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Comparison of Catalytic Units of Muscle and Liver Adenosine 3′,5′-Monophosphate Dependent Protein Kinases†

Hirohei Yamamura, Kaoru Nishiyama,*‡ Reiko Shimomura,§ and Yasutomi Nishizuka

ABSTRACT: Rabbit skeletal muscle adenosine 3′,5′-monophosphate (cAMP) dependent protein kinase is resolved into two fractions by ammonium sulfate fractionation followed by DEAE-cellulose and hydroxylapatite column chromatography. These fractions are activated by cAMP in a similar manner resulting in the release of common active protein kinase. The muscle active kinase shows essentially identical kinetic and catalytic properties with rat liver active protein kinase which is prepared as described previously (Kumon, A., Nishiyama, K., Yamamura, H., and Nishizuka, Y. (1972), *J. Biol. Chem. 247*, 3726). Both muscle and liver protein kinases phosphorylate the same specific seryl and threonyl residues of histone and protamine, and are capable of phosphorylating muscle glycogen phosphorylase *b* kinase and glycogen synthetase resulting in the activation and inactivation of the respective enzymes. The active kinases and regulatory proteins obtained from these tissues are crosswise reactive, and either one of these active kinases is inhibited by regulatory protein from the homologous as well as from the heterologous tissue. Upon isoelectrofocusing electrophoresis either muscle or liver active kinase is resolved further into two components of isoelectric points of pH 7.4 and 8.2. Nevertheless, each component is catalytically active and indistinguishable from the other in kinetic and catalytic properties. The two components show apparently the same molecular weights as estimated by gel filtration procedure. The exact nature of this microheterogeneity has remained unexplored.

A preceding report from this laboratory (Kumon *et al.*, 1972) has described that in rat liver multiple cAMP[1]-dependent protein kinases may be distinguished which differ from each other in their associated R proteins, and that cAMP activates all kinases by binding to R proteins in an allosteric manner and then releases apparently common active protein kinase. A preliminary survey has shown that at least two cAMP-dependent protein kinases may be obtained from most tissues and organs including rabbit skeletal muscle, rat brain, and bovine adrenal gland (Yamamura *et al.*, 1971a). Reimann *et al.* (1971a) have also described that two cAMP-dependent protein kinases obtained from rabbit skeletal muscle yield one type of active protein kinase upon sucrose density gradient centrifugation in the presence of the cyclic nucleotide. Comparative studies have revealed that active protein kinases obtained from various mammalian tissues including rabbit skeletal muscle and rat liver show closely similar kinetic and catalytic properties. The active kinases exhibit rather broad and identical substrate specificities, and incorporate the terminal phosphate of ATP into several regulatory enzymes and functional proteins. A plausible evidence may imply, at least in part, a molecular basis of pleiotropic action of cAMP in controlling several biochemical reactions in each tissue. The present paper is a full account for the preliminary report published earlier (Yamamura *et al.*, 1971b) and will describe the experiments of such comparison and cross-reactions of rabbit skeletal muscle and rat liver cAMP-dependent protein kinases as representatives.

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[1] Abbreviations used are: cAMP, adenosine 3′,5′-monophosphate: R protein, regulatory protein.

Experimental Section

Materials and Chemicals. Wistar albino rats (200–250 g) and domestic rabbits (3–3.5 kg) maintaining *ad libitum* on

CLEA laboratory chows were employed for the present studies. Calf thymus histone was prepared by the method of Johns (1967). Rat liver ribosomes were prepared as described by Honjo *et al.* (1971). Rabbit skeletal muscle glycogen phosphorylase *b* was prepared and recrystallized five times by the method of Fischer and Krebs (1962). This preparation was treated with charcoal to remove 5′-AMP before use. Rabbit skeletal muscle glycogen phosphorylase *b* kinase was prepared by the method of Krebs (1966). Rabbit skeletal muscle glycogen synthetase was prepared by the method of Villar-Palasi *et al.* (1966). These enzyme preparations were practically free of each other and of other interfering enzymes and proteins.

Human fibrinogen was kindly donated by Dr. S. Iwanaga, the Protein Research Institute, Osaka University. Salmon sperm protamine (lot 26B 8060), trypsin (type III), bovine serum albumin, and horse heart cytochrome *c* (type III) were obtained from Sigma. Bovine casein (Hammarsten) was purchased from Merck AG-Darmstadt. Egg yolk phosvitin was obtained from Mann. Human γ-globulin (fraction II) and ovalbumin (twice recrystallized) were obtained from Nutritional Biochemicals.

[γ-^{32}P]ATP was prepared by the method of Glynn and Chappel (1964). UDP-[U-^{14}C]glucose (227 Ci/mol) was a product of Radiochemical Center. Cyclic [^{3}H]AMP (4.47 Ci/mmol) was a product of New England Nuclear. The radiochemical purity of these compounds was examined by thin-layer chromatography before use. Other chemicals were obtained from commercial sources.

Rat Liver Protein Kinases. Two fractions of rat liver cAMP-dependent protein kinase (liver protein kinase B_1 and B_2) and one active protein kinase independent of the cyclic nucleotide (liver protein kinase B_0) were partially purified from the soluble fraction prepared in the presence of diisopropyl fluorophosphate by ammonium sulfate fractionation followed by DEAE-Sephadex and hydroxylapatite column chromatography as described previously (Kumon *et al.*, 1972). Both protein kinase B_1 and B_2 were shown to be dissociated by cAMP into common active protein kinase and different R proteins, and the latter active kinase was identified as protein kinase B_0 (Kumon *et al.*, 1972).

Rat Liver R Protein. A rat liver R protein was prepared from the soluble fraction by ammonium sulfate fractionation followed by calcium phosphate gel treatment, DEAE-cellulose, and hydroxylapatite column chromatography as described previously (Kumon *et al.*, 1972). The R protein prepared in this manner is the cAMP binding protein originally associated with protein kinase B_1. This preparation was essentially free of protein kinase and could bind approximately 50 pmol of cAMP/mg of protein. The R protein associated with protein kinase B_2 appears to be unstable and decomposed to a smaller inactive protein.

Assay of Protein Kinase. Protein kinase activity was assayed by measuring the radioactivity of [γ-^{32}P]ATP incorporated into an acid-precipitable material using a glass-fiber filter (Whatman GF 83) in the presence and absence of 4 μM cAMP as described previously (Kumon *et al.*, 1972). The specific activity of [γ-^{32}P]ATP employed was 30–80 × 10^3 cpm/nmol, and 100 μg of calf thymus whole histone was used as phosphate acceptor.

Assay of cAMP Binding Protein. cAMP binding protein was assayed by measuring the binding of cyclic [^{3}H]AMP using a Millipore filter (pore size, 0.45 μ) as described previously (Kumon *et al.*, 1972).

Assay of Activation of Glycogen Phosphorylase. The reaction mixture (0.10 ml) contained 2.5 μmol of Tris-maleate at pH 6.4, 0.5 μmol of magnesium acetate, 10 nmol of ATP, 8.4 μg of crystalline muscle glycogen phosphorylase *b*, 0.8 μg of muscle glycogen phosphorylase *b* kinase, and protein kinase. After incubation for 10 min at 30°, 0.03 ml of a solution containing 1.0 mg of glycogen and 0.5 μmol of $^{32}P_i$ (320 cpm/nmol) was added. The mixture was incubated for additional 15 min at 30°. Then, $^{32}P_i$ was precipitated as a triethylamine–phosphomolybdate complex by the method of Sugino and Miyoshi (1964), and the radioactive organic phosphate, namely, glucose 1-phosphate, remaining in the supernatant was determined after centrifugation for 10 min at 4000*g*.

Assay of Phosphorylation of Glycogen Phosphorylase. The reaction mixture (0.25 ml) contained 10 μmol of Tris-Cl at pH 7.0, 1 μmol of magnesium acetate, 2.5 nmol of [γ-^{32}P]ATP (6×10^4 cpm per nmol), 84 μg of crystalline muscle glycogen phosphorylase *b*, 1.4 μg of muscle glycogen phosphorylase *b* kinase, and protein kinase. After incubation for 10 min at 30°, the reaction was stopped by the addition of 10 ml of 10% trichloroacetic acid, and the radioactive acid-precipitable material was collected on a Millipore filter (pore size, 0.45 μ).

Assay of Conversion of I form to D Form of Glycogen Synthetase. The reaction mixture (0.06 ml) initially contained 2 μmol of Tris-Cl at pH 7.5, 0.2 μmol of magnesium acetate, 10 nmol of ATP, 2.5 μg of muscle glycogen synthetase, and protein kinase. After incubation for 10 min at 30°, 0.06 ml of a solution, containing 1 μmol of UDP-[U-^{14}C]glucose (7500 cpm/μmol), 0.5 μmol of EDTA, 3 μmol of Tris-Cl at pH 8.0, 1 mg of glycogen, was added and the mixture was incubated for additional 10 min at 30°. Another reaction was carried out with 1 μmol of glucose 6-phosphate as an additional ingredient in order to assay the total activity (I and D forms) of glycogen synthetase. The reaction was finally stopped by the addition of 10 ml of 75% ethanol, and the radioactive precipitate was collected on a glass-fiber filter (Whatman GF 83).

Determinations. The radioactivity of ^{32}P samples was determined using a Nuclear-Chicago Geiger Muller gas-flow counter, Model 4338, and that of ^{3}H and ^{14}C samples was determined using a Packard Tri-Carb liquid scintillation spectrometer, Model 3320, with Bray's solution (Bray, 1960). Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

Estimation of Molecular Weight. Molecular weight of the proteins was estimated by gel filtration on a Sephadex G-75 column (93 × 2.5 cm) by the method of Andrews (1964). Elution was performed upward at a flow rate of 3 ml/hr. The proteins used as standards were human γ-globulin, ovalbumin, and horse heart cytochrome *c*.

Tryptic Digestion and Acid Hydrolysis of Histone. Tryptic digestion and acid hydrolysis of enzymically fully phosphorylated radioactive histone preparations were carried out under the conditions specified previously (Kumon *et al.*, 1972).

Paper Chromatography and Paper Electrophoresis. Ascending paper chromatography and high-voltage paper electrophoresis were carried out on Toyo 51 filter paper under the conditions described previously (Kumon *et al.*, 1972).

Isoelectrofocusing electrophoresis was performed by the method described by Svensson (1961) using a 110-ml column (LKB Instruments) maintained at 4°. The pH gradient (pH 3–10) was established during electrophoresis following the sequential addition of ampholyte solutions (0.8% w/v) in a 0–48% (w/v) sucrose gradient. The protein sample was applied in the central ampholyte solution. Electrophoresis was con-

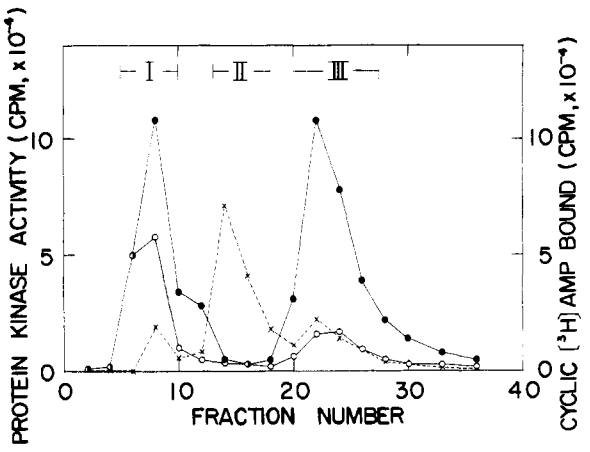

FIGURE 1: DEAE-cellulose column chromatography of rabbit skeletal muscle protein kinases. The enzyme solution (250 mg of protein, see text) was applied on a DEAE-cellulose column. Detailed experimental conditions were described in the text. (●,○), Protein kinase activity with and without cAMP (4 μM), respectively; (×) radioactivity of cyclic [³H]AMP bound.

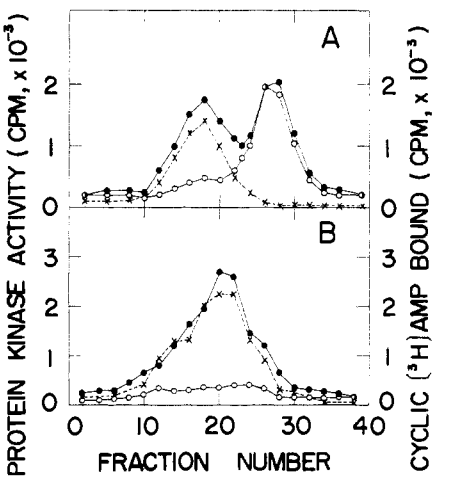

FIGURE 2: Separation of muscle protein kinase B_0, B_1, and B_2 by hydroxylapatite column chromatography. Detailed experimental conditions were described in the text. (A) Fraction I in Figure 1; (B) fraction III in Figure 1. (●,○) Protein kinase activity with and without cAMP (4 μM), respectively; (×) radioactivity of cyclic [³H]AMP bound.

tinued at 900 V over a period of 42 hr for equilibration. Fractions of 1.8 ml each were collected.

Autoradiography. An X-ray film, Type IX, Fuji Photo Film Co., was exposed for about 14 hr to the filter paper under test.

Results

Purification and Characterization of Rabbit Skeletal Muscle cAMP-Dependent Protein Kinases. PURIFICATION OF MUSCLE PROTEIN KINASES. A rabbit was sacrificed by exsanguination, and skeletal muscle was quickly removed and chilled in ice. All subsequent manipulations were performed at 0–4°. The muscle was minced and homogenized with a Waring blender with six volumes of 0.05 M Tris-Cl (pH 8.2), containing 5 mM EDTA and 6 mM 2-mercaptoethanol. The homogenate was centrifuged for 20 min at 20,000*g*. To the supernatant solution solid ammonium sulfate was added to a final concentration of 50% (35 g/100 ml). After centrifugation for 20 min at 20,000*g*, the precipitate was dissolved in a small quantity of 0.05 M Tris-Cl at pH 8.2 containing 5 mM EDTA and 6 mM 2-mercaptoethanol. The solution was dialyzed overnight against a large volume of 2 mM EDTA at pH 8.0 and was lyophilized. The powder was stored at −20°. The powder (1.2 g) was stirred for 30 min with 50 ml of 0.05 M Tris-Cl at pH 7.8 containing 5 mM EDTA and 0.05 M 2-mercaptoethanol, and the suspension was centrifuged for 10 min at 20,000*g*, and further for 3 hr at 65,000*g* with a Beckman L_3 ultracentrifuge. The clear supernatant solution was applied to a DEAE-cellulose (DE-52) column (15 × 2 cm) equilibrated with 0.02 M Tris-Cl at pH 7.5 containing 6 mM 2-mercaptoethanol. Elution was carried out with a 400-ml linear concentration gradient from 0 to 0.4 M NaCl in the same buffer, and fractions of 10 ml each were collected. When each fraction was assayed for both protein kinase activity and cAMP binding protein, several peaks of activity appeared as shown in Figure 1.

The protein kinase in fraction I (Figure 1, tubes 5–10) was applied directly to a hydroxylapatite column (3 × 1.5 cm) equilibrated with 0.03 M potassium phosphate at pH 7.5 containing 6 mM 2-mercaptoethanol. After the column was washed with 30 ml of the same buffer, elution was performed with a linear concentration gradient of potassium phosphate. The mixing chamber and reservoir each contained initially 80 ml of, respectively, 0.03 and 0.25 M potassium phosphate at pH 7.5 containing 6 mM 2-mercaptoethanol. When fractions of 4 ml each were collected and assayed for the kinase activity, two enzyme peaks appeared as shown in Figure 2A. The enzyme in the first peak was stimulated greatly by the addition of cAMP, whereas the enzyme in the second peak did not respond to the cyclic nucleotide. These enzymes are referred to as muscle protein kinase B_1 and B_0, respectively. Similarly, the protein kinase in fraction III (Figure 1, tubes 20–27) was chromatographed on a hydroxylapatite column under the same conditions as described for fraction I. As shown in Figure 2B, the enzyme was eluted from the column as a rather broad peak which did not coincide in its elution pattern with either protein kinase B_0 or B_1. This enzyme was stimulated by cAMP, and little activity was found in the absence of the cyclic nucleotide. The enzyme is referred to as muscle protein kinase B_2. When each fraction was assayed for the capacity to bind cAMP, protein kinase B_1 and B_2 but not protein kinase B_0 was able to do so. These enzyme preparations were dialyzed overnight against a large volume of 0.02 M Tris-Cl at pH 7.5 containing 6 mM 2-mercaptoethanol. The specific activities of protein kinase B_0, B_1, and B_2 increased by the above procedure about 250-, 20-, and 70-fold, respectively, starting from the 65,000*g* supernatant and with an overall yield of approximately 30% of the original total activity. In comparison to the fractionation procedure described by Reimann *et al.* (1971a) protein kinase B_1 and B_2 are probably identical with their fraction I and II, respectively.

PURIFICATION OF MUSCLE R PROTEIN. Fraction II (Figure 1, tubes 13–18) was applied to a hydroxylapatite column (3 × 1.5 cm) equilibrated with 10 mM potassium phosphate at pH 7.5, containing 6 mM 2-mercaptoethanol. After the column was washed with 30 ml of the same buffer, R protein was eluted as a sharp peak with 0.03 M potassium phosphate (pH 7.5), containing 6 mM 2-mercaptoethanol, and was dialyzed overnight against a large volume of 0.02 M Tris-Cl at pH 7.5

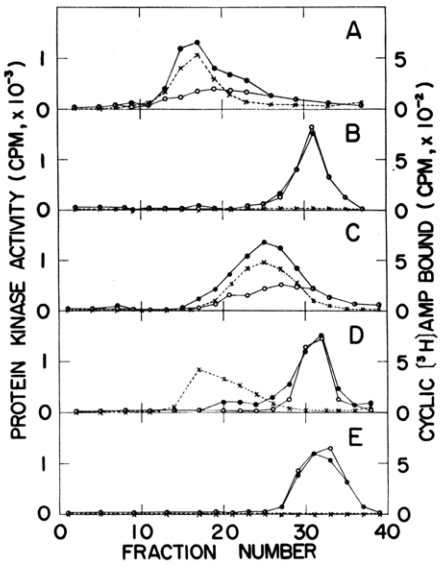

FIGURE 3: Hydroxylapatite column chromatography of rabbit skeletal muscle protein kinases in the presence and absence of cAMP. Each enzyme solution was applied to a hydroxylapatite column (diameter, 1.5 cm; length, 3 cm) equilibrated with 0.03 M potassium phosphate at pH 7.5 containing 6 mM 2-mercaptoethanol. Elution was carried out with a 120-ml linear concentration gradient from 0.03 to 0.25 M potassium phosphate at pH 7.5 containing 6 mM 2-mercaptoethanol. Fractions (3 ml each) were collected and assayed for both kinase activity and binding activity for cAMP. In expt B and D, each fraction was dialyzed extensively against a large volume of 0.02 M Tris-Cl at pH 7.5 containing 6 mM 2-mercaptoethanol and 10% glycerol to remove cAMP before being assayed. Solid and open circles represent protein kinase activity in the presence and absence of cAMP (1 μM), respectively. Dotted line indicates the radioactivity of cyclic [^{3}H]AMP bound. (A) Protein kinase B_1; (B) protein kinase B_1 in the presence of 1 μM cAMP; (C) protein kinase B_2; (D) protein kinase B_2 in the presence of 1 μM cAMP; (E) protein kinase B_0.

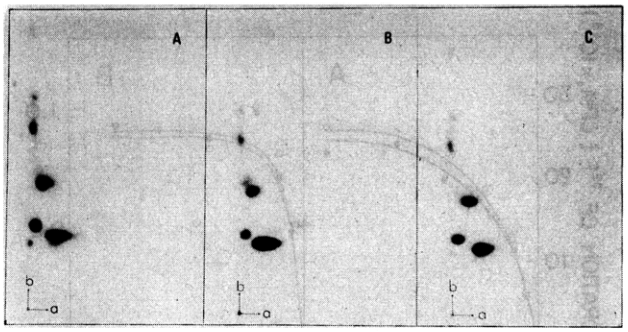

FIGURE 4: Autoradiography of the tryptic digests of radioactive histone preparations fully phosphorylated by muscle protein kinase B_0, B_1, and B_2. The tryptic digestion, paper chromatography and high-voltage paper electrophoresis were carried out under the conditions described under Experimental Section. (A) Protein kinase B_0; (B) protein kinase B_1; (C) protein kinase B_2.

containing 6 mM 2-mercaptoethanol. The R-protein preparation thus obtained was essentially free of protein kinase and could bind approximately 80 pmol of cAMP/mg of protein.

EFFECT OF cAMP ON MUSCLE PROTEIN KINASE B_1 AND B_2. Muscle protein kinase B_0, B_1, and B_2 exhibited identical substrate specificities and similar kinetic properties. The K_a values for cAMP of protein kinase B_1 and B_2 were estimated to be 6×10^{-9} and 8×10^{-9} M, respectively. The experiments diagrammed in Figure 3 were designed to show whether cAMP converted muscle protein kinase B_1 and B_2 to active protein kinases. When protein kinase B_1 was again chromatographed on a hydroxylapatite column, the enzyme was recovered as a single peak which was stimulated greatly by cAMP; the activity of binding cyclic AMP was found to coincide exactly with the kinase activity (Figure 3A). If, however, the same preparation was chromatographed on the column in the presence of the cyclic nucleotide (1 μM), the peak of kinase shifted considerably and appeared in the fraction corresponding to protein kinase B_0 (Figure 3B). Under these conditions the cAMP binding protein originally associated with protein kinase B_1 disappeared and remained adsorbed on this column. A similar set of experiments with protein kinase B_2 indicated that the enzyme was also recovered in the fraction corresponding to protein kinase B_0 when chromatographed in the presence of cAMP (Figure 3C,D). Figure 3E shows protein kinase B_0 chromatographed on the same column in the absence of cAMP; in the presence of the cyclic nucleotide this protein kinase gave an identical elution profile with that illustrated in Figure 3E.

PROPERTIES OF MUSCLE ACTIVE PROTEIN KINASES. The active protein kinases thus produced from muscle protein kinase B_1 and B_2 were indistinguishable from protein kinase B_0 by several criteria. None of the three active kinases bound or responded to cAMP. These kinases were inactivated rapidly in an exactly identical manner upon heat treatment at 50°, whereas both protein kinase B_1 and B_2 were stable. With a limited amount of histone as substrate, phosphate was incorporated up to the same level with either one of these active protein kinases. These kinases were shown to phosphorylate the same specific sites of histone as judged by the fingerprint technique. Calf thymus whole histone was fully phosphorylated separately with these kinases. The radioactive histone preparations were subjected to tryptic digestion and, subsequently, to paper chromatography followed by high-voltage paper electrophoresis. As illustrated in Figure 4, an identical pattern of radioactive peptides was obtained for each active protein kinase. These results indicate that in rabbit skeletal muscle, as has been described for rat liver (Kumon *et al.*, 1972), apparently common active protein kinase is present in at least two cAMP-dependent forms which differ from each other in their associated R proteins.

Comparison and Cross-reactions of Muscle and Liver Protein Kinases. The active protein kinase obtained from rabbit skeletal muscle as described above exhibited closely similar kinetic and catalytic properties to that obtained from rat liver. In the experiments described below muscle and liver protein kinase B_0 were mainly used for comparison unless otherwise specified.

PHOSPHATE ACCEPTOR PROTEINS. Muscle and liver protein kinase B_0 showed essentially the same substrate specificities, and phosphorylated preferentially histone. Salmon sperm protamine, bovine casein, and rat liver ribosomal proteins were approximately 12, 3, and 3%, respectively, as active in reaction rates as calf thymus histone. In addition, these kinases were equally active in phosphorylating rabbit skeletal muscle glycogen phosphorylase *b* kinase and glycogen synthetase as described below. Bovine serum albumin, ovalbumin, egg yolk phosvitin, human γ-globulin, and fibrinogen were inert as phosphate acceptors.

SITES OF PHOSPHORYLATION. Both muscle and liver protein kinase B_0 incorporated the terminal phosphate of ATP into preferentially seryl and some threonyl residues of various

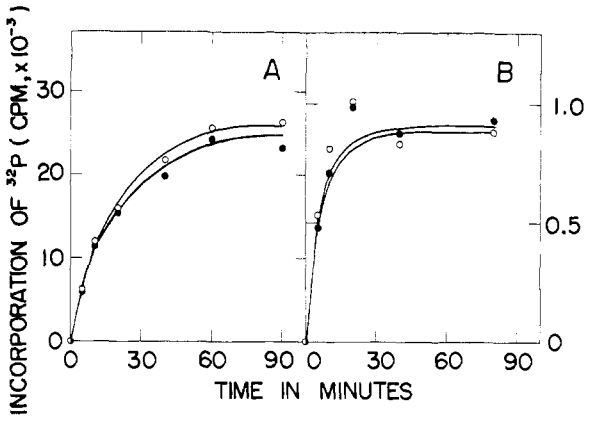

FIGURE 5: Phosphorylation of calf thymus histone and rat liver ribosomes with rabbit skeletal muscle and rat liver protein kinases. The reaction mixture (0.25 ml) contained 12 μmol of Tris-Cl at pH 7.0, 3 μmol of $MgCl_2$, 2.5 nmol of [γ-^{32}P]ATP (6×10^4 cpm/nmol), 1 nmol of cAMP, either 100 μg of calf thymus histone (A) or 400 μg of rat liver ribosomes (B), and 3 μg of either rabbit skeletal muscle or rat liver protein kinases B_0. Incubation was carried out at 30°. The acid-precipitable radioactivity was determined using a Millipore filter. (●,○) Rabbit skeletal muscle and rat liver protein kinase B_0, respectively.

TABLE 1: Effects of Rat Liver and Rabbit Skeletal Muscle Protein Kinases on Phosphorylation and Activation of Rabbit Skeletal Muscle Glycogen Phosphorylase *b* Kinase.[a]

Protein Kinase	Phosphorylase *b* Kinase	Phosphorylase *b*	$^{32}P_i$ Incorp into Protein Fraction (cpm)	$^{32}P_i$ Incorp into Glucose 1-Phosphate (cpm)
None	+	+	533	836
	−	+	0	0
	+	−	0	0
	−	−	0	0
Rat liver	+	−	1824	3510
	−	+	312	174
	+	−	180	167
	−	−	0	0
Rabbit skeletal muscle	+	+	1717	3495
	−	+	0	0
	+	−	13	0
	−	−	0	0

[a] Detailed experimental conditions were described under Experimental Section. Where indicated rat liver protein kinase B_0 (14 μg) and rabbit skeletal muscle protein kinase B_0 (3 μg) were added. The specific activity of muscle protein kinase B_0 employed for the experiments was 4.5 times higher than that of liver protein kinase B_0 with calf thymus histone as substrate.

acceptor proteins. For example, acid hydrolysis of the histone preparation phosphorylated by each protein kinase resulted in the formation of mainly phosphoserine, the amount of phosphothreonine produced was less than 3% of that of phosphoserine. With a limited amount of histone as substrate, phosphate was incorporated up to the same level with each protein kinase (Figure 5A). A similar result was obtained with rat liver ribosomes (Figure 5B). These protein kinases were shown to phosphorylate the same specific sites of histone as judged by the fingerprint procedure (also see Figure 8).

ACTIVATION OF GLYCOGEN PHOSPHORYLASE *b* KINASE. Rabbit skeletal muscle glycogen phosphorylase *b* kinase was shown first by Walsh *et al.* (1968) to be activated through phosphorylation by cAMP-dependent protein kinase obtained from the same tissue. In order to obtain additional evidence for the functional similarity of muscle and liver protein kinases, experiments were performed to ascertain whether rat liver protein kinase B_0 was crosswise reactive with muscle glycogen phosphorylase *b* kinase. The results summarized in Table I indicate that muscle as well as liver protein kinase B_0 phosphorylated glycogen phosphorylase *b* kinase, resulting in the consecutive activation of glycogen phosphorylase as judged by the incorporation of the terminal phosphate of ATP into an acid-precipitable material, and also by the phosphorylase activity in the absence of 5′-AMP. Some activities observed in the absence of protein kinase in this experiment appeared to be due to autocatalytic phosphorylation of glycogen phosphorylase *b* kinase as proposed by Krebs (1966), or due to the activated form of phosphorylase kinase which slightly contaminated the preparation.

INHIBITION OF GLYCOGEN SYNTHETASE. Yip and Larner (1969) reported that muscle and liver glycogen synthetase kinase reacted equally with muscle as well as liver glycogen synthetase. Soderling *et al.* (1970) and Villar-Palasi and Schlender (1970) proposed that glycogen phosphorylase *b* kinase kinase was identical with glycogen synthetase kinase in rabbit skeletal muscle. In confirmation of these observations, both muscle and liver protein kinase B_0 equally phosphorylated muscle glycogen synthetase, resulting in the conversion of I form to D form as shown in Figure 6.

CROSS-REACTION OF PROTEIN KINASE B_0 AND R PROTEIN. The active protein kinases and R proteins obtained from rabbit skeletal muscle and rat liver were crosswise reactive, and the activity of either one of these kinases was inhibited progressively by the addition of increasing amounts of R protein obtained from the homologous as well as from the heterologous tissue as shown in Figure 7. This inhibition was completely overcome by cAMP which resulted in full restoration of the original kinase activity. Each R-protein preparation *per se* was essentially free of enzymic activity.

COMPARISON OF OTHER PROPERTIES. Muscle and liver protein kinase B_0 showed closely similar kinetic properties. The apparent K_m value for ATP was 5×10^{-6} M in the presence of 3 mM $MgCl_2$ for both kinases as calculated by double-reciprocal plots of ATP concentration against histone phosphorylation. The maximum activity of these kinases was observed at pH 7 in 20 mM Tris-Cl. Both kinases were rather unstable and rapidly inactivated at 50° with an approximate half-life of 2 min.

Analysis with gel filtration on a Sephadex G-75 column revealed that muscle and liver protein kinase B_0 were indistinguishable and recovered in the same fraction. The molecular weight of these kinases was estimated to be about 3.5×10^4 under the conditions described under Experimental Section. A previous paper (Kumon *et al.*, 1972) proposed that molecular weight of liver protein kinase B_0 was about

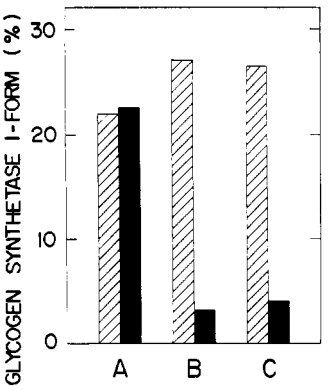

FIGURE 6: Conversion of I form to D form of rabbit skeletal muscle glycogen synthetase by rabbit skeletal muscle and rat liver protein kinases. Detailed experimental conditions were described under Experimental Section. Solid and shaded bars represent experimental values with and without ATP, respectively. The specific activity of muscle protein kinase B_0 employed here was 4.5 times higher than that of liver protein kinase B_0 with calf thymus histone as substrate. (A) Without protein kinase; (B) with rat liver protein kinase B_0 (14 μg); (C) with rabbit skeletal muscle protein kinase B_0 (3 μg).

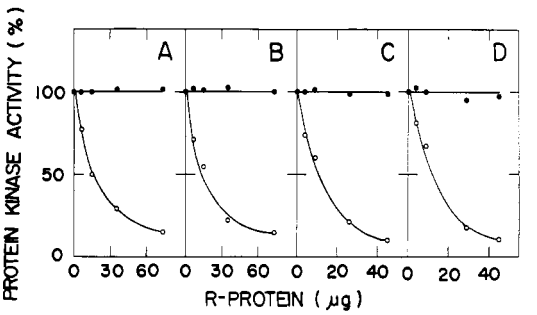

FIGURE 7: Effects of R protein and cAMP on protein kinases obtained from homologous and heterologous tissues. The R protein indicated was added to the assay mixture of active protein kinase from either rabbit skeletal muscle or from rat liver. (●,○) Protein kinase activity with and without cAMP (1 μM), respectively. (A) Rat liver protein kinase B_0 (0.62 μg) with rat liver R protein as indicated; (B) rabbit skeletal muscle protein kinase B_0 (0.65 μg) with rat liver R protein as indicated; (C) rat liver protein kinase B_0 (0.62 μg) with rabbit skeletal muscle R protein as indicated; (D) rabbit skeletal muscle protein kinase B_0 (0.65 μg) with rabbit skeletal muscle R protein as indicated.

3×10^4. Since the gel filtration procedure alone is not necessarily reliable, the exact molecular weight of the protein kinase will be reexamined using additional procedures after further purification of the enzymes.

ELECTROPHORETIC ANALYSIS OF ACTIVE PROTEIN KINASES. When muscle and liver protein kinase B_0 were subjected to isoelectrofocusing electrophoresis under the conditions specified under Experimental Section, each active kinase was resolved into two components of isoelectric points of pH 7.4 and 8.2 as shown in Figure 8. In the absence of cAMP muscle and liver protein kinase B_1 and B_2 were indistinguishable each having an isoelectric point of 5.2 under the same conditions. If, however, each preparation of muscle and liver protein kinase B_1 and B_2 was mixed with cAMP (1 μM) and then subjected to the electrophoresis, two components of isoelectric points of pH 7.4 and 8.2 were similarly obtained.

COMPARISON OF ELECTROPHORETICALLY DISTINCT TWO COMPONENTS OF ACTIVE PROTEIN KINASES. Comparative studies revealed that the two components derived from each active protein kinase showed essentially identical kinetic and catalytic properties, and incorporated the terminal phosphate of ATP into the same specific sites of histone and protamine as shown in Figure 9. In addition, both components were equally active in phosphorylating rabbit muscle glycogen phosphorylase *b* kinase as well as glycogen synthetase resulting in the activation and inactivation of the respective enzymes. These components exhibited essentially identical K_m value for ATP, heat stability, optimal pH, and optimal Mg^{2+} concentration. Both components were similarly inhibited by the addition of R protein from either muscle or liver, and the inhibition was completely overcome by cAMP.

The separate identity of the two components described above was demonstrated only by the electrophoretic procedure, and the chromatographic procedure itself was not responsible for the resolution of the two components. Each component obtained in this manner was subjected to repeated electrophoresis and usually obtained free of contamination of the other. Gel filtration on a Sephadex G-75 column was unable to separate the two components, and molecular weight of each component was estimated to be approximately 3.5×10^4.

Discussion

Two cAMP-dependent protein kinases have been obtained from various mammalian tissues such as rabbit reticulocyte (Tao *et al.*, 1970), bovine adrenal gland (Gill and Garren, 1970), rat liver (Eil and Wood, 1971; Chen and Walsh, 1971), rabbit skeletal muscle (Reimann *et al.*, 1971a,b), and rat brain (Miyamoto *et al.*, 1971). A preceding report (Kumon *et al.*, 1972) has shown multiple cAMP-dependent protein kinases obtained from rat liver which are all dissociated by the cyclic nucleotide into common active protein kinase and various R proteins. Similarly, the two rabbit skeletal muscle cAMP-dependent protein kinases described above appear to consist of common active protein kinase and different R proteins. However, it is unknown whether the two cAMP-dependent

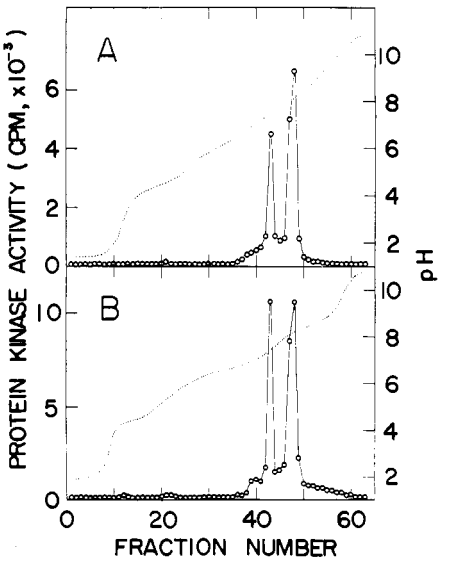

FIGURE 8: Isoelectrofocusing electrophoresis of rabbit skeletal muscle and rat liver protein kinases. Detailed experimental conditions were described under Experimental Section. Fractions were assayed for pH (- - - - -), and for protein kinase activity (——). (A) Rat liver protein kinase B_0; (B) rabbit skeletal muscle protein kinase B_0.

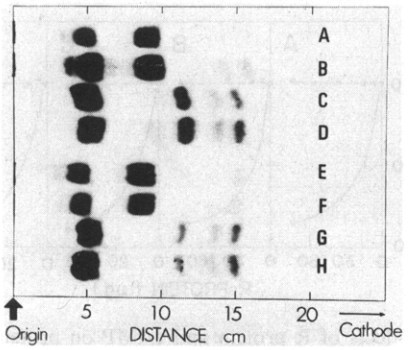

FIGURE 9: Autoradiography of the electropherogram of the tryptic digests of calf thymus histone and salmon sperm protamine fully phosphorylated separately by electrophoretically distinct two components of rabbit skeletal muscle and rat liver active protein kinases. The tryptic digestion, high-voltage electrophoresis, and autoradiography were carried out under the conditions described under Experimental Section. (A, B) Histone phosphorylated with rat liver components of isoelectric points of pH 7.4 and 8.2, respectively; (C, D) protamine phosphorylated with rat liver components of isoelectric points of pH 7.4 and 8.2, respectively; (E, F) histone phosphorylated with rabbit skeletal muscle components of isoelectric points of pH 7.4 and 8.2, respectively; (G, H) protamine phosphorylated with rabbit skeletal muscle components of isoelectric points of pH 7.4 and 8.2, respectively.

protein kinases are produced by dissociation of a single enzyme complex during the isolation procedure or exist *in vivo*.

The present studies have also shown that the active protein kinases obtained from rabbit skeletal muscle and rat liver are indistinguishable from each other, and exhibit closely similar kinetic and catalytic properties. These kinases show rather broad and identical substrate specificities and incorporate the terminal phosphate of ATP into the same specific sites of several phosphate acceptor proteins. In addition, each active protein kinase is converted to a cAMP-dependent form by the addition of R protein from the homologous as well as from the heterologous tissue. These results suggest that the protein kinases lack organ and species specificities and appear to be at least functionally identical enzymes. A vast array of evidence now available in the literature indicates that cAMP acts as an intracellular mediator of various hormones and regulates a variety of biochemical processes. The evidence presented in this paper may suggest that the action of hormone seems to be amplified by the successive activation of two consecutive enzymes, adenylate cyclase and protein kinase, resulting in the control of various biochemical reactions in each target tissue. However, this assumption may not necessarily exclude additional action of cAMP, and the exact nature of substrate proteins in each tissue has remained to be elucidated.

Chen and Walsh (1971) have recently reported that multiple active protein kinases may be obtained from rat liver soluble fraction using isoelectrofocusing electrophoresis. The present studies show that both muscle and liver active protein kinases partially purified in the presence of diisopropyl fluorophosphate are each resolved into two components of isoelectric points of pH 7.4 and 8.2. Nevertheless, these components are indistinguishable from each other in their kinetic and catalytic properties. This heterogeneity does not seem to represent simply artifact during the electrophoretic procedure, nor is it likely that one component is a subunit or an aggregated form of the other. No evidence has been available indicating that the two components are isozymes which exist indeed *in vivo*. It is possible that the heterogeneity represents artifact due to some modification such as proteolysis or deamidation of enzyme protein during the isolation procedure. The exact identity or similarity of the catalytic units of muscle and liver cAMP-dependent protein kinases as well as the precise nature of the two components described above will remain unexplored until homogeneous enzyme preparations are available.

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