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Multiple Forms of Hepatic Adenosine 3′:5′-Monophosphate Dependent Protein Kinase*

Lee-Jing Chen and Donal A. Walsh†

ABSTRACT: Cytoplasmic hepatic protein kinase is resolved into three fractions by chromatography on DEAE-Sephadex. Two of these fractions exhibit a sedimentation constant = 6.8 S, whereas the third component is 4.0 S. The activity of each 6.8S fraction is stimulated by adenosine 3′:5′-monophosphate (cAMP) whereas that of the smallest protein kinase (4.0 S) is independent of this nucleotide. The 6.8S protein kinase that is present in the highest proportion can be dissociated to yield two protein kinase fractions, each of 4.0 S, whose activity is independent of cAMP, and neither of which is identical with the 4.0S protein kinase isolated by DEAE-Sephadex chromatography. On the basis of reconstitution experiments and on work recently presented by several laboratories it is interpreted that each 4.0S component represents a species of catalytic subunit of cAMP-dependent protein kinase, whereas the 6.8S protein is a holoenzyme(s) composed of catalytic and nucleotide-binding regulatory subunits.

Adenosine 3′:5′-cyclic monophosphate has been shown to be the intracellular second messenger of a number of hormones that control hepatic metabolism (Sutherland and Rall, 1960; Robison *et al.*, 1968). The stimulation of hepatic glycogenolysis, gluconeogenesis, and lipolysis by glucagon and epinephrine can be mimicked by the action of cAMP.[1] In addition, the elevation of tissue levels of cAMP promoted by the action of either of these hormones precedes all other established metabolic events. Insulin, which promotes the reversal of each of these metabolic processes, has been shown to decrease the level of cAMP in liver (Exton *et al.*, 1966).

With the discovery of the cAMP-dependent protein kinase from skeletal muscle (Walsh *et al.*, 1968), it has been suggested that the initial step of many of the actions of this nucleotide is the activation of this enzyme. This enzyme has been found in a wide variety of tissues (Kuo and Greengard, 1969) and utilizes several different proteins as substrates. The enzyme catalyzes the phosphorylation of phosphorylase *b* kinase (Walsh *et al.*, 1971a) and glycogen synthetase (Schlender *et al.*, 1969) from skeletal muscle, hormone-sensitive lipase from adipose tissue (Corbin *et al.*, 1970; Huttunen *et al.*, 1970), histone from liver (Langan, 1968), RNA polymerase from *Escherichia coli* (Martelo *et al.*, 1970), and microtubules from brain (Goodman *et al.*, 1970).

The question then arises whether a single protein kinase initiates the various hepatic metabolic functions that are mediated by cAMP, or alternatively whether a number of isozymes are involved in its diversity of action. It was originally proposed by Brostrom *et al.* (1970) on the basis of studies of reversible inactivation and has since been demonstrated by several laboratories (Gill and Garren, 1970; Tao *et al.*, 1970; Kumon *et al.*, 1970; Reimann *et al.*, 1971a) that cAMP-de-

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[1] Abbreviations used are: cAMP, adenosine 3′:5′-cyclic monophosphate; *pI*, isoelectric point.

pendent protein kinase can be separated into a subunit binding the cyclic nucleotide and a catalytic subunit. Dissociation of the holoenzyme into these subunits results in deinhibition of the catalytic activity. This reaction is illustrated in eq 1 in which C refers to the catalytic subunit, R refers to the nucleotide-binding regulatory subunit, and CR refers to the holoenzyme.

$$\underset{\text{(inactive)}}{CR} + cAMP \rightleftharpoons \underset{\text{(active)}}{C} + R\text{-}cAMP \qquad (1)$$

In this communication we are able to show that at least three different catalytic subunits can be identified from rat liver cytosol. An initial characterization of their properties is described.

Materials and Methods

Protein kinase activity was assayed according to the method of Reimann *et al.* (1971b) with the following modifications: the assay mixture contained 2-(*N*-morpholino)ethanesulfonate buffer (pH 7.0, 2.5 μmoles), aminophylline (0.14 μmol), magnesium chloride (1 μmole), ATP (20 nmoles), histone (20 μg), and cAMP (where added, 0.2 nmole), in a total volume of 70 μl. The reaction was initiated by the addition of enzyme and the mixture was incubated for 25 min at 30°. Unless otherwise stated, protein kinase activity was determined utilizing histone f_{2b} as substrate. One unit of protein kinase activity is defined as that amount of enzyme which catalyzes the incorporation into protein of 1 pmole of phosphate/min under the standard assay conditions described. Lactate dehydrogenase, glutamate dehydrogenase, and glucose 6-phosphatase were determined by the methods of Kornberg (1955), Strecker (1955), and Swanson (1955), respectively. The unit of activity of each of these three enzymes is defined as that amount of enzyme which catalyzes the utilization of 1 μmole of substrate/min under the standard conditions. Protein was determined by the method of Lowry *et al.* (1951).

Cytoplasmic, mitochondrial, and microsomal fractions were obtained essentially by the procedure of Schneider (1948). Rat liver (4 g) was homogenized in 20 ml of 0.25 M sucrose containing 5 mM Tris-Cl (pH 7.5), 1 mM EDTA, and 10 mM β-mercaptoethanol (homogenization buffer) utilizing a Potter-Elvehjem homogenizer with a Teflon pestle. The homogenate was centrifuged for 10 min at 600*g*. The sediment was discarded and the supernatant was centrifuged for an additional 10 min at 8500*g*. The pellet, containing mitochondria, was suspended in 10 ml of the homogenization buffer and centrifuged at 8500*g* for 10 min. This washing procedure was repeated twice.

The postmitochondrial fraction obtained from the initial centrifugation at 8500*g* was centrifuged at 105,000*g* for 36 min to obtain the microsomal and cytoplasmic fractions. The microsomal pellet was washed twice by homogenization with 10 ml of homogenization buffer and separated by centrifugation at 105,000*g* for 36 min. The supernatant solution obtained from the initial centrifugation at 105,000*g* was used as the cytosol fraction.

The nuclear fraction was prepared essentially according to the method of Chauveau *et al.* (1956) as modified by Steele *et al.* (1965). Minced liver (4 g) was homogenized in 20 ml of 2.2 M sucrose containing 3 mM $CaCl_2$ with four strokes of a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 40,000*g* for 1 hr. The nuclear pellet was resuspended in 1.0 M sucrose containing 1 mM calcium acetate in a volume of 1 ml/g of original tissue. The homogenate was centrifuged at 3000*g* for 5 min, and the precipitate was used as the nuclear fraction. Disruption of the mitochondrial, microsomal, and nuclear fractions was achieved by resuspending the pellets in 5 mM Tris-Cl (pH 7.5), 1 mM EDTA, and 10 mM β-mercaptoethanol in a volume of 2 ml/g of original tissue, and freezing and thawing each suspension once. The total suspensions were used as the source of the enzymes.

Isoelectrofocusing electrophoresis was performed according to the method originally described by Svensson (1962) using a 110-ml column (LKB Instruments, Inc.) maintained at 0°. The pH gradient was established during electrophoresis following the sequential addition of ampholyte solutions (2% w/v) in a 0–47% (w/v) sucrose gradient. The protein solution was applied in the central ampholyte solution. Electrophoresis was initiated at 200 V, increased to 800 V over a period of 24 hr, and continued at this voltage for at least another 16 hr for equilibration. Upon completion of the electrophoresis, fractions were collected and assayed for protein kinase activity and for pH utilizing a Radiometer pH meter 4.

Histone fractions were prepared by method 1 described by Johns (1964) utilizing the modification described by Kincade and Cole (1966) for the removal of degradation products of fraction f_1. The amino acid composition of each of these fractions coincided closely with those reported (Johns, 1964).

Phosphorylase *b* was purified as described by DeLange *et al.* (1968). Hemoglobin was isolated by the method of Ingram (1955). The preparation of the heat-stable protein inhibitor of cAMP-dependent protein kinases has been described previously (Walsh *et al.*, 1971b). The regulatory subunit of skeletal muscle protein kinase was a gift of Drs. Krebs, Brostrom, and Corbin. [γ-^{32}P]ATP was prepared by the previously described modification (Walsh *et al.*, 1971a) of the method of Glynn and Chappell (1964). All other chemicals were commercial preparations.

Results

Fractionation of Rat Liver Protein Kinase. Initial experiments indicated that cAMP-dependent protein kinase activity is located almost entirely in the cytosol fraction of liver[2] (Table I). In this experiment the relative activities of lactate dehydrogenase, glutamate dehydrogenase, and glucose 6-phosphatase were used as a guideline for the extent of cross-contamination of cytosol, mitochondria, and microsomes, respectively. Negligible protein kinase activity could be detected in the mitochondria. The low level of activity of cAMP-dependent protein kinase that was detected in the microsomal and nuclear fractions approximately paralleled the distribution of lactate dehydrogenase that was used as a cytoplasmic marker. However, Siebert (1961) has reported that nuclei do contain a low level of lactate dehydrogenase. The presence of some cAMP-dependent protein kinase in the nuclei would be consistent with the observations of Langan (1969) of the *in vivo* phosphorylation of histone under the specific influence of glucagon. All other experiments described in this paper utilized rat cytosol as the source of protein kinase.

Cytoplasmic protein kinase is resolved into three fractions by chromatography on DEAE-Sephadex. For a typical experiment, male Sprague–Dawley rats weighing approximately 200 g, fed standard chow diet, were used as the source of the

[2] These observations of cellular distribution are in confirmation of results previously obtained by T. Langan (personal communication).

TABLE I: Subcellular Distribution of Hepatic Protein Kinase.

Cell Fraction[a]	Protein Kinase Act.[b] −cAMP Units × 10^{-3}/g of Tissue	Protein Kinase Act.[b] +cAMP Units × 10^{-3}/g of Tissue	Protein Kinase Act.[b] +cAMP Units × 10^{-2}/mg of Protein	Units/g of Tissue: Lactate Dehydrogenase	Units/g of Tissue: Glutamate Dehydrogenase	Units/g of Tissue: Glucose 6-Phosphatase
Cytosol	93	289	430	73.4	<0.05	<0.5
Mitochondria	<2	<2	<2	0.6	2.8	10
Microsome	5	13	9	2.6	<0.05	128
Nuclei	7	20	2	3.9	0.2	28

[a] Cell fractionation was performed according to the procedure described under Materials and Methods. [b] Protein kinase activity was assayed in either the presence or absence of 3×10^{-6} M cAMP (as indicated) utilizing histone f_{2b} as substrate. Each fraction was appropriately diluted to obtain initial reaction rates.

enzyme. Livers were homogenized in five volumes of 0.25 M sucrose containing 5 mM Tris-Cl (pH 7.5), 1 mM EDTA, and 10 mM β-mercaptoethanol, utilizing a Potter-Elvehjem homogenizer with a Teflon pestle. The homogenate was centrifuged at 105,000*g* for 1 hr and the resultant supernatant solution was passed through a two-layer cheesecloth. The filtrate (12 ml) was applied to a DEAE-Sephadex A-25 column (1.8 × 22 cm) which had been previously equilibrated with 5 mM Tris-Cl (pH 7.5), 1 mM EDTA, and 10 mM β-mercaptoethanol. The column was eluted with a linear gradient of sodium chloride in the same buffer. The elution profile of the DEAE-Sephadex column is shown in Figure 1. At least three fractions of enzymatic activity were resolved by this procedure. The first fraction, eluted between 0 and 0.06 M sodium chloride, is designated fraction I. The activity of this fraction is totally independent of cAMP. Two other fractions, designated II and III, were eluted between 0.08 and 0.25, and 0.27 and 0.36 M sodium chloride, respectively. The activities for each of these latter fractions were stimulated by cAMP. The elution profile of fraction II as assayed for protein kinase activity in the presence or absence of cAMP (Figure 1) is possibly indicative of the presence of more than a single enzymatic component. For the purposes of the study reported here no attempt has been made to resolve this fraction further by this technique. In comparison to the fractionation procedure described by Yamamura *et al.* (1970), fraction I is probably identical with their protein kinase B_2 whereas fractions II and III presumably rep-

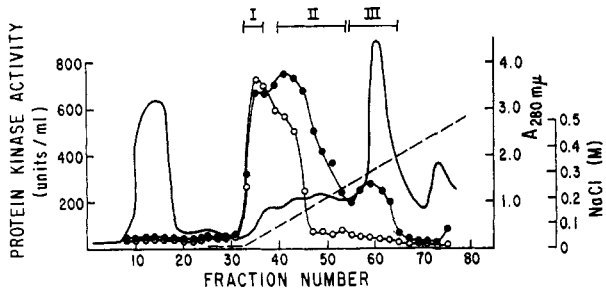

FIGURE 1: Fractionation of heptatic protein kinase by chromatography on DEAE-Sephadex. Hepatic cytosol (12 ml), prepared as described in the text, was applied to a column (1.8 × 22 cm) of DEAE-Sephadex A-25 which had been previously equilibrated with 5 mM Tris-Cl (pH 7.5), 1 mM EDTA, and 10 mM β-mercaptoethanol. The column was developed with a linear gradient of sodium chloride (0–0.5 M) in the same buffer. The total volume of the gradient was 100 ml. Fractions of 2 ml were collected. The salt concentration of the fractions (broken line) was calculated from determinations of conductivity utilizing a Radiometer CDM2C conductivity meter. Protein kinase activity was determined in either the presence (●) or absence (○) of cAMP. The optical density of each fraction is indicated by a solid line. Fraction tubes 33–37, 40–54, and 55–65 were used as the source of protein kinase fractions I, II, and III, respectively.

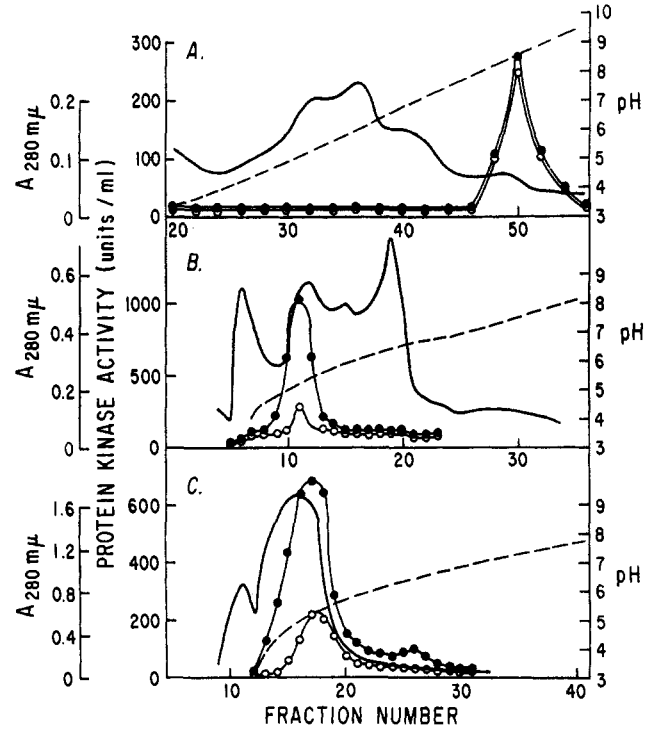

FIGURE 2: Isoelectrofocusing electrophoresis of hepatic protein kinase fractions I (A), II (B), and III (C). Electrophoresis was performed utilizing ampholyte solutions with a pH range of 3–10 for fraction I and 5–8 for fractions II and III. The protein kinase fractions applied to the column, I (3000 units), II (10,000 units), and III (14,000 units), were prepared as described in the text. Fractions (2 ml) were assayed for pH (broken line), absorption at 280 mμ (solid line), and protein kinase activity in the presence (●) or absence (○) of cAMP. All other details are described in the text.

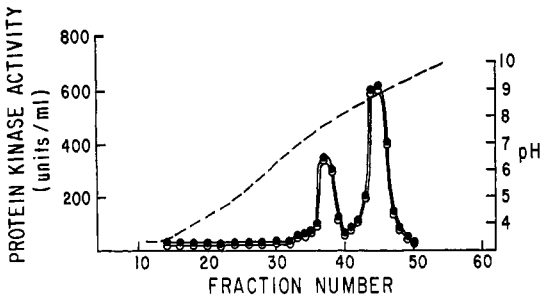

FIGURE 3: The effect of cAMP on the isoelectrofocusing electrophoretic pattern of hepatic protein kinase fraction II. Protein kinase fraction II (12,000 units) that had been subjected to prior electrophoresis under the conditions defined in the legend of Figure 2 was pooled and dialyzed against 5 mM Tris-Cl (pH 7.5), 1 mM EDTA, and 10 mM β-mercaptoethanol to remove ampholytes. The sample was incubated with 4×10^{-6} M cAMP at 4° for 10 min. This protein was then subjected to electrophoresis under conditions identical with those described in the legend of Figure 2. Fractions of 2 ml were assayed for pH (broken line), and for protein kinase activity in the presence (●) or absence (○) of cAMP.

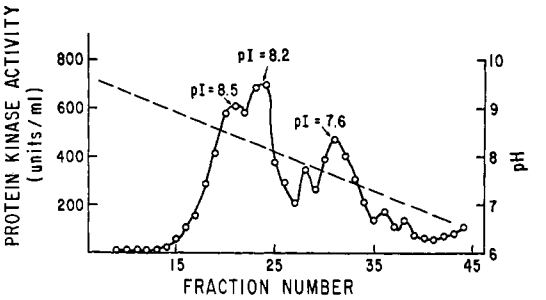

FIGURE 4: Resolution of heptatic protein kinase fractions I, II_A, and II_B by isoelectrofocusing electrophoresis. A mixture of protein kinase fraction I (5200 units) and fraction II (7600 units) each obtained by chromatography on DEAE-Sephadex (Figure 1) was incubated with 4×10^{-6} M cAMP at 4° for 10 min and subjected to electrophoresis under conditions described in the legend of Figure 2 except ampholyte solutions were used with a pH range of 7–10. Fractions of 2 ml were collected. Protein kinase activity was determined in the absence of cAMP.

resent a resolution into two components of their protein kinase B_1.

Isoelectrofocusing Electrophoresis of Hepatic Protein Kinases. The isolated fractions of protein kinase that were separated by chromatography on DEAE-Sephadex were subjected to the technique of isoelectrofocusing as described originally by Svensson (1962). Each fraction was dialyzed against 5 mM Tris-Cl (pH 7.5), containing 1 mM EDTA and 10 mM β-mercaptoethanol. The electrophoretic profile that was obtained for each fraction is presented in Figure 2. The isoelectric point of fraction I (Figure 2A) is pH 8.6. Fractions II (Figure 2B) and III (Figure 2C) are indistinguishable by this technique each having an isoelectric point of pH 5.2. Incubation of hepatic protein kinase fraction II with 4×10^{-6} M cAMP for 10 min at 4° produces a marked change in the observed electrophoretic profile. Under these conditions the protein kinase is resolved into two fractions of isoelectric points, pH 7.6 and pH 8.9, respectively (Figure 3). These fractions are designated II_A and II_B, respectively. The resolution of fraction II into two catalytic components is obtained either with enzyme that has been subjected to electrophoresis under the conditions of the experiment presented in Figure 2, as is demonstrated in Figure 3, or with material obtained directly from chromatography on DEAE-Sephadex. Whereas the activity of fraction II is markedly stimulated by cAMP ($K_a = 4 \times 10^{-8}$ M) the activities of II_A and II_B are independent of this nucleotide. This latter observation cannot be attributed to the contamination of cAMP in these fractions, the extent of which, determined utilizing [^{3}H]cAMP, was less than 1×10^{-10} M. In contrast to the results observed of the effect of cAMP on the electrophoretic profile of fraction II, incubation of the cyclic nucleotide with fraction I produced no effect on the isoelectric point of this enzyme.

These observations of the effect of cAMP on protein kinase fraction II are best interpreted on the basis of the known subunit structure of protein kinase as illustrated in eq 1 (Kumon *et al.*, 1970; Gill and Garren, 1970; Tao *et al.*, 1970; Reimann *et al.*, 1971a). Fractions II_A and II_B probably represent catalytic subunits from two species of protein kinase in fraction II. Fraction II is presumably the holoenzyme(s) composed of the component regulatory and catalytic subunits of the protein kinase. Fraction I may also represent a species of catalytic subunit, an interpretation similar to that presented by Kumon *et al.* (1970). A small difference in the isoelectric points of fractions I and II_B that has been consistently observed suggested that these may represent separate entities. This observation was further supported by the partial electrophoretic separation of these fractions. A mixture of fractions I and II was incubated with cAMP prior to isoelectrofocusing. The fractionation obtained is illustrated in Figure 4 which demonstrates the presence of fraction II_A ($pI = 7.6$), fraction I ($pI = 8.2$), and fraction II_B ($pI = 8.5$). The significance of other minor fractions obtained by this experiment is not known. The ionic compositions of the ampholyte solutions are such

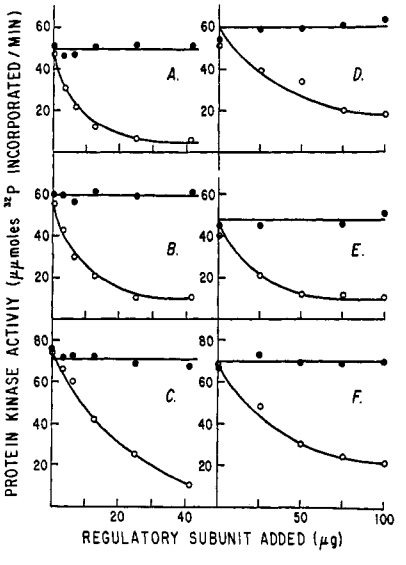

FIGURE 5: Reconstitution of cAMP dependency to the isolated catalytic subunits of hepatic protein kinases. The activities of fractions II_A (A and D), II_B (B and E), and I (C and F) were determined in the standard protein kinase assay system as described in the text in the presence of varying aliquots of the regulatory subunit of skeletal muscle protein kinase (A, B, and C) and of a fraction of hepatic protein isolated as described in the text (D, E, and F). These activities were determined in the presence (●) and absence (○) of cAMP. Fraction I was obtained by chromatography on DEAE-Sephadex (Figure 1). Fractions II_A and II_B were isolated by isoelectrofocusing electrophoresis (Figure 3). All other conditions are described in the text.

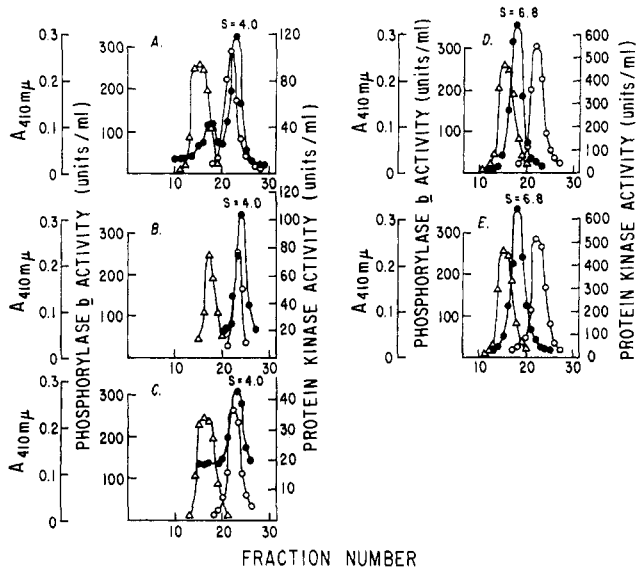

FIGURE 6: Ultracentrifugation of hepatic protein kinases in sucrose gradient. Hepatic protein kinase fractions I (A), II_A (B), II_B (C), II (D), and III (E) were centrifuged in a 5–20% w/v sucrose gradient at 39,000 rpm in a Beckman SW 40 rotor for 16.5 hr at 5°. At the termination of centrifugation each fraction was assayed for protein kinase activity (●). Sedimentation coefficients were determined by the method of Martin and Ames (1961) utilizing hemoglobin, 4.6 S, and phosphorylase *b*, 8.4 S, as internal standards in each experiment. Phosphorylase *b* activity (△) was determined by the method described by Krebs *et al.* (1964). Hemoglobin was measured by absorption at 410 mμ (○).

that minor differences in isoelectric points of proteins are observed when using ampholyte solutions that extend over different pH ranges. This is observed for the protein kinase fractions I and II_B when using ampholytes of pH range 3–10 (Figure 2 and 3) as compared to pH range 7–10 (Figure 4) and has also been observed with other proteins in this laboratory. Resolution of the catalytic components of fractions I and II has been obtained using ampholytes of pH range 3–10 yielding protein kinase fractions of isoelectric points 7.6 (II_A), 8.5 (I), and 8.9 (II_B). This latter experiment is difficult to reproduce consistently because of the lower resolving power of the wider pH-range electrophoresis. Resolution of these fractions has been accomplished at least three times by electrophoresis.

Reconstitution of cAMP-Dependent Hepatic Protein Kinases. The supposition expressed above that fractions II_A and II_B are each catalytic subunits of protein kinases is confirmed by the reconstitution of cAMP dependency on the addition of regulatory subunit. These experiments were performed utilizing the method described by Reimann *et al.* (1971a). The addition of the regulatory subunit isolated from rabbit skeletal muscle inhibited the activity of II_A (Figure 5A) and II_B (Figure 5B) when assayed in the absence of cAMP but was without effect on these activities assayed in the presence of cyclic nucleotide. The activity of fraction I assayed in the absence of cAMP is also inhibited by the addition of the protein kinase regulatory subunit (Figure 5C) as has been described by Kumon *et al.* (1970). The reestablishment of cAMP dependency to II_A, II_B, and I also occurs with the addition of a hepatic protein fraction which is probably identical with hepatic regulatory subunit. This latter protein was isolated from the electrofocusing experiment presented in Figure 3 by assaying the fractions obtained for their ability to restore cAMP dependency to fraction II_B. Addition of a protein fraction of isoelectric point pH 4.5 restored cAMP dependency to hepatic protein kinase fractions I, II_A, and II_B (Figure 5D, E, and F).

Sedimentation Characteristics of Hepatic Protein Kinases. The sedimentation characteristics of the isolated protein kinase fractions were examined by the method of Martin and Ames (1961). Calculations of sedimentation coefficients were determined by the utilization of hemoglobin (4.6 S) (Schachman and Edelstein, 1966) and skeletal muscle phosphorylase *b* (8.4 S) (Seery *et al.*, 1967) as internal standards in each of the sucrose gradient ultracentrifugations. Fraction I isolated by DEAE-Sephadex chromatography (Figure 1) and fractions II_A and II_B isolated by isoelectrofocusing (Figure 3) each exhibited a sedimentation constant of 4.0 S (Figure 6A–C). In contrast, the sedimentation coefficients of the cAMP-dependent protein kinases (fraction II and III) were each found to be 6.8 S (Figure 6D and E). Fraction I contained a small amount of protein kinase activity that had an *s* value of 6.8 S which presumably represents a contamination of fraction II in the preparation.

Effect of the Skeletal Muscle Heat-Stable Protein Inhibitor of Protein Kinases on Hepatic Protein Kinase Fractions. The purification and properties of a heat-stable protein that occurs in a wide range of tissues and inhibits the activity of cAMP-dependent protein kinases from skeletal muscle, cardiac muscle, adipose tissue, liver, and brain have been previously described (Walsh *et al.*, 1971b). The mechanism of action of this inhibitor involves a direct interaction with the protein kinase or some component thereof (Walsh *et al.*, 1971b). The heat-stable protein isolated from skeletal muscle inhibits the activity of both the hepatic cAMP-dependent protein kinase fractions II and III (Figure 7A and B) and all three of the hepatic protein kinase catalytic subunits, I, II_A, and II_B (Fig-

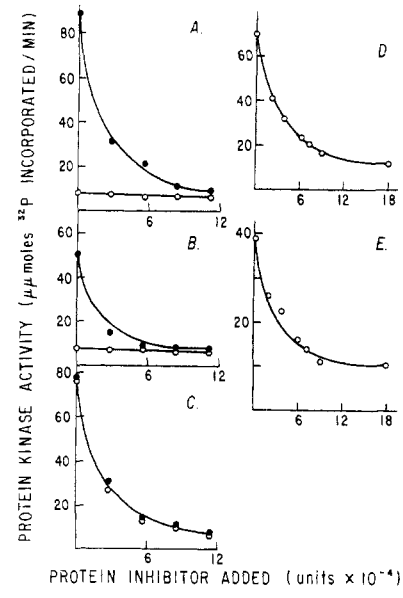

FIGURE 7: The effect of the heat-stable protein inhibitor of cAMP-dependent protein kinases on the activity of hepatic protein kinase fractions. The activities of fractions II (A), III (B), I (C), II_A (D), and II_B (E) were determined in the standard assay system in the presence of varying aliquots of the purified heat-stable protein inhibitor (Walsh *et al.*, 1971b) isolated from rabbit skeletal muscle, in either the presence (●) or absence (○) of cAMP. Units of inhibitor have been defined previously (Walsh *et al.*, 1971b). All other conditions are described in the text.

TABLE II: Substrate Specificity of the Catalytic Subunits of Hepatic Protein Kinase.[a]

Substrate	Protein Kinase Act. (Units/ml)		
	I	II_A	II_B
Histone f_{2a}	136	137	159
Histone f_{2b}	226	300	407
Histone f_3	162	139	189
Histone f_1	90	140	151
Histone mixture[b]	93	81	135

[a] Protein kinase activity was determined utilizing the standard reaction mixture described in the text. The reactions in which histone fractions f_{2a}, f_{2b}, and f_3 were utilized as substrates were terminated by the addition of 0.2 ml of bovine serum albumin (6.25 mg/ml) and 1.5 ml of 6.6% trichloroacetic acid. The extent of protein-bound phosphate was determined as described elsewhere (Walsh *et al.*, 1971a). The reactions in which histone fraction f_1 or the histone mixture was used were terminated by the addition of 0.2 ml of bovine serum albumin (6.25 mg/ml) and 1.5 ml of 24% trichloroacetic acid. The amount of protein-bound phosphate for these latter incubations was determined as described previously (Walsh *et al.*, 1971a) with the exception that the concentrations of trichloroacetic acid utilized were increased 3.6-fold for each procedure. [b] Type II_A prepared from calf thymus (Sigma Chemical Co.).

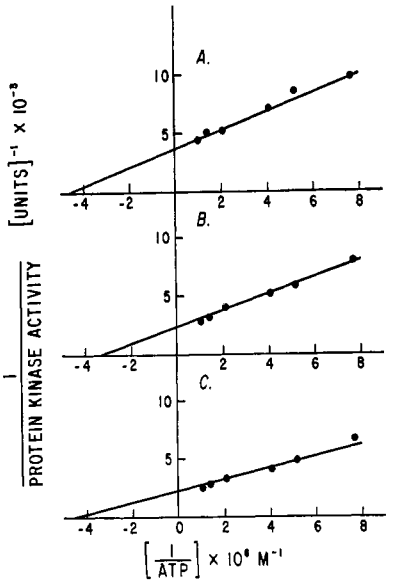

FIGURE 8: Double-reciprocal plots of initial velocity of protein kinase activity *vs.* ATP concentration for catalytic subunits II_A and II_B. The activity of each catalytic subunit was determined utilizing the standard assay system in the absence of cAMP with the exception that ATP concentration was varied as indicated. Magnesium chloride concentration was 10 mM. Protein kinase fractions used were A, fraction I; B, fraction II_A; and C, fraction II_B. Sources of these proteins are described in the text.

ure 7C–E).[3] In contrast to the effects observed with protein kinase regulatory subunit, inhibition of catalytic subunit activity by the heat-stable protein is not relieved by cAMP.

Preliminary Characterization of the Catalytic Activities of the Hepatic Protein Kinase Catalytic Subunits. A comparison of the catalytic activities of I, II_A, and II_B indicated an equivalent sensitivity with respect to the nucleotide substrate. Double-reciprocal plots of ATP concentration against the initial velocity of histone phosphorylation for fractions I, II_A, and II_B are depicted in Figure 8A, B, and C, respectively. In this study, magnesium chloride was 10 mM. The values of the apparent K_m for ATP of 2×10^{-5}, 3×10^{-5}, and 2×10^{-5} M obtained for each of these fractions respectively are identical within experimental error. A small difference is detected between protein kinase fractions I, II_A, and II_B with respect to protein substrate specificity. In the experiment presented in Table II the catalytic activity of each of these fractions is presented utilizing as substrates histone fractions f_1, f_{2a}, f_{2b}, f_3, and a commercial preparation of histone containing several histone fractions. The experiment was performed by adjusting the concentration of each protein kinase to give approximately equal activity with f_{2a} as substrate. The most significant difference observed was between fraction I and II_B with either histone fractions f_{2b} or f_1 as substrate. Each of the protein kinase fractions (I, II, II_A, II_B, and III) exhibited the same specificity with respect to the phosphorylation of a particular serine residue in f_1 histone as has been described previously by Langan (1969) for a hepatic protein kinase.

[3] Whereas previously it has been shown (Walsh *et al.*, 1971b) that with freshly prepared rat cytosol the inhibitor does not affect the protein kinase activity expressed in the absence of cAMP, it is now shown that it does inhibit fraction I. This is interpreted to means that fraction I is a dissociated form of the holoenzyme produced during purification, whereas the cAMP-independent activity of a freshly prepared cytosol is composed of many other protein kinases which would include fraction HK_2 identified by Langan (1971) and phosphorylase kinase (DeLange *et al.*, 1968).

Discussion

The evidence presented in this paper indicates the existence of multiple forms of hepatic protein kinase. At least two c AMP-dependent protein kinases have been detected by chromatography on DEAE-Sephadex. Multiple forms of cAMP-dependent protein kinase from skeletal muscle have been reported previously by Reimann *et al.* (1971b). The partially purified enzymes from this latter source were prepared by chromatography on DEAE-cellulose and differ in molecular weight as discerned by differences in sedimentation in a sucrose gradient (Reimann *et al.*, 1971b). The lower molecular weight species (4.8 S) would appear to be produced by degradation of the larger enzyme (6.8 S) (E. G. Krebs, C. O. Brostrom, and J. D. Corbin, personal communication). In contrast to the findings with the skeletal muscle enzyme, the enzymes isolated from liver exhibit identical sedimentation coefficients (6.8 S). The fraction of hepatic cAMP-dependent protein kinase that is present in the greatest quantity (fraction II) contains two different catalytic subunits (II_A and II_B). The resolution obtained on DEAE-Sephadex is insufficient to discern whether these different catalytic subunits originally comprised a single holoenzyme with two different catalytic components or alternatively, and more probably, two separate holoenzymes. Although protein kinase fraction II migrates as a single fraction by electrophoresis (Figure 2B), this should not be considered as a sufficient criteria of enzymatic homogeneity because by this technique no difference could be discerned between pro-

tein kinase fractions II and III. The cAMP-independent protein kinase (fraction I) is apparently not identical with either of the catalytic subunit components of fraction II. In addition to a partial resolution of fraction I and catalytic subunit II_B by isoelectrofocusing electrophoresis (Figure 4), these two components can also be distinguished on the basis of activity with different protein substrates (Table II). The identification of fraction I as a catalytic subunit of cAMP-dependent protein kinase is based on its inhibition by either rabbit skeletal muscle or rat liver regulatory subunit and by the heat-stable protein inhibitor characterized previously by Walsh *et al.* (1971b). Fraction I also exhibits a sedimentation coefficient identical with those of both hepatic catalytic subunits II_A and II_B, and also with that of the catalytic subunit of rabbit skeletal muscle (E. G. Krebs, C. B. Brostrom, and J. D. Corbin, personal communication). The true *in vivo* status of fraction I remains to be established. The question arises as to whether it may be produced by dissociation during the isolation procedure and also as to whether the activity of this fraction is regulated *in vivo* by a nucleotide-binding subunit. No knowledge is currently available concerning the relative amounts of catalytic and regulatory subunits in the cell.

cAMP has been implicated in a wide diversity of actions in the control of hepatic function. The finding of multiple forms of the hepatic protein kinase enzyme may lead in part to an explanation of this multiplicity of action of this nucleotide. Our understanding of the well established role of cAMP in the regulation of hepatic glycogen metabolism (Sutherland and Rall, 1958) has not yet been extended to the detailed enzymology that has been achieved for skeletal muscle (Krebs *et al.*, 1964; DeLange *et al.*, 1968; Riley *et al.*, 1968; Larner, 1967). In skeletal muscle a cAMP-dependent protein kinase controls both glycogenolysis (Walsh *et al.*, 1971a) and glycogenesis (Huijing and Larner, 1966; Soderling *et al.*, 1970). The many similarities that exist between glycogen metabolism in liver and skeletal muscle suggest that it is quite probable that this aspect of hepatic metabolism will likewise involve the regulation by cAMP-dependent protein kinase(s). Similarly, hepatic lipolysis is controlled by cAMP (Bewsher and Ashmore, 1966), potentially by a mechanism involving a protein kinase, as has been discerned for adipose tissue (Corbin *et al.*, 1970; Huttunen *et al.*, 1970). Hepatic gluconeogenesis is also regulated by cAMP (Exton and Park, 1967) by a mechanism not yet understood. Many aspects of the regulation of hepatic macromolecular synthesis involve the action of cAMP. Wicks (1971) has indicated a function for this nucleotide in a posttranscriptional event in the induction of both tyrosine transaminase and phosphoenolpyruvate carboxykinase, whereas the control of the induction of serine deaminase would appear to reside at the level of RNA synthesis (Jost and Sahib, 1971). The potential relationship of this latter observation to the elegant demonstration of the *in vivo* stimulation of phosphorylation of histone (Langan, 1969) remains to be elucidated. Although several of these effects of cAMP may not involve protein phosphorylation as appears probable in bacteria (Eron *et al.*, 1971; Emmer *et al.*, 1970), there are many potential sites for a role for cAMP-dependent protein kinases in the regulation of hepatic function.

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Site of Inactivation of Thyrotropin-Releasing Hormone by Human Plasma*

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ABSTRACT: Thyrotropin-releasing hormone (TRH) (synthetic, L-(pyro)Glu-L-His-L-Pro-NH_2) and its ^{14}C analog (L-(pyro)-Glu-L-[^{14}C]His-L-Pro-NH_2) after incubation with human plasma at 37° for 1 hr showed a 72% decrease in activity. After the recovery and purification, the peptide obtained exhibited different chromatographic and electrophoretic mobilities from TRH, but the amino acid composition was identical. The recovery of the hormone after incubation in terms of either amino acid analysis or radioactivity was 40–50%. An analogous experiment at 0° did not inactivate TRH and the recovery was 60%. The recovery of 40–50% of radioactivity from the purified Pauly-positive, inactive peptide showed that histidine is not cleaved away during incubation. Inactivated TRH did not show a free N terminus by Edman-dansyl procedure suggesting that the (pyro)glutamyl ring is also intact. On treatment with [^{14}C]diazomethane, the inactivated TRH was quantitatively converted into a [^{14}C]methyl ester, indicating a free C terminus. Inactivated TRH was compared chromatographically and electrophoretically to synthetic (pyro)Glu-His-Pro-OH and found to have the same mobilities. These suggest that during incubation with plasma, TRH undergoes cleavage of the amide group at the prolyl end which is probably the site of inactivation.

Previous reports from this laboratory (Nair *et al.*, 1970; Schally *et al.*, 1970) described the elucidation of the structure of thyrotropin-releasing hormone (TRH),[1] which controls the secretion of the thyrotropic hormone (TSH) from the anterior pituitary gland (Schally *et al.*, 1968). Determination of the structure of TRH as L-(pyro)Glu-L-His-L-Pro-NH_2 was paralleled by its successful synthesis (Bøler *et al.*, 1969; Flouret, 1970). The comparison of the chemical, spectroscopic (Nair *et al.*, 1970; R. M. G. Nair and A. V. Schally, in preparation[2]), and biological (Schally and Bowers, 1971; Bowers *et al.*, 1970a,b) properties of the synthetic L-(pyro)Glu-L-His-L-Pro-NH_2 and the natural porcine TRH proved that they are identical. The structure of ovine TRH was simultaneously shown by Burgus *et al.* (1969, 1970) to be also (pyro)Glu-His-Pro-NH_2. In the course of our previous investigations on the biological activity of TRH, we have undertaken a series of studies on inactivation of TRH by animal and human plasma (Redding and Schally, 1969). After the correct molecular structure of this neurohumor became known to us, it was thought that the rapid inactivation of TRH observed during its incubation with plasma needed further investigation as to how and at what particular site the inactivation occurred. An insight into the "site of inactivation" could shed more light on the metabolism and mechanism of action of this hormone which is now being extensively used in clinical studies (Bowers *et al.*, 1970a,b; Fleischer *et al.*, 1970; Herschman and Pittman, 1970).

Materials

Synthetic L-(pyro)Glu-L-His-L-Pro-NH_2, (pyro)Glu-His-Pro-OH, His-Pro-NH_2, His-Pro-NH_2-HCl, (pyro)Glu-His, and L-(pyro)Glu-L-[^{14}C]His-L-Pro-NH_2 (specific activity 0.7 mCi/mg) were obtained from Abbott Laboratories. The syn-

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[1] Abbreviations used are: TRH, thyrotropin-releasing hormone; TSH, thyrotropin-secreting hormone; DNS-Cl, dimethylaminonaphthalenesulfonyl chloride; PITC, phenyl isothiocyanate.

[2] A paper on the nmr and mass spectroscopic comparison of the free and derivatized porcine TRH and L-(pyro)Glu-L-His-L-Pro-NH_2 is sent to *Biochemistry*.