

Dephosphorylation of cAMP-dependent Protein Kinase Regulatory Subunit (Type II) by Calmodulin-dependent Protein Phosphatase

DETERMINANTS OF SUBSTRATE SPECIFICITY*

(Received for publication, September 3, 1985)

Donald K. Blumenthal[‡], Koji Takio, R. Scott Hansen, and Edwin G. Krebs[§]

From the Departments of Pharmacology and Biochemistry, Howard Hughes Medical Institute, University of Washington, Seattle, Washington 98195

Calmodulin-dependent protein phosphatase purified from bovine cardiac muscle catalyzed the rapid dephosphorylation of Ser-95 of bovine cardiac cAMP-dependent protein kinase regulatory subunit (R_{II}). The kinetic constants determined for the reaction ($K_m = 20 \mu\text{M}$; $V_{max} = 2 \mu\text{mol min}^{-1} \text{mg}^{-1}$) are comparable to those determined for other good substrates of this phosphatase. Because little is known about the determinants of substrate specificity for the calmodulin-dependent phosphatase, various phosphopeptides were used to investigate the structural features important for substrate recognition. Limited proteolysis of phospho- R_{II} with trypsin and chymotrypsin yielded fragments (residues 93-400 and 91-400, respectively) that were poor substrates, whereas digestion with *Staphylococcus aureus* V8 protease produced three phosphopeptides that were all dephosphorylated as rapidly as intact R_{II} . The sequence of the shortest phosphopeptide produced by *S. aureus* V8 protease was determined by sequence analysis to be Asp-Leu-Asp-Val-Pro-Ile-Pro-Gly-Arg-Phe-Asp-Arg-Arg-Val-Ser-Val-Cys-Ala-Glu, corresponding to residues 81-99 of R_{II} . Synthetic phosphopeptides corresponding to residues 81-99, 85-99, 90-99, and 91-99 were prepared to determine the minimum sequence necessary for substrate recognition. Only the 19-residue peptide (81-99) was dephosphorylated with kinetics comparable to R_{II} ($K_m = 26 \mu\text{M}$, $V_{max} = 1.7 \mu\text{mol min}^{-1} \text{mg}^{-1}$). Structural analysis of this peptide indicates that an amphipathic β -sheet structure may be an important structural determinant for some substrates of the calmodulin-dependent phosphatase.

Protein phosphorylation is known to be an important biochemical mechanism for the regulation of numerous physiological processes (reviewed in Ref. 1). The extent of phosphorylation of a given phosphoprotein depends upon the activities of both the protein kinase(s) and protein phosphatase(s) that catalyze the respective phosphorylation and dephosphorylation reactions. Cohen and co-workers (2, 3) have recently described a calmodulin-dependent phosphoprotein phosphatase that was purified to homogeneity from rabbit skeletal muscle. Work by that group and others indicates that this

enzyme is found in many other tissues, most notably brain, where the enzyme was shown to be identical to the calmodulin-binding protein known as calcineurin¹ (2, 4-6). The enzyme was found to have a very narrow substrate specificity compared to other known protein phosphatases (2, 3). Because of a report showing that calcineurin binds tightly to type II cAMP-dependent protein kinase from brain via the type II regulatory subunit (R_{II}^2 ; Ref. 7), the possibility that bovine cardiac calmodulin-dependent protein phosphatase might catalyze the dephosphorylation of autophosphorylated R_{II} from heart was investigated. The calmodulin-dependent phosphatase was found to rapidly dephosphorylate this substrate *in vitro*. This report describes this reaction, various factors which influence the reaction, and in particular, the features of R_{II} structure important for recognition as a substrate by the phosphatase.

MATERIALS AND METHODS

Preparation of Cardiac Calmodulin-dependent Protein Phosphatase—Calmodulin-dependent protein phosphatase was purified from fresh bovine heart ventricles following a modified procedure for cyclic nucleotide phosphodiesterase purification (8, 9). About 25 kg of ventricle was ground and then homogenized using buffer (2.5 liter/kg tissue) containing 40 mM Tris-Cl, pH 8.6, 15 mM 2-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged (5000 \times g) and filtered through glass wool; the supernatant was adjusted to pH 8.0 with 5 N NaOH, and phenylmethylsulfonyl fluoride was added (0.3 mM final). The extract was then batch-adsorbed to approximately 8 liters (packed volume) of DEAE-cellulose (Whatman DE52, equilibrated in 20 mM Tris-Cl, pH 8.0) by gently stirring for 30-40 min. The slurry was filtered and washed with 80-100 liters of 20 mM Tris-Cl, pH 8.0, 1 mM MgCl_2 , 15 mM 2-mercaptoethanol, 0.1 mM CaCl_2 , 0.1 M NaCl (buffer A) using gravity and aspirator suction. The DEAE-cellulose was mixed to a slurry (about 2 parts cellulose to 1 part buffer) and placed in a large Buchner funnel (35 \times 12 cm) fitted with wet-strengthened filter paper (Schleicher & Schuell). After careful packing, the "column" was further washed with 20 liters of buffer A (using gravity for packing and washing). Protein was eluted by addition of 4 mM EDTA (or 3 mM EGTA) to the wash buffer, and 1-liter fractions were collected at a flow rate of 6-9 liters/h. The fractions were pooled on the basis of cyclic nucleotide phosphodiesterase activity, which typically coeluted with calmodulin-dependent *p*-nitrophenylphosphatase activity. The pooled fractions (usually 4-5 liters total volume) were adjusted to 0.4 mM phenylmethylsulfonyl fluoride and 5 mM MgCl_2 (or 3 mM CaCl_2). Packed calmodulin-Sepharose (about 100 g containing 0.3-0.5 mg of calmodulin/g or 50 g containing 1-2 mg of calmodulin/g) pre-equilibrated in buffer A

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[‡] Supported as a postdoctoral fellow of the Muscular Dystrophy Association. Present address: Dept. of Biochemistry, University of Texas Health Center, Tyler, TX 75710.

[§] To whom correspondence should be addressed.

¹ The protein termed calmodulin-dependent protein phosphatase in this work has also been called protein phosphatase 2B, CaM-BP₈₀, calcineurin, and modulator-binding protein.

² The abbreviations used are: R_{II} , regulatory subunit of type II cAMP-dependent protein kinase; Mops, 3-(*N*-morpholino) propane-sulfonic acid; EGTA, [ethylenedis(oxyethylenenitrilo)]tetraacetic acid.

was added and gently stirred for 1–3 h. The resin was collected in a coarse sintered glass filter funnel (350 ml) and washed with 500 ml of buffer A. The filtrate and initial washes were combined and treated with additional calmodulin-Sepharose to adsorb any remaining calmodulin-binding proteins. A 1:1 slurry of the washed resin was loaded into a chromatography column (2.5-cm diameter) and washed further with buffer A until the eluent contained little protein ($>0.01 \mu\text{g/ml}$). Calmodulin-binding proteins were eluted with buffer A containing 1 mM EGTA. The protein peak was pooled and stored at 4 °C. Two calmodulin-Sepharose adsorptions and elutions were usually necessary to extract most of the calmodulin-binding proteins. The two pools were combined and usually contained 10–25 mg of protein. Cyclic nucleotide phosphodiesterase was removed from the calmodulin-binding protein mixture using an anti-calmodulin antibody (ACAP-1) affinity column (10). The EGTA-eluted protein from the calmodulin-Sepharose column was mixed with purified calmodulin (1 mg of calmodulin for every 2 mg of protein eluate) and adjusted to 1.5 mM CaCl_2 . The mixture was slowly applied to a column of ACAP-1-Sepharose (1.5 \times 8 cm, 10 mg of antibody/g of resin coupling density) or ACAP-1-Affi-Gel 10 (2 \times 8 cm, 10–15 mg/g coupling density) which had been equilibrated in buffer A. Protein-containing fractions which did not bind to the antibody affinity column were pooled and used for calmodulin-dependent phosphatase purification. This material was reapplied to a fresh antibody affinity column to remove any remaining phosphodiesterase, then adjusted to 2 mM EGTA, diluted 2-fold with 20 mM sodium phosphate, pH 6.8, and applied to a column of DEAE-cellulose (0.7 \times 5 cm equilibrated in 20 mM sodium phosphate, pH 6.8, 0.05 M NaCl, 1 mM dithiothreitol, 0.2 mM EGTA). The column was eluted with a linear gradient (50 ml) from 0.05 to 0.4 M NaCl. Calcineurin was conservatively pooled on the basis of analysis by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The yield was typically 2–5 mg of protein of which at least 90% was accounted for by the M_r 15,000 and 60,000 subunits of calcineurin. Sephacryl S-200 chromatography was used as an alternative fractionation procedure to the final DEAE-cellulose step and resulted in a similar preparation. The beef heart and brain subunits had similar electrophoretic mobilities except that the large subunit of the heart enzyme often appeared as a doublet in high resolution gels.

Preparation of Other Proteins and Peptides—Calmodulin was prepared from fresh and frozen bovine testes using a procedure outlined previously (11). Fresh bovine cardiac muscle was used for purification of cAMP-dependent protein kinase catalytic subunit (12) and type II regulatory subunit (13). Synthetic peptides were prepared by using a Beckman 990 automated peptide synthesizer and standard solid-phase techniques (14). Cleavage and side-chain deprotection were effected by using HF containing 20% anisole for 45 min at 0 °C. Peptides were purified by ion-exchange chromatography (SP-Sephadex, Pharmacia) and gel filtration (G-10, Pharmacia). Peptide sequence was verified by amino acid analysis and Edman degradation. Peptide purity was greater than 95% as estimated by reversed-phase high performance liquid chromatography. Prior to phosphorylation, peptide concentrations were determined by amino acid analysis.

Preparation of Phospho- R_{II} and Phosphopeptides— R_{II} and synthetic peptides were radioactively labeled with ^{32}P by incubating the protein or peptide with a 2-fold molar excess of [γ - ^{32}P]ATP (DuPont New England Nuclear, 200–400 cpm/pmol) and bovine cardiac cAMP-dependent protein kinase catalytic subunit (10–20 $\mu\text{g/ml}$) in the presence of 20 mM Mops, pH 7.0, 2 mM magnesium acetate, 15 mM β -mercaptoethanol at room temperature. The time course of phosphorylation was followed using a filter paper technique (15). The substrate was usually completely phosphorylated within 30 min; if not, more catalytic subunit was added. A stoichiometry of 1 mol of phosphate/mol of substrate was verified by comparing the amount of phosphate incorporated with that expected based on the concentration of substrate used and the specific activity of radioisotope. In the case of R_{II} , the reaction mixture was placed in dialysis tubing in a 1-liter Plexiglas chamber containing 15 mM β -mercaptoethanol, 2 mM Mops, pH 7.0, at 4 °C. Voltage (\sim 150 V) was applied across the chamber using 15-cm long platinum wire electrodes for 45–60 min. The buffer was changed and the process repeated three or four times before transferring the dialysis bag to a 4-liter container for overnight dialysis without applied current. The buffer for this last dialysis contained 20 mM Mops, pH 7.0, 15 mM β -mercaptoethanol. Nonprotein bound radioactivity was determined by precipitating a sample of the dialyzed protein with 10% trichloroacetic acid in the presence of 0.5 mg/ml bovine serum albumin. The amount of radioactivity remaining in the supernatant following a 3-min spin in an Eppendorf

microcentrifuge was typically 1% or less of the total radioactivity in the sample. The phosphoprotein sample thus prepared was stored at 4 °C until needed and showed no evidence of contaminating phosphatase activity. Synthetic phosphopeptide samples were treated with Dowex AG1-X8 as described by Wright *et al.* (16) to remove nonpeptide bound ^{32}P . Nonpeptide bound radioactivity was usually 0.5% or less of the total radioactivity. Labeled phosphopeptides were stored at -20 °C until needed.

Phosphatase Assays—Dephosphorylation of phospho- R_{II} and phosphopeptides was determined in duplicate in 50- μl reaction volumes from which 20- μl samples were removed at two time points (usually 5 and 15 min) following initiation of the reaction. All reactions were performed at 30 °C in the presence of 50 mM Mops, pH 7.0, 2 mM magnesium acetate, 2 mM manganese acetate, 1 μM calmodulin, 15 mM β -mercaptoethanol. Concentrations of other components were varied as required and are indicated where appropriate. Substrate concentration was determined by liquid scintillation counting using the specific radioactivity of the incorporated ^{32}P label. The concentration of phosphatase was adjusted to maintain linear rates of dephosphorylation with respect to time. All reactions included a 5-min preincubation before starting the reaction by addition of substrate. Reactions involving R_{II} were terminated by transferring the aforementioned 20- μl reaction mixture samples to 1.5-ml conical plastic centrifuge tubes containing ice-cold 10% trichloroacetic acid. Bovine serum albumin was immediately added to a concentration of 0.5 mg/ml to facilitate precipitation. The samples were spun for 3 min in an Eppendorf microcentrifuge and the supernatants counted to determine ^{32}P released. The procedure described below for phosphopeptides was also employed and gave identical results. In the case of reactions involving phosphopeptides, the 20- μl samples of reaction were quenched by adding the samples to 1.5-ml centrifuge tubes containing 75 mM phosphoric acid and placing on ice. The quenched samples were applied to 0.5 ml of AG50W-X8 (Bio-Rad) as described by Wright *et al.* (16), and the unadsorbed material collected, and counted in a liquid scintillation counter to determine the amount of ^{32}P released.

RESULTS

Dephosphorylation of Intact R_{II} —Calmodulin-dependent protein phosphatase purified from bovine cardiac muscle catalyzed the rapid dephosphorylation of autophosphorylated bovine cardiac R_{II} . Previous work has shown that autophosphorylation occurs at Ser-95 in this protein (17, 18), and data obtained from limited proteolysis (described below) are consistent with those reports. The kinetic parameters determined for the dephosphorylation reaction (K_m and V_{max} values of 20 μM , 2 $\mu\text{mol min}^{-1} \text{mg}^{-1}$, respectively, were obtained in the presence of 2 mM Mn^{2+} , 1 μM calmodulin, at pH 7.0, and 30 °C) are comparable to those reported for calmodulin-dependent phosphatases purified from other tissues using substrates such as protein phosphatase inhibitor-1, myosin light chains, α -subunit of phosphorylase kinase, G-substrate, DARPP-32, and protein K-F. (3, 19). Although all of the data presented herein were obtained with phosphatase purified from heart, the calmodulin-dependent protein phosphatase purified from bovine brain also rapidly dephosphorylated cardiac R_{II} (data not shown). Indeed, the substrate specificity of the heart calmodulin-dependent phosphatase appears to be quite similar to calmodulin-dependent phosphatases purified from other tissues. The heart enzyme preferentially dephosphorylates the α -subunit of phosphorylase kinase compared to the β -subunit (42) and catalyzes the dephosphorylation of phosphorylase *b* at a rate that is approximately 50-fold lower than the rate of R_{II} dephosphorylation. These substrate preferences are in accord with substrate specificities reported for the skeletal muscle enzyme (2, 3). Moreover, preparations of heart and brain enzyme showed similar patterns of substrate specificity when assayed with gizzard myosin light chains, *p*-nitrophenyl phosphate, and several different phosphotyrosine peptide and protein substrates (42).

The calmodulin-dependence of the enzyme purified from

heart was similar to that reported for preparations from other tissues (3, 6). In the absence of Mn^{2+} , the dephosphorylation of R_{II} was completely dependent on Ca^{2+} and calmodulin, with half-maximal activation occurring at 3 nM calmodulin (data not shown). In the presence of 2 mM Mn^{2+} , the rate of dephosphorylation was only partially dependent on calmodulin; addition of 1 μM calmodulin stimulated the enzyme 4–5-fold. The maximal rate of R_{II} dephosphorylation was observed with 2 mM Mn^{2+} , 1 μM calmodulin. The rate of dephosphorylation observed with 100 μM Ca^{2+} , 1 μM calmodulin, and no added Mn^{2+} was approximately 4-fold lower than this rate.

A number of different agents were tested for their ability to inhibit dephosphorylation of R_{II} (Table I). EGTA (2 mM) inhibited R_{II} dephosphorylation completely, indicating the importance of metal ions such as Ca^{2+} or Mn^{2+} in the reaction. Low concentrations of lanthanum chloride (100 μM) also inhibited dephosphorylation of R_{II} , but the mechanism of this inhibition is not clear. At the concentrations of La^{3+} used, it probably binds to both calmodulin and the B subunit of the enzyme. Lanthanum ion may also interact with the catalytic site of the enzyme since other metal ions are known to interact with this site (20–25). Trifluoperazine, a widely used calmodulin antagonist, effectively inhibited R_{II} dephosphorylation (~90% inhibition at 100 μM trifluoperazine, 1 μM calmodulin). Inorganic phosphate was also an effective inhibitor (complete inhibition of R_{II} dephosphorylation was achieved with 50 mM phosphate), but sodium fluoride was not found to inhibit R_{II} dephosphorylation significantly at concentrations as high as 3 mM.

The catalytic subunit of the cAMP-dependent protein kinase also effectively prevented R_{II} dephosphorylation when present at concentrations equal to those of R_{II} . Presumably this is because the kinase catalytic subunit binds R_{II} in the vicinity of the phosphorylation site and thereby prevents access by the phosphatase. Similar effects of the kinase catalytic subunit on R_{II} dephosphorylation have been observed with other protein phosphatases (26, 27) and may be of physiological significance with respect to the regulation of R_{II} dephosphorylation. The report that calcineurin binds to the holoenzyme form of brain cAMP-dependent protein kinase (7) suggests that this isozyme of R_{II} contains two distinct interaction sites for calcineurin. This does not appear to be the case for cardiac R_{II} since attempts to demonstrate complex formation between calcineurin, calmodulin, and reconstituted

heart Type II protein kinase were unsuccessful (28).

Dephosphorylation of R_{II} Fragments—Because the structural basis for the narrow substrate specificity of the calmodulin-dependent phosphatase is not well understood, it was of interest to prepare different proteolytic fragments of R_{II} to determine which features of structure might be important for recognition by the phosphatase. Previous studies (18, 29–31) have described the preparation and characterization of bovine cardiac R_{II} fragments using different proteolytic enzymes. Fig. 1 shows a diagram indicating the sites of proteolysis obtained when limiting amounts of different proteases are used. Chymotrypsin and trypsin cleave at sites just to the amino terminal side of Ser-95 and yield phosphorylated fragments which have molecular weights (using polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate) of approximately 37,000 (17, 30, 31). Both cAMP-binding domains are contained within these fragments (29, 32). Limited proteolysis with *Staphylococcus aureus* V8 protease cleaves after Glu-99 yielding a phosphorylated fragment which has an apparent molecular weight of 17,000 by gel electrophoresis and which consists of the amino terminal 99 residues of R_{II} (17, 31).

Patterns of proteolysis similar to those previously reported were obtained in the present study, except that more extensive digestion was obtained using *S. aureus* protease. Phosphopeptides from each proteolytic digestion were separated by gel filtration chromatography (Fig. 2) and analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. In the case of trypsin and chymotrypsin, the peaks indicated as being pooled in Fig. 2 exhibited apparent M_r of ~37,000 by gel electrophoresis (data not shown). With *S. aureus* protease three peaks of radioactivity were pooled which showed apparent M_r values on electrophoretic gels ranging from 2,000 (peak III) to 17,000 (peak I) (data not shown). The various pooled peaks shown in Fig. 2 were assayed for their ability to serve as substrates for the calmodulin-dependent phosphatase (Table II). The trypsin fragment was the poorest substrate, followed by the chymotrypsin fragment. Interestingly, all three fragments obtained by *S. aureus* protease digestion were dephosphorylated as rapidly as intact R_{II} .

Because all of the determinants for substrate specificity appeared to be present in the shortest fragment from *S. aureus* protease digestion (peak III), this material was subjected to more detailed investigation and peaks I and II from *S. aureus* protease digestion were not further characterized. The gel filtration pool containing the smallest fragment was subjected to purification by reversed-phase high performance liquid chromatography (Fig. 3). Although a number of peptides were present in the pool, only one had significant amounts of ^{32}P associated with it (Fig. 3). The high performance liquid chromatography fraction containing this peptide was subjected to

TABLE I

Effect of various inhibitors on R_{II} dephosphorylation by cardiac calmodulin-dependent phosphatase

Inhibitor ^a	Concentration	% inhibition
Sodium fluoride	0.3 mM	3
	1.0 mM	0
	3.0 mM	10
Sodium phosphate ^b	50 mM	100
C-subunit	(equimolar to R_{II})	94
Trifluoperazine	1 μM	10
	10 μM	51
	100 μM	87
EGTA or EDTA ^c	2 mM	100
$LaCl_3$	100 μM	50
	1 mM	100

^aDephosphorylation of ^{32}P - R_{II} was performed as described under "Materials and Methods." The concentration of ^{32}P - R_{II} and calmodulin in the reaction were 10 μM and 1 μM , respectively.

^bThe pH of the sodium phosphate solution was adjusted to 7.0 before adding to the assay mixture.

^cThe reaction mixture contained 2 mM Mg^{2+} , but Mn^{2+} and Ca^{2+} were omitted.

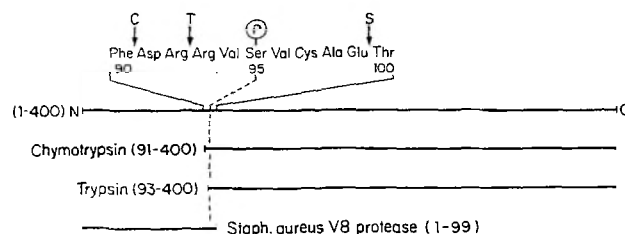


FIG. 1. Sites of limited proteolysis in phospho- R_{II} . The sequence around the autophosphorylation site of R_{II} (Ser 95) is shown with arrows indicating cleavage sites following proteolysis of autophosphorylated R_{II} with chymotrypsin (C), trypsin (T), and *S. aureus* V8 protease (S). A schematic representation of the resulting fragments is shown.

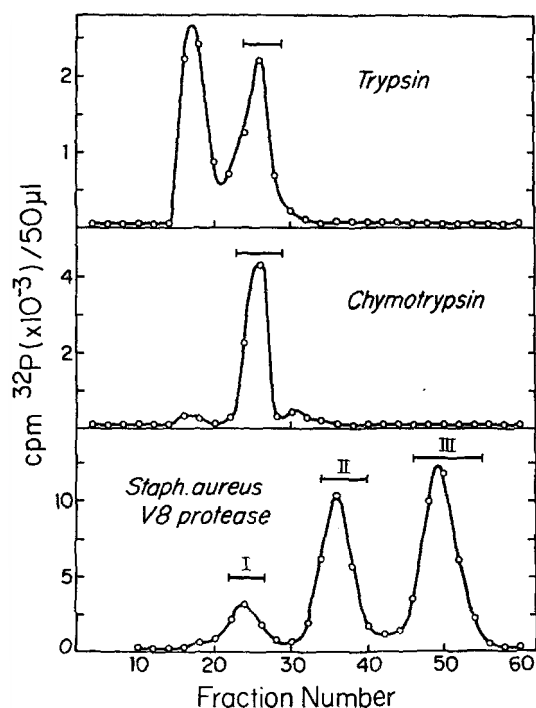


FIG. 2. G-75 chromatography of R_{II} proteolytic fragments. Following limited proteolytic digestion, samples were applied to G-75 (2.5 × 90 cm) equilibrated in 0.3 M ammonium acetate, pH 7.0, 15 mM β-mercaptoethanol, and the fractions (8 ml each) indicated by bars were pooled and lyophilized. Limited tryptic digestion was performed using a trypsin (L-1-tosyl-amido-2-phenylethyl chloromethyl ketone-trypsin, Worthington): ³²P-R_{II} ratio of 1:1000 (w/w) at 21°C for 5 min. Tosyl-L-lysine chloromethyl ketone (Sigma) and soybean trypsin inhibitor (Calbiochem-Behring) were used to stop proteolysis before chromatography. Proteolysis with α-chymotrypsin (Worthington) was performed on ice for 75 min at an enzyme/substrate ratio of 1:500 (w/w). Proteolysis was stopped by adding tosylamide 2-phenylethyl chloromethyl ketone (Sigma) and soybean trypsin inhibitor. Digestion with *S. aureus* V8 protease (Miles; enzyme/substrate ratio of 1:25) was allowed to proceed for 2 h on ice before adding phenylmethylsulfonyl fluoride (Sigma). The mixture was applied to a small column (0.7 × 24 cm) of cAMP-(N-6)-agarose (P-L Biochemicals) and the flow-through fractions applied to G-75. Pool I was used to obtain the data shown in Table II.

TABLE II

Dephosphorylation of R_{II} proteolytic fragments

Fragments were generated with the indicated protease and were purified by gel filtration chromatography (Fig. 2), dried under vacuum, and redissolved to give a final concentration of 8 μM in the dephosphorylation reaction. Rates of dephosphorylation of the fragments were determined as described under "Materials and Methods" and are reported relative to the rate of intact R_{II} dephosphorylation.

Fragment	Relative rate of dephosphorylation
Trypsin	<0.01
Chymotrypsin	0.08
<i>S. aureus</i> protease peak I	1.0
<i>S. aureus</i> protease peak II	1.0
<i>S. aureus</i> protease peak III	1.0

amino acid composition and sequence analysis and found to have the following sequence: Asp-Leu-Asp-Val-Pro-Ile-Pro-Gly-Arg-Phe-Asp-Arg-Arg-Val-Ser-Val-Cys-Ala-Glu. This is the smallest phosphopeptide expected from R_{II} using *S. aureus* protease (17).

Dephosphorylation of Synthetic Phosphopeptides—In order to define the minimum primary structure required for recognition by the calmodulin-dependent phosphatase, a series of

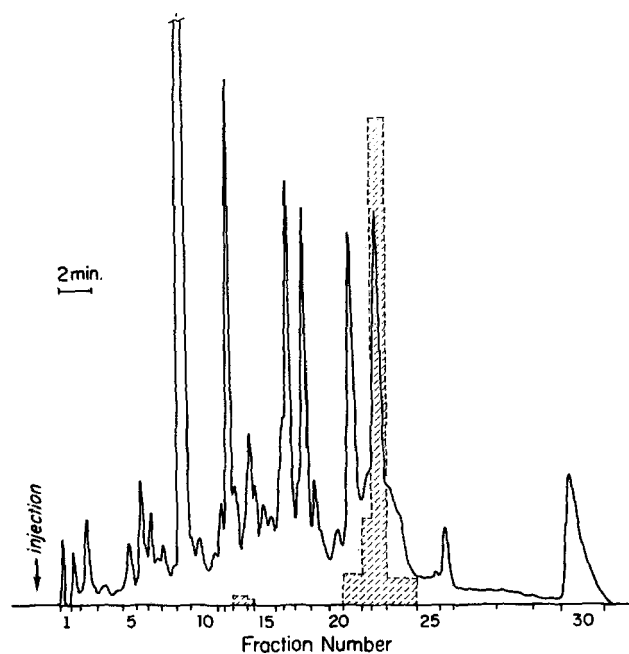


FIG. 3. Reversed-phase high performance liquid chromatography purification of G-75 peak III from *S. aureus* protease digestion. A sample (150 μl) from the pooled fractions indicated as peak III (Fig. 2, bottom panel) was applied to a Varian MicroPak MCH-10 reversed-phase column (0.8 × 50 cm, C-18). A gradient from 0.1% trifluoroacetic acid/H₂O to 60% of 0.08% trifluoroacetic acid/CH₃CN over 30 min (flow rate = 2 ml/min) was used to develop the chromatogram. Absorbance at 206 nm (solid line) was used to monitor eluted material, and fractions were collected by hand into scintillation vials for detection of ³²P by Cerenkov radiation (cross-hatched area). Fraction 23 contained 73% of all radioactivity collected (217,000 cpm by Cerenkov). This fraction was submitted for amino acid composition and sequence analysis.

TABLE III

Kinetic parameters for dephosphorylation of synthetic R_{II} phosphopeptides by cardiac calmodulin-dependent phosphatase

Phosphatase assays were performed in duplicate as described under "Materials and Methods" except that 100-μl reaction volumes were used. Samples (20 μl each) were removed at three time points (typically 5, 10, and 20 min) to determine reaction rates and to assure that linear rates were maintained throughout the course of the reaction. Another aliquot (20 μl) was removed and counted to determine substrate concentration. Kinetic parameters were determined graphically from double-reciprocal plots.

Peptide sequence ^a	<i>K_m</i>	<i>V_{max}</i>	<i>V_{max}/K_m</i>
	μM	nmol/min/mg	
DRRVSVAAE	400	130	0.32
FDRRVSVAAE	210	130	0.62
PIPGRFDRRVSVAAE	130	240	1.8
DLDVPIPGRFDRRVSVAAE	26	1700	65
R _{II}	20	2000	100

^a One-letter designations for amino acids are given in the legend to Fig. 4.

synthetic phosphopeptides were prepared. The kinetic parameters for these peptides are shown in Table III. The longest peptide synthesized corresponds in sequence to the 19-residue peptide fragment generated by *S. aureus* protease (*i.e.* peak III), except that Cys-97 has been replaced by alanine. The dephosphorylation of this peptide exhibits kinetic constants that are comparable to those observed for intact R_{II}. Reducing the length at the N terminus by 4 residues markedly reduces the rate at which the peptide is dephosphorylated. Both the *K_m* and *V_{max}* values are affected. Decreasing the peptide length

continues to alter both K_m and V_{max} values. The large difference in substrate competence observed between the 15- and 19-residue peptides (about a 30-fold difference in K_m/V_{max} ratio) is particularly interesting considering that the additional residues of the longer peptide are more than 10 residues away from the phosphoserine residue.

DISCUSSION

An important biochemical feature of the calmodulin-dependent protein phosphatase is its high degree of substrate specificity (2, 3, 19). Several lines of evidence suggest that higher order structure is an important determinant of substrate specificity for this enzyme. Comparison of the amino acid sequences of phosphorylation sites in phosphoproteins that are good substrates for this enzyme and those that are poor does not indicate any consistent features of primary structure which might be important for substrate recognition. Basic amino acid residues close to the phosphorylated residue are found in most of the good substrates but also in several phosphoproteins which are not good substrates. Three of the best substrates, phosphatase inhibitor-1, DARPP-32, and G-substrate, have homologous sequences that are somewhat unusual in that proline residues are found adjacent to the phosphorylation sites in these proteins and in that the phosphorylated residue is threonine (19, 33). Interestingly, G-substrate has two such sites that are dephosphorylated at quite different rates (approximately 20-fold) by the calmodulin-dependent phosphatase (19). The 12 residues immediately surrounding the two phosphorylation sites differ by only one amino acid (33). Moreover, other enzymes acting on these phosphorylation sites, including other type 2 protein phosphatases (19) and cGMP-dependent protein kinase (33), show little or no preference for either site. Taken together with the results in the present report, these data support the idea that higher order structures are important determinants for substrate recognition by the calmodulin-dependent protein phosphatase.

Structural analysis of the autophosphorylation site in R_{II} may indicate features of higher order structure important for substrate recognition, particularly when considered in relation to data obtained with synthetic phosphopeptide substrates. A structural profile for the shortest peptide sequence giving optimal kinetic constants (R_{II} peptide 81-99) is shown in Fig. 4. Secondary structural predictions based on Chou-Fasman probabilities (34-36) indicate a high probability of β -turn in the region containing the two prolyl residues. Immediately C-terminal to this region is a short segment predicted to form β -strand. The very C terminus might form α -helix, although it would probably be unstable since only one turn of helix is predicted. NMR studies by Rosevear and co-workers (38) using a synthetic heptapeptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly) with a sequence very similar to the sequence around the phosphorylation site in R_{II} indicate that an extended coil structure is likely to occur in this region of the molecule. The amino terminus of the nonadecapeptide appears to have no strong propensity for any periodic secondary structure as determined by Chou-Fasman analysis; however, consideration of the peptide's hydrophobicity profile (Fig. 4, bottom panel) indicates that the amino terminus might form a β -strand since alternating hydrophobic and hydrophilic residues are often found in β -strands at the surface of proteins (37, 39). The predicted occurrence of the secondary structural sequence β -strand, β -turn, β -strand indicates that the amino terminus of the nonadecapeptide might form a small segment of amphipathic antiparallel β -sheet structure. Although this structural model is tentative, it is strongly supported by data from

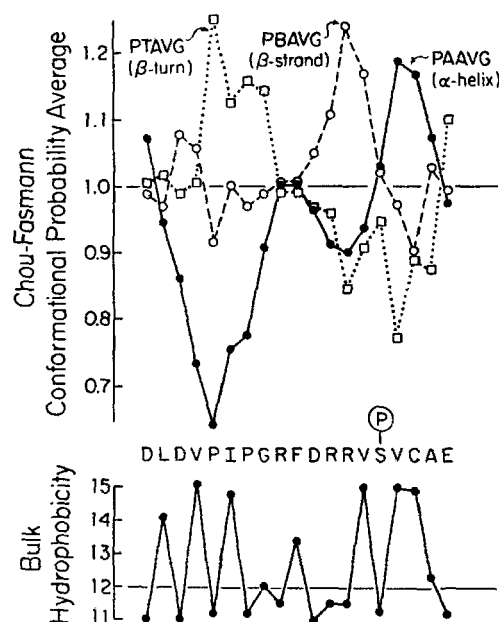


FIG. 4. Structural analysis of R_{II} phosphopeptide from *S. aureus* protease digestion. Chou-Fasman probabilities (34), averaged over 4 residues, were calculated for α -helix (\circ — \circ), β -strand (\square — \square), and β -turn (\triangle — \triangle) using the program of Corrigan and Huang (35) and data from Argos *et al.* (36). Bulk hydrophobicity values (37) were plotted without averaging (bottom panel). The peptide sequence is shown between the top and bottom panels using the following one-letter codes to designate each amino acid: A (alanine), R (arginine), D (aspartic acid), C (cysteine), E (glutamic acid), G (glycine), I (isoleucine), L (leucine), F (phenylalanine), P (proline), S (serine), and V (valine).

the dephosphorylation of the synthetic phosphopeptides (Table II). The 19-residue synthetic phosphopeptide, which is presumably capable of forming the proposed β -sheet structure, is a much better substrate than the 15-residue peptide which lacks the 4 residues necessary for completing the β -sheet. Considering that these 4 critical residues are quite distant in linear sequence from the phosphoserine residue and that they only increase the size of the peptide by about 25% lends support to the argument that these residues are important because of their participation in some sort of higher order structure.

Although the calmodulin-dependent phosphatase shows a high degree of substrate specificity with respect to phosphoserine and phosphothreonyl substrates, data from studies using several different phosphotyrosyl substrates of known sequence indicate a relative lack of specificity (42). Interestingly, a synthetic phosphopeptide corresponding in sequence to the best synthetic peptide employed in the present study, but containing phosphotyrosyl in place of phosphoserine, was no better a substrate than any of the other phosphotyrosyl substrates examined. Thus, the structural determinants important for substrate specificity appear to depend upon the nature of the phosphorylated residue in the protein or peptide.

Only a limited amount of data is available regarding the substrate specificities of other protein phosphatases using synthetic phosphopeptide substrates (16, 40, 41). The data from these various studies suggest that higher order structures may not be important determinants for these other phosphatases, since short peptides were readily dephosphorylated by the enzymes used in those studies. However, a number of phosphoprotein phosphatases have recently been described that have not been extensively studied using synthetic phosphopeptides and which may show substrate specificity re-

quirements that involve higher order structure. It will be interesting to study these various enzymes by techniques similar to those described here to determine whether or not this is the case.

In summary, the data obtained using autophosphorylated R_{II} as a model substrate indicate that the basis for the calmodulin-dependent protein phosphatase's high degree of substrate specificity may be a requirement for higher order structure. In the case of R_{II} , the higher order structure involved appears to be a small region of β -sheet adjacent to the phosphorylation site. Future experiments involving structural and enzymological characterization of other substrates for this enzyme should clarify whether this is a general structural feature required for substrate recognition.

Acknowledgments—We wish to acknowledge the use of facilities and equipment in the laboratory of Dr. Joseph Beavo, helpful discussions with Drs. Joseph Beavo and Rachel Klevit, technical assistance from Edwina Beckman, Floyd Kennedy, and Barbara Flug, and assistance from Evelyn Mercier in preparing this manuscript.

REFERENCES

- Cohen, P. (1982) *Nature* **296**, 613–620
- Stewart, A. A., Ingebritsen, T. S., Manalan, A., Klee, C. B., and Cohen, P. (1982) *FEBS Lett.* **137**, 80–84
- Stewart, A. A., Ingebritsen, T. S., and Cohen, P. (1983) *Eur. J. Biochem.* **132**, 289–295
- Yang, S.-D., Tallant, E. A., and Cheung, W. Y. (1982) *Biochem. Biophys. Res. Commun.* **106**, 1419–1425
- Tonks, N. K., and Cohen, P. (1983) *Biochim. Biophys. Acta* **747**, 191–193
- Tallant, E. A., and Wallace, R. W. (1985) *J. Biol. Chem.* **260**, 7744–7751
- Hathaway, D. R., Adelstein, R. S., and Klee, C. B. (1981) *J. Biol. Chem.* **256**, 8183–8189
- LaPorte, D. C., Toscano, W. A., and Storm, D. R. (1979) *Biochemistry* **18**, 2820–2825
- Martins, T. J., Mumby, M. C., and Beavo, J. A. (1982) *J. Biol. Chem.* **257**, 1973–1979
- Hansen, R. S., and Beavo, J. A. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79**, 2788–2792
- Blumenthal, D. K., Takio, K., Edelman, A. M., Charbonneau, H., Titani, K., Walsh, K. A., and Krebs, E. G. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82**, 3187–3191
- Peters, K. A., Demaille, J. G., and Fischer, E. H. (1977) *Biochemistry* **16**, 5691–5697
- Dills, W. L., Goodwin, C. D., Lincoln, T. M., Beavo, J. A., Bechtel, P. J., Corbin, J. D., and Krebs, E. G. (1979) *Adv. Cyclic Nucleotide Res.* **10**, 199–217
- Glass, D. B. (1983) *Methods Enzymol.* **99**, 119–139
- Roskoski, R. (1983) *Methods Enzymol.* **99**, 3–6
- Wright, D. E., Noiman, E. S., Chock, P. B., and Chau, V. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78**, 6048–6050
- Takio, K., Smith, S. B., Krebs, E. G., Walsh, K. A., and Titani, K. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79**, 2544–2548
- Takio, K., Smith, S. B., Krebs, E. G., Walsh, K. A., and Titani, K. (1984) *Biochemistry* **23**, 4200–4206
- King, M. M., Huang, C. Y., Chock, P. B., Nairn, A. C., Hemmings, H. C., Jr., Chan, K.-F. J., and Greengard, P. (1984) *J. Biol. Chem.* **259**, 8080–8083
- King, M. M., and Huang, C. Y. (1983) *Biochem. Biophys. Res. Commun.* **114**, 955–961
- King, M. M., and Huang, C. Y. (1984) *J. Biol. Chem.* **259**, 8847–8856
- Li, H. C., and Chan, W. W. S. (1984) *Eur. J. Biochem.* **144**, 447–452
- Merat, D. L., Hu, A. Y., Carter, T. E., and Cheung, W. Y. (1984) *Biochem. Biophys. Res. Commun.* **122**, 1389–1396
- Gupta, R. C., Khandelwal, R. L., and Sulakhe, P. V. (1984) *FEBS Lett.* **169**, 251–255
- Wolff, D. J., and Sved, D. W. (1985) *J. Biol. Chem.* **260**, 4195–4202
- Chou, C.-K., Alfano, J., and Rosen, O. M. (1977) *J. Biol. Chem.* **252**, 2855–2859
- Uno, I. (1980) *Biochim. Biophys. Acta* **631**, 59–69
- Klee, C. B., Krinks, M. H., Hathaway, D. R., and Flockhart, D. A. (1982) in *Calmodulin and Intracellular Ca^{2+} Receptors* (Kakiuchi, S., Hidaka, H. and Means, A. R., eds) pp. 303–312, Plenum Publishing Corp., New York
- Potter, R. L., and Taylor, S. S. (1979) *J. Biol. Chem.* **254**, 2413–2418
- Takio, K., Walsh, K. A., Neurath, H., Smith, S. B., Krebs, E. G., and Titani, K. (1980) *FEBS Lett.* **114**, 83–88
- Geahlen, R. L., Carmichael, D. F., Hashimoto, E., and Krebs, E. G. (1982) *Adv. Enzyme Regul.* **20**, 195–209
- Corbin, J. D., Sugden, P. H., West, L., Flockhart, D. A., Lincoln, T. M., and McCarthy, D. (1978) *J. Biol. Chem.* **253**, 3997–4003
- Aitken, A., Bilham, T., Cohen, P., Aswad, D. W., and Greengard, P. (1981) *J. Biol. Chem.* **256**, 3501–3506
- Chou, P. Y., and Fasman, G. D. (1978) *Annu. Rev. Biochem.* **47**, 251–276
- Corrigan, A. J., and Huang, P. C. (1982) *Comput. Programs Biomed.* **15**, 163–168
- Argos, P., Hanei, M., and Garavito, R. M. (1978) *FEBS Lett.* **93**, 19–24
- Cid, H., Bunster, M., Arriagada, E., and Campos, M. (1982) *FEBS Lett.* **150**, 247–254
- Rosevear, P. R., Fry, D. C., Mildvan, A. S., Doughly, M., O'Brian, C., and Kaiser, E. T. (1984) *Biochemistry* **23**, 3161–3173
- Kanehisha, M. I., and Tsong, T. Y. (1980) *Biopolymers* **19**, 1617–1628
- Titanji, V. P. K., Ragnarsson, U., Humble, E., and Zetterqvist, O. (1980) *J. Biol. Chem.* **255**, 11339–11343
- Shacter-Noiman, E., and Chock, P. B. (1983) *J. Biol. Chem.* **258**, 4214–4219
- Chan, C. P., Gallis, B., Blumenthal, D. K., Pallen, C. J., Wang, J. H., and Krebs, E. G. (1986) *J. Biol. Chem.*, in press