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Deep Quench: An Expanded Dynamic Range for Protein Kinase Sensors

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Protein kinases catalyze the phosphorylation of serine, threonine, and tyrosine residues in protein and peptide substrates. These enzymes have received considerable attention due to the relationship between aberrant kinase activity and an assortment of human afflictions. Specific and highly sensitive protein kinase sensors furnish a means to rapidly identify inhibitors, assess protein structure/function relationships, and correlate kinase activity with cellular behavior. A large number of kinase assays have been described; however, assays with fluorescent readouts are most easily applied to both in vitro and intracellular settings. GFP-labeled protein and fluorophore-labeled peptide substrates generally deliver, upon phosphorylation, a fluorescent response that varies from 10 to 60% to 2−9-fold, respectively.[1] By comparison, many fluorescent sensors developed for a variety of biomolecules (e.g., proteinases[2] and the detection of specific nucleotide sequences[3]) display enhancements of 25-fold and greater. A large dynamic range offers enhanced sensitivity, thereby furnishing a means to assess target biomolecule behavior under a variety of conditions. Unlike nearly all of the protein kinase assays reported to date,[4] the readout described in studies with proteinases[2] and molecular beacons[3] arises via relief of fluorescent quenching. We report herein an approach devised around the latter conceptual framework ("Deep Quench") which delivers a robust protein kinase-elicited fluorescent response.

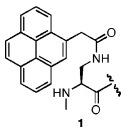
Our initial studies have focused on the strategy outlined in Scheme 1. A fluorophore-labeled kinase substrate (**A**) exhibits little or no fluorescence (**B**) in the presence of a quencher molecule. Upon phosphorylation, the peptide product (**C**) is sequestered by a phospho-Ser binding domain to form the complex **D**, which disrupts the interaction between peptide fluorophore and quencher. The latter should partially or completely restore the fluorescence of the starting peptide.

Pyrene was chosen to serve as the fluorophore on an amino acid sequence (Ac-GRTGRRFSYP-amide) recognized by the cAMP-dependent protein kinase (PKA).[5] We employed the phospho-Ser binding domain, 14-3-3τ, to serve as the sequestering agent since 14-3-3 domains display a high affinity for phospho-Ser-containing peptides (K_D < 100 nM).[6] The assay was constructed in the following stages:

1. Identification of quenching agents. Fluorescent quenching by a secondary dye is a commonly employed method used to study a wide assortment of biological phenomena.[7] However, we required a molecule that would quench fluorophore fluorescence by forming a noncovalent complex with a targeted protein kinase peptide substrate. A library of 47 commercially available dyes (Supporting Information) was assembled and analyzed for the ability to quench the fluorescence of a family of pyrene-substituted peptides **P1**−**P11** (Table 1). Pyreneacetic acid (Pyr) was attached at different sites along the PKA consensus sequence peptide via a substituted 2,3-diaminopropionic (Dap) residue **1** as well as to the N-terminus of the peptide via variable length linkers.

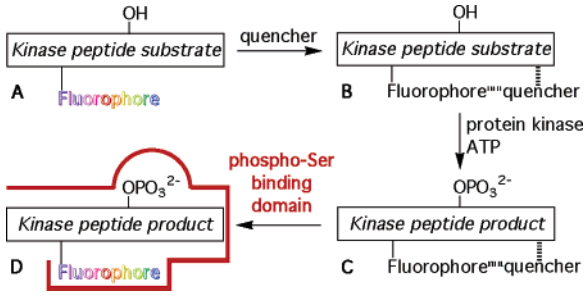
Table 1. Pyrene-Substituted Peptides **P1**−**P11** Containing Either Dap (Pyr) at the Indicated Internal Sites (**P1**−**P5**) or 1-Pyreneacetyl Appended to the N-Terminus of Peptides **P6**−**P11**

	Peptide
P1	Ac-GRTGRRFSDap(Pyr)P-amide
P2	Ac-GRTGRRDap(Pyr)SYP-amide
P3	Ac-GRTDap(Pyr)RRFSYP-amide
P4	Ac-GRDap(Pyr)GRRFSYP-amide
P5	Ac-Dap(Pyr)RTGRRFSYP-amide
P6	Pyr-β-Ala-GRTGRRFSYP-amide
P7	Pyr-Abu-GRTGRRFSYP-amide
P8	Pyr-Ava-GRTGRRFSYP-amide
P9	Pyr-Ahx-GRTGRRFSYP-amide
P10	Pyr-Aoc-GRTGRRFSYP-amide
P11	Pyr-miniPEG-GRTGRRFSYP-amide



1

Scheme 1. Enhanced Sensing of Protein Kinase Activity via a Deeply Quenched Kinase Peptide Substrate



Ten dyes were identified that serve as effective quenchers (≥40%) of pyrene fluorescence (5 μM peptide and 5 μM dye) for several of the peptides (Supporting Information), including Rose Bengal (**2**), Aniline Blue WS (**3**), and Ponceau S (**4**) (Chart 1). The latter, as well as the other lead quenchers, are negatively charged species. Complex formation of the quencher with the peptide is likely stabilized by electrostatic (positively charged Arg residues) and hydrophobic (fluorophore) interactions.

K_D values were acquired for the set of the 10 lead quenchers with peptide **P2** in order to obtain a target range for the quencher/peptide ratios to be employed in the subsequent assays (vide infra). These apparent K_D values were determined using the quenching of pyrene fluorescence as a barometer of peptide/quencher complex stability. Since the peptide/quencher pairs may interact via several different modes, not all of which might furnish efficient quenching, the actual K_D values could be tighter than suggested by the apparent dissociation constants. K_D values with peptide **P2** range from 2.8 ± 0.8 μM (Evans Blue) up to 19.6 ± 3.4 μM (Reactive Blue, Supporting Information). An inner filter effect (at high dye concentrations) was corrected as previously described.[8]

2. Identification of the lead pyrene−peptide/quencher pairs. The 11 pyrene-substituted peptides (**P1**−**P11** at 5 μM) were incubated with a 5-, 10-, 25-, and 50-fold molar excess of each of the 10 lead quenchers in the presence of PKA, ATP, and the phospho-Ser

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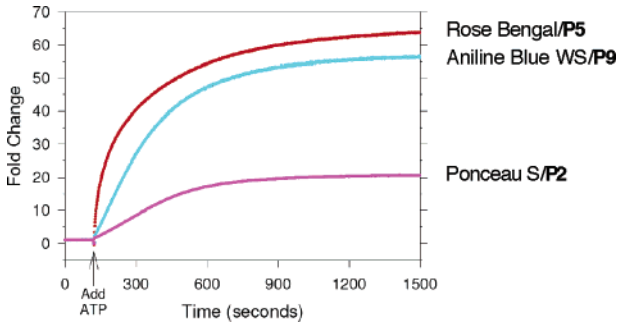
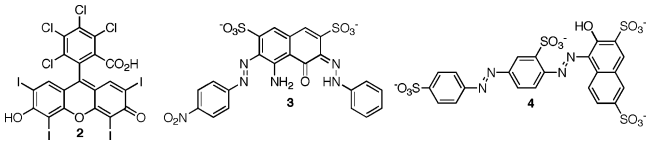


Figure 1. Fluorescence fold-change as a function of time in the presence of Rose Bengal/**P5** (25 μM/5 μM), Aniline Blue WS/**P9** (50 μM/5 μM), or Ponceau S/**P2** (250 μM/5μM) pairs. [PKA] were chosen for each pair so that the reaction would be completed within 30 min [Rose Bengal/**P5** (2.5 μM); Aniline Blue WS/**P9** (0.7 μM); Ponceau S/**P2** (10 nM)].

Chart 1. Lead Quenching Dyes of Pyrene−Peptide Fluorescence



binding domain 14-3-3τ. Several control experiments were performed, including conducting the assay in the absence of quenching agent. Under the latter conditions, only small enhancements in fluorescence (0−64%) were observed (Supporting Information). Since pyrene is an environmentally sensitive fluorophore, these results suggest that the phosphopeptide product binds to 14-3-3 in a manner that inserts pyrene into a modestly hydrophobic environment. At high molar excess dye ratios (>25-fold), pyrene emission is so deeply quenched that background fluorescence significantly contributes to the total fluorescence of the pyrene−peptide sample. Consequently, background subtraction was performed to establish a baseline upon which changes in fluorescence intensity could be quantified (Supporting Information).

Screening, using a multiwell plate reader, revealed several unique quencher/peptide pair combinations that exhibit robust fluorescence changes in response to phosphorylation: Aniline Blue WS **3** and **P9** peptide, Ponceau S **4** and **P2** peptide, and Rose Bengal **2** and **P5** peptide. A more detailed analysis was performed using a standard spectrofluorimeter (Figure 1). The Rose Bengal/peptide **P5** pair exhibits an unprecedented 64-fold phosphorylation-induced enhancement in fluorescence. The Aniline Blue WS/peptide P9 combination is nearly as robust (55-fold), while the Ponceau S/peptide **P2** pair is somewhat more subdued (21-fold). The apparent K_D values of the two most effective pairs (Rose Bengal/peptide **P5**: 0.40 ± 0.03 μM; Aniline Blue WS/peptide **P9**: 0.60 ± 0.03 μM) are significantly tighter than those obtained for the 10 lead dyes with peptide **P2** (Supporting Information).

The peptides **P2** (K_m = 7.1 ± 1.9 μM; V_{max} = 8.4 ± 1.2 μmol/min·mg), **P5** (K_m = 1.7 ± 0.4 μM; V_{max} = 5.7 ± 0.4 μmol/min·mg), and **P9** (K_m = 1.6 ± 0.9 μM; V_{max} = 7.1 ± 1.2 μmol/min·mg) are all effective PKA substrates in the presence of 14-3-3τ. In addition, we employed the Ponceau S/peptide **P2** combination to examine the inhibitory efficacy of the ATP analogue H-9[9] and a peptide fragment (14−22) of PKI,[10] a protein-based inhibitor of PKA. Under previously reported conditions ([ATP] = 10 μM), H-9 is a reasonably effective inhibitor (IC_{50} = 1.9 ± 0.2 μM) of PKA.[9] However, these conditions are nonphysiological since intracellular levels of ATP are typically above 1 mM. Under the latter conditions ([ATP] = 1 mM), the potency of H-9 is dramatically reduced (IC_{50} = 42 ± 1 μM), as expected for an ATP analogue. In addition, we examined the inhibitory efficacy of the PKI 14−22 peptide inhibitor under identical conditions using two different assays. Both the deep quench strategy (1.1 ± 0.1 μM) and the commonly employed radioactive ATP method (1.6 ± 0.2 μM) furnish nearly identical IC_{50} values.

In summary, we have established a new approach for eliciting robust fluorescent readouts of protein kinase activity. Future studies will include an extension of this methodology to other fluorophores and additional protein kinases. Indeed, a number of basophilic protein kinases phosphorylate sequences that are recognized by 14-3-3 domains. Furthermore, the strategy should be applicable to a wide array of tyrosine kinases with the ready availability of pTyr binding domains.

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Supporting Information Available: Experimental details of peptide synthesis and characterization, screens, and enzyme assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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