Caged Thiophosphotyrosine Peptides**

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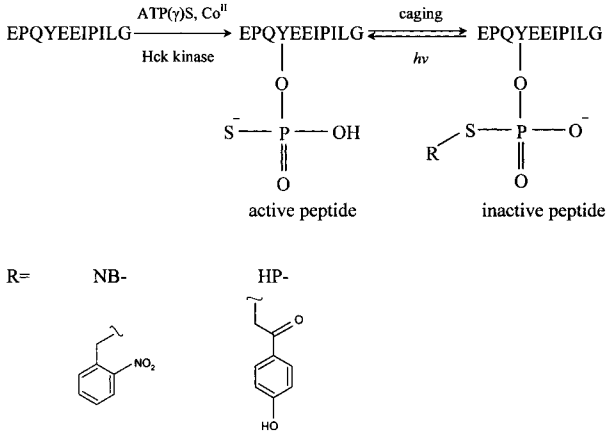
Caged reagents are molecules from which biological effectors are released by photolysis.[1, 2] In recent studies, caged peptides and proteins have received attention because of their potential importance in investigations of processes such as cell signaling.[3, 4] Although methods suited to specific situations have been explored,[5–10] we have focused our attention on general procedures to cage unprotected peptides and proteins in aqueous solutions.

Because of the relative ease of nucleophilic substitution on cysteine, this amino acid has been introduced into peptides by chemical synthesis and into proteins by mutagenesis as a site for modification by caging reagents.[3, 11–13] We recognized that most proteins involved in cell signaling are regulated by phosphorylation. Indeed, up to 30% of proteins in mammalian cells are phosphorylated and well over one percent of mammalian proteins are protein kinases.[14–16] Therefore, we reasoned that thiophosphoryl groups in peptides and proteins might also be utilized as sites for caging. This idea was readily demonstrated with thiophosphorylserine residues in peptides, by reaction with 2-nitrobenzyl bromide (NBB) to generate caged peptides that could be converted back into the thiophosphoryl peptides by near-UV light.[17] Unfortunately, we encountered difficulties with thiophosphoryltyrosine residues, which was especially troublesome because of the pivotal role of tyrosine phosphorylation in cell signaling.[18–23]

The initial difficulty centered around the enzymatic thiophosphorylation of model peptides. Herein, we show that tyrosine-containing peptides can be thiophosphorylated with cognate kinases by using divalent transition metal ions instead of Mg^{II} in the reaction buffer. The thiophosphorylated peptides can then be caged by reaction with electrophilic reagents such as NBB or *p*-hydroxyphenacyl bromide (HPB). The *p*-hydroxyphenacyl group has been used previously to cage molecules, including amino acids and oligopeptides, on carboxylate groups[24–27] and, recently, a protein phosphatase on a cysteine residue.[28] The mechanism of photorelease of substrates from *p*-hydroxyphenacyl carboxylate and phosphate esters has recently been examined.[27, 29, 30] In the present context, HPB is shown to be superior to the prevailing 2-nitrobenzyl reagents.

In attempting to prepare thiophosphotyrosine peptides, we were, in most cases, unable to detect high extents of thiophosphorylation with ATP(γ)S by using tyrosine kinases under conditions that work well when ATP itself is used.[31–33] For example, Abl, Src, and EGF and insulin receptor kinases[23] failed to thiophosphorylate peptide substrates. To explore this problem more thoroughly, we focused on EPQ**Y**EEIPILG. A related peptide, EPQ**Y**EEIPIYL, binds to Src homology (SH2) domains when phosphorylated and phosphorylated EPQ**Y**EEIPIA acts as an activator of Src kinase.[34] Divalent metal ions other than Mg^{II} have proved to be useful for promoting thiophosphorylation in other circumstances. For example, Cole and colleagues showed that k_{cat} for the phosphorylation of poly(Glu, Tyr) by C-terminal Src kinase (Csk) is hardly reduced for ATP(γ)S versus ATP in the presence of Ni^{II} and Co^{II}, while ATP(γ)S is far less effective in the presence of Mg^{II} and Mn^{II}.[33] Following this lead, we found that EPQ**Y**EEIPILG could be thiophosphorylated with Hck kinase in the presence of Co^{II} (Scheme 1).[35]



Scheme 1. Caging and uncaging of thiophosphotyrosine peptides. The caging reagents are 2-nitrobenzyl bromide (R = NB) and *p*-hydroxyphenacyl bromide (R = HP).

Thiophosphorylated EPQ**Y**EEIPILG was treated with a five-fold molar excess of HPB or NBB in the dark at room temperature in various aqueous buffers.[36] The reaction products were separated by high-pressure liquid chromatography (HPLC) and identified by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). By using ^{35}S-labeled EPQ$\mathbf{Y}_{\mathbf{Ps}}$EEIPILG, the yields of the two caged products could be determined by HPLC and scintillation counting: *p*-hydroxyphenacyl derivative EPQ$\mathbf{Y}_{\mathbf{Ps}}$**(HP)**EEIPILG, 90%; 2-nitrobenzyl derivative EPQ$\mathbf{Y}_{\mathbf{Ps}}$**(NB)**EEIPILG, 75%. In both cases, the final yield was little affected by the pH of the reaction buffer in the range pH 5.8 to 8.0. The stability of EPQ$\mathbf{Y}_{\mathbf{Ps}}$**(HP)**EEIPILG and EPQ$\mathbf{Y}_{\mathbf{Ps}}$**(NB)**EEIPILG was tested, after purification by HPLC. Both caged peptides were stable in aqueous solution at 37 °C for at least 24 h at pH values from 5.8 to 8.2.[37]

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EPQ$\mathbf{Y_{Ps}}$**(HP)**EEIPILG and EPQ$\mathbf{Y_{Ps}}$**(NB)**EEIPILG were uncaged by photolysis at 312 nm.[38] EPQ$\mathbf{Y_{Ps}}$EEIPILG was released from EPQ$\mathbf{Y_{Ps}}$**(HP)**EEIPILG in a yield of 50 to 70% at both pH 5.8 and pH 7.3. The $t_{1/2}$ values were 0.55 min at pH 5.8 and 0.52 min at pH 7.3, which correspond to product quantum yield (ϕ_P) values of 0.65 and 0.56 at the respective pH values. At pH 8.2, the yield was greatly reduced and several by-products were formed that could not be identified by MALDI-MS. Under the same conditions, EPQ$\mathbf{Y_{Ps}}$EEIPILG was released from EPQ$\mathbf{Y_{Ps}}$**(NB)**EEIPILG in 50 to 60% yield. The $t_{1/2}$ values were 5.5 min at pH 5.8 and 8.0 min at pH 7.3, which correspond to ϕ_P values of 0.37 and 0.25.

Finally, the caged peptides and uncaged products were tested in a biochemical assay, which measured the ability of the peptides to bind to an immobilized SH2 domain[39] (Figure 1). When ^{35}S-EPQ$\mathbf{Y_{Ps}}$-EEIPILG was incubated with excess Src SH2-GST attached to agarose,[40, 41] 27–31% of the radiolabel bound. When the caged ^{35}S-labeled peptides EPQ$\mathbf{Y_{Ps}}$**(HP)**EEIPILG and EPQ$\mathbf{Y_{Ps}}$**(NB)**EEIPILG were tested only 1–5% of the radioactivity was bound, which largely represents nonspecific binding, as determined with competing unlabeled peptide or by using an irrelevant peptide. After photolysis of EPQ$\mathbf{Y_{Ps}}$**(HP)**EEIPILG and EPQ$\mathbf{Y_{Ps}}$**(NB)**EEIPILG at 312 nm, in both cases, 25–31% of the radioactivity in the uncaged product, repurified by HPLC, was bound to the immobilized SH2 domains. When the uncaged product was not subjected to repurification, the extent of release as judged by Src SH2-agarose binding (corrected for binding efficiency) was consistent with the yield of 50–70% obtained by HLPC.

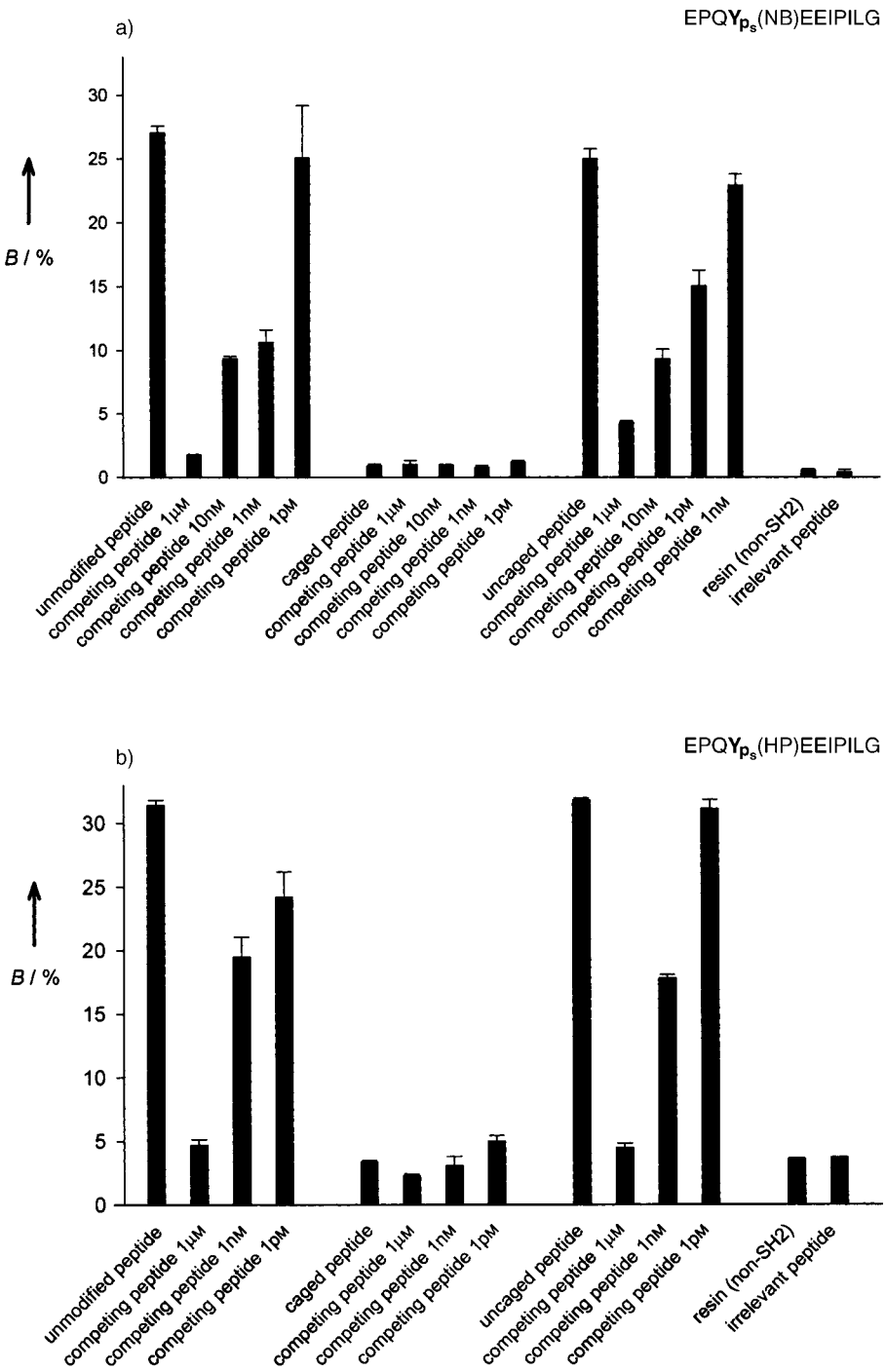

Figure 1. Binding of caged and uncaged peptides to a recombinant SH2 domain. a) EPQ$\mathbf{Y_{Ps}}$EEIPILG caged with NBB; b) EPQ$\mathbf{Y_{Ps}}$EEIPILG caged with HPB. ^{35}S-labeled caged or uncaged peptides were incubated with a Src SH2-SH3-GST fusion protein immobilized on glutathione–agarose beads. *B* is the percentage of the radioactivity retained by the beads. The bound peptides were eluted from the washed beads and assayed by scintillation counting. Competing peptide: EPQ$\mathbf{Y_P}$EEIPILG; irrelevant peptide: ^{35}S-labeled thiophosphoryl kemptide (LRRASLG).

Here, we have shown that an SH2-binding peptide can be enzymatically thiophosphorylated on tyrosine and then caged with either the NB or HP protecting group. The caged peptides are no longer able to bind to an SH2 domain, but this activity is restored upon photolysis. The NB and HP groups serve their purpose equally well when caging efficiency, the stability of the caged peptide, and the yield of functional peptide after uncaging are considered. However, the HP-protected peptide is about 10 times more sensitive to near-UV radiation, which can be attributed largely to higher ε values in the wavelength range used here and to a lesser extent to higher ϕ_P values. While the photosensitivity of a caged reagent ($\phi_P\varepsilon$) is most important for experiments in cell biology, where photochemical damage to living cells and tissues must be avoided, ϕ_P itself can be of primary importance in certain biophysical experiments, where processes must be triggered with a single flash of light. The values of ϕ_P determined here were in the same range as those measured for *p*-hydroxyphenacyl carboxylate and phosphate

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esters under various conditions in the neutral pH range (all $\phi_P > 0.2$).[27, 29, 30] The HP group generally has the additional advantage of much faster departure after absorption of a photon[27] compared with the NB group[42] and we expect that this finding will apply to thiophosphate esters caged on sulfur. At pH values above 8.0, the photolysis of EPQ**Y**$_{P_S}$**(HP)**EEIPILG produced by-products as well as the uncaged peptide. The pK_a of the *p*-hydroxyphenacyl group in water is $\sim$8. Zhang et al. found that the ϕ_P of *p*-hydroxyphenyl-2-oxoethyl acetate dropped to zero when the phenolic group was deprotonated in the ground state.[29] It might be possible to adjust the pK_a of hydroxyphenacyl reagents, and thereby ϕ_P, by judicious substitution of the aromatic ring. That work is in progress. In any event, with $\phi_P\varepsilon$ values of $\sim$5000, *p*-hydroxyphenacyl reagents are of indisputable utility and we are now extending our studies to signaling proteins thiophosphorylated on serine and tyrosine and to applications in cells.

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[35] The EPQ**Y**EEIPILG peptide (2 mM final) was thiophosphorylated with Hck kinase in buffer (100 μL) containing 50 mM Tris · HCl, pH 7.3, 0.015 % Brij 35, 50 μM *N*,*N*′-(1,2-ethanediyl)bis[(*N*-carboxymethyl)glycine] (EDTA), 10 mM $CoCl_2$. ATP(γ)S (Sigma, in 100 μL of water) was added in five-fold molar excess with respect to the peptide. Hck kinase (0.25 – 0.5 μg in 1 – 2 μL of storage buffer) was added and the mixture was incubated at 30 °C for 30 – 60 min. The thiophosphorylated peptide was purified by reverse-phase HPLC with a Vydac C18 column (linear gradient of 10 – 40 % CH_3CN; buffer A: 0.1 % CF_3COOH in water; buffer B: 0.075 % CF_3COOH in CH_3CN) and identified by MALDI-MS. To prepare ^{35}S-labeled peptide, 100 μCi ^{35}S-ATP(γ)S (Amersham) was used, diluted to a specific activity of 177 Ci mmol^{-1} with ATP(γ)S.
[36] The thiophosphorylated peptide EPQ**Y**$_{P_S}$EEIPILG was chemically modified with HPB or NBB in 100 mM sodium acetate buffer (pH 5.8), or 100 mM Tris · HCl (pH 7.3 or 8.0), at 25 °C for 30 min in the dark. Both reagents were prepared as 50 mM stock solutions in 95 % EtOH. The final concentration of the reagents was 1 mM, a five-fold molar excess with respect to the peptide. The caged products were purified by HPLC by using the reverse-phase column. The products were identified by MALDI-MS. EPQ**Y**$_{P_S}$EEIPILG could not be modified cleanly with 2-bromo-2-(2-nitrophenyl)acetic acid (BNPA) or 4,5-dimethoxy-2-nitrobenzyl bromide (DMNBB) (data not given).
[37] The radiolabeled caged peptides were stored at pH 5.8 (100 mM sodium acetate), pH 7.3 (100 mM Tris · HCl), and pH 8.2 (100 mM Tris · HCl) at both 4 °C and 37 °C for 24 h. No decomposition was detected by HPLC.
[38] Photolysis of HPLC-purified ^{35}S-labeled caged peptides at 4 nM concentration was carried out in volumes of 200 μL at pH 5.8 (100 mM sodium acetate), pH 7.3 (100 mM Tris · HCl), and pH 8.2 (100 mM Tris · HCl) in the wells of a 96-well plate (well size of 360 μL: 0.36 cm^2 × 1 cm) by irradiation 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, which was shielded by a glass band-pass filter to provide a window at 280 – 370 nm (Oriel 59154). The light intensity was measured by the irradiation of 6 mM $K_3Fe(C_2O_4)_3$ as described previously.[43] The photolysis products were removed at various time points during irradiation and subjected to HPLC analysis. The peptides were identified by MALDI-MS and quantified by scintillation counting. The half-time of photolysis ($t_{1/2}$) and product quantum yields (ϕ_P) were estimated by using $t_{1/2} = 0.3/\phi_P I_0 \varepsilon$.[3] Corrections were made for the spectral distribution of the lamp and absorption by the filter.
[39] A Src SH2-SH3-GST fusion protein was expressed in *E. coli* and purified.[44] The protein was immobilized on glutathione-Sepharose 4B beads (Pharmacia, No. 17 – 0756 – 01) as described previously.[40] ^{35}S-labeled peptides (10 μL, 50 nM), photolyzed where indicated, were incubated with SH2-SH3-GST agarose (20 μL slurry, 1.6 μM bound protein) in a total volume of 250 μL containing 50 mM Tris · HCl, pH 7.3, 200 mM NaCl, 10 mM 1,4-dithio-threitol (DTT), 0.05 % (w/v) bovine serum albumin for 5 h at room temperature and cooled to 4 °C. After collecting the flow-through, the beads were washed twice, within 1 min, at 4 °C with the incubation buffer (400 μL). Bound peptide was then eluted with CF_3COOH (0.1 %. 100 μL) and assayed by scintillation counting.
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