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Self-Reporting Fluorescent Substrates of Protein Tyrosine Kinases

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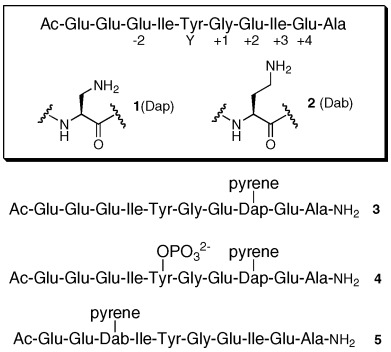
Probes that provide a continuous fluorescent readout of protein tyrosine kinase activity offer a direct means to observe kinase action in living cells, may serve in a diagnostic capacity as sensors of aberrant activity, and will undoubtedly prove invaluable in high-throughput screening assays. Several genetically encoded FRET-based proteins have been described that, upon tyrosine phosphorylation, display fluorescent changes up to 50%.[1a] A few peptide-derived reporters have been introduced as well, but these require nonphysiological levels of "helper" ions[1b] or proteins[1c] to observe a significant fluorescent change in response to tyrosine phosphorylation. We report herein a strategy that permits the peptide substrate to self-recognize and fluorescently report the phosphorylation of tyrosine residues. This approach has furnished peptide substrates that display a several-fold amplification of fluorescent intensity upon phosphorylation. In addition, these substrates can be conveniently used to examine kinase self-activation and activity under cellular-mimetic conditions.

The tyrosine aryl side chain is known to engage other aromatic species, including fluorophores, in $\pi-\pi$ stacking interactions.[2] Phosphorylation of the tyrosine moiety could alter the nature of, or possibly disrupt, these interactions, thereby leading to a perturbation of the photophysical properties of the aromatic binding partner. We employed pyrene in the latter capacity since the fluorescent properties of this fluorophore are sensitive to environmental conditions. Src and related tyrosine kinases catalyze the phosphorylation of the *tyrosine* moiety in acidic peptides, such as Ac-Glu-Glu-Glu-Ile-*Tyr*-Gly-Glu-Ile-Glu-Ala.[1c,3] We prepared a library of analogues of this peptide in which a pyrene substituent is appended off of L-2,3-diaminopropionic acid **1** (Dap) or L-2,4-diaminobutanoic acid **2** (Dab) residues at specific sites on the peptide chain encompassing the tyrosine moiety. Individual members of this library were subsequently incubated with Src, and fluorescent intensity followed as a function of time. Phosphorylation-induced changes range from a minimum of 1.8-fold up to nearly 5-fold (see Supporting Information). We chose two peptides for further evaluation, namely, the Dap-substituted derivative at Y+3 (**3**) (4.3-fold) and the Dab-modified analogue at Y−2 (**5**) (4.7-fold). The phosphorylated analogue of **3**, peptide **4**, was synthesized as well.

Both unphosphorylated and phosphorylated peptide derivatives were examined by NMR to assess whether the aromatic moieties of the pyrene and tyrosine residues are spatially proximate. The pyrene protons in the unphosphorylated peptide **3** exhibit pronounced nuclear Overhauser enhancements (nOes) with their tyrosine counterparts (Figure 1a). Furthermore, all of the aromatic and benzylic protons on the tyrosine side chain are shifted upfield, suggesting that the pyrene and tyrosine rings are engaged in a $\pi-\pi$ stacking interaction as opposed to an edge−face interaction.[4] With this in mind, we propose a tentative working model, as illustrated in Figure 2, of the interaction between the pyrene and tyrosine aromatic nuclei. In contrast to the results obtained for compound **3**, the corresponding phosphorylated peptide **4** exhibits only weak nOes between the two aryl substituents (Figure 1b). These results indicate that the phosphate moiety compromises the ability of the pyrene and tyrosine aryl groups to interact with one another and suggest that the enhanced pyrene fluorescence in **4** is a consequence of its phosphorylation-induced liberated state.



Peptides **3** and **5** serve as substrates for a variety of protein tyrosine kinases (see Table 1 and Supporting Information). Since Src recognizes the chosen peptide sequence, it is not surprising that other members of the Src kinase subfamily (SrcN1, SrcN2, Fyn, Fgr, Hck, Lck, Yes, LynA, and LynB) likewise utilize peptides **3** and **5** as substrates. In addition, other nonreceptor tyrosine kinases (Abl, Csk, and Fes/Fps) as well as receptor tyrosine kinases (FGFR, TrkA, and Flt3) phosphorylate both peptides. However, these peptides are by no means universal tyrosine kinase substrates since several enzymes (ZAP-70, c-Met, EGF, Eph, IR, MLK1) are unable to effectively catalyze the phosphorylation of either **3** or **5**. The amino acid sequence preferences of these noncompliant kinases

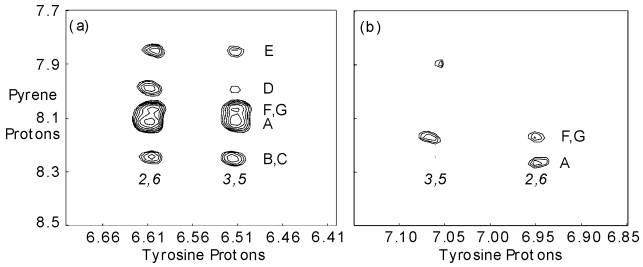


Figure 1. (a) Two-dimensional NOESY spectrum of the unphosphorylated peptide **3** showing nOes between the pyrene aromatic protons (for designations and assignments, see Figure 2 and tables in Supporting Information) and the tyrosine aromatic protons; nOes between the benzylic protons of the two aryl substituents are present as well (data not shown). (b) Two-dimensional NOESY spectrum of the phosphorylated peptide **4** showing weak nOes between only a few of the pyrene and tyrosine aromatic protons. A variety of mixing times were examined, with 450 ms furnishing optimal signal intensity.

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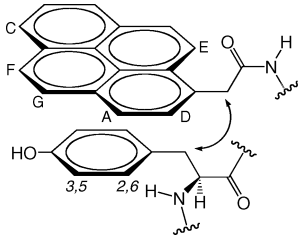

Figure 2. Schematic model of the interaction between the pyrene and phenol substituents based on the nOe and chemical shift data. The double-headed arrow indicates that nOes between the benzylic protons are observed as well.

Table 1. K_m and V_{max} Values for the Phosphorylation of Peptide **5** by Selected Tyrosine Kinases (see Supporting Information for a complete list of kinases and kinetic constants with peptide **3**)

tyrosine kinases	V_{max} (μmol/min·mg)	K_m (μM)
Src	2.4 ± 0.2	21 ± 3
FynT	0.41 ± 0.05	24 ± 4
Lck	2.1 ± 0.1	40 ± 1
Fes/Fps	4.1 ± 0.2	130 ± 10
FGFR	0.98 ± 0.09	80 ± 10
TrkA	2.9 ± 0.5	210 ± 40

are likely responsible for this behavior. In general, the phosphorylation of the Y−2 Dab derivative **5** proceeds with modestly lower K_m values than its Y+3 counterpart **3**. There are a number of possible explanations for the latter observation, with perhaps the simplest being that the various tyrosine kinases find the bulky Dap-pyrene moiety at Y+3 slightly more challenging to accommodate.

The fluorescent assay allows ATP concentrations to be employed that are identical to levels present in cells (1−10 mM). Unfortunately, low ATP concentrations (50 μM) can deceptively inflate the potency of protein kinase inhibitors since the vast majority are competitive with ATP.[5] For example, the pyrazolopyrimidine PP2 is a general inhibitor of the Src tyrosine kinase family.[6] In contrast to the low ATP concentrations employed in the latter studies, physiologically relevant ATP levels can be readily used with the pyrene−peptide substrates. We found that the IC_{50} of PP2 at 5 mM ATP is 4.1 ± 0.3 μM (Lck kinase), approximately 50-fold higher than the corresponding IC_{50} (86 ± 14 nM) at 50 μM ATP. These results confirm that ATP levels have a clear impact on the apparent efficacy of inhibitors that are competitive with ATP.

Tyrosine kinase activity is often regulated by autophosphorylation. Single fixed time point assays typically do not reveal whether the kinase is in its fully activated state. By contrast, the pyrene−peptide assay exposed a significant initial lag period in the progress curve for the Brk-catalyzed phosphorylation of pyrene−peptide **3**, which was initiated via the addition of ATP (Figure 3a). This observation is consistent with a report by Qiu and Miller, who established that Brk autophosphorylation enhances enzymatic activity.[7] By contrast, preincubation of Brk with ATP to ensure full enzyme activation, followed by addition of the pyrene−peptide substrate, furnished a reaction progress curve in which the lag phase is absent (Figure 3b). These results demonstrate that critical features hidden in discontinuous assays are readily revealed using the pyrene-based kinase reporters.

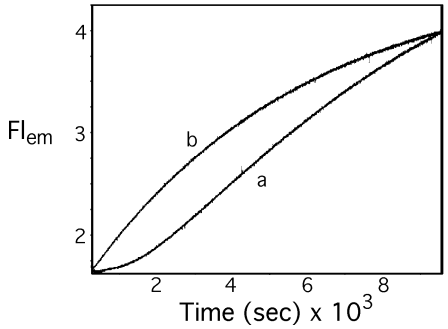

Figure 3. (a) Fluorescence emission (Fl_{em}) versus time for the Brk-catalyzed phosphorylation of peptide **3** initiated by addition of ATP. The biphasic reaction progress curve is highlighted by an initial lag period. (b) Fl_{em} versus time for the Brk-catalyzed phosphorylation of peptide **3** initiated by addition of pyrene−peptide **3**. Brk and ATP were preincubated for 120 min prior to addition of **3**.

In summary, we have developed a series of peptides that recognize and signal their phosphorylation status. These species are easily prepared in large quantities, can be modified with unnatural substituents to enhance potency and selectivity,[8] and can be caged at the site of phosphorylation,[9] which enables the investigator to control when the reporter is active. We are currently examining whether other fluorophores will noncovalently associate with tyrosine residues and subsequently fluorescently report the introduction of a phosphate group.

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Supporting Information Available: Experimental details of protein kinase sensor synthesis and characterization, and complete ref 3b. This material is available free of charge via the Internet at http://pubs.acs.org.

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