DISSOCIATION OF RABBIT MUSCLE CYCLIC AMP-DEPENDENT PROTEIN KINASE INTO CATALYTIC AND REGULATORY SUBUNITS BY *p*-CHLOROMERCURIBENZOATE AND METHYLMERCURIC CHLORIDE

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1. Introduction

Numerous studies have established that cyclic AMP-dependent protein kinases are composed of catalytic and regulatory subunits [1]. Binding of cyclic AMP to the regulatory subunit of the inactive holoenzyme causes dissociation into free regulatory subunits and active free catalytic subunits. Preincubation of a number of protein kinases with their protein substrates has also been shown to lead to their activation, probably by promoting dissociation of catalytic and regulatory subunits [2–5]. However there is little information on the nature of the interaction between the subunits in the intact holoenzyme, despite some evidence that sulfhydryl groups may be involved [6]. In this paper it is shown that preincubation of rabbit muscle protein kinase with low concentrations of *p*-chloromercuribenzoate (PCMB) or methylmercuric chloride causes dissociation of the holoenzyme into free catalytic and regulatory subunits.

2. Materials and methods

Rabbit muscle protein kinase was purified as described by Walsh et al. [7] up to the DE 52 cellulose chromatography stage. When assayed with whole calf thymus histone as described below in the presence of 10^{-6}M cyclic AMP the preparation catalysed the transfer of 2.7 nmol of phosphate to histone/min/mg of protein.

The standard kinase assay contained 20 μmoles Na–acetate, pH 6, 1 μmole $MgCl_2$, 0.1 mg of whole calf thymus histone (Sigma, type IIA), 5.4 nmol [α-^{32}P]ATP (specific activity 0.18–0.30 × 10^3 counts/min/pmol) and enzyme in a final volume of 100 μl. Assays were carried out at 30°C for the indicated times; reactions were stopped and incorporation of label into histone measured as described before [5].

Where indicated kinase assays were carried out in which the enzyme was preincubated with either PCMB or cyclic AMP before assaying enzymic activity. In these experiments protein kinase (4.8 μg protein) and 20 μmoles sodium acetate, pH 6, with or without PCMB or cyclic AMP, were preincubated in a final volume of 50 μl. At the end of the preincubation period the kinase assay was initiated by addition of 100 μl of a reaction mixture containing 1.5 times the amounts of $MgCl_2$, [α-^{32}P]ATP and histone as described above for the standard assay.

Cyclic AMP binding was measured in assays containing 20 μmol Na–acetate, pH 6, 12.5 nCi ^{3}H-cyclic (specific activity, 27.5 Ci/mmol) and enzyme in a final volume of 100 μl. After incubation for 60 min at 1°C the reaction mixture was diluted with 1 ml of 50 mM sodium acetate, pH 6 and filtered through Millipore filters (HAWP). After washing with 50 mM sodium acetate, pH 6 (2 × 1 ml) radioactivity was measured using Bray's [8] scintillation fluid.

Linear sucrose gradients (5–20%; 4.4ml) contained 50 mM sodium acetate buffer (pH 6) and were centrifuged at 2°C for 16 hr at 240 000 *g*. Fractions (4 drops) were collected and 20 μl samples were assayed for histone kinase activity (16 min, 30°C).

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3. Results and discussion

In Table 1 the effect of preincubation of muscle protein kinase on the basal activity, and on the response to cyclic AMP is shown. In the control assays stimulation by cyclic AMP was less than is normally observed, largely because of a 3.4-fold increase in basal activity during the 10 min preincubation. It is clear that cyclic AMP addition does not increase kinase activity in the presence of PCMB. Although preincubation with PCMB alone resulted in only a small increase in total kinase activity, an enhanced activity was observed when 2-mercaptoethanol was included in the assay. The sulfhydryl reagent completely reversed the inhibition by PCMB in the presence of cyclic AMP. This data is consistent with a model in which PCMB induces dissociation of the protein kinase holoenzyme into catalytic and regulatory subunits. However, because this does not lead to greatly enhanced enzyme activity in the absence of 2-mercaptoethanol, it is necessary to also postulate that PCMB is an inhibitor of the catalytic subunit.

This model was tested in an experiment in which enzyme pretreated with PCMB was fractionated by centrifugation on sucrose density gradients, also containing PCMB (fig.1). Comparison with the control

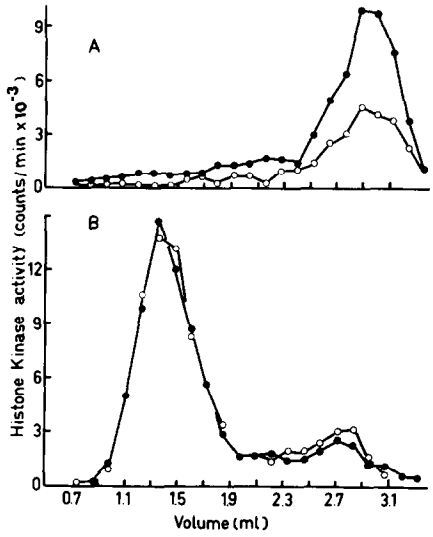

Fig. 1. Effect of PCMB on the sedimentation of rabbit muscle histone kinase activity in sucrose density gradients. A: protein kinase (28.8 μg protein) was preincubated with 10^{-5} M PCMB, 200 mM sodium acetate pH 6 (final vol 400 μl) for 10 min at 4°C before loading on a gradient also containing 10^{-5} M PCMB. After centrifugation assays were carried out in the absence (o–o–o) and presence (•–•–•) of 2 mercaptoethanol (10^{-3} M) as described in the Materials and methods section. B: control gradient; conditions were as described in frame A, but in the absence of PCMB. Assays were carried out in the absence (o–o–o) and presence (•–•–•) of 10^{-3} M 2-mercaptoethanol.

Table 1
Effect of PCMB on rabbit muscle protein kinase activity

Additions to reaction mixture			
First preincubation (5 min)	Second preincubation (5 min)	Assay (5 min)	Protein kinase activity (nmol/min/mg protein)
–	–	–	0.94
–	–	ME	0.94
–	cyclic AMP	–	3.07
–	cyclic AMP	ME	3.14
PCMB	–	–	1.14
PCMB	–	ME	2.21
PCMB	cyclic AMP	–	1.13
PCMB	cyclic AMP	ME	3.00

Assays were carried out as described in the Results section after preincubation for a total of 10 min. Where indicated, PCMB (10^{-5} M) and cyclic AMP (10^{-6} M) were added during preincubation, and 2-mercaptoethanol (ME; 10^{-3} M) during the assay of protein kinase activity.

gradient indicates that the inclusion of PCMB caused the dissociation of the holoenzyme into a lower molecular weight catalytic component. The catalytic activity in the gradient containing PCMB was enhanced by 2-mercaptoethanol, indicating that the mercurial is an inhibitor of the catalytic subunit. Further control experiments indicated that the sedimentation pattern of the catalytic activity in the presence of PCMB, corresponded exactly with that of the cyclic nucleotide-independent activity obtained after centrifugation in the presence of cyclic AMP (10^{-6}M). In the centrifugation system used, cyclic AMP binding activity sedimented at a position intermediate to the holoenzyme and the dissociated catalytic subunit. Assays of cyclic AMP binding after centrifugation in the presence of PCMB showed only traces of binding activity indicating a labilization of the regulatory subunit by the mercurial. Although PCMB (10^{-5} M)

caused only a small decrease in binding activity when standard assays of cyclic AMP binding at 1°C were carried out for 60 min, preincubation of holoenzyme with PCMB (10^{-5}M) for 21 hr decreased cyclic AMP binding by more than 50% compared with controls preincubated without PCMB.

The sucrose density gradient experiments reported in fig.1 were repeated but replacing PCMB with 10^{-5}M methylmercuric chloride. Similar results were obtained, indicating that this alkylmercury derivative also caused the dissociation of holoenzyme into catalytic and regulatory subunits. Further details of the inhibitory effects of both PCMB and methylmercuric chloride on the histone kinase activity of the isolated catalytic subunit are shown in fig.2.

The data clearly shows that interaction of both PCMB and methylmercuric chloride with the rabbit muscle holoenzyme causes its dissociation into catalytic and regulatory subunits, and implies that sulfhydryl groups are involved in the interaction between these subunits. The situation is analogous to the dissociation of *E. coli* aspartate transcarbamylase into catalytic and regulatory subunits by *p*-mercuribenzoate [9]. The dissociative and inhibitory effects of methylmercuric chloride are of particular interest in view of its ecological importance and identification as a neurological toxin in man [10,11], and because of the proposed involvement of cyclic nucleotides in brain function [12–15].

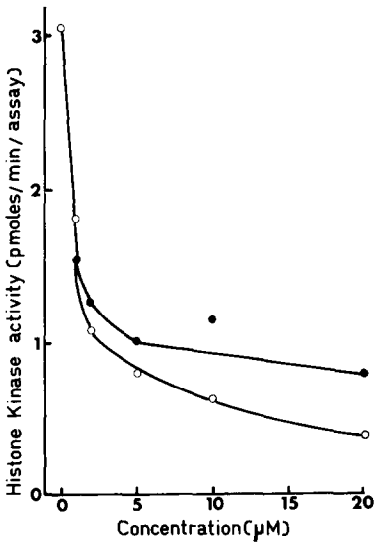

Fig.2. Inhibition of isolated catalytic subunit by PCMB and methylmercuric chloride. To obtain isolated catalytic subunit, rabbit muscle protein kinase (240 μg protein) was centrifuged on a sucrose density gradient containing 8×10^{-7} M cyclic AMP. Peak fractions containing histone kinase activity were pooled. Assays were carried out for 10 min as described in the Materials and methods section and contained a final concentration of 4×10^{-8} M cyclic AMP (derived from the gradient).

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