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Fluorogenic Peptide Substrates for Serine and Threonine Phosphatases

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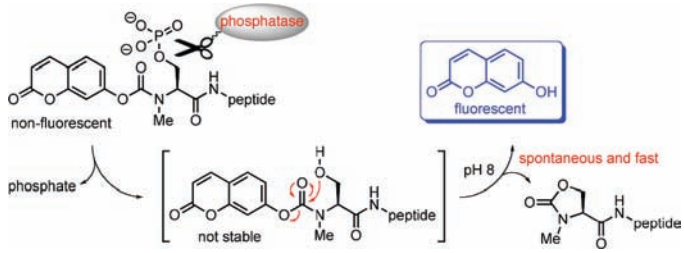
ABSTRACT



A new fluorescent assay for Ser/Thr protein phosphatases has been developed. Hydrolysis of a phosphoSer residue liberates the Ser hydroxyl group, which induces a cyclization reaction on the *N*-terminal carbamate and releases a fluorescent reporter. Sequence selectivity is observed using several peptide substrates against alkaline phosphatase (ALP), bacteriophage λ protein phosphatase (λ-PPase), and *vaccinia* H1 related phosphatase (VHR). These studies suggest that the assay could be a useful tool for profiling the substrate specificities of medicinally important phosphatases.

Serine and threonine protein phosphatases (Ser/Thr PPases) are key regulators of signal transduction and play essential roles in cell proliferation, division, and apoptosis in eukaryotes.[1] The specificities of PPases are controlled by a number of regulatory proteins that form heterodimeric complexes with the catalytic domains. These regulatory proteins influence both catalytic activity and subcellular localization.[2] The abnormal function of Ser/Thr PPases are implicated in a variety of human diseases including asthma, myocardial infarction, and immunosuppression.[3] Consequently, there is a great deal of interest in imaging their activity *in vivo*, understanding their specificities, and discovering selective inhibitors as therapeutic agents. New assays that are highly sensitive, amenable to high throughput screening applications, and can be used to profile the substrate specificities of PPases would be valuable tools to accomplish these goals.

To date, the substrate specificities of Ser/Thr PPases have not been comprehensively examined; published work employs a limited number of variants of known peptide sequences.[4] However, it is clear from inhibition studies with natural products and their analogs that a high degree of selectivity can be engineered into inhibitors.[3] Peptide substrates show significant differences in activities for particular sequences. Thus, important information may be gained about the biological activity of Ser/Thr PPases by studying their

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sequence selectivities. These data will be useful for the design of synthetic PPase inhibitors. Traditional PPase assays employ malachite green to detect inorganic phosphate, or use ^{32}P-labeled substrates. More recent assays have been developed that rely on changes in fluorescence, fluorescence polarization, or luminescence or changes in a substrate's susceptibility to cleavage by proteases.[5] Herein, we report a new PPase assay that incorporates a number of attractive features including high sensitivity, ability to measure kinetics in real time, and substrates that contain a natural phosphoserine residue rather than an activated phosphate ester such as *p*-nitrophenyl phosphate. This new assay is also amenable to substrate profiling and high throughput screening applications.

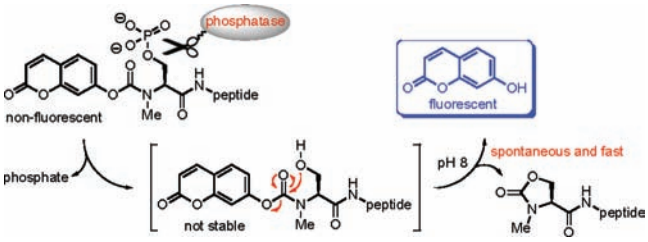

Figure 1. Outline of the Ser/Thr PPase assay.

Figure 1 shows the general outline of the assay. The substrates incorporate a coumaryl group connected to the *N*-terminus of a phosphoSer-containing peptide through a carbamate linkage. Hydrolysis of the phosphate ester by a phosphatase generates the free Ser hydroxyl group, which undergoes a spontaneous intramolecular 5-membered ring closure onto the carbonyl group of the carbamate. This cyclization liberates 7-hydroxycoumarin, which generates a strong fluorescent signal.

During the development of this assay, we required substrates that are stable under the aqueous conditions of the enzymatic reactions but give a fast ring closure upon hydrolysis of the phosphate ester. We first examined the stability of model substrates **1** and **2**. The carbamate linkage in compound **1a** hydrolyzed in pH 8 buffer with a half-life of 30 min, while compound **2a** was fully hydrolyzed after only several minutes. Since carbamate hydrolysis occurs by deprotonation of the *N*-H group and elimination of 7-hydroxycoumarin to give the corresponding isocyanate, replacing the *N*-H with an *N*-Me group should decrease the rate of background hydrolysis. We found that the *N*-Me derivatives **3** and **5** were stable (hydrolysis rate <0.5 nM s^{-1}). By contrast, **4a**, which contains a free Ser hydroxyl group,

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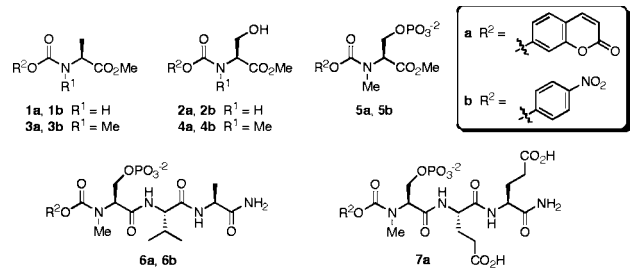

Figure 2. Structures of the substrates.

undergoes fast, spontaneous cyclization (rate =115 nM s^{-1}) to liberate 7-hydroxycoumarin.

We examined the kinetics of substrate hydrolysis using three phosphatases: alkaline phosphatase (ALP), bacteriophage λ protein phosphatase (λ-PPase), and *vaccinia* H1 related phosphatase (VHR). ALP is a nonspecific enzyme that hydrolyzes phosphate esters from a wide variety of substrates. λ-PPase is homologous with the *N*-terminal half of protein phosphatase 1 (PP1) and is commonly used as a model PPase. Finally, VHR is a duel specificity phosphatase that hydrolyzes both phosphoTyr and phosphoSer/phosphoThr-containing substrates. Figure 3 shows the fluorescence spectra

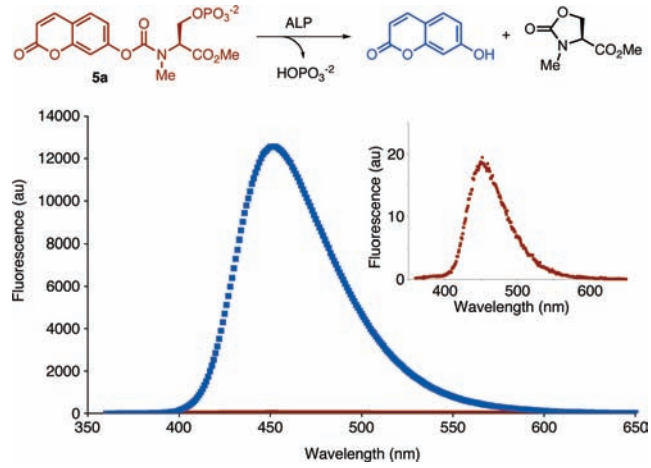

Figure 3. Fluorescence spectra of compound **5a** (50 mM in Tris buffer, pH 8.0) before (brown points and insert) and after (blue points) treatment with ALP (λ_{ex} = 340 nm, λ_{em} = 452 nm).

of the simplest substrate **5a**. Fluorescence intensity increases by 650-fold upon hydrolysis of the substrate by ALP. These results demonstrate that the assay has a large dynamic range, and will provide good sensitivity. We also examined substrates such as **5b** that liberate *p*-nitrophenol. Hydrolysis of these compounds can be monitored by UV spectroscopy. Kinetic parameters for **5a** and **5b** with ALP are shown in Table 1.

To increase noncovalent interactions of the substrates with phosphatases, we prepared tripeptides **6** and **7**. These

Table 1. Kinetic Data for Substrates **5**−**7**

enzyme	substrate	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($M^{-1}s^{-1}$)
ALP	**5a**	0.17 ± 0.02	380 ± 70	430
	5b	0.62 ± 0.7	820 ± 400	760
	6a	5.5 ± 0.6	41 ± 13	134 000
	6b	7.9 ± 0.9	400 ± 120	19 600
	7a	4.1 ± 0.3	70 ± 20	58 500
λ-PPase	**6a**	0.015 ± 0.001	130 ± 40	135
	6b	0.018 ± 0.02	770 ± 320	23
	7a	0.0031 ± 0.0004	170 ± 40	18
VHR	**6a**	0.041 ± 0.004	220 ± 80	186
	6b	0.083 ± 0.007	790 ± 210	105
	7a	NA[a]	NA[a]	NA[a]

[a] No detectable activity with 2 mM **7a** over 30 min.

substrates were synthesized using Fmoc solid phase peptide synthesis procedures. The *N*-terminus of each peptide was capped with *p*-nitrophenyl chloroformate or coumaryl chloroformate, prepared from 7-hydroxycoumarin and triphosgene. The sequence of substrate **6** (pSVA) is derived from the regulatory subunit of protein kinase A, which is a known substrate for VHR.[6] In compound **7a**, this sequence has been changed to pSEE to alter the electrostatic interactions with the enzymes. Compounds **6a**, **6b**, and **7a** are all excellent substrates for ALP (Table 1). The tripeptides **6a** and **6b** show 300-fold and 25-fold improvement in k_{cat}/K_m over their single amino acid analogs **5a** and **5b**. Two general observations can be made comparing the coumaryl and *p*-nitrophenyl substrates. First, the k_{cat}/K_m values for the coumaryl-containing substrate **6a** are higher than for the *p*-nitrophenyl analog **6b**. This difference typically reflects the higher affinity of **6a** for the phosphatases when compared to **6b**. Second, the accuracy of the kinetic measurements is higher with the fluorescent substrate **6a**.

The tripeptides are also substrates for λ-PPase. This enzyme shows a 6-fold preference for the pSVA sequence in **6a**. By contrast, VHR is highly selective. Compound **6a**, which contains the natural sequence from the regulatory subunit of protein kinase A, is a substrate with a k_{cat}/K_m value of 186 M^{-1} s^{-1}. On the other hand, compound **7a** with the sequence pSEE does not react with VHR, even at high concentrations and over extended reaction times. We performed computer modeling studies (Hyperchem) to rationalize these observations. As shown in Figure 4, substrate **6a** was docked into the active site of VHR (C124S). We used the crystal structure of a bound phosphopeptide (PDB 1J4X) as a guide to correctly position the substrate in the active site.[6b] This model suggests that the coumaryl group of the substrate binds in a pocket defined by Arg158 and Asn41, while the phosphate ester of the phosphoSer side chain contacts Arg130 and Ser124. The side chains of Val and Ala interact with the acidic patch on the phosphatase. If substrate **6a** is replaced by **7a**, the side chains of the two Glu residues on **7a** form repulsive electrostatic interactions

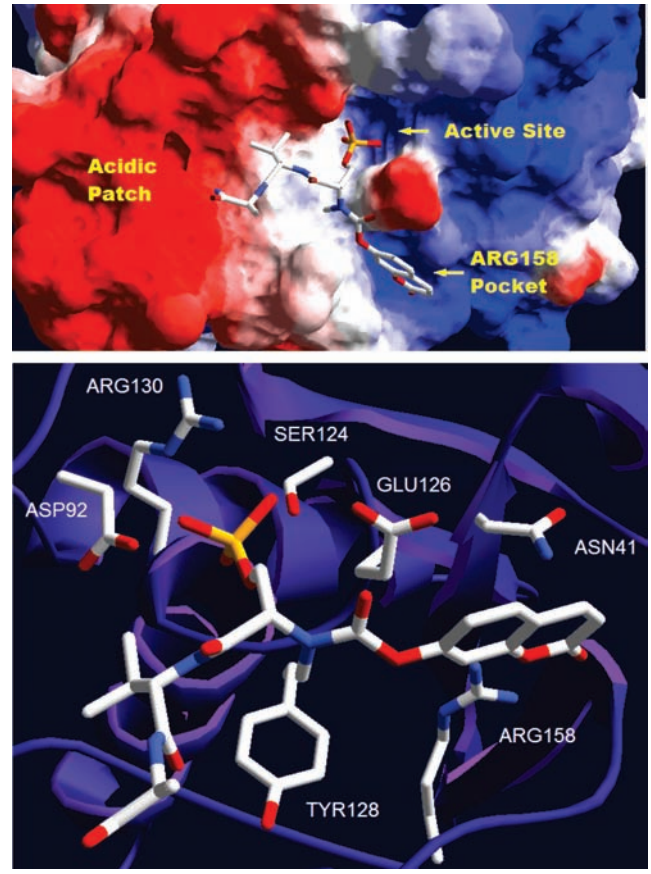

Figure 4. (Top) Model showing the interactions between substrate **6a** and the electrostatic surface potential of VHR (C124S). (Bottom) Detailed view showing substrate **6a** docked into the active site.

with the acidic patch, and in particular with Asp92. This electrostatic repulsion causes **7a** to be a nonsubstrate for VHR.

As a final validation of this assay we measured the activity of two know inhibitiors that have been reported in the literature. RK-682 (Figure 5) has a reported IC_{50} value of

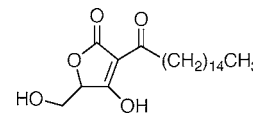

Figure 5. Structure of RK-682.

2.0 μM against VHR at pH 6.5 using *p*-nitrophenyl phosphate as the substrate.[7] Using **6a** as the substrate, we measured the IC_{50} value to be 3.6 μM at pH 8.0. Sodium vanadate inhibits alkaline phosphatase with a K_i of 2.1 μM.[8] Using compound **5b** as the substrate, we measured the IC_{50} value to be 1.6 μM. In both cases, the similarity between reported

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and measured inhibition demonstrates that this new fluorescent assay for PPases provides accurate inhibition data.

We have developed a new continuous assay for Ser/Thr PPases that relies on a cyclization reaction to liberate a fluorescent reporter. The assay shows good sensitivity, and the substrates are straightforward to prepare by solid phase peptide synthesis. We have observed sequence selectivity using several peptide substrates against ALP, λ-PPase, and VHR. In its current form, the assay is useful for probing interactions between a phosphatase and the amino acids that are on the *C*-terminal side of the phosphoSer residue of the substrate. We are currently working to modify the substrates so that they provide information about interactions with *N*-terminal residues as well. This assay should be a useful tool for profiling the substrate specificities of a variety of medicinally important phosphatases.

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Supporting Information Available: Experimental procedures and spectroscopic data for all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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