 DG desalting gel (#150-0738) and Micro Bio-Spin columns

(67) Knighton, D. R.; Zheng, J.; Ten Eyck, L. F.; Xuong, N.-H.; Taylor, S. S.; Sowadski, J. M. *Science* **1991**, *253*, 407−414.
(68) Knighton, D. R.; Zheng, J.; Ten Eyck, L. F.; Xuong, N.-H.; Taylor, S. S.; Sowadski, J. M. *Science* **1991**, *253*, 414−420.
(69) Steinberg, R. A.; Cauthron, R. D.; Symcox, M. M.; Shuntoh, H. *Mol. Cell. Biol.* **1993**, *13*, 2332−2341.
(70) Cauthron, R. D.; Carter, K. B.; Liauw, S.; Steinberg, R. A. *Mol. Cell. Biol.* **1998**, *18*, 1416−1423.
(71) Cheng, X.; Ma, Y.; Moore, M.; Hemmings, B. A.; Taylor, S. S. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 9849−9854.
(72) Zheng, J.; Knighton, D. R.; Xuong, N.-H.; Taylor, S. S.; Sowadski, J. M.; Ten Eyck, L. F. *Protein Sci.* **1993**, *2*, 1559−1573.
(73) Curley, K.; Lawrence, D. S. *J. Am. Chem. Soc.* **1998**, *120*, 8573−8574.
(74) Ghosh, M.; Ichetovkin, I.; Song, X.; Condeelis, J. S.; Lawrence, D. S. *J. Am. Chem. Soc.* **2002**, *124*, 2440−2441.

containing Bio-Gel P-6 resin (#732-6222) were from Bio-Rad. Ni^{2+}−NTA magnetic agarose beads [binding capacity: 300 μg mL^{-1} for 6xHis-tagged DHFR protein (24 kDa); 5% suspension, #36113] and Ni^{2+}−NTA agarose were from Qiagen. Fluorescent dye-labeled kemptide (LRRASLG) (PepTag A1 peptide, #V5340) was from Promega. 5-thio-2-nitrobenzoic acid (TNB)-thiol agarose (TNB-thiol, loading: 5 μmol of TNB/mL of settled gel; capacity: 10−15 mg of reduced human IgG/mL of gel, # 204099) was from Pierce. Restriction enzymes were purchased from New England Biolabs. Oligonucleotide primers were synthesized by Integrated DNA Technology. DNA was sequenced at Lone Star Labs. Unless otherwise indicated, additional reagents were from Sigma.

Kinase Assay: Fluorescence Method. Kemptide (LRRASLG) labeled with a fluorescent dye (PepTag A1 peptide) was used in a rapid, sensitive, but nonquantitative, kinase assay. The net charge of the peptide is changed from +1 to −1 upon phosphorylation by the C subunit of PKA. The phosphorylated peptide was rapidly separated from the nonphosphorylated peptide in a 0.8% agarose gel; the peptides migrated toward opposite electrodes. In brief, buffer (10 μL) containing 25 mM Tris·HCl, 30 mM $MgCl_2$, 3 mM ATP, and PepTag A1 peptide (1.52 nmol) (pH 7.4) was warmed to 30 °C before incubation with prewarmed C subunit [25 μL in 25 mM Tris·HCl, 0.2 mg mL^{-1} BSA (pH 7.4)] for 10 min at 30 °C. The reaction was stopped by heating at 95 °C for 10 min before the addition of 80% glycerol (2 μL). The reaction mixture was then resolved in an 0.8% agarose gel at 100 V for 15−20 min. The gel was prepared and run in 50 mM Tris·HCl (pH 8.0). Kinase activity was roughly estimated from the intensity of the band of phosphorylated kemptide, determined from a photograph taken on a transilluminator.

Kinase Assay: Radioisotopic Method. The radioisotopic kinase assay used kemptide (LRRASLG) as the substrate.[75−77] To 10X buffer [5 μL, 500 mM MOPS, 100 mM $MgCl_2$, 1 mg mL^{-1} BSA, 1% Nonidet P-40 (pH 6.8)] was added ATP [10 μL, 1 mM containing [γ-^{32}P]ATP at 550 dpm $pmol^{-1}$] and kemptide (10 μL, 1 mM, in water). Kinase [25 μL, ∼2.5 ng in 10 mM MOPS, 10 mM DTT, and 0.2 mg mL^{-1} BSA (pH 6.8)] was prewarmed to 30 °C and mixed with the buffer containing ATP and kemptide. The mixture was incubated at 30 °C for 10 min. A portion (20 μL) was spotted onto phosphocellulose paper (2 × 2 cm), which was immediately immersed into 1 L of 75 mM phosphoric acid. The paper was washed four times, for 10 min each time, with 1 L of 75 mM phosphoric acid. The radioactivity on the paper was measured by liquid scintillation counting.

Construction of the C_α C199A/C343A Gene Encoding an N-Terminal Hexahistidine Tag. A plasmid, pRSET-B/C_αC199A/C343A containing a gene encoding the C199A/C343A mutant of the mouse C_α subunit of PKA was a gift from Dr. Fredrich W. Herberg (Ruhr-Universitat, Bochum, Germany). To insert a sequence encoding a hexahistidine tag at the 5′ end of the gene, pRSET-B/C_αC199A/C343A was first linearized with *Hind*III and then used as the template for PCR with the following primers: 5′-CAT ATG CAT CAT CAT CAT CAT CAT GGC AAC GCC GCC GCC GCC AAG AAG G-3′ (sense), 5′-AAG CTT ATC AAA ACT CAG TAA ACT CCT TGC-3′ (antisense). The PCR product was cloned into the TOPO-TA vector (Invitrogen). The purified plasmid was digested with *Nde*1 and *Hind*III, and the resulting insert was ligated into pRSET-B (Invitrogen), which had been cut with the same enzymes, to yield pRSET-B/$C_\alpha H_6$/C199A/C343A. The sequence of the new C_α gene was verified after ligation into the pRSET-B vector.

Expression and Purification of the Nonphosphorylated Protein. Recombinant nonphosphorylated C_α mutant protein, designated H_6-(T^{197})C199A/C343A, $T^{197}C_\alpha$ for short, was expressed in the presence of the specific PKA inhibitor *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H89). In brief, *E. coli* BL21(DE3), freshly transformed with pRSET-B/$C_\alpha H_6$/C199A/C343A, was used to inoculate 10 mL of Luria−Bertani (LB) medium (Difco) containing 100 μg mL^{-1} ampicillin (LB/amp). After 8 h at 37°C, the starter culture was added to a shaker flask containing 1 L LB/amp and grown to an optical density at 600 nm (OD_{600}) of 0.3−0.4. The temperature was then shifted from 37 to 24 °C. When the OD_{600} reached 0.6−0.7, protein expression was induced by the addition of 0.2 mM isopropyl β-D-thiogalactoside (IPTG). To obtain nonphosphorylated protein, 40 μM H89, which prevents autophosphorylation, was added to the culture medium at the time of induction from a 50 mM stock solution in DMSO.[70,71] The culture was grown for an additional 6 h at 24 °C.

Cells were harvested by centrifugation (5000*g* for 25 min at 4 °C). The pellet from a 1 L culture was resuspended in 10 mL of ice-cold lysis-wash buffer [25 mM sodium phosphate, 20 mM imidazole, 200 mM NaCl, 40 μM H89, 1 mM benzamidine, and 200 μM PMSF (pH 8.0)], and passed two times through a prechilled French press at 8000 psi. The lysate was clarified by centrifugation at 20000*g* for 30 min at 4 °C. The supernatant was mixed with metal chelate affinity matrix (1 mL, Ni^{2+}−NTA agarose, #30210, Qiagen) and rotated overnight at 4 °C. The suspension was then poured into a disposable column (Econo-Pac, #732-1010, Bio-Rad). The flow-through was collected for analysis, and the beads were washed with 30 bead volumes of the lysis-wash buffer. The bound protein was then eluted with 5 bead volumes of elution buffer [25 mM sodium phosphate, 100 mM imidazole, and 200 mM NaCl (pH 8.0)]. The eluate was dialyzed extensively at 4 °C against storage buffer [25 mM sodium phosphate, 10% glycerol, 10 mM NaCl, 1 mM benzamidine and 200 μM PMSF (pH 7.0)], and stored at −80 °C. The protein concentration in the dialysate was determined by measuring the absorbance at 280 nm (with $A_{0.1\%} = 1.26$, calculated from the amino acid composition). The yield of $T^{197}C_\alpha$ was very low at 150 μg for 10 L of culture. The protein appeared as a single band in an SDS−polyacrylamide gel and had a specific catalytic activity of 1.8 ± 0.3 μmol min^{-1} mg^{-1}.

Thiophosphorylation of $T^{197}C_\alpha$. Nonphosphorylated $T^{197}C_\alpha$ was thiophosphorylated with PDK1 in the presence of ATP(γ)S to yield $P_ST^{197}C_\alpha$. Each reaction (1 mL) contained 1.2 μM $T^{197}C_\alpha$, 25 mM sodium phosphate, 150 ng PDK1, 100 μM ATP(γ)S, 1% Prionex, 0.1 mM EDTA, 10 mM $MgCl_2$, and 10 mM DTT (pH 7.0). PDK1 (5 ng μL^{-1}) was stored in 50 mM Tris·HCl, 0.1 mM EGTA, 0.1% 2-mercaptoethanol, 150 mM NaCl, 270 mM sucrose, 1 mM benzamidine, 200 μM PMSF, and 1 mg mL^{-1} BSA (pH 7.5). After 45 min at 30 °C, the reaction was continued for an additional 2−3 h at 4 °C. To prepare ^{35}S-labeled $P_ST^{197}C_\alpha$, 100 μM [^{35}S]ATP(γ)S at a specific activity of 13 200 dpm $pmol^{-1}$ was used.

Confirmation of Thiophosphorylation of $T^{197}C_\alpha$ by Western Blotting. $P_ST^{197}C_\alpha$ was subjected to SDS−polyacrylamide electrophoresis. Protein in the gel was electrotransferred to a nitrocellulose membrane in transfer buffer [25 mM Tris·HCl, 200 mM glycine, 20% methanol (pH 8.5)]. The membrane was washed with Tris-buffered saline [TBS, 25 mL, 20 mM Tris·HCl, 137 mM NaCl (pH 7.6)] for 5 min at room temperature. To block nonspecific binding sites, the membrane was then incubated in blocking buffer (25 mL of TBS containing 0.1% Tween-20 and 5% w/v nonfat dry milk) for 1 h at room temperature, before it was washed three times with TBS/T buffer (15 mL of TBS containing 0.1% Tween-20) for 5 min.

The primary antibody (10 μL), a monoclonal antibody against the thiophosphate-epitope, or a polyclonal antiphosphothreonine antibody, was diluted 1000-fold in dilution buffer (10 mL, TBS/T containing 5% BSA) and then incubated with the membrane with gentle agitation overnight at 4 °C. After washing three times for 5 min with TBS/T (15 mL), the membrane was incubated with HRP-conjugated anti-mouse IgG (1:2000 dilution, for the detection of thiophosphothreonine residues), or HRP-conjugated anti-rabbit IgG (1:2000 dilution, for the detection of phosphothreonine residues) in blocking buffer (10 mL) with gentle agitation for 1 h at room temperature. After washing three

(75) Cook, P. F.; Neville, M. E.; Vrana, K. E.; Hartl, F. T.; Roskoski, R. *Biochemistry* **1982**, *21*, 5794.
(76) Roskoski, R. *Methods Enzymol.* **1983**, *99*, 3−6.
(77) Cheley, S.; Bayley, H. *Biochemistry* **1991**, *30*, 10246−10255.

times for 5 min with TBS/T (15 mL), the membrane was incubated with LumiGLO (10 mL) with gentle agitation for 1 min at room temperature. The membrane was then wrapped in Saran wrap and placed on X-ray film.

Confirmation of Thiophosphorylation of $T^{197}C_\alpha$ by Gel Shift Electrophoresis. A portion of a ^{35}S-thiophosphorylation reaction (1 μL) was incubated with 15 mM IASD, a sulfhydryl-directed gel-shift reagent,[78] in 200 mM sodium phosphate (pH 8.0) in a final volume of 10 μL for 10 min at room temperature, in the presence or absence of 1% SDS. IASD was added from a 100 mM stock in water. The reaction was stopped by adding DTT to 20 mM from a 240 mM stock solution in water, followed by one volume of 2X Laemmli sample buffer.[79] The protein was then subjected to electrophoresis in a 12% SDS−polyacrylamide gel and visualized by autoradiography. As controls, 1−2.5 μg of unphosphorylated $T^{197}C_\alpha$ (the cysteine-free mutant) or phosphorylated wild-type catalytic subunit (with two cysteine residues) was incubated with 15 mM IASD after treatment with 1 mM DTT, and then analyzed by SDS−polyacrylamide gel electrophoresis and Coomassie blue staining.

Estimation of the Extent of Thiophosphorylation by TCA Precipitation. Trichloroacetic acid (TCA) (20%) containing 20 μg BSA (200 μL) was added to a thiophosphorylation reaction (100 μL), which had been catalyzed by PDK1 as described above. The mixture was incubated for 15 min on ice, before centrifugation at 12000*g* for 10 min. To remove free nucleotide, the pellet was reprecipitated three times by adding 30% TCA (200 μL) immediately after solubilization with 50 mM NaOH (100 μL). The radioactivity in the reprecipitated pellet was measured by scintillation counting. The extent of thiophosphorylation was expressed as pmol of ATP(γ)S incorporated per pmol of C subunit.

Removal of ATP(γ)S and DTT from $P_ST^{197}C_\alpha$ by Gel Filtration. Low-mass thiols were removed from $P_ST^{197}C_\alpha$ by passage through Bio-Gel P-6 DG prior to purification of the thiophosphorylated protein with TNB-thiol agarose. Bio-Gel P-6 DG desalting gel was prepared in 25 mM sodium phosphate, 400 mM NaCl, 1mM EDTA, and 1% Prionex (pH 6.5). The gel was packed into a column of 5-mL bead volume (18 cm height). The products of a thiophosphorylation reaction (1 mL containing ~50 μg kinase) were loaded onto the column. After the first 1 mL of eluate had been discarded, $P_ST^{197}C_\alpha$ was eluted with 1.5 mL of the same buffer. $P_ST^{197}C_\alpha$ elution was monitored by measuring the kinase activity in each fraction by the fluorescence method.

Purification of $P_ST^{197}C_\alpha$ with TNB-Thiol Agarose. To separate the thiophosphorylated C subunit from nonthiophosphorylated protein, TNB (5-thio-2-nitrobenzoic acid)thiol agarose, which contains an activated disulfide bond, was coupled to the thiophosphorylated protein. $P_ST^{197}C_\alpha$ was then eluted with 20 mM β-mercaptoethanol. Specifically, the TNB-thiol agarose was first treated with equilibration buffer [EB: 25 mM sodium phosphate, 400 mM NaCl, 2 mM EDTA, and 0.05% Tween 20 (pH 6.5)]. The eluant from the gel filtration column (see above, 1.5 mL) containing 0.2 mg mL^{-1} BSA (added from a 30 mg mL^{-1} stock in water) was incubated with TNB-thiol agarose (200 μL settled volume) in a 2-mL tube with gentle agitation for 2 h at 4 °C. After removal of the supernatant, the beads were transferred to a spin filter (Rainin, #7016-024) and washed three times with EB (400 μL), and twice with washing buffer [400 μL: 25 mM sodium phosphate, 400 mM NaCl, 2 mM EDTA, 10 mM imidazole, 0.05% Tween 20 (pH 8.0)]. The bound thiophosphorylated protein was liberated with elution buffer [5 × 400 μL: 25 mM sodium phosphate, 20 mM β-mercaptoethanol, 200 mM NaCl, 10 mM imidazole and 0.4 mg mL^{-1} BSA (pH 8.0)] at 4 °C. Portions of the flow-through (1.5 μL), wash (2 μL), and eluate (2 μL) were saved for kinase assays.

Removal of β-Mercaptoethanol and Concentration of $P_ST^{197}C_\alpha$ with Ni^{2+}−NTA Magnetic Agarose Beads. Ni^{2+}−NTA magnetic agarose beads were used to remove βME and concentrate the $P_ST^{197}C_\alpha$ protein. The beads were first treated with equilibration buffer [25 mM sodium phosphate, 200 mM NaCl, 10 mM imidazole, and 0.1 mg mL^{-1} BSA (pH 8.0)]. Portions of the eluate from the TNB-thiol agarose (1 mL), containing 10 mM imidazole, were mixed with the Ni^{2+}−NTA beads (250 μL of 5% bead suspension) and rotated for 5−6 h at 4 °C. The beads were transferred to a single tube and washed four times with 80 bead vols of the same buffer without BSA. The protein was then recovered with elution buffer [4 × 0.8 bead volumes: 25 mM Tris·HCl, 100 mM imidazole, 200 mM NaCl, and 1% Prionex (pH 7.3)]. Portions of the supernatant (10 μL), wash (10 μL), and eluate (0.5 μL) were saved for kinase assays.

Caging of $P_ST^{197}C_\alpha$ with 4-Hydroxyphenacyl Bromide. $P_ST^{197}C_\alpha$ (1 μM) was reacted with 1 mM 4-hydroxyphenacyl bromide in 25 mM Tris·HCl, 200 mM NaCl, 2 mM EDTA, and 1% Prionex (pH 7.3) (135 μL) for 15 min at 25 °C in the dark. The caging reagent was added from a 100 mM stock solution in 95% ethanol. The reaction was stopped by adding 200 mM DTT in water to a final concentration of 20 mM. To remove excess reagents, the reaction mixture (150 μL) was applied to a Bio-Gel P6 desalting column (1-mL bead volume) which had been equilibrated with 25 mM Tris·HCl, 1% Prionex, 2 mM EDTA, 10 mM DTT (pH 7.3). The first 200 μL of eluate was discarded, and the next 300 μL containing the caged C subunit HP-$P_ST^{197}C_\alpha$ was collected and assayed for kinase activity by using the ^{32}P method. As controls, the activities of $P_ST^{197}C_\alpha$ and $T^{197}C_\alpha$ were also determined.

Activation of HP-$P_ST^{197}C_\alpha$ by Photolysis. ^{35}S-labeled HP-$P_ST^{197}C_\alpha$ [30 nM in 200 μL of 25 mM Tris·HCl, 2 mM EDTA (pH 7.3)] was photolyzed in the wells of a microtiter plate (well size: 0.36 cm^2 × 1 cm, 360-μL volume). Irradiation was performed with a 30-W UV lamp (2200 μW cm^{-2}, peak emission 312 nm, Cole-Parmer E-09815-22). The lamp was set 1.5 cm above the sample. A glass band-pass filter (Oriel #59154) provided a wavelength window from 280 to 370 nm. Prior to photolysis, the UV light was stabilized for at least 20 min. The activity of the protein before and after irradiation was determined by kinase assay by the ^{32}P method. C subunit (2.5 ng in 2 μL) from the photolysis reaction was immediately added to buffer (23 μL) containing 10 mM MOPS, 10 mM DTT, and 0.2 mg mL^{-1} BSA (pH 6.8) for the assay.

Determination of Product Quantum Yields. ^{35}S-labeled HP-$P_ST^{197}C_\alpha$ was irradiated as described above for 0, 0.5, 1, 1.5, 2.5, 5, and 10 min. Duplicate samples (150 μL) in coupling buffer [200 μL final volume: 25 mM Tris·HCl, 2 mM EDTA, 400 mM NaCl, and 0.2 mg mL^{-1} BSA (pH 7.3)] were immediately applied to TNB-thiol agarose (50 μL bead volume) and gently agitated for 2 h at 4 °C. The deprotected protein with a restored thiophosphate group was expected to couple to the beads. After collecting the flow-through, the beads were washed four times with 25 mM Tris·HCl, 1 mM EDTA (pH 7.3) (400 μL). The attached protein was then eluted with 20 mM DTT in 25 mM Tris·HCl, 1 mM EDTA (pH 7.3) (4 × 200 μL). The radioactivity in each fraction was measured by scintillation counting. ^{35}S-labeled $P_ST^{197}C_\alpha$ was irradiated in parallel as a control.

The half-time of photolysis ($t_{1/2}$) and product quantum yields (ϕ_p) were estimated by using $t_{1/2} = 0.3/\phi_p I_0 \epsilon$, which applies at low light absorption ($A < 0.1$), where I_0 is the incident light intensity (mmol photons s^{-1} cm^{-2}) and ϵ is the extinction coefficient (M^{-1} cm^{-1}).[4] The photoreduction of potassium ferrioxalate (6 mM) was used as a standard[80] to measure the total light intensity over the entire wavelength range after passage through the filter. We assumed that all the incident light was absorbed by the ferrioxalate, $\phi_P = 1.25$, and $\epsilon_{510} = 11\,050$ M^{-1} cm^{-1} for the 1,10-phenanthroline complex of Fe(II).[80] Using $\Delta\lambda = 10$ nm windows, the distribution of I_0 as a function of wavelength was then obtained by normalizing the total light intensity to the emission spectrum of the lamp and transmittance characteristics of the filter. In

(78) Krishnasastry, M.; Walker, B.; Braha, O.; Bayley, H. *FEBS Lett.* **1994**, *356*, 66−71.
(79) Laemmli, U. K. *Nature* **1970**, *227*, 680−685.
(80) Hatchard, C. G.; Parker, C. A. *Proc. R. Soc. London A* **1956**, *235*, 518−536.

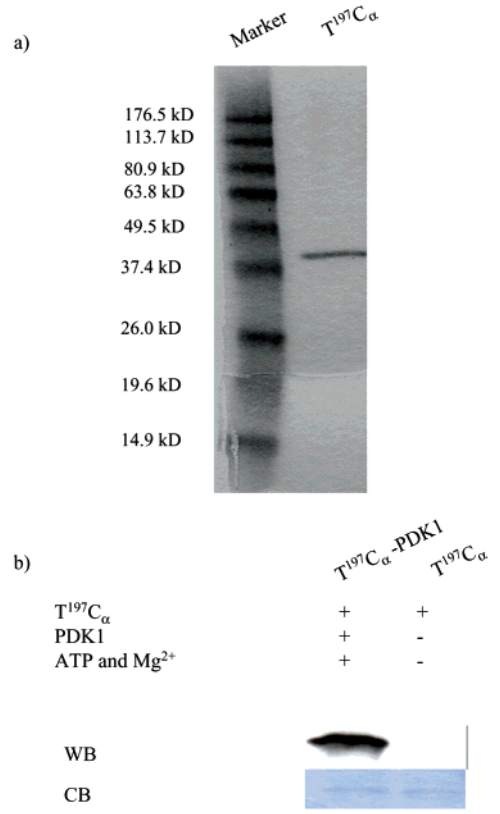

Figure 2. Purification of $T^{197}C_\alpha$. (A) Ni^{2+}−NTA agarose chromatography. Unphosphorylated $T^{197}C_\alpha$, which carries a hexahistidine tag, was purified from an *E. coli* lysate by using Ni^{2+}−NTA agarose as described in the Experimental Section. A 12.5% SDS−polyacrylamide gel stained with Coomassie blue is shown. (B) $T^{197}C_\alpha$ does not contain threonine phosphate. Purified $T^{197}C_\alpha$ was phosphorylated with PDK1. This material and unphosphorylated $T^{197}C_\alpha$ were subjected to electrophoresis in a 12.5% SDS−polyacrylamide gel. After transfer to a nitrocellulose membrane, the proteins were probed with an antiphosphothreonine antibody. Upper panel: western blot. Lower panel: Coomassie blue staining.

applying $\phi_p = 0.3/t_{1/2}\epsilon I_0$, we used a value for ϵI_0 that was the sum of the products of the extinction coefficients for the 4-hydroxyphenacyl group and the I_0 values over the applicable wavelength range.

Results

Expression and Purification of a Nonphosphorylated C Subunit of Protein Kinase A, $T^{197}C_\alpha$. It had been reported that a nonphosphorylated C subunit of PKA can be obtained by expression in *E. coli* in the presence of the inhibitor H89, which prevents autophosphorylation.[70,71] Unfortunately, we found that H89 significantly suppresses the growth of the host bacteria and the overall level of protein expression. Therefore, to facilitate recovery and purification of the protein, we placed a sequence encoding a hexahistidine tag at the N terminus of a gene encoding the C199A/C343A mutant of the mouse C_α subunit (Figure 1b). The construct was ligated into the plasmid pRSET-B, which contains an inducible T7 promoter. Immediately after induction with IPTG, H89 was added to a concentration of 40 μM and growth was continued for 6 h at 24 °C. The expression of $T^{197}C_\alpha$ as soluble protein was low. After passage of the cells through a French press, the C subunit, $T^{197}C_\alpha$, was present in the supernatant, and it was purified by using Ni^{2+}−NTA agarose (Figure 2a). The yield was 150 μg of protein for 10 L of culture. The phosphorylation status of the protein at threonine was analyzed by Western blotting with an antibody against phosphothreonine. A faint signal was detected for $T^{197}C_\alpha$ (Figure 2b, lane 1), which was greatly strengthened after phosphorylation with PDK1 and ATP (Figure 2b, lane 2). The faint signal was probably the result of nonspecific binding, as the molecular weight markers were also visible on the blots (data not shown).

Thiophosphorylation of the C subunit, $T^{197}C_\alpha$, by PDK1. For many years, it was thought that the C subunit of PKA is activated in vivo by autophosphorylation at Thr-197 in an intermolecular reaction.[69] This notion, has been challenged by recent studies in which 3-phosphoinositide-dependent kinase 1 (PDK1) has been proposed as the physiological activator.[71] In vitro, PDK1 phosphorylates the C subunit of PKA exclusively at Thr-197 at a much faster rate than that of autophosphorylation.[70,71]

In the present study, PDK1 was used to thiophosphorylate $T^{197}C_\alpha$, and the modification of the protein was examined by Western blotting with an antibody against thiophosphate (Figure 3a). Thiophosphate was detected in the C subunit when phosphorylation was carried out with ATP(γ)S (Figure 3a, left panel). When PDK1 was omitted, the extent of thiophosphorylation, in this case presumably autothiophosphorylation, was much lower (Figure 3a, left panel). The signal for the phosphorylated wild-type C subunit was weak, indicating that the antithiophosphate antibody does not cross-react with phosphorylated proteins (Figure 3a, right panel). In contrast to the influence of metal ions on the thiophosphorylation of tyrosine residues,[58] Co^{2+} and Mn^{2+} were found to be much less effective compared to Mg^{2+} (data not shown).

A chemical test was made to further confirm the presence of thiophosphate in the C subunit. IASD is a sulfhydryl-directed gel-shift reagent, which was predicted to react with the thiophosphate group. Indeed, IASD modification of $T^{197}C_\alpha$ yielded a slower-migrating band upon SDS−polyacrylamide gel electrophoresis (Figure 3b). In a parallel experiment, IASD had no effect on the mobility of the phosphorylated cysteine-free protein $T^{197}C_\alpha$, whereas a shift was observed when wild-type catalytic subunit, which contains two cysteine residues, was treated with IASD (Figure 3b).

Activation of $T^{197}C_\alpha$ by PDK1-Catalyzed Thiophosphorylation. Thiophosphorylation of $T^{197}C_\alpha$ with PDK1 resulted in a 4.7-fold increase in activity (Figure 3c). After exposure to conditions intended to allow autothiophosphorylation, the activity of the C subunit was close to the residual activity of nonphosphorylated $T^{197}C_\alpha$ (Figure 3c), consistent with the finding that autophosphorylation[69,70] and autothiophosphorylation (Figure 3a) are slow. The finding that thiophosphorylated $T^{197}C_\alpha$, $P_ST^{197}C_\alpha$, was less active than phosphorylated $T^{197}C_\alpha$, $P_OT^{197}C_\alpha$, (obtained by expression in the absence of H89) and phosphorylated wild-type C subunit reflects a slow rate of thiophosphate transfer from ATP(γ)S, rather than an intrinsically lower specific activity of $P_ST^{197}C_\alpha$. The extent of thiophosphorylation was measured by using [^{35}S]ATP(γ)S and found to be 0.25 mol of ^{35}S per mol of C subunit. That thiophosphorylation was substoichiometric was confirmed when the specific activity of purified $P_ST^{197}C_\alpha$ was determined (see below). Attempts to increase the extent of thiophosphorylation by prolonging the incubation with PDK1 were not made because of the potential lability of the C subunit.

Purification of the Thiophosphorylated C Subunit, $P_ST^{197}C_\alpha$. To separate $P_ST^{197}C_\alpha$ from unthiophosphorylated C

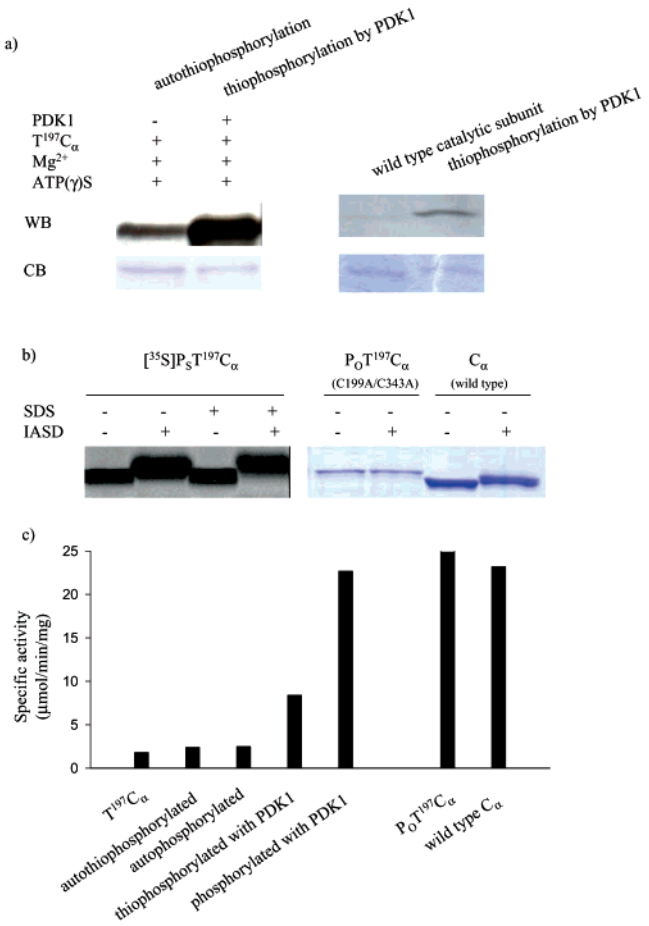

Figure 3. Thiophosphorylation of $T^{197}C_\alpha$ with PDK1. (A) Detection of thiophosphothreonine by western blotting. After thiophosphorylation, the reaction mixture containing $T^{197}C_\alpha$ was subjected to SDS−polyacrylamide gel electrophoresis. Left panel, western blot with the antithiophosphothreonine antibody. Right panel, western blot of the phosphorylated wild-type C subunit and thiophosphorylated $T^{197}C_\alpha$. WB, blot; CB, Coomassie blue staining. (B) ^{35}S-thiophosphorylated $T^{197}C_\alpha$ treated with IASD and analyzed by gel-shift electrophoresis. A portion of a thiophosphorylation reaction (1 μL) carried out with [^{35}S]-ATP(γ)S was incubated with 15 mM IASD in 200 mM sodium phosphate (pH 8.0) in a final volume of 10 μL for 10 min in the presence or absence of 1% SDS. The reaction was stopped by adding DTT to 20 mM, followed by one volume of 2X Laemmli gel loading buffer. The protein was then analyzed by electrophoresis in a 12.5% SDS−polyacrylamide gel and autoradiography (left panel). As controls, $P_OT^{197}C_\alpha$ (1.0 μg, a cysteine-free protein, from cells grown in the absence of H89) or wild-type C subunit (3.0 μg) (with two cysteine residues) were incubated with 15 mM IASD after treatment for 5 min with 1 mM DTT, and then analyzed by SDS−polyacrylamide gel electrophoresis and Coomassie blue-staining (right panel). (C) Activation of the enzyme as determined with the ^{32}P kinase assay. The specific activities of C subunits obtained by various means are recorded. $P_OT^{197}C_\alpha$ was obtained from cells grown in the absence of H89. Wild-type C_α was obtained by expression in *E. coli*. The averages of duplicate determinations are shown.

subunit, TNB-thiol agarose was used to capture the thiophosphorylated protein. Prior to the TNB-thiol agarose treatment, reducing reagent was removed from the protein by gel filtration. About 70% of the thiophosphorylated protein bound to the activated agarose, as determined by using ^{35}S-labeled material. Kinase activity found in the flow-through was largely due to the residual activity of nonthiophosphorylated C subunit. $P_ST^{197}C_\alpha$ was eluted with 20 mM β-mercaptoethanol. The eluted $P_ST^{197}C_\alpha$ was very dilute. Further, it was necessary to remove the β-mercaptoethanol before the caging reaction. Both concentration and removal of the reducing agent were accomplished

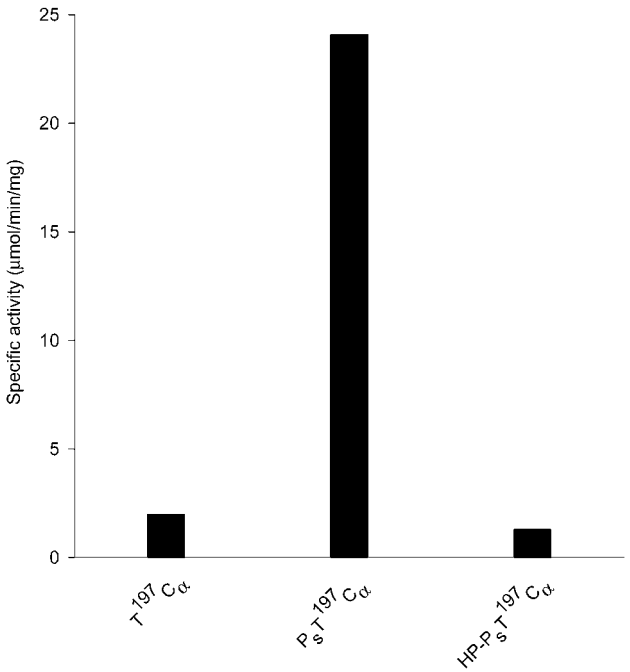

Figure 4. Inactivation of $P_ST^{197}C_\alpha$ by modification with 4-hydroxyphenacyl bromide. $P_ST^{197}C_\alpha$ (1 μM) that had been purified with TNB-thiol agarose and subsequently concentrated with Ni^{2+}−NTA magnetic agarose beads was caged by treatment with 4-hydroxyphenacylbromide in 25 mM Tris·HCl, 150 mM NaCl, 2 mM EDTA, 1% Prionex (pH 7.3) for 15 min at 25 °C in the dark. The reaction was stopped with 20 mM DTT. After the removal of the excess reagents by gel filtration over Bio-Gel P6, the kinase activity of the modified C subunit, HP-$P_ST^{197}C_\alpha$, was assayed by the ^{32}P method. The averages of duplicate determinations are shown. The activities of the nonphosphorylated $T^{197}C_\alpha$ and the thiophosphorylated, but not caged, $P_ST^{197}C_\alpha$ were also determined.

by using Ni^{2+} magnetic beads. The concentration was 16-fold, and 80% of the protein was recovered with a specific kinase activity similar to that of phosphorylated wild-type C subunits (Figure 4). The concentrated $P_ST^{197}C_\alpha$ was caged directly, avoiding prolonged storage.

Caging of $P_ST^{197}C_\alpha$ with 4-Hydroxyphenacyl Bromide, HPB. Purified and concentrated $P_ST^{197}C_\alpha$ was caged by reaction with 1 mM 4-hydroxyphenacyl bromide (HPB) at pH 7.3 for 15 min at 25 °C. Excess reagents were removed from the protein by gel filtration. The modified protein, HP-$P_ST^{197}C_\alpha$, had a specific kinase activity toward the peptide substrate kemptide of 1.4 ± 0.1 μmol min^{-1} mg^{-1} ($n = 4$), compared with 24.2 ± 0.9 μmol min^{-1} mg^{-1} ($n = 4$) before the reaction with HPB, which amounts to a reduction of (17± 1)-fold .

The residual activity of a C subunit caged at Cys-199 with nitrobenzyl bromide was reduced from 5 to 8% of the initial value to 3 to 5% of that value by *N*-ethylmaleimide treatment,[45] most likely because a fraction of the sulfhydryl groups were unmodified in the caging reaction. To determine whether the HPB modification of $P_ST^{197}C_\alpha$ was complete, the protein was incubated with 5 mM NEM for 15 min at room temperature in the dark. No further reduction in activity occurred (data not shown). In addition, the extent of binding of the HPB-modified $P_ST^{197}C_\alpha$ to TNB-thiol agarose was not greater than the extent of nonspecific binding, further suggesting that the HPB reaction was complete. Together, these results suggest that HP-$P_ST^{197}C_\alpha$ has an intrinsic residual activity. Therefore, the treatment with *N*-ethylmaleimide was omitted from the preparation. In a control experiment, the specific activity of phosphorylated $P_OT^{197}C_\alpha$

was unaffected by treatment with HPB. The caged protein, HP-$P_ST^{197}C_\alpha$, was stable when stored in 25 mM Tris·HCl, 10 mM DTT, 2 mM EDTA, 1 mg mL^{-1} BSA (pH 7.3), for 15 h at 4 °C in the dark, as judged by the restoration of activity upon photolysis (see below).

Activation of HP-$P_ST^{197}C_\alpha$ by Photolysis. When HP-$P_ST^{197}C_\alpha$ was photolyzed at pH 7.3 with near-UV light (peak emission 312 nm), $P_ST^{197}C_\alpha$ was regenerated. First, we showed that the irradiated protein contained a free thiophosphate group as judged by its ability to bind to TNB-thiol agarose. Before photolysis, ~2.4% of ^{35}S-labeled HP-$P_ST^{197}C_\alpha$ bound to the activated agarose, which is close to the value for nonspecific binding (Figure 5a). After photolysis for 10 min, ~58% of radioactivity was bound. When this result was normalized to take into account the fact that 65−70% of $P_ST^{197}C_\alpha$ binds to TNB-thiol agarose, it was concluded that 85−90% of the HP-$P_ST^{197}C_\alpha$ was deprotected with a half-time of 1.6 min (Figure 5a). From these data, the product quantum yield was determined to be $\phi_P = 0.21$. In control experiments, it was shown that the coupling of $P_ST^{197}C_\alpha$ to TNB-thiol agarose was unaffected by irradiation (Figure 5a).

Second, the specific activity of the uncaged C subunit was measured. The increase in catalytic activity reached a maximum of (15 ± 1)-fold ($n = 4$) in 10 min with a half-time of 1.5 min (Figure 5b). The activity of $P_ST^{197}C_\alpha$ was unaffected by irradiation (Figure 5b).

Discussion

We show here that a catalytic subunit of PKA, an archetypal kinase, can be selectively caged at Thr-197, a critical activating phosphorylation site. An unphosphorylated form of the C subunit, $T^{197}C_\alpha$, was obtained by expression in *E. coli* in the presence of H89, a kinase inhibitor that prevents autophosphorylation. $T^{197}C_\alpha$ was then thiophosphorylated at Thr-197 to yield $P_ST^{197}C_\alpha$ in a reaction catalyzed by PDK1, a kinase, which is most likely the physiological activator of the C subunit. After purification, the specific kinase activity of $P_ST^{197}C_\alpha$ toward a peptide substrate was closely similar to that of the phosphorylated wild-type protein. $P_ST^{197}C_\alpha$ was caged by alkylation of the sulfur atom of the thiophosphoryl group with 4-hydroxyphenacyl bromide (HPB), which resulted in an ~17-fold reduction in catalytic activity. The residual catalytic activity was shown to be an intrinsic property of the caged C subunit, HP-$P_ST^{197}C_\alpha$, rather than contamination, for example, by uncaged protein. Upon photolysis at 312 nm, the activity of the C subunit was restored to 85−90% of its original value. These results are comparable with those obtained with C subunits caged by other means. For example, the C subunit was caged at Cys-199 with 2-nitrobenzyl bromide,[45] and the residual activity (5−8%) was further reduced by treatment with *N*-ethylmaleimide (to 3−5%). After photolysis, the specific activity was increased by 20 to 30-fold. The C subunit was also caged at the same position by using a peptide affinity reagent containing a 2-nitrobenzyl alkylating group.[73] The activity of the modified protein was reduced to 2% of the initial value, and a 25-fold increase in activity was observed after photolysis.

The expression of $T^{197}C_\alpha$ in *E. coli* in the presence of H89 was low. Nevertheless, by using an N-terminal His tag to facilitate recovery, sufficient C subunit was obtained for our purposes (~150 μg per 10 L of culture). We originally planned

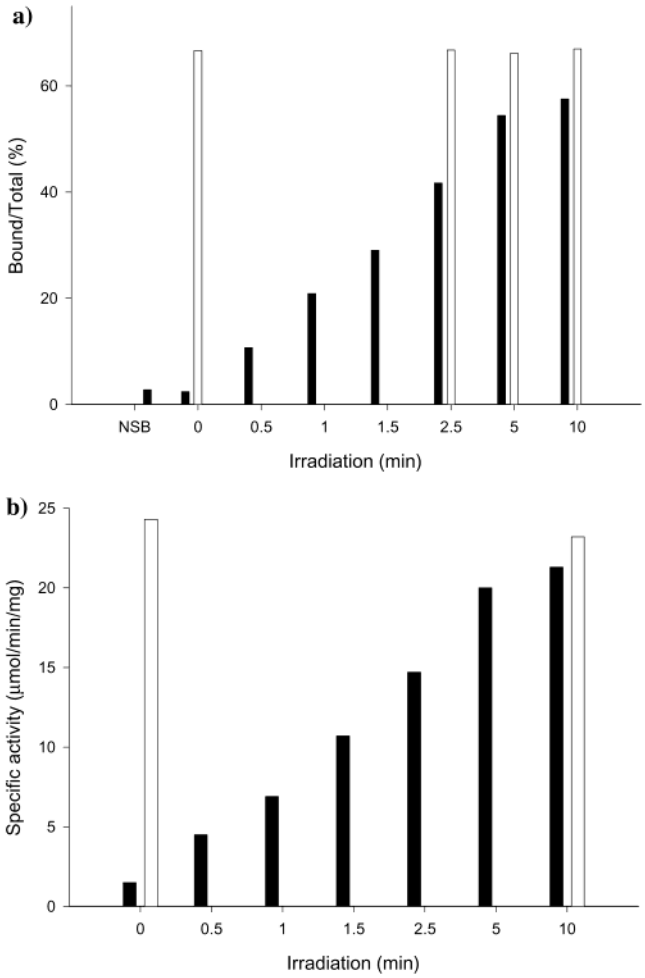

Figure 5. Activation of the caged kinase, HP-$P_ST^{197}C_\alpha$, by photolysis. (A) Capture of the photolysis product, $P_ST^{197}C_\alpha$, with TNB-thiol agarose beads. HP-$P_ST^{197}C_\alpha$ (30 nM) in 25 mM Tris·HCl, 2 mM EDTA (pH 7.3) was irradiated in 200-μL portions for the times indicated. At each timepoint, a sample (150 μL) was adjusted to 200 μL so that the final buffer composition was 25 mM Tris·HCl, 2 mM EDTA, 400 mM NaCl, 2 mM EDTA, 0.2 mg mL^{-1} BSA (pH 7.3). The solution was immediately mixed with TNB-thiol agarose (50-μL bead volume) and agitated gently for 2 h at 4 °C. After collecting the flow-through, the beads were washed with 25 mM Tris·HCl, 1 mM EDTA (pH 7.3) (4 × 400 μL). The bound protein was then eluted with 20 mM DTT in 25 mM Tris·HCl, 1 mM EDTA (pH 7.3) (4 × 200 μL). The radioactivity in each fraction was measured by scintillation counting. ^{35}S-labeled $P_ST^{197}C_\alpha$ that had not been caged was irradiated in parallel. The averages of duplicate determinations are shown. Filled bars: HP-$P_ST^{197}C_\alpha$; NSB: binding to underivatized agarose (BioRad #162-0134); unfilled bars: $P_ST^{197}C_\alpha$. (B) Time course of uncaging of HP-$P_ST^{197}C_\alpha$ as determined by kinase assay. An irradiated sample (2 μL, see Experimental Section) was removed at the times indicated and assayed for kinase activity by the ^{32}P-method. The averages of duplicate determinations are shown. Filled bars: HP-$P_ST^{197}C_\alpha$; unfilled bars: $P_ST^{197}C_\alpha$.

to thiophosphorylate the C subunit by using an autophosphorylation reaction.[69] However, during the course of our work, PDK1 was found to be an excellent candidate for the physiological PKA kinase.[71] PDK1 rapidly phosphorylates the C subunit exclusively at Thr-197, and therefore it was adopted for our studies. By contrast, autophosphorylation is slow.[69−71] For example, when unphosphorylated C subunit was incubated with a high concentration of active phosphorylated C subunit, the reaction took 6 h to complete at 30 °C.[70] In the case of thiophosphorylation of tyrosine, we found that several kinases performed poorly when Mg^{2+} was the divalent metal ion, but Co^{2+} and Ni^{2+} could be used instead.[58] For thiophosphorylation

of the C subunit by PDK1, Mg^{2+} worked satisfactorily although the rate of thiophosphorylation was slower than that of phosphorylation.

After caging with the 4-hydroxyphenacyl group, the specific kinase activity of $P_ST^{197}C_\alpha$ is greatly reduced, although not to zero: ~5% of the activity remains. Thr-197 is in the "activation loop" of the C subunit, which is critical for the correct organization of the active site because of its interactions with several residues from both lobes of the protein (Figure 1a). The C subunit has two conformations: open and closed.[67,68,72,81] For example, when an inhibitor peptide is bound, the enzyme displays a "closed" conformation, in which the cleft between the two lobes is relatively narrow, and the phosphate on Thr-197 interacts directly with Arg-165 and Lys-189, in the large lobe, and His-87 in the small lobe. In the absence of the peptide, the protein assumes an "open" conformation with a wider cleft between the two lobes. In this form, the interaction between His-87 in the small lobe and the phosphate of Thr-197 is broken, and the distance between the two residues increases to 5.95 Å. In addition, the conserved catalytic loop (residues 165−171) is required for catalysis, and its correct orientation is crucial. The phosphoryl group on Thr-197 contacts the loop via an electrostatic interaction with Arg-165, and it is likely that this interaction is disrupted in the caged C subunit. Therefore, most probably, once the C subunit is caged at Thr-197, either the active site is faultily assembled, or the enzyme cannot cycle efficiently through the open and closed forms, which is required for catalysis.

The human genome contains sequences that encode around 1000 protein kinases. Many of these kinases are activated by phosphorylation at sites corresponding to Thr-197 in the C subunit of PKA.[82] These sites are modified either by heterologous kinases or in autophosphorylation reactions. For example, the cyclin-dependent kinase CDK2 is phosphorylated at Thr-161,[83] and the MAP kinase ERK2 is phosphorylated at Thr-183 (and Tyr-185).[84] Therefore, the work performed here should be directly applicable to such cases. Other kinases and signaling proteins are activated by phosphorylation at other sites. Indeed, it is hard to find an example of a protein involved in cell signaling that is not regulated by phosphorylation. It follows that the procedures demonstrated in this work should be adaptable to components of virtually any signaling pathway.

We chose to use the 4-hydroxyphenacyl group to cage $P_ST^{197}C_\alpha$. This reagent[6,52−56,58] is one of several (see, e.g.,[85,86]) that have been developed recently to replace the established 2-nitrobenzyl reagents. In the present work, the reagent performed well, producing a protein caged on thiophosphate that could be uncaged with a ~15-fold increase in activity. While the 4-hydroxyphenacyl group and its derivatives have been used to cage small molecules including ATP, phosphate, glutamic acid, GABA, and various peptides,[6,52−54,56,58] there has been only one brief report of a protein caged by this means. In the latter, a protein tyrosine phosphatase was caged at the active-site cysteine; 30−80% of the activity was recovered upon photolysis.[46] Here we describe a more extensive study involving a more generally applicable procedure. Our work included the determination of the product quantum yield, $\phi_P = 0.21$. This value is similar to ϕ_P values obtained for 4-hydroxyphenacyl carboxylate and phosphate esters, which are in the range $\phi_p = 0.2-0.4$,[6,52−54,56,87,88] with the exception of glutamate caged on the γ carboxylate for which $\phi_p = 0.08$.[52]

It is the product of the extinction coefficient and quantum yield ($\epsilon\phi_p$) at wavelengths compatible with the preparation under investigation that determines the utility of caged reagents in biological experiments. At 312 nm, the value of $\epsilon\phi_p = 1575$ for HP-$P_ST^{197}C_\alpha$ is very favorable. For example, 2-nitrobenzyl reagents, which have been applied widely to biological preparations, have $\epsilon\phi_p \approx 550$ at 308 nm at pH 7.5,[88] while 7-hydroxycoumarin-4-ylmethyl (Bhc) has $\epsilon\phi_p \approx 330$ at 365 nm at pH 7.2.[85] The normal physiological concentration of PKA is around $10^{-6}-10^{-7}$ M.[89] Therefore, in cell biological experiments there should be no appreciable screening of the activating radiation by the caged protein. Additional advantages of the 4-hydroxyphenacyl group include the formation of a spiroketone upon photolysis, which is hydrolyzed to the unreactive 4-hydroxyphenylacetic acid.[55,59] This photoproduct does not compete for incident light because of the blue-shift in absorbance. In addition, photodeprotection is rapid after the absorption of a photon ($k \approx 10^7-10^8$ s^{-1}),[53,88] compared with the release from 2-nitrobenzyl reagents ($k \approx 1-10^3$ s^{-1}).[51] Rapid deprotection is important where an event must be quickly triggered, which is the case for certain biophysical measurements, such as investigations of protein folding[36,37] or the initiation of enzyme turnover in time-resolved crystallography.[90]

In cell biological experiments, what matter usually are the lack of residual activity and the extent of activation upon irradiation, measured as the fold increase in activity. It should be noted that a 10-fold increase in activity can be produced, for example, by photolysis of a preparation with 1% activity to yield 10% activity, or by a preparation with 10% activity to yield 100% activity. HP-$P_ST^{197}C_\alpha$ does have significant residual activity (~5%), which appears to be an intrinsic property of the protein. Therefore, it will be necessary to take care to use the lowest possible amount of this reagent, for example, for microinjection into target cells for subsequent photoactivation. Gratifyingly, the extent of activation of HP-$P_ST^{197}C_\alpha$, at ~15-fold, is excellent and approaches the theoretical maximum given the limitation imposed by the residual activity. The high proportion of activation (~85−90% of the molecules) is again useful for biophysical measurements of ensembles, where the properties of the major fraction of the molecules are monitored.

The results obtained in this work suggest several improvements that might be made to the caged C subunit. First, it might be possible to lower the residual activity by modifying the protein with a larger or charged reagent. Second, the use of

(81) Zheng, J.; Knighton, D. R.; Ten Eyck, L. F.; Karlsson, R.; Xuong, N.; Taylor, S. S.; Sowadski, J. M. *Biochemistry* **1993**, *32*, 2154−2161.
(82) Johnson, L. N.; Noble, M. E. M.; Owen, D. J. *Cell* **1996**, *85*, 149−158.
(83) Jeffrey, P. D.; Russo, A. A.; Polyak, K.; Gibbs, E.; Hurwitz, J.; Massagué, J.; Pavletich, N. P. *Nature* **1995**, *376*, 313−320.
(84) Canagarajah, B. J.; Khokhlatchev, A.; Cobb, M. H.; Goldsmith, E. J. *Cell* **1997**, *90*, 859−869.
(85) Furuta, T.; Wang, S. S. H.; Dantzker, J. L.; Dore. T. M.; Bybee, W. J.; Callaway, E. M.; Denk, W.; Tsien, R. Y. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 1193−1200.
(86) Ando, H.; Furuta, T.; Tsien, R. Y.; Okamoto, H. *Nat. Genet.* **2001**, *28*, 317−324.
(87) Zhang, K.; Corrie, J. E. T.; Munasinghe, R. N.; Wan, P. *J. Am. Chem. Soc.* **1999**, *121*, 5625−5632.
(88) Giebel, S.; Barth, A.; Amslinger, S.; Jung, A. H.; Burzik, C.; Clarke, R. J.; Givens, R. S.; Fendler, K. *Biophys. J.* **2000**, *79*, 1346−1357.
(89) Bossemeyer, D.; Kinzel, V.; Reed, J. cAMP-Dependent Protein Kinase: Structure, Function and Control. In *Protein Phosphorylation*; Marks, F., Ed.; VCH: Weinheim, 1996; pp 37−79.
(90) Stoddard, B. L.; Cohen, B. E.; Brubaker, M.; Mesecar, A. D.; Koshland, D. E. *Nat. Struct. Biol.* **1998**, *5*, 891−897.

reagents with higher ϵ values or with the absorption maximum pushed further toward the visible might be less damaging in the presence of cells or tissues. In this context, experiments with the 3-methoxy and 3,5-dimethoxy derivatives of 4-hydroxyphenacyl bromide show promise (unpublished results), although the photochemistry of these protecting groups differs and the ϕ_P values of oxygen esters are lower.[55] Finally, at least in principle, there is no need to use a cysteine-free protein. We have previously shown that the reactive monoanion of the thiophosphoryl group ($pK_a \approx 5.3$) can be modified in the presence of a free cysteine in a peptide,[4,63] and this manipulation might be extended to proteins.

Acknowledgment. This work was supported by grants from the NIH (NS26760) and the Robert A. Welch Foundation (A-1335) to H.B and the University of Kansas to R.S.G. We thank R.A. Steinberg and X. Cheng for their advice on preparing unphosphorylated C subunit, P.G. Conrad and A. Jung for the synthesis of 4-hydroxyphenacyl bromide, and S. Conlan for help with the figures.

JA020405E