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STUDIES ON A CYCLIC AMP-DEPENDENT PROTEIN KINASE FROM RAT THYROID GLAND

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Summary

We have confirmed the presence of cyclic AMP-dependent protein kinase activity (ATP:protein phosphotransferase, EC 2.7.1.37) in the soluble protein fraction of rat thyroid homogenates, and have purified the enzyme with a 20% yield by gel filtration on Sephadex G-200 followed by ion exchange chromatography on DEAE-cellulose. Kinase activity was assayed in a standard system with [γ-^{32}P]ATP and a mixed fraction of calf thymus histones at pH 6.5, 30°C, in the presence and absence of cyclic AMP. Two peaks of cyclic AMP-dependent protein kinase activity were resolved by gel filtration and each was further purified on DEAE-cellulose and designated DK_I and DK_{II}: specific activities were, respectively, 5171 and 2080 units/mg protein, representing an approximate 700-fold purification of activity based on assay of the crude extracts. However, crude thyroid extracts reveal only 1/10 of their inherent kinase activity, probably due to the presence of ATPase activity, and in addition, to interaction of the large amounts of thyroglobulin with the histone phosphoryl acceptor. A unit of activity is defined as 1 pmol phosphate transferred from phosphoryl donor to acceptor per min. The two kinases were stable for up to 1 month when stored at 4°C. The K_m with respect to cyclic AMP is $6.1 \cdot 10^{-8}$ M for DK_I and $3.1 \cdot 10^{-8}$ M for DK_{II}, and with respect to ATP, in the presence and absence of cyclic AMP ($1 \cdot 10^{-6}$ M), respectively, is $1.0 \cdot 10^{-5}$ M and $1.2 \cdot 10^{-5}$ M for DK_I, and $0.93 \cdot 10^{-5}$ M and $0.75 \cdot 10^{-5}$ M for DK_{II}. These values are similar to those reported for cyclic nucleotide dependent protein kinases from other tissues. Molecular weights estimated by gel filtration are 230 000 and 152 000 for DK_I and DK_{II} respectively. Sedimentation coefficients of 8.7 for DK_I and 6.6 for DK_{II} were estimated by centrifugation of the material in the linear sucrose density gradients. Enzymatic profiles of the gradients of the heavier kinase consistenly showed a small peak corresponding to 6.6 S. Although the data might suggest that multiple forms of protein kinase exist in the rat thyroid, they are also consistent with the presence of a kinase in several states of aggregation peculiar to the purification procedure. Both DK_I

and DK_{II} when incubated with cyclic AMP were converted to 4.8 S material that was essentially completely nucleotide independent, consistent with the well-described disaggregation of the catalytic subunit(s) from protein kinase holoenzyme.

Introduction

It is widely accepted that many hormonal effects are mediated by changes the intracellular concentration of cyclic 3′,5′-adenosine monophosphate [1]. Thyrotropin has been shown to alter the thyroidal content of cyclic AMP and its dibutyryl derivative can mimic the hormonal actions of Thryotropin in the thyroid gland [4—9]. The discovery of a cyclic AMP-dependent protein kinase (ATP:protein phosphotransferase, EC 2.7.1.37) in skeletal muscle [10] and subsequently in a wide variety of tissues [11] led to the proposal that the diverse actions of cyclic AMP are mediated through regulation of the activity of this enzyme. Cyclic AMP-dependent protein kinase has been identified and purified from the thyroids of several mammalian species [12—19] and its activation by the addition of thyrotropin to beef and sheep thyroid slices described [20]. The laboratory rat has been used extensively to study thyroid gland activity in a variety of physiological states and serves as a model of mammalian thyroid function. The present study confirms the presence of cyclic AMP-dependent protein kinase activity in the particulate-free extracts of rat thyroid gland, describes several factors present in the rat thyroid that influence the protein kinase activity and describes the partial purification and some properties of the cyclic AMP-dependent protein kinase from rat thyroid glands.

Materials and Methods

Thyroids were obtained from male Long-Evans rats, 70—120 days old. ^{32}P-labeled inorganic phosphate in dilute HCl was purchased from Amersham-Searle Corp. Mixed calf thymus histrones type II-A, protamine, α-casein, cyclic AMP, ATP (disodium salt) and theophylline were all purchased from Sigma Chemical Co. Sephadex G-200 and DEAE-cellulose (Whatman DE52) were purchased from Pharmacia Fine Chemicals and Reeve Angel Co. respectively.

Protein kinase assay

Cyclic AMP-dependent protein kinase activity was assayed by a modification of the procedure of Corbin and Reimann [21]. The total volume of the reaction mixture was 100 μl which in addition to enzyme contained the following components, unless otherwise indicated: glycerolphosphate buffer (50 mM, pH 6.5), magnesium acetate (10 mM), theophylline (2 mM), ethylene glycol bis(β-aminoethylether) *N,N′*-tetraacetic acid (0.3 mM, [γ-^{32}P]ATP (0.1 mM, $1 \cdot 10^6$ cpm), histone (100 μg) and, where indicated, cyclic AMP (1 μM). The reaction was initiated by the addition of enzyme after a 5-min pre-incubation of the other ingredients. The reaction was allowed to proceed in a shaking water bath at 30°C for 10 min and then a 50 μl sample of each reaction

mixture was pipetted onto a Whatman 3 mM filter paper disc (24 mm in diameter) which was immediately dropped into ice-cold 5% phosphotungstic acid (5—10 ml per disc) which was stirred at slowest speed of a magnetic stirrer for 15 min. This was followed by 3 washings with 5% phosphotungstic acid at room temperature, dehydration of the discs in 95% ethanol (5—10 ml per disc) for 5 min and finally in the same volume of petroleum ether for 5 min. The discs were dried in air and then counted in a Packard Tri-Carb liquid Scintillation Counter, each in 10 ml of toluene-base scintillation solution (5.0 g/l of 2,5-diphenyloxazole and 0.2 g/l of 1,4-bis-2-(5-phenyloxozolyl)benzene). Enzymatic activity was expressed as kinase units, one unit representing the transfer of 1 pmol of phosphate from phosphoryl donor to phosphoryl acceptor per min. Protein concentrations were determined by the method of Lowry [22] or by absorbance at 230 or at 280 nm.

Data shown in the illustrative material are typical of experiments each performed at least two and sometimes three times. The points in the figures are means of duplicate assays with the exception of data presented for the enzymatic profiles pertaining to the purification procedures, in which cases only single points assays were performed.

Rat thyroid protein kinase purification

100 male Long-Evans rats were lightly anesthetized with ether, bled by syringe from the abdominal aorta, and the thyroids were removed and dissected free of fat and parathyroid tissue. The thyroids were kept frozen on solid CO_2 during the collection period. Frozen rat thyroids could be stored for up to 2 weeks at −80°C with no loss of enzymatic activity. The thyroids were homogenized at 4°C in a motor-driven glass homogenizer with 2 vol. of a buffer solution pH 7.5 designated TEM, containing Tris (50 mM), ethylenediaminetetraacetic acid (EDTA) (1 mM) and 2-mercaptoethanol (15 mM). All subsequent purification steps were carried out at 4°C. The thyroid homogenate was centrifuged at 5 000 × *g* for 30 min in an SS-34 rotor in a Sorvall RC-2 centrifuge and the supernatant solution was collected and recentrifuged at 100 000 × *g* for 90 min in a SW 56 Ti rotor in a Beckman L5-65 Ultracentrifuge. The supernatant solution from the high speed spin was applied to a 2.6 × 55-cm Sephadex G-200 column equilibrated in TEM buffer, and descending chromatography initiated at a flow rate of 20 ml/h. Two peaks of cyclic AMP-dependent kinase activity were resolved and the fractions of each pooled, concentrated by ultrafiltration on a Diaflo PM-10 membrane filter, and rechromatographed separately on Sephadex G-200.

Each of the two rechromatographed kinases was concentrated by ultracentrifugation (at this stage of purification the active fractions could be stored at −20°C for up to 2 months with no detectable loss of activity), and then applied to a 0.9 × 20 cm DEAE-cellulose column equilibrated in TEM buffer. The column was washed with TEM buffer until the absorption of the eluate at 280 nanometers returned to baseline. Elution of the cyclic AMP-dependent kinase activity was achieved with a linear NaCl gradient (0—400 mM) in TEM buffer. The fractions containing enzyme activity were pooled, concentrated by ultracentrifugation and dialyzed to remove NaCl. (Cyclic AMP-dependent protein kinase activity could be kept at 4°C for up to one month with little loss of activity.)

Sucrose density gradient centrifugation

Enzyme samples of 100—300 μl were layered on linear sucrose density gradients (4.0 ml, 2—10% in TEM buffer) in nitro-cellulose tubes (Beckman Instrument Co.). Centrifugation, unless otherwise indicated, was in a Beckman SW 56 TI rotor at 45 000 rev./min for 16.8 h at 4°C in a L5-65 Beckman ultracentrifuge. After centrifugation the bottom of each centrifuge tube was pierced and 20 equal fractions collected. The fractions were assayed for protein kinase activity, and the sedimentation coefficients calculated by the method of McEwen [23]; this method in our hands gave *S*-values virtually the same as known values of five standard proteins.

Synthesis of [^{32}P]ATP

This was by the method of Post and Sen [24] and a modification by Walsh et al. [25]. $Mg_2 \cdot$ ATP and $Mg_2 \cdot$ ADP were incubated for 30 min in a reaction mixture containing $^{32}P_i$, 3-phosphoglycerate, 3-phosphoglyceraldehyde dehydrogenase, phosphoglycerate kinase and NAD. The [^{32}P]ATP formed was adsorbed to Norit A which was collected on millipore filters, eluted with 0.15 M NH_4OH in 50% ethanol and then concentrated in a rotary evaporator; the specific activity was approx. 5 Ci/mmol, less than 1% of the label was free inorganic phosphate and less than 1% was ADP. The labelled ATP was stored at −20°C.

Results

Protein kinase activity was assayable in the soluble fraction of rat thyroid glands and was stimulated 2- to 4-fold by the addition of cyclic AMP ($1 \cdot 10^{-6}$ M). However, large amounts of flocculent precipitate were present in the reaction tubes containing thyroid cytosol and histone. These precipitates were presumed to reflect the ionic interaction of polycationic histone with polyanionic thyroglobulin, both proteins present in abundance in the reaction vessels. Dilution of the 100 000 × *g* supernatant (1 : 10 or 1 : 100) reduced or eliminated the flocculent precipitate and resulted in increases in the apparent specific activity of the protein kinase with increase in the cyclic AMP-dependence from 4- to 12-fold (Table I). However, the small extent of labelling of the histone in assay mixtures of the 100-fold diluted extract made it difficult to calculate the enzymatic activity accurately. The findings in like experiments in which 10 mM NaF was added to the reaction mixture to inhibit ATPase were generally similar. The presence of NaF resulted in increases in the apparent total activity above that occurring as a result of dilution. Experiments in which purified thyroglobulin was added to the reaction mixtures showed that thyroglobulin depressed the phosphorylating ability of rat thyroid protein kinase in the standard kinase assay (McIlroy, P.J., personal communication). To look for evidence of ATPase activity in the 100 000 × *g* supernatant the reaction mixtures were examined for the presence of $^{32}P_i$ at the end of the standard kinase assay. To 50 μl of the reaction mixture sufficient Norit A was added in the presence of 5% phosphotungstic acid to adsorb residual ATP and precipitate the phosphorylated proteins. The mixtures were then centrifuged to pellet the Norit and precipitated proteins and an aliquot of the clear supernatant

TABLE I

KINASE ACTIVITY IN CRUDE EXTRACTS OF RAT THYROID GLAND

Cyclic AMP-dependent kinase activity is expressed as kinase units, 1 unit representing the transfer of 1 pmol of phosphate per min. Histone concentration was 100 μg/100 μl. ATP concentration was $1 \cdot 10^{-4}$ M. Data reported as means of duplicate determinations.

	Cyclic AMP-dependent protein kinase (units/mg protein)			
	NaF (10 mM)		—	
	$1 \cdot 10^{-6}$ M cyclic AMP	—	$1 \cdot 10^{-6}$ m cyclic AMP	—
Thyroid extract (from 2.54 g tissue)	9.83	2.52	2.83	0.53
1 : 10 dilution thyroid extract	80.48	7.21	60.39	4.84
1 : 100 dilution	123.69	13.16	122.11	16.32

counted for ^{32}P by liquid scintillation counting (see Materials and Methods). Initially all of the ^{32}P label was present as γ-labeled [^{32}P]ATP and the presence of $^{32}P_i$ indicates ATP degradation (Table II). Appropriate correction was made for the very small amount of $^{32}P_i$ initially contaminating the [^{32}P]ATP stock. In the absence of NaF, the very dilute thyroid 100 000 × *g* supernatant showed 96% of the ^{32}P label as P_i. 10 mM NaF only partially inhibited the conversion of labeled ATP to $^{32}P_i$ at all dilutions of the thyroid 100 000 × *g* supernatant. These data show that sufficient ATPase activity, partially inhibitable by 10 mM NaF, is present in the soluble fraction of rat thyroid gland to significantly alter the ATP concentration during the 10-min course of the protein kinase assay. Addtionally, it would appear that sufficient thyroglobulin is present to sequester quantities of the phosphoryl acceptor protein (histone) and depress the observable protein kinase activity. To study the rat thyroidal protein kinase unobscured by the "inhibiting" factors present in the 100 000 × *g* supernatant of thyroid glands purification of the activity was attempted.

Table III presents the protocol and typical results of the purification procedure adopted. To arrive at an estimate of the total kinase activity in the

TABLE II

DISTRIBUTION OF ^{32}P LABEL AS [^{32}P]ATP AND $^{32}P_i$ AFTER 10-min STANDARD KINASE ASSAY

Initial concentration of [γ-^{32}P]ATP was $1 \cdot 10^{-4}$ M. [^{32}P]ATP was determined by subtraction of the $^{32}P_i$ not absorbed to Norit A from the total ^{32}P label present at the end of the kinase assay in the presence of $1 \cdot 10^{-6}$ M cyclic AMP. Data reported as means of quadruplicate determinations ±S.E.

	NaF (10 mM)		—	
	[^{32}P]ATP (M) × 10^5	$^{32}P_i$ (M) × 10^5	[^{32}P]ATP (M) × 10^5	$^{32}P_i$ (M) × 10^5
Thyroid extract (from 2.54 g tissue)	0.99 ± 0.42	8.5 ± 0.4	0.67 ± 0.35	9.6 ± 0.3
1 : 10 dilution thyroid extract	5.80 ± 0.35	3.8 ± 0.4	3.46 ± 0.22	6.5 ± 0.2
1 : 100 dilution	9.37 ± 0.08	0.6 ± 0.08	9.00 ± 0.08	1.0 ± 0.08

TABLE III

PURIFICATION OF CYCLIC AMP-DEPENDENT PROTEIN KINASE OF RAT THYROID

Concentration of cyclic AMP, when used, was $1 \cdot 10^{-6}$ M, that of histone 100 μg/100 μl, and of ATP $1 \cdot 10^{-4}$ M. 1 unit of kinase activity represents 1 pmol of phosphate transferred per min. These are the results of a typical representative experiment, and the values are the means of duplicate determinations.

	Volume (ml)	Protein (mg)	Cyclic AMP-dependent protein kinase (units)	Specific activities (units/mg protein)			Fold purification	Recovery (%)
				Basal (—cyclic AMP)	Total (+cyclic AMP)	cyclic AMP-dependent		
1. 100 000 × *g* supernatant from 2.54 *g* tissue	4.0	432		11 *	86 *	75 *		
2. Sephadex G-200								
K_I	4.3	43	20 163	65	533	468	6	63
K_{II}	4.3	25	12 064	81	566	485	6	37
3. Rechromatography Sephadex G-200								
K_I	4.3	16.7	20 359	161	1 380	1 219	16	63
K_{II}	4.3	6.4	9 532	306	1 795	1 489	20	30
4. DEAE-cellulose								
DK_I	4.0	1.1	5 688	783	5 954	5 171	70	18
DK_{II}	3.5	0.5	1 040	332	2 412	2 080	28	3

* Estimated specific enzyme activity calculated from the line 2 of this table with the assumption that gel filtration on Sephadex allowed full recovery of all of the activity of crude extract. See text.

soluble fraction of the thyroid unobscured by the inhibiting effects of histone-thryroglobulin complexes and ATPases, we make the simplifying assumption that the initial gel filtration allows quantitative recovery of the kinase and that the enzyme activity is fully expressed. The total number of kinase units present after gel filtration divided by the initial protein concentration is referred to as the estimated specific enzyme activity of the 100 000 × *g* supernatant. Initial gel filtration on Sephadex G-200 (Fig. 1) resolved two cyclic AMP-dependent protein kinases designated K_I and K_{II} with apparent molecular weights, respectively, of 230 000 and 152 000, representing increases in specific enzyme activith of approx. 7-fold over the corrected specific enzyme activity of the crude extract. The pooled and concentrated active fractions K_I and K_{II} were rechromatographed, separately, on the Sephadex G-200 column which resulted in a further 2.5-fold increase in specific activities, with little loss of total activity. The highly cyclic AMP-dependent kinases were further purified separately by ion-exchange chromatography on DEAE-cellulose (Fig. 2). Each kinase appeared as a single peak of activity with the peak fraction eluting at 150 mM NaCl in TEM buffer. These fractions were designated DK_I and DK_{II} and had specific activities, respectively, of 5171 units/mg and 2082 units/mg representing a 70-fold purification of the corrected activity of the crude extract. No ATPase activity could be detected in the purified kinases. A comparison of the specific activities of these purified enzymes directly with that of the undiluted extract would mistakenly indicate a purification of approx. 700-fold. The DK_I and DK_{II} enzymes showed only very minimal loss of activity when stored for up to one month at 4° C.

The rate of ^{32}P incorporation catalyzed by the purified kinase DK_I as the

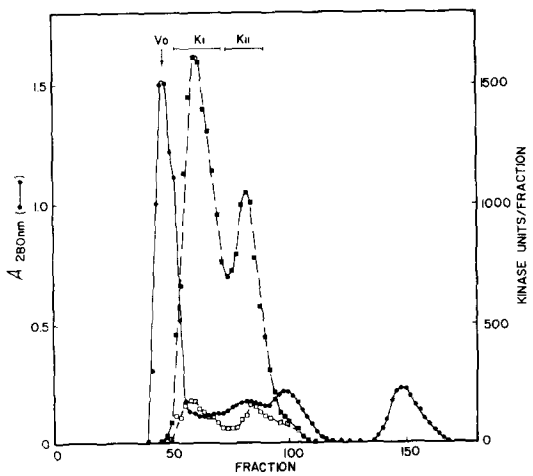

Fig. 1. Sephadex G-200 gel filtration of 100 000 × *g* supernatant of rat thyroid homogenate. 4.0 ml representing extract from 100 rat thyroids was applied to a 2.6 × 55 cm column equilibrated with TEM buffer pH 7.5 at 4°C. Descending chromatography with TEM buffer at a flow rate of 20 ml/h was initiated and 2-ml fractions collected. The 2 peaks of cyclic AMP-dependent kinase activity were designated K_I and K_{II}; fractions of each were pooled as indicated and concentrated by ultrafiltration on a PM-10 Diaflo membrane filter. V_0 indicates the column void volume. All procedures were carried out at 4°C. 1 unit of kinase activity represents 1 pmol transferred per min. ■, +cyclic AMP; □, −cyclic AMP.

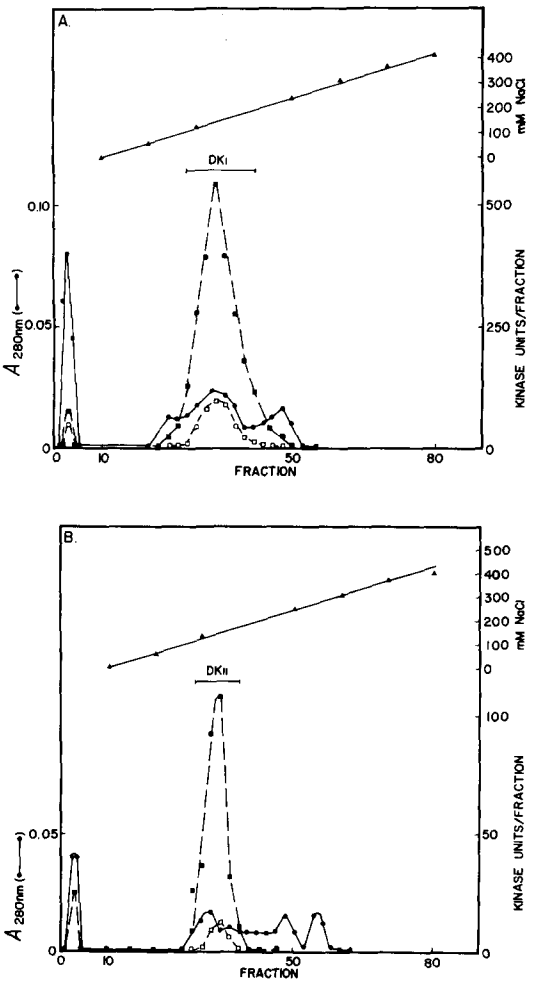

Fig. 2. DEAE-cellulose chromatography of protein kinases K_I and K_{II} resolved on Sephadex G-200 and rechromatographed on the same column. DEAE-cellulose was equilibrated in TEM buffer pH 7.5 at 4°C. Each kinase was applied separately (2A refers to rechromatographed K_I and 2B to rechromatographed K_{II}) to a 0.9 X 20 cm column, the column washed at a flow rate of 30 ml/h until the A_{280} was near baseline, and then developed with a 0—400 mM NaCl linear gradient in TEM buffer. Fractions containing activity were pooled, dialyzed against TEM buffer, concentrated by ultracentrifugation on a PM-10 Diaflo filter and designated DK_I and DK_{II}. Fraction volumes of 5 ml were collected during sample application and subsequent column washing, and of 3 ml during linear salt gradient elution. 1 unit of kinase activity represents 1 pmol phosphate transferred per min. ■, +cyclic AMP; □, —cyclic AMP.

concentration of histone was altered showed saturation kinetics with a K_m for histone of 100 μg/100 μl calculated from data plotted as reciprocals according to the Lineweaver-Burk equation. The enzymatic activity of kinase DK_I under standard conditions was a linear function of enzyme concentration up to 12.5 mg. protein/100 μl.

When ATP concentration was varied from $3 \cdot 10^{-7}$ M to $1 \cdot 10^{-3}$ M, K_m for DK_I with respect to ATP (calculated from a Lineweaver-Burk plot) is $1 \cdot 10^{-5}$ M in the presence of cyclic AMP and $1.2 \cdot 10^{-5}$ M in the absence of the

cyclic nucleotide. For DK_{II} the corresponding values are $0.93 \cdot 10^{-5}$ and $0.75 \cdot 10^{-5}$ M.

The enzymatic activity of DK_I and DK_{II} as the concentration of cyclic AMP was varied from 10^{-10} to 10^{-4} M in the presence of saturating concentrations of ATP followed Michaelis-Menten kinetics. Values of K_m for DK_I and DK_{II} with respect to cyclic AMP calculated from Lineweaver-Burk plots of the data are $6.1 \cdot 10^{-8}$ M and $3.1 \cdot 10^{-8}$ M, respectively.

When protamine or casein was substituted for histone as the phosphoryl acceptor in the assay of kinase DK_I in the presence of cyclic AMP, the extent of phosphorylation during 10 min was only 13% and 2% respectively, of that of histone; in the absence of the cyclic nucleotide the corresponding values were 21% and 7%. Endogenous proteins present in the soluble fraction of the rat thyroid, principally thyroglobulin, appear to be poor substrates for the enzyme; ^{32}P transferred to protein in reaction tubes which contained all the assay components except histone was only 10% of that in the complete assay mixture.

Protein kinase activity of DK_I and DK_{II} each of which appeared as a single peak eluting from DEAE-cellulose at the same NaCl concentration, was analyzed in linear sucrose density gradients (2—10%). Fig. 3 shows the enzymatic profiles of the gradients and indicates that DK_I, the higher molecular weight kinase of the two originally separated on Sephadex, sedimented as 8.7 S with a shoulder of 6.6 S. When DK_I and DK_{II} were stored at 4°C in TEM buffer for two weeks prior to analysis in the gradients, the major component of DK_I sedimented as 6.7 S with a shoulder of 8.1 S and DK_{II} had a large component of the heavier 8.1 S activity (Figs. 4a and b). These results suggest that the two peaks of kinase separated initially on Sephadex represent inter-

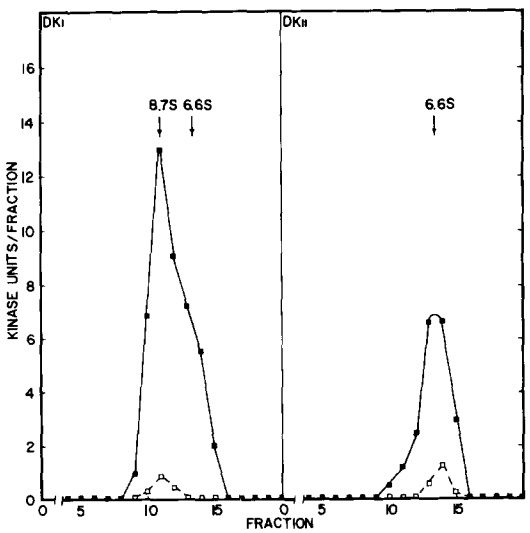

Fig. 3. Kinase activity profile in linear sucrose density gradients. Centrifugation was for the 16 h at 33 500 rev./min at 4°C in an SW Ti rotor. 200 µl of enzyme solution DK_I or DK_{II} was applied to 4.0 ml 2—10% linear sucrose density gradient in TEM buffer pH 7.5 at 4°C. Kinase activity was assayed by the standard assay (see Materials and Methods). ■, +cyclic AMP; □, —cyclic AMP.

convertible (polymeric) forms of the same enzyme. Fig. 4 shows, in addition, that incubation of both DK_I and DK_{II} with cyclic AMP prior to ultracentrifugation in sucrose density gradients caused a shift of the major portion of the enzymatic activity to 4.8 S, consistent with the well-described [27] disaggregation of the catalytic subunit(s) from the protein kinase holoenzyme. The lack of nucleotide dependency of the 4.8 S enzyme seen in Fig. 4 is also consistent with the notion that the 4.8 S activity is attributable to the catalytic subunit of the enzyme.

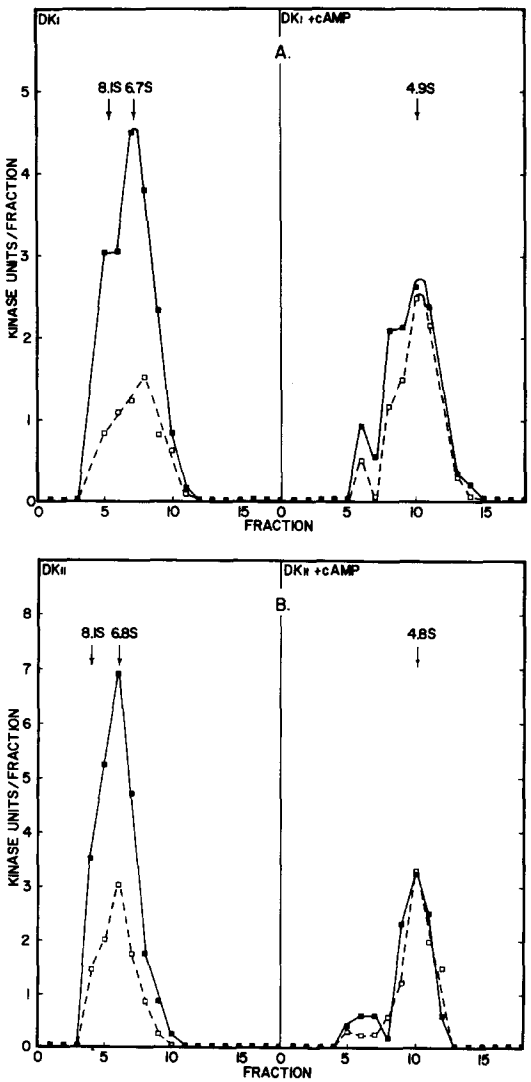

Fig. 4. Kinase activity profiles in linear sucrose density gradients. Centrifugation was for 16.8 h at 45 000 rev./min at 4°C in an SW 56 Ti rotor. 4A. 200 µl of an enzyme solution of DK_I incubated at 30°C for 10 min in the presence or absence of $1 \cdot 10^{-6}$ M cyclic AMP, was applied to 4.0 ml 2—10% linear sucrose density gradients in TEM buffer pH 7.5 at 4°C. 4B. Data for kinase DK_{II}. Conditions were as described for Fig. 4A. Kinase activity was assayed by the standard assay (see Materials and Methods) ■, +cyclic AMP; □, —cyclic AMP.

Discussion

This report confirms the existence in rat thyroid extract of cyclic AMP-stimulatable protein kinase activity [12], and describes its partial purification and some of its properties. The results emphasize the caveat of Corbin et al. [28] that assay of protein kinase activity in crude tissue extracts is subject to sources of serious error. The cyclic AMP-dependence and the total amount of assayable enzyme increased, the latter enormously so, in extracts diluted 10- or 100-fold. Also, total assayable kinase activity in a given volume of extract was more than 10 times greater after an initial purification of the enzyme on Sephadex. Addition of NaF to the assay tubes increased the amoung both of assayable nucleotide-dependent and independent activity in the whole and in the 10-fold diluted extract, but not in the 100-fold diluted extract. The results suggested that kinase activity of the extract was being masked by the presence of ATPase(s), and experiments testing for the appearance of P_i in the assay mixtures confirmed this to be the case. These findings with rat thyroid extracts agree with those reported for rat adipose tissue by Corbin et al. [28] who showed that ATPase activity of the preparation was very inhibitory to protein kinase at the ATP concentration used, and that inclusion of NaF in the assay mixture minimized the ATPase effect, and that nucleotide dependency and total activity increased when the crude extract was diluted. In contrast, Field al. [20], although they did not assay directly for ATPase, found that NaF inhibited kinase activity of pig thyoid homogenates and that dilution of the homogenates resulted in little or no change in either total units of kinase enzyme or in the ratio of nucleotide independent to dependent activity. This puzzling discrepancy while unexplained might possibly be related to the low concentration of ATP used in the Field assay, namely $5 \cdot 10^{-6}$ M, approximately 1/3 of the K_m, while in the present work and that of Corbin et al., ATP was added to give concentration, respectively, 10 and 20 times the K_m.

The presence of phosphohistone phosphatase activity in the crude extract could result in under-estimation of the total protein kinase activity. In addition, in assays of thyroid extracts which contain high concentrations of thyroglobulin, the flocculent precipitates which form very likely remove large quantities of histone substrate from solution and lead to low estimates of enzyme activity. As we have pointed out in Results, failure to find full expression of the kinase activity of crude extracts leads to a tremendous over-estimate of the overall fold-purification.

Another point to be mentioned is that the thyroid extract used as starting material for protein kinase purification in our experiments is the supernatant solution from a high-speed centrifugation (100 000 $\times g$ for 90 min) from which appreciable amounts of membrane-associated protein had been removed. The specific activity of the kinase in such a solution would be higher than in the more crude extracts (prepared from low-speed centrifugation) used by other workers [12,14,29,30] who have purified protein kinases. The increase in specific activity achievable in our purification would thus be correspondingly lower, the high-speed centrifugation itself representing a purification step.

Multiple forms of cyclic AMP-dependent protein kinase of differing molecular weights have been reported to be present in a given tissue [13—15,17]. This

was true, also, in the case of the rat thyroid glands examined in the present experiments. However, we have suggested in the Results section that the two kinases separated on Sephadex G-200 are perhaps interconvertible forms of the same enzyme comprising different states of aggregation of the holoenzyme and of the regulatory and catalytic subunits.

Swillins et al. [31] have called into question the meaning of K_m values determined from conventional graphical representation of kinetic data for enzymes such as protein kinases that are activated when they dissociate. In addition, the meaning of K_m for a system using mixed histones is questionable. Nevertheless, those values still seem to be useful to compare the properties and behavior of nucleotide dependent protein kinases from different tissues. In this regard, the magnitude of K_m with respect to cyclic AMP (10^{-8} M) and ATP (10^{-5} M) reported here for the rat thyroidal enzyme activity are in agreement with those determined for protein kinases by others [13—15,17,18,29].

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