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EVIDENCE THAT RABBIT MUSCLE PROTEIN KINASE HAS TWO KINETICALLY DISTINCT BINDING SITES FOR ADENOSINE 3'; 5'-CYCLIC MONOPHOSPHATE

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SUMMARY: Two distinct populations of binding sites for cyclic AMP are associated with the regulatory moity of cyclic AMP dependent protein kinase (E.C. 2.7.1.37), as judged from the kinetics of the interaction between the nucleotide and the binding protein. The two types of sites were present at the proportion 1:1. The rate of dissociation of bound cyclic AMP was more rapid for one type of site than for the other type. High ionic strength accentuated the difference in the rate of dissociation of cyclic AMP from the two sites.

The two binding sites and protein kinase activity copurified during the entire procedure for preparation of protein kinase holoenzyme. The kinetic properties of each of the two sites and the proportion between them was the same in a highly purified preparation of the regulatory moiety of protein kinase and in binding protein freshly prepared in the presence of protease-inhibitor.

The cyclic AMP dependent protein kinases have been extensively studied because of their key role in the mediation of the intracellular effects of cyclic AMP (1, 2, 3 for reviews). The two main isozyme forms of cyclic AMP dependent protein kinase in mammalian tissues can be separated by chromatography on DEAE-cellulose (4). Both the isozyme form eluted at low ionic strength (type I) and that eluted from DEAE-cellulose at higher ionic strength (type II) is composed of two regulatory and two catalytic subunits (5,3).

Interaction between cyclic AMP and the holoenzyme results in the generation of two free active catalytic subunits and the binding of two molecules of cyclic AMP to the regulatory subunit dimer (2, 5).

The possibility that the two binding sites for cyclic AMP on each regulatory subunit dimer are dissimilar seems not to have been investigated. The present paper reports two distinct populations of binding sites for cylic AMP associated with cyclic AMP dependent protein kinase I from rabbit skeletal muscle.

EXPERIMENTAL

Preparation of binding protein from rabbit skeletal muscle. The preparation of protein kinase I holoenzyme routinely used for binding

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studies was purified about 500-fold through the following steps:

Homogenization, precipitation with polyethyleneglycol, DEAE-cellulose chromatography and precipitation with 55% satd. $(NH_4)_2 SO_4$ as described (9) followed by chromatography on Ultrogel AcA34 (L.K.B., Bromma, Sweden) and Hydroxylapatite (Bio-Gel H.T. from Bio. rad., Richmond, Calif., U.S.A.), reprecititation with polythylene-glycol (24 g/100 ml sample) and ultracentrifugation on density gradients of glycerol (10-30% w/v).

The regulatory moiety of protein kinase I was separated from the catalytic moiety by incubation of the above preparation with cyclic [^{3}H]AMP followed by DEAE-cellulose chromatography.

To prepare protein kinase I under conditions reducing the likelihood of extensive proteolysis, muscle from a well-fed rabbit was homogenized for 30 sec (1/10 w/v) in the presence of 0.5 mM phenylmethyl-sulfonylfluoride (Sigma, Mo., U.S.A.). The supernatant obtained by centrifuging at 30 000 x g av. for 20 min was immediately subjected to DEAE-cellulose chromatography. The first DEAE-cellulose fractions containing protein kinase I were devoid of adenosine-binding activity and were used for kinetic studies less than 12 h after the rabbit had been killed.

Assay procedures. The assay for protein kinase activity as well as the $(NH_4)_2 SO_4$-precipitation/membrane filtration method to study the binding to proteins of cyclic [^{3}H]AMP and [^{3}H] adenosine have been described previously (6). In the experiments of Figs. 1 & 2 the binding of cyclic [^{3}H]AMP was assayed either at 0°C for 2 h in 50 mM Hepes-NaOH pH 7.2/30 mM EDTA/800 mM NaCl/20 mM 2-mercaptoethanol/albumin (0.5 mg/ml)/heat-stable protein fraction (0.3 mg/ml)/10 µM adenosine/10 µM erythro-9-(2-hydroxy-3-nonyl) adenine and 10 nM cyclic [^{3}H]AMP, conditions favouring the binding to the regulatory moiety of protein kinase I (6), or for 2 h at 37°C in 30 mM Mes-NaOH pH 6.0/4 mM EDTA/500 mM KCl/20 mM 2-mercaptoethanol/albumin (0.5 mg/ml)/1 mM cyclic GMP and 2.5 µM cyclic [^{3}H]AMP (1 Ci/mmol). conditions favouring the binding of the nuclotide to cyclic AMP/adenosine binding protein (7). Unlabelled adenosine was present to supress binding of cyclic [^{3}H]AMP to cyclic AMP/adenosine binding proteins and cyclic GMP to supress the binding to protein kinase. Erythro-9- (2-hydroxy-3-nonyl) adenine and EDTA were present to inhibit the breakdown of adenosine and cyclic AMP by adenosine deaminase and cyclic AMP phosphodiesterase, respectively. Albumin, 2-mercaptoethanol, EDTA (8) and heat-stable protein fraction (9) were present to stabilize the regulatory moiety of protein kinase I.

Plotting of kinetic data. The data of the dissociation rate experiments were plotted semilogarithmically (Figs. 3 & 4) to linearize first order kinetics.

RESULTS

Separation of cyclic AMP dependent protein kinase I from other binding proteins for cyclic AMP in skeletal muscle extract. Rabbit skeletal muscle in addition to cyclic AMP dependent protein kinase contains cyclic AMP-adenosine binding proteins (Fig. 1 & 2a) of the type found in erythrocytes (10) and liver (11, 12, 13). Protein kinase I can be completely resolved from that class of binding proteins by a combination of polyethylene-glycol fractionation (Fig. 1) and DEAE-

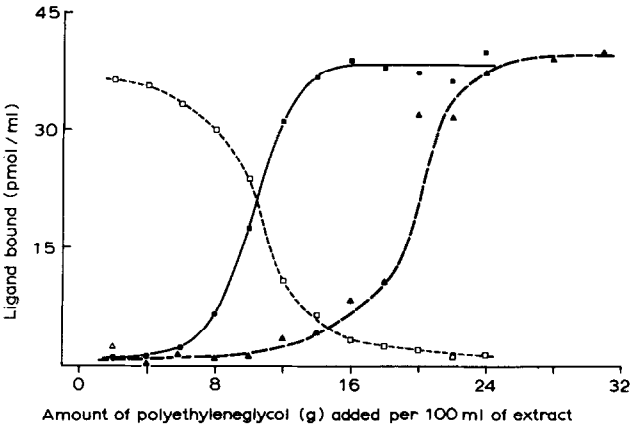

Fig. 1. Polyethyleneglycol-precipitation of binding proteins from rabbit skeletal muscle. To samples of muscle extract, obtained as described (9) except for the absence of polyethyleneglycol from the homogenization buffer, was added the amounts of polyethyleneglycol 6000 indicated on the abscissa, and the resulting precipitates collected by centrifugation (20 000 x g av. for 30 min) 1 h later.
The sediments were dissolved in 10 mM Tris-HCl pH 7.5/1 mM EDTA/ 20 mM 2-mercaptoethanol. Samples from the supernatant (open symbols) or the redissolved sediment (closed symbols) were incubated under conditions (see Exp. sect.) favouring the binding of cyclic [^{3}H]AMP to protein kinase (□,■). Other samples of the redissolved sediments were incubated with 0.5 µM [^{3}H]adenosine/0.5 µM erythro-9-(2-hydroxy-3-nonyl) adenine for 3 h at 0°C (▲). The amount of bound ligand is expressed per ml of the original extract.

cellulose chromatography (Fig. 2b). That procedure also removed cyclic AMP phosphodiesterase activity (9). The cyclic [^{3}H]AMP binding activity and protein kinase activity copurified during all the purification steps for protein kinase I subsequent to DEAE-cellulose chromatography, and after preincubation with cyclic AMP all the binding activity was eluted as a single symmetrical peak from DEAE-cellulose at the position expected for the regulatory moiety of protein kinase I (2, 13).

Dissociation rate experiments. There was a biphasic rate of exchange between bound labelled and free unlabelled cyclic AMP, each of the phases obeying first order kinetics. At high ionic strength the difference between the two phases was especially marked, the first phase being accelerated, and the second phase retarted (Fig. 3). When unlabelled cyclic AMP was not added more than 90% of the cyclic [^{3}H]AMP bound at zero time remained bound after 60 h at 0°C or 40 min at 37°C (Fig. 3b) for all the incubations. More than 95% of the initially bound cyclic [^{3}H]AMP was exchangeable at 37°C.

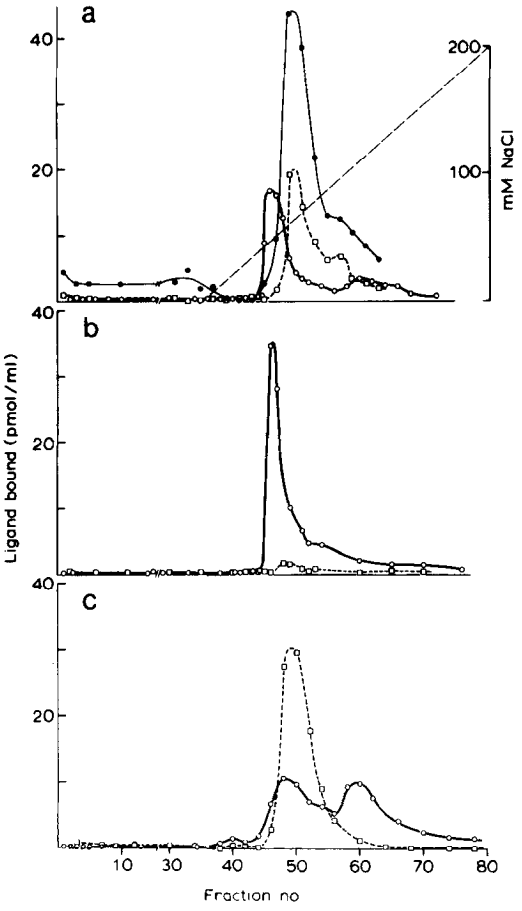

Fig. 2. DEAE-cellulose chromatography of polyethyleneglycol-fractions from muscle extract

a) Extract of rabbit skeletal muscle (20 g) prepared as described (9) was diluted to 0.2 l with water and subjected to DEAE-cellulose chromatography (9).

b) The fraction of extract from rabbit skeletal muscle (40 g) precipitated between 6.5 and 14 g of polyethyleneglycol/100 ml was dissolved in 0.2 l of column equilibration buffer and chromatographed as above.

c) The supernatant after precipitation with 14 g of polyethyleneglycol/100 ml was diluted to 0.4 l with water and chromatographed as above.

Binding of cyclic [^{3}H]AMP was assayed under conditions favouring binding to the regulatory moiety of protein kinase (o) or cyclic AMP/adenosine binding proteins (•), as described in the Exp. Sect. The binding of [^{3}H] adenosine was assayed as described in the legend to Fig. 1 (□). The results are given as pmol of ligand bound/ml faction (20 ml). The NaCl-gradient used to elute the enzyme activities is shown by a dashed line in a).

Increasing the concentration of NaCl, KCl, or Na_2 EDTA increased the initial rate of exchange of the bound cyclic [^{3}H]AMP. Na_2 EDTA was more efficient in this regard than the other salts; NaCl and KCl were equipotent. The inclusion of 10 mM Mg^{2+} or Ca^{2+} or the use of

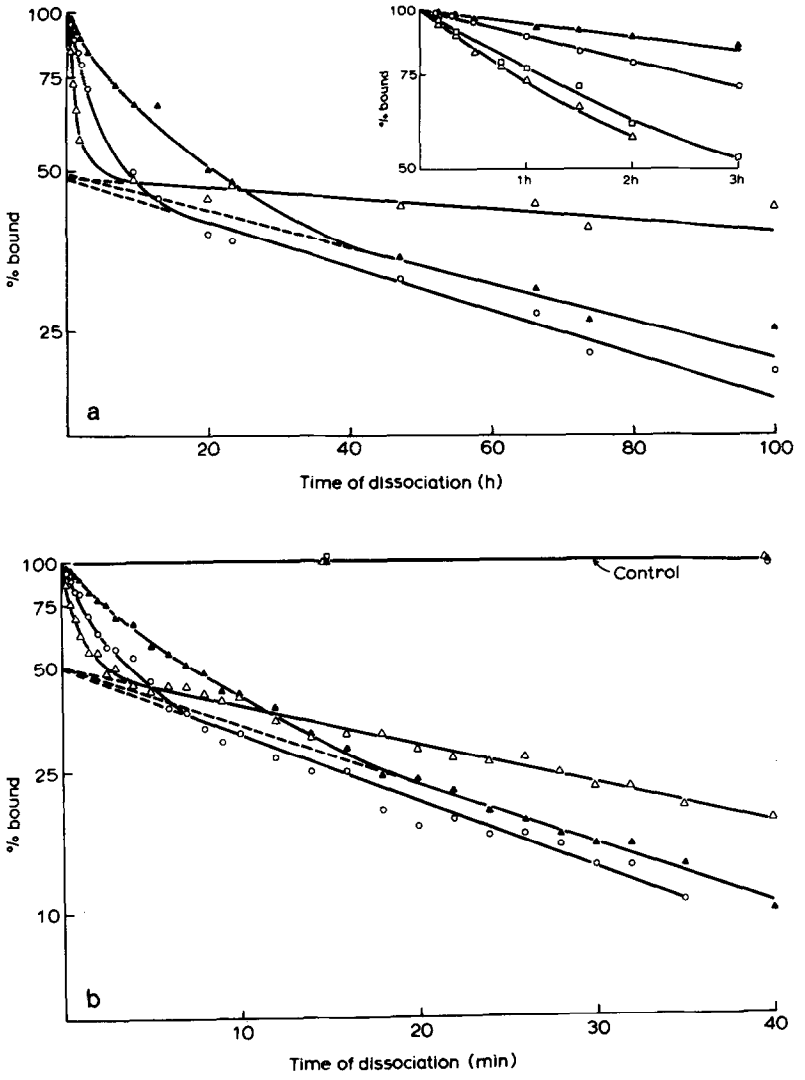

Fig. 3. The dissociation rate of complexed cyclic [^{3}H] AMP as a function of ionic strength and temperature. Protein kinase I was preincubated for 1 h at 0°C (a) or for 15 min at 37°C (b) in the presence of 0.1 µM cyclic [^{3}H] AMP, albumin (0.5 mg/ml), heat-stable protein fraction (0.3 mg/ml) and either 15 mM Hepes-NaOH pH 7.0/1 mM EDTA/20 mM 2-mercaptoethanol (▲), the same buffer with 150 mM KCl (o), or 50 mM Hepes-NaOH pH 7.0/ 30 mM EDTA/20 mM 2-mercaptoethanol with 0.8 M (□) or 3.2 m (Δ) NaCl. The preincubate was divided in two portions. One portion was mixed with 1/100 its volume of 10 mM unlabelled cyclic AMP (at zero time) and samples removed for determination of cyclic [^{3}H] AMP bound at the time points indicated on the abscissa. The other portion served as control of the stability of the binding reagent.
a) Experiment conducted at 0°C. The initial dissociation rate is shown in the inset.
b) Experiment at 37°C. The amount bound in samples of control incubates is shown in the top part of the figure.

Mes- or glycyl-glycine-buffers instead of Hepes did not significantly affect the dissociation rate.

A tenfold increase of the concentration of albumin and heat-stable protein or their omission (the loss of binding sites occuring in the absence of those stabilizing proteins being corrected for) did not alter the rate of exchange of bound cyclic [^{3}H]AMP.

The free regulatory moiety of protein kinase I, as well as kinase subjected to prolonged preincubation with cyclic [^{3}H]AMP at high ionic strength and kinase prepared so as to minimize proteolysis all showed the same shape of the dissociation-rate curve (Fig. 4) as the routinely used preparation of protein kinase I (Fig. 3a).

DISCUSSION

The kinetics of the interaction between cyclic [^{3}H]AMP and highly purified protein kinase I (Figs. 3 & 4) strongly suggested the presence of two distinct and equally large populations of binding sites for cyclic AMP associated with protein kinase I. The dissociation rate was biphasic at physiological pH, ionic strength and temperature (Fig. 3b) as well as under all other conditions tested. Since protein kinase I preincubated for 60 h in the presence of a saturating concentration of cyclic [^{3}H]AMP as well as enzyme freshly prepared in the presence of protease-inhibitor both showed the same biphasic exchange between bound labelled and free unlabelled cyclic AMP, trapping of isotope during the incubation or progressive modification of the binding protein occurring during incubation or purification were unlikely.

The presently described binding sites could be separated (Figs. 1 &2) from a class of cyclic AMP binding proteins not associated with protein kinase (10-13); but cochromatographed with cyclic AMP dependent protein kinase activity on Ultrogel and hydroxylapatite and cosedimented with it on density gradient centrifugation. After preincubation in the presence of cyclic AMP both binding sites were eluted from DEAE-cellulose at the ionic strength expected for the regulatory moiety of protein kinase I. Those results as well as recently conducted experiments (S.O. Døskeland, unpublished observations) showing that the binding of cyclic AMP to the two sites occurs concerted when protein kinase I is in the holoenzyme form and that there is interaction between the sites in preparations of the regulatory subunit dimer of protein kinase I, indicate that the presently described binding sites are associated with protein kinase I and probably reside on the same regulatory subunit dimer.

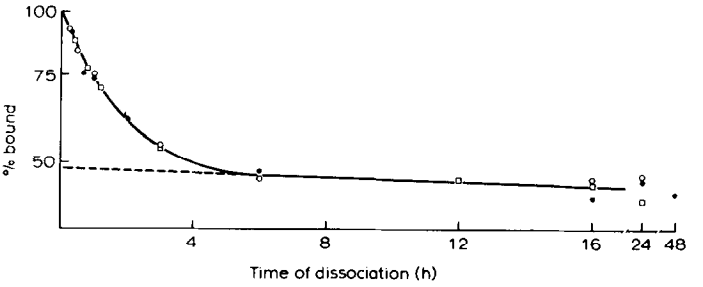

Fig. 4. The dissociation rate of cyclic [^{3}H] AMP complexed to various preparations of binding protein. The rate of exchange at 0°C of bound cyclic [^{3}H] AMP and excess unlabelled nucleotide added at zero time in 50 mM Hepes-NaOH pH 7.0/30 mM EDTA/3.2 M NaCl/20 mM 2-mercaptoethanol/albumin (0.5 mg/ml) and heat-stable protein (0.3 mg/ml) is plotted as a function of time.
a) Binding protein was one of the control-incubations (o) described in the legend to Fig. 3a (kept for 60 h a 0°C in the Hepes/3.2 M NaCl-buffer just described).
b) Binding protein was freshly prepared (see Exp. sect.) protein kinase I (□), preincubated for 30 min at 0°C with 0.5 μM cyclic [^{3}H] AMP before the addition of unlabelled cyclic AMP:
c) Binding protein was the free regulatory moiety of protein kinase I (●), prepared as described in the Exp. sect.

A strong interaction between identical binding sites cannot explain the two phases of the dissociation curves (Fig. 3), since these were obtained under conditions (saturation of all binding sites) when the interaction would be the same at all stages of the dissociation of bound labelled cyclic AMP.

Earlier studies of the dissociation rate of the complex between cyclic AMP and the regulatory moiety of protein kinase I have focused on the increase of dissociation rate occurring at high temperature in the presence of Mg-ATP and the catalytic subunit of protein kinase, and no heterogeneity of binding sites has been noted (14, 15).

The biological significance of the presence of two kinetically distinct binding sites for cyclic AMP associated with protein kinase I is so far unknown. The effect of occupancy of either or both of the two binding sites on the reassociation of the protein kinase subunits will be studied.

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