A generic medium throughput activity assay procedure for serine–threonine protein phosphatases using ^{14}C-labelled *N*-acetyl-Arg-Arg-Ala-Thr(P)-Val-Ala

Jonathan Sanvoisin,[a] John R. Pollard,[a] Pantea Hormozdiari,[a] Walter H. J. Ward[b] and David Gani*[a]

[a] *School of Chemistry, Haworth Building, The University of Birmingham, Edgbaston, Birmingham, UK B15 2TT*
[b] *AstraZeneca, Mereside, Alderley Park, Macclesfield, Cheshire, UK SK10 4TG*

Received (in Cambridge, UK) 9th January 2001, Accepted 24th May 2001
First published as an Advance Article on the web 4th July 2001

A new and sensitive activity assay for the Ser-Thr protein phosphatases PP1 and PP2A based upon the hydrolysis of the synthetic radiolabelled phosphopeptide substrate *N*-acetyl-Arg-Arg-Ala-Thr(P)-Val-Ala, is described. The protocol is also applicable to the assay of PP2B and PP2C activity. The radiolabelled phosphopeptide is stable and can be stored for prolonged periods without deterioration or loss of radioactivity offering advantages over the use of ^{32}P-labelled substrates. The assay method involves the separation of phosphopeptide substrate from the peptide alcohol product by anion exchange chromatography. The separation protocol is not sensitive to the presence of inorganic phosphate anion or metal cations. Using PP1 as the enzyme the radiochemical assay procedure afforded a V_{max} value of 17 (±2) μM s^{-1} [28 (±3) μM s^{-1} μg^{-1}] and a K_M value of 3.7 (±0.9) mM for the substrate Ac-Arg-Arg-Ala-Thr(P)-Val-Ala with significantly greater accuracy than for a Malachite Green based assay. Inorganic phosphate was shown to be a competitive product inhibitor (K_i = 1.6 mM) and nodularin was found to be a potent competitive inhibitor (K_i = 0.19 nM) for PP1 with respect to the phosphopeptide substrate. This assay procedure was employed to determine the mode and magnitude of the inhibition of PP1 by the nodularin analogue described in the previous article [M. E. O'Donnell, J. Sanvoisin and D. Gani, *J. Chem. Soc., Perkin Trans. 1*, 2001 (DOI:10.1039/b100402f)]. The enzyme PP2A afforded a V_{max} value of 2.8 (±0.2) μM s^{-1} [45 (±2) μM s^{-1}μg^{-1}] and a K_M value of 4.1 (±0.7) mM for the radiolabelled substrate.

Introduction

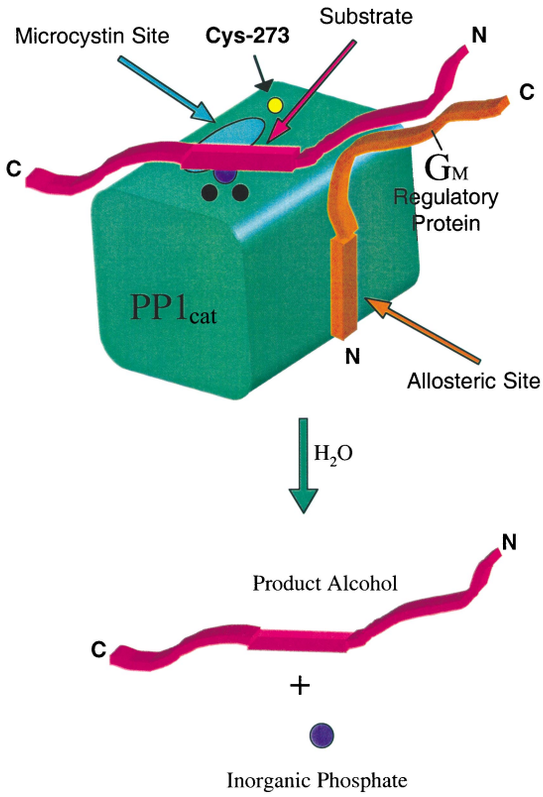
The balanced transfer of phosphoryl groups from one entity to another as catalysed by phosphatases and kinases is the basal mechanism by which cellular function is controlled.[1–3] Some 4% of the eukaryotic genome codes for kinases and phosphatases[4] and when the fine balance between phosphorylation and dephosphorylation is upset, cellular dysfunction occurs leading to a range of diseased states.[5,6] In the cytoplasm of eukaryotic cells, most, if not all, phosphoseryl–phosphothreonyl protein phosphatase activity can be accounted for by just four different catalytic subunit types, PP1, PP2A, 2B and 2C.[7] *In vivo* a catalytic subunit is targeted towards a specific phosphoprotein substrate by a regulatory subunit[8,9] but it is now clear that some substrates are dephosphorylated by more than one type of catalytic subunit (Scheme 1). Some regulatory subunits, for example inhibitor 1 and DARPP are natural PP1 inhibitors and are themselves phosphothreonine proteins. Intriguingly, these are deactivated as inhibitors through the action of a Ser-Thr protein phosphatase.[10] Many regulatory subunits also bind through the occupancy of an allosteric site on PP1 which enhances the catalytic activity of the enzyme towards specific phosphoprotein substrates but depresses activity with respect to others.[11] Thus, the control of dephosphorylation is extremely complex and unravelling the intricacies calls for the availability of a range of specific inhibitors and allosteric site ligands.

PP1 and PP2A are highly homologous and display 49% identity in amino acid sequence.[12] The residues forming the active site are identical and regions of each protein that bind to the inhibitor microcystin are highly conserved in X-ray crystal structures of the PP1–microcystin complex[13,14] and a PP2A–microcystin homology model.[15,16] From this comparison it is apparent why PP1 and PP2A activities are inhibited similarly by such compounds as okadaic acid, tautomycin and nodularin which possess a diverse range of structures.[17–19]† In order to better understand the intracellular roles of these two related catalytic subunits it is desirable to be able to substantially suppress the activity of one or the other selectively through the use of cell-permeable selective or specific inhibitors. There are known natural products that are selective for PP2A inhibition, but none of these are specific. For example, the tumour promoter okadaic acid is 10^4 times more active against PP2A than PP1.[20] At the present time there are no known small molecule inhibitors that display even nearly equivalent levels of selectivity for PP1 inhibition, although a number of groups, including our own, have been working towards discovering such entities. To date, the best reported selectivities are for microcystin analogues that display approximately 7-fold greater potency towards PP1 than PP2A as determined by comparison of IC_{50} values.[21] Generally there are significant difficulties in comparing the potency of inhibitors for PP1 and PP2A. This is due to the tendency to measure IC_{50} values, rather than K_i values, see below. Complications in analysis and interpretation are caused by using a single substrate concentration or, a narrow range of substrate concentrations, in assessing very tight-binding inhibitors. Moreover, the conditions used for efficacy determinations have varied widely from one laboratory to another. Differences include divalent metal ion

† Also see the two preceding articles in this series in refs. 25 and 26.

DOI: 10.1039/b100403o

This journal is © The Royal Society of Chemistry 2001



Scheme 1 Cartoon showing expected arrangement of inhibitor and substrate binding sites and the mode of enzyme activation and substrate specificity control by the regulatory protein G_M in the hydrolysis of the phosphopeptide substrate.

concentration, substrate concentration, pH, the type of substrate used and the method for the measurement of activity.

To support work in our own laboratories on the development of specific inhibitors of PP1 and PP2A using nodularin analogues[22–26] and mechanistic studies of the catalytic reaction and its allosteric modification,[27] we identified a requirement for a reliable and generic assay protocol suitable for both PP1 and PP2A. Here we describe a sensitive radiochemical assay suitable for assessing activity in each of the four Ser-Thr protein phosphatase catalytic subunit types. The assay is based upon the hydrolysis of *N*-[1-^{14}C]acetyl-Arg-Arg-Ala-Thr(P)-Val-Ala and circumvents many of the problems associated with established activity assay procedures using either ^{32}P-labelled phosphorylated protein substrates,[28] Malachite Green[29,30] or, *p*-nitrophenyl phosphate.[31] A comparison with a protocol based on the detection of inorganic phosphate using Malachite Green is also provided. The assay described here was used to determine the mode of binding and the efficacy of inhibition for inorganic phosphate, product alcohols and nodularin analogues described in the previous article[26] and in the determination of the direct hydrolytic ternary complex mechanism for the enzyme.[27]

Results and discussion

The most commonly used assay procedure for PP1 involves monitoring the loss of ^{32}P-labelled inorganic phosphate from the ^{32}P-labelled phosphorylated protein substrate, usually ^{32}P-glycogen phosphorylase a. Of course, different ^{32}P-labelled protein substrates are needed to assay the other protein phosphatases. A major drawback is the relatively short half life of ^{32}P (14.3 days) which necessitates the repeated preparation of the ^{32}P-labelled substrate and the performance of high numbers of control experiments which must be run simultaneously to compensate for the decay of the radioactivity in calibrating enzymic activity.[28] Such drawbacks have promoted minimalist approaches to obtaining inhibition data and, indeed, the majority of inhibition data published[32–34] for the natural products microcystin, nodularin and okadaic acid, where ^{32}P-labelled substrates were used, quote only IC_{50} values. Assay conditions often vary between laboratories making it difficult to compare results. A stopped fluorescence assay has also been used based upon the derivatisation of the components of the incubation mixture with fluorescamine. Here, the phosphorylated peptidic substrate and dephosphorylated products were separated by gel electrophoresis and the fluorescence yield in each derivative was quantified by gel densitometry.[7] The technique is useful but time consuming for multiple determinations and has a limit of detection for peptidic substrate above 0.02 nmol. Unfortunately, continuous fluorescence assays that have been developed for tyrosine phosphatases[35] that measure the fluorescence difference in phosphorylated tyrosine peptide substrates and their phenolic products cannot be used for Ser-Thr phosphatases.

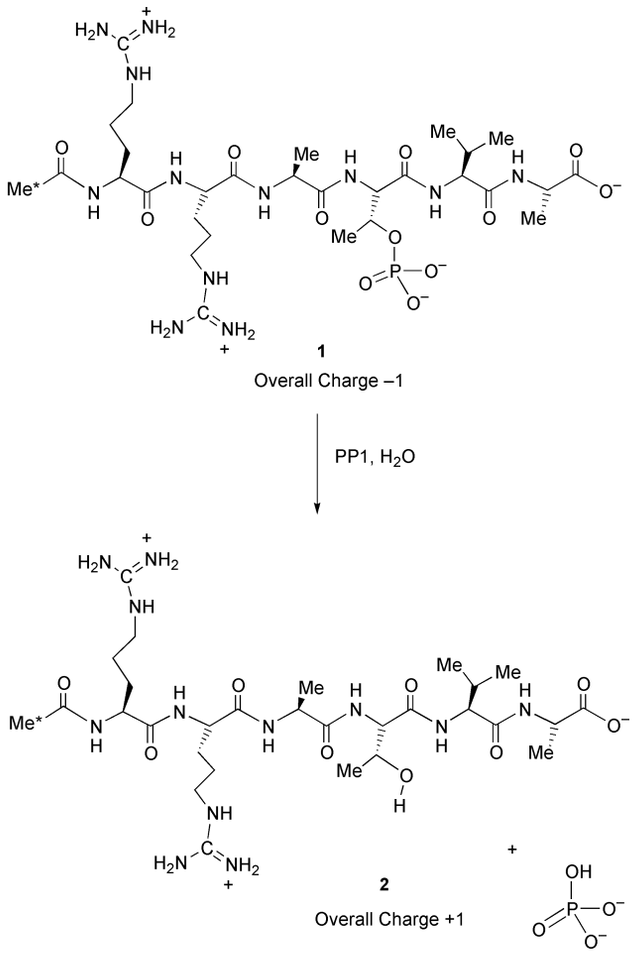
Several established assay techniques measure P_i product release, either directly or indirectly. The most widely used of these derivatises the product as the phosphomolybdate complex. In the presence of the basic dye, Malachite Green, the complex gives an intensely coloured chromophore ($\varepsilon = 90000$ M^{-1} cm^{-1}) with λ_{max} at 660 nm.[29,30] This assay technique is extremely sensitive to contaminating traces of P_i derived from, for example, distilled water, glass-ware and non-specifically hydrolysed substrate. Moreover, the overall sensitivity of the method is low and is inappropriate for the detection of nanomolar concentrations of P_i.[30] A continuous coupled assay for the detection of P_i has also been developed using purine nucleoside phosphorylase with the chromophoric substrate 7-methyl-6-thioguanosine ($\lambda_{max} = 360$ nm).[31] As for any assay which is dependent upon measuring P_i, it can not be used where P_i is present at the start of the reaction, for example, to establish the magnitude and mode of product inhibition by P_i, or, under physiological conditions where millimolar concentrations of P_i are present.[36] The ability to measure enzyme activity in the presence of P_i was important to our on-going studies[27] and hence all methods based upon Pi detection were deemed inappropriate.

The most useful generic assay for PP1 measures the hydrolysis of *p*-nitrophenyl phosphate (*p*-NPP) to *p*-nitrophenolate spectrophotometrically. The assay procedure has the advantage of monitoring hydrolysis directly and continuously but unfortunately *p*-NPP is a very poor substrate for $PP1_{cat}$ ($K_M = 18$ mM). *p*-NPP resembles the structure of the natural substrates poorly and displays an optimum activity at pH 8.0. Phosphoprotein substrates are processed most efficiently at pH 7.0[20] although, as yet, it is not known whether the phosphate group of the substrate binds to the enzyme as the dianion or the monoanion for either PP1 or PP2A. PP2B (calcineurin) which possesses an essentially identical active-site to PP1 and PP2A may process substrates ionised as the monoanionic phosphate esters.[37] Nevertheless, *p*-NPP has been used to obtain K_i values for microcystin LR, okadaic acid, calyculin A and tautomycin for both PP1 and PP2A.[20] The values were obtained by fitting the dose response data to the Morrison equation at single substrate concentrations and complete kinetic evaluations of the inhibitory characteristics were not obtained. Clearly achieving the near saturating concentrations of *p*-NPP required for good kinetic data is not trivial below pH 8.0 given its high K_M value.

In consideration of the drawbacks associated with existing assay procedures and our desire to be able to compare the inhibitory properties of products and potentially selective inhibitors against PP1 and PP2A directly,we set out to devise a new generic assay. While the natural substrates for these multi-domain enzyme systems are themselves proteins, it was known that the activity of the protein phosphatases could be measured using small synthetic phosphorylated peptides as substrates.[38–42] It had been shown that short, 6–25 residue, phosphopeptides

are moderate to good substrates for PP1 as well as PP2A, B and C.[39] The other potential benefits of using small peptides over proteins were that they could be readily prepared in high purity and be simply modified to introduce structural changes into the substrate.[41] Such compounds, whether substrates or inhibitors, would be valuable probes for mapping the active site structure of the protein and for testing the mechanism of the enzyme.[42] The six residue phosphorylated peptide Arg-Arg-Ala-Thr(P)-Val-Ala had been shown to be a good substrate for PP2A and PP2C and a moderate substrate for PP1 and PP2B.[39,40] Thus, a single assay procedure, designed around this phosphorylated substrate offered potential utility in measuring the activity of all four main Ser-Thr protein phosphatases.

We considered preparing the phosphopeptide substrate Arg-Arg-Ala-Thr(P)-Val-Ala with a ^{14}C-label as such a strategy would permit long-term storage, compared to ^{32}P-labelled phosphopeptides, and allow a single batch of substrate of known specific activity to be used for several kinetic experiments, without the need for re-synthesis. In order to provide maximum assay sensitivity and also minimise errors due to non-enzymic substrate hydrolysis, we wished to be able to measure the phosphatase activity directly, rather than indirectly, through difference calculations. Therefore, a method for reliably separating the phosphopeptide substrate from the product alcohol was required. Since preferred peptide substrates for PP1 and PP2A typically possess two positively charged N-terminal Arg residues,[40] a dianionic phosphothreonine residue and opposite charges for the ammonium and carboxylate groups at the extremes of the molecule, such substrates are charge neutral overall. The *N*-terminal acetylation of the substrate sequence Arg-Arg-Ala-Thr(P)-Val-Ala was known not to compromise substrate activity for PP2A[38] and this acetylated phosphopeptide (**1**) would possess an overall single negative charge at *ca*. pH 7.0–8.0, the optimum pH for PP1 and PP2A activity. Given that the hydrolysis of the phosphate ester would result in the formation of a peptide alcohol (**2**) possessing an overall single positive charge, we opted to employ an ion exchange resin that would only bind to the negatively charged substrate molecule. Thus, the amount of eluted radioactivity would correlate directly with the extent of hydrolysis (Scheme 2).



Scheme 2

The strongly basic anion exchange resin Dowex **1**, which contains a resin-bound trimethylbenzylammonium moiety, seemed appropriate. Test trials showed a good separation of the phosphopeptide (**1**) from the peptide alcohol (**2**). In order to introduce a radiolabel into the peptide, we opted to use a late stage *N*-terminal acetylation since [1-^{14}C]acetic anhydride is commercially available. The purified radiolabelled phosphopeptide was then used to optimise the assay procedure as described below.

The ^{14}C-labelled phosphorylated hexapeptide substrate [*acetyl*-1-^{14}C]Ac-Arg-Arg-Ala-Thr(P)-Val-Ala was synthesised following the method previously communicated by Hormozdiari and Gani.[43] The resin-bound hexapeptide Arg(pmc)-Arg-(pmc)-Ala-Thr-Val-Ala was constructed using solid-phase synthesis on Wang resin and was then *N*-acetylated using [1-^{14}C]acetic anhydride. Excess unlabelled acetic anhydride was used to ensure complete acetylation. The resin-bound *N*-acetylated peptide was subsequently phosphorylated with bis(pentafluorophenyl) chlorophosphate.[43,44] This reagent was known to be a highly active phosphorylating agent where both of the pentafluorophenyl protecting groups could be removed under the acidic conditions used for peptide cleavage from the resin. The ^{14}C-radiolabelled hexapeptide substrate was diluted with non-labelled acetylhexapeptide such that the final specific activity of the mixture was 0.64 mCi mmol^{-1}.

A simple and rapid method for the separation of phosphorylated hexapeptide substrate from the dephosphorylated product was developed based on centrifugation–anion exchange chromatography, utilising the difference in negative charge of the two molecules. Anion exchange spin columns, suitable for centrifugation in a microcentrifuge, were modified Promega DNA purification columns (see Experimental). Under the conditions of the assay the phosphorylated ^{14}C-labelled *N*-acetylhexapeptide bound tightly to the resin, whilst the dephosphorylated ^{14}C-labelled *N*-acetylhexapeptide did not bind and could be recovered by elution. This provided a very simple and rapid method for separating the substrate and product from enzyme incubations. Control experiments at 30 μM substrate concentration indicated no leakage through the column.

The time course for hydrolysis was monitored in initial experiments using a substrate concentration of 2.52 mM and approximately 0.01 units of PP1activity. Aliquots were removed at regular time intervals over a total period of 20 minutes and the reaction was quenched by adding the aliquot to an equivalent volume of aqueous KF. The solution was then subjected to anion exchange spin chromatography. In separate experiments it was shown that the presence of KF did not affect the binding of either the substrate or the product to the Dowex-1 × 200 spin columns. The eluted dephosphorylated product was analysed for ^{14}C-content by scintillation counting and the radioactivity was compared to that of an equivalent amount of unreacted substrate, the $t = 0$ reading, to give the extent of conversion at any given time. The resulting data showed that the enzyme catalysed reaction was linear over the first 2 minutes and proceeded to 60% conversion after 30 minutes. This protocol formed the basis for the stopped assay where conditions were varied and where the reactions were quenched after 1 and 2 minutes.

The new assay protocol was first utilised for the determination of V_{max} and K_M for Ac-Arg-Arg-Ala-Thr(P)-Val-Ala in a direct comparison to the Malachite Green assay. The variation of initial rate with substrate concentration was

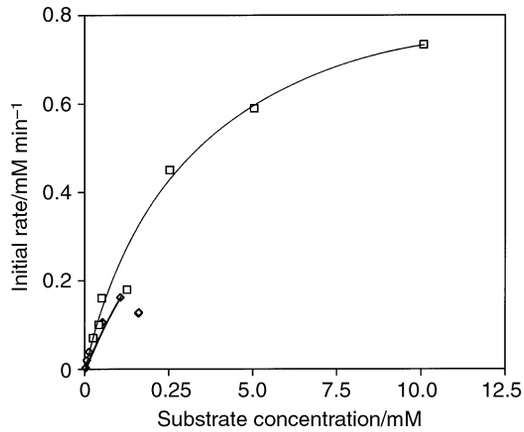


Fig. 1 Michaelis–Menten plots of initial rate data from Malachite Green (diamonds) and ^{14}C-radiolabelled (squares) assays.

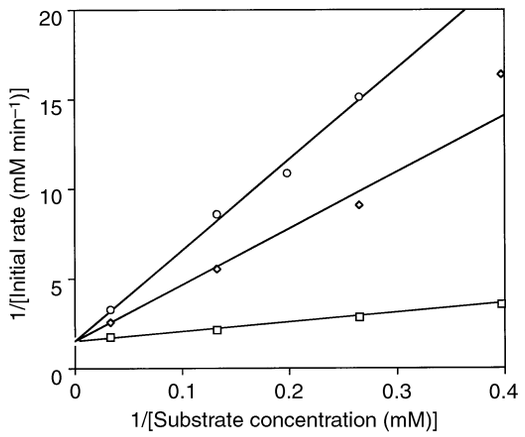


Fig. 2 Lineweaver–Burk plot of phosphate inhibition data. Substrate concentration over the range 2.25 to 30.18 mM at three phosphate concentrations, Squares = 0 mM, Diamonds = 5 mM and Circles = 10 mM.

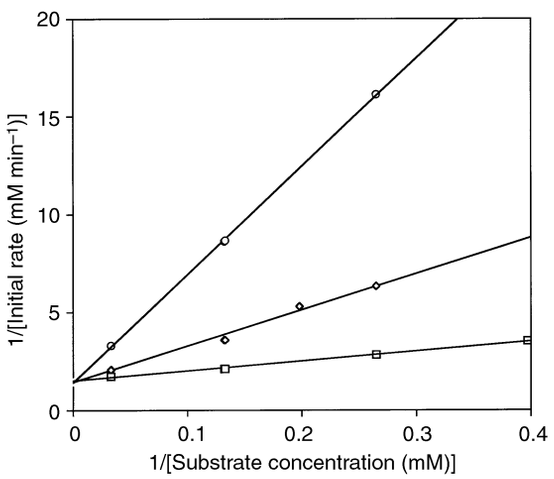


Fig. 3 Lineweaver–Burk plot of nodularin inhibition data. Substrate concentration over the range 2.25 to 30.18 mM at three nodularin concentrations, Squares = 0 nM, Diamonds = 0.2 nM and Circles = 1 nM.

determined from duplicate assays and the resulting plot is shown in Fig. 1. The data were analysed by non-linear regression analysis to afford values of V_{max} and K_M for the phosphorylated ^{14}C-labelled *N*-acetylhexapeptide with PP1 of 17 (±2) μM s^{-1} [28 (±3) μM s^{-1} μg^{-1}] and 3.7 (±0.9) mM respectively. The assay was repeated using PP2A to afford a V_{max} of 2.8 (±0.2) μM s^{-1} [45 (±2) μM s^{-1} μg^{-1}] and a K_M of 4.1 (±0.7) mM. The Malachite Green assay gave values for V_{max} and K_M of 14 (±2.3) μM s^{-1} [23 (±4) μM s^{-1} μg^{-1}] and 1.5 (±0.8) mM, respectively, for PP1. The comparison of the two protocols demonstrated that the new assay is not just easier to perform but is also more accurate and is applicable to both PP1 and PP2A. The large errors in the Malachite Green assay arise largely from P_i associated with high substrate concentrations. This limits the maximum usable concentration of substrate to 2 mM, whereas for the radiochemical assay the usable concentration range extends beyond 11 mM, three times the K_M value.

One of the important advantages of the ^{14}C-labelled *N*-acetylhexapeptide assay protocol is its ability to be utilised in the presence of inorganic phosphate. Preliminary tests showed that P_i concentrations up to 10 mM had no adverse effects on the performance of the ion exchange resin in the separation of the product and substrate peptides. Therefore, the new assay protocol was used to assess the mode and magnitude of P_i product inhibition. Variations in the initial rate for the PP1-catalysed hydrolysis reaction with varying substrate concentration at each phosphate concentration were fitted by non-linear regression analysis to obtain values for V_{max}(app) and K_M(app) (see Fig. 2). V_{max} was unaffected by P_i concentration indicative of competitive inhibition. A plot of V_{max}/K(app) against P_i concentration gave a K_i value of 1.6 (±0.9) mM.

The radiochemical assay was also used to determine the inhibitor characteristics of nodularin to provide direct comparison with the literature where an IC_{50} value of 1 nM has been reported.[17] Initial rate data at varying substrate concentration for each nodularin concentration were fitted by non-linear regression analysis. V_{max} was unaffected by nodularin concentration in accord with a competitive mode of inhibition with respect to the substrate (see Fig. 3) and a plot of V_{max}/K(app) against nodularin concentration gave a K_i value of 0.19 (±0.05) nM. These results confirm that the new assay can be used with confidence to provide kinetic data for the PP1-catalysed dephosphorylation reaction. Exactly the same protocols were used for the determination of mode and magnitude of inhibition displayed by nodularin analogues described in the second article[26] in this series.

In conclusion, the results show that the new assay based on the rapid separation of radiolabelled *N*-acetyl-Arg-Arg-Ala-Thr(P)-Val-Ala and *N*-acetyl-Arg-Arg-Ala-Thr-Val-Ala provides a useful kinetic tool for assessing PP1 and PP2A activity and potentially, other Ser-Thr protein phosphatases. Moreover, the protocol allows for a direct comparison of the activities of inhibitors for PP1 and PP2A and is sensitive and flexible enough to be used over the wide range of substrate concentrations required for the determination of K_i values. The assay can be used when P_i is present and can be used for the rapid screening of inhibitors in a single determination mode at high substrate concentration. Such protocols could be employed in medium throughput screens and could be extended to any or all of the Ser-Thr protein phosphatases by simply performing parallel assays containing the radiolabelled substrate and the required target phosphatase enzyme(s). This assay system is now being used in the evaluation of new selective inhibitors based upon nodularin, (see previous articles[25,26]), in mechanistic studies,[27] and in the determination of the catalytic modifications caused by allosteric regulatory modifiers (see Scheme 1).

Experimental

General

Magnesium chloride hexahydrate, tris, manganese(II) dichloride, *p*-nitrophenyl phosphate, trifluoroacetic acid, [1-^{14}C]-acetic anhydride, triethylsilane, isopropylthio-β-D-galactopyranoside (IPTG) and dithiothreitol were purchased from

Sigma Chemical Co. (Poole, Dorset). A recombinant *Escherichia coli* which expresses PP1 was used as a source of PP1 and was a gift from Dr Raj Beri of AstraZeneca, Alderley Park. PP2A (purified from human red blood cells) was purchased from TCS Biologicals Ltd (Claydon, Bucks). Ni-NTA resin was purchased from Quiagen, Ltd. (Crawley, Sussex). Fmoc amino acids, Fmoc Ala-Wang resin and PyBOP were purchased from Novabiochem (CN Biosciences UK, Beeston Nottingham). Bis(pentafluorophenyl) chlorophosphate was prepared as described by Hormozdiari and Gani[43] using a modification of a patented procedure.[45] All other chemicals were of analytical grade or were purified prior to use. Radioactivity was measured on a Packard 2500TR liquid scintillation analyser using Packard Emulsifier Safe™ scintillation fluid.

Purification of recombinant PP1

The catalytic subunit of PP1 used in all experiments was an *N*-terminal hexaHis tagged recombinant human protein expressed in *E. coli*. The recombinant cells were grown in 1 dm^3 batches in Luria broth and expression was induced with IPTG. After cell lysis the hexaHis tagged PP1 was purified in one step on an Ni-NTA resin column. The pure protein was characterised and its properties were compared to those reported for the wild-type enzyme. The protein displayed the expected mobility on SDS-PAGE and showed comparable requirements for activity for divalent metal ions and a preference for manganese ions. The protein which catalysed the hydrolysis of *p*-NPP, was sensitive to nodularin inhibition in the nanomolar range and was affected by two classes of PP1-specific allosteric activator. Specific activity was 20 units mg^{-1} when diluted to 0.1 μg cm^{-1} in tris buffer at pH 7.5 containing 0.2 μm $MnCl_2$. Full details will be reported elsewhere. For long-term storage at −20 °C, the enzyme was dialysed into 50% aqueous glycerol.

Synthesis of substrates

Ac-RRAT(P)VA. The hexapeptide Arg(pmc)-Arg(pmc)-Ala-Thr-Val-Ala-Wang resin was prepared using α-amino group Fmoc protection and PyBOP coupling chemistry on an automated solid-phase peptide synthesiser. The amino acids and coupling reagents were used in a 2-fold excess and arginine, threonine and valine residues were all subject to double coupling programmes.

To Arg(pmc)-Arg(pmc)-Ala-Thr-Val-Ala-Wang resin (246.8 mg, 0.1 mmol) in dimethylformamide (2 cm^3) was added acetic anhydride (0.1 cm^3, 0.3 mmol) and the mixture gently stirred under dry argon for 1 h at room temperature. The resin was filtered and washed with dimethylformamide (10 × 5 cm^3) and then re-suspended in dry dichloromethane (2 cm^3). Dimethylaminopyridine (12 mg, 0.1 mmol), triethylamine (1 cm^3, 12 mmol,) and bis(pentafluorophenyl) chlorophosphate (1.7 g, 2 mmol) were then added. The reaction was stirred under dry argon for 24 h and the resin was then washed alternately with dichloromethane and methanol (5 cm^3 each ×10). To the resin was then added trifluoroacetic acid–water–triethylsilane (90:5:5, 10 cm^3) and the mixture stirred for 3 h at room temperature. The resin was removed by filtration and was washed with water (2 × 2 cm^3). The filtrate was concentrated to 0.5 cm^3 under reduced pressure and then diethyl ether (50 cm^3) was added to precipitate the phosphopeptide. The supernatant solution was decanted and the peptide was washed with further portions of diethyl ether (3 × 50 cm^3). The phosphopeptide was then purified by anion exchange chromatography on Dowex 1 × 200 (1 × 3 cm). The column was washed with water [3 column volumes (CV)] and the phosphopeptide was eluted with 0.1 mol dm^{-3} trifluoroacetic acid (3 CV). The crude phosphopeptide was further purified by reversed phase C18 HPLC (1 × 25 cm) eluting with a gradient of 100% water–trifluoroacetic acid (99.1:0.1) to 100% acetonitrile–trifluoroacetic acid–water (99.7:0.05:0.25) over 30 CV. The phosphopeptide eluted with a retention value of 4.4 CV to afford, upon lyophilisation, a white solid in 56% yield (38 mg). δ_H(300 MHz; ^2H_2O) 0.96 [6 H, d, *J* 6.3, CH(CH_3)$_2$ (Val)], 1.20 [3 H, d, *J* 6.3, CH_3 (Thr)], 1.38 [6 H, m, 2 × CH_3 (Ala)], 1.58 [4 H, m, 2 × $CH_2CH_2CH_2$ (Arg)], 1.69 [4 H, m, 2 × CH_2CH (Arg)], 1.93 (3 H, s, acetyl CH_3) and 2.00 [1 H, m, β-CH (Val)], 3.11 [4 H, m, 2 × CH_2NH (Arg)], 4.00–4.55 (7 H, m, α and β CH); δ_C(75.5 MHz; ^2H_2O) 18.54 and 20.19 [Val CH(CH_3)$_2$-(Val)], 19.03 and 20.35 [2 × Ala CH_3(Ala)], 20.75 [CH_3 (Thr)], 24.10 (acetyl CH_3), 26.87 [2 × $CH_2CH_2CH_2$(Arg)], 30.70 [2 × $CH_2CH_2CH_2$ (Arg)], 32.86 [CH (Val)], 43.07 [2 × CH_2NH (Arg)], 51.21 [2 × α-CH (Ala)], 55.56 and 55.93 [2 × α-CH (Arg)], 61.07 [β-CH (Thr)], 61.79 [α-CH_3 (Val)], 74.27 [α-CH (Thr)], 159.29 [2 × CN (Arg)], 173.20 (CH_3CO), 175.20–177.42 (5 C amide CO) and 178.70 (COOH); δ_P(121.5 MHz; ^2H_2O) −0.01; *m*/*z* (FAB+) 795 (M^+).

A sample of the phosphopeptide (0.8 mM) in tris buffer (30 mM, pH 7.5) was dissolved in 70% ^2H_2O in water and placed in an NMR tube. After the addition of PP2A the dephosphorylation was found to be complete in 45 min by ^1H NMR spectroscopy (monitored by observing the signals for the threonine CH_3).

[acetyl-1-^{14}C]Ac-RRAT(p)VA. [*acetyl*-1-^{14}C]Ac-Arg-Arg-Ala-Thr(P)-Val-Ala was prepared in a manner identical to that described for the synthesis of unlabelled phosphopeptide using [1-^{14}C]acetic anhydride (16 mCi $mmol^{-1}$ total ^{14}C = 1.6 mCi) as the acylating agent. The labelled phosphopeptide was purified by anion exchange chromatography on Dowex 1 × 200 as before and further HPLC purification afforded a white solid in 22% chemical yield (18 mg) and 18% radioactive yield (0.32 mCi). The TLC R_f of 0.25 (silica gel, 10% conc. NH_4OH in methanol) and the HPLC (column and gradient as for unlabelled material) retention volume of 4.3 CV were identical with the mobilities of the unlabelled material. *m*/*z* (FAB$^+$) 796 (M^+).

Malachite Green assay

Preparation of the Malachite Green reagent.[29] A solution of Malachite Green dye (1.5 g) in HCl (0.2 M, 780 ml) was added to a solution of ammonium molybdate (10.5 g) in HCl (5 M, 220 cm^3) with vigorous stirring. The resulting solution was filtered and stored in the dark until required. K_2HPO_4 (181.87 mg) was dissolved in water (25 cm^3) to afford a standard solution of 1.67 mM inorganic phosphate. Aliquots of this solution (0, 25, 50, 75, 100 mm^3) were mixed with Malachite Green reagent (1 cm^3) and the colour was allowed to develop for 10 min before the absorbance was measured at 660 nm. The data were plotted as nmol of phosphate in the assay medium *versus* absorbance.

Time–course. The substrate Ac-Arg-Arg-Ala-Thr(P)-Val-Ala solution (5 mm^3, 10 mg cm^{-3}, 12.57 mM) and enzyme (5 mm^3, 0.05 units) were incubated at 25 °C in 50 mM tris buffer at pH 7.5 containing 0.5 M NaCl, 1 mM $MgCl_2$, 0.2 mM $MnCl_2$, and 0.5 mM DTT (15 mm^3), [total volume 25 mm^3 (final concentrations substrate 2.52 mM, tris 30 mM, NaCl 0.3 M, Mg^{2+} 0.6 mM, Mn^{2+} 0.12 mM and DTT 0.3 mM)] for 3 min. The reaction was quenched by the addition of the Malachite Green reagent (1 cm^3) and the colour was allowed to develop for 10 min. The absorbance was measured at 660 nm. This predetermination facilitated the choice of a suitable duration for the time–course experiment. The assay procedure was repeated using a total volume of 125 mm^3 and aliquots of 25 mm^3 were removed and were quenched after 1, 2, 5 and 10 min by the addition of Malachite Green reagent. The absorbance data were converted into nmol of phosphate released by comparison with the calibration curve to afford a time–course for hydrolysis.

Kinetics. The assay procedure above was followed using enzyme solution (5 mm^3, 0.05 units) and 50 mM tris buffer at pH 7.5 containing 0.5 M NaCl, 1 mM $MgCl_2$, 0.2 mM $MnCl_2$, and 0.5 mM DTT (15 mm^3) and various substrate concentrations in the range 0.1–5.04 mM, in a total volume of 25 mm^3. The reaction was incubated at 25 °C for 3 min (while the rate was still constant) and was then quenched by the addition of Malachite Green reagent (1 cm^3). The absorbance data were used to calculate an initial rate for each substrate concentration. The initial rate data were analysed by non linear regression analysis, (see Fig. 1) to give values for V_{max} and K_M for the substrate Ac-Arg-Arg-Ala-Thr(P)-Val-Ala with PP1.

Radiochemical assay

The radiochemical stopped assay was carried out routinely using anion exchange columns as described in the following general method.

General procedure. [Acetyl 1-^{14}C]Ac-Arg-Arg-Ala-Thr(P)-Val-Ala [12 mm^3 of a 10 mg cm^{-3} stock (specific activity 0.64 mCi $mmol^{-1}$)] and enzyme extract (12 mm^3, 0.01 units) were incubated at 25 °C in 50 mM tris buffer at pH 7.5 containing 0.5 M NaCl, 1 mM $MgCl_2$, 0.2 mM $MnCl_2$, and 0.5 mM DTT (36 mm^3) in a total volume of 60 mm^3. Aliquots (25 mm^3) were removed after 1 and 2 min and the reaction quenched by the addition of an equal volume of 0.2 M KF. Unreacted substrate and dephosphorylated product were separated by anion exchange chromatography using spin microcentrifuge columns prepared from Promega DNA purification columns. The original filtration membrane was replaced with a small plug of glass wool and the column was packed with Dowex 1 × 200 resin (100 mg). The resin was prewashed with 1 M NaOH (1 CV, 300 mm^3) and then distilled water (5 CV, 1.5 cm^3) and the washings were completely removed from the columns by centrifugation (Microcentaur at 2000 rpm for 15 s). The quenched aliquots of the incubation solution were loaded on to the prepared columns and the unbound dephosphorylated product was eluted with 4% ethanol in water (3 × 200 mm^3) with centrifugation between each addition. The combined eluents were added to Emulsifier Safe scintillation fluid (3.5 cm^3) and the ^{14}C-content was determined by scintillation counting. It was found that the most reproducible results were obtained when fresh Dowex 1 × 200 resin was used for each sample.

Time–course. A time–course for the PP1-catalysed reaction was performed using the radiochemical assay in a total volume of 160 mm^3 containing 32 mm^3 of PP1 solution and 32 mm^3 of substrate solution in 96 mm^3 buffer. The reactions were started by the addition of enzyme. Several 25 mm^3 aliquots were removed at intervals over 20 min and were quenched and processed as described above. A control reference for $t = 0$ min was obtained by preparing a solution containing 0.5 M KF (25 mm^3), buffer (20 mm^3) and substrate (5 mm^3) and treating this in the same way as for the quenched aliquots. A control reference equivalent to 100% substrate conversion was obtained by diluting substrate stock solution (5 mm^3) with distilled water (600 mm^3) and then adding to this scintillation fluid (3.5 cm^3) for ^{14}C-content determination by scintillation counting. The radioactive content for each time point was corrected by subtracting a control for $t = 0$ min. This was then converted to % conversion by reference to the value for 100%.

Kinetics. The radiochemical assay procedure above was followed using enzyme solution (12 mm^3, 0.01 units) in 50 mM tris buffer at pH 7.5 containing 0.5 M NaCl, 1 mM $MgCl_2$, 0.2 mM $MnCl_2$, and 0.5 mM DTT (36 mm^3) and various concentrations of substrate (from 0.25 mM to 10.08 mM) in a total volume of 60 mm^3. Reactions were initiated by the addition of enzyme and aliquots (25 mm^3) were removed and quenched at $t = 1$ and 2 min and were processed as described above. All reactions were carried out in duplicate and the 2 time points were plotted as percentage conversion *versus* time. The initial rate of reaction was calculated for each substrate concentration from a linear regression through $t = 0$ and the two time points and rate data were analysed by non-linear regression analysis to give values of K_M and V_{max} (see Fig. 1).

Phosphate inhibition. The radiochemical assay procedure above was followed using enzyme (12 mm^3, 0.01 units) in 50 mM tris buffer at pH 7.5 containing 0.5 M NaCl, 0.2 mM $MnCl_2$, and 0.5 mM DTT (24 mm^3) and P_i solution (12 mm^3), to give final phosphate concentrations of between 0 and 10 mM, and various concentrations of substrate over the range 2.25 to 30.18 mM in a total volume of 60 mm^3. Reactions were initiated by the addition of enzyme and aliquots (25 mm^3) were removed and quenched at $t = 1$ and 2 min and were processed as described above. All reactions were carried out in duplicate. Initial rate data for each phosphate concentration *versus* substrate concentration were analysed by non-linear regression to afford values for V(app) and V/K_M(app). These values were plotted against inhibitor concentration to obtain a K_i value for P_i.

Nodularin inhibition. The radiochemical assay procedure above was followed using enzyme (12 mm^3, 0.01 units) in 50 mM tris buffer at pH 7.5 containing 0.5 M NaCl, 0.2 mM $MnCl_2$, and 0.5 mM DTT (24 mm^3), nodularin solution (12 mm^3), to give final nodularin concentrations between 0 and 1 nM, and various concentrations of substrate over the range 2.25 to 30.18 mM, all in a total volume of 60 mm^3. The enzyme was incubated at 25 °C with the nodularin for 1 min before the reaction was initiated by the addition of the substrate. Aliquots (25 mm^3) were removed after 1 and 2 min after initiation and were quenched and processed as described above. All reactions were carried out in duplicate and initial rate data were treated as described for the P_i inhibition experiment above to obtain a value for K_i for nodularin.

Inhibition by nodularin analogues. The radiochemical assay procedure described above was employed using the same substrate concentrations and appropriate inhibitor concentrations. The K_i values and the modes of inhibition were determined and are reported in the previous article.[26]

PP2A kinetics

The time course and kinetics experiments were repeated as described for PP1 but using purified PP2A (source: human red blood cells). The enzyme preparation contained 0.1 milliunits cm^{-3}.

Acknowledgements

The authors wish to thank Dr R. Beri for providing a recombinant strain of *E. coli* expressing PP1; Drs C. Hamilton and M. Abbott for preliminary work on the characterisation of the protein and for discussion; Dr M. O'Donnell for useful suggestions and Dr M. Akhtar for scientific support. We thank the BBSRC Biomolecular Sciences Committee for grant B/49/BO7992 and AstraZeneca for a Strategic Research Fund grant and a CASE award to P. H.

References

1 L. N. Johnson and D. Barford, *Annu. Rev. Biophys. Biomol. Struct.*, 1997, **22**, 199.
2 D. Gani and J. Wilkie, in *Structure and Bonding*, eds. H. A. Hill, P. Sadler and A. H. Thompson, Springer-Verlag, Heidelberg, 1997, p. 133.

3 N. Berndt, *Front. Biosci.*, 1999, **4**, d22.
4 Z. Jia, *Biochem. Cell. Biol.*, 1997, **75**, 17.
5 M. Bollen and P. T. W. Cohen, *Biochem. J.*, 1998, **333**, 253.
6 T. Kawatabe, A. J. Muskin and S. J. Korsmeyer, *Nature*, 1997, **385**, 454.
7 A. Takai and G. Mieskes, *Biochem. J.*, 1991, **775**, 233.
8 E. Y. C. Lee, L. Zhang, S. Zhao, Q. Wei, J. Zhang, Z. Q. Qi and E. R. Belmonte, *Front. Biosci.*, 1999, **4**, d270.
9 C. G. Armstrong, M. J. Doherty and P. T. W. Cohen, *Biochem. J.*, 1998, **336**, 699.
10 G. A. Nimmo and P. Cohen, *Eur. J. Biochem.*, 1978, **87**, 353.
11 D. F. Johnson, G. Moorhead, F. B. Caudwell, P. Cohen, Y. H. Chen, M. X. Chen and P. T. W. Cohen, *Eur. J. Biochem.*, 1996, **239**, 317.
12 N. Berndt, D. G. Campbell, F. B. Caudwell, P. Cohen, E. F. da Cruz e Silva, O. B. da Cruz e Silva and P. T. W. Cohen, *FEBS Lett.*, 1987, **223**, 340.
13 G. J. Barton, P. T. W. Cohen and D. Barford, *J. Mol. Biol.*, 1994, **235**, 763.
14 J. Goldberg, H.-B. Huang, Y.-G. Kwon, P. Greengard, A. C. Nairn and J. Kurinan, *Nature*, 1995, **376**, 745.
15 J. R. Bagu, B. D. Sykes, M. M. Craig and C. F. B. Holmes, *J. Biol. Chem.*, 1997, **272**, 5087.
16 M. K. Lindvall, P. M. Pihko and A. M. P. Koskinen, *J. Biol. Chem.*, 1997, **272**, 23312.
17 R. E. Honkanen, M. Dukelow, J. Zwiller, R. E. Moore, B. S. Khatra and A. L. Boynton, *Mol. Pharmacol.*, 1991, **40**, 577.
18 M. Runnegar, N. Berndt, S.-M. Kong, A. L. Lee and L. Zhang, *Biochem. Biophys. Res. Commun.*, 1995, **216**, 162.
19 A. McClusky, C. Taylor, R. J. Quinn, M. Suganuma and H. Fujiki, *Bioorg. Med. Chem. Lett.*, 1996, **6**, 1025.
20 A. Takai, K. Sasaki, H. Nagai, G. Mieskes, M. Isobe, K. Isono and T. Yasumoto, *Biochem. J.*, 1995, **306**, 657.
21 J. B. Aggen, J. M. Humphrey, C. M. Gauss, H. B. Huang, A. C. Nairn and A. R. Chamberlain, *Bioorg. Med. Chem.*, 1999, **7**, 453.
22 A. P. Mehrotra and D. Gani, *Tetrahedron Lett.*, 1996, **37**, 6915.
23 A. P. Mehrotra, K. L. Webster and D. Gani, *J. Chem. Soc., Perkin Trans. 1*, 1997, 2495.
24 A. B. Maude, A. P. Mehrotra and D. Gani, *J. Chem. Soc., Perkin Trans. 1*, 1997, 2513.
25 First paper in this series: K. L. Webster, A. B. Maude, M. E. O'Donnell, A. P. Mehrotra and D. Gani, *J. Chem. Soc., Perkin Trans. 1*, 2001, (DOI: 10.1039/b100401h).
26 Preceding paper: M. E. O'Donnell, J. Sanvoisin and D. Gani, *J. Chem. Soc., Perkin Trans. 1*, 2001, (DOI: 10.1039/b100402f).
27 J. Sanvoisin and D. Gani, *Bioorg. Med. Chem. Lett.*, 2001, **11**, 471.
28 Y. Kim and H. R. Matthews, *Anal. Biochem.*, 1993, **211**, 28.
29 K. Itaya and M. Ui, *Clin. Chem. Acta*, 1966, **14**, 361.
30 H. H. Hess and J. E. Derr, *Anal. Biochem.*, 1975, **63**, 607.
31 Q. Cheng, W.-X. Wang and S. D. Killilea, *Anal. Biochem.*, 1995, **226**, 68.
32 J. H. Connor, T. Kleeman, S. Barik, R. E. Honkanen and S. Shenolikar, *J. Biol. Chem.*, 1999, **274**, 22366.
33 L. Shi, W. W. Carmichael and P. J. Kennelly, *J. Biol. Chem.*, 1999, **274**, 10039.
34 Z. Zhang, S. Zhao, F. Long, L. Zhang, G. Bai, H. Shima, M. Nagao and E. Y. C. Lee, *J. Biol. Chem.*, 1994, **269**, 16997; R. Samy, H. Y. Kim, M. Brady and P. T. Toogood, *J. Org. Chem.*, 1999, **64**, 2711.
35 C. GarciaEchevenera and D. H. Rich, *Bioorg. Med. Chem. Lett.*, 1993, **3**, 1601.
36 H. S. Tenenhouse and C. R. Scriver, *J. Clin. Invest.*, 1975, **55**, 644.
37 B. L. Martin and D. J. Graves, *J. Biol. Chem.*, 1986, **261**, 14545.
38 P. Hormozdiari, PhD thesis, University of St Andrews, 1996.
39 L. A. Pinna and A. Donella-Deana, *Biochim. Biophys. Acta*, 1994, **1222**, 415.
40 A. Donella-Deana, M. H. Krinks, M. Ruzzene, C. Klee and L. A. Pinna, *Eur. J. Biochem.*, 1994, **219**, 109.
41 M. P. Lutz, D. I. Pinon and L. J. Miller, *Anal. Biochem.*, 1994, **220**, 268.
42 B. L. Martin, S. Luo, A. Kinlanar, M. Chen and D. J. Graves, *Arch. Biochem. Biophys.*, 1998, **359**, 179.
43 P. Hormozdiari and D. Gani, *Tetrahedron Lett.*, 1996, **37**, 8227.
44 M. Hillier, PhD thesis, University of St Andrews, 2000.
45 R. H. Boschan and J. P. Holder, UAP 3,341,630/1967.