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Biochemistry

A synthetic peptide substrate specific for casein kinase II

(protein kinase/protein kinase specificity/peptide phosphorylation)

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ABSTRACT **A synthetic peptide having the sequence Arg-Arg-Arg-Glu-Glu-Glu-Thr-Glu-Glu-Glu was found to serve as a convenient substrate for the protein kinase generally referred to as casein kinase II. The enzyme exhibited an apparent K_m of 500 μM for the peptide, as compared to an apparent K_m of 50 μM for casein. The maximum velocities for phosphorylation of the peptide and of casein were similar. The peptide was not phosphorylated by any of eight other protein kinases, all of which were shown to be active toward their known substrates. The peptide was used to monitor activity during steps in the purification of casein kinase II from bovine liver. These experiments demonstrated that with this peptide it is now possible to obtain specific measurements of casein kinase II activity in crude enzyme preparations.**

Casein kinase II is a protein kinase that has unclear cellular functions (reviewed in ref. 1). This enzyme catalyzes the phosphorylation of serine and threonine residues in many different proteins (2–10), but most of the presently known casein kinase II-catalyzed reactions are not accompanied by apparent functional changes in the substrates (4, 5, 7, 10–12). Casein kinase II has been found in many different cell types, including fungal cells (13), plant cells (14), and cells from a number of animal tissues (15–17). Thus, although the role of the enzyme is uncertain, its physiological importance is suggested by its widespread distribution.

Casein, which is probably not a physiological substrate for casein kinase II (1), is the protein most commonly used to assay the enzyme's activity *in vitro*. However, many other protein kinases also catalyze the phosphorylation of casein, including the cAMP-dependent protein kinase, phosphorylase kinase, the insulin-receptor and epidermal growth factor (EGF)-receptor kinases, smooth-muscle myosin light chain kinase, and casein kinase I, another enzyme that historically has been assayed by using casein. It follows that any phosphate incorporated into casein in reactions using an impure enzyme may be due to the sum of activities from more than one protein kinase. In order to study the cellular function and regulation of casein kinase II, it was desirable to be able to monitor activity solely due to casein kinase II under conditions in which more than one kinase was present. For this it was deemed necessary to develop a substrate that would be specific for casein kinase II.

In this report we describe a synthetic peptide that serves as a substrate for casein kinase II but not for any of eight other protein kinases tested. With this peptide it was possible to carry out specific measurements of casein kinase II activity in crude preparations, as demonstrated by its use in monitoring activity during steps in the purification of the enzyme from bovine liver.

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MATERIALS AND METHODS

Materials. The *t*-butyloxycarbonyl derivatives of arginine and glutamic acid were from Vega Biochemicals (Tucson, AZ). The *t*-butyloxycarbonyl derivative of threonine and the glutamic acid polystyrene resin were from Peninsula Laboratories (San Carlos, CA). [γ-^{32}P]ATP was from New England Nuclear. Heparin, heparin agarose, phosphoserine, H2B histone, and phosphothreonine were from Sigma. Purified whole casein was from Matheson, Coleman and Bell (Norwood, OH). Phosphotyrosine and a wheat germ lectin-purified preparation of human placental EGF- and insulin-receptors (18) were gifts from Linda Pike of this laboratory. Chicken gizzard myosin light chains and chicken gizzard myosin light chain kinase were gifts from Arthur Edelman of this laboratory. Protein kinase C was a gift from Yasutomi Nishizuka (Kobe University School of Medicine, Kobe, Japan). Other kinases [catalytic subunit of the cAMP-dependent protein kinase from bovine heart (19), bovine lung cGMP-dependent protein kinase (20), and rabbit skeletal muscle phosphorylase kinase (21)] and substrates [phosphorylase b (22), Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly (Arg-Arg-SRC-peptide, which has the amino acid sequence of the site of phosphorylation of the tyrosine residue in the transforming protein pp60^{src} of Rous sarcoma virus) (23), and Leu-Arg-Arg-Ala-Ser-Leu-Gly (designated Kemptide) (24)] used in this study were prepared as previously described.

Peptide Synthesis. A peptide having the structure Arg-Arg-Arg-Glu-Glu-Glu-Thr-Glu-Glu-Glu, which will be referred to in this paper as "the synthetic peptide," was synthesized by using the *t*-butyloxycarbonyl derivatives of Arg(NO_2), Glu(Bzl), Thr(Bzl), and Glu(Bzl)-*O*-resin on a Beckman 990B automated solid-phase peptide synthesizer. The coupling reaction conditions have been described (ref. 25; see also ref. 26). Cleavage of the peptide from the resin and removal of side-chain protecting groups were done simultaneously in HF (23). The deprotected peptide was purified on DEAE-Sephadex with a linear gradient of 0.05–2 M ammonium acetate (pH 7.0) and then desalted by Sephadex G-10 chromatography in 30% (vol/vol) acetic acid. The identity of the peptide was confirmed by amino acid composition and sequence analysis.

Protein Kinase Activity Assays. In assays for various protein kinases, several procedures and conditions were identical as follows. Reactions were started by the addition of enzyme (unless stated otherwise in referenced procedures) and were stopped by spotting an aliquot of the reaction mixture onto paper and immediately placing in acid as described below. All kinase assays were carried out at 30°C. Control reactions without substrates were performed at the same time

Abbreviations: EGF, epidermal growth factor; Arg-Arg-SRC-peptide, Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly.

as reactions containing substrates, and any counts obtained from the control reactions were subtracted from those obtained when substrate was present. This provided a measure of ^{32}P incorporated into the added substrate without interference from kinase autophosphorylation reactions or from phosphorylation of substrates present in the enzyme preparations.

Casein kinase II reactions were carried out in a final volume of 50 μl containing 100 μM [γ-^{32}P]ATP (10–1000 cpm/pmol), 10 mM $MgCl_2$, an aliquot of crude enzyme (3 μl) or an appropriate amount of partially purified enzyme, 50 mM 3-(*N*-Morpholino)propanesulfonic acid (Mops) (pH 7.0), and 150 mM NaCl. The amounts of substrates and other constituents and the duration of reactions are specified in the figure legends. Casein kinase I reactions were carried out as for casein kinase II. Casein and the synthetic peptide were used as substrates at respective final concentrations of 2.2 mg/ml and 2 mM. Reactions were allowed to continue for 20 min. cAMP-dependent protein kinase and cGMP-dependent protein kinase reaction mixtures had a final volume of 40 μl and contained 180 μM [γ-^{32}P]ATP (≈100 cpm/pmol), 6 mM $MgCl_2$, ≈1 μg of catalytic subunit per ml, 25 mM Tris (pH 7.4), and 125 μM Kemptide or 100 μM H2B histone or 1 mM synthetic peptide. The reactions were continued for 3 min. Assays with smooth muscle myosin light chain kinase contained 200 μM [γ-^{32}P]ATP (≈100 cpm/pmol), 10 mM $MgCl_2$, 2 μg of enzyme per ml, 100 mM Tris (pH 7.5), 1 μM calmodulin, 100 μM $CaCl_2$, 90 μM KCl, and 5 μM mixed myosin light chains or 2 mM synthetic peptide. The reactions were continued for 15 min. Protein kinase C reactions contained 200 μM [γ-^{32}P]ATP, 25 mM $MgCl_2$, 1 μg of protein kinase C, 20 mM Tris (pH 7.4), 100 μM $CaCl_2$, 0.1 mg of bovine serum albumin per ml, and 27 μM mixed myosin light chains or 2 mM synthetic peptide. These reactions were continued for 30 min. Phosphorylation reactions with the insulin and EGF receptors were performed as described by Pike *et al.* (18) for phosphorylation of Arg-Arg-SRC. The final concentrations of Arg-Arg-SRC and the synthetic peptide were 2 mM. The reactions were for 15 min. Phosphorylase kinase reactions were carried out as described by Shenolikar *et al.* (27). The final concentration of phosphorylase *b* was 4 mg/ml, and that of the synthetic peptide was 3 mM.

Phosphorylated products in all reactions, except those using phosphorylase *b* as the substrate, were measured by the method of Glass *et al.* (28). Briefly, an aliquot of the reaction mixture was spotted on a 2-cm^2 piece of Whatman P81 paper, which was washed three times for 4 min in 75 mM H_3PO_4, dried with a warm air blower, put in a vial with scintillation fluid, and assayed for ^{32}P. Labeled phosphorylase *a* was measured by spotting an aliquot of the reaction mixture on squares of Whatman ET31 paper and washing for 10 min in cold 10% trichloroacetic acid and then twice for 15 min in 5% trichloracetic acid. The papers were dried and assayed for radioactivity as above.

Purification of Casein Kinase II. Casein kinase II was purified by using a modification of the method of Carmichael *et al.* (5). All procedures were performed at 4°C. One kilogram of bovine liver was homogenized in a blender with 2.5 vol of 30 mM Mops, pH 7.0/1 mM EDTA/1 mM dithiothreitol (buffer A) containing 0.5 mM phenylmethanesulfonyl fluoride and then was centrifuged for 60 min at 8,000 × *g*. The supernatant was stirred for 2 hr with 350 ml (settled volume) of phosphocellulose equilibrated in buffer A, and the resultant mixture was poured into a sintered glass filter funnel. The phosphocellulose resin was washed with 3 liters of buffer A, followed by 700 ml of buffer A containing 1.1 M NaCl. The high-salt eluate contained phosphorylating activity towards the peptide and casein and was dialyzed overnight against 20 liters of buffer A. A settled volume of 75 ml of heparin-agarose was stirred with the dialyzed fraction for 1 hr. The slurry was poured into a column, washed with 1 liter of buffer A, and eluted with a 350-ml linear gradient of 0–0.5 M $(NH_4)_2SO_4$. The fractions containing kinase activity were pooled and then dialyzed overnight against 20 liters of buffer A. The dialyzed fraction was mixed for 1 hr with 75 ml (settled volume) of DEAE-cellulose equilibrated in buffer A, and the resin was then washed with 250 ml of buffer A, poured into a column, and eluted with a 300-ml linear gradient of 0–0.8 M NaCl in buffer A. The eluate was collected in 2-ml fractions, and the fractions containing kinase activity toward the peptide were pooled, dialyzed overnight against buffer A containing 0.3 M NaCl, and stored at 4°C. At this stage the enzyme was only partially pure, as evidenced by a number of Coomassie blue-stained bands on sodium dodecyl sulfate/polyacrylamide gels.

Phosphoamino Acid Analyses. Casein kinase II reaction mixtures containing either synthetic peptide or without substrate were hydrolyzed in evacuated ampules containing 6 M HCl for 2 hr at 110°C. Phosphoserine, phosphothreonine, and phosphotyrosine (50 μg each) were added to the hydrolysates. The samples were dried, dissolved in 100 μl of water, and 2 μl of each were spotted on Eastman cellulose thin-layer plates and electrophoresed at pH 3.6 for 2 hr at 500 V. The standards were located by ninhydrin staining, and radioactive spots were visualized by autoradiography.

RESULTS

Phosphorylation of a Synthetic Peptide by Casein Kinase II. The synthetic peptide Arg-Arg-Arg-Glu-Glu-Glu-Thr-Glu-Glu-Glu was found to serve as a substrate for casein kinase II from bovine liver. The phosphorylation reaction proceeded linearly for at least 1 hr under the conditions used (Fig. 1). At high enzyme concentrations, the peptide was phosphorylated to a stoichiometry of essentially 1 mol of phosphate per mol of peptide (not illustrated). Examination of an acid hydrolysate of the phosphorylated peptide by high-voltage electrophoresis showed that threonine was the phosphorylated amino acid (Fig. 2). Fig. 3 shows the Lineweaver–Burk plot for phosphorylation of the synthetic peptide by casein kinase II. From this plot, the K_m of casein kinase II for the peptide was determined to be 500 μM. Phosphorylation of casein was measured under the same conditions as phosphorylation of the peptide and the K_m for casein was determined to be 10-fold lower than the K_m for the peptide (Fig. 3). The maximum velocities for phosphorylation of the peptide and

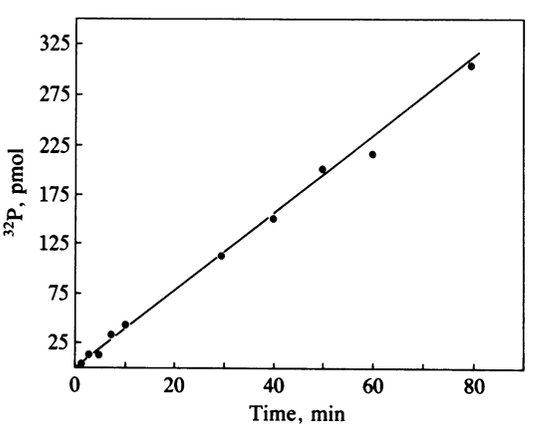

FIG. 1. Time course of the phosphorylation of the synthetic peptide by casein kinase II. The phosphorylation reactions contained 100 ng of partially purified casein kinase II and 0.5 mM peptide and were carried out at 30°C. Reactions were stopped after the indicated time, and the phosphate incorporated into the synthetic peptide was quantitated as described.

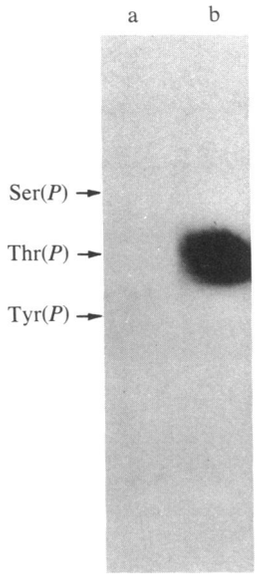

FIG. 2. Phosphoamino acid analysis of the synthetic peptide phosphorylated by casein kinase II. Phosphorylation reaction mixtures containing 10 ng of partially purified casein kinase II and either no substrate (lane a) or 200 nmol of peptide (lane b) were hydrolyzed, subjected to high-voltage electrophoresis, and autoradiographed as described. The arrows indicate the positions of phosphoamino acid standards.

of casein, also determined from the Lineweaver–Burk plots, were similar (Fig. 3).

Inhibition of Phosphorylation of the Synthetic Peptide by Heparin. Heparin is known to be a potent inhibitor of the phosphorylation of casein by casein kinase II (29, 30). Under the conditions used in the present study, a K_i for heparin inhibition of casein phosphorylation was found to be 6 nM and a similar K_i was observed for heparin inhibition of the casein kinase II-catalyzed phosphorylation of the synthetic peptide (not illustrated). The inhibitor was found to be competitive with respect to the peptide substrate.

Specificity of Phosphorylation of the Synthetic Peptide by Casein Kinase II. Eight protein kinases in addition to casein kinase II were tested for their abilities to phosphorylate the synthetic peptide. For each of the enzymes tested, control reactions were carried out by using a known substrate for the enzyme in place of the peptide. As shown in Table 1, all of the kinases were able to transfer phosphate to their known substrates. However, under identical conditions none of the kinases other than casein kinase II could phosphorylate the peptide to any measurable extent (Table 1).

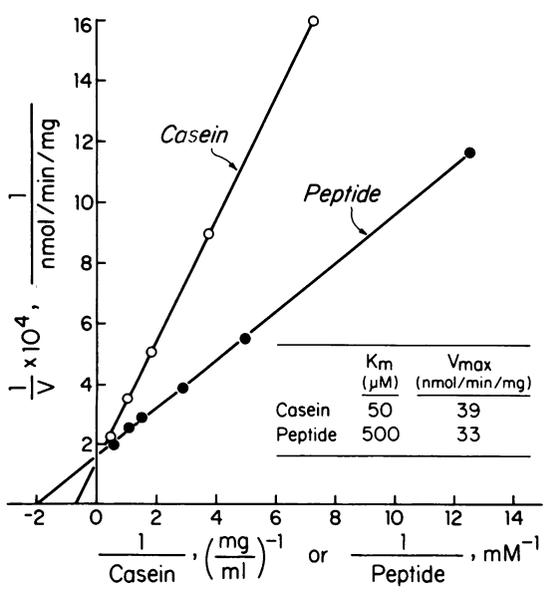

FIG. 3. Lineweaver–Burk plots of the phosphorylation of the synthetic peptide and casein by casein kinase II. Phosphorylation reactions were carried out for 6 min as described. Partially purified casein kinase II was at a concentration of 100 ng/50 μl.

Use of the Specific Peptide Substrate to Assay Impure Cell Fractions for Casein Kinase II Activity. Casein is the substrate usually used to measure casein kinase II activity *in vitro*, but use of this substrate does not provide an accurate measure of casein kinase II activity in crude fractions because many other kinases also can phosphorylate casein. To see if use of the synthetic peptide to measure kinase activity could resolve casein kinase II activity from other casein-phosphorylating activities in a crude system, the peptide and casein were used in side-by-side assays of fractions obtained during the purification of casein kinase II from bovine liver. After homogenization, centrifugation, and phosphocellulose chromatography, the fraction that was obtained was next applied to heparin-agarose resin. The elution profile from this resin is shown in Fig. 4. This profile demonstrated the ability of the peptide to distinguish casein kinase II activity from other kinase activity directed toward casein. There were two major peaks of kinase activity toward casein but only one peak of kinase activity toward the peptide. The peak of activity toward the peptide was superimposed on one of the peaks and occurred at a salt concentration that elutes casein kinase II from heparin-agarose. The relative yield of casein kinase II for this chromatographic step was 20% when activity toward the peptide was used in the calculations and 13% when activity toward casein was used. The apparently higher yield of enzyme using peptide phosphorylation in the calculations is due to the fact that the total activity applied to the heparin-agarose column was less when measured with

Table 1. Activity of various kinases towards Arg-Arg-Arg-Glu-Glu-Glu-Thr-Glu-Glu-Glu

Kinase	Control substrate	Phosphate incorporated, pmol: Control substrate	Phosphate incorporated, pmol: Peptide
cAMP-dependent protein kinase	Kemptide	959	<1
cGMP-dependent protein kinase	Histone H2B	255	<1
Phosphorylase kinase	Phosphorylase *b*	1027	<1
Protein kinase C	Smooth muscle myosin light chains	902	<1
Smooth muscle MLCK	Smooth muscle myosin light chains	676	<1
Insulin-stimulated kinase	Arg-Arg-SRC	28	<1
EGF-stimulated kinase	Arg-Arg-SRC	62	<1
Casein kinase I	Casein	2637	<1
Casein kinase II	Casein	2977	1048

The abilities of various kinases to phosphorylate the synthetic peptide were tested. At the same time, control reactions were carried out with a known acceptor substrate for each enzyme in place of the synthetic peptide. The amount of phosphate transferred by each kinase to the synthetic peptide or to the control substrate was determined as described. The casein kinase II reactions contained either peptide at 0.9 mM or casein at 2.2 mg/ml and lasted for 15 min. Substrate concentrations and lengths of reactions for all other kinase assays are reported in *Materials and Methods*. MLCK, myosin light chain kinase.

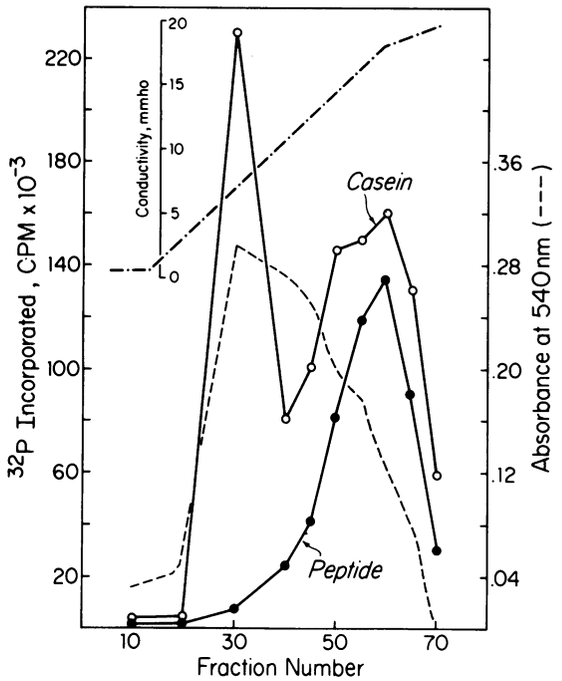

FIG. 4. Purification of casein kinase II by heparin-agarose chromatography. Details of the column, elution gradient, and measurement of kinase activity towards peptide (●) and casein (○) are as described. Absorbance at 540 nm was measured after allowing 2 μl of the fraction to react with the Coomassie blue reagent of Bradford (31). Fractions 50–70 were pooled.

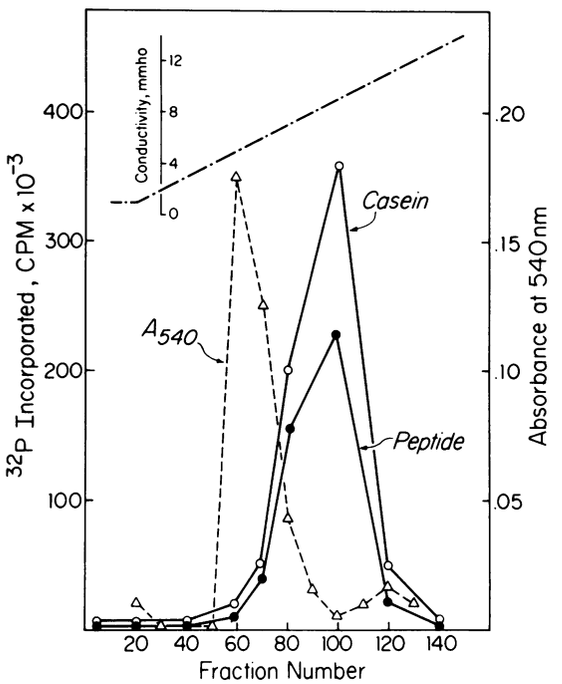

FIG. 5. Purification of casein kinase II by DEAE-cellulose chromatography. Details of the column, elution gradient, and measurement of kinase activity towards peptide (●) and casein (○) are as described. Absorbance at 540 nm was measured as described in the legend of Fig. 4. Fractions 80–110 were pooled.

the peptide as the substrate than with casein as the substrate. The total activities differed because peptide phosphorylation provided a measure of only casein kinase II activity, whereas casein phosphorylation provided a measure of the activities of all kinases capable of phorphorylating casein. The fractions that contained casein kinase II were pooled, applied to DEAE-cellulose, and then eluted from this resin. Fig. 5 shows the elution profile, which consisted of coincident peaks of activity toward the peptide or casein. The relative yield of kinase after this step was the same when it was calculated with either activity toward the peptide or toward casein. At this stage in the purification, it appears that all of the casein kinase activity was casein kinase II activity.

DISCUSSION

The objective of the present study was to synthesize a peptide that would serve as a substrate for casein kinase II and be specific for this enzyme. In approaching the problem, we took into account several features of the sequences around casein kinase II phosphorylation sites in protein substrates. The most striking feature of the sequences is their high content of acidic amino acids. Data reported by Pinna *et al.* (32) showed that the sites of casein kinase II phosphorylation in α_{S1}-, α_{S2}-, and β-casein are located either in regions containing several glutamate or aspartate residues or within strings of serine-phosphate residues. Accordingly, the phosphorylatable amino acid in the peptide synthesized in this study was surrounded by six glutamates. It was anticipated that the presence of these glutamates would prevent the cAMP-dependent protein kinase from phosphorylating the peptide, since studies have shown that acidic residues are negative determinants in substrate recognition for that enzyme (24, 33). This expectation proved to be correct because no phosphate was transferred from ATP to the peptide by the cAMP-dependent protein kinase. The cGMP-dependent protein kinase (34) and phosphorylase kinase, two other enzymes for which acidic residues in substrates appear to be negative determinants, also did not phosphorylate the peptide.

The presence of acidic residues alone was not considered sufficient to render a substrate specific for casein kinase II because acidic residues serve as positive determinants in substrates for several other protein kinases. For example, there is evidence that acidic residues may be important for substrate recognition by the insulin- and EGF-receptor kinases (35) and also for substrate recognition by casein kinase I (36). The ability to design an acidic peptide that would not be phosphorylated by these kinases relied on the choice of the residue at the phosphorylation site. Casein kinase II has been shown to phosphorylate both serine and threonine residues in proteins (32), so either of these amino acids were candidates for placement in the peptide. Threonine was chosen because casein kinase I has been shown to phosphorylate only serine residues in proteins (1), and the insulin- and EGF-receptor kinases have been shown to phosphorylate only tyrosine residues in proteins and synthetic peptides (18). That casein kinase I did not phosphorylate the peptide provides further evidence for this enzyme's specificity towards serine. That the receptor kinases did not phosphorylate the peptide provides further evidence for their specificities toward tyrosine.

Protein kinase C and smooth muscle myosin light chain kinase have as yet less-defined substrate specificities, except that they are both able to transfer phosphate to serine and threonine residues. However, neither of them could phosphorylate this peptide. Thus, of all the kinases tested, the peptide was phosphorylated only by casein kinase II. The possibility that some kinase not included in this representative survey could phosphorylate the peptide remains.

The three arginines were added to the peptide to allow for simple measurement of peptide phosphorylation by the phosphocellulose paper assay of Glass *et al.* (28). Adding arginines to a synthetic peptide so that it would adhere to phosphocellulose paper is a device originally used by Casnellie *et al.* (23) in their design of a peptide substrate for tyro-

sine protein kinases. Whether these arginines affect the kinetic parameters for phosphorylation of the peptide by casein kinase II is unknown.

It generally has been observed that the K_m values of kinases for their synthetic peptide substrates are higher than for their protein substrates, and these observations may reflect the importance of higher-order structure in substrate recognition. The K_m of casein kinase II for this synthetic peptide, 500 μM, was 10-fold higher than the K_m for casein. This value for the peptide lies in the middle of the range of K_m values observed for other kinases with synthetic peptide substrates. For example, the K_m of the insulin receptor kinase for the tyrosine-containing peptide Arg-Arg-SRC is 2000 μM (18) and the K_m of the cAMP-dependent protein kinase for Kemptide is 20 μM (24).

The need for a substrate that was specific for casein kinase II arose because it was desirable to assay casein kinase II activity in systems in which more than one kinase was present. The synthetic peptide reported herein appears to have fulfilled this need. The usefulness of the peptide for assaying casein kinase II activity in crude systems was demonstrated by the ability of the peptide to distinguish casein kinase II activity from other kinase activities in crude liver preparations. Thus, more accurate measures of yields and specific activities during casein kinase II purification and less ambiguous results on studies of casein kinase II regulation should be afforded by the use of the specific peptide substrate instead of casein. Because the peptide has a single phosphorylation site, the peptide also may be applied to the determination of the mechanism of casein kinase II-catalyzed reactions.

Concurrent with this study another laboratory has applied synthetic peptides to investigations of casein kinase II (personal communication, Lorenzo A. Pinna; also ref. 37).

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