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Characterization of the Phosphotyrosyl Protein Phosphatase Activity of Calmodulin-dependent Protein Phosphatase*

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Calmodulin-dependent protein phosphatase from bovine brain and heart was assayed for phosphotyrosine and phosphoserine phosphatase activity using several substrates: 1) smooth muscle myosin light chain (LC_{20}) phosphorylated on tyrosine or serine residues, 2) angiotensin I phosphorylated on tyrosine, and 3) synthetic phosphotyrosine- or phosphoserine-containing peptides with amino acid sequences patterned after the autophosphorylation site in Type II regulatory subunit of the cAMP-dependent protein kinase. The phosphatase was activated by Ni^{2+} and Mn^{2+}, and stimulated further by calmodulin. In the presence of Ni^{2+} and calmodulin, it exhibited similar kinetic constants for the dephosphorylation of phosphotyrosyl LC_{20} (K_m = 0.9 μM, and V_{max} = 350 nmol/min/mg) and phosphoseryl LC_{20} (K_m = 2.6 μM, V_{max} = 690 nmol/min/mg). Dephosphorylation of phosphotyrosyl LC_{20} was inhibited by phosphoseryl LC_{20} with an apparent K_i of 2 μM. Compared to the reactions with phosphotyrosyl LC_{20} as the substrate, reactions with phosphotyrosine-containing oligopeptides exhibited slightly higher K_m and lower V_{max} values. The reaction with the phosphoseryl peptide based on the Type II regulatory subunit sequence exhibited a slightly higher K_m (23 μM), but a much higher V_{max} (4400 nmol/min/mg) than that with its phosphotyrosine-containing counterpart. Micromolar concentrations of Zn^{2+} inhibited the phosphatase activity; vanadate was less potent, and 25 mM NaF was ineffective. The study provides quantitative data to serve as a basis for comparing the ability of the calmodulin-dependent protein phosphatase to act on phosphotyrosine- and phosphoserine-containing substrates.

The calmodulin-dependent protein phosphatase, also known as calcineurin, was originally purified as a major calcium- and calmodulin-binding protein from bovine brain (1, 2). Subsequent investigations indicated that this protein was identical to a calmodulin-dependent protein phosphatase (designated protein phosphatase 2B) purified from rabbit skeletal muscle (3, 4). Based on immunological and immunohistochemical data (5, 6), it is known that this enzyme is located predominantly in the brain but also in a variety of other mammalian tissues. Purification of the phosphatase from bovine brain (1, 2, 7), rabbit skeletal muscle (3, 8), bovine heart (9, 10), and most recently human platelets (11) has been reported. The enzyme is found to consist of two subunits having different molecular weights. Subunit A (M_r ~ 61,000) interacts with calmodulin and contains the catalytic site (12–15); subunit B (M_r ~ 15,000) binds up to four Ca^{2+} with high affinity (13, 15).

Physiological substrates for the calmodulin-dependent protein phosphatase have not been established. Under *in vitro* conditions, the enzyme catalyzes the dephosphorylation of a variety of proteins phosphorylated at serine or threonine residues (4, 8, 14–19), but exhibits a high catalytic efficiency toward only a few of these phosphoproteins. This enzyme is thus considered to have a very narrow substrate specificity compared to other known protein phosphatases (17, 20). Recent reports have indicated that the calmodulin-dependent protein phosphatase also exhibits activity toward free phosphotyrosine and *p*-nitrophenyl phosphate (21, 22), phosphotyrosyl casein (23), and the epidermal growth factor receptor (24), but detailed comparisons of phosphotyrosyl and phosphoseryl dephosphorylation reactions have not been performed under comparable conditions. The question is still open, therefore, of whether the calmodulin-dependent phosphatase exhibits kinetic characteristics suggesting that it might be capable of catalyzing the dephosphorylation of phosphotyrosyl and phosphoseryl/threonyl proteins *in vivo*.

In the present report, we present a detailed characterization and comparison of the phosphoseryl and phosphotyrosyl phosphatase activities of the calmodulin-dependent phosphatase using defined substrates. All of the protein and peptide substrates used were of known sequence and contained phosphoserine or phosphotyrosine residues at characterized sites. Moreover, in one instance, peptide substrates were constructed so that phosphoserine or phosphotyrosine was present in an identical peptide sequence. Kinetic constants obtained for the reactions with different substrates were used as a basis for comparisons. In light of evidence (25) indicating that the phosphatase exists in at least two main isozymic species (neuronal and non-neuronal), the experiments were performed using preparations of enzyme purified from bovine brain and cardiac muscle. In addition, activation by divalent cations (Ni^{2+} and Mn^{2+}) in the presence of calmodulin, and the inhibition by various common phosphatase inhibitors (F^-, Zn^{2+}, and VO_4^{3-}) of both phosphoseryl and phosphotyrosyl phosphatase activities of the enzyme were investigated. The results of these studies indicate that the calmodulin-dependent phosphatase can, under certain conditions, catalyze the dephosphorylation of phosphotyrosyl substrates at rates comparable to those seen with phosphoseryl substrates.

EXPERIMENTAL PROCEDURES

Materials—Epidermal growth factor was purchased from Collaborative Research and angiotensin I was purchased from Sigma. [γ-^{32}P]

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ATP was obtained from Du Pont/New England Nuclear. All other chemicals were of reagent grade. The R_{II}[1] peptides, R_{II}-(81–99)-peptide and [Tyr^{95}]R_{II}-(81–99)-peptide, were prepared using a Beckman 990 automated peptide synthesizer and standard solid-phase techniques (19) and subsequently purified by reversed-phase high-performance liquid chromatography. Human epidermoid carcinoma A431 cells were subcultured and membranes prepared according to Brautigan *et al.* (26).

Preparation of Proteins—Calmodulin-dependent protein phosphatase was purified from bovine brain according to the procedure described by Sharma *et al.* (7) and from bovine cardiac muscle by a modified procedure described elsewhere (19). Calmodulin was purified from fresh and frozen bovine testes (27). Smooth muscle regulatory myosin light chain (LC_{20}) was purified from chicken gizzard as previously described (28). Smooth muscle myosin light chain kinase was purified from chicken gizzard (29), and the catalytic subunit of cAMP-dependent protein kinase was purified from bovine heart (30) according to published procedures. Monoclonal antibody against the B subunit of the brain calmodulin-dependent protein phosphatase (VA_1) was obtained from mouse as described elsewhere (31).

Preparation of Phosphorylated Substrates—LC_{20} was phosphorylated at two tyrosine sites (tyrosine 142 and 155) using an A431 cell membrane preparation as previously described (28). The 90-μl reaction mixture contained 4 μg of A431 membranes, 100 ng of epidermal growth factor, 0.1% Nonidet P-40, 2.5 mM $MnCl_2$, 30 mM HEPES, pH 7.4, 100 μM sodium orthovanadate, 600 μM [γ-^{32}P]ATP (specific activity 800–1200 cpm/pmol) and 10 μM LC_{20}. Protein-bound radioactivity throughout the course of the phosphorylation reaction was followed using a filter paper technique as previously described (28). Stoichiometry of phosphate incorporation was calculated based on the specific activity of the radioisotope, the amount of protein-bound radioactivity, and the amount of substrate added to the reaction. After 120 min at 30 °C, the reaction mixture was subjected to electrodialysis against 2 mM MOPS, pH 7.0, 1 mM dithiothreitol, and 0.1 M urea to remove unincorporated ^{32}P. The resultant mixture was then equilibrated with 25 mM MOPS, pH 7.0, 1 mM dithiothreitol, and 0.3 M NaCl by extensive dialysis. The [^{32}P]phosphotyrosyl LC_{20} used in dephosphorylation experiments was estimaed to contain 1.5–2.0 mol of ^{32}P/mol of protein.

Smooth muscle LC_{20} was also phosphorylated at a serine residue (serine 19) in a separate reaction using smooth muscle myosin light chain kinase as described (25, 32). The phosphorylation reaction was carried out in 50 mM MOPS, pH 7.0, 0.2 mM $CaCl_2$, 10 mM magnesium acetate, 1 μM calmodulin, 1 mM dithiothreitol, 1 mM [γ-^{32}P]ATP (specific activity 400–500 cpm/pmol), 2 μg/ml myosin light chain kinase, and 10 μM LC_{20} at 30 °C for 120 min. Excess [γ-^{32}P]ATP was removed as described above. The [^{32}P]phosphoseryl LC_{20} was estimated to contain 1 mol of ^{32}P/mol of protein.

Angiotensin I and [Tyr^{95}]R_{II}-(81–99)-peptide peptides were phosphorylated at their respective tyrosine residues using A431 cell membranes as described for phosphotyrosyl LC_{20} above. Phosphopeptides were separated from non-peptide-bound ^{32}P by chromatography on AG 1-X2 acetate (Bio-Rad) columns using 1 N acetic acid. After evaporation under reduced pressure, the phosphopeptides were resuspended in water, and the solutions were adjusted to neutral pH. Phosphate incorporation, as indicated by the filter paper assay, was found to be approximately 0.2 mol of ^{32}P/mol of peptide. This necessitated further purification since most of the peptides in the mixtures were in the unphosphorylated forms, which might cause product inhibition in the subsequent dephosphorylation reactions. The phosphorylated and unphosphorylated forms were separated by reversed-phase high-performance liquid chromatography using a gradient of acetonitrile in the presence of trifluoroacetic acid. Fractions were pooled according to radioactivity and concentrated by evaporation under reduced pressure. The stoichiometry of phosphorylation was estimated to be greater than 0.8 mol of ^{32}P/mol of peptide.

R_{II}-(81–99)-peptide was phosphorylated at its serine residue using bovine cardiac cAMP-dependent protein kinase catalytic subunit in a reaction containing 50 mM MOPS, pH 7.0, 5 mM magnesium acetate, 1 mM dithiothreitol, 2 mM [γ-^{32}P]ATP (specific activity 200–400 cpm/pmol), 0.028 mg/ml catalytic subunit, and 1 mM peptide, at 30 °C for 120 min. The stoichiometry was determined to be 1 mol of ^{32}P/mol of peptide. Non-peptide-bound ^{32}P was removed as described for the phosphotyrosyl peptides above.

Dephosphorylation Assay—Calmodulin-dependent protein phosphatase (brain or heart enzyme) was routinely preincubated for 4 min at 30 °C at a concentration of 0.11 mg/ml in the presence of 0.1 mg/ml bovine serum albumin, 1 mM divalent metal cation (as indicated), and 50 mM MOPS, pH 7.0, before subsequent addition to the dephosphorylation reaction mixtures. Phosphatase activity was assayed at 30 °C in 50 mM MOPS, pH 7.0, 1 mM dithiothreitol, 1 μM calmodulin, 1 mM divalent metal ion, and varying amounts of phosphorylated substrates in a total volume of 40–80 μl. The enzyme concentration in each reaction was adjusted (0.3–0.56 μg/ml) so that reaction rates remained linear for 3–5 min, and substrate depletion never exceeded 10%. Total ^{32}P in the substrates was determined by directly counting a 10-μl aliquot of the substrate preparation. Background ^{32}P released was determined by incubating substrates under identical reaction conditions without added phosphatase. To terminate the reactions with phosphoprotein substrates, a 20-μl aliquot of the incubation mixture was added to 125-μl of ice-cold 10% trichloroacetic acid. Bovine serum albumin was immediately added to a final concentration of 0.5 mg/ml to facilitate precipitation. Radioactivity in the trichloroacetic acid supernatant was then determined in Aquasol (Du Pont/New England Nuclear) by liquid scintillation counting. Background radioactivity was less than 5% of total radioactivity. To terminate reactions with phosphopeptide substrates, an aliquot of the reaction mixture was added to 0.5 ml of 5% trichloroacetic acid, 0.1 M potassium phosphate and set on ice. $^{32}P_i$ released was separated from phosphopeptides using AG-50W-X2 (Bio-Rad) resin as outlined by Manalan and Klee (16). Eluents were collected and radioactivity quantitated by liquid scintillation counting. Background radioactivity was less than 1% of total radioactivity. Units of activity are expressed in terms of nanomoles of $^{32}P_i$ released per minute per milligram of protein.

For experiments with inhibitors, the compounds were first preincubated at varying concentrations with the phosphatase and 1 mM divalent cation as described above. The same inhibitor concentrations were then maintained in the subsequent assays. For reactions with phosphotyrosyl peptide substrates, the phosphorylated peptides were only purified up to the step before high-performance liquid chromatography (see above, "Preparation of Substrates"); the substrate mixture thus contained unphosphorylated peptides. For experiments with the monoclonal antibody VA_1, the phosphatase was first preincubated with 1 mM Ni^{2+} and 0.2 mg/ml bovine serum albumin for 4 min at 30 °C before addition of the antibody at a ratio of 1:2 (enzyme:antibody, w/w) to the mixture. After a further incubation of 5 min, aliquots were transferred and assayed for phosphatase activity as outlined above.

Other Methods—Protein concentrations were determined according to Bradford (33), using bovine serum albumin as standard. Specific activity of [γ-^{32}P]ATP used in phosphorylation reactions was estimated after dilution of stock [γ-^{32}P]ATP (specific activity = 2500 Ci/mmol) with nonradioactive ATP. Total radioactivity was determined by liquid scintillation counting. The concentration of ATP was measured enzymatically using the method of Lamprecht and Trautschold (34).

RESULTS

Activators of Phosphatase Activity: Nickel, Manganese, and Calmodulin—The calmodulin-dependent protein phosphatase showed low phosphotyrosyl and phosphoseryl phosphatase activities in the presence of 5 mM EDTA, without added activators (data not shown). Short periods of preincubation with divalent cations stimulated enzyme activity, as has been shown in previous reports (21, 22, 35). Specifically, the dephosphorylation of both phosphotyrosyl substrates (phosphotyrosyl LC_{20} and angiotensin) and phosphoseryl substrates (phosphoseryl LC_{20} and R_{II}-(81–99)-peptide) were found to be stimulated by preincubating the enzyme with 1 mM Ni^{2+} or 1 mM Mn^{2+}. Activation by Ni^{2+} was nearly maximal (60%

[1] The abbreviations used are: R_{II}, type II regulatory subunit of cAMP-dependent protein kinase; LC_{20}, the 20,000-dalton myosin regulatory light chain from chicken gizzard; R_{II}-(81–99)-peptide, a synthetic peptide having the sequence corresponding to residues 81–99 of R_{II}; [Tyr^{95}]R_{II}-(81–99)-peptide, R_{II}-(81–99) peptide having a tyrosine substituted for residue 95; VA_1, monoclonal antibody against B subunit of brain calmodulin-dependent protein phosphatase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid.

increase) by 4 min at 30 °C, and remained constant for at least 30 min. Activation by Mn^{2+} was very slight (15% increase) over a 30-min period. Preincubation with either action resulted in maximal activation of phosphatase activity at a concentration of 1 mM, but was inhibitory at 10 mM. Calmodulin dependence of the enzyme could be demonstrated with both phosphotyrosyl and phosphoseryl substrates. In the presence of 1 mM Ni^{2+} or Mn^{2+}, 1 μM calmodulin caused a further 2–3-fold activation of the enzyme; addition of 100 μM trifluoperazine completely inhibited this stimulation (data not shown). Based on these results, a standard phosphatase assay protocol was established (see "Experimental Procedures"). The enzyme was routinely preincubated with 1 mM divalent cation and 1 μM calmodulin for 4 min at 30 °C. In the subsequent phosphatase assays, these same concentrations of activators were maintained. These experimental conditions were chosen so that the enzyme could be studied in its maximally activated state. It should be emphasized, however, that although strong activation is achieved by heavy metals *in vitro*, it is still open as to whether or not they have a physiological role.

Inhibitors of Phosphatase Activity: Zinc, Vanadate, Fluoride, and Antibody VA_1—Three commonly used phosphatase inhibitors were tested for their ability to inhibit the dephosphorylation of phosphotyrosyl and phosphoseryl substrates by the calmodulin-dependent protein phosphatase. Zinc ion, at micromolar concentrations, has been reported to inhibit a phosphotyrosyl protein phosphatase from Ehrlich ascites tumor cells (36). Vandate ion is known to inhibit a variety of enzymatic reactions involving phosphate transfer (37), but has also been reported to be a specific phosphotyrosyl protein phosphatase inhibitor. Swarup *et al.* (38) reported that 10 μM vanadate caused over 80% inhibition of a phosphotyrosyl protein phosphatase activity, whereas phosphoseryl protein phosphatase activity was not affected. Fluoride is a widely recognized inhibitor of phosphoseryl protein phosphatases, but has also been reported to have a slight stimulatory effect on phosphotyrosyl protein phosphatases (26). In the present study, the inhibitors were added to both the preincubation and assay mixtures. As shown in Fig. 1*A*, Zn^{2+} inhibited the Ni^{2+} and calmodulin-stimulated phosphatase activity at micromolar concentrations. Fifty per cent inhibition was observed at 3–5 μM Zn^{2+} with phosphotyrosyl substrates, and at 9–14 μM Zn^{2+} with phosphoseryl substrates. Zinc ion also inhibited the Mn^{2+}- and calmodulin-stimulated phosphatase activity (data not shown). Ten micromolar Zn^{2+} inibited the dephosphorylation of the two phosphotyrosyl substrates by 75%, and inhibited the dephosphorylation of the two phosphoseryl substrates by 60%. In contrast, vanadate was much less effective than Zn^{2+} in inhibiting the Ni^{2+}- and calmodulin-stimulated phosphatase activity (Fig. 1*B*). Fifty per cent inhibition was observed at concentrations of 500–800 μM with most substrates tested. However, vanadate appeared to be more effective when Mn^{2+} was the activator compared to Ni^{2+}. One millimolar vanadate inhibited the Mn^{2+}- and calmodulin-stimulated phosphatase activity toward all four substrates used in Fig. 1 by 75–80% (data not shown). These results are quite different from that of Swarup *et al.* (38). However, we have used purified enzyme and substrate preparations in the present study, whereas the above cited investigation dealt with a partially purified phosphatase preparation from A431 cell membranes. Sodium fluoride, at a concentration of 25 mM, was not inhibitory nor stimulatory toward either the Ni^{2+}/calmodulin or the Mn^{2+}/calmodulin-activated enzyme (data not shown). We conclude from these results that Zn^{2+} is a more effective inhibitor of the calmodulin-dependent protein phosphatase than vanadate, but neither compound is a specific inhibitor for phosphotyrosyl protein phosphatases.

A monoclonal antibody developed against the B subunit of the brain calmodulin-dependent protein phosphatase (31), and designated VA_1, caused similar inhibition of the phosphotyrosyl and phosphoseryl phosphatase activities. When tested at a single concentration against the Ni^{2+}/calmodulin-activated brain enzyme, VA_1 caused a 50% inhibition of the dephosphorylation of phosphotyrosyl LC_{20} and a 40% inhibition of the dephosphorylation of phosphoseryl R_{II}-(81–99)-peptide (data not shown).

Kinetic Parameters—Table I summarizes the kinetic con-

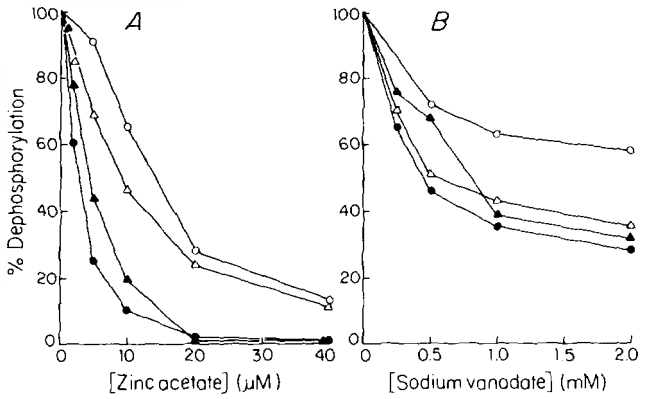

FIG. 1. **The effects of increasing concentrations of (*A*) zinc and (*B*) vanadate ions on the activity of brain calmodulin-dependent protein phosphatase.** Dephosphorylation of phosphotyrosyl LC_{20} (0.8 μM, ▲), phosphoseryl LC_{20} (0.8 μM, △), phosphotyrosyl [Tyr^{95}]R_{II}-(81–99)-peptide (1 μM, ●), and phosphoseryl R_{II}-(81–99)-peptide (11 μM, ○) were measured in the presence of 1 mM Ni^{2+} and 1 μM calmodulin as described under "Experimental Procedures." The inhibitors were present in both the 4-min preincubation and 5-min assay periods at the indicated concentrations. ^{32}P released in the absence of inhibitors was taken as 100% and was in the range of 4000–12000 cpm.

TABLE I

Kinetic parameters of dephosphorylation for phosphotyrosyl and phosphoseryl substrates by brain calmodulin-dependent protein phosphatase

Rates of dephosphorylation were determined in the presence of 1 μM calmodlin and 1 mM of either Ni^{2+} or Mn^{2+} as described under "Experimental Procedures." The ranges of substrate concentrations were 0.3–15 μM for LC_{20}, 0.8–14 μM for [Tyr^{95}]R_{II}-(81–99)-peptide, 3–86 μM for angiotensin I, and 3–140 μM for R_{II}-(81–99)-peptide. Enzyme concentrations (0.03–14 μg/ml) were adjusted so that the reaction rates remained linear for 3–5 min. The K_m and V_{max} for each substrate were determined from direct linear plot (39) of the free ^{32}P release data. The values shown are means of two to three separate determinations from a single batch of enzyme preparation. For each of these means, S.E. value was less than 10% of the mean.

Substrate	Ni^{2+}/calmodulin			Mn^{2+}/calmodulin		
	K_m	V_{max}	V_{max}/K_m	K_m	V_{max}	V_{max}/K_m
	μM	nmol/min/mg		μM	nmol/min/mg	
1) Phosphotyrosyl substrates						
LC_{20}	0.9	350	388.9	5.3	10	1.9
[Tyr^{95}]R_{II}-(81–99)-peptide	2.5	250	100.0	3.0	7	2.3
Angiotensin 1	6.1	274	44.9	26.0	12	0.5
2) Phosphoseryl substrates						
LC_{20}	2.6	690	265.4	9.2	360	39.1
R_{II}-(81–99)-peptide	23.0	4400	191.3	44.0	6000	136.4

stants for the dephosphorylation of phosphotyrosyl and phosphoseryl substrates by the brain calmodulin-dependent protein phosphatase. In general, the enzyme exhibited lower K_m values with phosphotyrosyl substrates and exhibited higher V_{max} values with the phosphoseryl substrates. Phosphotyrosyl LC_{20} appeared to be the best phosphotyrosine-containing substrate tested in the present study; the K_m was lower and the V_{max} higher than for the small phosphotyrosyl peptides. As LC_{20} can be phosphorylated on two tyrosine sites, and since we have not characterized the dephosphorylation reaction with respect to individual sites, the kinetic constants reported here represent a composite rate of dephosphorylation of the two sites. For the Ni^{2+}/calmodulin-activated enzyme, phosphotyrosyl LC_{20} was a more favorable substrate than phosphoseryl LC_{20}, as indicated by the higher V_{max}/K_m ratio. However, the trend was reversed with the two synthetic peptides based on the sequence of R_{II}. Under identical experimental conditions, phosphoseryl R_{II}-(81–99)-peptide was a better substrate than phosphotyrosyl [Tyr^{95}]R_{II}-(81–99)-peptide; the V_{max}/K_m ratio was 2-fold higher for the former.

The difference in V_{max}/K_m ratio between phosphotyrosyl and phosphoseryl substrates was more pronounced with the Mn^{2+}/calmodulin-activated enzyme. The ratio was 20-fold higher with phosphoseryl LC_{20} than with phosphotyrosyl LC_{20}, and 59-fold higher with the phosphoseryl R_{II}-(81–99)-peptide than with the phosphotyrosyl [Tyr^{95}]R_{II}-(81–99)-peptide. Activation by Ni^{2+} and calmodulin resulted in higher V_{max}/K_m ratios in comparison to Mn^{2+} and calmodulin. Except in the case of phosphoseryl R_{II}-(81–99)-peptide, this was primarily the result of a marked increase in V_{max}. In general, the enzyme exhibited more favorable kinetic constants for phosphotyrosyl substrates in the presence of Ni^{2+} and calmodulin, whereas the enzyme appeared to favor phosphoseryl substrates in the presence of Mn^{2+} and calmodulin.

Nonradioactive phosphoseryl LC_{20} was found to be an effective inhibitor of the dephosphorylation of phosphotyrosyl LC_{20}. When assayed in the presence of Ni^{2+} and calmodulin, at phosphotyrosyl concentrations of 1 and 2 μM (see Table I for K_m values), 1.5–6 μM of phosphoseryl LC_{20} caused inhibition ranging from 30 to 60% (data not shown). Although the limited data did not permit rigorous kinetic analysis for the exact nature of the inhibition, we nevertheless were able to demonstrate a clear competition between the two types of substrates. The estimated K_i was 2 μM, assuming competitive inhibition.

Brain Versus Cardiac Enzyme—No major differences were found between the activities of the enzymes purified from brain *versus* heart. The calmodulin dependence of phosphatase activity and the activation by Ni^{2+} and Mn^{2+} were very similar for both types of enzyme preparations (data not shown). The kinetic data shown in Table I for the five different substrates were obtained from experiments using a single preparation of the brain enzyme. Other preparations of brain enzyme occasionally showed specific activities 4–5-fold lower than those found with the first preparation. For example, using one of the latter preparations, the K_m values for phosphotyrosyl LC_{20} were similar to those shown in Table I, but the V_{max} values were 4–5-fold lower. (K_m = 1.1 μM, V_{max} = 87 nmol/min/mg in the presence of Ni^{2+}/calmodulin, and K_m = 5 μM, V_{max} = 2 nmol/min/mg in the presence of Mn^{2+}/calmodulin). However, the kinetic constants obtained using three separate enzyme preparations from cardiac tissues did not vary significantly. For phosphotyrosyl LC_{20}, these values (K_m = 2 μM, V_{max} = 66 nmol/min/mg with Ni^{2+}/calmodulin; and K_m = 4.6 μM, V_{max} = 8 nmol/min/mg with Mn^{2+}/calmodulin) were very similar to those found with the second preparation of brain enzyme, which showed lower specific activity. It is likely that the variability in specific activity seen with different preparations of brain enzyme and the difference in specific activities between the most active brain and heart preparations are the results of enzyme denaturation occurring during purification and handling.

DISCUSSION

The calmodulin-dependent protein phosphatase shows a high degree of specificity with substrates containing phosphoserine and phosphothreonine (4, 8, 14–19), but can also dephosphorylate phosphotyrosine-containing molecules (21–24). Since the work to date regarding phosphotyrosyl substrates has been rather preliminary, it has been difficult to assess the possible importance of the phosphotyrosyl protein phosphatase activity of this enzyme. In the present study, we used a variety of defined peptide and protein substrates to make thorough and direct comparisons of the phosphotyrosyl and phosphoseryl phosphatase activities of the calmodulin-dependent phosphatase. In addition, with the finding that a synthetic phosphoserine-containing peptide based on the structure of the autophosphorylation site of Type II regulatory subunit of the cyclic AMP-dependent protein kinase is an excellent substrate for the phosphatase (19), the opportunity presented itself to use a phosphotyrosine analogue of the peptide in a comparative study of this type. It was reasoned that this approach could provide more meaningful information that might be obtained by comparing the action of the phosphatase on a randomly selected set of phosphoserine (threonine)- and phosphotyrosine-containing proteins.

The five substrates employed in the present study represent a diverse array of peptide and polypeptide molecules, with differing amino acid sequences and varying chain length. All of these substrates are of known sequence and contain phosphoserine or phosphotyrosine residues at defined sites (see Table II). The two substrates, phosphoseryl LC_{20} and phosphotyrosyl LC_{20}, have identical overall primary structure. They differ in their phosphorylated amino acids and thus also the amino acid sequences around the phosphorylation sites. In contrast, the two synthetic peptides based on R_{II} have identical sequence around the phosphorylation sites and differ only in the phosphorylated amino acids. In a previous paper (19), it has been shown that R_{II} is among the few known favored phosphoseryl substrates of the calmodulin-dependent protein phosphatase (8, 17, 25). The kinetic constants for the 19-residue peptide R_{II}-(81–99)-peptide are comparable to those of the intact R_{II} protein (19); (also see Table I). In experiments using synthetic peptides with sequences based on R_{II}-(81–99)-peptide in which peptide chain length was varied, comparison of kinetic constants indicated that higher order structures are important for recognition of phosphoseryl protein substrates. Theoretical consideration of the primary structure suggests that the *N* terminus of R_{II}-(81–99)-peptide has a potential to form an amphipathic β-sheet structure. In the present study, the [Tyr^{95}]R_{II}-(81–99)-peptide could presumably also form the necessary β-sheet structure. However, it was not a particularly good substrate; the K_m values were 10-fold lower than that of R_{II}-(81–99)-peptide, but the V_{max} values were very much lower (Table I). This peptide was also not a significantly better substrate than either of the other two phosphotyrosyl substrates examined. Due to lack of additional well-characterized phosphotyrosyl substrates, it is at present difficult to speculate on the structural determinants of substrate specificity, but the data suggest that they differ with phosphotyrosyl *versus* phosphoseryl substrates. Consistent with this conclusion are the observations that the phos-

TABLE II

Amino acid sequences surrounding the phosphorylation sites of protein and peptide substrates

Substrates	Sequence
Gizzard LC_{20}	Ser-Ser-Lys-Arg-Ala-Lys-Ala-Lys-Thr-Thr-Lys-Lys-Arg-Pro-Gln-Arg-Ala-Thr-Ser-Asn-Val-Phe P 19 Phe-Thr-Asp-Glu-Glu-Val-Asp-Glu-Met-Tyr-Arg-Glu-Ala-Pro-Ile-Asp-Lys-Lys- P 142 Gly-Asn-Phe-Asn-Tyr-Val-Glu-Phe-Thr-Arg P 155
Angiotensin I	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu P 4
R_{II}-(81–99)-peptide	Asp-Leu-Asp-Val-Pro-Ile-Pro-Gly-Arg-Phe-Asp-Arg-Arg-Val-Ser-Val-Ala-Ala-Glu P 95
[Tyr^{95}]R_{II}-(81–99)-peptide	Asp-Leu-Asp-Val-Pro-Ile-Pro-Gly-Arg-Phe-Asp-Arg-Arg-Val-Tyr-Val-Ala-Ala-Glu P 95

photyrosyl and phosphoseryl phosphatase activities of the enzyme are differentially activated by Ni^{2+} *versus* Mn^{2+}. This raises the question of whether the two phosphatase activities can be attributed to the same catalytic site of the enzyme; however, the results of the substrate competition experiments suggest that phosphoseryl LC_{20} and phosphotyrosyl LC_{20} interact with the same catalytic site. Additional experiments are needed to clarify this point.

Our study offers several lines of evidence against the possibility that the dephosphorylation of phosphotyrosyl substrates and phosphoseryl substrates is catalyzed by two different enzymes. First, both phosphotyrosyl and phosphoseryl phosphatase activities were activated by Ni^{2+} and Mn^{2+} and were similarly dependent on calmodulin. Second, quite similar K_m and V_{max} values were obtained for the two types of substrates with enzyme prepared from two distinct tissues (brain and heart) using different purification procedures (7, 19). Third, dephosphorylation of phosphotyrosyl LC_{20} can be inhibited by phosphoseryl LC_{20}. Finally, an antibody against the brain enzyme causes similar inhibition of the phosphatase activity with both phosphotyrosyl and phosphoseryl substrates. Taken together, these data strongly support the view that the phosphotyrosyl protein phosphatase activity described herein is an integral property of the calmodulin-dependent protein phosphatase.

The present study provides quantitative data to serve as a basis for comparing the ability of the calmodulin-dependent protein phosphatase to act on phosphotyrosine- and phosphoserine-containing substrates. However, it is still not possible to draw any final conclusions concerning the physiological significance of the dephosphorylation of phosphotyrosine-containing substrates by this enzyme. Our present results clearly indicate that further work in this area is warranted. Important in this respect would be the examination of cellular proteins identified as natural substrates of protein tyrosine kinases as these become available. The finding that both phosphotyrosyl and phosphoseryl protein phosphatase activities reside in the same enzyme protein is intriguing. The possible mechanisms by which a protein phosphatase with dual activities might regulate the coordinate dephosphorylation of phosphotyrosine and phosphoserine/threonine residues on proteins await further investigations.

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