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A Phos-tag-based magnetic-bead method for rapid and selective separation of phosphorylated biomolecules

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ABSTRACT

A simple and efficient method based on magnetic-bead technology has been developed for the separation of phosphorylated and nonphosphorylated low-molecular-weight biomolecules, such as nucleotides, phosphorylated amino acids, or phosphopeptides. The phosphate-binding site on the bead is an alkoxide-bridged dinuclear zinc(II) complex with 1,3-bis(pyridin-2-ylmethylamino)propan-2-olate (Phos-tag), which is linked to a hydrophilic cross-linked agarose coating on a magnetic core particle. All steps for the phosphate-affinity separation are conducted in buffers of neutral pH with 50 μL of the magnetic beads in a 1.5-mL microtube. The entire separation protocol for phosphomonoester-type compounds, from addition to elution, requires less than 12 min per sample if the buffers and the zinc(II)-bound Phos-tag magnetic beads have been prepared in advance. The phosphate-affinity magnetic beads are reusable at least 15 times without a decrease in their phosphate-binding ability and they are stable for three months in propan-2-ol.

1. Introduction

Phosphorylated biomolecules with low molecular weights, such as nucleotides and phosphopeptides, have attracted much interest in the fields of metabolomics and phosphoproteomics [1,2]. Profiling of nucleotides and their pharmaceutically relevant analogs is analytically challenging because of their chemical and biological instability and their characteristic activities over wide ranges of concentrations in biological systems. Although many methods have been developed for determining nucleotide levels, few are capable of comprehensive nucleotide profiling at low concentrations in small volumes or from small weights of samples. Therefore, a rapid and comprehensive method that permits the separation of various nucleotides from complex samples containing breakdown enzymes and various substrates that react with nucleotides is desirable for the purpose of profiling [3].

Currently, a high-performance liquid chromatography–mass spectrometry (HPLC–MS) technique using a reverse-phase column or an ion-exchange column linked to a mass spectrometer is the method most widely used in microanalyses of nucleotides [4,5], but the separations are time consuming. On the other hand, large-scale identification of phosphorylated amino acids in proteins has become possible through dramatic advancements in mass spectrometry, coupled with improvements in instrumentation and the development of various methods for the pre-separation of phosphopeptides produced by enzymatic digestion [6–8]. One of the most frequently used method is immobilized metal-ion affinity chromatography (IMAC), in which an anionic phosphate ligand is captured at an open coordination site of an Fe(III)– or Ga(III)–chelate complex [9–12]. However, this technique requires acidic conditions for selective binding and elution of phosphopeptides, because less-labile metal hydroxide complexes form under neutral or basic conditions. Furthermore, like phosphopeptides, acidic groups in peptides can also form stable complexes, resulting in nonspecific binding of many acidic peptides as a result of their greater binding constants with the metal complexes.

Abbreviations: AcOH, acetic acid; ADP, adenosine 5′-diphosphate; AMP, adenosine 5′-monophosphate; ATP, adenosine 5′-triphosphate; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; BSA, bovine serum albumin; cyclic AMP, adenosine 3′,5′-cyclic monophosphate; dApT, 2′-deoxyadenylylthymidine dinucleotide; dCpT, 2′-deoxycytidylylthymidine dinucleotide; dGpT, 2′-deoxyguanylylthymidine dinucleotide; EDTA, ethylenediaminetetraacetic acid; FMN, riboflavin 5′-phosphate; FT, flow-through fraction; HPLC, high-performance liquid chromatography; IMAC, immobilized metal-ion affinity chromatography; NAD, nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide 2′-phosphate; NHS, *N*-hydroxysuccinimide; Phos-tag, phosphate-binding tag molecule; pSer, phosphoserine; pThr, phosphothreonine; pTyr, phosphotyrosine; *RT*, retention time; TFA, trifluoroacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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An alternative chromatographic technique for more-selective enrichment of phosphopeptides involves the use of a metal oxide (TiO_2) as a solid stationary phase [13–15]. The TiO_2 particles are modified by surface treatment with reagents such as 2,5-dihydroxybenzoic acid or lactic acid to confer selectivity to phosphates. As a result, the chemical structure of the phosphate-binding site is less well characterized than those of IMAC systems.

Another method that is widely used is immunoaffinity precipitation with an antiphosphopeptide antibody immobilized on highly porous crosslinked agarose beads or magnetic polymer beads [16–18]. However, the antibody-based procedure has the drawback that it is incapable of comprehensive enrichment of phosphopeptides. In addition, the immunoaffinity beads are physically and chemically unstable, and they are therefore unsuitable for repeated use or for long-term storage. Currently, many studies on the phosphoproteome use a combination of these enrichment strategies to obtain the purest sample possible.

We have recently reported several original methods for analyzing phosphorylated biomolecules, such as phosphopeptides, phosphorylated proteins, nucleotides, or phospholipids, by the use of a phosphate-binding tag molecule (Zn^{2+}–Phos-tag); this is an alkoxide-bridged dinuclear zinc(II) complex of 1,3-bis[bis(pyridine-2-ylmethyl)amino]propan-2-olate [19]. To date, technologies based on derivatives of Phos-tag have contributed extensively to the development of a wide range of practical procedures for studies on phosphorylated biomolecules. The methods that have been developed include a phosphate-affinity chromatographic technique for the enrichment of phosphorylated proteins and phosphopeptides [20,21], a surface plasmon-resonance technique for the detection of phosphopeptides [22], a microarray method for high-throughput profiling of protein kinase activities in cell lysates [23], a technique for chemiluminescent detection for phosphorylated proteins on a Western-blotting membrane [24], and a phosphate-affinity polyacrylamide gel electrophoresis technique for detecting shifts in the mobilities of phosphorylated proteins and oligonucleotides in comparison with those of their nonphosphorylated counterparts [24,25].

Here, we describe the design and development of novel phosphate-affinity magnetic beads (Zn^{2+}–Phos-tag magnetic beads) for the rapid and simple separation of phosphorylated biomolecules. Phosphate groups on the biomolecules are reversibly captured on the beads through bidentate coordination between the phosphate dianion and the immobilized Zn^{2+}–Phos-tag molecule at neutral pH values in aqueous media.

2. Materials and methods

2.1. Materials

N-Hydroxysuccinimide-activated magnetic beads (NHS Mag Sepharose) were purchased from GE Healthcare (Little Chalfont, UK). A phosphorylated Src peptide (Thr-Ser-Thr-Glu-Pro-Gln-pTyr-Gln-Pro-Gly-Glu-Asn-Leu), a phosphorylated insulin receptor peptide (Thr-Arg-Asp-Ile-pTyr-Glu-Thr-Asp-Tyr-Tyr-Arg-Lys), and their nonphosphorylated counterparts were purchased from AnaSpec (San Jose, CA, USA). A monophosphopeptide (Phe-Gln-pSer-Glu-Glu-Gln-Gln-Gln-Thr-Glu-Asp-Glu-Leu-Gln-Asp-Lys) and a tetraphosphopeptide (Arg-Glu-Leu-Glu-Glu-Leu-Asn-Val-Pro-Gly-Glu-Ile-Val-Glu-pSer-Leu-pSer-pSer-pSer-Glu-Glu-Ser-Ile-Thr-Arg), both derived from bovine β-casein, were both purchased from AnaSpec (San Jose, CA, USA). Bovine β-casein, bovine serum albumin (BSA), trypsin (proteomics grade), 2′-deoxyadenosine, *O*-phospho-L-tyrosine (phosphotyrosine), tyrosine, *O*-phospho-L-serine (phosphoserine), L-serine, *O*-phospho-L-threonine (phosphothreonine), L-threonine, adenosine 5′-triphosphate (ATP), adenosine 5′-diphosphate (ADP), adenosine 5′-monophosphate (AMP), riboflavin, riboflavin 5′-phosphate (FMN) sodium salt, nicotinamide adenine dinucleotide (NAD), and nicotinamide adenine dinucleotide 2′-phosphate (NADP) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Ethylenediaminetetraacetic acid (EDTA) disodium salt, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol (Bis-Tris; $pK_a = 6.5$ for its protonated species), tris(hydroxymethyl)aminomethane [Tris; 2-amino-2-(hydroxymethyl)propane-1,3-diol; $pK_a = 8.1$ for its protonated species), creatine{*N*-[amino(imino)methyl]-*N*-methylglycine}, and phosphocreatine {*N*-[imino(phosphonoamino)methyl]-*N*-methylglycine} were purchased from Nacalai Tesque (Kyoto, Japan). A diamine spacer, 4,7,10-trioxatridecane-1,13-diamine was purchased from Tokyo Kasei (Tokyo, Japan). All chemical reagents and solvents used were of the highest commercial quality and were used without further purification. All aqueous solutions were prepared by using deionized and distilled water.

2.2. Apparatus

UV–visible spectra were recorded on a V-630 spectrophotometer (Jasco; Tokyo, Japan) at 25.0 ± 0.1 °C. ^1H (500 MHz) NMR spectra were recorded on an LA500 spectrometer (JEOL; Tokyo, Japan) at 25 °C with tetramethylsilane (in $CDCl_3$) as the internal reference. Measurements of pH were performed with a LAQUA F-72 pH meter (Horiba; Kyoto, Japan) with a 9618-10 combination pH electrode (Horiba) that was calibrated by using standard buffers (pH 4.01 and 6.86) at 25 °C. A freeze–dry system (Eyala FDU-1200 and CVE-2000; Koshigaya, Japan) with an oil-free vacuum pump (Ulvac DISL-101; Miyazaki, Japan) was used. Samples were shaken by using a MicroMixer E-36 benchtop shaker (Taitec; Koshigaya, Japan) or a Thermomixer Comfort (Eppendorf AG; Hamburg, Germany).

2.3. HPLC analysis

HPLC was performed by using a JASCO HPLC system (Tokyo, Japan) consisting of a column oven (CO-2060), a UV detector (UV-2070), a degasser (DG-2080-53), and two pumps (PU-2080 plus); the flow rate was 1.0 mL/min and the temperature was 40 °C, except as noted. The columns used were of the ion-exchange type [DEAE-2SW; 4.6 mm internal diameter (i.d.) × 250 mm] (TSKgel; Tokyo, Tosoh)] or the reverse-phase type (CAPCELL PAK C18 UG80; 4.6 mm i.d. × 150 mm) (Shiseido; Yokohama, Japan). Analyses of the adenosine nucleotides AMP [retention time (*RT*) = 3.9 min], ADP (*RT* = 4.7 min), and ATP (*RT* = 5.6 min) were conducted by using the anion-exchange column under isocratic conditions [eluent: 0.50 M phosphoric acid–acetonitrile (4:1)] with detection at 260 nm. Riboflavin (*RT* = 4.5 min) and flavin mononucleotide (FMN; *RT* = 3.5 min) were analyzed by using the reverse-phase column under isocratic conditions [eluent: 10 mM aqueous NaH_2PO_4/NaOH (pH 5.5)–methanol (13:7)] at a flow rate of 0.8 mL/min and 37 °C, and with detection at 445 nm. The analysis of NAD (*RT* = 9.3 min) and NADP (*RT* = 2.9 min) was conducted by using the reverse-phase column under isocratic conditions (eluent: 10 mM aqueous KH_2PO_4/H_3PO_4, pH 3.5) with detection at 260 nm. Creatine (*RT* = 1.9 min) and phosphocreatine (*RT* = 1.4 min) were analyzed by using the reverse-phase column under isocratic conditions [eluent: 10 mM aqueous KH_2PO_4/H_3PO_4 (pH 3.5)–acetonitrile (17:3) containing 2.0 mM of sodium dodecyl sulfate] with detection at 210 nm. Tyrosine (*RT* = 14.6 min), phosphotyrosine (*RT* = 3.4 min), and phenyl phosphate dianion (*RT* = 5.3 min) were analyzed by using the reverse-phase column with 0.10% (v/v) aqueous TFA as the eluent and detection at 254 nm. The tryptic digest of β-casein was analyzed by using the reverse-phase column under gradient conditions [eluent: 0.10% (v/v) aqueous TFA–0.10% (v/v) TFA

in acetonitrile from 9:1 to 7:13 over 30 min] with detection at 215 nm: peaks at *RT* values of 8.5 and 13.6 min were assigned, respectively, to the monophosphorylated and tetraphosphorylated peptides derived from β-casein. A mixed sample of a tryptic digest of bovine serum albumin (BSA) and the synthetic peptides was analyzed by using the reverse-phase column under gradient conditions [eluent: 0.10% (v/v) aqueous TFA–0.10% (v/v) TFA in acetonitrile, from 19:1 to 3:2 over 30 min] with detection at 215 nm. The retention times of the synthetic peptides were 13.7 min for the phosphorylated Src peptide, 14.3 min for the phosphorylated insulin receptor peptide, 16.8 min for nonphosphorylated Src peptide, and 16.1 min for the nonphosphorylated insulin receptor peptide.

2.4. Synthesis of the amino-pendant Phos-tag ligand

A novel amino-pendant Phos-tag ligand attached to a hydrophilic 15-atom spacer, *N*-[3-{2-[2-(3-aminopropoxy) ethoxy]ethoxy}propyl]-6-{[{3-[bis(pyridin-2-ylmethyl)amino]-2-hydroxypropyl}(pyridin-2-ylmethyl)amino]methyl}nicotinamide, was synthesized by a similar method to that reported previously [20], except for the length of the spacer. The final step for the attachment of the spacer was performed as follows. The symmetric diamino triether 4,7,10-trioxatridecane-1,13-diamine (2.68 g, 12.2 mmol) was added to methyl 6-{[{3-[bis(pyridin-2-ylmethyl)amino]-2-hydroxypropyl}(pyridin-2-ylmethyl)amino]methyl}nicotinate (1.25 g, 2.4 mmol) in MeOH (10 mL). The mixture was refluxed for 15 h and then the solvent was evaporated. The oily residue was dissolved in $CHCl_3$ (100 mL) and the resulting solution was washed with distilled water (50 mL). The organic fraction was dried over Na_2SO_4 and then concentrated. The residue was purified by gel column chromatography [silica gel (50 g; Fuji Silisia DM1020 NH), $CHCl_3$–MeOH (100:1)] to give amino-pendant Phos-tag as a pale-yellow oil; yield: 1.33 g (77%); TLC [Merck TLC plate NH_2 F_{254s}, eluent: $CHCl_3$–MeOH (25:1)] R_f = 0.44 (one spot); ^{1}H NMR ($CDCl_3$): δ = 1.64 (quin, J = 6.5 Hz, 2H, NCCHCO), 1.88 (m, 2H, m, CONCCHC), 2.54–2.69 (m, 4H, NCHCCN), 2.72 (t, J = 6.5 Hz, 2H, NCHCC), 3.43–3.54 (m, 4H, NCCCHO), 3.54–3.68 (m, 10H, OCHCHO, CONCH), 3.79–3.98 (m, 9H, NCCHCN, PyCHN), 7.08–7.15 (m, 3H, PyH), 7.33 (d, J = 7.8 Hz, 3H, PyH), 7.45 (d, J = 8.0 Hz, 1H, PyH), 7.54–7.61 (m, 3H, PyH), 7.77 (br s, 1H, CONH), 8.05 (d, J = 8.0 Hz, 1H, PyH), 8.49 (d, J = 5.0 Hz, 3H, PyH), 8.88 (s, 1H, PyH); HPLC [reverse-phase column, MeCN–5 mM aqueous $HClO_4$ (3:22), 254 nm], *RT* = 10.3 min (single peak); purity > 99.5%.

2.5. Immobilization of the Phos-tag ligand on the magnetic beads

The amino-pendant Phos-tag ligand (2.0 μmol, 1.4 mg) was mixed with NHS Mag Sepharose (0.10 mL), which has *N*-hydroxysuccinimidoyl carboxylic ester groups (8–14 μmol/mL-beads) immobilized on Sepharose-coated magnetic beads (diameter: 37–100 μm), and the mixture was incubated in a 1.5-mL microtube containing propan-2-ol (0.50 mL) for 1 h at room temperature (*ca.* 25 °C) with mild shaking, then allowed to stand for 12 h at 4 °C. The amount of immobilized Phos-tag ligand was determined to be 8.0 μmol/mL-bead from the amount of unreacted Phos-tag ligand (1.2 μmol) remaining in the propan-2-ol solution, as determined by HPLC. After removing the liquid fraction on a magnetic stand, the remaining *N*-hydroxysuccinimide carboxyl ester groups were converted into Tris-bound amide moieties or carboxylate groups by treatment with an aqueous solution of 1.0 M Tris–acetic acid (AcOH) (pH 7.6, 0.50 mL) at room temperature for 12 h. The liquid fraction was removed and the magnetic beads were washed twice with propan-2-ol (0.50 mL). The volume of the Phos-tag-bound beads was almost identical to that of the original NHS Mag Sepharose beads. The resulting Phos-tag magnetic beads were stored in propan-2-ol (0.50 mL) at 4 °C.

2.6. Tryptic digestion of β-casein and bovine serum albumin

The protein (β-casein or BSA) was subjected to tryptic digestion by mixing a solution of the protein (1.0 mg) in water (0.10 mL) with 0.10 M aqueous $(NH_4)_2CO_3$–H_2CO_3 (pH 8.0, 0.40 mL) containing 20 μg of proteomic-grade trypsin for 12 h at 37 °C. Large molecules, such as the trypsin and partially digested proteins, were removed by ultrafiltration through a Microcon YM-10 ultrafilter unit at 14,000 × *g* for 40 min. The resulting filtrate was freeze dried and stored below 0 °C.

2.7. Phosphate-affinity separation by using Phos-tag magnetic beads

The basic protocol for the separation of phosphorylated biomolecules at room temperature under normal gravity is as follows. An appropriate volume of a suspension of Phos-tag magnetic beads (50 μL of beads in the Zn^{2+}-free form) in propan-2-ol is placed in a 1.5-mL microtube. By using a magnetic stand, the beads are amassed on the wall of the microtube within 5 s, and the storage solution is then removed by pipetting. The beads are resuspended in 0.10 M Bis-Tris–AcOH buffer (pH 6.8; 0.10 mL) on a benchtop shaker (30 s) and then the liquid is removed again. The washing operation is repeated and the beads are resuspended in 0.10 M Bis-Tris–AcOH buffer (pH 6.8; 0.10 mL) containing 0.10 M $Zn(OAc)_2$ and shaken for 3 min. The liquid is discarded and the Zn^{2+}-containing beads (Zn^{2+}–Phos-tag beads) are washed twice with 0.10 M Bis-Tris–AcOH buffer (pH 6.8; 0.10 mL), and the washings are discarded. A sample solution consisting of 0.10 M Bis-Tris–AcOH buffer (pH 6.8) containing phosphorylated and nonphosphorylated biomolecules is then added to the Zn^{2+}–Phos-tag beads (50 μL) in the microtube. The mixture is incubated with shaking for 3 min at room temperature. The beads are amassed magnetically and the liquid is removed as the flow-through fraction (FT). The beads are resuspended in 0.10 M Bis-Tris–AcOH buffer (pH 6.8; 0.20 mL) and the slurry is shaken for 30 s. The beads are amassed once more and the liquid is isolated. This washing operation is repeated three times and the washings are collected each time as a series of washing fractions (W1–W3). The beads are then washed once more with distilled water (0.20 mL) to give the final washing fraction (W4). To elute any phosphorylated biomolecules bound to the Zn^{2+}–Phos-tag, a pyrophosphate buffer consisting of 0.10 M $Na_4P_2O_7$–AcOH (pH 7.0; 0.10 mL) is added to the microtube and mixed for 30 s. This eluting operation is repeated up to five times. The beads are amassed magnetically and the liquid is isolated each time to give the respective elution fraction, E1–E5. Recoveries of phosphorylated biomolecules in the various fractions were analyzed by HPLC and/or UV spectrophotometry (see below). All the phosphate-separation experiments were conducted at least in triplicate. The recovery of a phosphorylated sample by applying the basic protocol was reproducible to within 10%, and an average value was estimated in each case. To recycle the beads, the final pyrophosphate-bound Zn^{2+}–Phos-tag beads are converted into the Zn^{2+}-free form by washing three times with 0.10 M EDTA–NaOH buffer (pH 7.0) for 15 min at room temperature. After the washing solution has been discarded, the beads are stored in propan-2-ol at 4 °C. Alternatively, the washed beads can be treated with an excess of $Zn(AcO)_2$, as discussed in the basic protocol, and stored in the Zn^{2+}-bound form in propan-2-ol at 4 °C for subsequent reuse.

3. Results and discussion

3.1. Characteristics of Zn^{2+}–Phos-tag magnetic beads

The magnetic beads with pendant dinuclear zinc(II) Phos-tag complexes (Zn^{2+}–Phos-tag magnetic beads) were prepared by mixing the zinc(II)-free Phos-tag beads with a large excess of zinc(II) ion in 0.10 M Bis-Tris–AcOH buffer (pH 6.8). Under these conditions, the Zn^{2+}–Phos-tag moiety should contain a 1:1 acetate-bound dinuclear zinc(II) species with a net charge of +2 [19]. The Zn^{2+}–Phos-tag moiety is connected to the crosslinked agarose around the magnetic core particle through a hydrophilic 15-atom spacer. The volume of a Zn^{2+}–Phos-tag magnetic bead swollen in water is almost the same as that of the zinc(II)-free bead. The Zn^{2+}–Phos-tag magnetic beads hold 10 ± 2 μL of swelling water per 50 μL of beads magnetically amassed on the wall of a 1.5-mL microtube under normal gravity. If 100 μL of an aqueous solution is added to 50 μL of the beads and then the liquid fraction is discarded, about 10 vol% of the solution will remain around the swollen beads. Fig. 1 shows the preparative scheme and the structure of the Zn^{2+}–Phos-tag magnetic bead. The phosphate-binding capacity of the beads was determined as a phosphate-binding amount (μmol of phosphate per mL of beads). Since the phenyl phosphate dianion [PhOP(=O)$(O^-)_2$] shows a strong affinity (K_d = 25 nM at 25 °C) for the unsubstituted Zn^{2+}–Phos-tag molecule [19], we used this dianion as a standard phosphate in this study. By performing the basic protocol illustrated in Fig. 2, except for the use of an elution buffer of 0.10 M aqueous NaH_2PO_4–NaOH (pH 7.0), we obtained a flow-through fraction (FT), four washing fractions (W1–W4), and five elution fractions (E1–E5) for a sample solution that originally contained 0.80 μmol of phenyl phosphate dianion, equal to two equivalents of the Zn^{2+}–Phos-tag moiety immobilized on 50 μL of the beads. This determination was performed in quadruplicate. The amount of the phosphate in each of the fractions was analyzed by reverse-phase HPLC. All of the phenyl phosphate dianion added was recovered in the various fractions. The average percentage recoveries of phenyl phosphate dianion were as follows: 52% in the FT, 16% in W1, 5% in W2, 1% in W3, 0% in W4, 15% in E1, 7% in E2, 3% in E3, 1% in E4, and 0% in E5. This distribution pattern, which showed two peaks in the recovery at the FT and the E1 fractions, is similar to that for the separation of an excess of phosphate by using Phos-tag-linked agarose gel [20], thereby confirming the presence of phosphate-affinity sites on the beads. From the amounts of phenyl phosphate dianion in the elution fractions, the phosphate-binding capacity was determined to be 4.1 ± 0.4 μmol/mL of beads (0.2 μmol per 50 μL of beads). The value is almost half that of the immobilized Phos-tag moiety (8.0 μmol/mL of beads), possibly as a result of the presence of some steric hindrance around the binding site. When the amount of phenyl phosphate dianion was half the phosphate capacity (0.1 μmol per 50 μL of beads), no phenyl phosphate dianion was detected in the FT fraction or the four washing fractions W1–W4, and all the phosphate was recovered in the elution fractions E1–E4. With Zn^{2+}-free Phos-tag beads, all the phenyl phosphate dianion appeared in the FT, W1, and W2 fractions. In addition, phosphate-selective binding by Zn^{2+}–Phos-tag magnetic beads (50 μL) was confirmed by a similar experiment using a mixed sample of AMP (0.10 μmol) and its dephosphorylated analog, 2′-deoxyadenosine (0.10 μmol), dissolved in 0.10 mL of 0.10 M Bis-Tris–AcOH (pH 6.8). Fig. 3 is representative of the separation results obtained by using an elution buffer of 0.10 M NaH_2PO_4–NaOH (pH 7.0). The two adenine derivatives were clearly separated: the adenosine was all eliminated in the FT and washing fractions W1–W3 (black columns in Fig. 3), and 97% of AMP was eluted in the five elution fractions E1–E5 (white columns in Fig. 3). When a 10 times diluted sample containing the same amounts of AMP and 2′-deoxyadenosine in 1.0 mL of the sample buffer was used (each at 0.10 mM), a similar AMP-selective distribution was observed with 50 μL of the beads. Because more than 98% of AMP (0.10 μmol in 1.0 mL) was captured by the beads within 1 min at room temperature, we therefore selected a period of 3 min for our basic protocol to ensure that complete binding occurred. A similar fast equilibrium for binding of phosphate to Zn^{2+}–Phos-tag has been previously demonstrated by a real-time phosphatase assay using micromolar concentrations of a fluorescent Zn^{2+}–Phos-tag derivative [26]. The total time required for the basic protocol, from the addition of a target phosphate sample to elution, is very short: less than 12 min. A further advantage is that all the steps can be conducted in aqueous solutions at neutral pH. The ability of the beads to bind phosphotyrosine remained almost constant after they had been reused 15 times in one day, and the standard deviation in the recoveries of phosphotyrosine in the elution fractions was less than 5%. Furthermore, beads in the free Phos-tag ligand form or in the Zn^{2+}-bound form that had been stored in propan-2-ol at 4 °C showed no decrease in phosphate affinity or capacity after three months or more.

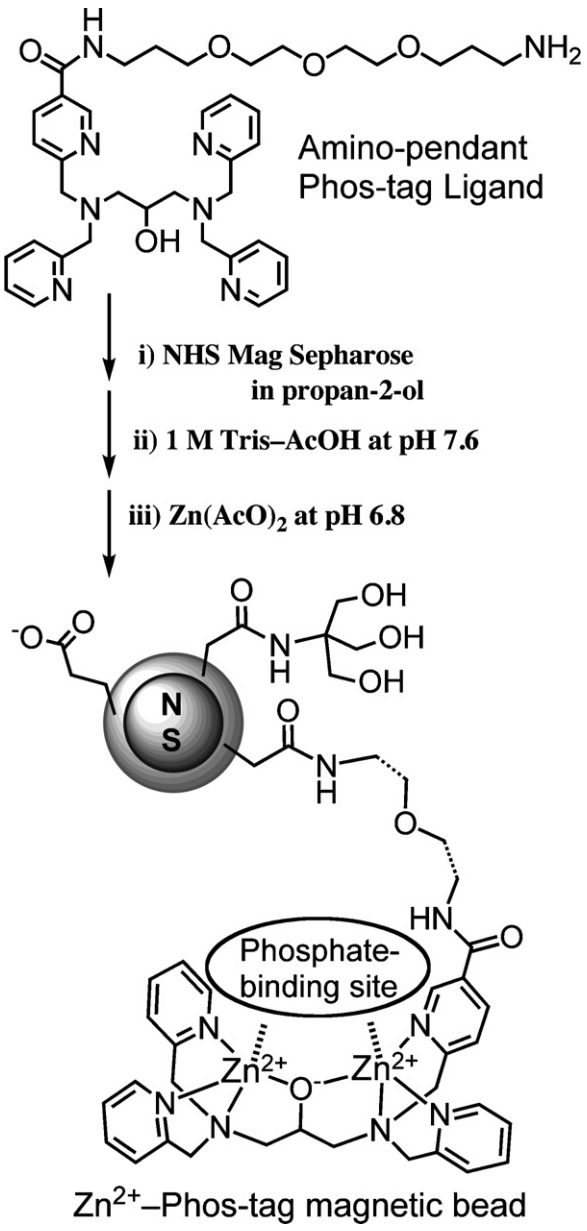

Fig. 1. Scheme for the preparation of Zn^{2+}–Phos-tag magnetic beads.

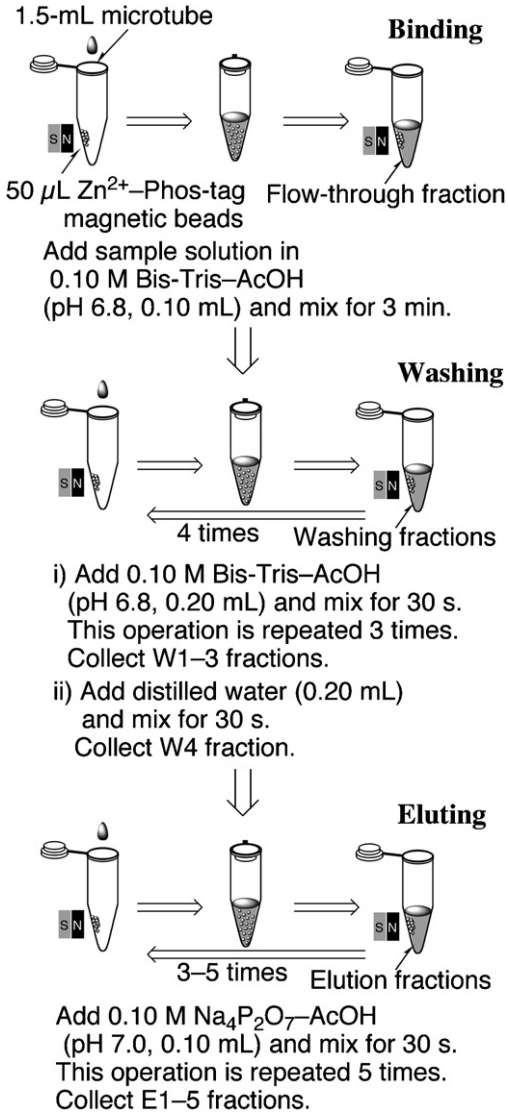

Fig. 2. Basic protocol for the separation of phosphorylated biomolecules by using 50 μL of Zn^{2+}–Phos-tag magnetic beads and a 1.5-mL microtube at room temperature.

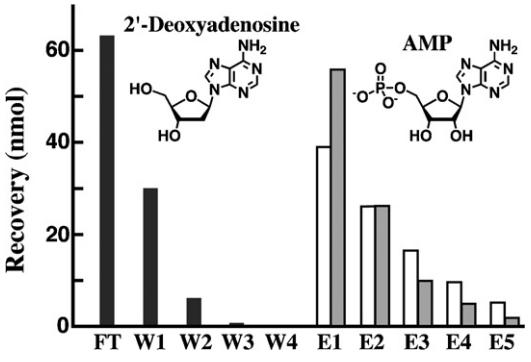

Fig. 3. Recoveries of 2′-deoxyadenosine (black columns) and AMP (white or gray columns) by using 50 μL of Zn^{2+}–Phos-tag magnetic beads. The sample solution (pH 6.8, 0.10 M Bis-Tris–AcOH, 0.10 mL) contained equivalent amounts (0.10 μmol) of each compound. The elution buffer solution (pH 7.0, 0.10 mL × 5) was 0.10 M aqueous NaH_2PO_4–NaOH (white columns) or 0.10 M aqueous $Na_4P_2O_7$–AcOH (gray columns).

3.2. Phosphate affinities of Zn^{2+}–Phos-tag magnetic beads

To estimate the relative affinity of Zn^{2+}–Phos-tag magnetic beads for various phosphorylated compounds with low molecular weights, we performed competitive experiments between phenyl phosphate dianion and the following dianionic phosphates: FMN [pK_2 = 6.2, where pK_2 is the second pK_a of the phosphate group at 25 °C (27)], AMP (pK_2 = 6.2), *O*-phosphotyrosine (pK_2 = 5.6), *O*-phosphoserine (pK_2 = 5.8), *O*-phosphothreonine (pK_2 = 5.6), and phosphocreatine [pK_a = 2.7, pK_2 = 4.5, pK_a = 11.2 (protonated guanidyl group)]. The experimental procedure for the preparation of 50 μL of Zn^{2+}–Phos-tag magnetic beads bound to 0.10 μmol phenyl phosphate dianion was the same as the basic protocol up to the elution step. In a subsequent equilibration step, an aqueous solution (0.10 mL) containing a certain concentration of a competitive substrate and 0.10 M Bis-Tris–AcOH (pH 7.0) was used as an eluting buffer solution. After addition of the buffer, the resultant slurry was incubated at 25 °C for 5 min on a benchtop shaker to permit competitive replacement of the bead-bound phenyl phosphate dianion with the other phosphate dianions. In the absence of an additional phosphate, no elution of phenyl phosphate dianion was observed. Furthermore, no degradation, such as hydrolysis of the phosphorylated molecules to their nonphosphorylated counterparts, was observed under the experimental conditions, even with a prolonged incubation time of 1 h. We determined the value of $C_{1/2}$, the concentration of the additional phosphate required to remove half the phenyl phosphate dianion from the beads. At $C_{1/2}$, the equilibrated concentration of phenyl phosphate dianion in the elution fraction was 0.50 mM (equivalent to 50 nmol in 0.10 mL) and the amount remaining bound to the beads was 50 nmol. From the concentrations and the $C_{1/2}$ values, the relative affinity between the phosphates and Zn^{2+}–Phos-tag magnetic beads were estimated. The smaller is the value of $C_{1/2}$, the larger is the affinity. We obtained the following values for $C_{1/2}$: phosphocreatine, 200 mM; *O*-phosphothreonine, 73 mM; *O*-phosphoserine, 46 mM; *O*-phosphotyrosine, 18 mM; AMP, 15 mM; and FMN, 7.4 mM. All these values of $C_{1/2}$ are much larger than the equilibrated concentration of 0.50 mM of unbound phenyl phosphate dianion in the solution. The hydrophobicity and lower steric hindrance of the phenyl group probably contribute to its preferential binding, even to Zn^{2+}–Phos-tag moieties entrapped within the confined spaces of the beads. Of the phosphate dianions other than phenyl phosphate dianion, FMN showed the strongest affinity for the magnetic beads, possibly as a result of the more-hydrophobic interactions between the aromatic groups of FMN and the Phos-tag moiety. This fact is consistent with the structure of the Zn^{2+}–Phos-tag complex of the 4-nitrophenyl phosphate dianion, as determined by X-ray diffractometry, in which the pyridyl group of Phos-tag and the phenyl group face one another, suggesting the presence of a strong π–π hydrophobic interaction [19]. Phosphocreatine [$(HO)_2P(=O)NHC(=NH)N(Me)CH_2CO_2H$], which at pH 7 is a polyionic moiety containing a carboxylate monoanion and a phosphate dianion with an adjacent guanidinium monocation, showed a much weaker affinity for Zn^{2+}–Phos-tag magnetic beads. The electron-withdrawing nature of the guanidinium group reduces the electron density on the phosphate dianion, resulting in a decrease in the pK_2 value of the phosphate (to 4.5) and a consequent reduction in its ability to coordinate to the Zn^{2+}–Phos-tag moiety. With regard to the hydrophilic inorganic phosphates, the $C_{1/2}$ values are 60 mM for HPO_4^{2-} (pK_a values: 11.7, 6.7, and 2.0) and 3.0 mM for hydrogen pyrophosphate ($HP_2O_7^{3-}$; pK_a values 8.4, 6.0, 1.8, and <1) at pH 7. The stronger affinity for hydrogen pyrophosphate might be due to its greater anionic charge of −3 and its ability to chelate Zn^{2+} ($K_{ZnL} = [Zn^{2+}–P_2O_7^{4-}]/[Zn^{2+}][P_2O_7^{4-}] = 10^{8.7}\ M^{-1}$ [27]), which cause partial removal of zinc(II) ions from the Zn^{2+}–Phos-tag moiety, as in the case of the strong metal chelator EDTA. When a buffer

solution of 0.10 M aqueous $Na_4P_2O_7$–AcOH (pH 7.0) was used as the elution buffer, the greater recovery (57%) of AMP in the E1 fraction was observed (Fig. 3; gray columns) than that using the buffer of NaH_2PO_4–NaOH (Fig. 3; white columns). We therefore adopted this as our first-choice buffer in the basic protocol. Contrary to expectation, the phosphate-mimetic phosphatase inhibitor dihydrogen vanadate monoanion ($H_2VO_4^-$) (pK_a values for H_3VO_4: 13.0, 7.9, 3.8 [27]) showed no elution activity at concentrations of up to 50 mM in the same competitive experiment. This fact is convenient, as it permits the separation of phosphorylated biomolecules in the presence of the phosphatase inhibitor. Although the immobilized Zn^{2+}–Phos-tag moieties on the magnetic beads should possess a certain range of K_d values for phosphate molecules, the competitive adsorption method permits the estimation of relative affinities of various phosphate compounds under common experimental conditions.

3.3. Separation of various types of nucleotides

To evaluate the relative affinities of the dianion adenosine 5′-monophosphate (AMP; pK_2 = 6.2), the trianion adenosine 5′-diphosphate (ADP; pK_2 = 4.0, pK_3 = 6.4), and the tetraanion adenosine 5′-triphosphate (ATP; pK_3 = 4.1, pK_4 = 6.5), a mixed sample of these three nucleotides (each at 0.10 μmol) in 0.10 M Bis-Tris–AcOH buffer (pH 6.8) was subject to a separation experiment according to the basic protocol to obtain the usual ten fractions (FT, W1–W4, and E1–E5). Fig. 4A shows the amounts of the three nucleotides in each of the fractions. The standard deviations of the recoveries of those nucleotides were less than 5%. Because the total amounts of the phosphates (0.30 μmol) exceeds the binding capacity (0.20 μmol of phenyl phosphate dianion per 50 μL of beads), significant overflows of the three nucleotides were observed. A comparison of the recoveries showed that the affinity increases in the order ATP < ADP < AMP. Although the electric charge on ATP is the largest (−4), the affinity of the dianionic AMP is almost three times greater than that of ATP. This may be due to the more-favorable hydrophobic interaction with AMP, the adenyl group of which can approach closer to the Phos-tag ligand, as well as to the larger steric hindrance of the hydrated triphosphate group of ATP. When smaller amounts of the three nucleotides (50 nmol/0.10-mL sample buffer) were used, almost all the nucleotides were captured and collected in the elution fractions (see Fig. 4B).

Previously, we have shown by means of real-time kinase profiling using a fluorescent Phos-tag derivative that the presence of an excess of Mg^{2+} ion, which is essential for kinase reactions, decreases the binding activity of ATP to Zn^{2+}–Phos-tag [28]. Bearing this fact in mind, we examined the effect of adding 5.0 mM Mg^{2+} ion to the sample and to the washing buffers in a separation experiment using 0.50 mM of each nucleotide (50 nmol). As expected, significantly greater overflows were observed in comparison with those in the absence of Mg^{2+} ion (Fig. 4B and C). Nucleotide anions (L^{n-}) can form 1:1 complexes with Mg^{2+} ion with K_d values (where $K_d = [Mg^{2+}][L^{n-}]/[Mg^{2+} - L^{n-}]$) of 87 μM for ATP ($n$ = 4), 0.68 mM for ADP (n = 3), and 11 mM for AMP (n = 2) at 25 °C [27]. From the K_d and pK_a values, and from the concentration (0.50 mM) of the nucleotides in the sample buffer (pH 6.8) containing 5 mM of free Mg^{2+} ion, it was possible to estimate the reduced chemical activities of the Mg^{2+}-unbound nucleotides to be 8 μM for tetraanionic ATP, 57 μM for trianionic ADP, and 290 μM for dianionic AMP. Thus, the remarkable increase of the overflow of ATP is probably the result of the considerable decrease in its chemical activity (*ca.* 1/60) in comparison with that of ADP and AMP. However, large amounts of the last two nucleotides remained bound on the beads in the presence of Mg^{2+} ion, indicating that the Zn^{2+}–Phos-tag beads possess strong phosphate-binding sites with K_d values in a micromolar concentration range. In fact, when a solution of tenfold diluted nucleotides (i.e., 50 μM each of ATP, ADP, and AMP) in 1.0 mL of the 0.10 M Bis-Tris–AcOH sample buffer was used, the phosphate-selective distribution in the washing and elution fractions was almost identical to that shown in Fig. 4B.

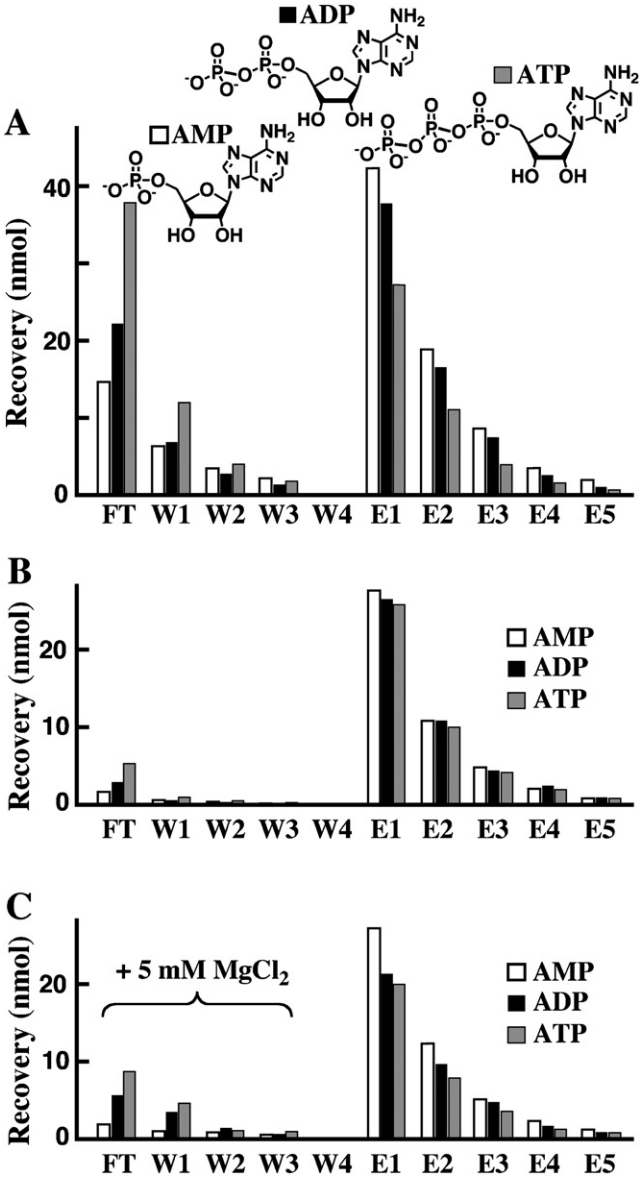

Fig. 4. Recoveries of AMP (white columns), ADP (black columns), and ATP (gray columns) using 50 μL of Zn^{2+}–Phos-tag magnetic beads. The elution buffer solution was 0.10 M aqueous $Na_4P_2O_7$–AcOH (pH 7.0, 0.10 mL × 5). (A) The sample solution (pH 6.8, 0.10 M Bis-Tris–AcOH, 0.10 mL) contained 0.10 μmol of the each nucleotide. (B) The sample solution (pH 6.8, 0.10 M Bis-Tris–AcOH, 0.10 mL) contained 50 nmol of the each nucleotide. (C) The sample solution (pH 6.8, 0.10 M Bis-Tris–AcOH, 0.10 mL) contained 50 nmol of each nucleotide and 5.0 mM $MgCl_2$. The washing buffer (pH 6.8, 0.10 M Bis-Tris–AcOH, 0.20 mL × 3) contained 5.0 mM $MgCl_2$.

In contrast, we confirmed that there is no affinity between Zn^{2+}–Phos-tag magnetic beads and phosphodiester-type monoanionic nucleotides [ROP(=O(O^-)OR′; 0.10 μmol/50 μL of beads) such as adenosine 3′,5′-cyclic monophosphate (cyclic AMP; λ_{max} = 259 nm, $\varepsilon = 1.5 \times 10^4\ M^{-1}\ cm^{-1}$), 2′-deoxyadenylylthymidine dinucleotide (dApT; λ_{max} = 260 nm, $\varepsilon = 2.1 \times 10^4\ M^{-1}\ cm^{-1}$ [30]), 2′-deoxyguanylylthymidine dinucleotide (dGpT; λ_{sh} = 251 nm, $\varepsilon = 2.0 \times 10^4\ M^{-1}\ cm^{-1}$ [30]), or 2′-deoxycytidylylthymidine dinucleotide (dCpT; λ_{max} = 267 nm, $\varepsilon = 1.6 \times 10^4\ M^{-1}\ cm^{-1}$ [30]), resulting in their complete recovery

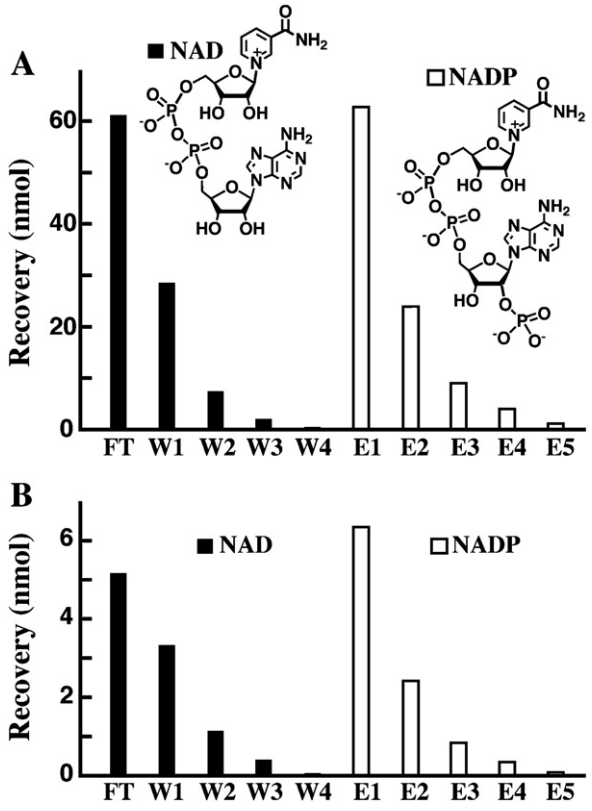

Fig. 5. Recoveries of NAD (black columns) and NADP (white columns) using 50 μL of Zn^{2+}–Phos-tag magnetic beads. The elution buffer solution was 0.10 M aqueous $Na_4P_2O_7$–AcOH (pH 7.0, 0.10 mL × 5). (A) The sample solution (pH 6.8, 0.10 M Bis-Tris–AcOH, 0.10 mL) contained 0.10 μmol of the each nucleotide. (B) The sample solution (pH 6.8, 0.10 M Bis-Tris–AcOH, 0.10 mL) contained 10 nmol of the each nucleotide.

in the FT and washing fractions. These results are consistent with the much lower affinity between the diphenyl phosphate monoanion [$(PhO)_2P(=O)O^-$; $K_d > 50$ mM] and the unsubstituted Zn^{2+}–Phos-tag molecule compared with that between the phenyl phosphate dianion [$PhOP(=O)(O^-)_2$; $K_d = 25$ nM] and the unsubstituted Zn^{2+}–Phos-tag molecule in aqueous solution at 25 °C [19]. In addition, we noted that the four DNA bases 2′-deoxyadenine, thymine, 2′-deoxyguanine, and 2′-deoxycytosine have no affinity for the Zn^{2+}–Phos-tag molecule under the experimental conditions. Previously, we have shown that phosphate-affinity electrophoresis on a polyacrylamide gel containing immobilized Zn^{2+}–Phos-tag molecules can produce clean separations between various oligonucleotides or DNA oligomers with 3′- and/or 5′-terminal phosphate groups and their nonphosphorylated counterparts [25]. The ability of the Zn^{2+}–Phos-tag moiety to bind selectively to compounds with terminal phosphate group suggests that the magnetic bead method might be useful in the purification of oligonucleotides carrying terminal phosphate groups.

Finally, we examined the separation of another group of nucleotides containing pyrophosphate diester moieties [$ROP(=O)(O^-)$–O–$P(=O)(O^-)OR'$] by using NAD (nicotinamide adenine dinucleotide) and its 2′-phosphorylated counterpart (NADP) as typical examples; both these compounds ($\lambda_{max} = 259$ nm, $\varepsilon = 1.69 \times 10^4\ M^{-1}\ cm^{-1}$) act as coenzymes for various biological redox reactions. We conducted a separation experiment on a mixed sample of NAD and NADP (each at 0.10 μmol in 0.10 mL of the sample buffer). All the fractions were analyzed by HPLC using the reverse-phase column. Fig. 5A shows the results for the distribution; these confirm that a distinct separation did occur, with more than 99% recovery of each of the nucleotides. The standard deviations in the recoveries of the nucleotides were less than 7%. To evaluate the separation efficiency for smaller amounts of sample, a mixed sample of 10 nmol of the each nucleotide in 0.10 mL of the sample buffer was subjected to the same experiment. The amounts of nucleotides in each fraction are shown in Fig. 5B. The complete separation of NAD and NADP suggests that no binding of NAD occurred, even in the presence of *ca.* 20 equivalents of phosphate-free binding sites in Zn^{2+}–Phos-tag. These facts demonstrate that pyrophosphate diester dianions have no affinity for the Phos-tag moiety under the experimental conditions. Therefore, the Phos-tag-based magnetic bead method can be used in the comprehensive purification of phosphomonoester-type nucleotides [$ROP(=O)(O^-)_2$] by enrichment in the elution fractions, and of phosphodiester-type nucleotides [$ROP(=O(O^-)OR'$] and pyrophosphate diester-type nucleotides [$ROP(=O)(O^-)$–O–$P(=O)(O^-)OR'$] by collection in the flow-through and washing fractions.

3.4. Separation of phosphorylated biomolecules related to the phosphoproteome

To date, phosphotyrosine (pTyr), phosphoserine (pSer), and phosphothreonine (pThr) have attracted the most attention in relation to phosphoproteomic research on cellular signaling in higher eukaryotes. However, many other phosphorylated amino acid residues, such as phosphohistidine, phospholysine, phosphoarginine, phosphocysteine, phosphoaspartic acid, and phosphoglutamic acid, are equally important, although their biological roles are less well understood. In the biochemical analysis of phosphorylated proteins containing these unusual residues, sites of phosphorylation are frequently missed owing to the acid lability of the P—N and P—O bonds. Because the Zn^{2+}–Phos-tag magnetic bead method can be conducted under mild conditions and at neutral pH values, we attempted to apply the method to the separation of phosphorylated biomolecules related to the phosphoproteome. As described in Section 3.2, we have demonstrated that Zn^{2+}–Phos-tag magnetic beads binds to three *O*-phosphorylated amino acids (pTyr, pSer, and pThr) and to phosphocreatine [$(HO)_2P(=O)NHC(=NH)N(Me)CH_2CO_2H$], an analog of *N*-phosphorylated arginine, and we estimated their $C_{1/2}$ values by means of phosphate-exchange experiments. Incidentally, the carboxylic–phosphoric acid anhydride acetyl phosphate ($AcOPO_3^{2-}$), an analog of phosphoaspartic acid and phosphoglutamic acid, can be detected as a stable 1:1 Zn^{2+}–Phos-tag complex in aqueous solution by means of MALDI-TOF mass spectrometry [29]. Because pTyr and phosphocreatine are commercially available and are capable of being analyzed quantitatively by HPLC, we used these phosphates as typical samples. Whereas pTyr has a strong affinity for the Zn^{2+}–Phos-tag magnetic beads, phosphocreatine has a much weaker affinity. As its chemical stability is similar to that of phosphorylated arginine [31], phosphocreatine is stable at neutral pH, but is extremely unstable at pH values below 4, as a result of acid-catalyzed hydrolysis, and its half-life at pH 3.6 and 30 °C is 43 min [32]. First, we examined the separation of pTyr by using a mixed sample containing equimolar amounts (0.10 μmol) of pTyr and Tyr (each at 1.0 mM) according to the basic protocol. The distribution results showed a distinct separation and almost complete recovery of pTyr in the elution fractions. The FT and W1–W3 fractions contained 62, 28, 8, and 2 nmol of Tyr, respectively, with none in W4. The E1–E5 fractions contained 67, 22, 8, 3, and 0 nmol of pTyr, respectively. To examine the case when the amount of sample was much smaller than the phosphate-binding capacity of the beads (0.20 μmol per 50 μL of beads), we subjected a sample containing 10 nmol each of pTyr and Tyr in 1.0 mL of the sample buffer to the same experiment. Although the sample concentration was decreased by a factor of 100 to 10 μM, a similar distribution pattern to that shown at 1.0 mM was observed. The amounts of the

pTyr eluted in the E1–E5 fractions were 6.5, 2.4, 0.9, 0.2, and 0 nmol, respectively.

Similarly, we examined the separation of phosphocreatine (0.10 μmol) from its nonphosphorylated counterpart, creatine (0.10 μmol). Because acetate ion interferes with the HPLC analysis of creatine, we used a sample buffer consisting of 0.10 M aqueous Bis-Tris–HCl (pH 6.8) and an elution buffer consisting of 0.10 M aqueous $Na_4P_2O_7$–HCl (pH 7.0). The results showed a phosphate-selective distribution, but with slight leakages (3 nmol) of phosphocreatine in the FT and W1 fractions. All the creatine molecules were eliminated in the FT and W1–W3 fractions. The amounts of phosphocreatine eluted in the E1–E5 fractions were 57, 22, 10, 5, and 3 nmol, respectively. After the separation procedure had been interrupted at the first elution step (E1), the suspension of the beads was allowed to stand for 12 h at room temperature and then the subsequent steps were performed to obtain the E1–E5 fractions. HPLC analysis of the eluting fractions showed that the phosphocreatine molecules remained intact, resulting in the same recoveries as those reported above. In other words, the Zn^{2+}–Phos-tag magnetic beads showed no detectable acceleration of the hydrolysis of phosphocreatine under the experimental conditions. Furthermore, when we conducted a separation experiment by using a mixed sample of 50 nmol pTyr and 50 nmol phosphocreatine with 50 μL of beads, we achieved complete capture and efficient elution of both the phosphate molecules in the E1–E5 fractions, in a similar manner to that of the mixed sample of AMP, ADP, and ATP.

Finally, to examine the performance of the Zn^{2+}–Phos-tag magnetic bead method in the analysis of phosphopeptides, we examined the separation of phosphopeptides from a tryptic digest. A well-known phosphorylated protein, β-casein (MW 24 kDa, 5 nmol) was used as the first example; this should provide, in theory, 16 peptides containing two phosphoserine peptides. One of these is the monophosphopeptide Phe-Gln-pSer-Glu-Glu-Gln-Gln-Gln-Thr-Glu-Asp-Glu-Leu-Gln-Asp-Lys (P1) and another is the tetraphosphopeptide Arg-Glu-Leu-Glu-Glu-Leu-Asn-Val-Pro-Gly-Glu-Ile-Val-Glu-pSer-Leu-pSer-pSer-pSer-Glu-Glu-Ser-Ile-Thr-Arg (P2). By following the basic protocol using 50 μL of the beads, except with the addition of 0.10 M NaCl to the washing buffer, we obtained ten fractions, consisting of 0.10 mL of FT, 0.20 mL each of W1–W3, 0.20 mL of W4, and 0.10 mL each of E1–E5. The presence of the neutral salt NaCl ensured complete elimination of nonphosphorylated peptides. The percentage recoveries of the phosphopeptides in each fraction were determined by HPLC. Peak assignments were made by using the commercially available standard phosphopeptides P1 and P2. Because the chromatography stage had to be performed at an acidic pH (see Section 2.3), the samples were acidified with TFA to below pH 3 immediately before injection. Fig. 6A shows typical chromatograms for the sample solution, flow-through fraction (FT), and an elution fraction (E1). Clearly, both phosphopeptides were efficiently separated from their nonphosphorylated counterparts. The recoveries of P1 and P2 in the E1 fraction were 69% and 71%, respectively. The remaining phosphopeptides on the beads were eluted in the E2 and E3 fractions, resulting in total recoveries of more than 95%.

Next, we examined a more-complex sample (0.10 μL) containing two synthetic samples of naturally occurring phosphotyrosine peptides (each at 5 nmol), their nonphosphorylated counterparts (more than twice the amount), and a tryptic digest of the nonphosphorylated protein BSA (MW 66 kDa, 5 nmol). This digest is generally used as a standard nonphosphorylated impurity in phosphate-specificity measurements [33]. The phosphopeptides were a phosphorylated Src peptide 521–533 (a phosphotyrosine acidic peptide; P3) and a kinase domain of insulin receptor-2 (a phosphotyrosine neutral peptide; P4), the sequences of which are

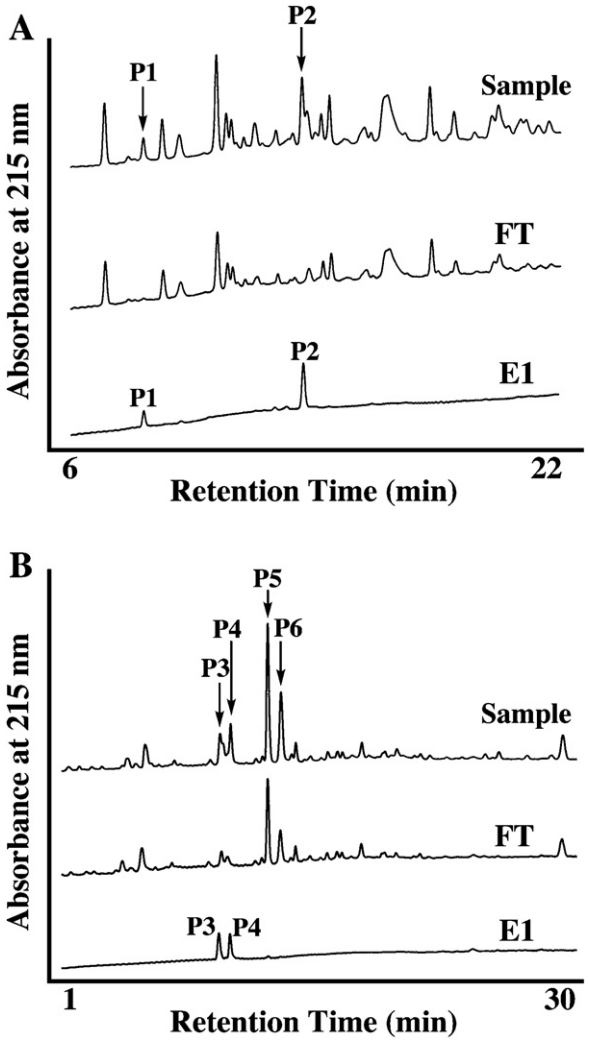

Fig. 6. Comparison of reverse-phase HPLC for peptide analysis. (A) The tryptic digest of β-casein (5 nmol) in the sample solution (upper chromatogram), in the flow-through fraction (middle chromatogram), and in the elution fraction (E1, lower chromatogram). P1 and P2 are the mono- and tetraphosphoserine peptides, respectively. The washing buffer for the W1–W3 steps consisted of 0.10 M Bis-Tris–AcOH and 0.10 M aqueous NaCl (pH 6.8, 0.20 mL × 3). (B) A peptide mixture of the tryptic digest of BSA and synthetic peptides in the sample solution (upper chromatogram), in the flow-through fraction (middle chromatogram), and in the elution fraction E1 (lower chromatogram). P3, P4, P5, and P6 are the phosphorylated Src peptide (5 nmol), the phosphorylated insulin receptor peptide (5 nmol), the nonphosphorylated insulin receptor peptide, and the nonphosphorylated Src peptide, respectively. The washing buffer for the W1–W3 steps consisted of 0.10 M Bis-Tris–AcOH and 0.10 M aqueous NaOAc (pH 6.8, 0.20 mL × 3).

discussed in Section 2.1. The separation experiment was conducted by a similar protocol to that for the digest of β-casein. Because a few percent of the nonphosphorylated insulin receptor peptide remained on the beads after the washing step using 0.10 M NaCl, we changed the additive in the washing buffer to 0.10 M NaOAc. Fig. 6B shows typical chromatograms for the sample and for the FT and E1 fractions. All the nonphosphorylated peptides, including the nonphosphorylated P5 and P6, were eliminated, and both the phosphopeptides were collected in the elution fractions. P3 and P4 both showed the same distribution pattern in the E1, E2, and E3 fractions, with recoveries of 68, 24, and 8%, respectively. Thus, the Zn^{2+}–Phos-tag magnetic beads can comprehensively and reversibly capture various phosphorylated compounds related to the phosphoproteome under mild conditions, such as a neutral

pH and temperatures between 10 and 30 °C. If, however, the total concentration of phosphorylated species, including inorganic phosphate and phosphomonoester-type biomolecules, in a sample solution is not known, the size of the sample or the volume of the beads should be optimized so as not to lose phosphorylated target molecules, especially those with a relatively low affinity for Zn^{2+}–Phos-tag magnetic beads.

4. Conclusions

We have demonstrated a novel procedure for the simple and comprehensive separation of phosphomonoester-type biomolecules such as nucleotides, phosphorylated amino acids, or phosphopeptides by using the magnetic bead method based on the Phos-tag technology. The beads were synthesized by coupling a primary amine derivative of the Phos-tag ligand to an NHS-activated crosslinked agarose coating on a magnetic core. Under optimal conditions, the phosphate capacity was 4 μmol of phenyl phosphate dianion per milliliter of beads, and the phosphate-selective elution yield was almost 100%. The operation time for sample collection in the elution fractions was less than 12 min, which is shorter than those reported for the other phosphate-affinity purification methods, such as IMAC or immunoaffinity precipitation. Because the neutral pH buffer system and the short separation time produce less damage to the separated molecules, the collected phosphorylated biomolecules should be ideal for use in many downstream applications. Furthermore, the beads are sufficiently stable to permit their multiple use and their long-term storage for more than three months. Thus, the Phos-tag-based magnetic bead method can be successfully used for the purification and isolation of various phosphorylated biomolecules with high recovery yields and high purities, and it should provide the means to perform a variety of experiments in metabolomics and phosphoproteomics by coupling with various down-stream applications including advanced mass spectrometry-based technology.

Acknowledgments

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