Adenosine 3':5'-Cyclic Monophosphate-Dependent Protein Kinase in Brown Fat from Newborn Rabbits

CHANGES IN THE BINDING OF ADENOSINE 3':5'-CYCLIC MONOPHOSPHATE AFTER PREINCUBATION OF THE TISSUE WITH NORADRENALINE OR INCUBATION OF THE ENZYME WITH ADENOSINE TRIPHOSPHATE

By BRIAN L. KNIGHT

*Medical Research Council Lipid Metabolism Unit, Hammersmith Hospital, Ducane Road,
London W12 0HS, U.K.*

(Received 23 June 1975)

The equilibrium binding of cyclic AMP to a 150-fold purified preparation of protein kinase, when expressed as the reciprocal of bound against the reciprocal of free cyclic AMP, gave a plot consisting of two straight lines. The values of apparent K_b given by these lines were lowered by preincubating the intact tissue with noradrenaline or incubating the enzyme preparation with Mg^{2+} plus ATP. This effect was reversed by incubating the preparation (which contained some phosphatase impurities) with Mg^{2+} alone. None of these procedures affected the maximal binding of cyclic AMP. During incubation of the enzyme with Mg^{2+} plus ATP, the terminal phosphoryl group was incorporated into protein, over 40% being present in the kinase itself. This phosphate was removed during incubation of the preparation with Mg^{2+} alone. The validity of expressing cyclic AMP binding as a double-reciprocal plot is discussed, and the experimental plots are compared with those derived theoretically. The results suggest that protein kinase in brown fat is present in two forms, one with an apparent K_b for cyclic AMP of approx. 250 nM (dephosphorylated) and one with an apparent K_b of approx. 14 nM (phosphorylated). Preincubation of the tissue with noradrenaline results in phosphorylation of the kinase and an increase from 15% to 45% in the proportion of the higher-affinity form.

Cyclic AMP-dependent protein kinase has been found in all eukaryotic cells so far studied and is known to phosphorylate a number of important regulatory enzymes (Langan, 1973) and thereby to influence their activity. Stimulation of protein kinase is the only direct action of cyclic AMP that has yet been conclusively demonstrated. However, the substrate specificity of purified protein kinases is too broad to explain adequately the complexity and variety of the hormonal responses that have been shown to be mediated by cyclic AMP (Robison *et al.*, 1971). Thus consideration must be given to the possibility that the rate of phosphorylation of regulatory enzymes is ultimately determined not only by the concentration of cyclic AMP, but also by factors such as the availability of protein substrate (Langan, 1973), the nature and local concentration of the nucleotide substrate (Knight & Skala, 1975; Ventimiglia & Wool, 1974) and the presence of protein kinase-modulator proteins (Donnelly *et al.*, 1973a,b; Majumder, 1974). A further factor of potential importance has been revealed by Erlichman *et al.* (1974), who found that the regulatory unit of protein kinase could itself be phosphorylated, and that this phosphorylation affected the response of the enzyme to cyclic AMP. Autophosphorylation of protein kinase was confirmed

by Maeno *et al.* (1974), but it is not known whether this process influences the effects of cyclic AMP within intact cells.

In the present paper it is shown that changes in the characteristics of the binding of cyclic AMP to protein kinase which occur during the incubation of brown fat *in vitro* can be reproduced by submitting the partially purified enzyme to conditions that would be expected to alter its degree of phosphorylation.

Methods

Brown fat was obtained from the interscapular and cervical regions of 2-day-old rabbits which had been left with their mother until the morning of the experiment. The rabbits were killed by breaking their necks. Tissue from a number of rabbits was pooled and either homogenized directly or cut into small pieces and incubated at 37°C (with shaking) in Krebs-Ringer bicarbonate buffer, pH 7.4 (Umbreit *et al.*, 1964), containing half the recommended concentration of Ca^{2+} , and 3% bovine serum albumin (fraction V). The gas phase was $O_2 + CO_2$ (95:5). After 2 h incubation, noradrenaline (5 µg/ml) was added to some of the flasks and the incubation stopped 15 min later. At the end of

the incubation the tissue was rapidly separated from the medium by filtration under suction and placed in ice-cold homogenization medium. It was then immediately blotted, minced through a stainless-steel mincer and homogenized in 5 vol. of ice-cold 0.25M-sucrose containing 1mM-EDTA, pH 7.0, in a glass homogenizer tube with a close-fitting Teflon pestle. Protein kinase was purified, at 4°C, by acid precipitation, $(\text{NH}_4)_2\text{SO}_4$ precipitation and DEAE-cellulose chromatography as described previously (Knight & Fordham, 1975). The kinase eluted from DEAE-cellulose was dialysed (20 vol. with two changes) against 20mM-Mes buffer, pH 6.5 [approx. 30mM-2-(N-morpholino)ethanesulphonic acid adjusted to pH 6.5 with NaOH solution and diluted to 20mM] and concentrated by ultrafiltration through a Pellicon (type PS) membrane.

Protein kinase activity, protein-bound ^{32}P and the binding of cyclic ^3H AMP were assayed as described previously (Knight & Fordham, 1975). Autophosphorylation of the kinase preparation was performed by incubation at 37°C with 10mM-MgCl₂ and 50mM-ATP.

Histone-Sepharose was prepared as described by Corbin *et al.* (1972b). Samples (2–5 ml) were applied to a column (0.9cm × 7cm) of histone-Sepharose. The column was washed with 10 ml of 20mM-Mes buffer, pH 6.5 (containing 1mM-EDTA) and eluted with a 40 ml linear gradient (0–500mM) of NaCl in the same buffer; 2 ml fractions were collected. In experiments where it was necessary to dissociate the kinase, the sample was incubated at 0°C for 30 min with 5 μM -cyclic ^3H AMP (approx. 1000 c.p.m./pmol) before being applied to the column. The column was then washed with 3 ml of 20mM-Mes buffer, pH 6.5, containing 5 μM -cyclic ^3H AMP followed by 10 ml of buffer without cyclic AMP (Corbin *et al.*, 1972b). The column was eluted as before. Each fraction was assayed for protein kinase activity and cyclic AMP binding (with 2 μM -cyclic ^3H AMP). In these experiments protein-bound ^{32}P was assayed in each fraction by filtering a portion (0.5 ml) through a cellulose ester filter, and washing and counting the filter for radioactivity as described for the cyclic AMP-binding assay.

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

Non-labelled nucleotides, histone (type IIA) and Sepharose (type 4B-200) were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Cellulose ester filters (type HA, 0.45 μm pore size) and Pellicon ultrafiltration membranes (type PS) were from Millipore Corp., Bedford, Mass., U.S.A. Cyclic $[8\text{-}^3\text{H}]\text{AMP}$ and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ were purchased from The Radiochemical Centre, Amersham, Bucks., U.K.

Results

Cyclic AMP-dependent protein kinase was purified 150-fold from brown fat of newborn rabbits by acid precipitation, $(\text{NH}_4)_2\text{SO}_4$ precipitation (0–50% saturation) and DEAE-cellulose chromatography as described previously (Knight & Fordham, 1975). The double-reciprocal plot of equilibrium binding of cyclic AMP against cyclic AMP concentration for this kinase is biphasic. The binding characteristics

Fig. 1. Equilibrium binding of cyclic AMP to partially purified protein kinase from brown fat that had been preincubated with noradrenaline (a), without noradrenaline (b) or not preincubated (c).

Pieces (2–5mg wet wt.) of brown fat were either homogenized directly or incubated at 37°C in gassed Krebs-Ringer bicarbonate buffer, pH 7.4, in the presence or absence of noradrenaline (5 $\mu\text{g}/\text{ml}$) as described in the Methods section. The tissue was then homogenized in ice-cold 0.25M-sucrose containing 1mM-EDTA, pH 7.0, and protein kinase was purified by acid and $(\text{NH}_4)_2\text{SO}_4$ precipitation and DEAE-cellulose chromatography. Equilibrium binding of cyclic ^3H AMP was assayed as described previously (Knight & Fordham, 1975).

Table 1. *Effect of preincubation of brown fat on the values of 'apparent K_b ' for cyclic AMP binding to partially purified protein kinase*

Double-reciprocal plots of bound to free cyclic AMP were not linear (Fig. 1) and were resolved into two components. Values of apparent K_b of each component were obtained from the intercepts for three preparations from tissue which had not been incubated, three preparations from tissue which had been preincubated without noradrenaline and five preparations from tissue which had been preincubated with noradrenaline (5 μ g/ml). Results are given as means $\pm$ S.E.M.

	Apparent K_b (nM)	
	Component 1	Component 2
Not preincubated	71.3 $\pm$ 9.5*	147.2 $\pm$ 6.4
Preincubated without noradrenaline	44.3 $\pm$ 5.8*	146.9 $\pm$ 4.3
Preincubated with noradrenaline	22.5 $\pm$ 1.9*	111.5 $\pm$ 11.0

* Significantly different from each other ($P < 0.05$).

are not altered after a further 10-fold purification of the protein kinase, and the non-linearity cannot be explained by the presence of unassociated cyclic AMP-binding protein, catalytic subunits, endogenous substrates or inhibitor proteins (Knight & Fordham, 1975). A discussion on the use of this plot to express the results of cyclic AMP binding to protein kinase is given below.

The 'apparent K_b ' values for both components of cyclic AMP binding were lower in tissue which had been preincubated with noradrenaline (Fig. 1a) than in tissue which had not been incubated (Fig. 1c), or which had been preincubated without noradrenaline (Fig. 1b). The results from a number of different preparations are shown in Table 1. The differences between values of 'apparent K_b ' for the components with the lower affinity for cyclic AMP were all statistically significant. There was no effect of preincubation of noradrenaline on the maximal binding of cyclic AMP when related to the kinase activity (approx. 2 fmol per kinase activity of 1 pmol/min).

When the partially purified kinase was incubated with [γ - 32 P]ATP, radioactivity was incorporated into trichloroacetic acid-precipitable proteins (Fig. 2). The radioactivity was released from the protein during incubation at 37°C for 20 h with 0.25 M-NaOH, but not with cold NaOH or hot trichloroacetic acid. Thus protein-bound phosphate had the properties expected of a phosphoester of serine or threonine, which are the residues normally phosphorylated by cyclic AMP-dependent protein kinase (Langan, 1973). Phosphorylation was essentially complete after 1 h at 37°C, during which time the catalytic activity of the protein kinase was not altered (Fig. 2).

Fig. 2. *Time-course of the autophosphorylation of a preparation of partially purified protein kinase from brown fat*

The protein kinase preparation was incubated at 37°C in the presence of 10 mM-MgCl₂ and 50 μ M-[γ - 32 P]ATP (200–300 c.p.m./pmol). After 3 h the preparation was put at 0°C and left overnight. [γ - 32 P]ATP (50 μ M) was re-added and the preparation incubated at 37°C in the presence (●) or absence (○) of 10 mM-NaF. At various times duplicate samples were taken for assay of protein-bound 32 P (●, ○) and kinase activity (▲).

Fig. 3. *Equilibrium binding of cyclic AMP to preparations of protein kinase which had been incubated for 1 h at 37°C with 10 mM-MgCl₂ (○) or 10 mM-MgCl₂ plus 50 μ M-ATP (●)*

Protein kinase was prepared from tissue which had not been preincubated (a) and from tissue which had been preincubated with noradrenaline (b).

The enzyme preparation still contained some ATPase (adenosine triphosphatase) and phosphatase activities, since no 32 P remained bound to protein after a further 17 h at 0°C. Re-addition of [γ - 32 P]ATP and incubation at 37°C resulted in renewed phosphorylation. The maximum was lower than that observed on the previous day by a percentage similar to the loss of enzymic activity, and was not affected by the presence of fluoride (Fig. 2).

In order to discover whether phosphorylation or dephosphorylation could affect the binding of cyclic AMP to protein kinase, preparations of the enzyme were incubated at 37°C with Mg^{2+} plus ATP. With a preparation from brown fat which had not been preincubated it was just possible to separate two components of binding with apparent K_b values of 240 and 125 nM (Fig. 1c). After incubation for 1 h with Mg^{2+} alone, only one component of cyclic AMP binding was present, with an apparent K_b of 250 nM (Fig. 3a). However, after incubation with Mg^{2+} plus ATP, which promotes phosphorylation (Fig. 2), two components of binding with apparent K_b values of 26 and 133 nM were clearly distinguishable (Fig. 3a). There was no effect of either Mg^{2+} or ATP on the binding characteristics if the preparation was not incubated at 37°C. Similar experiments with a protein kinase preparation from tissue which had been preincubated with noradrenaline are shown in Fig. 3(b). In this case incubation at 37°C for 1 h with Mg^{2+} alone raised the apparent K_b values of each component from 15 and 75 nM to 21 and 116 nM respectively. After incubation with Mg^{2+}

plus ATP only one component of binding, with an apparent K_b of 14 nM, was present. With both preparations the maximal binding of cyclic AMP after incubation with Mg^{2+} alone was similar to that with Mg^{2+} plus ATP, indicating that the loss of one of the components of binding was not associated with a decrease in the total binding capacity.

All of the ^{32}P which was incorporated into endogenous proteins by the preparations of protein kinase was retained on a column of histone-Sepharose (Fig. 4a). It was removed from the column as two major peaks by a linear gradient of NaCl. One of these peaks, containing approx. 40% of the recovered radioactivity, was eluted in the same fractions as the cyclic AMP-dependent protein kinase activity and cyclic AMP-binding proteins. The other peak, eluted by a lower concentration of NaCl, contained very little kinase activity or binding proteins. If the protein kinase was dissociated into its subunits by preincubation with cyclic AMP before being applied to the column, the peak of cyclic AMP-dependent kinase activity was greatly decreased (Fig. 4b). However, little of the dissociated catalytic activity or binding proteins could be detected. This instability of dissociated subunits of protein kinase from adipose tissues when separated by sucrose-gradient centrifugation or affinity chromatography has been observed previously (Corbin *et al.*, 1972a; Knight & Fordham, 1975). The loss of the peak of cyclic AMP-dependent protein kinase was accompanied by a decrease in the minor peak of protein-bound ^{32}P and a shift in its position to that of the detectable cyclic AMP-binding proteins (Fig. 4b). The major peak of protein-bound ^{32}P was not affected by preincubation of the kinase preparation with cyclic AMP. These results are consistent with the possibility that about 40% of the ^{32}P which was incorporated into protein by the kinase preparations was present in the protein kinase itself, probably located in the regulatory subunit.

Fig. 4. Chromatography of the phosphorylated protein kinase preparation on histone-Sepharose

The protein kinase preparation was incubated for 3 h at 37°C with 10 mM- $MgCl_2$ plus 50 μM - $[\gamma\text{-}^{32}P]ATP$ (200–300 c.p.m./pmol). It was either applied directly to a column (0.9 cm $\times$ 7 cm) of histone-Sepharose (a) or incubated for 30 min at 0°C with 5 μM -cyclic $[^3H]AMP$ before being applied to the column (b). The columns were washed and eluted with linear gradients (0–500 mM) of NaCl as described in the Methods section. Fractions were assayed for protein-bound ^{32}P (●), protein kinase activity in the presence (▲) and absence (△) of cyclic AMP and for cyclic AMP binding (○).

Discussion

The results presented here show that the characteristics of the binding of cyclic AMP to partially purified protein kinase could be altered by incubating the enzyme preparation with ATP to promote phosphorylation of endogenous proteins, or with Mg^{2+} to promote their dephosphorylation. The binding characteristics could also be affected by the conditions imposed on the tissue before homogenization. Preincubation of the tissue with noradrenaline caused changes in the binding characteristics which were qualitatively similar to those produced by incubating the enzyme with ATP. These changes could be reversed by incubating the enzyme with Mg^{2+} alone.

Interpretation of these results is not straightforward. Indeed, there is no interpretation of the kinetics of the binding of cyclic AMP to protein kinase which adequately reconciles current theory with experimental data. Protein kinase is made up of regulatory subunits, which bind cyclic AMP, and catalytic subunits which are inhibited in the holoenzyme complex. The binding of cyclic AMP to the regulatory subunits causes the complex to dissociate, thus releasing catalytic activity (Langan, 1973). The proportion of the complex that is dissociated (and therefore cyclic AMP that is bound) would be expected to be determined by the equilibrium between the concentrations of holoenzyme, cyclic AMP, free regulatory and catalytic subunits and, possibly, of a holoenzyme-cyclic AMP intermediate. Even in the simplest case, where there are equal proportions of regulatory and catalytic subunits and the intermediate complex is ignored, this equilibrium gives a complicated relationship between free and bound cyclic AMP (Swillens *et al.*, 1974; Mackenzie & Stellwagen, 1974). However, under the conditions normally used for the assay of cyclic AMP binding, the results are usually found to fall on a straight line when expressed as double-reciprocal or Scatchard (1949) plots (Walton & Garren, 1970; Gilman, 1970; Walsh *et al.*, 1971; Donovan & Oliver, 1972; Guthrow *et al.*, 1972; Montague & Howell, 1972; Jastorff & Bar, 1973; Sanborn *et al.*, 1973). These plots are derived from a simplified equilibrium equation which does not include the free catalytic subunit. Non-linearity has occasionally been observed, but at lower concentrations of cyclic AMP than those used here (Sanborn *et al.*, 1973; Mackenzie & Stellwagen, 1974; Pierre & Loeb, 1972).

Thus, in the light of these previously published observations, it could be concluded that the non-linearity of the present double-reciprocal plots is due to the presence of two components of binding and not to an unsuitable treatment of the data. A number of observations support this conclusion. The results do not conform to the kinetics of the simplest equation including the catalytic subunit. Also straight double-reciprocal plots, giving both the highest and lowest observed values of apparent K_b , were obtained after incubating the kinase preparations with Mg^{2+} or Mg^{2+} plus ATP (Fig. 3). The binding characteristics of preparations containing a mixture of kinases with these affinities for cyclic AMP are very similar to those obtained experimentally. A mixture containing 40% of a kinase with a K_b of 14 nm and 60% of a kinase with a K_b of 250 nm gives a curved double-reciprocal plot which, when experimental points are used, would be interpreted as two straight lines with apparent K_b values of 20 and 64 nm (Fig. 5a). Decreasing the proportion of the 14 nm kinase increases the apparent K_b of each of the components of the curve (Figs. 5b and 5c). Thus the lowering of the apparent

Fig. 5. Theoretical double-reciprocal curves of cyclic AMP binding to a preparation of protein kinase containing two enzymes with different affinities for cyclic AMP

Calculations were based on the equilibrium equation with holoenzyme and cyclic AMP on one side and bound cyclic AMP on the other. At each concentration of cyclic AMP the cyclic AMP bound in a system with a K_b of 14 nm was added to that bound in a system with a K_b of 250 nm. The calculations were performed for mixtures containing 40% (a), 20% (b) and 10% (c) of the higher-affinity component.

K_b values observed after preincubation of the tissue with noradrenaline, or incubation of the enzyme preparation with ATP, can be explained by an increase in the relative proportion of a kinase with a high affinity for cyclic AMP. Since there was no change in the total cyclic AMP bound, the kinase with the lower affinity for cyclic AMP must, on this hypothesis, have been converted into the higher-affinity form. It can be seen from Fig. 5 that the plot curves rapidly as it approaches the y-axis. Since the number of points to be included in the calculation of the lower-affinity K_b was decided visually, the values obtained can only be approximate.

The best interpretation of these results is that cyclic AMP-dependent protein kinase in brown fat can exist in two forms with different affinities for

cyclic AMP. Incubation of the tissue with noradrenaline results in the conversion of the low-affinity form into the high-affinity form. A comparison between Figs. 1 and 5 indicates that the high-affinity form accounted for about 5% of the total in non-incubated tissue, 15% in tissue incubated without noradrenaline and 45% in tissue incubated with noradrenaline. The demonstration that this effect can be reproduced by incubating the partially purified enzyme with Mg^{2+} plus ATP, and reversed by incubating it with Mg^{2+} alone, strongly suggests that the higher-affinity kinase is the phosphorylated form of the lower-affinity kinase. Radioactivity was, indeed, incorporated from [γ - ^{32}P]ATP into protein kinase, probably into the regulatory subunit (Fig. 4), and could then be removed by phosphatase impurities in the enzyme preparation. These observations do not, however, give any indication of the total amount of phosphate present in the enzyme. Unfortunately the protein kinase eluted from histone-Sepharose was extremely unstable and did not survive dialysis or incubation at 37°C, so that a conclusive study of the phosphorylation of the kinase could not be performed.

The observation that the affinity of protein kinase for cyclic AMP can be altered within intact cells provides another mechanism by which the hormonal response of a tissue could be modified. The necessity for ATP in the assay of kinase activity prevents any direct demonstration of a change in the response of the enzyme to cyclic AMP. However, at a given concentration of cyclic AMP, a change in the amount of free catalytic activity corresponding to a change in the amount of cyclic AMP bound would explain some anomalous results which have been obtained with brown fat. In particular, it is possible to explain how, with low concentrations of noradrenaline or in the presence of insulin or propranolol, lipolysis can be stimulated without any increase in the concentration of cyclic AMP (Knight, 1974). The mechanism by which noradrenaline could affect the degree of phosphorylation of protein kinase and its affinity for cyclic AMP remains obscure. It has been shown that insulin and noradrenaline increased the phosphorylation of certain proteins in adipose tissue by a process which does not involve cyclic AMP (Benjamin & Singer, 1974). It would be interesting to discover whether one of these proteins was the kinase itself.

I thank Mrs. Rosemary A. Fordham for her excellent technical assistance and Dr. N. B. Myant for his help and encouragement.

Results

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