A phosphotyrosine-containing quenched fluorogenic peptide as a novel substrate for protein tyrosine phosphatases

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Mca-Gly-Asp-Ala-Glu-Tyr(PO_3H_2)-Ala-Ala-Lys(DNP)-Arg-NH_2, where Mca is (7-methoxycoumarin-4-yl)acetyl and DNP is 2,4-dinitrophenyl, was synthesized as a fluorogenic substrate for protein tyrosine phosphatases (PTPs). In the peptide, the fluorescent Mca group is quenched efficiently by the DNP group. Although the fluorescence intensity of the substrate was practically unchanged upon PTP-catalysed dephosphorylation, it increased approx. 120-fold upon subsequent treatment with chymotrypsin. Analysis by HPLC showed that chymotrypsin cleaved only the dephosphorylated substrate at the Tyr-Ala bond. Thus with the aid of chymotrypsin, dephosphorylation of the substrate can be measured fluorometrically. A strictly linear correlation was observed between PTP concentration and dephosphorylation rate. The fluorogenic substrate was dephosphorylated by some PTPs much more rapidly than the corresponding ^{32}P-labelled substrate used for comparison, whereas alkaline phosphatase dephosphorylated the two substrates at similar rates. The fluorogenic substrate is therefore more specific for PTPs than the radiolabelled substrate. The assay with the fluorogenic substrate could be applied to the estimation of kinetc parameters and measurement of PTP activity in crude-enzyme preparations. The lower detection limit of our assay (1 μM substrate in 200 μl of reaction mixture) was estimated to be 0.2–0.4 pmol, whereas it was estimated to be about 1 pmol in the assay that used ^{32}P-labelled peptide (specific radioactivity of approx. 1000 c.p.m./pmol). Our assay is simple, specific, highly sensitive and non-radioisotopic, and hence would contribute greatly to the development of PTP biology.

Key words: chymotrypsin, dephosphorylation, fluorimetric assay, non-radioisotopic assay, resonance-energy transfer.

INTRODUCTION

Protein tyrosine phosphatases (PTPs), which catalyse the dephosphorylation of phosphotyrosine residues, are involved in many biologically important processes [1–5]. PTP activity is usually measured using phosphotyrosine-containing proteins or peptides as specific substrates. Dephosphorylation of these substrates can be measured in several ways. The released phosphate is measured by colorimetry as a Malachite Green–phosphomolybdate complex [6]. This method can detect sub-nmol amounts of phosphate but is greatly distorted by endogenous phosphate in crude-enzyme preparations. Another assay, which takes advantage of the increase in absorbance or fluorescence upon dephosphorylation, permits continuous monitoring of dephosphorylation [7,8]. The lower detection limit of the spectrophotometric method is in the nmol range, whereas the fluorimetric method is nearly two orders of magnitude more sensitive. Although the continuous monitoring seems especially useful in kinetic studies, proteins in crude-enzyme preparations or proteins added as enzyme stabilizers to the assay mixture interfere with the measurements, since the assay is conducted at a wavelength where proteins absorb and fluoresce. A similar assay is presented that uses phosphotyrosine as a substrate [9]. Quantification of the dephosphorylated peptide by HPLC [9,10] and use of anti-phosphotyrosine antibodies to detect remaining phosphopeptides [10,11] may be highly sensitive but are time-consuming and laborious, and hence are not convenient for practical use. Probably the most sensitive and widely employed assay uses ^{32}P-labelled substrates [12]. The radioactive phosphate released from [^{32}P]phosphotyrosine residues is measured after organic-solvent extraction as a phosphomolybdate complex or separation from unreacted protein substrate by acid precipitation. Although the lower detection limit of the radioisotopic assay depends on the specific radioactivity of [^{32}P]phosphate, sub-pmol to pmol amounts of phosphate may be detectable if the specific radioactivity is about 1000 c.p.m./pmol. Despite the high sensitivity of the radioisotopic assay, the preparation and use of radiolabelled substrates have some drawbacks: (i) the phosphorylation of proteins or peptides, which uses appropriate tyrosine kinases and [γ-^{32}P]ATP, is not stoichiometric in most cases, resulting in lot-to-lot variation in the degree of phosphorylation; (ii) phosphorylation may occur at more than one tyrosine residue in a protein, making the interpretation of the experimental data complicated; (iii) the short half-life of ^{32}P (14 days) makes it difficult to use the same lot over a long period of time; and (iv) special care must be taken to avoid unnecessary exposure to radiation. The drawbacks associated with the PTP assays described above seem to constitute some of the reasons why research on protein dephosphorylation has lagged behind that of protein phosphorylation. Therefore development of a new assay procedure that is non-radioisotopic, at least as sensitive as existing radioisotopic assays, and compatible with contaminants such as inorganic phosphate and proteins, is strongly desired.

In the present study, we have developed a novel non-radioisotopic method for the assay of PTPs (Scheme 1 shows the principle of the assay). The assay uses a fluorogenic phosphopeptide substrate internally quenched by resonance-energy transfer. The substrate contains a fluorophore group, (7-methoxycoumarin-4-yl)acetyl (Mca), and a quencher group, 2,4-dinitrophenyl (DNP) [13], in addition to a phosphotyrosine

Abbreviations used: PTP, protein tyrosine phosphatase; Mca, (7-methoxycoumarin-4-yl)acetyl; DNP, 2,4-dinitrophenyl; Fmoc, 9-fluorenylmethoxycarbonyl; PyBOP, benzotriazol-1-yl-oxytris-pyrrolidinophosphonium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; PP, protein phosphatase.

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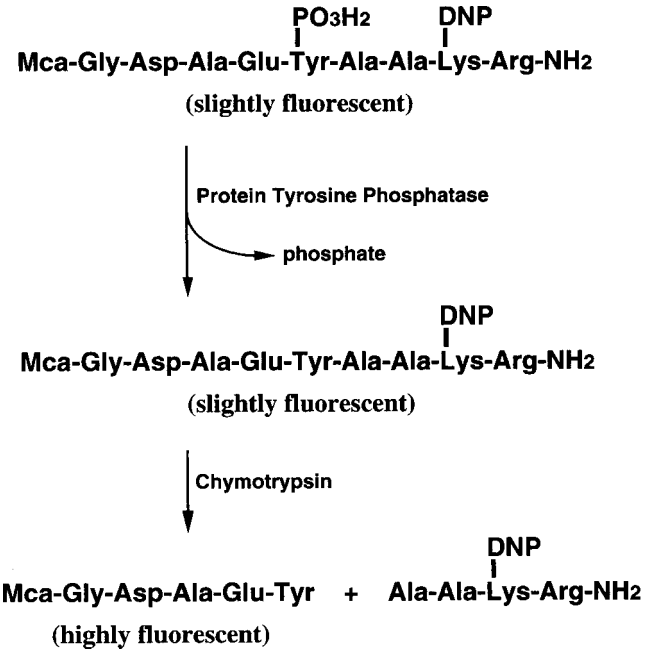

Scheme 1 Principle of PTP assay

residue located between these groups. PTP-catalysed dephosphorylation of this peptide and subsequent chymotryptic cleavage of the dephosphorylated species resulted in a considerable fluorescence enhancement. This article describes the synthesis and evaluation of the fluorogenic phosphopeptide substrate for use in the sensitive assay of PTPs. In addition, the data obtained with our assay were compared with those obtained from a conventional radioisotopic assay.

MATERIALS AND METHODS

Materials

YOP PTP (recombinant *Yersinia enterocolitica* Yop51*), T-cell PTP (recombinant TCΔC11), LAR PTP (recombinant leucocyte-antigen-related D1), protein phosphatase 1 (recombinant PP1α) and Abelson murine leukaemia virus (Abl) protein tyrosine kinase (recombinant) were from New England Biolabs. Alkaline phosphatase (bovine intestinal mucosa), Lys(DNP) and 7-methoxycoumarin-4-acetic acid were from Sigma. Chymotrypsin (bovine pancreas, three-times crystallized) was from Worthington and dissolved in 1 mM HCl before use. [γ-^{32}P]ATP (4500 Ci/mmol) was from ICN Biomedicals. Fmoc-Tyr(PO_3H_2) (where Fmoc is 9-fluorenylmethoxycarbonyl) was from Calbiochem-Novabiochem. Fmoc-Lys(DNP) was prepared by reaction of Lys(DNP) with Fmoc *N*-hydroxysuccinimide ester. Other Fmoc-amino acid derivatives and resins were from Watanabe Chemical Industries, Hiroshima, Japan. Mca-Gly was prepared by coupling of glycine ethyl ester and 7-methoxycoumarin-4-acetic acid using benzotriazol-1-yl-oxytris-pyrrolidinophosphonium hexafluorophosphate (PyBOP) and 1-hydroxybenzotriazole (HOBt), followed by removal of the ethoxy group by saponification. Okadaic acid and microcystin-LR were from Wako Pure Chemical Industries, Osaka, Japan. Crude extracts of *Porphyromonas gingivalis* cells and rat pancreas and submaxillary gland were prepared as follows. The cells and tissues were homogenized in twice their weight of 10 mM Tris/HCl (pH 7.2) containing 5 mM EDTA, 2 mM dithiothreitol and 0.15 M NaCl. After centrifugation of the homogenate, the supernatant was used for experiments.

Synthesis of substrates

The fluorogenic phosphopeptide had the structure Mca-Gly-Asp-Ala-Glu-Tyr(PO_3H_2)-Ala-Ala-Lys(DNP)-Arg-NH_2. The sequence of this peptide was similar to that around the phosphotyrosine residue in pp60^{src}, the Rous sarcoma virus-transforming protein [14], and more similar to that of a peptide used by Casnellie et al. [15] as a tyrosine protein kinase substrate. The peptide was synthesized by manual Fmoc solid-phase methodology on a Rink amide methylbenzhydrylamine resin (0.49 mmol/g). Fmoc-Tyr(PO_3H_2) with the side chain unprotected was introduced directly essentially as described by Ottinger et al. [16]. Side-chain-protecting groups were *t*-butyl ester for Asp and Glu, and 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulphonyl for Arg. Elongation of the peptide chain was achieved using 5 Equiv. of Fmoc-amino acid derivatives (or Mca-Gly in the final coupling step) with 5 Eq each of PyBOP and HOBt, and 10 Equiv. of *N*,*N*-di-isopropylethylamine as coupling reagents. The peptide was cleaved from the resin and the side chain deprotected with trifluoroacetic acid/thioanisole/water/phenol/ethanedithiol (84:5:5:3:3, by vol.) for 2 h. The crude peptide precipitated by the addition of ether was collected, dried and purified by reversed-phase HPLC with a linear gradient of acetonitrile. Amino acid analysis of the purified peptide after hydrolysis in 6 M HCl at 110 °C for 24 h gave the expected molar ratios: Gly, 1.00; Asp, 0.98; Ala, 2.90; Glu, 0.98; Tyr, 0.73; and Arg, 0.96. The purified peptide (M_r 1441) also gave the desired mass by fast-atom-bombardment MS: $(M+H)^+$, 1442. Stock solutions were made in DMSO, which could be stored without any detectable deterioration for months at −20 °C. The concentration of the stock solution was determined using the molar absorption coefficient of the peptide at pH 6.6 (ϵ_{410}, 7300 $M^{-1}\cdot cm^{-1}$).

The radiolabelled peptide that was used for comparison in the radioisotopic assay was prepared by enzymic peptide phosphorylation as follows. The phosphorylation reaction was carried out in 650 μl of 50 mM Tris/HCl (pH 7.5) containing 0.6 mM Gly-Asp-Ala-Glu-Tyr-Ala-Ala-Lys-Arg-NH_2 (synthesized by a conventional solid-phase methodology), 10 mM $MgCl_2$, 1 mM EGTA, 1.5 mM dithiothreitol, 0.01 % (v/v) Brij 35, 0.15 mM sodium vanadate, 0.77 mM [^{32}P]ATP (1.16 Ci/mmol) and 5000 units (manufacturer's definition) of Abelson murine leukaemia virus protein tyrosine kinase. After incubation at room temperature for 25 h, an additional 5000 units of the kinase was added. After further incubation for 23 h, 0.5 ml of 1 M acetic acid was added. The mixture was then applied to a small column (1 ml) of phosphocellulose (P11, Whatman) and the column was washed exhaustively with 0.5 M acetic acid until almost no radioactivity was detected. Subsequently, the phosphorylated peptide was eluted from the column with 0.15 M aqueous ammonia. No attempt was made to separate the phosphorylated peptide from the unphosphorylated one that co-eluted with it. From the radioactivity of the effluents, the degree of phosphorylation was found to be about 20 %. The phosphopeptide-containing fractions were lyophilized and dissolved in water. The concentration of the phosphopeptide was determined on the basis of the specific radioactivity of [^{32}P]ATP used.

Assay of phosphatases

Activities of PTPs, protein phosphatase (PP)1 and crude-enzyme preparations were measured in 20 mM 3,3-dimethylglutarate

buffer (pH 6.6). Alkaline phosphatase activity was measured in 20 mM carbonate buffer (pH 9.5). Each buffer contained 0.01 % (w/v) BSA and an appropriate substrate. For PP1 assay, 1 mM $MnCl_2$ was also included. Phosphatase activity was determined using *p*-nitrophenyl phosphate (10 mM) as a substrate, and 1 unit of phosphatase was defined as the amount of enzyme that hydrolysed 1 nmol of the substrate in 1 min. The release of *p*-nitrophenol was monitored continuously at 405 nm. All assays were performed at 25 °C.

Dephosphorylation of the fluorogenic peptide was measured with a fluorescence spectrophotometer (Hitachi F-3000) at excitation and emission wavelengths of 328 and 395 nm, respectively, using a 5-mm × 5-mm square microcuvette. Bandwidths were set at 5 nm (excitation and emission) or 1.5 nm (excitation) and 10 nm (emission). In the continuous fluorescence monitoring, the latter set of bandwidths was always employed, since gradual increases and decreases in fluorescence intensity were observed for the fluorogenic peptide and Mca-Gly-Asp-Ala-Glu-Tyr (a chymotryptic fragment of dephosphorylated peptide) respectively, when exposed continuously to UV irradiation at 5 nm bandwidth (results not shown).

Dephosphorylation of the radiolabelled peptide was measured according to the method of Shacter [17]. The sample (200 μl) was mixed with 800 μl of 5 mM silicotungstate/1 mM H_2SO_4 to stop the dephosphorylation reaction. Then, 160 μl of 5 % (w/v) ammonium molybdate/2 M H_2SO_4 and 1.2 ml of 2-methyl-1-propanol/toluene (1:1, v/v) were added. After vortexing and centrifugation, 0.5 ml of the organic phase was withdrawn, mixed with a scintillation cocktail and the radioactivity was estimated. The total c.p.m. extracted into the organic phase was calculated by multiplying the observed c.p.m. by 2.3, as described in [17].

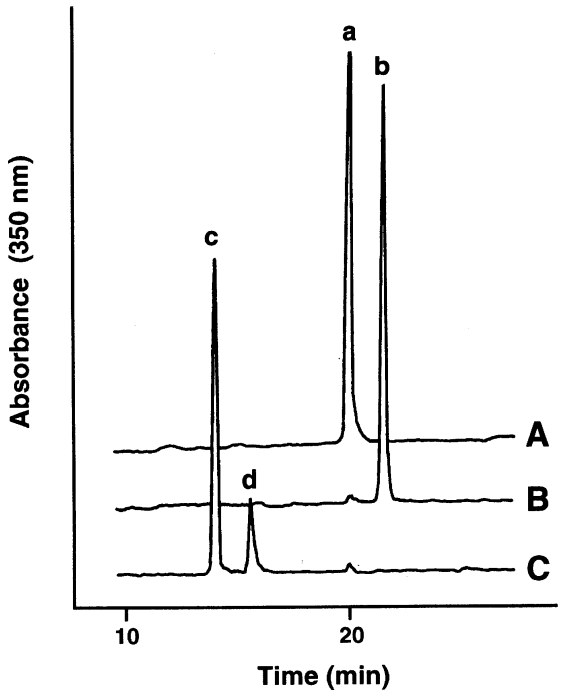

Figure 1 Analysis of reaction products by HPLC

Samples (100 μl) were applied to a column (4.6 mm × 150 mm) of Capcell Pak C_8 (Shiseido, Tokyo, Japan) equilibrated with 10% (v/v) aqueous acetonitrile containing 0.05% (v/v) trifluoroacetic acid, and eluted with a linear gradient of 10–60% acetonitrile containing 0.04% trifluoroacetic acid over 30 min at 1 ml/min. Trace A, Mca-Gly-Asp-Ala-Glu-Tyr(PO_3H_2)-Ala-Ala-Lys(DNP)-Arg-NH_2; trace B, YOP PTP hydrolysate of A; trace C, chymotrypsin hydrolysate of B.

RESULTS

HPLC analysis of enzymic products of the fluorogenic peptide

To verify that the enzymic reactions shown in Scheme 1 actually take place, we analysed the reaction products by HPLC (Figure 1). Elution was monitored at 350 nm, where both Mca and DNP groups have absorption. When the fluorogenic phosphopeptide (10 μM) was incubated with YOP PTP (250 units/ml) at pH 6.6 for 30 min, a new peak (Figure 1, peak b) appeared with almost complete disappearance of the original peak (Figure 1, peak a). This new peak had the same retention time as an authentic sample of the corresponding dephosphorylated peptide. Subsequent treatment with chymotrypsin (0.05 %, w/v) at pH 6.6 for 30 min gave two new peaks (Figure 1, peaks c and d) having the same retention times as authentic samples of Ala-Ala-Lys(DNP)-Arg-NH_2 and Mca-Gly-Asp-Ala-Glu-Tyr, respectively, with complete disappearance of peak b (Figure 1). Treatment of the fluorogenic phosphopeptide with chymotrypsin alone (without pretreatment with a PTP) did not change the chromatogram at all (results not shown), indicating that chymotrypsin was specific exclusively for the newly generated tyrosine residue. During the course of the enzymic treatment described above, the fluorescence intensity of the reaction mixture was also measured (diluted 10-fold for measurement). Although, as expected, practically no change in fluorescence intensity was observed on dephosphorylation, approx. 120-fold increase was observed on treatment with chymotrypsin. The fluorescence intensity after chymotrypsin treatment was the same as that of 1 μM Mca-Gly-Asp-Ala-Glu-Tyr. Thus these results confirm the reactions shown in Scheme 1.

Effect of enzyme concentration on dephosphorylation rate

We examined whether the rate of dephosphorylation of the fluorogenic peptide is dependent on enzyme concentration. Enzymic reactions were allowed to proceed in a microcuvette fitted in the fluorescence spectrophotometer, during which time the fluorescence intensity was monitored continuously (Figure 2A). The fluorogenic peptide (1 μM) was allowed to react with various concentrations of YOP PTP in 200 μl of reaction mixture. After incubation for 1 min, the reaction was stopped by the addition of sodium vanadate (0.1 mM, final concentration). Chymotrypsin (0.05 % final concentration, w/v) was then added to restore quenched fluorescence. The fluorescence intensity reached a plateau in about 15 s. Since the increase in fluorescence intensity can be converted directly into the concentration of the dephosphorylated species (using Mca-Gly-Asp-Ala-Glu-Tyr as a reference), the results obtained above were plotted in terms of the degree of dephosphorylation as a function of enzyme concentration (Figure 2B). Dephosphorylation of as little as 1 % of substrate was detected, and a linear correlation was observed up to at least 30 % dephosphorylation.

A parallel experiment was conducted for comparison under the same conditions (1 μM substrate in 200 μl of reaction mixture) with a corresponding radiolabelled peptide, Gly-Asp-Ala-Glu-Tyr($^{32}PO_3H_2$)-Ala-Ala-Lys-Arg-NH_2. As Figure 3 shows, a curve similar to that obtained with the fluorogenic peptide (Figure 2B) was obtained, although a departure from linearity was observed at low enzyme concentrations for unknown reasons. Also in this

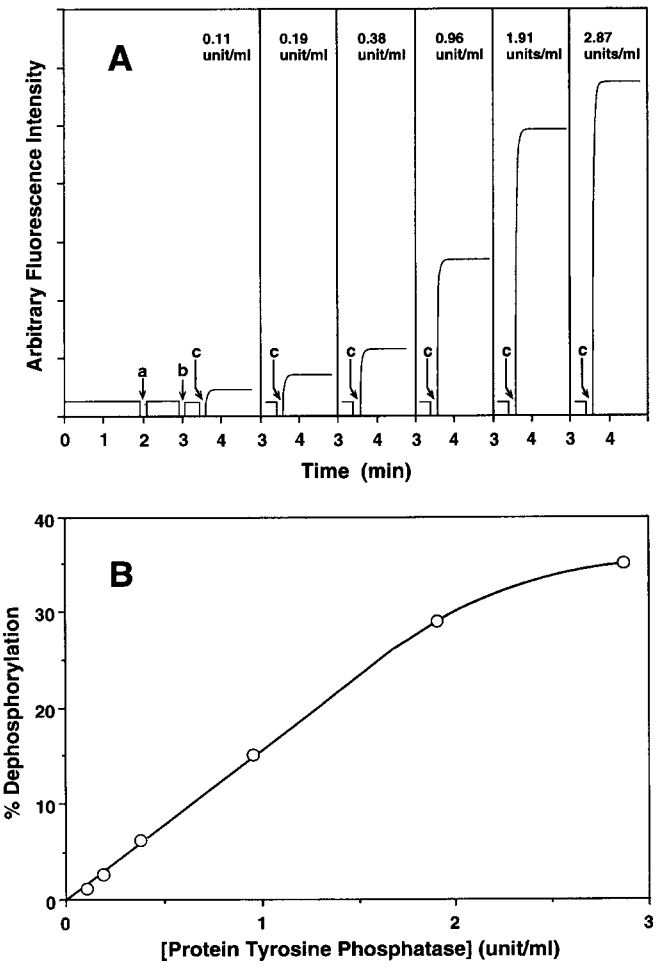

Figure 2 Dependence of the rate of fluorogenic peptide dephosphorylation on enzyme concentration

(**A**) Continuous monitoring of the change in fluorescence intensity. To 200 μl of 1 μM fluorogenic peptide in a microcuvette was added 2 μl each of YOP PTP solution (a), 10 mM sodium vanadate (b) and 5% (w/v) chymotrypsin (c) as indicated. (**B**) A plot on the basis of the results obtained in (**A**).

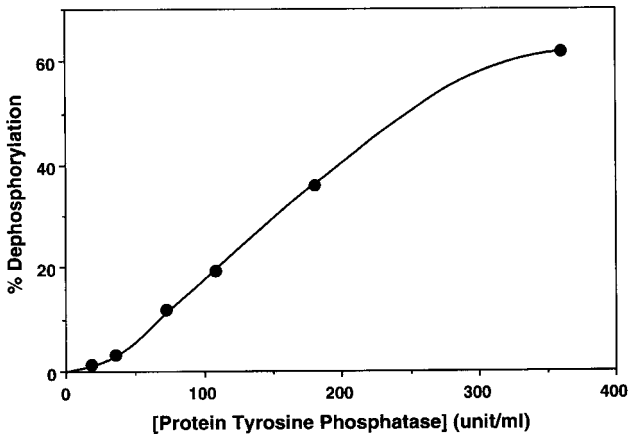

Figure 3 Dependence of the rate of ^{32}P-labelled peptide dephosphorylation on enzyme concentration

To 200 μl of 1 μM ^{32}P-labelled peptide (1070 c.p.m./pmol) was added 2 μl of YOP PTP solution. After 1 min, the reaction mixture was processed as described in the Materials and methods section. The background (no enzyme) radioactivity recovered in 0.5 ml of organic phase was 450 c.p.m.

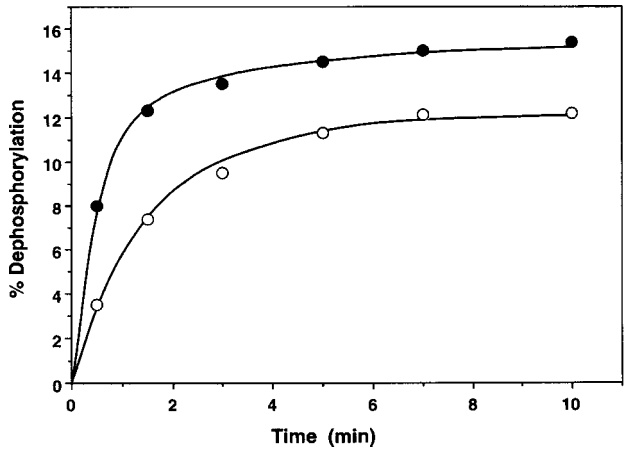

Figure 4 Time course of dephosphorylation

To 2 ml of 1 μM fluorogenic peptide or ^{32}P-labelled peptide (1120 c.p.m./pmol) was added 20 μl (0.77 unit for the former peptide and 163 units for the latter peptide) of YOP PTP solution. Aliquots (200 μl) were withdrawn at indicated time intervals and treated as follows. For the fluorogenic peptide, aliquots were added to 2 μl of 10 mM sodium vanadate, and, after approx. 30 s, 2 μl of 5% (w/v) chymotrypsin was added before the increase in fluorescence intensity was measured. For the labelled peptide, aliquots were processed as described in the Materials and methods section. ○, Fluorogenic peptide; ●, labelled peptide.

assay dephosphorylation of about 1% of the substrate could be detected. Comparison of the two calibration curves (Figures 2B and 3) revealed that the fluorogenic peptide was two orders of magnitude more sensitive to dephosphorylation by YOP PTP than the radiolabelled peptide.

Time course of dephosphorylation

The degree of YOP-PTP-catalysed dephosphorylation of the fluorogenic peptide (1 μM) was determined at time intervals on the basis of the increase in fluorescence intensity. As Figure 4 shows, the degree of dephosphorylation seemed to be linear up to 1 min. The deviation from linearity thereafter may have been due to the inactivation of the enzyme. The radiolabelled peptide gave a similar time course of dephosphorylation. The results obtained here and above demonstrate that the fluorogenic peptide can be used as a substrate for the quantitative measurement of PTP activity.

Kinetics experiment

As an example of the possibilities of application for the fluorogenic substrate, kinetic constants were determined for the YOP-PTP-catalysed dephosphorylation. The increase in fluorescence intensity in 1 min, which was taken as a measure of initial velocity, was measured at several substrate concentrations. The fluorescence measurement may have been affected by quenching due mainly to an inner filter effect, especially at high concentrations of substrate. We therefore examined this effect by measuring the increase in fluorescence intensity on addition of a reference compound, Mca-Gly-Asp-Ala-Glu-Tyr (0.1 μM final

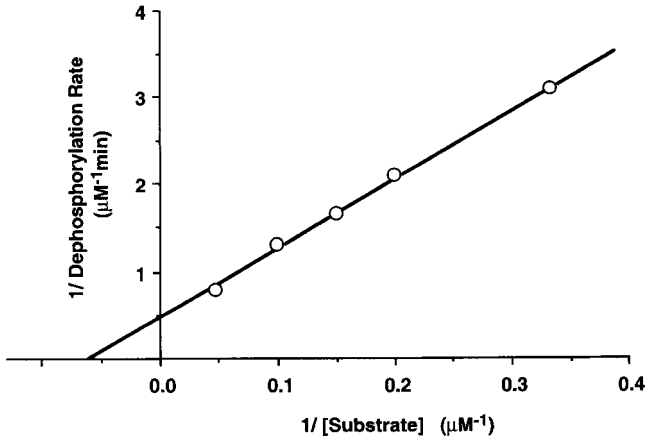

Figure 5 Determination of kinetic constants for the fluorogenic peptide dephosphorylation

Dephosphorylation of the fluorogenic peptide was measured as in Figure 2(A) at substrate concentrations of 3, 5, 6.8, 10 and 20 μM. The reaction mixture contained 0.72 unit of YOP PTP/ml.

concentration), to the substrate solution at the concentrations employed in the kinetic experiment. The fluorescence intensity of the reference compound decreased with increasing substrate concentrations, being 57 % of the unquenched value at 20 μM (the highest concentration employed in the kinetics experiment). A K_m of 16 μM and a V_{max} of 2.9 nmol/unit per min were obtained from a double-reciprocal plot (Figure 5) made by using the fluorescence data after correction for the inner filter effect.

We also attempted to estimate kinetic parameters for the radiolabelled substrate. The dephosphorylation rate increased linearly with substrate concentration up to 1 μM, but decreased suddenly thereafter (results not shown). The radiolabelled substrate preparation used here contained large amounts of unphosphorylated peptide (about four times the phosphorylated one), which had failed to be phosphorylated by the kinase. It is possible that this unphosphorylated peptide served as an inhibitor. We therefore measured dephosphorylation of the radiolabelled substrate (1 μM) in the presence of a high concentration (100 μM) of unphosphorylated peptide, and found that the unphosphorylated peptide had essentially no inhibitory effect (results not shown). The decreased dephosphorylation rate may be interpreted in terms of substrate inhibition, although the exact reason awaits further investigation. Extrapolation of the linear portion of the double-reciprocal plot gave a line passing very close to the origin (results not shown). Thus the kinetic parameters could not be determined accurately.

Comparison with other phosphatases

Dephosphorylation of the fluorogenic substrate was examined with several phosphatases. The radiolabelled substrate was also used for comparison. Both substrates were used at 1 μM. As shown in Table 1, the fluorogenic substrate was dephosphorylated by all the PTPs examined and a serine/threonine-specific protein phosphatase, PP1. The PP1 preparation used here is a recombinant α-isoform, and is reported to have an intrinsic activity toward phosphotyrosine residues [18]. To confirm this, we examined the effects of okadaic acid [19] and microcystin-LR [20], strong inhibitors of PP1, on the tyrosine phosphatase activity of the recombinant PP1. The tyrosine phosphatase activity was measured with the fluorogenic substrate (1 μM) in the presence of various concentrations of the inhibitors. Almost complete (over 95 %) inhibition was observed with micromolar concentrations of okadaic acid and nanomolar concentrations of microcystin-LR (results not shown), confirming that the observed tyrosine phosphatase activity was inherent in the recombinant PP1. The radiolabelled substrate was also dephosphorylated by the PTPs and PP1, but much more slowly. On the other hand, alkaline phosphatase dephosphorylated the two substrates at similar rates.

Table 1 Dephosphorylation of fluorogenic and ^{32}P-labelled peptides by various phosphatases

Dephosphorylation of the fluorogenic peptide was measured as in Figure 2(A) except that the reaction with alkaline phosphatase was stopped with 0.2 mM (final concentration) sodium vanadate. Dephosphorylation of the radiolabelled peptide (880 c.p.m./pmol) was measured as in Figure 3.

	Dephosphorylation rate (pmol/unit per min)	
Enzyme	Fluorogenic peptide	Radiolabelled peptide
YOP PTP	160	1.5
T-cell PTP	670	3.1
LAR PTP	74	5.7
PP1	26	0.05
Alkaline phosphatase	0.4	0.3

Application to crude-enzyme preparations

Assays suitable for measuring PTP activities in crude-enzyme preparations would be useful especially for physiological studies in which changes in enzyme activities in biological samples are measured. Application of our assay procedure to crude-enzyme preparations, however, may be hampered by at least two endogenous factors. One is proteolytic activity that may degrade the substrate, particularly between the fluorophore and the quencher, and the other is substances such as chymotrypsin inhibitors or high concentrations of proteins that may disturb efficient chymotryptic cleavage of the dephosphorylated substrate. In order to examine these possibilities, we assayed PTPs in crude-enzyme preparations from different origins that are rich in proteolytic activities. The fluorogenic substrate (196 μl of 1 μM solution) was incubated with the crude extracts (4 μl each) of pancreas (10 min), submaxillary gland [21] (6 min) and *P. gingivalis*, a suspected pathogen of adult periodontitis [22] (10 min), during which the change in fluorescence intensity was monitored continuously. The fluorescence intensity increased steadily with all the crude extracts, suggesting proteolytic cleavage of a peptide bond(s). The time course for the *P. gingivalis* extract is shown as a representative result in Figure 6. The increase in fluorescence intensity in 10 min (Figure 6, portion a) corresponded to proteolytic cleavage of as little as 1.2 % of the substrate. On the other hand, the amount of substrate dephosphorylated during this period, which was estimated from the burst of fluorescence intensity (Figure 6, portion b) on addition of chymotrypsin, was 24 %. If at least a part of portion a (Figure 6) was due to the proteolytic cleavage of the dephosphorylated species that occurred during the incubation, it must be added to portion b for more accurate estimation of dephosphorylation rate. In the case of the *P. gingivalis* extract, however, the ratio of b to a was high and hence the proteolytic cleavage was practically insignificant. Use of appropriate protease inhibitors is re-

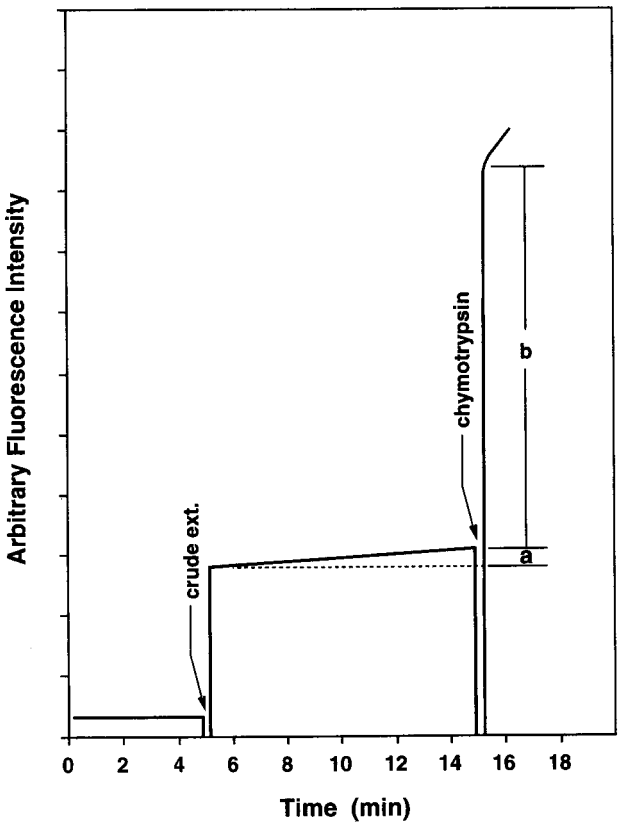

Figure 6 Measurement of PTP activity in the crude extract of *P. gingivalis* cells

To 196 μl of 1 μM fluorogenic peptide was added 4 μl (0.37 phosphatase unit) of *P. gingivalis* crude extract. After incubation for 10 min, 2 μl of 15% (w/v) chymotrypsin was added. Sodium vanadate was not used to stop the reaction, since it was only partially effective at 0.1 mM. Higher concentrations of vanadate were not examined, since it is a strong fluorescence quencher. See the Results section for details of portions a and b.

commended when the ratio b/a is low. In the case of pancreas and submaxillary gland, the rates of proteolytic cleavage were slower and the rates of dephosphorylation were higher (results not shown), and hence the ratios b/a were much higher. The burst of fluorescence intensity following addition of chymotrypsin (0.15% final concentration, w/v) ceased within 15 s for the three crude extracts. Thus although the crude extracts used here may have contained some chymotrypsin-inhibiting substances, their effects were too small to make the assay difficult. When the rate of chymotryptic cleavage of the dephosphorylated substrate is extremely reduced, such a difficulty may be overcome by increasing the concentration of chymotrypsin.

DISCUSSION

The radioisotopic PTP assay that uses [^{32}P]phosphotyrosyl peptides or proteins as specific substrates is most commonly employed probably because of significant advantages over other published methods. These are that it is not only highly sensitive but also relatively easy to conduct and compatible with endogenous substances in crude samples such as inorganic phosphate and proteins. However, despite these advantages, the assay procedure has some drawbacks, as described in the Introduction. Our fluorimetric assay was therefore developed in an attempt to overcome these drawbacks. Both the fluorimetric and radioisotopic assays performed in this study could detect dephosphorylation of about 1% (i.e. 2 pmol) of the substrate when 1 μM substrate in 200 μl of reaction mixture is used (Figures 2 and 3). Although not fully explored in this study, the sensitivity may be higher. In the fluorimetric assay, an increase in fluorescence as small as one-tenth to one-fifth of the background fluorescence (signal-to-noise ratio of approx. 10–20) should be detectable by appropriately selecting the full scale of the chart recorder. This would indicate a sensitivity to detect dephosphorylation of about 0.1–0.2% of the substrate. Therefore, the lower detection limit would be about 0.2–0.4 pmol. In the radioisotopic assay performed for comparison, when the specific radioactivity of the substrate was 1070 c.p.m./pmol, the observed background radioactivity recovered in 0.5 ml of 2-methyl-1-propanol/toluene was 450 c.p.m. (described in the legend to Figure 3). At this level of background, counts of at least 400–500 c.p.m. above background may be required for reliable detection. Thus the lower detection limit would be about 1 pmol. Our fluorimetric assay is therefore superior to or at least comparable in sensitivity with assays using ^{32}P-labelled substrates. The fluorogenic substrate was dephosphorylated by some PTPs much more quickly than the corresponding radiolabelled substrate (Table 1) and was more specific for PTPs than the radiolabelled substrate, since alkaline phosphatase did not discriminate between the two substrates (Table 1). Derivatization with the fluorophore and quencher had a favourable effect on the peptide, although it is not certain whether such an effect is commonly observed for any phosphotyrosyl peptide. The fluorimetric assay could be applied to the measurement of PTP activity in crude-enzyme preparations without any noticeable difficulties (e.g. Figure 6). Addition of inorganic phosphate (up to 0.2 M) and BSA (up to 0.2%) to the assay mixture did not affect the fluorescence measurement (results not shown). The fluorogenic substrate has outstanding advantages over ^{32}P-labelled substrates in that the compound is stoichiometrically phosphorylated and stable enough to allow the use of the same lot over long periods of time, thus eliminating the need for time-consuming and laborious substrate preparation at regular intervals. The fluorimetric assay does not involve separation of released inorganic phosphate from the unreacted phosphopeptide, which is essential to radioisotopic assays, and can be performed even in a single cuvette (Figures 2, 5 and 6). Taking these features together, it is no overstatement to say that our fluorimetric assay procedure is superior to existing radioisotopic assays and would greatly contribute to the development of the research on protein dephosphorylation.

The assay principle presented in this article can be applied to the investigation of PTP substrate specificity, which would provide useful information, especially for the preparation of fluorogenic substrates that are highly selective for particular physiologically important PTPs. Such a study is best conducted by determining kinetic constants for the dephosphorylation of fluorogenic substrates with different amino acid sequences, since PTP substrate specificity is at least in part controlled at the primary structure level [23–26]. A limitation in preparing such substrates is that Tyr, Phe and Trp should not be incorporated between a fluorophore and a quencher, since chymotrypsin is specific for the cleavage of peptide bonds at the carboxyl side of these residues. However, specific substrates for a particular PTP, if not the best, may be prepared by properly arranging amino acid residues other than these three. A recombinant form of PP1 had tyrosine phosphatase activity dephosphorylating our fluorogenic substrate (Table 1), and this activity was blocked by low concentrations of okadaic acid and microcystin-LR. It is therefore expected that our assay can be used, by taking advantage of

this nature, as a valuable non-radioisotopic method for detecting these naturally occurring toxins.

In the present article we have only described a discontinuous assay (activity measurement at time intervals) of PTPs. One might think that it would be possible to monitor dephosphorylation continuously if chymotrypsin is included in advance in the substrate solution prior to addition of a PTP sample. For such a continuous assay, sufficient amounts of chymotrypsin should be included to completely cleave the dephosphorylated species promptly (within a few s). Our preliminary experiment showed that at least 0.15% (w/v) chymotrypsin in reaction mixture is required to completely cleave less than 1 μM dephosphorylated species within 5 s at pH 6.6. However, it is highly probable that PTPs are unstable at such a high chymotrypsin concentration. In fact, when 0.15% chymotrypsin was included in the assay mixture, YOP PTP lost its activity by approx. 50% in 1 min even in the presence of 0.01% BSA. Consequently, we cannot but regard the continuous assay as unreliable as long as the present fluorogenic substrate is used. We are now making efforts to design new substrates that are suitable for continuous assays, i.e. those with much more elevated sensitivity to chymotryptic cleavage after dephosphorylation.

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