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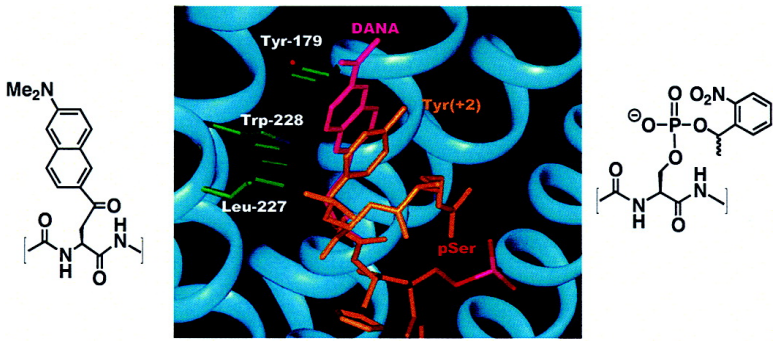

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Fluorescent Caged Phosphoserine Peptides as Probes to Investigate Phosphorylation-Dependent Protein Associations

M. Eugenio Vázquez, Mark Nitz, Justine Stehn, Michael B.Yaffe, and Barbara Imperiali*

Department of Chemistry and Department of Biology, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, Massachusetts 02139

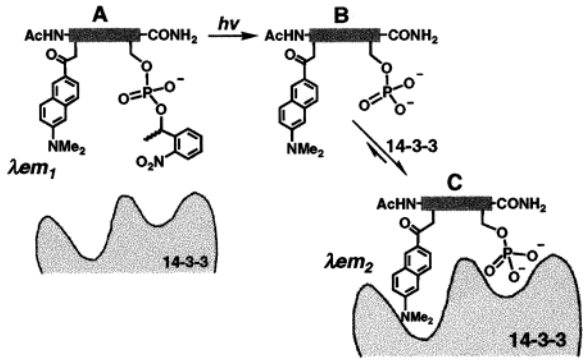
Received March 16, 2003; E-mail: imper@mit.edu

Signaling mechanisms involving protein phosphorylation play a key role in regulating many cellular processes, including cell cycle regulation, cell migration, and membrane transport.[1] The elucidation of complex macromolecular networks involved in such processes is a central challenge to modern biology, and the development of new chemical tools for the study of such pathways constitutes an increasingly important field of biological chemistry.

The 14-3-3 proteins comprise a highly conserved family of proteins,[2] which are essential intermediates in cell cycle regulation, acting through phosphorylation-dependent protein−protein interactions.[3] Herein, we present a powerful method for the study of such processes that combines the advantages of two recently introduced chemical probes: first, a class of photolabile caged phosphoamino acids[4] and, second, DANA,[5] a residue with a fluorescent solvatochromic side chain, which provides a sensitive indicator of protein−protein interactions. The general utility of these probes relies on the inability of caged phosphopeptides to bind to their cognate protein partners, coupled with the use of the fluorescent amino acid to report the binding event upon uncaging of the phosphate protecting group (Scheme 1). While the use of caged thiophosphorylated peptides and proteins has been reported,[6] this represents the first example of the use of caged phosphopeptides for the study of phosphorylation-dependent biological interactions.

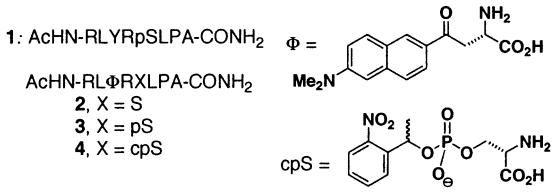
Caged molecules are substances that include a key functionality masked by a protecting group that can be deprotected with the application of an external signal, usually UV light.[7] Upon uncaging, active species that produce specific biological responses can be generated with spatial and temporal control.[8] Fluorescent probes, on the other hand, have found widespread applications because of the potential for imaging biological processes with high selectivity and sensitivity. Of particular interest are the environment-sensitive fluorophores that change spectral properties upon changes in environment. 6-Propionyl-2-(dimethylamino)naphthalene (PRODAN), first introduced by Weber and Farris,[9] is an example of an environmentally sensitive fluorophore.[10] Upon transfer to more hydrophobic environments, there is a marked blue shift in the emission maximum wavelength, and the quantum yield of the PRODAN fluorophore increases.[11] In this report, the PRODAN-based amino acid, 6-(2-dimethylaminonaphthoyl)alanine (DANA), is used to probe the phosphorylation-dependent binding of an octapeptide to the 14-3-3 ζ isoform.[5] On the basis of previously reported peptide library screening that identified the optimal sequence for 14-3-3 ζ binding[12] and the X-ray structure of 14-3-3 bound with octapeptide **1**,[13] we synthesized peptides **2**, **3**, and **4**, incorporating DANA and the key serine residue in three forms: unmodified serine, phosphoserine, and caged phosphoserine (Chart 1). Phosphoserine was caged as the 1-(2-nitrophenyl)ethyl derivative. This caging group has been previously used to mask biologically active molecules including divalent calcium,[14] nucleotide derivatives,[8] and a variety of peptides[15,16] and proteins.[17] The photochemical release of these species occurs with reasonable quantum efficiencies at wavelengths above 300 nm.[7]

Scheme 1 [a]



[a] (A) Caged phosphopeptide is unable to bind target protein 14-3-3, DANA exhibits a λ_{em1}max = 522 nm. (B) Irradiation of caged phosphopeptide releases free phosphoserine-containing peptide. (C) Released phosphoserine-peptide binds to the protein, thereby modulating the fluorescence properties of the DANA amino acid λ_{em2}max = 501 nm.

Chart 1. Sequence of 14-3-3-binding Peptide; **2**, **3**, and **4**: Peptides Examined in This Study (Φ = DANA and cpS = Caged Phosphoserine)



The position of DANA within the sequence was chosen to maximize the change in environment of the DANA side chain upon binding, while minimizing perturbations of the phosphopeptide−protein interface to maintain a high binding constant. The X-ray structure shows that in the bound complex the pSer(−2) Tyr is buried inside a hydrophobic pocket formed by four amino acid side chains (Val, Tyr, Leu, and Trp). On the basis of this structure, first-order studies using restrained molecular dynamics of the phosphopeptide **3**/14-3-3 ζ complex suggested that substitution of the tyrosine by DANA should have a minimum impact on the binding properties of the modified peptide.

Synthesis of peptides **2** and **3** was achieved using standard Fmoc-based solid-phase peptide synthesis (SPPS) protocols.[18] Synthesis of peptide **4** was based on an interassembly approach integrated into the SPPS scheme developed in our group.[4] Briefly, the peptide was assembled up to the modified serine residue, which was incorporated without side-chain protection. Subsequently, successive phosphitylation and oxidation afforded the desired caged phospho-

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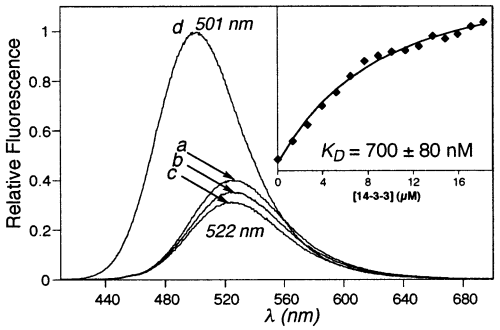

Figure 1. Fluorescence spectra of **2** and **3** (2 μM). (a) Peptide **2** in absence of 14-3-3 ζ (6.25 μM). (b) Peptide **2** in the presence of 14-3-3 ζ. (c) Peptide **3** in absence of 14-3-3 ζ. (d) Peptide **3** in the presence of 14-3-3 ζ. Insert shows the fluorescence titration of **3** (300 nM) with 14-3-3 ζ.

serine. The remaining peptide synthesis, resin deprotection, purification, and analysis were completed following standard procedures.

Incubation of 14-3-3 ζ with the unphosphorylated peptide **2** did not induce any change in the fluorescence spectrum (Figure 1b). In contrast, phosphopeptide **3** showed a dramatic increase in the emission intensity upon addition of 14-3-3 ζ; moreover, a shift of the maximum emission wavelength was observed from 522 nm in the free form to 501 nm when bound to 14-3-3 ζ (Figure 1d); this shift is consistent with the expected change of the local environment of PRODAN upon complexation. This represents a significant advantage over the increase in quantum yield reported for other fluorophores (such as nitrobenzoxazole) which fail to show a shift in emission wavelength,[19] as ratiometric analysis can be applied. The change in fluorescence also allowed determination of the binding constant of peptide **3** with 14-3-3 ζ. Titration of **3** with a 14-3-3 solution resulted in a series of spectra that could be fit to a 1:1 binding model. The observed K_D is 700 $\pm$ 80 nM, which is consistent with previously reported values for similar peptides.[12]

After establishing that the fluorescence properties of free and bound peptide **3** were distinct, it was then possible to assess the binding of **4** to 14-3-3 ζ before and after photolysis. Incubation of peptide **4** with 14-3-3 ζ did not produce any change in fluorescence.[20] In this case, the key phosphoserine recognition determinant of 14-3-3 ζ failed to recognize the phosphopeptide in the masked form. In contrast, irradiation of the mixture of **4** and 14-3-3 ζ with UV light (365 nm) (see Figure 2) released the phosphoserine which then enabled binding to 14-3-3 ζ, as indicated by the new increased emission maximum at 501 nm. Uncaging of peptide **4** was confirmed by HPLC analysis; irradiation over a 5 min time course effects complete generation of free phosphopeptide. As a control, irradiation of peptide **4** in absence of 14-3-3 ζ failed to produce any change in the fluorescence spectrum.

In conclusion, we have shown that uncaging of 1-(2-nitrophenyl)ethyl phosphoserine peptides can be used to release bioactive species, and we have also demonstrated that DANA-containing peptides can be used to monitor binding events in vitro. Designed peptides containing these two unnatural amino acid probes represent valuable tools for the study of phosphorylation-dependent interactions within complex signaling networks since the binding of such species can be modulated with spatial and temporal control by photolysis, and at the same time be localized using the reporting fluorescent functionality.

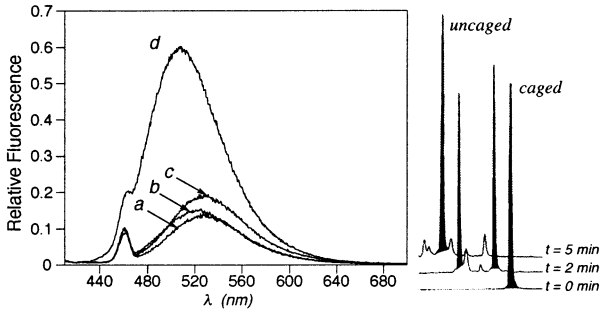

Figure 2. Fluorescence spectra of **4** (300 nM). (a) In absence of 14-3-3 ζ. (b) In the presence of 1.35 μM 14-3-3 ζ protein. (c) In absence of 14-3-3 ζ after 5 min irradiation. (d) In presence of 1.35 μM 14-3-3 ζ after UV irradiation. HPLC traces showing the uncaging of **4** (2 μM) at different UV irradiation times.

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Supporting Information Available: Characterization of peptides **2**, **3**, and **4**, fluorescence spectra, HPLC traces, and molecular modeling (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(20) The binding constant of peptide **4** for 14-3-3 ζ is estimated to be at least 100-fold higher than that of peptide **3**. See Supporting Information for experimental details.

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