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PROTEIN KINASES FROM LIVER MITOCHONDRIA OF TUMOUR-BEARING RATS

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Summary

The mitochondria of liver of Yoshida ascites tumour-bearing rats contained two forms of protein kinase distinguishable on the basis of their kinetic properties, substrate specificity and responses to cyclic adenosine 3′,5′-monophosphate (cAMP). One of these (kinase I) was activated 2—3 fold by cAMP while the other form (kinase II) was insensitive to the action of cAMP. Kinase I which was selective towards histone F1 as substrate was obtained as a homogeneous preparation and was observed to have a molecular weight of 170 000 by Sephadex G-150 gel filtration. Protein kinase II appeared to be a smaller protein with molecular weight of 54 000 and was specific towards acidic proteins namely casein and phosvitin. Protein kinases isolated from liver mitochondria of normal rats showed variations in respect to elution profile of DEAE-cellulose and electrophoretic mobility. The preparation corresponding to kinase I did not show stimulatory responses to cAMP.

Introduction

Protein kinases which catalyse the phosphorylation of phosphoproteins, histones and protamines have been found in a number of mammalian tissues [1—5] and in bacteria [6]. A striking feature of this phosphorylation process is its stimulation by cyclic adenosine 3′,5′-monophosphate (cAMP) and this has stimulated several recent investigations on the mechanism of its action [1—3,7—9]. It has been shown that phosphokinase from rabbit skeletal muscle contained a regulatory subunit which could bind this cyclic nucleotide tightly, thus causing dissociation of this subunit from the catalytic subunit [8]. It is recognised that levels of cAMP and protein kinases have involvement in the regulation of me-

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tabolism of glycogen [10] and lipids [11,12] and in DNA transcription processes [13,14].

It has recently been reported that intraperitoneal administration of cAMP or theophylline could cause retardation of tumour in mice and inhibition of growth of cultured mammalian cells [15—18]. On the other hand, high rates of phosphate renewal accompanied by enhanced protein kinase activity observed in malignancy suggested a role for this enzyme [19,20]. These observations may thus seem to be conflicting in respect of the involvement of cAMP in activating protein kinase during tumour growth.

Previous studies from this laboratory have indicated that protein kinase activity was very high in the livers of animals bearing ascites tumour than in the tumour itself or in liver of normal animals [19]. This enzyme was found to be localised mainly in the mitochondria with comparatively less activity in the cytosol fraction [20]. The present report describes differential responses of molecular forms of mitochondrial protein kinases to cAMP and their possible role in the phosphorylation of proteins in the host liver. It was also observed that these forms were differing from their counterparts isolated from the normal liver.

Materials and Methods

Materials. Casein (Hammersten) was purchased from Mann Research Laboratories Inc., New York, and protamine sulfate, bovine serum albumin, disodium salt of ATP, adenosine 3′,5′-monophosphate (cAMP) and DEAE-cellulose (anion exchanger, capacity 0.95 meq/g) from Sigma Chemicals Co., U.S.A. Phosvitin was isolated from fresh hen egg yolk by the method of Ramachandran and Sampathkumar [21]. Histone subfractions F1, F2a, F2b and F3 were isolated from rat liver nuclei by the procedure of Johns [22].

Adenosine triphosphate (ATP) labelled with ^{32}P in γ-position was prepared as described by Pawse et al. [23].

Preparation of protein kinase. Male rats (Wistar) of 175—200 g body weight fed on stock laboratory diet were used in these investigations.

Yoshida Sarcoma (ascites) tumour strains were obtained from the collection maintained at the Cancer Research Institute, Tata Memorial Centre, Bombay. The tumour in male rats was maintained by the intraperitoneal transplantation of the ascites fluid on every fourth day.

Protein kinase was prepared from liver mitochondria of the tumour-bearing rats or from control rats by the procedure reported earlier [20]. The entire mitochondrial fraction was free of cytosol contamination as assessed by the absence of the activity of marker enzyme lactate dehydrogenase.

Assay of protein kinase. The assay conditions employed were as described earlier [20]. The system contained, in a total volume of 1.0 ml, 2 mg of the substrate, 50 μmol of Tris · HCl buffer pH 7.4, 1.0 μmol [γ-^{32}P]ATP ($1—2 \cdot 10^5$ cpm/μmole of phosphate), 0.4 μmol of theophylline, 1 μM cAMP (when added) and the enzyme containing 20—45 μg of protein. The reaction was carried out at 37° C for 15 min and stopped with trichloroacetic acid at 25% final concentration. The activity of the enzyme has been expressed as nmol of phosphorous incorporated into the recovered protein and specific activity as the

transfer of radioactive phosphate catalysed by 1 mg of the enzyme protein in 15 min incubation time.

Analytical ultracentrifugation. Sedimentation velocity experiments were performed with a Beckman model E ultracentrifuge equipped with Schlieren optics in An-D rotor at 59 780 rev./min at 20° C. Photographs of the sedimenting patterns were taken at various time intervals after the rotor reached constant speed.

Molecular weight determination. The molecular weight of protein kinases was estimated by gel filtration on Sephadex G-150 by the method of Andrews [24]. The Sephadex column (93 × 2.5 cm) equilibrated with 0.05 M Tris · HCl buffer, pH 7.4 was calibrated by using standard proteins including aldolase, bovine serum albumin, ovalbumin, trypsin inhibitor and cytochrome *c*.

Protein concentration was estimated either spectrophotometrically at 280 nm or by the method of Lowry et al. [25]

Results

Multiplicity of protein kinase in host liver mitochondria

The recoveries of the protein and the enzyme activity obtained during purification are summarised in Table I. Protein kinase was released from the mitochondrial membrane by ultrasonication and purified by procedures described earlier [20]. The treatment of mitochondria with 0.1% detergent or with high-ionic-strength buffer did not cause release of the activity [20] thus excluding the possibility of the cytosol enzyme bound to the particulate fraction as observed by Keely et al. [26]. Two different proteins such as histone F1 and casein were employed as substrates for the enzyme assay in presence and absence of cAMP to evaluate the extent of purification at each stage. The crude extract obtained with ultrasonicated mitochondria did not respond to the action of cAMP. The stimulatory influence of this compound was, however, detected on further precipitation with ammonium sulphate. Fig. 1(A) shows the elution profile on DEAE-cellulose of the preparation obtained at 0—50% saturation of ammonium sulphate. The enzyme activity was resolved into two distinct peaks at 0.13 and 0.31 M concentration of NaCl as reported earlier [20]. These fractions were arbitrarily called as kinase I and kinase II respectively. The former was stimulated 2—3-fold by cAMP and preferentially phosphorylated histone F1 while the latter was cAMP-independent and had substrate specificity towards acidic phosphoproteins, phosvitin and casein. The extent of purification as well as the enzyme recoveries therefore appeared different with respective substrates. Thus protein kinase I showed 274-fold purification in the presence of cAMP as compared to 142-fold purification of kinase II. The specific activity of kinase II with casein as substrate was found to be more than that of kinase I with histone F1 as substrate.

The activities of kinase I and II were tested with various substrates such as histone fractions, protamine and acidic phosphoproteins namely casein and phosvitin as shown in Table II. As already stated above, kinase I showed preferential activity for histone F1 as compared to other proteins. The extent of stimulation of this reaction by cAMP was more than 2-fold with histone F1 though enhancement in the enzyme activity by this nucleotide was also ob-

TABLE I

PURIFICATION OF PROTEIN KINASES FROM LIVER MITOCHONDRIA OF TUMOUR-BEARING RATS

The details of the purification procedure have been published earlier [20]. The activity was assayed using histone F1 and casein as phosphate recipients as stated in the text. The specific activity of the enzyme is expressed as nmol of phosphorus incorporated into substrate proteins/mg of enzyme proteins/15 min incubation time. The values in parentheses are those obtained when casein was employed as the substrate. The enzyme recoveries are calculated from the total activity observed with these respective substrates.

Fraction	Total protein (mg)	Total activity		Specific activity		Extent of purification		Recovery (%)	
		—cAMP	+cAMP	—cAMP	+cAMP	—cAMP	+cAMP	—cAMP	+cAMP
Mitochondrial suspension	950	19.95 (93.1)	27.55 (120.7)	0.021 (0.098)	0.029 (0.127)	—	—	—	—
Ultrasonicated extract	375	214.5 (452.3)	241.1 (533.6)	0.572 (1.206)	0.643 (1.423)	27.2 (12.3)	22.2 (11.2)	100 (100)	100 (100)
Ammonium sulphate fractionation (0—50% saturation)	120	124.8 (325.7)	215.0 (369.2)	1.040 (2.714)	1.792 (3.077)	49.5 (27.7)	61.8 (24.2)	58.1 (72)	89.1 (69.1)
DEAE-cellulose chromatography									
Protein kinase I	6.2	20.9 (8.0)	49.4 (12.62)	3.371 (1.285)	7.965 (2.036)	160.5 (16.0)	274.7 (16.0)	9.7 (1.7)	20.4 (2.3)
Protein kinase II	10.4	2.1 (145.3)	2.1 (145.9)	0.198 (13.971)	0.201 (14.024)	9.4 (142.6)	6.9 (142.6)	0.99 (32.1)	0.8 (27.3)

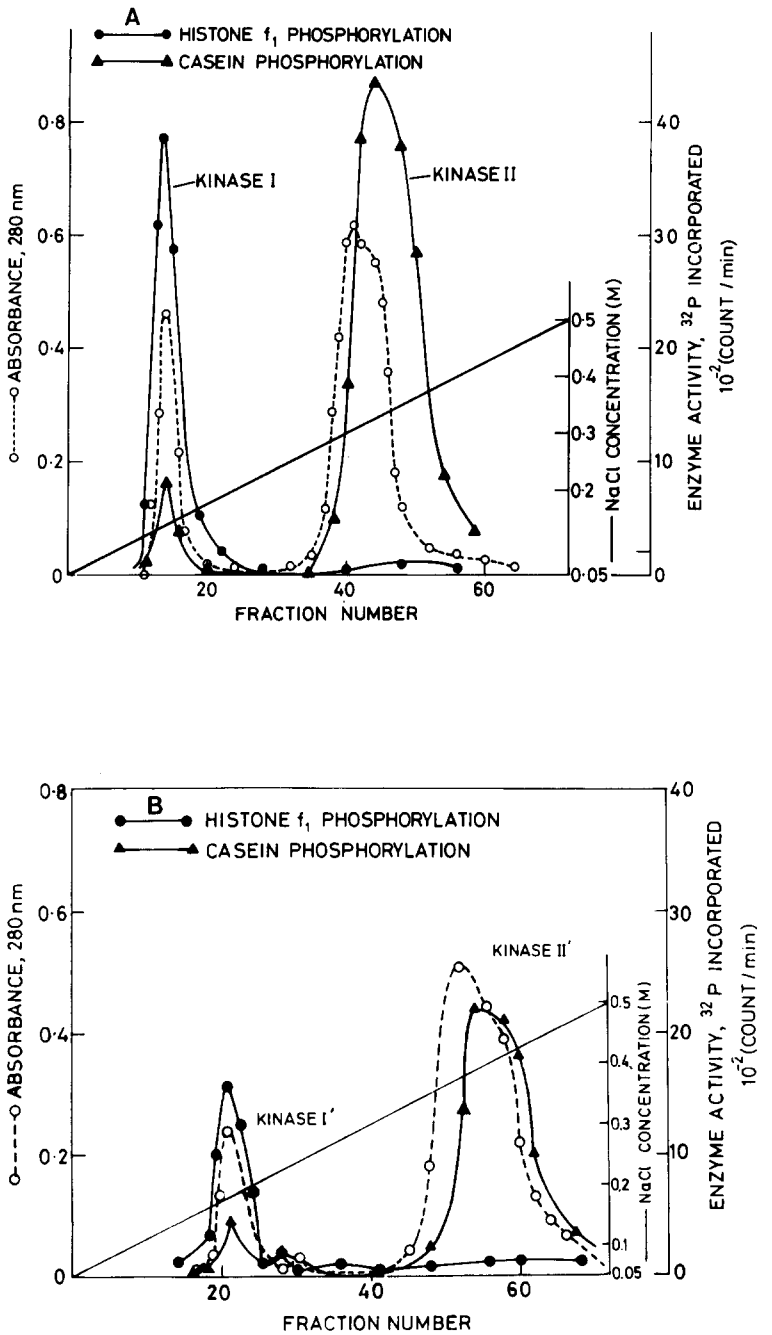

Fig. 1. DEAE-cellulose chromatography of host liver (A) and normal rat liver (B) mitochondrial protein kinases. The enzyme preparation, 50 mg/ml for A and 30 mg/ml for B obtained after ammonium sulfate precipitation (0—50% saturation) and subsequent dialysis, was applied to a column (2 × 60 cm) of DEAE-cellulose equilibrated with 0.05 M Tris · HCl buffer pH 7.4. An increasing gradient of NaCl (0.05—0.5 M) was set up for elution and the eluate was collected in 3.5 ml fractions, aliquots of which were assayed for protein concentration and the kinase activity in presence of cAMP with histone F1 or casein as described in the text.

served with other substrates though to a lesser extent. Kinase II catalysed the transfer of phosphate into casein and phosvitin and did not require cAMP for maximal activity. Both the preparations were inactive towards protamine sulfate and serum albumin, although they exhibited differences in specificity towards basic or acidic proteins as substrates.

Variations in normal liver mitochondria

The elution pattern of the enzyme from normal rat liver mitochondria on DEAE-cellulose chromatography is shown in Fig. 1(B). The enzyme resolved into two peaks eluting at 0.18 and 0.39 M NaCl concentration under similar conditions. The fractions from normal rat liver mitochondria were designated as kinases I′ and II′ respectively.

The activity profile of kinase II′ did not coincide with the protein peak (Fig. 1B) thus indicating that this preparation was still heterogeneous. On the other hand, kinase I′ was observed to be homogeneous on polyacrylamide gel electrophoresis (Fig. 2) though the electrophoretic mobility of kinase I′ was different from that of kinase I from host liver. Kinases I′ and II′ acted similarly in respect of substrate specificity, phosphorylating histone F1 and casein respectively. However, the cAMP dependency of kinase I′ examined with different substrates showed that this was not stimulated in contrast to kinase I of host liver (Table II).

Properties of protein kinases

The extent of stimulation of kinase I from host liver by cAMP is shown in Fig. 3 with maximal at $1 \cdot 10^{-6}$ M concentration, with both the substrate viz., histone F1 and casein. The response was sigmoidal with respect to cAMP concentrations. However, the nucleotide did not stimulate protein kinase II activity (Fig. 3). Also, there was no measurable phosphate incorporation into histone F1 with this preparation.

Fig. 4 shows the effect of cAMP on the activities of kinase I and kinase II observed as a function of pH employing Tris · maleate and Tris · HCl buffers re-

TABLE II

SUBSTRATE SPECIFICITY OF MITOCHONDRIAL PROTEIN KINASE

The details of assay system are stated in the text. 2 mg of each of these substrate proteins were employed in the reaction mixture and the specific activity of the enzyme obtained is expressed in this table. Figures in parentheses indicate the extent of stimulation by cAMP as percentage of original activity.

Substrate	Host liver				Normal liver			
	Kinase I		Kinase II		Kinase I′		Kinase II′	
	−cAMP	+cAMP	−cAMP	+cAMP	−cAMP	+cAMP	−cAMP	+cAMP
Phosvitin	1.66	2.55(153)	16.03	17.32(108)	0.79	0.70(88)	3.91	3.85(98)
Casein	1.51	2.13(141)	13.40	12.68(94)	0.94	0.90(95)	3.47	3.64(104)
Histone F1	3.42	7.62(222)	0.18	0.20(111)	1.63	1.71(104)	0.31	0.21(68)
Histone F2a	0.93	1.24(133)	Nil	Nil	0.26	0.24(92)	Nil	Nil
Histone F2b	1.88	3.59(190)	0.20	0.22(110)	0.09	0.07(77)	Nil	Nil
Histone F3	0.63	0.81(132)	Nil	Nil	0.19	0.14(73)	0.07	0.07(100)

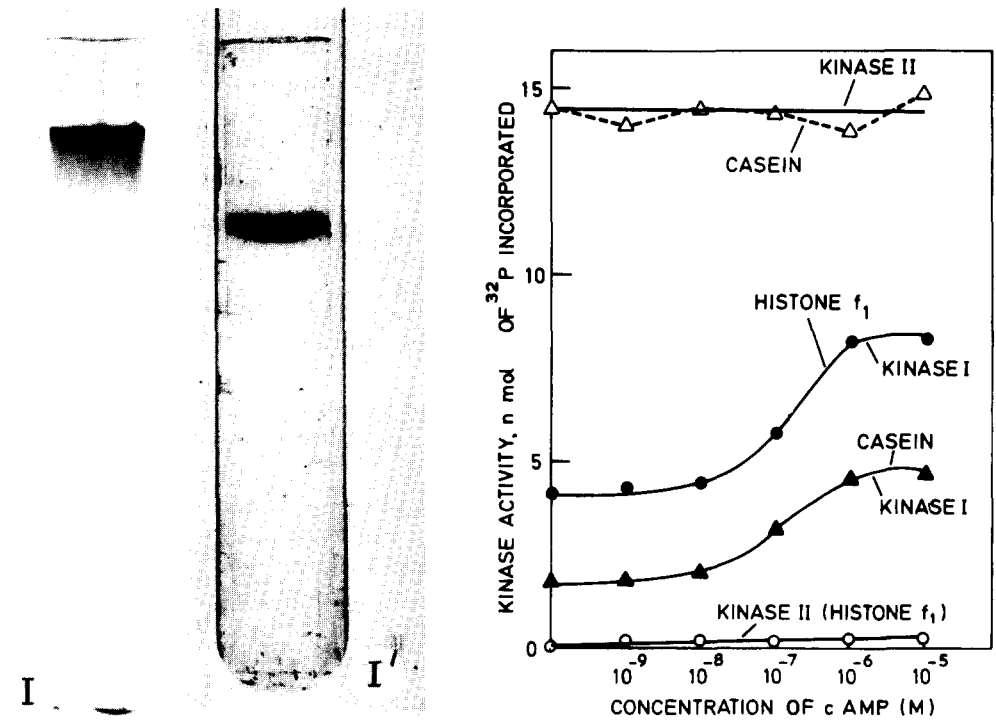

Fig. 2. Acrylamide gel electrophoresis of purified liver mitochondrial kinase of ascites tumour-bearing rat (kinase I) and normal rat (kinase I'). Analytical gel electrophoresis was performed as described by Davis [27] at 3 mA/tube for 40 min by using gels containing 7% (w/v) final concentration of polyacrylamide.

Fig. 3. Effect of varying concentrations of cAMP on phosphorylation by protein kinase fractions. Histone F1 and casein were employed as substrates for kinases I and II. Other assay conditions were as stated in the text.

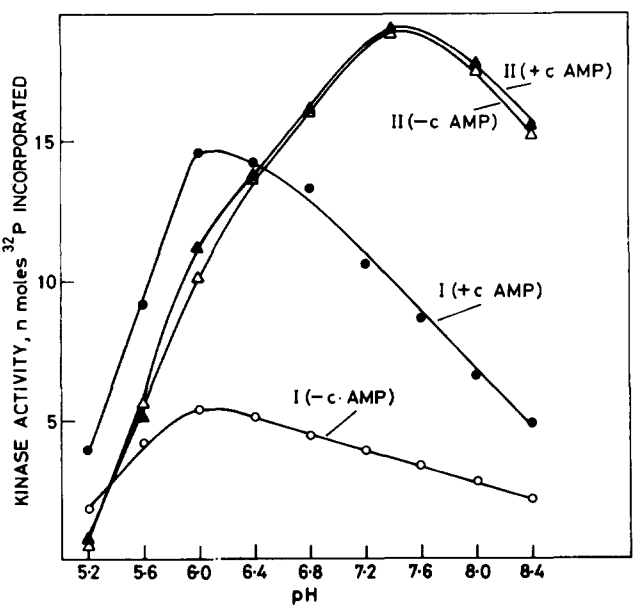

Fig. 4. Effect of pH on activity of protein kinases I and II with histone F1 and casein respectively as substrates in presence and absence of cAMP.

spectively in the range of 5.5—8.4. Kinase I had an optimum at pH 6.0 whereas kinase II showed maximal activity at pH 7.4. The pH optima for normal rat liver, kinases I′ and II′ were similar to those of the corresponding forms in host liver. The optimal pH of cAMP-dependent protein kinase I with respect to the substrate histone was slightly lower than that reported by Corbin et al. [28] for adipose tissue kinase but similar to that of mammary gland enzyme [4]. Most of the experiments with kinase I and kinase II were therefore carried out at pH 6.0 and 7.4 respectively. The activities of both the preparations were linear with the time of incubation up to 30 min. In the case of kinase II, however, the activity tended to fall after this period. The extent of phosphate incorporation into the respective substrates was also linear with the concentrations (0—100 μg) of the enzyme protein with both these preparations.

The apparent K_m values of Kinase I for histone F1 in presence and absence of cAMP were 0.71 and 0.45 mg/ml respectively, while that of kinase II with casein as substrate was 0.83 mg/ml.

The divalent cation (Mg^{2+}) required by protein kinase could be replaced by Mn^{2+} or Fe^{2+} to obtain activities to the extent of 95 and 45% respectively. The activity of kinase I from the host liver was observed to be stimulated by Co^{2+} to the same magnitude as that obtained with cAMP. Co^{2+} did not cause stimulation of kinase I′ from normal rat liver.

Aliquots of the enzyme (0.1 ml) were kept at two different temperatures (40 and 50°C) maintained in a water bath for 15 min and the tubes containing the aliquots were chilled quickly by keeping in an ice-bucket at 0°C. The activity was tested in the usual assay system and the data obtained are presented in Fig. 5. Protein kinase II was relatively more stable than protein kinase I even at 50°C. The sensitivity of kinase I towards heat was greater when cAMP was added to the system. This is in contrast to kinase I′ of normal rat liver which was more heat-stable than kinase I even in presence of cAMP.

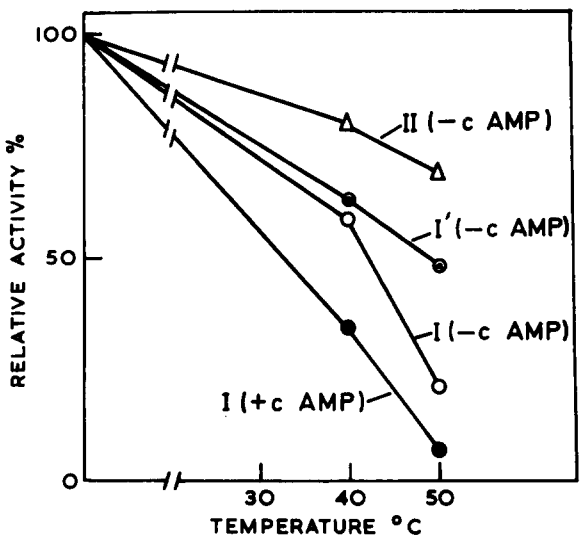

Fig. 5. Effect of temperature on protein kinase I, I′ and II.

Structural aspects of kinase I of host liver

Kinase I, which was sensitive to cAMP was used to study the structural aspects. This preparation found to be homogeneous by various criteria of purity such as rechromatography on DEAE-cellulose, absorption spectra and polyacrylamide gel electrophoresis, was taken up for its dissociation by cAMP [29]. The enzyme was applied to a column of DEAE-cellulose (1 × 3 cm), previously equilibrated with 0.05 M Tris · HCl buffer, pH 7.4. After washing with 20 ml of the buffer, the catalytic protein was eluted with 10 ml of the buffer containing 10^{-5} M cAMP. Residual cAMP was then washed off the resin with 20 ml of 0.12 M Tris · HCl buffer, pH 7.4. The protein binding with cAMP was then eluted with 10 ml of 0.3 M Tris · HCl buffer, pH 7.4. The two protein components were then separately dialysed against four changes of 0.05 M Tris · HCl buffer, pH 7.4.

Protein kinase I, preincubated with 10^{-5} M of cAMP prior to DEAE-cellulose chromatography exhibited two protein components on ultracentrifugation as shown in Fig. 6 with $S_{20,w}$ values of 4.07 and 5.24 respectively. The catalytically active component of the enzyme obtained by subsequent DEAE-cellulose chromatography gave a single symmetrical peak with $S_{20,w}$ value of 4.1. The addition of 10 μmol of Co^{2+} to kinase I resulted in partial precipitation of the protein. It was however observed that protein kinase activity with histone F1 was contained in supernatant while the precipitate did not show any activity. The supernatant fraction of Co^{2+}-treated kinase I on ultracentrifugation showed a single component differing in its $S_{20,w}$ value of 4.9. The catalytically active fraction retained its substrate specificity, preferentially phosphorylating histone F1 and was not stimulated by the addition of cAMP.

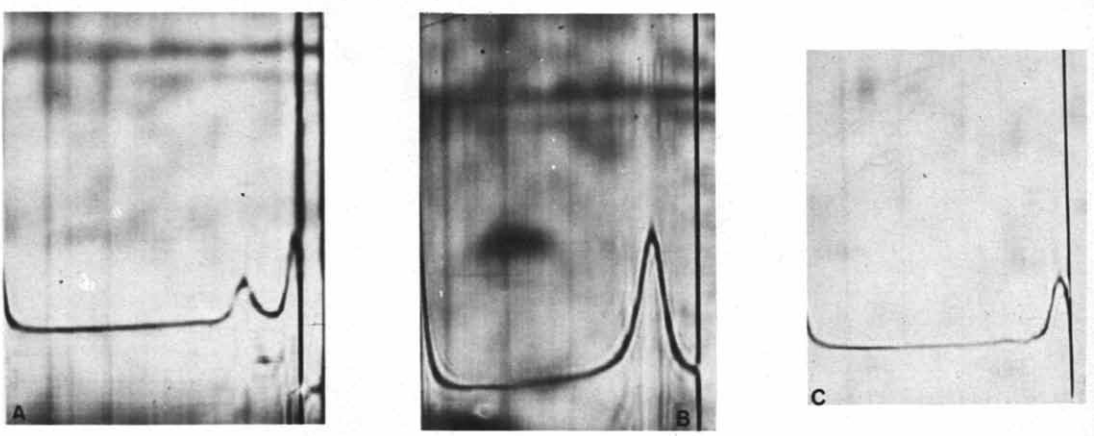

Fig. 6. Sedimentation velocity patterns of kinase I subunit fractions. The sedimentation movements shown, was right to left and the temperature during the run was 20° C. (A) Kinase I (2.5 mg/ml) preincubated with 10^{-5} M cAMP in 0.05 M Tris · HCl buffer, pH 7.4; (B) catalytically active component (3.7. mg/ml) obtained after cAMP treatment and subsequent DEAE-cellulose chromatography; and (C) catalytically active solution (2.5 mg/ml) obtained after treatment with 10 μmol of Co^{2+} ions.

TABLE III

MOLECULAR WEIGHT DETERMINATION OF MITOCHONDRIAL PROTEIN KINASE

Molecular weight was determined by Sephadex G-150 gel-filtration technique.

Preparation	Molecular weight
Kinase I (undissociated)	170 000
Catalytic unit	2 × 37 500
Regulatory unit	86 000
Kinase II	54 000

The molecular weights of enzymatically active and inactive components of kinase I, obtained by cAMP dissociation, were found to be 37 500 and 86 000 respectively by Sephadex G-150 gel filtration technique (Table III). The enzyme before dissociation was estimated to have molecular weight of 170 000 (Fig. 7). The catalytically active fraction obtained after Co^{2+} dissociation had the molecular weight of 76 000, suggesting that this could be a dimer.

Though the cAMP independent kinase II was heterogeneous on polyacrylamide gel electrophoresis, the molecular weight of this preparation was estimated to be only 54 000.

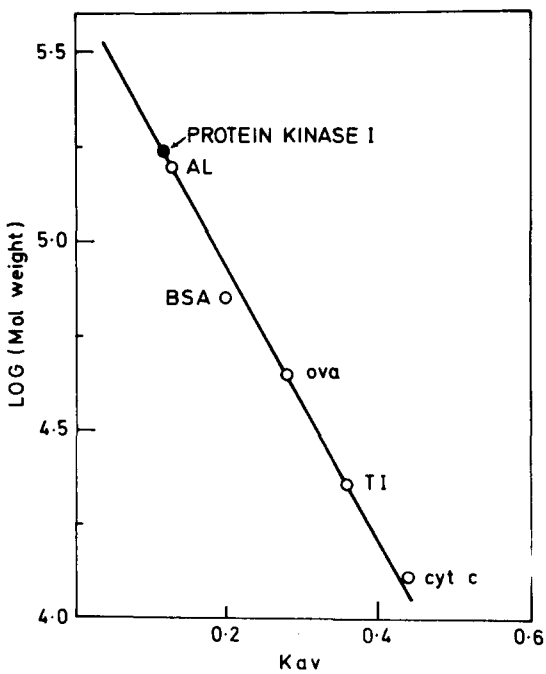

Fig. 7. Molecular weight determination of mitochondrial protein kinases of host liver by Sephadex G-150 gel filtration. BSA, bovine serum albumin; ova, ovalbumin; cyt c, cytochrome *c*; TI,

Discussion

The foregoing results point to the multiplicity of rat liver mitochondrial protein kinase which responded differentially to cAMP. It is interesting to note that kinases I and II have different substrate specificities and kinetic properties. These forms also showed subtle variations as compared to the corresponding forms in the normal system. The responses to cAMP of kinase I appeared to be similar to those reported for the enzyme from other tissues [3,28]. The sigmoidal activation of this preparation in relation to concentration of cAMP points to subunit interactions, probably similar to that reported for rabbit skeletal muscle enzyme [8]. The activation by cAMP of kinase I was most effective with histone as substrate, though the observed residual activity of this preparation with other proteins was also stimulated by the cyclic nucleotide. The activity of kinase II on the other hand was not dependent of cAMP and this preparation phosphorylated only acidic proteins. There was no evidence to show that kinase II was a subunit of protein kinase I.

Rats undergoing malignancy were used in these studies since liver mitochondria of these animals exhibited high protein kinase activity [20]. Chen and Walsh [30] in their studies on intracellular localisation of protein kinase in rat liver, had not detected the activity in the mitochondrial fraction. This could be ascribed to their using histone F2b as substrate for assaying the enzyme. It is apparent from the present studies that one of the forms did not act on this substrate while the other showed comparatively low activity.

Kinase I, sensitive to cAMP was observed to dissociate into catalytically active and inactive components either by cAMP or by Co^{2+} ions. Studies on protein kinases from other sources have suggested that the number of catalytic subunits could vary while there was only one regulatory subunit [3,29]. The ultracentrifugation studies on kinase I in presence of cAMP also indicated presence of two protein components. The addition of Co^{2+} to the enzyme caused precipitation of enzymatically inactive protein, leaving the active component in solution thus showing only one peak in the ultracentrifugation pattern. It has been reported with other kinases that compounds like histones, monovalent ions, alkali and chaotropic salts can also cause dissociation of the enzyme [31—33]. Protein kinases are known to have a high affinity for cAMP, binding firmly and irreversibly to the regulatory component [3,8]. The effect with Co^{2+} also appeared to be similar and it was not possible to reassociate the fraction precipitated by the metal ions with the active component in solution.

Protein kinase I′ from normal rat liver mitochondria, despite showing similarity with its counterpart in the host liver, in respect of substrate specificity, responded differently to cAMP. The protein kinases of hepatoma tissue culture cells have also been reported to be less responsive to cAMP [34]. It has been suggested that malignancy is associated with low effective levels of cAMP [35]. The cytosol fractions of hepatoma cells was also deficient in cAMP binding sites [34,36]. The present results indicate that propagation of Yoshida ascites tumour cells in rats could cause alterations in the charge of protein kinase I which reflected in some of the functional characteristics. A shift in the elution profile of protein kinase has been reported by Criss et al. [37] in Morris hepatoma, while the present work was in progress. The presence of a unique nuclear

protein kinase fraction and a specific phosphoprotein substrate in neoplastic tissue nuclei, which are not found in normal rat liver, have also been reported [38]. There is increasing recognition that neoplasia can cause profound alterations in liver isozyme patterns [39]. Nuclear phosphoproteins have been shown to have involvement in gene regulation [40]. Similar situations may arise in view of the existence of distinct species of DNA [41] and phosphoproteins in mitochondria [2,42—44]. The modifications of protein kinases observed in the present studies may therefore have some bearing on protein synthesis in that organelle during the propagation of tumour cells.

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