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Characterization of PKAC α enzyme kinetics and inhibition in an HPLC assay with a chromophoric substrate

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Abstract

Here we describe a convenient, inexpensive, and non-hazardous method for the measurement of kinase activity of the catalytic subunit of cAMP-dependent protein kinase (PKAC α). The assay is based on the separation of a substrate peptide labeled with a strong chromophore from the phosphorylated product peptide by high-performance liquid chromatograph (HPLC) and quantification of the product ratiometrically at a wavelength in the visual spectrum (Vis). The utility and reliability of the HPLC-Vis assay were demonstrated by characterizing the kinetic parameters (K_M , V_{max}) of the new Rh-MAB-Kemptide substrate, a commercially prepared TAMRA-Kemptide substrate, and ATP as well as the potency (IC_{50} , K_i) of the known PKAC α inhibitors H89 and PKI(5-24). The advantages of this assay are that it is convenient and inexpensive, uses readily synthesized or commercially available substrates that are shelf-stable, uses a common piece of laboratory equipment, and does not require any hazardous materials such as radioactive γ -³²P-ATP. The assay format is also highly flexible and could be adapted for testing of many different kinases by changing the peptide substrate sequence.

Keywords

Kinase Assay; cAMP-Dependent Protein Kinase; Kinetics Assay; Inhibition Assay; HPLC; Chromophore

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1. Introduction

The biochemical study of human kinases and the development of kinase inhibitors are important and active areas of biomedical research. Kinases are highly-studied as drug targets due to their central role in cellular signal transduction cascades and their implication in multiple disease states including cancer,[1] inflammation,[2] rheumatoid arthritis,[3] cardiac disease,[4] Parkinson's disease,[5] and Alzheimer's disease.[6] There are currently 28 FDA-approved kinase inhibitors that target 63 different kinases. [7–9] These clinically-validated kinases, however, represent only 12% of the 518-member human kinome, demonstrating that kinase biochemistry and inhibitor discovery will likely be active fields for many years to come.

The prolific research into kinases as therapeutic targets for multiple diseases states has given rise to an equally large proliferation of kinase assay technologies, particularly those used for high-throughput screening. In general, these assay technologies use proprietary methods for detection of the phosphorylated product and often utilize a development reagent such as a proteolytic enzyme, metal-labeled antibody or bead, or coupled enzyme systems. [10] These specialty reagents make these assays, which are mostly sold in kits, expensive to use and they often have an inflexible format for varying important parameters like substrate concentration.

The use of radiolabeled γ - ^{32}P -ATP assays, the gold standard for kinase assays,[11] is also becoming increasingly problematic in the academic environment due to increased regulations and issues with shared and mixed use laboratory spaces. These types of issues have prevented us from using γ - ^{32}P -ATP in our work and sent us in search of alternative technologies for kinase assays in our academic environment.

In our work to develop novel substrate competitive kinase inhibitors,[12–14] we have used an NADH-coupled enzyme assay [15] for kinetics analyses and the commercially available Z'-LYTE FRET-based assay [16] for inhibition studies. In our hands, we have found that each of these assays has significant drawbacks. While the reagents for the NADH-coupled enzyme assay are commercially available and the assay has a flexible format, we have found that changes in the activity of the coupled enzymes over time can cause a significant lack of reproducibility. And while the Z'-LYTE assay is commercially available, it is expensive, uses a proprietary enzymatic development reagent, and lacks format flexibility. This assay is best used for high-throughput screening and is not optimal of medium throughput use for inhibitor discovery in an academic laboratory setting. We also find the inability to *directly* measure the product formation to be a limitation for both of these assay technologies.

Given the limitation of the assay technologies that we have been using and our inability use ^{32}P assays, we wished to develop a new biochemical assay for kinase phosphotransfer activity. As we began this task, we determined that our new assay needed to meet the following criteria: 1) a single assay technology that could be used for both kinetic analyses and inhibition studies; 2) a method that *directly* measures product phosphorylation without using radiolabeled ^{32}P ; 3) an assay that utilizes readily available and inexpensive reagents in

a flexible format; and 4) an assay that utilizes standard laboratory equipment and does not require special instrumentation or supplies like proprietary well plates.

We hypothesized that an endpoint assay that utilizes a strong chromophore with a unique absorbance wavelength, such as rhodamine B, together with reverse phase high performance liquid chromatography (RP-HPLC) to separate and quantify the peptide substrate and phosphorylated product, would meet all of these criteria. In formulating this hypothesis, we were encouraged by previous reports of HPLC-UV kinase assays,[17, 18] which demonstrated that substrate and phosphorylated peptide could be separated, and a by fluorescence-TLC kinase assay.[19] Here we report the development of an HPLC-Vis assay and its use to perform a kinetic analysis and inhibition study with the catalytic subunit of cAMP-dependent protein kinase (PKAC α).

2. Materials and methods

2.1 Reagents

All amino acids (including 4-(methylamino)butyric acid, MAB), peptide coupling reagents, and 2-Cl-trityl resin, were purchased from Chem-Impex. Rhodamine B was purchased from Santa Cruz Biotechnology. Bulk and HPLC grade solvents were purchased from VWR and dry solvents were purchased from Sigma-Aldrich in SureSeal bottles. TAMRA-Kemptide was custom synthesized by GenScript and used as received. All assay reagents, including buffer salts, ATP, and MgCl₂ were of molecular biology grade and purchased from Sigma-Aldrich. H89 was purchased from Selleck Chem and PKI(5-24) was purchased from Alfa Aesar.

2.2 Recombinant Protein Production

2.2.1 Plasmid Design and Synthesis—The plasmid for the expression of recombinant human PKAC α was prepared by GenScript using a combination of gene synthesis and molecular biology (see Supplementary Information for gene insert sequence, full plasmid sequence, and resulting protein sequence). First, a protein sequence for full length recombinant PKAC α (Gly1 to Phe350) was designed to include an N-terminal (His)₆ tag followed immediately by a TEV cleavage site (ENLYFQ/G), where the remaining glycine residue left after TEV cleavage became the first glycine in the processed protein sequence. To the codons for this sequence were added a 5'-NdeI restriction site (which includes a start codon), two stop codons, a 3'-HindIII restriction site. This entire gene insert was synthesized, cloned into the pET-17b vector, and the vector sequenced to confirm the correct incorporation of the insert.

2.2.2 Protein Expression, Isolation, and Purification—To express PKAC α , competent *E. coli* cells were transformed with the plasmid described above, selecting with ampicillin (50 mg/L). Bacterial cells were grown in auto induction media at 37 °C for 16 hr. Following growth, cells were spun down to a pellet at 6000 rpm for 10 min, lysed into buffer (25 mM Tris, pH 8, 300 mM NaCl, and 10 mM imidazole), allowed to equilibrate for 30 min at ambient temperature, and debris removed by centrifugation at 20,000 rpm for 30 min. The soluble fraction was then loaded onto a Ni-NTA column, washed with 5 column volumes of

the loading buffer, and purified (His)₆-PKAC α eluted with 100 mM imidazole. The purified protein was then treated with TEV protease (1 wt%; 1 mg TEV protease per 100 mg PKAC α) overnight at room temperature to remove the (His)₆ tag. Following TEV cleavage, the kinase was dialyzed into storage buffer (25 mM phosphate buffer with 10% glycerol and 1 mM DTT), aliquoted, and stored at -80 °C until use.

2.3 High performance liquid chromatography instrument, columns, and gradients

All HPLC experiments were carried out on an Agilent LC 1200 system with a degasser, quaternary pump, vial and well plate autosampler, column heater, and diode array detector. All data was collected and processed with Agilent ChemStation software. In all HPLC runs, absorbance was monitored at 560 nm, 550 nm, 280 nm, and 210 nm. For preparatory scale purification of Rh-MAB-Kemptide, the system was operated in semi-prep mode using a Restek Ultra C18 5 μ m, 10 $\times$ 150mm column, and a flow rate of 3 mL/min. The compound was purified using a gradient of water with 0.1% formic acid (Solvent A) and acetonitrile with 0.1% formic acid (Solvent B); Gradient: 0–1 min, 5% B; 1–10 min, 5–45% B; 10–15 min, 45–65% B; 15–16 min, 65–5% B. For kinetics and inhibition assays, the system was operated in analytical mode using a Restek Ultra C18 3 μ m, 2.1 $\times$ 100mm column and a flow rate of 0.3 mL/min. Assays were analyzed using a gradient of water with 0.1% formic acid (Solvent A) and acetonitrile with 0.1% formic acid (Solvent B); Gradient: 0–0.5 min, 5% B; 0.5–9.0 min, 5–77% B; 9.0–9.5 min, 77% B; 9.5–10 min, 77–5% B; 10–13 min, 5% B. % Phosphorylation was determined by integrating both the substrate and product peaks measured at 560 nm.

2.4 Synthesis of Rh-MAB-Kemptide Substrate

Rh-MAB-Kemptide was synthesized using an Fmoc solid phase synthesis strategy. 2-Cl-Trityl resin (250 mg, 1.1 meq/g loading) was first loaded manually by stirring with Fmoc-Gly-OH (297.31 mg, 1 mmol) and diisopropylethylamine (0.17 mL, 1 mmol) in dichloromethane (2.5 mL) at room temperature for 3 hours. Following washing with DCM (2 $\times$ 5 mL) and drying under vacuum, the Fmoc-Gly-2-Cl-trityl resin (0.16 mg, 0.1 mmol) was loaded into a CEM Liberty Blue microwave peptide synthesizer. Subsequent amino acids Leu, Ser, Ala, Arg, Arg, and Leu (0.2 M in DMF) were incorporated using a standard coupling cycle (deprotection: 20% piperidine in DMF; coupling: 0.2 M DIC and 0.2 M HOBt; washes: DMF) at 50 °C, with double couplings used for Arg. Following the deprotection of the Fmoc from the Leu, the resin was removed from the peptide synthesizer and the N-terminus coupled to the previously reported rhodamine-MAB-NHS ester fragment [12] (250 mg, 0.5 mmol, see Fig. S1 in the Supplementary Information) with NEt₃ (0.14 mL, 1 mmol) in DMF (5 mL). Following removal of excess reagents and washing with DMF (2 $\times$ 5 mL), the Rh-MAB-Kemptide peptide was cleaved from the resin by treatment with global deprotection cocktail (95% TFA, 2.5% water, 2.5% TIS; 5 mL) at room temperature for 4 hours and the product was isolated by precipitation with ether. The crude pink solid was purified by RP-HPLC to afford pure Rh-MAB-Kemptide, which was lyophilized to a pink solid and characterized by MALDI-MS (C₆₅H₉₉N₁₆O₁₂⁺, [M+H]⁺ calculated: 1296.77, found: 1296.94).

2.5 UV/Vis characterization of strongly chromophoric peptide substrates

Samples of Rh-MAB-Kemptide and TAMRA-Kemptide were prepared in 25 mM phosphate buffer (pH = 7.4) at a final concentration of 100 μ M. Triplicate samples (80 μ L) of the chromophoric peptide substrates and buffer alone (as control) were pipetted into a 96-well plate (Greiner #675101). According to the manufacturer's specifications, 80 μ L volumes in these plates have a path length of 0.5 cm. The UV/Vis spectra were collected on a FlexStation 3 (Molecular Devices) plate reader (200–800 nm, 1 nm step). Absorbance data was corrected for background and Beer's Law was used to calculate the extinction coefficient (ϵ) at both λ_{max} and 560 nm.

2.6 Assay Conditions

2.6.1 Kinetics Assays for Kemptide analogues—Kinetics assays were performed in 96-well plates at 25 °C with a total reaction volume of 400 μ L. Kinase reactions contained PKAC α (0.25 nM), ATP (1 mM), MgCl₂ (10 mM), and Kemptide analogues **1** or **2** (50 – 1.56 μ M, 2-fold dilutions) in 50 mM MOPS (pH 7.4). Reactions were initiated by the addition of the Kemptide analogue, run for 10 minutes, and then quenched with 5% phosphoric acid (80 μ L). Kinase reactions were carried out in triplicate for each concentration of Kemptide analogues **1** or **2**. The 96-well plate was then loaded into the HPLC autosampler and each kinase reaction analyzed by the method described above using the 2.1 $\times$ 100mm C18 column. The volume of the injection was varied from 5 μ L to 100 μ L such that 0.5 nmoles of chromophore-labeled peptide was loaded and analyzed for each kinase reaction. Following HPLC analysis, the % phosphorylation was determined for each Kemptide analogue concentration by integrating the area of the peaks for the substrate and product at 560 nm. % Phosphorylation data were converted to velocities and plotted against the concentrations of the Kemptide analogue. Curve fitting and the determination of kinetics parameters were performed in Prism 7 (Graphpad) using non-linear regression to the Michaelis-Menten equation. Three biological replicates were carried out for each curve and the mean and SEM for each kinetic parameter were determined.

2.6.2 Kinetics Assay for ATP—Kinetics assays for ATP were performed using the same protocol and workflow as above, with modifications of the kinase reaction conditions. Kinetics assays were performed in 96-well plates at 25 °C with a total reaction volume of 200 μ L. Kinase reactions contained PKAC α (0.5 nM), Rh-MAB-Kemptide (**1**, 25 μ M), MgCl₂ (10 mM), and ATP (400 – 6.25 μ M, 2-fold dilutions) in 50 mM MOPS (pH 7.4). Reactions were initiated by the addition of ATP, run for 15 minutes, and then quenched with 5% phosphoric acid (100 μ L). Kinase reactions were carried out in triplicate for each concentration of ATP. Kinase reactions were analyzed by HPLC and kinetics parameters determined as described above. Three biological replicates were carried out for each curve and the mean and SEM for each kinetic parameter were determined.

2.6.3 Inhibition Assays—Inhibition assays were performed in 96-well plates at 25 °C with a total reaction volume of 200 μ L. Kinase reactions contained PKAC α (0.5 nM), ATP (25 μ M), MgCl₂ (10 mM), Rh-MAB-Kemptide (**1**) (10 μ M), inhibitor (H89 or PKI(5-24), 16 μ M – 15 pM, 4-fold dilutions) in 50 mM MOPS (pH 7.4). For H89, reactions were initiated by the addition of ATP after a 5-min pre-incubation of inhibitor with PKAC α and the other

reaction components. For PKI(5-24), reactions were initiated by the addition of Rh-MAB-Kemptide (**1**) after a 5-min pre-incubation of inhibitor with PKAC α and the other reaction components. Reactions were run for 1 hour and then quenched with 5% phosphoric acid (100 μ L). Kinase reactions were carried out in duplicate for each concentration of each inhibitor. The 96-well plate was then loaded into the HPLC autosampler and each kinase reaction analyzed by the method described above using the 2.1 $\times$ 100mm C18 column (80 μ L injection volume, 0.5 nmoles of chromophore-labeled peptide). Following HPLC analysis, the % phosphorylation was determined for each inhibitor concentration by integrating the area of the peaks for the substrate and product at 560 nm. % Phosphorylation data were normalized to the no inhibitor control well, converted to % Inhibition, and plotted against the log of the concentrations of the inhibitor. Curve fitting and the determination of the IC₅₀ were performed in Prism 7 (Graphpad) using non-linear regression to the four-parameter log(inhibitor) vs. response equation. K_i's were calculated using the Cheng-Prusoff equation and the K_M for Rh-MAB-Kemptide (**1**). Three biological replicates were carried out for each curve and the mean and SEM for the IC₅₀ and K_i for each inhibitor were determined.

2.7 Characterization of phosphorylation product by Mass Spectrometry

To definitively prove the identity of the phosphorylated product, a large-scale kinase reaction was run, the resultant product concentrated on a C18 SPE cartridge, and the product characterized by mass spec. The large-scale reaction was performed in a microcentrifuge tube at 25 °C with a total reaction volume of 1000 μ L. The kinase reaction contained PKAC α (4 nM), ATP (1 mM), MgCl₂ (10 mM), and Rh-MAB-Kemptide (**1**) (100 μ M) in 50 mM MOPS (pH 7.4). The reaction was initiated by the addition of Rh-MAB-Kemptide (**1**), run for 1 hour, and then quenched with 5% phosphoric acid (250 μ L). The reaction was analyzed by HPLC using the 2.1 $\times$ 100mm C18 column (5 μ L injection volume, ~0.5 nmoles of chromophore-labeled peptide) and the conversion measured to be ~80%. The entire quenched reaction was then loaded onto a C18 SPE cartridge, flushing first with 10% ACN in water followed by elution of chromophore-labeled peptide by methanol. The eluted product was concentrated to a dry film on a rotovap. The dry film was resuspended in a minimal volume of ACN with 0.1% acetic acid and analyzed by direct injection on a LCQ DecaXP Max mass spectrometer. ESI-MS (C₆₅H₁₀₀N₁₆O₁₅P⁺, [M+H]²⁺ calculated: 688.37, found: 688.46).

3. Results

3.1 Design, Synthesis, and Characterization of strongly chromophoric Kemptide analogues

The design concept for our assay requires a kinase substrate peptide that contains a strong chromophore with a unique absorbance wavelength that can be made in relatively large quantities (10–50 mg) for relatively low cost. For our work with PKAC α , we have chosen the standard Kemptide peptide as the substrate sequence (Fig. 1). As the chromophore, we have chosen to use rhodamine B, based on its strong absorbance at ~560 nm, low cost (~\$0.50/g), and our previous experience with making rhodamine B-labeled peptide inhibitors. [12] To join the rhodamine B chromophore to the peptide, we utilized a methylaminobutyric acid (MAB) linker. Combining these elements yields the Rh-MAB-Kemptide substrate (**1**, Fig. 1), which we synthesized using solid-phase peptide synthesis. The chromophore was

appended to the N-terminus of the substrate peptide using the Rh-MAB-NHS ester fragment (See Fig. S1 of the Supplementary Information) following a previously published protocol. [12] Following purification by prep-scale RP-HPLC, Rh-MAB-Kemptide (**1**) was characterized by MALDI-MS.

To demonstrate the utility of the assay for those not able to synthesize a custom chromophore-labeled peptide, we also had a TAMRA-Kemptide substrate (**2**, Fig 1) synthesized by GenScript. This peptide also meets our criteria of having a strong chromophore with a unique absorbance wavelength and it is readily available from contract research organizations (CROs).

With Rh-MAB-Kemptide (**1**) and TAMRA-Kemptide (**2**) in hand, we then turned to characterizing the absorbance spectra for each substrate. The UV/Vis spectra for Rh-MAB-Kemptide (**1**) and TAMRA-Kemptide (**2**) were collected and the λ_{max} for each strongly chromophoric substrate peptide determined (Fig. 2 and Table 1). Both chromophores showed strong absorbance at 560 nm, which was the wavelength chosen for our HPLC-Vis assay. The extinction coefficients (ϵ) for both Rh-MAB-Kemptide (**1**) and TAMRA-Kemptide (**2**) are $>35,000 \text{ M}^{-1}\text{cm}^{-1}$ at both 560 nm and λ_{max} , respectively, demonstrating that both chromophores have a strong absorbance that will be useful in the HPLC-Vis assay.

3.2 Development of the HPLC method

We next turned to developing an HPLC method that would elute the chromophoric substrate and product peptides in as short a time as possible while still maintaining separation between the peaks. After extensive optimization, we arrived at a 13-minute method using a $2.1 \times 100 \text{ mm}$ C18 column, which offered the best compromise between method time and peak resolution. The gradient for the method includes an $\sim 8.5\%$ per minute increase in acetonitrile for 8.5 minutes, a hold at a maximum of 77% acetonitrile, and a final 3-minute hold at the end to re-equilibrate the column at 5% acetonitrile. All of these parameters were extensively optimized and imperative for the success of the method. A faster gradient than the one specified results in poor separation of substrate and product peaks. The substrate and product peptides elute just before and during the hold at 77% acetonitrile, respectively. And failure to include the final re-equilibration period leads to poor separation and break through elution of chromophoric peptide in subsequent runs.

The elution of Rh-MAB-Kemptide (**1**) and TAMRA-Kemptide (**2**) using the method that we have developed are shown in Fig. 3. Both substrates show a strong signal at 560 nm ($>1,000$ mAU) when 0.5 nmoles of chromophore-labeled peptide are injected from solutions at concentrations suitable for kinase assays ($10 \mu\text{M}$). Both Rh-MAB-Kemptide (**1**) and TAMRA-Kemptide (**2**) elute at the end of the gradient portion of the method, with retention times of 8.45 and 7.81 minutes, respectively. Interestingly, substitution of the Kemptide peptide substrate with rhodamine B (or TAMRA) significantly increases the hydrophobicity of the compounds, making them elute at much later retention times than unlabeled peptide. This is advantageous for the kinase assay, as the chromophore-labeled substrate and products peptides will elute later than and farther away from other polar assay components.

An HPLC analysis of a kinase reaction using Rh-MAB-Kemptide (**1**) and recombinant human PKA α is shown in Fig. 4. A strong signal can be seen for both the substrate and product peaks at 560 nm after injection of 0.5 nmoles of chromophore-labeled peptide. The substrate and product peaks are clearly resolved (retention times of 8.43 and 8.84 minutes, respectively) and can be easily quantitated by integration of the area under the curves, which shows that 33.6% phosphorylation was reached in this reaction, corresponding to 3.36 μ M (or 0.672 nmoles) of phospho-Rh-MAB-Kemptide product. Importantly, both the substrate and product peaks are detected at a unique wavelength compared to the other assay components. The HPLC trace at 210 nm shows the other assay components such as the protein, ATP, and ADP. Use of TAMRA-Kemptide (**2**) under the same assay conditions yields similar results (data not shown).

To conclusively demonstrate that the peak at a retention time of 8.84 minutes is the phospho-Rh-MAB-Kemptide product, we ran a large-scale kinase reaction, isolated the product using a C18 SPE cartridge, and analyzed it by mass spectrometry (see Materials and Methods). The experimental mass spec data matched the expected data for phospho-Rh-MAB-Kemptide (see Fig. S3 of the Supplementary Information). We also ran spike experiments by adding additional Rh-MAB-Kemptide (**1**) to verify that the peak at the retention time of 8.43 minutes was in fact the substrate (data not shown).

3.3 Kinetics Analysis of PKA α with the HPLC-Vis assay using chromophoric Kemptide analogues

With a method in hand for the HPLC-Vis kinase assay that could separate and directly quantify phospho-Kemptide product, we turned to utilizing the method to analyze the enzyme kinetics of PKA α . We first ran a series of kinase reactions with varying concentrations of Rh-MAB-Kemptide (**1**) and TAMRA-Kemptide (**2**), at a fixed concentration of ATP, in order to determine the kinetic parameters for each of these peptide substrates (see Materials and Methods). Following kinase reactions, percent phosphorylation data was transformed to velocity, and the kinetics parameters were determined by fitting the data to the Michaelis-Menten equation (Fig. 5A & B, Table 2). The $K_{M,Peptide}$ for both Rh-MAB-Kemptide ($9.74 \pm 0.88 \mu$ M) and TAMRA-Kemptide ($7.25 \pm 0.94 \mu$ M) are comparable to the $K_{M,Peptide}$ reported for Kemptide using a radiolabeled 32 P assay ($12.8 \pm 2.5 \mu$ M).[19] It should be noted that the $K_{M,Peptide}$ for Kemptide in radiolabeled 32 P assays has a wide range of values in the literature, generally 10 – 40 μ M, depending on the exact assay conditions.[19, 20] A previous report of the use of a similar 5-TAMRA-Kemptide substrate in a TLC-fluorescence assay showed that appending the 5-TAMRA to the N-terminus of Kemptide significantly decreased the $K_{M,Peptide}$ (to $2.0 \pm 0.5 \mu$ M).[19] Under our assay conditions, we see a similar decrease for TAMRA-Kemptide (**2**), but a less pronounced one for Rh-MAB-Kemptide (**1**). Our hypothesis is that the methylaminobutyric acid (MAB) linker in **1** moves the rhodamine B farther away from the enzyme, preventing the chromophore from affecting the binding of the peptide substrate and therefore $K_{M,Peptide}$. Similar to the $K_{M,Peptide}$, the $V_{max,Peptide}$ for both Rh-MAB-Kemptide ($21.75 \pm 3.45 \mu$ mol/min/mg) and TAMRA-Kemptide ($10.16 \pm 1.85 \mu$ mol/min/mg) are within the range of reported $V_{max,Peptide}$ values in the literature for Kemptide ($6.4 \pm 0.4 - 20.2 \pm 0.5 \mu$ mol/min/mg).[19–21]

We also determined the kinetic parameters for ATP using the HPLC-Vis assay by varying the ATP concentration at a constant concentration of Rh-MAB-Kemptide (**1**) (see Materials and Methods). The $K_{M,ATP}$ ($16.93 \pm 1.27 \mu M$) in the HPLC-Vis assay was comparable to the $K_{M,ATP}$ using a radiolabeled ^{32}P assay ($17.2 \pm 1.8 \mu M$). [19, 20, 22, 23] Similarly, the $V_{max,ATP}$ ($4.52 \pm 0.33 \mu mol/min/mg$) in the HPLC-Vis assay was consistent with $V_{max,ATP}$ determined using a radiolabeled ^{32}P assay ($10.7 \pm 2.4 \mu M$). [20]

Collectively, these data demonstrate that the HPLC-Vis assay using Rh-MAB-Kemptide (**1**) is able to accurately measure the kinetics of PKAC α .

3.4 Studies of PKAC α Inhibitors with the HPLC-Vis assay using chromophoric Kemptide analogues

The final task in our work was to develop an inhibition assay using the HPLC-Vis method and Rh-MAB-Kemptide (**1**). For our inhibition assay, we set the concentration of Rh-MAB-Kemptide (**1**) at $10 \mu M$ ($\sim K_M$) and the concentration of ATP at $25 \mu M$ ($\sim 1.5 \times K_M$) so that the same assay conditions could be used for both ATP-competitive and peptide substrate-competitive inhibitors.

We first used the HPLC-Vis assay to measure the inhibition of PKAC α by PKI(5-24), which is a known peptide substrate-competitive inhibitor. [24] A series of kinase reactions in a 96-well plate were treated with varying concentrations of PKI(5-24), followed by determination of % phosphorylation for each well, calculation of % inhibition, and curve fitting to produce IC_{50} curves (Fig. 7A). The IC_{50} for PKI(5-24) in the HPLC-Vis assay was determined to be 7.4 ± 1.2 nM and was transformed into a K_i of 3.7 ± 0.6 nM (Table 3). This K_i in the HPLC-Vis assay is nearly identical to the reported K_i of 2.3 nM for PKI(5-24) from the literature. [25, 26] We then used the HPLC-Vis assay to measure the inhibition of PKAC α by the ATP-competitive inhibitor H89. [29] The same procedure as above was used except with varying concentrations of H89. The IC_{50} for H89 in the HPLC-Vis assay was determined to be 63.3 ± 4.5 nM and was transformed into a K_i of 25.5 ± 1.8 nM (Fig. 7B and Table 3). The literature value for the K_i of H89 is typically cited as 48 nM, from the work of Hidaka and coworkers. [30] A close reading of this work, however, reveals that the assay utilized histone IIB protein as the substrate, not Kemptide, and therefore this K_i value is not applicable to assays that use Kemptide peptide. A summary of the data for inhibition of PKAC α by H89 in assays using Kemptide is shown in Table 4. [16, 19, 27, 28] In these various methods, the IC_{50} for H89 varies from 18 – 135 nM and the K_i has been reported to be 15.8 nM and 103 nM using different methods. In three of the four methods, only an IC_{50} or K_i were reported, and as the K_M of the substrate with PKAC α was not reported, the other parameter could not be calculated. Our IC_{50} and K_i for H89 inhibition of PKAC α from the HPLC-Vis assay are consistent with these reported values.

Collectively, these data demonstrate that the HPLC-Vis assay using Rh-MAB-Kemptide (**1**) is able to accurately measure the inhibition of PKAC α by both ATP-competitive and peptide substrate-competitive inhibitors.

4. Discussion

In this present work, we describe a kinase assay that uses an HPLC-Vis method with a strongly chromophoric substrate. We have utilized PKA α as the model kinase for our method and shown that two variants of the PKA α substrate that are labeled on the N-terminus with a chromophore, Rh-MAB-Kemptide (**1**) and TAMRA-Kemptide (**2**), can be utilized. The assay method has several benefits, including: the use of non-toxic, inexpensive, readily available, and shelf-stable reagents, substrates and supplies; a flexible format that can be used for both kinetics and inhibition studies; the use of a standard and common piece of laboratory equipment (HPLC); and the ability to directly separate and quantify the amount of phosphorylated product generated in the experiments. Using this HPLC-Vis assay with Rh-MAB-Kemptide (**1**), we have shown that it can accurately measure both the kinetics parameters (K_M , V_{max} , k_{cat}) of the kinase as well as the potency (IC_{50} , K_i) of both ATP-competitive and peptide substrate-competitive inhibitors.

HPLC-based assays for kinases have been reported previously in the literature. [17, 18] Most existing methods measure the substrate and phosphorylated product peptides in the UV spectrum, typically at 210 or 220 nm, using the intrinsic absorbance of the peptide backbone and have a lower limit of detection of >10 pmoles.[17] However, HPLC assays at these wavelengths suffer from inherently low signal (due to the low intrinsic absorbance of peptides), potential interference from other assay components that also absorb in the UV spectra, and interference from baseline drift when gradients are used. The method described here overcomes these limitations by using a strong chromophore appended to the N-terminus of the peptide substrate and a unique wavelength of 560 nm for detection of substrate and product. Under the conditions used for the kinetics and inhibition assays, the lower limit of detection was 5 pmoles of phosphorylated Rh-MAB-Kemptide. However, we did not attempt to find or optimize the absolute lower limit of detection, which we estimate could be as low as 1 pmole. Detection at the unique wavelength of 560 nm prevents the interference from other assay components and any drift in the baseline from absorbance of the mobile phase. An additional benefit is that the rhodamine B and TAMRA chromophores are hydrophobic, moving the substrate and phosphorylated product to a longer retention time well away from other assay components.

The use of N-terminal labeled substrate peptides in kinase assays has also been reported in the literature, including with rhodamine.[10] In most cases, these labeled substrates and the kinase reaction products are quantified by fluorescence, which enables a lower limit of detection than our assay. However, in order to differentiate between unphosphorylated substrate and phosphorylated product, a development step (such as a proteolytic enzyme) or an expensive proprietary reagent (such as a metal-chelating bead or Tb/Eu-containing antibody) are needed, making these other assay technologies expensive. The one assay technology that circumvents this requirement to differentiate between unphosphorylated substrate and phosphorylated product is the Caliper assay,[28] which uses microfluidic chips and on-chip electrophoresis to separate the substrate and product, followed by quantification by fluorescence. The Caliper assay, however, requires an expensive single-purpose instrument and proprietary chips as consumables, both of which prevent its general and wide-spread use, especially in academic labs.

The only limitation for our HPLC-Vis kinase assay is the throughput. Our goal for this work was to develop a low- to medium-throughput assay that would allow us to carry out kinetics analyses and characterize the inhibition of small rounds of analogues (1–5 compounds). We have developed a workflow where we run our assays in 96-well plates during the work day, load those plates into a well-plate compatible HPLC autosampler, and conduct the analysis of each data point overnight. The main time limitation is the length of the HPLC gradient method (13 minutes). This time limitation could be overcome by using a UPLC instrument with a smaller diameter column or potentially using a shorter column (2.1×50mm) on an HPLC instrument.

Despite the throughput limitation, it should be noted that this is a highly versatile assay that could be adapted for testing of many different kinases simply by changing the peptide sequence. Combined with the ability to have substrates of this type synthesized by CROs, this could be considered an off-the-shelf assay for anyone in the kinase field.

In summary, we have developed an HPLC-Vis kinase assay method that utilizes inexpensive and non-toxic assay reagents and a common piece of laboratory equipment. We have also demonstrated, using PKA α and the substrate Rh-MAB-Kemptide (**1**), that this assay method can accurately measure the kinetics parameters and inhibition by both ATP-competitive and peptide substrate-competitive inhibitors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.
Structures of Rh-MAB-Kemptide (**1**) and TAMRA-Kemptide (**2**), PKACα substrates with strong chromophores.

Fig 2. UV/Vis spectra of Rh-MAB-Kemptide (1) and TAMRA-Kemptide (2). Both samples are 100 μ M in 25 mM phosphate buffer (pH 7.4).

Fig. 3. HPLC traces of Rh-MAB-Kemptide (**1**) and TAMRA-Kemptide (**2**) at 560 nm. Both samples are 10 μ M in 25 mM phosphate buffer (pH 7.4). HPLC analysis was carried out on the 2.1 $\times$ 100mm C18 column (50 μ L injection volume, 0.5 nmoles) as described in the Materials and Methods section. Retention times were 8.45 min for Rh-MAB-Kemptide (**1**) and 7.81 min for TAMRA-Kemptide (**2**).

Fig. 4.

HPLC trace of a kinase reaction with Rh-MAB-Kemptide (**1**) at multiple wavelengths.

Kinase reaction conditions were: PKAC α (0.5 nM), Rh-MAB-Kemptide (**1**) (10 μ M), ATP (25 μ M), MgCl₂ (10 mM), in 50 mM MOPS (pH 7.4) in a 200 μ L final volume for 1 hour. HPLC analysis was carried out on the 2.1 $\times$ 100mm C18 column (80 μ L injection volume, 0.5 nmoles) as described in the Materials and Methods section. Retention times were 8.43 min for Rh-MAB-Kemptide (**1**) and 8.84 min for phospho-Rh-MAB-Kemptide. Phosphorylation as measured at 560 nm was 33.6%.

Fig. 5. Kinetic analysis of the phosphorylation of Rh-MAB-Kemptide (**A**) and TAMRA-Kemptide (**B**) by recombinant human PKAC α at a fixed concentration of ATP for determination of $K_{M,\text{Peptide}}$ and other kinetic parameters.

Fig. 6. Kinetic analysis of the phosphorylation of Rh-MAB-Kemptide by recombinant human PKACα at varying ATP concentrations for determination of $K_{M,ATP}$ and other kinetic parameters.

Fig. 7.
Inhibition of recombinant human PKAα by PKI(5-24) (A) and H89 (B).

Table 1

UV/Vis spectral data for strongly chromophoric Kemptide peptide substrates.

Compound	λ_{max} (nm)	ϵ ($\text{M}^{-1}\text{cm}^{-1}$)	
		@ λ_{max}	@ 560 nm
Rh-MAB-Kemptide (1)	566	39,296	37,220
TAMRA-Kemptide (2)	554	38,988	36,186

Table 2

Kinetic parameters^a of phosphorylation of fluorescent Kemptide peptide substrates with recombinant human PKA α .

	K_M (μM)	V_{max} ($\mu mol/min/mg$)	k_{cat} (min^{-1})	k_{cat}/K_M ($\mu M^{-1}min^{-1}$)
Rh-MAB- Kemptide (1)	9.74 ± 0.88	21.75 ± 3.45	870 ± 138	90.7 ± 22.7
TAMRA- Kemptide (2)	7.25 ± 0.93	10.16 ± 1.85	406 ± 74	57.4 ± 16.5
ATP	16.93 ± 1.27	4.52 ± 0.33	362 ± 26	21.4 ± 1.22

^aKinetic parameters are the mean and SEM from three independent experiments ($n = 3$), where each independent experiment was performed in triplicate.

Table 3

Data for inhibition of PKAC α by PKI(5-24) and H89 measured in the HPLC-Vis assay with Rh-MAB-Kmeptide (**1**).^a

Inhibitor	IC ₅₀ (nM)	K _i (nM) ^b
PKI(5-24)	3.7 $\pm$ 0.6	25.5 $\pm$ 1.8
H89	7.4 $\pm$ 1.2	63.3 $\pm$ 4.5

^aInhibition data are the mean and SEM from three independent experiments (n = 3), where each independent experiment was performed in duplicate.

^bK_i's were calculated using Cheng-Prusoff equation and the K_M's from Table 2.

Table 4

Literature data for inhibition of PKAC α by H89 using Kemptide as a substrate.

Assay Method	IC ₅₀	K _i	[ATP]	Ref
³² P	135 nM	n.r. ^a	100 μ M	[27]
Z'-LYTE	18 nM	n.r. ^a	10 μ M	[16]
Caliper	n.r. ^a	103 nM	multiple ^b	[28]
fluorescence-TLC	100 nM	15.8 nM ^c	100 μ M	[19]

^a n.r. = not reported.

^b [ATP] used were 50, 75, and 100 μ M.

^c K_i was calculated using Cheng-Prusoff equation and reported K_M.