The substrate specificity of different forms of polycation-stimulated (PCS\(_H\), PCS\(_S\), and PCS\(_L\)) phosphorylase phosphatases and of the catalytic subunit of the MgATP-dependent protein phosphatase from rabbit skeletal muscle was investigated. This was done, with phosphorylase \(\alpha\) as the reference substrate, using the synthetic phosphopeptides patterned after the phosphorylated sites of pyruvate kinase (type L) (Arg-Glu-Ala-Ser\(^{32P}\)-Val-Ala (S\(_L\)), and its Thr\(^{32P}\) substitute (T\(_L\)), inhibitor-1 (Arg-Pro-Thr\(^{32P}\)-Pro-Ala (T\(_3\)), Arg-Glu-Pro-Thr\(^{32P}\)-Pro-Ala (T\(_4\)), and its Ser\(^{32P}\) substitute (S\(_4\))), and some modified phosphopeptides (Arg-Glu-Ala-Thr\(^{32P}\)-Thr\(^{32P}\)-Pro-Ala (T\(_5\)) and Arg-Pro-Pro-Thr\(^{32P}\)-Pro-Ala (T\(_6\))), all phosphorylated by cyclic AMP-dependent protein kinase. In addition, casein (Thr\(^{32P}\) and Thr\(^{32P}\)-Ala (T\(_2\))) and its Thr\(^{32P}\) substitute (T\(_2\)), all phosphorylated by cyclic AMP-dependent protein kinase. The PCS phosphatases show a striking preference for the T\(_4\) configuration, PCS\(_S\) being the least efficient. The catalytic subunit of the MgATP-dependent phosphatase was almost completely inactive toward all these substrates. As shown for the PCS\(_H\) phosphatase, and comparing with T\(_4\), the two proline residues flanking the Thr\(^{32P}\) in T\(_4\) and T\(_5\), just as in inhibitor-1, drastically impaired the dephosphorylation by lowering the \(V_{\text{max}}\) and not by affecting the apparent \(K_m\). The C-terminal proline (as in T\(_2\)) by itself represents a highly unfavorable factor in the dephosphorylation. The critical effect of the sequence X-Thr\(^{32P}\)-Pro or Pro-Thr\(^{32P}\)-Pro (T\(_4\), T\(_5\), T\(_6\), and inhibitor-1) can be overcome by manganese ions. The additional finding that this is not the case with the Pro-Ser\(^{32P}\)-Pro sequence (S\(_4\)) suggests that the effect of Mn\(^{2+}\) is highly substrate specific. These observations show the considerable importance of the primary structure of the substrate in determining the specificity of the protein phosphatases.

Four protein phosphatases are presumed to play an important role in dephosphorylating the major proteins involved in the control of general metabolism. Based on the enzyme-directed regulation of activity they have been classified as MgATP-dependent, polycation-stimulated, Mg\(^{2+}\)-dependent protein phosphatases and calcineurin (1). Among these we have studied in more detail the structure and regulation of the MgATP-dependent protein phosphatase (2, 3) and the polycation-stimulated phosphorylase phosphatase (4), the only enzymes in mammalian tissue extracts that possess significant activity toward phosphorylase \(\alpha\) (5, 6), a substrate of choice because of its single phosphorylation site. It is now generally accepted that the free catalytic subunits of these enzymes do not exist as such in vivo. An inactive, activated, and spontaneously active form of the MgATP-dependent protein phosphatase can be recognized (1), while according to their native apparent molecular weights and substrate specificity four different forms of polycation-stimulated (PCS\(^{\ast}\)) phosphorylase phosphatases have been described (4): PCS\(_H\), PCS\(_S\), PCS\(_H\), and PCS\(_L\).

Considerable information is available on the potential physiological substrates of the MgATP-dependent phosphatase, most of which have Ser-P as the target for dephosphorylation (5); however, this enzyme is also able to dephosphorylate the threonine residue in the subunit modulator protein during the activation process (7) and Thr-11 and Ser-117 of troponin I at similar rates (8). The different forms of polycation-stimulated phosphorylase phosphatases were only recently purified, but it is clear that dephosphorylation affects Thr-P as well as Ser-P, since, for instance, the PCS\(_H\) enzyme represents a major inhibitor-1 phosphatase (Thr-P) and is, contrary to the other PCS phosphatases and their catalytic subunit, a highly specific deinhibitor phosphatase (9, 10) in which Ser-P is involved. The MgATP-dependent and PCS phosphatases have broad and overlapping substrate specificities (5). However, a few substrates such as phosphatase inhibitor-1, pyruvate kinase, the \(\alpha\)-subunit of phosphorylase kinase, and casein phosphorylated on threonine are among the preferred targets for the polycation-stimulated phosphorylase phosphatases (4, 5). This remarkable site recognition is not due to the phosphoamino acid residue itself, since this is a threonine in inhibitor-1 and casein, and a serine in the \(\alpha\)-subunit of phosphorylase kinase. Hence, the PCS phosphatases are particularly interesting enzymes for substrate specificity studies, since not only two phosphorylated amino acids are involved, but the subunit structure of the PCS phosphatase seems to determine strongly the specificity of the enzyme.

In the present study, synthetic peptides, corresponding to some physiological substrates of protein phosphatases but containing different phosphorylated amino acid residues as well as variations in the surrounding amino acid sequence,
have been used to shed more light on the primary site requirements of different protein phosphatases. Such an approach also provides useful information on the importance of the molecular integrity of the physiological substrates as well as on the significance of the subunit structure of the phosphatases and on the effects small ligands may exert on the interaction between enzyme and phosphorylated substrates. Some of the present data have been published in a preliminary form (11).

Experimental Procedures

Materials—Rabbit skeletal muscle phosphorylase b (12), PCS\textsubscript{P}, PCSH\textsubscript{−}, PCSV\textsubscript{−} and PCS\textsubscript{−} phosphatase inhibitor-1 (13) were prepared according to published methods. The catalytic subunits of the MgATP-dependent phosphatase and the polycation-stimulated phosphorylase phosphatases were prepared by a procedure (14) modified as described (15). Casein kinase-2 was isolated from rat liver cytosol as described (16). Phosphorylase b kinase (17) and the catalytic subunit of cyclic AMP-dependent protein kinase from bovine cardiac muscle (18) were gifts of D. A. Walsh (Davis, U.S.A.). The synthetic peptides (19) and "whole casein" (20) were prepared as described. [γ\textsuperscript{32}P]ATP was purchased from the Radiochemical Centre, Amersham.

Substrate Phosphorylations—[\textsuperscript{32}P] Labeled phosphorylase a (21) and inhibitor-1 (22) were prepared as described.

The synthetic peptides were routinely phosphorylated by incubation with 200 µM [γ\textsuperscript{32}P]ATP (500-400 cpm/pmol), 200 mM MES buffer (pH 6.5), 100 mM MgCl\textsubscript{2}, 1.2 units of the catalytic subunit of cyclic AMP-dependent protein kinase, and 0.1 mg/ml synthetic peptide in a final volume of 250 µl. After 2 h, the reaction was terminated and the [γ\textsuperscript{32}P]ATP removed by addition of acetic acid and application of the sample onto phosphocellulose paper (23). The peptide extraction from the phosphocellulose paper was done using 3-4 ml of 3 M HCl with a yield of 60-80% of the total phosphopeptide adsorbed. The HCl was evaporated and removed by washing the samples twice with water. After drying, the phosphopeptides were dissolved in a buffer of 20 mM Tris-HCl, 0.5 mM dithiothreitol, at pH 7.4. The specific radioactivity of [32P] labeled peptide concentration was made on this assumption. The phosphorylation varied between 60 and 80% for the hexapeptide S\textsubscript{2}, already known as the best casein for the cyclic AMP-dependent protein kinase (18), between 10 and 40% for T\textsubscript{4}, and between 5 and 20% for all the other peptides employed: S\textsubscript{1}, S\textsubscript{3}, S\textsubscript{5}, T\textsubscript{5}, and T\textsubscript{6} (Table I).

Casein was phosphorylated by incubating for 1 h at 37°C whole casein (final concentration, 5 mg/ml) in 1 ml of a medium containing: 10 µM [γ\textsuperscript{32}P]ATP (specific radioactivity about 150 cpm/pmol), 12 mM MgCl\textsubscript{2}, 100 mM Tris-HCl buffer, pH 7.5, 100 mM NaCl, and about 50-100 ng of purified casein kinase-2. The reaction was stopped with trichloroacetic acid (final concentration, 10%). The precipitated protein was washed 3 times with 4 ml of 10% trichloroacetic acid, dissolved in 1 ml of 50 mM Tris-HCl, pH 7.5, and dialyzed overnight against 50 mM Tris-HCl, pH 7.5, in order to remove all the trichloroacetic acid. The ratio threonine-[\textsuperscript{32}P]/serine-[\textsuperscript{32}P] in whole casein using casein kinase-2 was higher than 1, as expected (24).

Protein Phosphatase Assay Procedures—Dephosphorylation of the phosphopeptides and proteins was performed by incubation of 10 µl aliquots of the phosphorylated substrate at 30°C for 30 min in the presence of the phosphatase indicated as described in Methods. One unit of protein phosphatase was defined as the amount of enzyme which released 1 nmol of [\textsuperscript{32}P] phosphate/min at 30°C in a 30-µl assay containing 1 mg/ml [\textsuperscript{32}P]phosphatase. The reaction was terminated by addition of 1.2 ml of 1:1 (v/v) isobutyl alcohol-toluene and 0.8 ml of a solution containing 5 mM silicotungstate and 1 mM H\textsubscript{2}SO\textsubscript{4}; the [\textsuperscript{32}P] phosphate was liberated as a phosphomolybdc complex and determined as described in Ref. 25.

When a time course of dephosphorylation was followed the total assay volume was 60 µl. The final concentration of the phosphopeptides was kept at 7 µM, while the concentrations of protein phosphatases were expressed as units of phosphorylase a phosphatase activity. At different time intervals 10-µl aliquots were taken, and the reaction was stopped as described.

The time course of dephosphorylation of phosphopeptides S\textsubscript{2} and T\textsubscript{4} differing only in the nature of the phosphorylated amino acid, by PCS\textsubscript{H} phosphatase is illustrated in Fig. 1A. It clearly shows the importance of the phosphorylated amino acid on phosphatase activity, since T\textsubscript{4} is dephosphorylated at

![Fig. 1. Time-dependent dephosphorylation of the synthetic phosphopeptides by the PCS\textsubscript{H} phosphatase. The phosphopeptides (7 µM) were incubated in the presence of 0.1 unit/ml (panel A) or 1 unit/ml (panel B) PCS\textsubscript{H} phosphatase and phosphatase activity measured as described under "Materials and Methods." (\textsuperscript{32}P)-Phosphate release is expressed as a percentage of the estimated amount of substrate present.](image)

### Table I

| Peptide | Average | S\textsubscript{1} | S\textsubscript{2} | S\textsubscript{3} | S\textsubscript{4} | S\textsubscript{5} | T\textsubscript{1} | T\textsubscript{2} | T\textsubscript{3} | T\textsubscript{4} | T\textsubscript{5} | T\textsubscript{6} | Inhibitor-1 |
|---------|---------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|
|         |         | Arg-Arg-Pro-Ser(\textsuperscript{32}P)-Pro-Ala | Arg-Arg-Ala-Ser(\textsuperscript{32}P)-Val-Ala | Arg-Arg-Pro-Thr(\textsuperscript{32}P)-Pro-Ala | Arg-Arg-Ala-Thr(\textsuperscript{32}P)-Pro-Ala | Arg-Arg-Ala-Thr(\textsuperscript{32}P)-Val-Ala | Arg-Arg-Pro-Thr(\textsuperscript{32}P)-Val-Ala | Arg-Arg-Pro-Thr(\textsuperscript{32}P)-Pro-Ala | Arg-Arg-Pro-Thr(\textsuperscript{32}P)-Val-Ala | Arg-Arg-Pro-Thr(\textsuperscript{32}P)-Pro-Ala | Arg-Arg-Pro-Thr(\textsuperscript{32}P)-Val-Ala | Arg-Arg-Pro-Thr(\textsuperscript{32}P)-Pro-Ala |
|         |         | 5-20% | 60-80% | 5-20% | 5-20% | 5-20% | 10-40% | 5-20% | 5-20% | 5-20% | 5-20% | 100% |

Structure of the phosphorylation site of inhibitor-1 and of the synthetic peptides and degree of phosphorylation obtained.
Specificity of the PCS Phosphorylase Phosphatases

TABLE II
Dephosphorylation rates of phosphopeptide and phosphoprotein substrates by variable amounts of different protein phosphatases (pmol min\(^{-1}\) mL\(^{-1}\))

| Phosphatases          | Substrates | S₁ | S₂ | T₁ | T₂ | T₃ | T₄ | T₅ | I₁ | Casein |
|-----------------------|------------|----|----|----|----|----|----|----|----|-------|
|                       |            | units/mL |    |    |    |    |    |    |    |       |
| PCS₉ phosphatase       |            | 0.06  | 16 | 8  | 10 | ND | ND | ND | ND |       |
|                       |            | 0.12  | 605 | ND | ND | 10 | 5  |    |    |       |
|                       |            | 0.25  | 990 | ND | 3  | 14 | 7  |    |    |       |
|                       |            | 0.5   | 2408 | 7  | 47 | 16 |    |    |    |       |
|                       |            | 2     | 156 | 48 |    | 27 | 128 |    |    |       |
|                       |            | 5     | 253 | 72 |    | 56 | 176 |    |    |       |
|                       |            | 10    | 205 | 86 |    |    |    |    |    |       |
| PCS₈ phosphatase       |            | 0.06  | 17 | 1  | 10 | ND | ND | ND | ND |       |
|                       |            | 0.12  | 1359| 3  | 14 | 7  |    |    |    |       |
|                       |            | 0.25  | 2408| 7  | 47 | 16 |    |    |    |       |
|                       |            | 0.5   | 27  | 128|    |    |    |    |    |       |
|                       |            | 2     | 56  | 176|    |    |    |    |    |       |
|                       |            | 5     | 253 | 72 |    |    |    |    |    |       |
|                       |            | 10    | 15  | 63 |    |    |    |    |    |       |
| PCS₉ phosphatase       |            | 0.06  | ND  | ND | ND | ND | ND | ND | ND |       |
|                       |            | 0.12  | ND  | ND | ND | ND | ND | ND | ND |       |
|                       |            | 0.25  | ND  | ND | ND | ND | ND | ND | ND |       |
|                       |            | 0.5   | ND  | ND | ND | ND | ND | ND | ND |       |
|                       |            | 2     | 325 | 9  | 34 |    |    |    |    |       |
|                       |            | 5     | 478 | 15 | 63 |    |    |    |    |       |
|                       |            | 10    | 62  | 13 |    |    |    |    |    |       |
| MgATP-dependent phosphatase (active catalytic subunit) | | | | | | | | | | |
|                       |            | 1     | ND  | ND | ND | ND | ND | ND | ND |       |
|                       |            | 5     | ND  | ND | ND | ND | ND | ND | ND |       |
|                       |            | 10    | ND  | ND | ND | ND | ND | ND | ND |       |

The concentration, calculated on the basis of (\(^{32}\)P)phosphate, was 7 \(\mu\)M for the synthetic phosphopeptides, 1 \(\mu\)M for inhibitor-1, and 2 \(\mu\)M for casein. Dephosphorylation with the release of less than 1 pmol. min\(^{-1}\) mL\(^{-1}\) is indicated as not detectable (ND).

a rapid rate, reaching 50% within 20 min while dephosphorylation of its phosphoseryl counterpart S₂ is almost negligible.

To determine the influence of the amino acid sequence on the efficiency of dephosphorylation by PCS₉ phosphatase, other synthetic phosphopeptides have been tested (Fig. 1B). Apparently, the replacement of the two amino acids alanine and valine flanking the phosphoamino acid with two proline residues drastically lowered the PCS₉ phosphatase activity (compare T₃ with T₄ and S₄ with S₅). It should be stressed that to obtain significant dephosphorylation of the phosphoseryl peptides the concentration of the enzyme is 10-fold higher than in the experiment shown in Fig. 1A.

The effects of structural modifications on the peptide by the dephosphorylation rate by PCS₉ and PCS₈ phosphatase and by the catalytic subunits of the PCS and the MgATP-dependent phosphatases are summarized in Table II, where the dephosphorylation rates of (\(^{32}\)P-Thr)casein and (\(^{32}\)P-Thr)inhibitor-1 are also reported. Two main conclusions can be drawn: 1) PCS₉ phosphatase was the enzyme displaying the highest activity toward all substrates tested; and 2) phosphopeptide T₄ was by far the best substrate for all the PCS phosphatases, but particularly for PCS₉ phosphatase. Moreover, the dephosphorylation rate of all phosphopeptides dropped to very low levels with the PCS₈ phosphatase and became undetectable with the catalytic subunit of MgATP-dependent protein phosphatase.

From both Fig. 1 and Table II, it appears that a structural modification which dramatically impairs the dephosphorylation efficiency is the presence of two proline residues around the Thr-P in T₄, such as in T₃ and T₅ as well as in inhibitor-1. As shown in Table III for the PCS₉ phosphatase, such an impairment is accounted for by a much lower \(V_{max}\) for the peptides T₁ and T₃ and for inhibitor-1 in comparison with the \(V_{max}\) for T₄. Interestingly, the \(K_m\) and \(V_{max}\) values for the T₁, T₃, and inhibitor-1 were quite comparable.

Which of the proline residues adjacent to the phosphothreonine represents the unfavorable factor on the phosphatase activity has also been studied. Table II shows that the single proline C-terminal to phosphothreonine (as in T₂) caused the major loss of phosphatase activity, while the enzyme activity was practically not affected by the presence of the N-terminal proline (as in T₁). The structural requirements for threonyl peptide phosphorylation by cyclic AMP-dependent protein kinase (19) and dephosphorylation by PCS₉ phosphatase were not completely identical; while prolyl residues influenced both processes in a quite comparable manner, the length of the N-terminal basic stretch and the overall structural integrity of inhibitor-1 dramatically improved the phosphorylation efficiency while...
having no appreciable effect on the dephosphorylation rate.

The effect of Mn2+ and polycations such as protamine on the activity of the phosphatases toward phosphopeptides was also investigated. Table IV shows that Mn2+ increased the phosphatase activity in all cases, but especially toward the phosphopeptides which, because of the presence of proline, were very poor substrates for all the protein phosphatases tested (e.g. T1, T2, and T3) (4-8-fold increase of the phosphatase activity). In particular, Mn2+ was required to obtain detectable dephosphorylation of phosphopeptides by the catalytic subunit of the MgATP-dependent phosphatase, which otherwise was inactive on these substrates (see also Table II). These data are consistent with the finding (2) that the active catalytic subunit of the MgATP-dependent phosphatase was able to dephosphorylate inhibitor-1 only in the presence of Mn2+. These results also suggest that, in the case of PCSH phosphatase, a stereospecific interaction between Mn2+ and the sequence Pro-Thr(P)-Pro or at least X-Thr(P)-Pro in the short peptides as well as in inhibitor-1, was responsible for optimizing the phosphatase reaction. The peptide dephosphorylation by all the PCS phosphatases was not affected by protamines (not shown), but these results obtained with small peptides do not exclude the enzyme-directed polycation stimulation seen with phosphatase a and inhibitor-1.

**DISCUSSION**

Seven small synthetic phosphopeptides partially related to the phosphorylation site of inhibitor-1 and pyruvate kinase (Table I), phosphorylated by cyclic AMP-dependent protein kinase, have been investigated as substrates for different forms of polycation-stimulated (PCS) phosphorylase phosphatases (PCSh, PCSi, and PCSc) and the catalytic subunit of the MgATP-dependent protein phosphatase. The results obtained support the following main conclusions: 1) unlike the catalytic subunit of the MgATP-dependent phosphatase, the PCS phosphatases readily dephosphorylated the same peptides with kinetic constants comparable with those of protein substrates; 2) PCSH and PCSi phosphatases exhibited a remarkable preference for the phosphothreonyl peptide Arg-Ala-Thr(P)-Val-Ala (T4) over its phosphoserine derivative; 3) the primary structure of the phosphoepitope played a considerable role in determining the phosphatase activity; and 4) Mn2+ ions dramatically improved the dephosphorylation efficiency of just those peptides exactly reproducing the phosphorylation site of phosphatase inhibitor-1.

The first conclusion can be drawn from Table II, showing that while the PCS phosphatases were more or less active toward all the phosphopeptides, the catalytic subunit of the MgATP-dependent phosphatase (at equivalent phosphorylase phosphatase activity) was practically unable to dephosphorylate any one of the phosphopeptides tested. These data support the conclusion that the structural features of our synthetic phosphopeptides were very unfavorable for the MgATP-dependent phosphatase activity. In particular, the negative results with peptides reproducing the phosphorylated site of inhibitor-1 support the concept that this protein as such was not a physiological substrate of the MgATP-dependent protein phosphatase. It is possible that an unfavorable feature shared by inhibitor-1 and all our peptides was the presence of arginine residues starting from the second residue on the N-terminal side of the phosphorylated amino acid. Actually, such a feature cannot be found in the majority of known physiological substrates of the MgATP-dependent phosphatase (5). So Arg-Ala-Thr(P)-Val-Ala (T5), reproducing the phosphorylated site of rat liver pyruvate kinase (type I), and the homologous threonyl-substituted Arg-Ala-Thr(P)-Val-Ala (T6) hexapeptides could be used to discriminate between the catalytic subunits of the MgATP-dependent and the PCS phosphatases (PCSc), since under comparable conditions they were dephosphorylated to an appreciable extent only by the latter enzyme.

Among the different forms of the polycation-stimulated phosphorylase phosphatases, PCSi phosphatase displayed the lowest activity toward the phosphosubstrates tested (see Table II). Even if the PCS phosphatases share a similar substrate specificity, the highest enzyme activity was shown by the PCSH phosphatase, while the threonyl peptide T1 was the preferred substrate for all PCS phosphatases. The PCSH phosphatase could be resolved by Mono Q fast protein liquid chromatography into two enzyme forms, PCSh and PCSc, and the homologous threonyl-substituted Arg-Ala-Thr(P)-Val-Ala (T6) hexapeptide could be used to discriminate between the catalytic subunits of the MgATP-dependent and the PCS phosphatases (PCSc), since under comparable conditions they were dephosphorylated to an appreciable extent only by the latter enzyme.

**TABLE IV**

| Enzyme activity of the PCSH and the MgATP-dependent phosphatase toward the Ser(P)- and Thr(P)-containing peptides and inhibitor-1 (I1) in the presence or absence of Mn2+ (pmol min⁻¹ ml⁻¹) |
|---|---|---|
| Phosphatase activity | Control | + 2 mM MnCl₂ |
| Phosphatases | Substrates | units/ml |
| PCSh phosphatase | S₁ | 14 | 21 |
| | S₂ | 121 | 161 |
| | T₁ | 20 | 171 |
| | T₂ | 14 | 60 |
| | 0.1 | 511 | 574 |
| | 2.1 | 1025 | 1496 |
| | T₄ | 30 | 114 |
| | I₁ | 61 | 187 |
| MgATP-dependent phosphatase | 10 | S₁ | ND | 5 |
| 10 | S₂ | ND | 5 |
| 10 | T₁ | ND | 29 |
| 10 | T₂ | 34 | 222 |
| 10 | T₄ | ND | 14 |
| 10 | I₁ | ND | 12 |
was the substrate (Tables II and IV), indicating that the integrity of the substrate molecule could not overcome the negative effect due to the C-terminal Pro. Mn\(^{2+}\) ions in general improved the PCSH phosphatase activity, but the effect was most pronounced with the phosphosubstrates which had the critical sequence X-Thr-Pro or Pro-Thr-Pro. The additional finding that the phosphatase activity with peptide S\(_2\), having two proline residues surrounding the Ser(P), was not significantly increased indicated that the stimulatory effect of Mn\(^{2+}\) was substrate specific and required a phosphothreonyl residue.

The activity of the MgATP-dependent phosphatase in the presence of Mn\(^{2+}\) was more evident with T\(_1\), than with T\(_0\), or inhibitor-1. This could point to different kinds of interaction between the two types of enzyme and the substrates when Mn\(^{2+}\) is present. Using a cardiac protein phosphatase (consisting apparently of 88% PCSH phosphatase and 12% catalytic subunit of protein phosphatase-I instead of the holoenzymes), which with due care was substrate specific and required a phosphothreonyl residue, the activity of the MgATP-dependent phosphatase in the presence of Mn\(^{2+}\) was about 1 order of magnitude higher than those we report now. This difference could be caused by the C-terminal Pro. Mn\(^{2+}\) ions in general were capable of mimicking the effect of this cation.

It would be tempting to assume that a prolyl residue adjacent to the C-terminal side of the target one has been shown to prevent also the fast phosphorylation of synthetic peptides by cyclic AMP-dependent protein kinase (19). However, such a hindrance is obviously overcome in the overall structure of inhibitor-1, which was phosphorylated much more readily than the hexa- and octapeptides reproducing its phosphorylation site. This was not so for the PCS phosphatases, which showed comparable kinetic constants with the phosphopeptides T\(_1\) and T\(_0\) as well as with inhibitor-1 (Table III). Cyclic AMP-dependent protein kinase obviously prefers seryl peptides over similar threonyl derivatives (19), whereas the PCS phosphatases exhibited a remarkable preference for the phosphothreonyl over the phosphoserine peptides (Fig. 1 and Table II). This finding and the ability of PCS\(_0\) and PCS\(_1\) phosphatase to dephosphorylate \((32P)-32P\)-casein phosphorylated by casein kinase-2 are intriguingly reminiscent of protein phosphatase-T, a phospho-

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