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PARTIAL PURIFICATION AND PROPERTIES OF A CYCLIC 3′,5′-AMP-INDEPENDENT PROTEIN KINASE FROM RAT LIVER

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Summary

1. A cyclic 3′,5′-AMP-independent protein kinase (ATP : protein phosphotransferase, EC 2.7.1.37) from rat liver cytosol was partially purified and characterized. Purification by $(NH_4)_2SO_4$ precipitation, DEAE-cellulose, Bio Gel A-0.5 m and cellulose phosphate chromatography increased the specific activity about 700-fold.

2. An endogenous protein substrate was closely associated with the protein kinase and was not separable from this enzyme up to the cellulose phosphate stage. After phosphorylation, chromatography with Bio Gel A-0.5 m partially separated this endogenous phosphoprotein from the enzyme activity; this dissociation had no apparent effect on kinase activity with casein or phosvitin as substrates, or on the apparent molecular weight of the enzyme (approx. 158 000).

3. This protein kinase with casein, phosvitin, or the endogenous substrate was totally insensitive to the thiol reagents, *p*-hydroxymercuribenzoate, 5,5′-dithiobis(2-nitrobenzoic acid), iodoacetamide, and *N*-ethylmaleimide. The enzyme was also unaffected by cyclic 3′,5′-AMP, heat-stable protein kinase inhibitor, and the regulatory subunit of a cyclic 3′,5′-AMP-dependent protein kinase.

Introduction

Protein kinases (ATP : protein phosphotransferases, EC 2.7.1.37) catalyze the transfer of γ-phosphate from ATP to a variety of proteins. Since the discovery of a cyclic 3′,5′-AMP-dependent protein kinase in rabbit skeletal muscle by Walsh et al. [1], similar enzymes have been found in many tissues [2—5],

Abbreviations: Cyclic 3′,5′-AMP is adenosine 3′,5′-cyclic phosphate; EGTA is [ethylenebis(oxyethylenenitrilo)]tetraacetic acid.

and it is now generally accepted that the action of cyclic 3′,5′-AMP is mediated by these enzymes [6,7]. Thus, cyclic 3′,5′-AMP-dependent protein kinases have attracted much attention as mediators of certain hormonal actions [8].

Protein kinases which are not cyclic 3′,5′-AMP dependent, on the other hand, catalyze the phosphorylation of acidic proteins or phosphoproteins such as casein and phosvitin. Although partially purified from a variety of tissues [9—13], these enzymes have been less extensively studied and their physiological roles are not clearly established.

The present work was initiated as an extension of our earlier studies with rat liver acetyl-CoA carboxylase [14] and was prompted by a report by Carlson and Kim [15] suggesting that this enzyme is regulated by a phosphorylation-dephosphorylation reaction analogous to that described for pyruvate dehydrogenase [16].

In our search for a phosphorylated form of acetyl-CoA carboxylase, we isolated a second activity peak for this enzyme using DEAE-cellulose chromatography. This second enzyme peak was associated with a major protein peak and could be labeled in vivo with ^{32}P. Considering that this fraction might include the "inactive phosphorylated" form of acetyl-CoA carboxylase, we proceeded with further purification of the protein fraction, which was subsequently shown by immunoprecipitation and determination of biotin content to be unrelated to acetyl-CoA carboxylase. Instead, it proved to be a protein kinase which was independent of cyclic 3′,5′-AMP and was very closely associated with an endogenous protein substrate.

This report describes the purification procedure and some properties of this enzyme.

Experimental

Materials

Phosvitin, protamine, histones, protein kinase inhibitor, ATP, cyclic 3′,5′-AMP, and cyclic 3′,5′-GMP were from Sigma Chemical Co.; sodium caseinate and fat-free diet from Nutritional Biochemical Corp.; phosphorylase *b* from Worthington Biochemical Corp.; and bovine serum albumin from Reheis Chemical Co. Bio Gel A-0.5 m was obtained from Bio-Rad Laboratories, and cellulose phosphate from Schleicher and Schuell Co. [Ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA) was obtained from Eastman Kodak Co. [α-^{32}P]-ATP and [γ-^{32}P]ATP (2—10 Ci/mmol) were products of New England Nuclear Corp.

Male Sprague-Dawley rats weighing 200—400 g were fed a fat-free diet for 4 days, starved for 48 h, and then refed the fat-free diet for 48 h prior to killing. This dietary regimen was followed because it increases acetyl-CoA carboxylase activity 10—20-fold [17] and the isolation of the protein kinase was an outgrowth of our studies with this enzyme. In other experiments the rats were fasted and refed fat-free diet as described above; each rat then received 2 ml of sesame oil by oral intubation as well as 4 mCi ^{32}P (as KH_2PO_4 in 0.9% NaCl) intravenously. The animals were killed 6 h later and the livers were isolated for further analysis of protein, acetyl-CoA carboxylase activity and protein-bound radioactivity.

Protein kinase assay

Activity was determined by measurement of the enzymatic transfer of ^{32}P from [γ-^{32}P]ATP to casein, phosvitin, or endogenous substrate. Unless otherwise indicated, the assay for kinase activity contained the following components in a final volume of 0.1 ml: 30 mM sodium phosphate buffer (pH 5.7), 10 mM $MgSO_4$, 50 μM [γ-^{32}P]ATP, ($5 \cdot 10^5$—$10 \cdot 10^5$ cpm), 5 μM mercaptoethanol, 1.2 mg sodium caseinate, and the enzyme source, usually purified through the Bio Gel A-0.5 m stage. Blanks contained no enzyme. The mixture was incubated at 37°C for 10 min, at which time 50 μl were transferred to a 2.3 cm Whatman 3 MM paper disc under a stream of hot air (70°C). The discs were washed as described by Mans and Novelli [18] with minor modifications consisting of a 10-fold increase in the volume of trichloroacetic acid and ether/ethanol mixture used for washing, to minimize non-protein-bound radioactivity. The washed discs were dried and counted in 10 ml of scintillation mixture in a liquid scintillation counter (LS 35S Beckman Instruments, Inc.). The blanks, which gave counts of 400—800 cpm, were used as controls. A comparison of this procedure with the trichloroacetic acid precipitation method described by Soderling et al. [19] gave identical results.

One unit of enzyme was defined as the amount of enzyme catalyzing the transfer of 1 nmol of ^{32}P from [γ-^{32}P]ATP to casein in 10 min per 0.1 ml of standard assay mixture.

The reaction followed zero-order kinetics (see Fig. 1A) for at least 10 min if the amount of enzyme was below 0.3 unit per assay. When phosvitin was used as the substrate, the rate was higher (2.0-fold) than with casein under the same conditions.

Phosphorylation of endogenous substrate

The reaction mixture for the determination of endogenous phosphorylation activity was identical to that used for standard kinase assays except that 30 mM phosphate buffer (pH 6.3) was used, no casein or phosvitin was present, and the ATP content was reduced to 10 μM. The reaction rate for endogenous phosphorylation was not linear (Fig. 1B). Even at 0°C the reaction proceeded at an appreciable rate over a long period of time. Overall rates of phosphorylation were higher with higher ATP concentrations. The values given for endogenous phosphorylation should therefore be considered as relative values only, since the standard conditions were suboptimal. These conditions were selected, however, becuase optimal sensitivity in terms of total cpm measured for a given amount of enzyme and [γ-^{32}P]ATP could be obtained. One unit of endogenous phosphorylation was defined as 1 nmol of ^{32}P incorporated per assay sample per 10 min of incubation.

Purification of kinase

(a) Purification through $(NH_4)_2SO_4$ step. Table I summarizes a typical preparation. Rats were killed by asphyxiation in a CO_2 chamber and livers were removed immediately and chilled in a buffer at pH 7.5 consisting of 0.1 M phosphate and 0.07 M $KHCO_3$. Livers were homogenized in 1.5 volumes (w/v) of buffer in 50 ml Potter-Elvehjem Teflon homogenizers for 3 min. This and all subsequent steps were carried out at 0—4°C. The homogenate was centrifuged

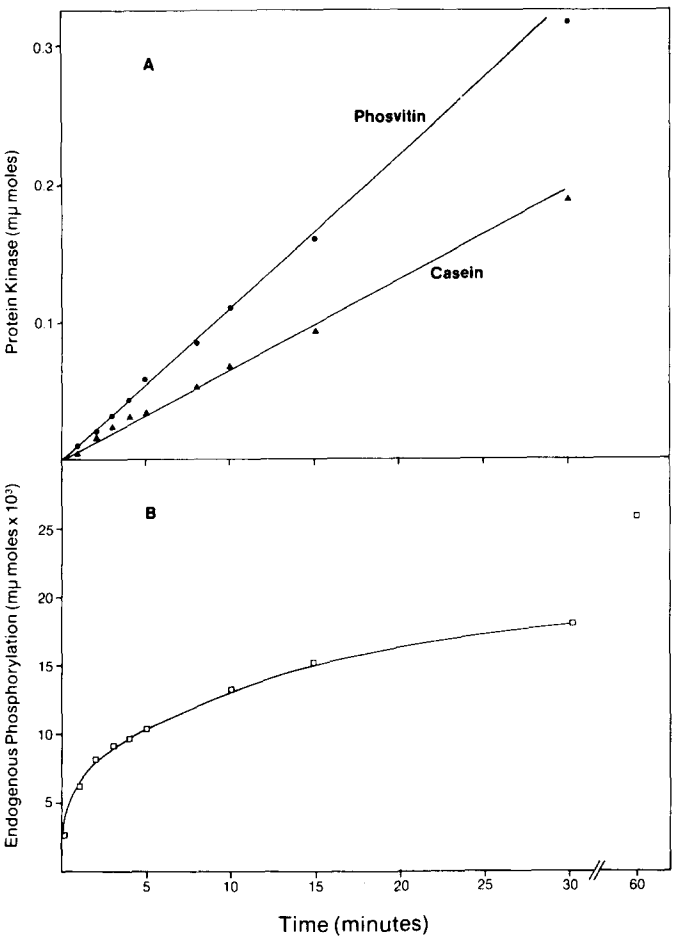

Fig. 1. (A) Time course of protein kinase reaction with casein or phosvitin as substrates. Under the standard assay conditions, detailed in Experimental, the reaction was initiated by the addition of 25 μl of protein kinase solution corresponding to.0.85 μg protein [20], purified through the Bio Gel A-0.5 m stage; this contained 172 units/mg kinase activity with casein as substrate. The protein-bound ^{32}-P activity was measured at the indicated time intervals and expressed as nmol of ^{32}P from [γ-^{32}P]ATP per 0.1 ml assay mixture. 1 nmol was equivalent to 33 000 cpm. Values were corrected for the zero time control (approx. 700 cpm), which was the same as a blank containing no enzyme. (B) Time course of endogenous phosphorylation. Conditions as detailed in A, except that 1 nmol of ATP per 0.1 ml assay mixture was used, and enzyme protein was 12.5 μg per assay. Values were corrected for the blank (no enzyme). Under these conditions the endogenous phosphorylation for 10 min was proportional to the amount of enzyme (data not shown) and was equivalent to 12 500 cpm/12.5 μg of enzyme solution.

at 10 000 × *g* for 15 min, the pellet discarded, and the supernatant centrifuged at 105 000 × *g* for 60 min. To the supernatant $(NH_4)_2SO_4$ (243 mg/ml) was added (40% saturation), adjusting the pH to 7.5 as needed with concentrated KOH. After 30 min of gentle stirring, the precipitate was collected by centrifugation at 27 000 × *g* for 30 min. The pellet was resuspended to about one-third the volume of the original supernatant in 0.05 M potassium phosphate buffer (pH 7.5) containing 10 mM citrate, 5 mM mercaptoethanol and 0.1 mM EDTA (buffer A) and dialyzed overnight against 4 l of the same buffer. The dialyzed

TABLE I

PURIFICATION OF PROTEIN KINASE AND ENDOGENOUS PHOSPHORYLATION ACTIVITY FROM RAT LIVER

One unit of protein kinase activity is defined as the amount of enzyme which catalyzes the transfer of 1 nmol of ^{32}P from [γ-^{32}P]ATP to casein in 10 min under the standard assay conditions. Endogenous phosphorylation activity units are nmol of ^{32}P incorporated into enzymes protein from [γ-^{32}P]ATP in 10 min as detailed under Experimental. Protein was determined by the method of Lowry et al. [20] with bovine serum albumin as standard.

Fraction	Volume (ml)	Protein (mg)	Protein kinase		Endogenous phosphorylation	
			Total activity	Specific activity	Total activity	Specific activity
10 000 × *g*-supernatant	503	23 943	125 000	5.2	915	0.04
105 000 × *g*-supernatant	422	15 023	89 000	5.9	532	0.04
0—40% $(NH_4)_2SO_4$ precipitate	142	4 110	58 000	14.1	547	0.13
DEAE-cellulose	24	256.8	9 936	38.7	182.4	0.71
Bio Gel A-0.5 m	55	70.1	9 835	140.3	187.0	2.7
Cellulose phosphate eluates	94	2.8	9 637	3441	0	0

material was centrifuged at 105 000 × *g* for 30 min; the pellet was washed three times by repeated homogenization and centrifugation, using small volumes of buffer A and combining the supernatants with the main fraction.

(b) DEAE-cellulose chromatography. The dialyzed $(NH_4)_2SO_4$ fraction was applied to a freshly recycled DEAE-cellulose column (3.5 × 70 cm bed volume) equilibrated with buffer A (Fig. 2). The column was then washed with 100 ml of the same buffer. Elution was carried out with a linear gradient obtained by mixing 500 ml of buffer A with 500 ml of 0.25 M potassium phosphate buffer (pH 7.5) containing 10 mM citrate, 5 mM mercaptoethanol and 0.1 mM EDTA; fractions of 9 ml were collected at a flow rate of 1 ml/min. At tube No. 111 a second linear gradient was applied, obtained by mixing 500 ml each of 0.1 M KCl and 1.0 M KCl in buffer A. All fractions were assayed for kinase and endogenous phosphorylation activity (with and without cyclic 3′,5′-AMP) and for protein content [20] (see Figs. 2 and 3). Fractions 173—188 were combined and concentrated to about 5 ml by Amicon ultrafiltration using a PM 10 membrane. The concentrated material was diluted to 50 ml with buffer A and reconcentrated; this process was repeated twice more to bring the final concentration of KCl below 10 mM.

(c) Bio Gel A-0.5 m chromatography. A 5 ml fraction of the material after DEAE-cellulose purification was placed on a Bio Gel A-0.5 m column (2 × 83 cm bed volume) equilibrated with 0.05 M potassium phosphate buffer (pH 7.5) containing 10 mM citrate and 0.5 M KCl. Fractions of 2.0 ml were collected at a flow rate of 15 ml/h. Aliquots from each fraction were assayed for protein content, kinase and endogenous phosphorylation activity (Fig. 4). Fractions 40—60 were pooled and subjected to Amicon processing as above to remove the KCl and concentrate the material.

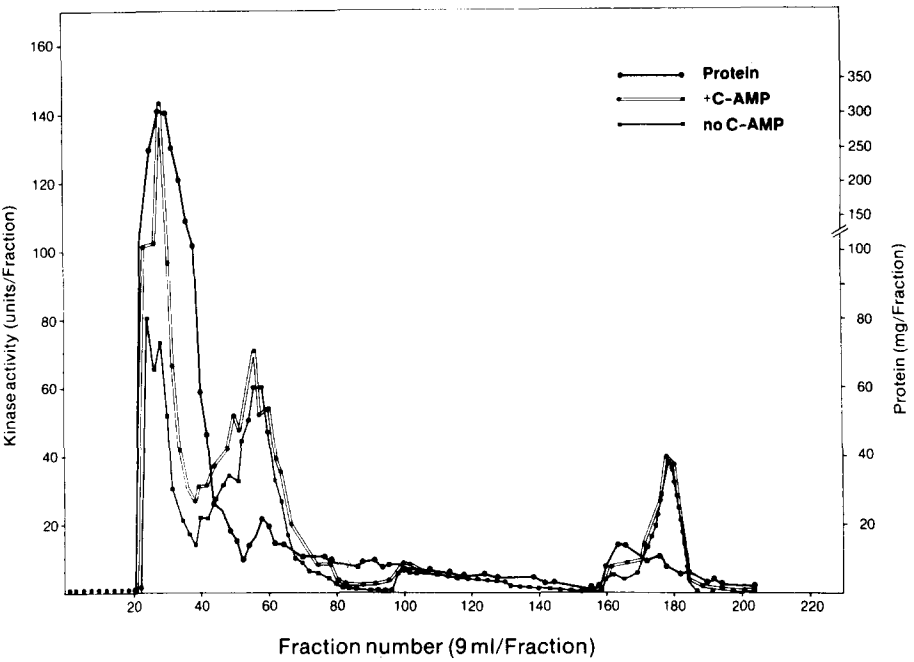

Fig. 2. DEAE-cellulose chromatography. Elution patterns of protein kinase activity with and without cyclic 3',5'-AMP ($1 \cdot 10^{-5}$ M). Technical details are given in the text.

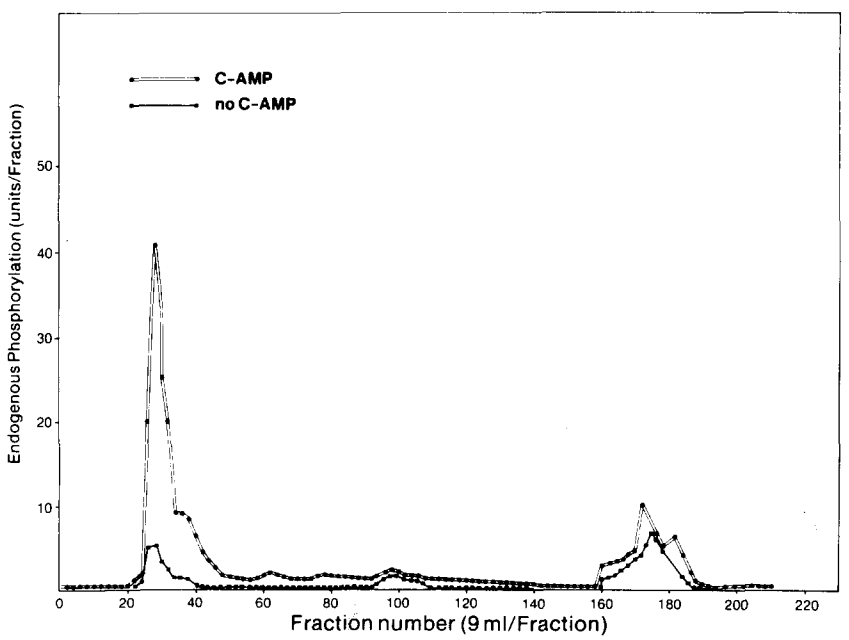

Fig. 3. Patterns of endogenous phosphorylation activity in DEAE-cellulose fractions with and without cyclic 3',5'-AMP ($1 \cdot 10^{-5}$ M). Conditions identical to those in Fig. 2 (same experiment).

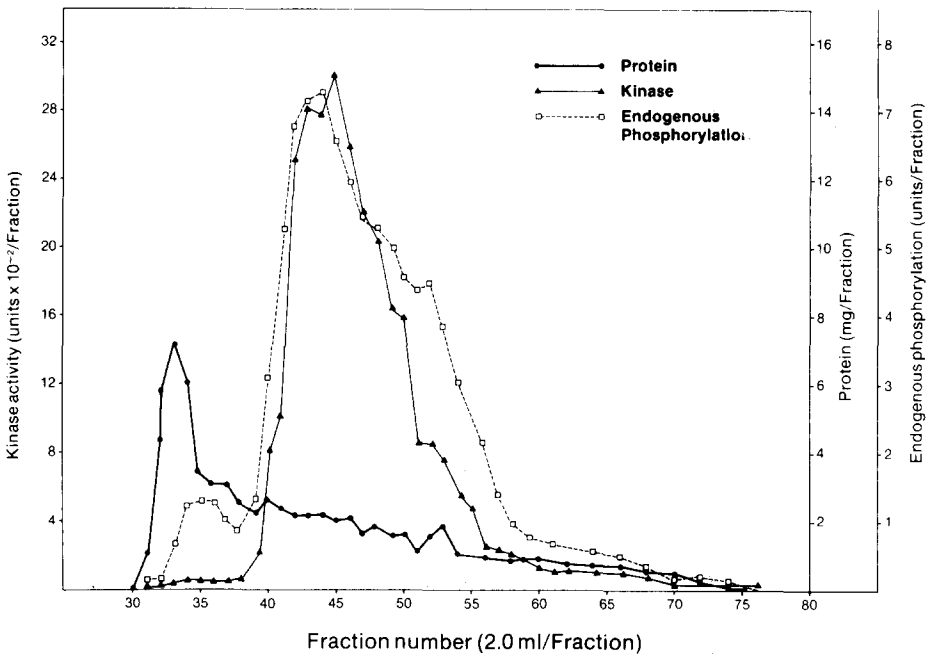

Fig. 4. Bio Gel A-0.5 m chromatography of protein kinase. A kinase solution purified through the DEAE-cellulose stage, containing (in 5.0 ml) 3480 units of kinase; 76 units of endogenous phosphorylation activity and 107 mg of protein was applied to the column (2 × 83 cm). Details are given in the text. The void volume (V_0) of the column was determined with Dextran Blue (V_0 = 38 ml); the total volume (V_t) was determined with ^3H_2O (V_t = 172 ml).

(d) Cellulose phosphate chromatography. After purification on Bio Gel A-0.5 m column, a 2.0 ml fraction of the kinase was applied to a cellulose phosphate column (6 × 1.5 cm bed volume), which was previously recycled with 0.1 M NaOH, water (until neutral), 0.1 M HCl, and water (until neutral) and finally equilibrated with buffer A. After the enzyme was adsorbed to the column, it was eluted with buffer A, collecting 2.0-ml fractions at a flow rate of 15 ml/h. After 30 ml were collected, the elution was continued with the same buffer containing 0.25 M NaCl until tube No. 25. A linear gradient was then instituted, obtained by mixing 35 ml each of 0.5 M NaCl and 1.2 M NaCl in buffer A. After this gradient was achieved, elution was continued with 1.2 M NaCl. All the fractions were assayed as before for protein kinase and endogenous phosphorylation activity. No endogenous phosphorylation could be detected in any of the individual fractions or after mixing the eluted protein peak with the kinase activity peak (see Fig. 5).

Fractions with kinase activity contained 98% of the activity of the starting material, and were pooled and concentrated by Amicon filtration as described above. This purification step provided a 25-fold increase in the specific activity of the kinase, but the enzyme proved to be very unstable at this stage. Table I summarizes the purification steps and the yields for a typical preparation of this kinase.

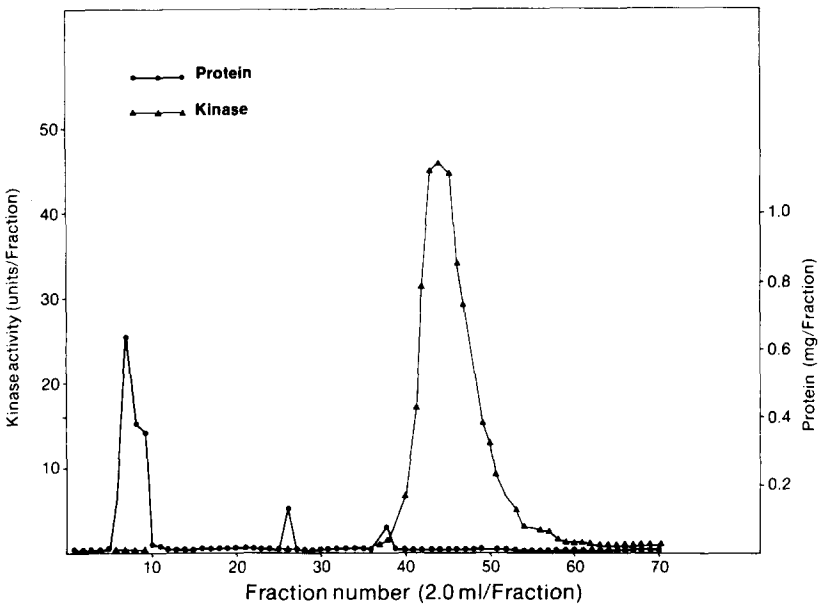

Fig. 5. Cellulose phosphate chromatography of protein kinase. 2.0 ml of protein kinase solution purified through the Bio Gel A-0.5 m stage, containing 356 units of kinase, 7 units of endogenous phosphorylation activity and 2.54 mg protein, were applied to a cellulose phosphate column (1.5 X 6 cm) equilibrated and eluted as detailed in the text.

Polyacrylamide gel electrophoresis studies were undertaken to establish purity, but we were unable to define the conditions under which this enzyme would penetrate the gel.

Results

Regulation of acetyl-CoA carboxylase by phosphorylation-dephosphorylation as suggested by Carlson and Kim [15] could explain a number of observations related to short term regulation of acetyl-CoA carboxylase, thus adding lipogenesis to the list of metabolic processes known to be regulated by phosphorylation-dephosphorylation reactions, which already includes lipolysis, glycogenesis, and glycogenolysis.

We felt that if this mechanism were indeed operative on acetyl-CoA carboxylase, oil feeding under conditions of maximal acetyl-CoA carboxylase activity (such as fasting and refeeding with a fat-free diet) should favor the formation of the phosphorylated inactive form of acetyl-CoA carboxylase [17]. In experiments such as the one described in the legend to Fig. 6, rats were given oil and $KH_2{}^{32}PO_4$ orally and the 0—30% $(NH_4)_2SO_4$ fraction of liver cytosol was chromatographed on a DEAE-cellulose column. It became apparent from such experiments that a major protein peak eluted with 0.5 M KCl contained protein-bound ^{32}P as well as acetyl-CoA carboxylase activity separable from the main peak of acetyl-CoA carboxylase, which was eluted earlier.

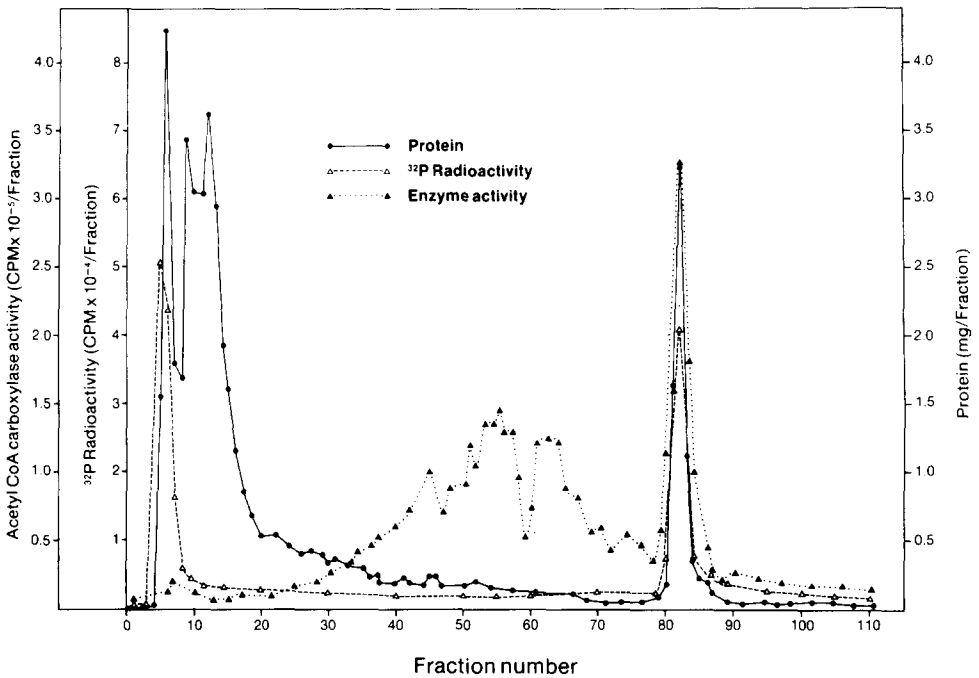

Fig. 6. DEAE-cellulose chromatography of hepatic acetyl-CoA carboxylase from rats treated with ^{32}P in vivo. Two male rats, fasted and refed with fat-free diet for 48 h, each received 2 ml of sesame oil by intubation and 0.4 ml of $KH_2{}^{32}PO_4$ solution in 0.9% NaCl (corresponding to 4 mCi of ^{32}P) intravenously. 6 h later they were sacrificed and the $(NH_4)_2SO_4$ fraction of the liver cytosol was prepared as described by Inoue and Lowenstein [21]. After overnight dialysis against three changes of buffer A, the $(NH_4)_2SO_4$ fraction was centrifuged and the supernatant solution (30 ml) was adsorbed onto a DEAE-cellulose column (2 × 13 cm). A linear gradient obtained by mixing 200 ml of 0.04 M and 200 ml of 0.25 M phosphate buffers (pH 7.5), containing in addition 10 mM citrate, 5 mM mercaptoethanol and 0.5 mM EDTA, was used for elution. After 365 ml in 5-ml fractions were collected, the elution was continued with buffer A containing 0.5 M KCl. Fractions were assayed for protein [20], acetyl-CoA carboxylase activity [17], and protein-bound radioactivity [18].

There was a possibility that this ^{32}P-containing peak might represent the phosphorylated form of acetyl-CoA carboxylase, which either had a low specific activity or was inactive but associated with small amounts of the active form of acetyl-CoA carboxylase. Further chromatographic separation on either a second DEAE-cellulose column or Sepharose 2B column showed, however, that the acetyl-CoA carboxylase activity was separable from the ^{32}P-labeled protein peak and that this acetyl-CoA carboxylase was kinetically indistinguishable from the main peak of the enzyme. Furthermore, immunoprecipitation with an acetyl-CoA carboxylase-specific antibody * and biotin determinations [21] on the separated ^{32}P-labeled protein peak showed that it was unrelated to acetyl-CoA carboxylase. Instead, this protein peak was found to possess protein kinase activity and to be associated with an endogenous protein substrate. This kinase was purified to study some of its properties and to test the possibility of phosphorylation in vitro of acetyl-CoA carboxylase or other enzymes.

* Courtesy of Dr. P.W. Majerus, Washington University, St. Louis, Mo., U.S.A.

Properties of protein kinase

Unlike other protein kinases present in liver cytosol, the activity of this enzyme on casein or the endogenous substrate was unaffected by cyclic 3′,5′-AMP (Figs. 2 and 3). An endogenous substrate was tightly associated with the protein kinase and was eluted from Bio Gel A-0.5 m as a complex with an apparent molecular weight of 158 000. The same apparent molecular weight was obtained with Sephadex G-200 chromatography, with a similar coincidence of the kinase and endogenous phosphorylation activities (Fig. 7). However, if the Bio Gel A-0.5 m fraction was exhaustively autophosphorylated as detailed in the legend to Fig. 8 and then subjected to Bio Gel A-0.5 m column chromatography, the ^{32}P-labelled protein was at least partially dissociated from the kinase activity (Fig. 8). It is of interest that there was no change in the apparent molecular weight of the kinase component on dissociation from the endogenous phosphorylated substrate. The kinase, however, became unstable after this dissociation from the endogenous substrate. The same lability was found following cellulose phosphate chromatography (Fig. 5). The endogenous substrate was not eluted from the cellulose phosphate column whether phosphorylated or not, but the purified kinase fraction obtained was extremely labile even when

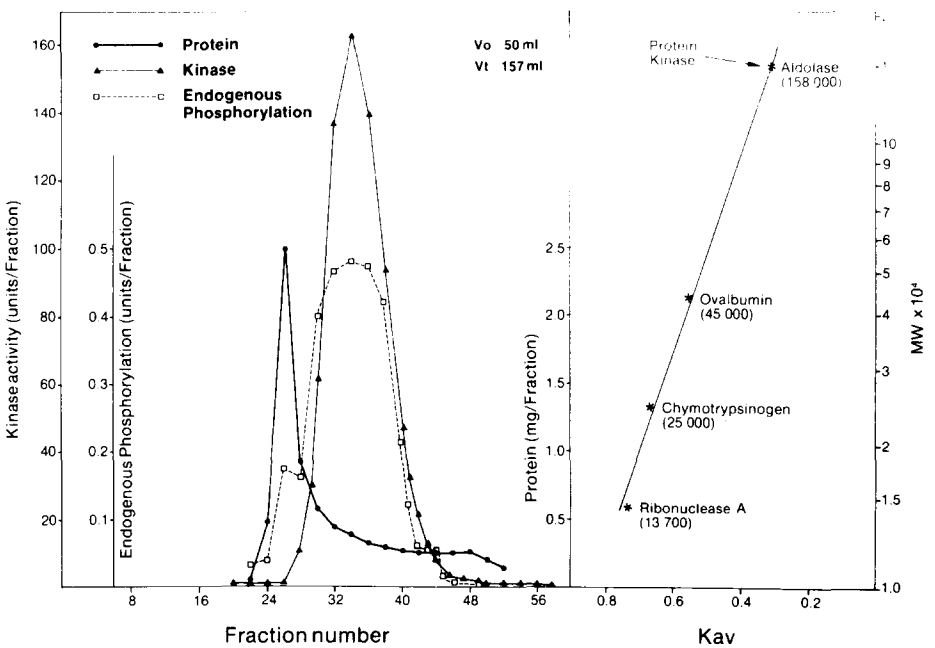

Fig. 7. Molecular weight determination of protein kinase on Sephadex G-200. The column (2.0 × 80 cm) was equilibrated with 0.05 M phosphate buffer (pH 7.5) containing 10 mM citrate and 0.5 M NaCl. Void volume was determined with blue dextran and total volume with ^3H_2O. The flow rate was adjusted to 29 ml/h. Protein kinase solution (2 ml) purified through the DEAE-cellulose stage, containing 42 mg of protein, 1391 units of kinase activity and 30.4 units of endogenous phosphorylation activity, was placed on the column. 2.5-ml fractions were collected and aliquots were assayed for protein kinase, endogenous phosphorylation and protein. The column was first calibrated with 10 mg each of chymotrypsinogen (M_r = 25 000) and aldolase (M_r = 158 000) in 2.0 ml buffer containing $1 \cdot 10^5$ cpm of ^3H_2O; 10 mg each of ribonuclease A (M_r = 13 700), and ovalbumin (M_r = 45 000) in 2.0 ml buffer were chromatographed separately for the determination of K_{av}.

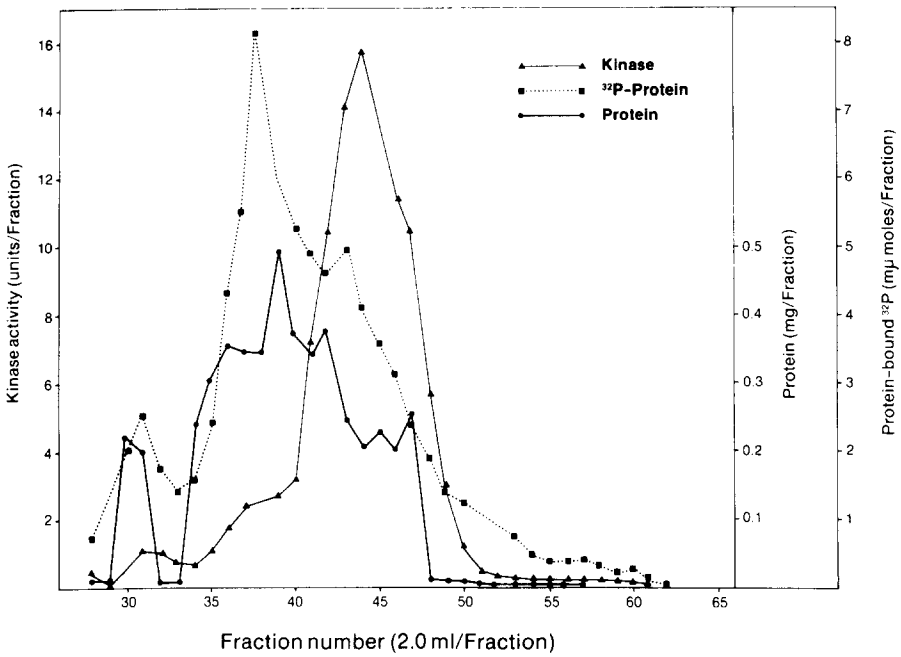

Fig. 8. Bio Gel A-0.5 m chromatography of kinase and endogenous phosphorylated phosphoprotein. A kinase fraction purified through the Bio Gel A-0.5 m stage, containing 6.35 mg protein, and 890 units of kinase activity, was incubated in a total volume of 10 ml of a mixture containing the following: 30 mM phosphate buffer (pH 7.5), 50 μM [γ-^{32}P]ATP, ($1.75 \cdot 10^7$ cpm/μmol), 30 mM $MgCl_2$, and 5 μM mercaptoethanol. The reaction was allowed to proceed at 34°C for 40 min, and 25-μl aliquots were taken at intervals for determination of protein-bound ^{32}P. After 40 min the mixture contained 19.7 nmol of bound ^{32}P per mg protein (125 nmol total). The solution was dialyzed for 3 days against several changes of 0.05 M phosphate buffer (pH 7.5), containing 0.5 M KCl and 10 mM citrate. The dialyzed material was concentrated by Amicon ultrafiltration to 4.5 ml and assayed for kinase activity (915 units total) and protein-bound ^{32}P (126 nmol total). It was then chromatographed on a Bio Gel A-0.5 m column (2 × 83 cm) under conditions identical to those in Fig. 4. Fractions were assayed for kinase, ^{32}P bound to protein, and protein. Void volume (V_0) and total volume (V_t) were 30 and 172 ml, respectively. Based on assays of individual fractions, a total of 79.5 nmol of protein-bound ^{32}P, 103.7 units of protein kinase and 3.9 mg of protein were recovered. After standing overnight at 0°C, however, pooling and Amicon concentration of the kinase-containing fractions (41—49) yielded only 12.8 units of total kinase activity.

stored under liquid nitrogen. Enzyme purified through the Bio Gel A-0.5 m step was therefore used for most of the experiments in this study.

Substrates

As shown in Table II, phosvitin gave rates of phosphorylation with this protein kinase about 2.0 times higher than did casein, and histone V a rate about two-thirds that of casein. Protamine and histone, VI and VIII-S were poorly phosphorylated (Table II). Phosphorylase *b*, lactate dehydrogenase, fructose-1,6-diphosphatase *, purified acetyl-CoA carboxylase, bovine serum albumin and collagen were not phosphorylated.

^{32}P from [α-^{32}P]ATP was not incorporated into either the endogenous sub-

* Courtesy of Dr. O. Tsolas, Roche Institute of Molecular Biology, Nutley, N.J., U.S.A.

TABLE II

PHOSPHORYLATION OF PROTEINS BY PROTEIN KINASE

Enzyme solution equivalent to 1.55 μg protein was present in each assay. Each substrate protein was present at 1.2 mg per 100 μl assay mixture. All other conditions as described under Experimental. The results are expressed as nmol of ^{32}P incorporated.

Substrate proteins	Control	Control plus cyclic 3′,5′-AMP	Control plus cyclic 3′,5′-GMP
None (endogenous phosphorylation)	0.068	0.070	0.086
Phosvitin	1.915	2.195	1.890
Casein	0.930	1.040	1.050
Histone III-S	0.140	0.180	0.180
Histone V	0.650	0.715	0.600
Histone VI	0.101	0.085	0.066
Histone VII	0.400	0.410	0.390
Histone VII-S	0.029	0.030	0.072
Protamine	0.049	0.048	0.032
Phosphorylase *b*	0.068	—	—

strate or into casein, thus eliminating the possibility that the entire nucleotide was bound and suggesting γ-^{32}P transfer. The ^{32}P bound to protein was not released during subsequent incubation at 30°C with 0.25 M HCl for 19 h or by treatment with cold or hot trichloroacetic acid. Similar incubation with 0.25 M NaOH resulted in complete release of the protein-bound ^{32}P. Treatment with 0.1 M hydroxylamine at pH 7.0, for 10 min at 30°C, released less than 5% of the protein-bound ^{32}P. These results suggest that the phosphoryl moiety was attached to the protein in an ester linkage to serine or threonine.

The effects of other nucleotides and nucleosides on the standard kinase reaction with casein and phosvitin were studied. 100 μM of ADP, GDP, GTP and ITP reduced the rate of ^{32}P incorporation by approx. 60, 40, 35 and 30%, respectively. It was not clear from these experiments whether the apparently lower rates were due to a transfer of ^{32}P from protein to ADP or GDP or to direct inhibitory effect on the kinase. GTP and ITP may compete for ATP as substrates, although not favorably.

Effect of metals, EDTA, citrate, EGTA, and NaF on kinase activity

This kinase had an absolute requirement for Mg^{2+}. At concentrations ranging from 6—60 mM, neither Mn^{2+} nor Zn^{2+} could replace Mg^{2+} in the phosphorylation reaction using either the endogenous substrate or casein. Similarly, low concentrations of Ca^{2+} (0.5—5.0 mM) had no effect on kinase activity; higher concentrations were inhibitory. At concentrations of 0.1—15 mM EGTA, which is known to inhibit phosphorylase kinase by binding Ca^{2+} [22], had no effect on this protein kinase. This observation, in conjunction with the inability of the enzyme to phosphorylate phosphorylase *b*, eliminates the possibility that it is phosphorylase kinase.

Co^{2+} had little effect at 0.2 mM, but at 2.5 mM caused 40% inhibition of kinase activity. Potassium citrate and EDTA were without effect at concentrations up to 20 mM. NaF at 10 and 20 mM inhibited kinase activity 50 and 75%, respectively.

Cyclic 3',5'-AMP independence of protein kinase activity

Cyclic 3',5'-AMP at concentrations ranging from 10^{-4}—10^{-8} M had no effect on either endogenous phosphorylation or the phosphorylation of casein, phosvitin and histones with ATP concentrations ranging from 50—500 μM (Table II). Furthermore, protein kinase inhibitor at concentrations of 0.1—1.0 mg/100 μl assay mixture had no inhibitory effect. In fact, there was a 2-fold increase in the endogenous phosphorylation in the presence of the protein kinase inhibitor. Addition of the regulatory subunit of the cyclic 3',5'-AMP-dependent protein kinase from cardiac muscle * at concentrations of 3.5—7 μg per 100 μl assay mixture was without effect on kinase activity, eliminating the possibility that we were dealing with a catalytic subunit of a cyclic 3',5'-AMP-dependent protein kinase.

Kinetic parameters

Double reciprocal plots of initial rates of casein phosphorylation by this kinase for varying concentrations of Mg^{2+} (0.3—20 mM) showed a K_m of 2.0 mM for Mg^{2+} under standard assay conditions. Higher concentrations of Mg^{2+} were inhibitory, 30 and 60 mM causing 50 and 90% inhibition, respectively. Similarly the K_m determined for ATP was 0.3 mM and for casein 0.17 mg/100 μl assay mixture.

Effects of pH and temperature

Casein phosphorylation was optimal at pH 5.7. Phosphorylation of the endogenous substrate was evident over a pH range 4—8, with activity at pH 4 75% of that observed at the optimal pH 6.3.

The optimal temperature for phosphorylation of the endogenous substrate was 35°C; higher temperatures up to 46°C caused some reduction of phosphorylation. There was no change in the rate of phosphorylation of casein or phosvitin at temperatures ranging from 37—46°C.

Effects of thiol reagents

Alkylating, mercaptide-forming, or oxidizing thiol reagents had no effect on protein kinase activity at concentrations 1—100 μM. Even at higher concentrations iodoacetamide (12.5 mM), *N*-ethylmaleimide (12.5 mM), *p*-hydroxymercuribenzoate (1.0 mM) and 5,5'-dithio-bis(2-nitrobenzoic acid) (1.0 mM) had little effect on enzyme activity.

Discussion

The multiplicity of cyclic 3',5'-AMP-dependent protein kinases from rat liver cytosol is evident from the work of Kumon et al. [23] and Chen and Walsh [24]. The former have also purified a cyclic 3',5'-AMP-independent kinase which, in the absence of cyclic 3',5'-AMP, was inhibited by the regulatory subunit of a cyclic 3',5'-AMP-dependent kinase [23].

The enzyme described in this report was unaffected by the regulatory subunit protein purified from cardiac muscle or by protein kinase inhibitor, suggesting that it was not a catalytic subunit of a cyclic 3',5'-AMP-dependent pro-

* Courtesy of Dr. O.M. Rosen, Albert Einstein College of Medicine, Bronx, N.Y., U.S.A.

tein kinase. Our enzyme preparation was not activated by Ca^{2+} or inhibited by EGTA [22], nor could it phosphorylate phosphorylase *b*; it was therefore unrelated to phosphorylase kinase *b*. This protein kinase differs from the phosvitin kinase purified by Goldstein and Hasty [25] from rooster liver in the relative rates of phosvitin and casein phosphorylation, the K_m for ATP and its inability to utilize GTP as a phosphoryl donor. A phosvitin kinase purified from rat liver cytosol by Baggio et al. [26] has not been characterized for comparison to our preparation.

At present the function of this hepatic kinase is unknown. Schlender and Reimann [27] have recently reported on a glycogen synthetase I kinase independent of cyclic 3′,5′-AMP which was isolated from rabbit kidney medulla. Huang and his co-workers [28] have also pointed out that muscle glycogen synthetase was phosphorylated more extensively when phosphorylation by a cyclic 3′,5′-AMP-independent kinase preceded the phosphorylation by a cyclic 3′,5′-AMP-dependent kinase. The hepatic kinase preparation described here may play a role in the phosphorylation of glycogen synthetase I.

The phosphorylation of the endogenous protein was of interest. This protein was inseparable from the protein kinase activity at several steps of the purification procedure and co-precipitated with it during electrofocusing at pH 4.8 (data now shown). Phosphorylation of this protein proceeded rapidly (even at 0°C). The protein-bound phosphate formed is not acid labile and therefore was unlike the phosphates of intermediate phosphoryl enzymes such as citrate lyase (EC 4.1.3.8) or nucleoside diphosphate kinase (EC 2.7.4.6) [29]. There are many proteins, in a variety of tissues, whose endogenous phosphorylation and dephosphorylation are dependent or independent of cyclic 3′,5′-AMP [30—32]. Increased as well as decreased phosphorylation of endogenous substrates has been observed following the addition of cyclic 3′,5′-AMP. The phosphorylation of the endogenous protein associated with the hepatic kinase preparation was not affected by cyclic 3′,5′-AMP. This phosphoprotein has not yet been identified and its role in the liver remains unknown. Several possibilities are open to investigation: it may be a regulatory subunit of a cyclic 3′,5′-AMP-dependent kinase and as such it should bind cyclic 3′,5′-AMP. It may be a regulatory subunit of a different enzyme or it may itself possess some as yet unidentified enzymatic activity. The function of this protein, whatever it may be, may depend on whether it is phosphorylated or dephosphorylated. We do not know whether it is present in other tissues, or whether the levels in liver vary under different nutritional conditions.

It seems quite certain that the endogenous phosphoprotein was essential to the stability of the protein kinase. The enzyme was remarkably stable at 0°C for weeks when it was not dissociated from its endogenous substrate. Following dissociation, the protein kinase became extremely labile even when stored in liquid N_2; other compounds such as ATP, casein, phosvitin, serum albumin, or sucrose could not substitute for this protein in stabilizing the enzyme.

Phosphorylation of the endogenous protein favored dissociation from the protein kinase to which it was otherwise firmly bound. This dissociation may be of physiological significance in terms of making binding sites on the protein kinase available to other enzyme protein(s) whose activity might be regulated by phosphorylation.

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