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N^{ω}-Phosphoarginine Phosphatase (17 kDa) and Alkaline Phosphatase as Protein Arginine Phosphatases[1]

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Seven synthetic polymers, $(Glu_4, Tyr)_n$, $(Arg)_n$, (Arg, Pro, Thr)$_n$, (Arg-Gly-Glu)$_6$, (Arg-Gly-Phe)$_6$, (Glu-Arg-Gly-Phe)$_5$, and (Ala-Leu-Arg-Arg-Ile-Arg-Gly-Glu-Arg)$_2$, were treated with phosphoryl chloride to phosphorylate their Tyr, Thr, and Arg residues. Protamines and histones were phosphorylated similarly. These phosphorylated peptides were examined as to whether or not they serve as substrates for intestinal alkaline phosphatase [EC 3.1.3.1] and liver N^{ω}-phosphoarginine phosphatase [Kuba, M., Ohmori, H., and Kumon, A. (1992) *Eur. J. Biochem.* 208, 747-752]. Phosphorylated polyarginine was hydrolyzed with a lower K_m with alkaline phosphatase than with N^{ω}-phosphoarginine phosphatase, while the phosphorylated forms of (Arg-Gly-Phe)$_6$ and culpeine were better substrates for N^{ω}-phosphoarginine phosphatase. When (Arg, Pro, Thr)$_n$ and culpeine were phosphorylated chemically after treatment with phenylglyoxal, these phosphorylated peptides were worse substrates for N^{ω}-phosphoarginine phosphatase than for alkaline phosphatase. Moreover, the results of proton-decoupled ^{31}P NMR analysis indicated that N^{ω}-phosphoarginine phosphatase released P_i from N^{ω}-phosphoarginine residues of phosphopeptides. These results indicate that both phosphatases function as protein arginine phosphatases in different manners, and that N^{ω}-phosphoarginine phosphatase is useful for selectively detecting N^{ω}-phosphoarginine residue in peptides containing various kinds of phosphorylated amino acids.

Key words: alkaline phosphatase, N^{ω}-phosphoarginine phosphatase, phenylglyoxal treatment, ^{31}P-NMR, protein arginine phosphatase.

N^{ω}-Phosphoarginine (P-Arg) occurs in either a free form or an associated form. Free P-Arg is synthesized *in vivo* by arginine kinase [EC 2.7.3.3] in invertebrates and is regarded as an immediate buffer of myoplasmic ATP (*1*). However, in mammalian tissues, arginine kinase and P-Arg are replaced by creatine kinase [EC 2.7.3.2] and phosphocreatine, respectively, and there is no possibility that free P-Arg plays any role in ATP metabolism. On the other hand, the associated form occurs as the P-Arg residues of protein molecules like rat myelin basic protein (*2*) and viral capsidic protein (*3, 4*). Concerning the metabolism of these P-Arg residues, it is likely that protein arginine kinase and protein arginine phosphatase participate in the phosphorylation of Arg residues and in the dephosphorylation of P-Arg residues, respectively. Until now, only two protein arginine kinases have been reported to occur in mammalian tissues (*5, 6*). One of them has been partially purified on a calmodulin affinity column from mouse leukemia cells and phosphorylates the Arg residues of histone H3 (*6*). However, such a protein arginine phosphatase that removes P_i from the P-Arg residues of histone H3 remains to be found.

Recent reports from our laboratory demonstrated that alkaline phosphatase [EC 3.1.3.1] (*7*) and N^{ω}-phosphoarginine phosphatase (PAPase) of 17 kDa (*8, 9*) are able to release P_i from P-Arg, suggesting that these phosphatases are candidates for protein arginine phosphatases. In this study, we examined whether or not these phosphatases could dephosphorylate various peptides containing P-Arg residues.

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Abbreviations: E, efficiency (ratio of V_{max} to K_m); P-Arg, N^{ω}-phosphoarginine; P-His, 3-phosphohistidine; P-Lys, 6-phospholysine; P-Ser, *O*-phosphoserine; P-Thr, *O*-phosphothreonine; P-Tyr, *O*-phosphotyrosine; PAPase, N^{ω}-phosphoarginine phosphatase; peptide ALRRIRGER, (Ala-Leu-Arg-Arg-Ile-Arg-Gly-Glu-Arg)$_2$; peptide ERGF, (Glu-Arg-Gly-Phe)$_6$; peptide EY, $(Glu_4, Tyr)_n$; peptide R, $(Arg)_n$; peptide RGE, (Arg-Gly-Glu)$_6$; peptide RGF, (Arg-Gly-Phe)$_6$; peptide RPT, (Arg, Pro, Thr)$_n$; ϕ-culpeine, culpeine treated with phenylglyoxal; ϕ-peptide RPT, (Arg, Pro, Thr)$_n$ treated with phenylglyoxal.

MATERIALS AND METHODS

Materials—Peptides: Three synthetic peptides, $(Glu_4, Tyr)_n$ (peptide EY), $(Arg)_n$ (peptide R), and (Arg, Pro, Thr)$_n$ (peptide RPT), sperm protamines (culpeine and salmine) from herring and salmon, calf thymus histones (histones V-S and VIII-S), and P-Arg were purchased from Sigma. Peptide EY is a 39 kDa random copolymer, consisting of Glu and Tyr in a molar ratio of 3.9:1.0. Peptide R is a 43 kDa homopolymer of Arg. Peptide RPT is a random

copolymer of 7.4 kDa, containing Arg, Pro, and Thr in a molar ratio of 52 : 17 : 26. Four sequential copolymers, that is, (Arg-Gly-Glu)$_6$ (peptide RGE), (Arg-Gly-Phe)$_6$ (peptide RGF), (Glu-Arg-Gly-Phe)$_5$ (peptide ERGF), and (Ala-Leu-Arg-Arg-Ile-Arg-Gly-Glu-Arg)$_2$ (peptide ALRRIRGER) were obtained from Peptide Institute, Osaka.

Enzymes: Bovine intestinal alkaline phosphatase (type 1-S) was obtained from Sigma. It hydrolyzes *O*-phosphoserine (P-Ser), *O*-phosphothreonine (P-Thr), and *O*-phosphotyrosine (P-Tyr) in addition to P-Arg (*7*). PAPase was purified partially from rat liver cytosol to the superfine Sephadex G-75 gel filtration step (*9*), its purity being 25%. The PAPase preparation dephosphorylated P-Arg alone, *i.e.* it did not hydrolyze phosphocreatine, 6-phospholysine (P-Lys), 3-phosphohistidine (P-His), P-Ser, P-Thr, or P-Tyr (*8*). When alkaline phosphatase or PAPase was incubated at 30°C in a 100 ml reaction mixture comprising 50 mM Tris-HCl (pH 7.5) and 1 mM P-Arg in the absence and presence of 10 mM 2-mercaptoethanol, respectively, alkaline phosphatase, or PAPase released P_i at the velocity of 96 or 222 nmol/min/mg protein, respectively. Since the specific activity of PAPase for P-Arg was 2.3-fold higher than that of alkaline phosphatase, every comparative assay in this study was carried out employing an amount of phosphatase having the same activity for P-Arg, that is, 2.0 μg of alkaline phosphatase or 0.85 μg of PAPase in each assay. The amount of P_i released into the reaction mixture was determined by the malachite green method (*8*).

Chemical Synthesis of Phosphorylated Peptides—Phosphorylated peptides were prepared by the P-Arg synthesis method (*10*). Briefly, 25 mg of a synthetic peptide or protein were dissolved in 600 μl of 7 N sodium hydroxide, followed by the dropwise addition of phosphoryl chloride (300 μl, 3.4 mmol) and 10 N sodium hydroxide (1.2 ml) over a period of 30 min. The temperature of the mixture was maintained at between 0 and 5°C by periodic immersion in an ice bath and the pH was kept at 13-14, as monitored with indicator paper. The mixture was dispersed sometimes by sonication if necessary. After the addition of phosphoryl chloride, the mixture was stirred for 1 h, followed by centrifugation at 2,000×g for 5 min. The supernatant was stored for the next step. The precipitate was extracted with another 0.5 ml of 20 mM Tris-HCl (pH 8.5) and 0.2 M sodium chloride, followed by centrifugation. This extraction was repeated twice more. The combined supernatant of about 3 ml was applied to a superfine Sephadex G-25 column (1×100 cm) equilibrated with 20 mM Tris-HCl (pH 8.5) and 0.2 M sodium chloride. Fractions of 2.3 ml each were collected. The results of one gel filtration are given in Fig. 1. Phosphorylated peptide RGF (P-peptide RGF) was eluted in fractions 13-16, while phosphoryl chloride and P_i were eluted in fractions 20-30 (Fig. 1). Phosphorylated peptides of high molecular mass were dialyzed against 20 mM Tris-HCl (pH 8.5) before use and were free of P_i. The protein concentrations of phosphorylated peptides were determined as follows: phosphorylated peptides of unknown concentration and the corresponding peptides (standard peptides) of known concentration were hydrolyzed at 110°C for 24 h in 6 N hydrochloride. After evaporation of the hydrochloride from the hydrolyzates *in vacuo*, the latter were subjected to coloring with ninhydrin reagent, according to the method of McGrath (*11*). The absorbance of the phosphorylated peptides at 570 nm was compared with that of the standard peptides. The protein concentrations of enzymes were determined by the method of Lowry *et al.* (*12*).

Double Modification of Peptide RPT and Culpeine with Phenylglyoxal and Phosphoryl Chloride—Before the phosphorylation of peptide RPT and culpeine with phosphoryl chloride, the Arg residues of these peptides were modified with phenylglyoxal, a diketone reagent specific for the guanidino groups of Arg residues (*13*). Briefly, 25 mg of each peptide was dissolved in 1 ml of 0.5 M borate (pH 9.0) containing 80 mg of phenylglyoxal. The mixture was incubated at 30°C for 2 h. These peptides treated with phenylglyoxal were phosphorylated further chemically with phosphoryl chloride to obtain phosphopeptides containing P-Thr residues (and P-Ser residues in the case of culpeine) and Arg residues modified with phenylglyoxal at the guanidino groups. These phosphopeptides were designated tentatively as P-ϕ-peptide RPT and P-ϕ-culpeine to distinguish them from P-peptide RPT and P-culpeine, respectively. The extents of phosphorylation of P-ϕ-peptide RPT and P-ϕ-culpeine were 31% and 8% of those of P-peptide RPT and P-culpeine, respectively.

Determination of Acid-Labile Phosphorus and Total Phosphorus in Phosphorylated Peptides—Acid-labile phosphorus in each phosphorylated peptide preparation was determined by measuring P_i released on boiling of the preparation for 10 min in 0.1 N hydrochloride.

For the assaying of total phosphorus in a phosphorylated peptide preparation, it was converted to ash as described by Ames (*14*). The amount of P_i in the ash was determined by the malachite green method (*8*). The amount of phosphopeptides was expressed as either the amount of total phosphorus or the amount of protein per assay. Upon

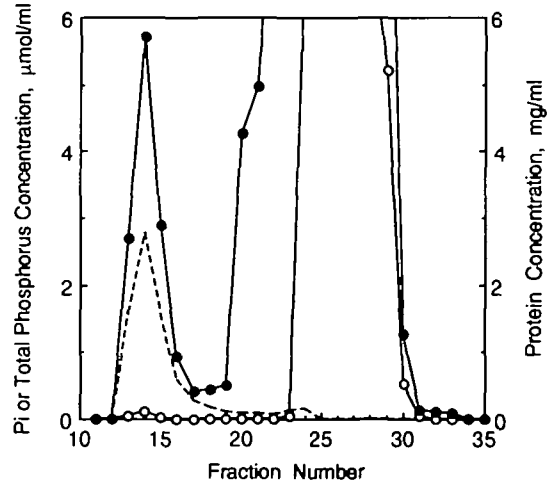

Fig. 1. **Sephadex G-25 column chromatography of reaction mixtures containing phosphoryl chloride and peptide RGF.** Twenty-five milligrams of peptide RGF and 3.4 mmol of phosphoryl chloride were incubated under the conditions described under "MATERIALS AND METHODS." The supernatant (3 ml) of the reaction mixture was applied to a superfine Sephadex G-25 column (1×100 cm) equilibrated with 20 mM Tris-HCl (pH 8.5) and 0.2 M sodium chloride. Fractions of 2.3 ml were collected. The concentrations of protein (broken line), P_i (open circles), and total phosphorus (closed circles) were determined according to the methods described under "MATERIALS AND METHODS." Fractions 13-15 were combined as the P-peptide RGF preparation.

TABLE I. **V_{max}, K_m, and efficiencies of alkaline phosphatase and PAPase for various phosphorylated substrates.** The velocity of phosphatase activity was determined by assaying the amount of P_i released into a 0.1 ml reaction mixture containing alkaline phosphatase (2 μg) or PAPase (0.85 μg) and various amounts of phosphopeptides as described under "MATERIALS AND METHODS." The V_{max} and K_m values are means for five experiments.

Substrates	Alk. phosphatase			PAPase			$\frac{E_p}{E_a}$
	V_{max}[a]	K_m[b]	E_a[c]	V_{max}[a]	K_m[b]	E_p[c]	
P-Amino acids							
P-Arg	0.10	0.29	0.34	0.30	0.34	0.88	2.56
P-Ser	0.63	0.23	2.74	0.02	n.d.[d]	n.d.	n.d.
P-Thr	0.56	0.22	2.55	0.01	n.d.	n.d.	n.d.
P-Tyr	0.59	0.48	1.23	0.01	n.d.	n.d.	n.d.
P-Synthetic peptides							
P-$(Glu_4, Tyr)_n$	0.24	0.20	1.20	0.01	n.d.	n.d.	n.d.
P-peptide ALRRIRGER	0.50	1.01	0.50	0.40	0.36	1.10	2.22
				0.91	1.82	0.50	1.01
P-$(Arg\text{-}Gly\text{-}Glu)_6$	0.57	0.48	1.19	0.63	0.42	1.50	1.26
P-$(Arg\text{-}Gly\text{-}Phe)_6$	0.09	0.71	0.13	1.05	1.74	0.60	4.62
P-$(Glu\text{-}Arg\text{-}Gly\text{-}Phe)_5$	0.46	0.72	0.64	1.43	1.78	0.80	1.25
P-$(Arg, Pro, Thr)_n$	0.11	0.78	0.14	0.83	2.41	0.34	2.48
P-$(Arg)_n$	0.40	0.24	1.67	0.40	1.87	0.21	0.13
P-Natural peptides							
P-Salmine	0.19	0.57	0.33	0.54	0.79	0.68	2.06
P-Culpeine	0.31	1.59	0.19	0.69	0.87	0.79	4.16
P-Histone V	0.10	0.30	0.33	0.17	0.51	0.33	1.00
P-Histone VIII	0.14	0.35	0.40	0.17	0.35	0.49	1.23

[a] V_{max} is expressed in μmol/min/mg protein. [b] K_m is expressed as the millimolar concentration of total phosphorus of the phosphorylated peptide in the reaction mixture. [c] Efficiency, E, is the ratio of V_{max} to K_m. E_a and E_p denote the efficiencies of alkaline phosphatase and PAPase, respectively. [d] n.d., not determined.

determination of the K_m values of the two phosphatases for the various substrates in Table I, the millimolar concentration of total phosphorus was employed as the unit of substrate concentration.

Identification of P-Tyr and P-Thr in Phosphorylated Peptides—To verify the existence of P-Tyr residues in the P-peptide EY preparation, the preparation was hydrolyzed at 155°C for 35 min in 5 N potassium hydroxide and then neutralized with perchloric acid (*15*). After centrifugation of the neutralized hydrolyzate, the supernatant was subjected to high voltage paper electrophoresis at 50 V/cm with a buffer solution (pH 3.5) containing 0.22% pyridine and 2.2% acetic acid. Similarly, for the detection of P-Thr residues in the P-peptide RPT preparation, the preparation was hydrolyzed at 110°C for 4 h in 6 N hydrochloric acid, followed by evaporation of the hydrochloric acid *in vacuo* (*16*). After solubilization of solid materials, the hydrolyzate was subjected to high voltage paper electrophoresis using a buffer solution (pH 1.9) containing 2% formic acid and 8% acetic acid. The spots of P-Tyr and P-Thr on the paper were stained with ninhydrin reagent. Incubation of P-peptide EY and P-peptide RPT with alkaline phosphatase resulted in the reduction of P-Tyr and P-Thr, respectively.

Identification of P-Arg Residues in Phosphorylated Peptides by ^{31}P NMR—Contrary to P-Tyr and P-Thr, there is no way to isolate P-Arg from phosphorylated peptides by acid or alkaline hydrolysis. In this study, the P-Arg residues of the phosphorylated peptides were identified by proton-decoupled ^{31}P NMR (*17*); P-Arg, P-peptide ALRRIRGER, P-peptide RGE, and P-peptide RGF were incubated in the absence and presence of PAPase, and then ^{31}P NMR spectra of the reaction mixtures were recorded with a JEOL NMR-lambda 400 spectrometer operating in the Fourier transform mode at 161.70 MHz, with proton irradiation at 399.65 MHz. Chemical shifts were corrected as to 0 ppm positioned at the corrected value of 85% orthophosphoric acid.

RESULTS

Protein arginine kinase is known to phosphorylate histone H3 at four Arg residues, three of which are localized at positions 128, 129, and 131 in the C-terminal region (*6*). Various synthetic peptides containing a phosphorylatable sequence similar to the C-terminal region were phosphorylated chemically, and then employed as substrates for alkaline phosphatase and PAPase (Fig. 2). Figure 2A shows the results of hydrolysis of P-peptide ALRRIRGER that contains the C-terminal sequence (126 to 134) of histone H3. Both phosphatases released 17-20 nmol of P_i in 240 min from 18 μg of P-peptide ALRRIRGER that contained 109 nmol phosphorus per assay. According to Table I including various kinetical values for both phosphatases, alkaline phosphatase exhibited V_{max} of 0.50 (μmol of P_i released per min per mg enzyme) and K_m of 1.01 (millimolar concentration of total phosphorus) for P-peptide ALRRIRGER. On the other hand, the concentration curve of PAPase for P-peptide ALRRIRGER showed a biphasic change (data not shown), giving two V_{max} and K_m sets for this peptide. One V_{max} and its K_m were 0.40 μmol/min/mg and 0.36 mM, respectively, which were comparable to those of PAPase for P-Arg (Table I). The other V_{max} and its K_m were 0.91 μmol/min/mg and 1.82 mM, respectively.

Arg-Gly-Glu is a partial sequence of peptide ALRRIRGER, and a phosphorylated copolymer of this motif, P-peptide RGE, also acted as a substrate for either alkaline phosphatase or PAPase (Fig. 2B). P_i release from P-peptide RGE by PAPase was 60% of that by alkaline phosphatase. However, when Glu in P-peptide RGE was replaced with Phe, PAPase released 2.4-fold more P_i from P-peptide RGF

than alkaline phosphatase in 240 min (Fig. 2C). Oppositely, a modified form of P-peptide RGF with an additional Glu, P-peptide ERGF, gave results similar to those on the hydrolysis of P-peptide RGE (Fig. 2D). The V_{max} of alkaline phosphatase for P-peptide RGF was the lowest among the V_{max}s for various phosphopeptides in Table I. This tendency of P-peptide RGF is reflected as a high ratio of E_p to E_a, 4.62 (Table I). These results suggested that the removal of Glu from P-peptides RGE and ERGF rendered them poor substrates for alkaline phosphatase but not for PAPase. Consequently, P-peptide RGF seemed to be a unique substrate that is favorable for PAPase but unfavorable for alkaline phosphatase.

Figure 3A shows the enzymatic hydrolysis of a phosphorylated form of a natural substrate, P-salmine, which contains P-Arg clusters interrupted by neutral amino acids, P-Ser or P-Thr. The two phosphatases showed apparently similar activity toward P-salmine. However, P-peptide R, with an uninterrupted cluster of P-Arg residues, was found to be a poor substrate for PAPase (Fig. 3B). Kinetical analysis revealed that PAPase exhibited an eightfold higher K_m for P-peptide R than alkaline phosphatase (Table I). This difference in the K_m value for P-peptide R between the two phosphatases brought about the lowest ratio of E_p to E_a, 0.13. Therefore, P-peptide R was a second unique substrate distinguishing the two phosphatases from each other.

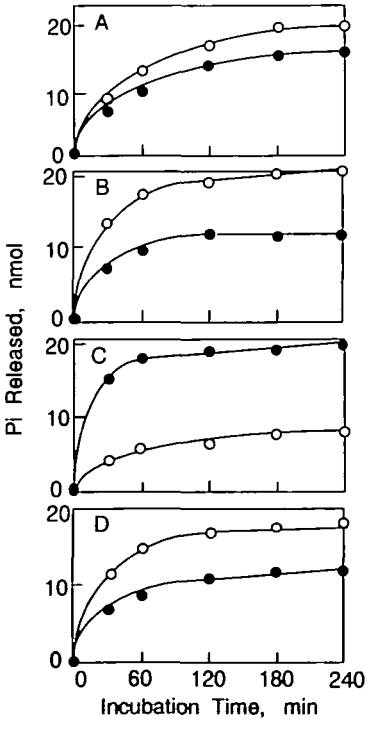

Fig. 2. **Time course of P_i release on incubation of P-peptide ALRRIRGER and its derivatives with phosphatases.** Each incubation was carried out with 2.0 μg of alkaline phosphatase and 0.85 μg of PAPase, that released 5.7 nmol of P_i from 1 mM P-Arg in 30 min. Peptides (amounts of total phosphorus/acid-labile phosphorus/peptide in 0.1 ml reaction mixture): A, P-peptide ALRRIRGER (109 nmol/66 nmol/130 μg); B, P-peptide RGE (58 nmol/44 nmol/84 μg); C, P-peptide RGF (57 nmol/35 nmol/103 μg); D, P-peptide ERGF (59 nmol/45 nmol/143 μg). Phosphatases: alkaline phosphatase (open circles); PAPase (closed circles).

Two other unique peptides showing different substrate specificities were P-peptide EY and P-culpeine (Table I). As expected from results of P-Tyr hydrolysis by phosphatases, PAPase did not release P_i from P-peptide EY, while alkaline phosphatase dephosphorylated the same peptide.

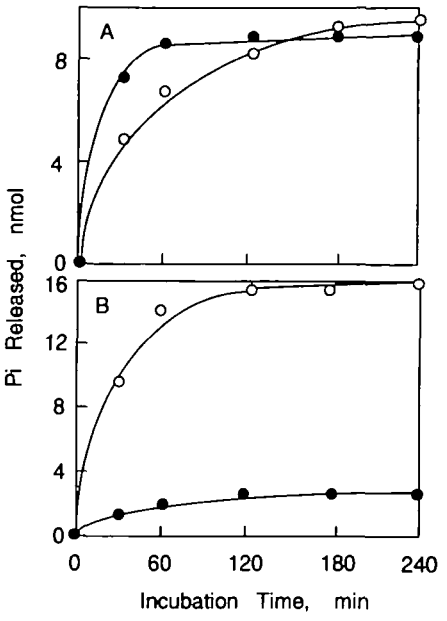

Fig. 3. **Time course of P_i release on incubation of P-salmine and P-peptide R with phosphatases.** Incubations were carried out as described under "MATERIALS AND METHODS." Peptides (amounts of total phosphorus/acid-labile phosphorus/peptide in 0.1 ml reaction mixture): A, P-salmine (76 nmol/42 nmol/47 μg); B, P-peptide R (31 nmol/21 nmol/23 μg). Phosphatases: alkaline phosphatase (2.0 μg, open circles); PAPase (0.85 μg, closed circles).

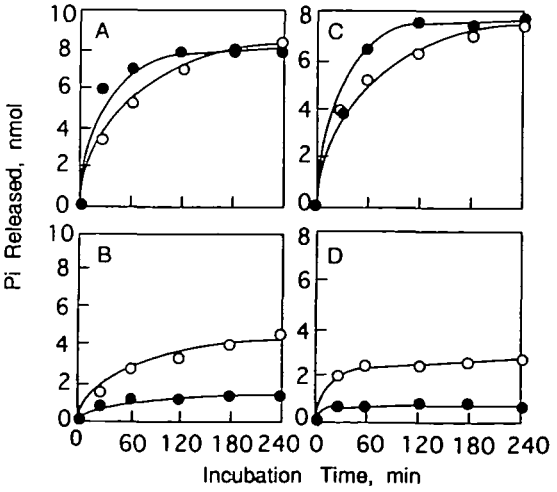

Fig. 4. **Time course of P_i release on incubation of P-peptide RPT, P-culpeine, P-ϕ-peptide RPT, and P-ϕ-culpeine with phosphatases.** Incubations were carried out as described under "MATERIALS AND METHODS." Peptides (amounts of total phosphorus/acid-labile phosphorus/peptide in 0.1 ml reaction mixture): A, P-peptide RPT (58 nmol/20 nmol/60 μg); B, P-ϕ-peptide RPT (17 nmol/4 nmol/53 μg); C, P-culpeine (54 nmol/44 nmol/129 μg); D, P-ϕ-culpeine (5 nmol/2 nmol/100 μg). Phosphatases: alkaline phosphatase (2.0 μg, open circles); PAPase (0.85 μg, closed circles).

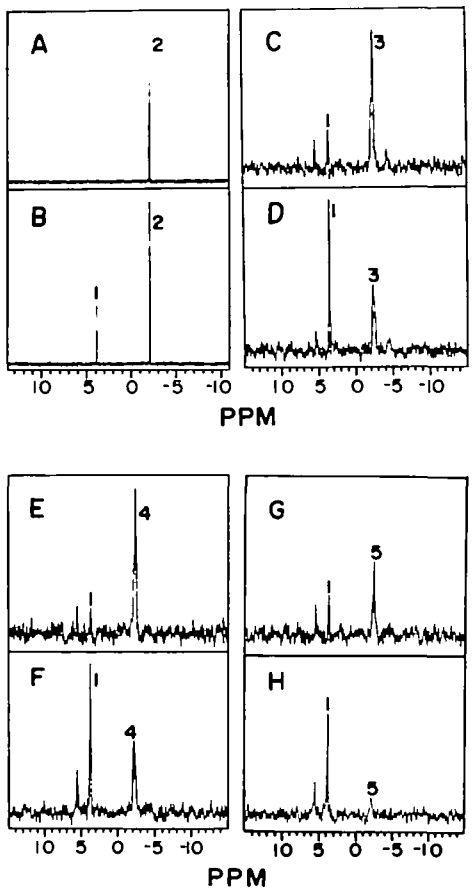

Fig. 5. **Proton-decoupled ^{31}P NMR spectra of P-Arg and P-peptides in the absence and presence of PAPase.** P-Arg (5 μmol/1.3 mg, A and B), P-peptide ALRRIRGER (0.9 μmol as total phosphorus/1.07 mg protein, C and D), P-peptide RGE (2 μmol as total phosphorus/2.91 mg protein, E and F), and P-peptide RGF (1.7 μmol as total phosphorus/3.09 mg protein, G and H) were added to a 0.5 ml mixture comprising 20 mM Tris-HCl (pH 7.5), 2 mM dithiothreitol in the absence (A, C, E, and G) or presence (B, D, F, and H) of 20 μg PAPase. After incubation of the reaction mixtures at 30°C for 6 h, they were lyophilized and then subjected to proton-decoupled ^{31}P NMR analysis. Sweep width: A and B, 4.00 kHz; C-H, 5.99 kHz. Scans: A, 462 times; B, 360 times; C-H, 4,096 times. Peak assignments: 1, P_i; 2, P-Arg; 3, P-peptide ALRRIRGER; 4, P-peptide RGE; 5, P-peptide RGF.

In the case of P-culpeine, alkaline phosphatase exhibited a twofold higher K_m than PAPase, and the latter exhibited a twofold higher V_{max} for P-culpeine than the former, resulting in a high ratio of E_p to E_a, 4.16.

The next experiment was carried out to demonstrate that the origins of P_i released by alkaline phosphatase and PAPase were different (Fig. 4). The upper part of Fig. 4 shows the time course of phosphatase-induced hydrolysis of P-peptide RPT and P-culpeine which contained phosphorylated Arg, Thr, and Ser residues. The phosphatases released P_i from P-peptide RPT (Fig. 4A) or P-culpeine (Fig. 4C) to almost the same extents. The lower part of Fig. 4 shows the P_i release from P-ϕ-peptides whose Arg residues were almost all unphosphorylated due to modification with phenylglyoxal, and whose Ser or Thr residues were phosphorylated; the P_i release from P-ϕ-peptide RPT (Fig. 4B) or P-ϕ-culpeine (Fig. 4D) by alkaline phosphatase was 50 or 30% of that from P-peptide RPT (Fig. 4A) or P-culpeine (Fig. 4C), respectively. Similarly, the P_i release from P-ϕ-peptide RPT (Fig. 4B) or P-ϕ-culpeine (Fig. 4D) by PAPase was 13 or 10% of that from P-peptide RPT (Fig. 4A) or P-culpeine (Fig. 4C), respectively. These results indicated that phenylglyoxal treatment had a more depressive effect on the P_i release by PAPase than that by alkaline phosphatase (Fig. 4, B and D), suggesting that alkaline phosphatase released P_i from P-Ser, P-Thr, and P-Arg residues, while PAPase only dephosphorylated P-Arg residues.

Figure 5 shows the proton-decoupled ^{31}P NMR spectra of the phosphopeptides incubated with and without PAPase. The P-Arg signal was observed at −2.07 ppm (Fig. 5A). The addition of PAPase decreased the P-Arg signal by 30% with a concomitant increase of the P_i signal at 3.90 ppm (Fig. 5B). Similarly, P-peptide ALRRIRGER signal was observed at −2.44 ppm, near the position of P-Arg (Fig. 5C), and 20% of the phosphopeptide signal was converted to a P_i signal by PAPase (Fig. 5D). Other results also indicated that PAPase dephosphorylated 30% of P-peptide RGE (Fig. 5, E and F) and 60% of P-peptide RGF (Fig. 5, G and H). All these results indicated that PAPase itself released P_i from the P-Arg residues in each phosphopeptide.

DISCUSSION

This paper indicated that alkaline phosphatase and PAPase dephosphorylated all the examined phosphopeptides with the exception of P-peptide EY with PAPase. For example, alkaline phosphatase exhibited a slightly lower K_m value for P-peptide R than that for P-Arg, while its V_{max} for P-peptide R was fourfold higher than that for P-Arg. This tendency of alkaline phosphatase toward P-peptide R was reflected in a high E_a value (1.67), as compared with the E_a value for P-Arg (0.34). Similarly, PAPase exhibited a twofold higher V_{max} for P-peptide RGE than that for P-Arg, and the E_p values for P-peptide RGE and P-Arg were 1.50 and 0.88, respectively (Table I), indicating that P-peptide RGE was a better substrate for PAPase than P-Arg. These results support the ideas that both phosphatases have more preferable peptide substrates than P-Arg, and that both phosphatases are able to function as protein arginine phosphatases.

The two phosphatases showed different substrate specificities, as indicated by the ratio of E_p to E_a (Table I); P-peptide EY was dephosphorylated only by alkaline phosphatase, while alkaline phosphatase exhibited higher affinity for P-peptide R than PAPase ($E_p/E_a = 0.13$). On the contrary, PAPase showed higher affinity, and a higher V_{max} for P-culpeine ($E_p/E_a = 4.16$) and a higher V_{max} for P-peptide RGF ($E_p/E_a = 4.62$) than alkaline phosphatase. These differences suggest that PAPase acts as a protein arginine phosphatase distinct from alkaline phosphatase.

Moreover, the authors want to indicate the possibility that PAPase is a useful tool for the identification of P-Arg residues in phosphorylated proteins. This proposal is based on the following findings: (i) it is very difficult to isolate P-Arg from phosphorylated peptides by conventional acid or alkaline hydrolysis, because of the instability of the nitrogen-phosphorus bonds in P-Arg residues on the hydrolysis. (ii) PAPase does not hydrolyze phosphorylated amino acids other than P-Arg (*8*). (iii) PAPase released P_i only

from P-Arg residues of phosphopeptides, as shown in this study.

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