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THE SEPARATION, PROPERTIES AND POSSIBLE SUBUNIT COMPOSITION OF ADENOSINE 3′,5′-MONOPHOSPHATE-DEPENDENT PROTEIN KINASES IN BROWN ADIPOSE TISSUE

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Summary

Two 8.5-S protein kinases (ATP : protein phosphotransferase EC 2.7.1.37) and one 6.6-S protein kinase were purified 500—1000-fold from the acid-soluble fraction of brown adipose tissue. The catalytic properties of the kinases were similar. Each kinase was activated by cyclic AMP and had two components of cyclic AMP binding. In the presence of 200 nM cyclic AMP, undissociated kinase activity sedimented at 7.7 or 5.5 S. Free catalytic activity (3.2 S) could be detected but was unstable. Free regulatory units could not be detected. The 8.5-S protein kinase was dissociated by freezing and thawing to a 7.7-S variety with loss of the higher affinity component of binding. The 7.7-S kinase was sedimented through linear gradients of sucrose containing different concentrations of cyclic AMP. At each concentration, kinase activity lost from the holoenzyme peak (% of original) was identical with the amount of cyclic AMP bound at equilibrium (% of maximum). Similar experiments on the 8.5-S kinase showed that the binding component with higher affinity was not associated with the release of catalytic activity. The results were consistent with the proposal that the kinases isolated contained one more cyclic AMP binding subunit than catalytic subunit (3 : 2 for 8.5 S and 2 : 1 for 6.6 S) and that this extra subunit was released to give an equal number of subunits of each type before catalytic activity was liberated.

Introduction

Cyclic AMP-dependent protein kinase is a complex of proteins, some with catalytic activity (catalytic subunits) and some with the ability to bind cyclic AMP (regulatory subunits). The activity of catalytic subunits is inhibited by their association with regulatory subunits. The binding of cyclic AMP to the regulatory units causes the complex to dissociate, thus releasing catalytic activ-

ity [1—6]. It is now thought that many of the regulatory effects of cyclic AMP are exerted through the stimulation of protein kinase, which phosphorylates and influences the activity of the target enzymes [7—18]. Recently it has been demonstrated that many tissues contain more than one cyclic AMP-dependent protein kinase [19—26]. This paper reports the separation and purification of a number of protein kinase complexes from brown adipose tissue, and a study of their physical, catalytic and cyclic AMP-binding porperties. The exact correspondence between cyclic AMP binding and the release of catalytic activity has been proved, and the results used to obtain an estimate of the subunit composition of the various complexes.

Methods and Materials

Brown fat was obtained from the interscapular and cervical regions of 2-day-old Dutch rabbits which had been left with their mother until the morning of the experiment. The rabbits were killed by breaking their necks. The fractionation of brown fat proteins was performed at 4°C through the acid precipitation, $(NH_4)_2SO_4$ precipitation and DEAE-cellulose chromatography steps described by Reimann et al. [19].

Ultracentrifugation and determination of sedimentation constants were performed essentially by the method of Martin and Ames [27]. The gradients were centrifuged in a Beckman SW 50.1 rotor at 37 000 rev./min for 16 h at 3°C and fractions (0.15-ml) collected by upward displacement [27].

For purification and determination of molecular weights by gel-filtration, samples (2 ml) were applied to a column (1.5 cm × 50 cm) of Sephadex G-200 which had been equilibrated at 4°C in 10 mM Tris buffer containing 1 mM EDTA (pH 7.0). The column was eluted with the same buffer at a flow rate of approx. 15 ml/h, and 1.5-ml fractions were collected. Molecular weights were determined by the method of Andrews [28], with ribonuclease, cytochrome *c*, albumin (crystalline, monomer), yeast alcohol dehydrogenase and blue dextran as standards.

Protein kinase activity was assayed essentially by Method B described by Reimann et al. [19]. Histone (15 mg/ml) was the protein substrate, and glass-fibre rather than filter-paper was used to collect the protein-bound ^{32}P. The reaction was started by the addition of the sample. Unless specified, protein kinase activity refers to activity in the presence of 2 μM cyclic AMP. The volume of sample was chosen so that kinase activity was always linear with respect to time and protein concentration. The binding of cyclic AMP was assayed at 0°C in 75 mM potassium phosphate buffer (pH 7.0), with cyclic [^3H] AMP as substrate (for concentration, see legends to tables and figures). The method of Gilman [29] was used to separate free from bound cyclic AMP at equilibrium. Under the conditions used, binding was a linear function of protein concentration and equilibrium was reached within 2 h. Protein was determined by the method of Lowry et al. [30].

The significance of the difference between mean values was tested using tables of Student's *t*. The method of least squares was used to determine the line of best fit for experimental points.

DEAE-cellulose (DE11) and glass-fibre discs (Type GF/C) were obtained

from Whatman Biochemicals, Maidstone, Kent, U.K. Cellulose ester filters (Type HA, 0.45-μm pore size) and Pellicon ultrafiltration membranes (Type PS) were obtained from Millipore Corporation, Bedford, Mass., U.S.A. Sephadex G-200 was from Pharmacia Fine Chemicals, Uppsala, Sweden. ATP, cyclic AMP, crystalline albumin, histone (Type IIA) and cytochrome *c* (horse heart) were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A., and other enzymes from C.F. Boehringer und Soehne, Mannheim, Germany. Cyclic [8-^{3}H]AMP (ammonium salt) and [γ-^{32}P]ATP (sodium salt) were from the Radiochemical Centre, Amersham, Bucks, U.K.

Results

Separation, partial purification and properties of protein kinase

Cyclic AMP-dependent protein kinase in brown fat was almost exclusively present in the 50 000 × *g* supernatant fraction. Approx. 20% of this kinase activity precipitated at pH 5.2 (acid precipitate fraction). About half of the acid-soluble activity was precipitated at a saturation of $(NH_4)_2SO_4$ of 45% (0—45 fraction) and one third at a saturation of between 50 and 60% (50—60 fraction). Each of the fractions was applied to a column of DEAE-cellulose which was eluted with a linear gradient of potassium phosphate. Two peaks of cyclic AMP-dependent protein kinase activity were eluted, which will be referred to as Peak I and Peak II, after the convention adopted by Reimann et al. [19]. The majority of the protein kinase from brown fat was eluted by approx. 250 mM potassium phosphate (Peak II). Only in the 0—45 fraction was there significant kinase activity in the Peak I position, and this only accounted for approx. 5% of the total. The fractions eluted from DEAE-cellulose will be referred to by the $(NH_4)_2SO_4$ fraction from which they were derived, followed by the peak number. The protein kinase activity in the Peak II fractions was purified 100—150-fold over that in the original homogenate.

The catalytic properties of the protein kinases in the three Peak II preparations were similar. The aparent K_m for histone (with $2 \cdot 10^{-4}$ M ATP) was approx. 2.5 mg/ml, and that for ATP (with 6 mg/ml histone) was approx. $1.75 \cdot 10^{-5}$ M. It has been shown previously that Mg^{2+} decreases the apparent K_m for ATP without affecting the maximal velocity [31]. The increase in activity caused by cyclic AMP gave a straight line with a slope of approx. 1.5 when expressed in the form of a Hill plot, with a half-maximal increase at approx. 100 nM cyclic AMP.

The effect of cyclic AMP on the equilibrium binding to a typical fraction is shown in Fig. 1. It is clear that there were two components of binding which differed in their affinity for cyclic AMP. Values for the apparent K_b and maximal binding of the two components of fractions from a number of different preparations are shown in Table I. It can be seen that the apparent K_b for the components with the lower affinity for cyclic AMP (Component 2) and the total binding of cyclic AMP were similar for each fraction. However, there were significant differences between fractions in the apparent K_b and maximal binding of the component with higher affinity (Component 1). The ratio of the total binding of cyclic AMP to that of Component 1 was 3.0 for Fraction 0—45 II, 1.7 for Fraction 50—60 II, and 2.1 for acid precipitate II.

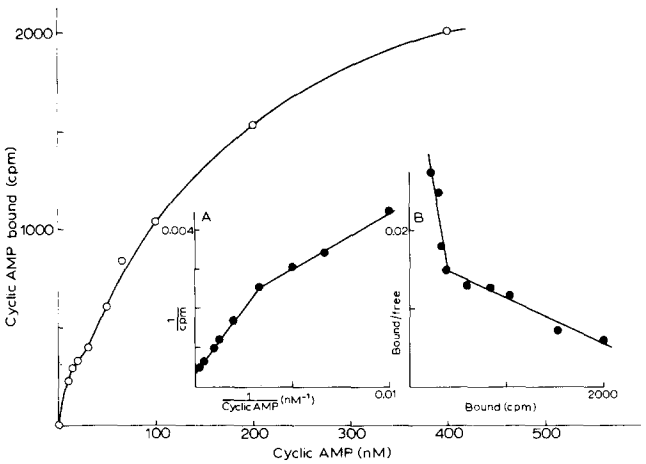

Fig. 1. The equilibrium binding of cyclic AMP to protein kinase with various concentrations of cyclic AMP. The binding of cyclic AMP to a 0-45 II preparation was assayed at pH 7.0 as described in the Methods section. The inserted figures show the same results in the form of a double reciprocal plot (A) or a Scatchard plot (B).

The protein kinase activity and cyclic AMP-binding ability of Fraction 0—45 II sedimented through a linear gradient of sucrose as a single peak with a sedimentation constant of 8.5 S (Fig. 2A). The 50—60 II (Fig. 2C) and acid precipitate II fractions gave two peaks of activity and binding, one at 8.5 S and another at approx. 6.7 S.

The various kinases were separated and purified by gel filtration on Sephadex G-200. The activity of the 0—45 II fraction was eluted in a peak which corresponded to a molecular weight of approx. 160 000. The 50—60 II fraction

TABLE I

THE BINDING OF CYCLIC AMP TO FRACTIONS CONTAINING PROTEIN KINASE ACTIVITY ELUTED FROM DEAE-CELLULOSE COLUMNS

Values for the apparent K_b and maximal binding of cyclic AMP to the protein kinase preparations were calculated from double reciprocal plots of equilibrium binding against cyclic AMP concentration. The double reciprocal plot of each of the Peak II preparations was biphasic (Fig. 1). Apparent K_b values are given for each of the components of binding. Maximal binding was calculated for the component with the lower apparent K_b (Component 1) and for the total cyclic AMP bound. Protein kinase activity was assayed in the presence of $2 \cdot 10^{-4}$ M ATP, $2 \cdot 10^{-6}$ M cyclic AMP and 15 mg/ml histone. Results are given as mean ±S.E. of 4 or 5 different preparations.

Fraction	Concn. of cyclic AMP for half maximal binding (nM)		Maximal binding per unit of kinase activity (fmol bound per activity of 1 pmol/min)	
	Component 1	Component 2	Component 1	Total
0—45 I	5.9 ± 0.7*	—	—	—
0—45 II	25.6 ± 4.5*	128.0 ± 17.4	1.76 ± 0.25	5.25 ± 0.56
50—60 II	50.3 ± 5.1*	116.7 ± 11.3	3.17 ± 0.07**	5.34 ± 0.10
Acid precipitate II	10.1 ± 1.0*	115.7 ± 7.1	2.94 ± 0.25**	6.31 ± 0.40

* Significantly different ($P < 0.05$, Student's t test) from each other.
** Significantly different ($P < 0.05$, Student's t test) from 0—45 II.

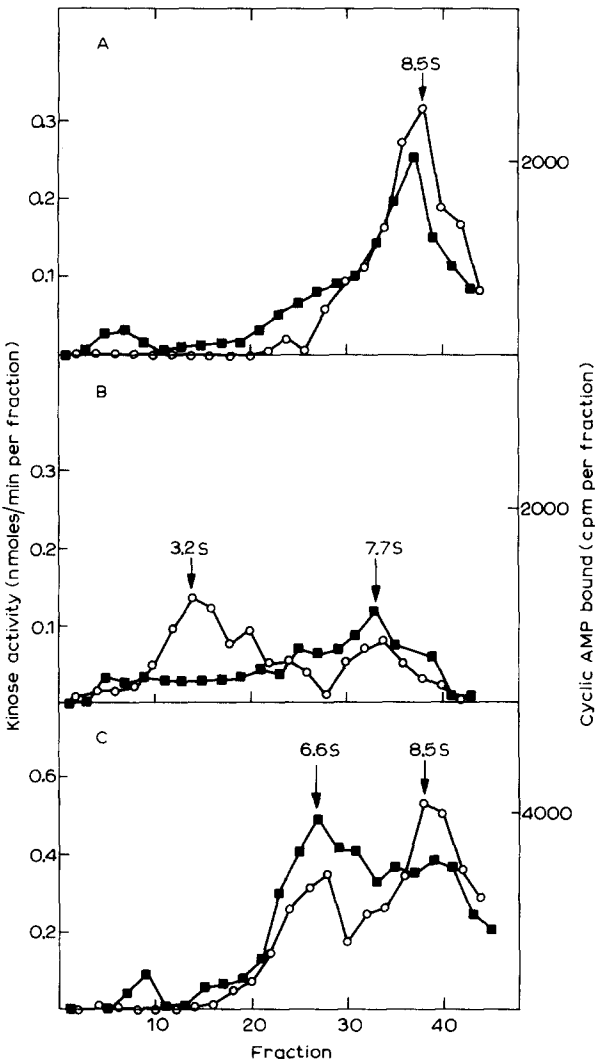

Fig. 2. Sucrose-density centrifugation of protein kinase preparations in the presence and absence of cyclic AMP. Samples were centrifuged through linear gradients of sucrose in the presence (B) and absence (A and C) of 200 nM cyclic AMP (see text for details). Preparations used were 0.45 II (A and B) and 50—60 II (C). Fractions were assayed for protein kinase in the presence of 2 μM cyclic AMP (○) and for cyclic [^{3}H] AMP binding (■) (400 nm cyclic AMP of the same specific activity as that used for the preincubation of samples).

gave two peaks of activity with molecular weights of approx. 160 000 and 90 000. The peaks from gel filtration were further separated and purified by sucrose-gradient centrifugation. At the end of this procedure the degree of purification, with respect to the original homogenate, was 1230-fold for the 8.5-S kinase from Fraction 0—45 II, 525-fold for the 8.5-S component of Fraction 50—60 II and 930-fold for the 6.6-S component of Fraction 50—60 II. A satisfactory purification and separation of the kinases of the acid precipitate II fraction could not be achieved.

TABLE II

SEDIMENTATION CONSTANTS AND MOLECULAR WEIGHTS OF PROTEIN KINASE PREPARATIONS, ESTIMATED FROM SUCROSE-DENSITY CENTRIFUGATION AND GEL FILTRATION ON SEPHADEX G-200

Sedimentation constants were estimated from the sedimentation of standard proteins (human haemoglobin and alcohol dehydrogenase) run with the samples. Approximate molecular weights calculated from the sedimentation constants are shown in parentheses. Molecular weights were obtained by gel filtration on a column (50 cm × 1.5 cm) of Sephadex G-200 which had been previously calibrated with proteins of known molecular weight. Binding of cyclic AMP per unit of kinase activity was the ratio, for each peak, of cyclic [^{3}H] AMP bound (400 nM cyclic AMP) to kinase activity assayed in the presence of 2 μM cyclic AMP.

Fraction	Sucrose gradient		Gel filtration	
	Sedimentation (S)	Binding per unit of kinase activity (fmol bound per kinase activity of 1 pmol/min)	Mol. wt	Binding per unit of kinase activity (fmol bound per kinase activity of 1 pmol/min)
0—45 II	8.5	1.62	160000	1.83
50—60 II	8.5	1.99	160000	1.94
	6.6	2.74	85000	2.80

The 8.5-S kinase obtained after gel filtration and gradient centrifugation of the 0—45 II fraction retained the cyclic AMP-binding characteristics of the parent sample. On repeated freezing and thawing the component of binding with the higher affinity for cyclic AMP was lost, and the kinase activity and binding ability sedimented with a sedimentation constant of 7.7 S. Thus it is probable that both components of binding are associated with a single protein kinase complex. Both the 8.5-S and the 6.6-S kinases from Fraction 50—60 II had two components of cyclic AMP binding. The apparent K_b values for the 6.6-S kinase were 25 and 90 nM, whereas those for the 8.5-S kinase were 10 and 80 nM.

It can be seen from Fig. 2C that the ratio of binding to activity for the 8.5-S and 6.6-S peaks was different. This difference was unaltered during the further purification by gel filtration. A summary of the sedimentation constants, approximate molecular weights and ratios of cyclic AMP binding to kinase activity of the peaks obtained from sucrose density-gradient centrifugation and gel filtration is shown in Table II. Values of binding to activity are lower than those shown in Table II since Mes rather than glycerophosphate was used as buffer in the assay of kinase activity. There was good agreement between binding to activity ratios of samples obtained from gradient centrifugation and the corresponding samples from gel filtration. The ratio of binding to activity was 35% higher for the 6.6-S component of Fraction 50—60 II than for the heavier component of the same fraction.

Dissociation of protein kinase by cyclic AMP

Fig. 2B shows the sedimentation pattern of a Fraction 0—45 II kinase through a gradient containing 200 nM cyclic AMP. As in white fat [32], free cyclic AMP-binding proteins could not be detected. However, in brown fat, unlike white fat, free catalytic subunits were also unstable. The recovery of free

kinase activity, which sedimented at 3.2 S (Fig. 2B), was always low and in many experiments could not be detected at all. The activity which remained associated with cyclic AMP binding sedimented more slowly than the activity of the same sample in the absence of cyclic AMP (Fig. 2B). The sedimentation constant of the remaining, undissociated, protein kinase in the presence of 200 nM cyclic AMP was approx. 7.7 S, which is similar to that of the kinase which had lost the high affinity binding component by freezing and thawing. Under similar conditions the undissociated kinase from the 6.6-S variety sedimented at 5.5 S. The evidence thus suggested that low concentrations of cyclic AMP could cause a partial dissociation of the protein kinase complex by splitting off a protein which had a high affinity for cyclic AMP. It was clearly of interest to discover whether the removal of this binding protein was associated with the release of catalytic activity. The sample was therefore incubated at 0°C with cyclic-[^{3}H]AMP for 2 h. Some of the incubation mixture was then taken to determine the amount of cyclic AMP bound, and the remainder (0.2 ml) was layered on to a linear sucrose gradient containing the same concentration of cyclic AMP and the same buffer at 0°C. The gradient was then centrifuged and the amount of kinase activity in the holoenzyme peak was determined (with 2 μM cyclic AMP). No attempt was made to separate the 8.5-S peak from the 7.7-S peak. This procedure was followed at various concentrations of cyclic AMP for the approx. 1000-fold purified, 8.5-S kinase from gel filtration of Fraction 0—45 II and for the similarly purified, 7.7-S kinase obtained by freezing and thawing the 8.5-S variety. It can be seen from Fig. 3 that the 7.7-S kinase exhibited only one component of cyclic AMP binding. At each concentration of cyclic AMP the kinase activity lost from the holoenzyme peak (expressed as a percentage of that present in the absence of cyclic AMP) was identical with the amount of cyclic AMP bound at equilibrium (expressed as a percentage of the maximum). Similar experiments with the 8.5-S kinase revealed that the component of binding with the higher affinity for cyclic AMP

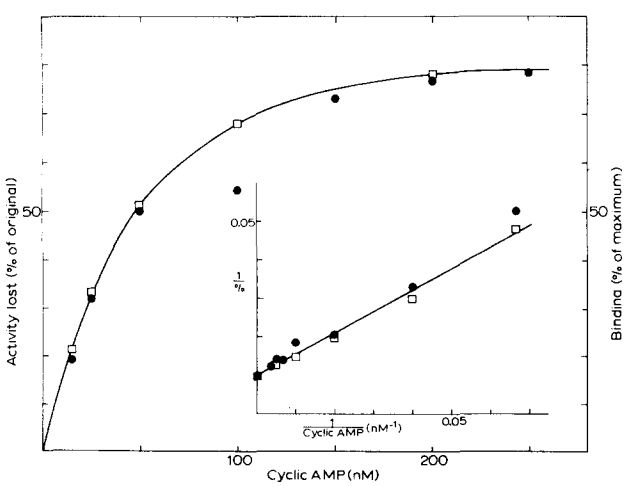

Fig. 3. Equilibrium binding of cyclic AMP (□) and the release of catalytic activity from the holoenzyme peak (●) of a 7.7-S kinase with different concentrations of cyclic AMP. See text for details. The inserted figure shows the same results as a double reciprocal plot.

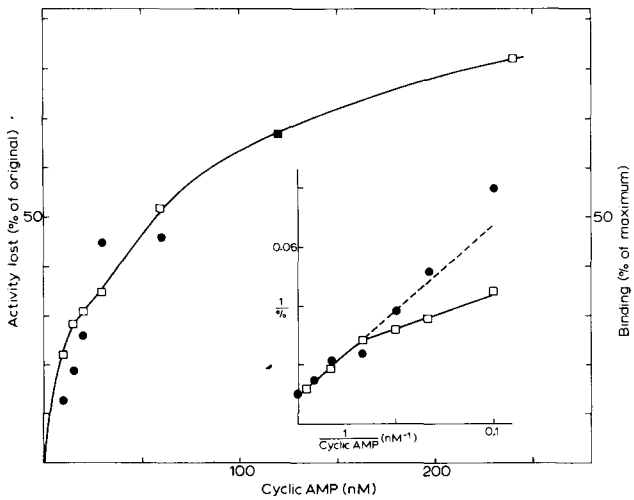

Fig. 4. Equilibrium binding of cyclic AMP (□) and the release of catalytic activity from the holoenzyme peak (●) of an 8.5-S kinase with different concentrations of cyclic AMP. See text for details. The inserted figure shows the same results as a double reciprocal plot.

was not associated with the release of catalytic activity. At low concentrations of cyclic AMP the amount of catalytic activity lost from the holoenzyme peak was less than would be expected from the amount of cyclic AMP bound (Fig. 4). Indeed, on a double reciprocal plot the dissociation of the kinase followed the line of the component of binding with the lower affinity for cyclic AMP.

Discussion

The results presented here show that there are two main varieties of cyclic AMP-dependent protein kinase in brown fat, with sedimentation constants of 6.6 S and 8.5 S. Three different protein kinases which sedimented at 8.5 S could be distinguished by differences in binding constant and solubility in acid. The significance of the differences in binding constant cannot be ascertained from the present results, but it is unlikely that the 1000-fold purified protein kinases still contained impurities that could affect the binding of cyclic AMP. Both components of binding were associated with a single kinase complex (see Results). The sensitivity to cyclic AMP was not influenced by the presence of endogenous substrates since none of the fractions exhibited kinase activity in the absence of added histone. Moreover, the close correspondence between cyclic AMP binding and the release of catalytic activity from the purified complexes (Fig. 3) shows that these preparations were free of any unassociated cyclic AMP-binding proteins or catalytic subunits.

It can be seen from Table III that the ratio of cyclic AMP binding to catalytic activity of the 6.6-S kinase variety was higher than that of the 8.5-S varieties. This might indicate that the 8.5-S complex contained a greater proportion of catalytic subunits than the 6.6-S complex, and could lead to an estimate of the proportions of the subunits in the complexes. Before conclu-

sions can be drawn from this observation it must be shown that the difference was not caused by some impurity. As has been discussed previously, the preparations did not contain endogenous substrates, free catalytic subunits or cyclic AMP-binding proteins. The heat-stable protein kinase inhibitor [33] has not yet been considered. Its presence in the 6.6-S variety could account for the high ratio observed. However, although inhibitory activity was present in the crude homogenate, the majority of it remained soluble in $(NH_4)_2 SO_4$ and none could be detected in any of the fractions eluted from DEAE-cellulose. Moreover, the relationship between activity and concentration of sample in the assay was linear for the final preparations up to the limit of the assay.

The use of the ratios of cyclic AMP-binding to catalytic activity to obtain an indication of the subunit composition of the protein kinase complexes can thus be reasonably justified. To do this it will be assumed that each catalytic subunit has the same maximal activity. Also it is necessary to define a binding subunit (not necessarily a complete regulatory subunit) as the smallest protein that can bind cyclic AMP, and to assume that each binding subunit binds the same amount of cyclic AMP. The relationship between the binding to activity ratios (1.35 : 1) is consistent with a ratio of binding to catalytic subunits of 3 : 2 for the 8.5-S variety and 2 : 1 for the 6.6-S variety. This interpretation is supported by the observations on the ratio of the total amount of cyclic AMP bound to the maximal binding of Component 1. This ratio was 3.0 for the 8.5-S kinase in Fraction 0—45 II, 2.9 for the 8.5-S kinase in Fraction 50—60 II and 2.1 for the 6.6-S kinase of Fraction 50—60 II. One binding subunit is lost on freezing and thawing, or with low concentrations of cyclic AMP, to give complexes with ratios of 2 : 2 (7.7 S) and 1 : 1 (5.5 S).

The interesting features of this proposal are that the isolated complexes contain one more binding subunit than catalytic subunit, and that the extra binding subunit is released to give an equal proportion of each subunit before catalytic subunits are liberated. It would be predicted from this model that the component of binding with the higher affinity for cyclic AMP, which was present in both forms but was lost on freezing and thawing, was not associated with the release of catalytic activity. This was checked by sedimenting the kinases through gradients containing different concentrations of cyclic AMP. At each concentration, kinase activity lost from the holoenzyme peak of the 7.7-S kinase (expressed as % of original) was identical with the amount of cyclic AMP bound (expressed as % of maximal). We believe that this is the first conclusive demonstration that cyclic AMP binding corresponds exactly to the release of catalytic activity. Similar experiments with the 8.5-S kinase (Fig. 4) showed that, at low concentrations of cyclic AMP, the release of catalytic activity was less than the binding of cyclic AMP, thus confirming the prediction made above. The model of subunit composition proposed here for brown fat is in general agreement with the results from a homogeneous preparation of protein kinase from beef heart muscle obtained by Erlichman et al. [34]. They concluded that this kinase (mol. wt 174 000; 6.8 S) was composed of two catalytic subunits and one cyclic AMP-binding protein containing two polypeptide chains of equal size.

The function of the component of binding with high affinity for cyclic AMP, which is not associated with the release of catalytic activity, is difficult

to assess. It may be a non-physiological association brought about during the extraction procedure, or it may have a physiological role, perhaps to "buffer" the release of catalytic activity from minor fluctuations in the tissue concentration of cyclic AMP.

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