CYCLIC 3',5'-NUCLEOTIDE PHOSPHODIESTERASE

Demonstration of an Activator

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SUMMARY. The activity of a mixture of a purified and a crude cyclic 3',5'-nucleotide phosphodiesterase was greater than the summed activities of the individual preparations. The crude enzyme contained an activator, which was removed from the purified enzyme during purification. The activator, isolated free of phosphodiesterase activity, effectively reconstituted the activity of the purified enzyme. The relative inactivity of purified phosphodiesterase was due to removal of the activator from the enzyme.

INTRODUCTION

Cyclic AMP is believed to be a mediator of a variety of different hormones (1). Cyclic 3',5'-nucleotide phosphodiesterase catalyzes the hydrolysis of this nucleotide to 5'-AMP. Thus the activity of phosphodiesterase is critically related to the tissue levels of cyclic AMP, and therefore to the extent and duration of the related hormonal action.

In an attempt to elucidate mechanisms regulating tissue levels of cyclic AMP, we have studied factors which affect phosphodiesterase activity (2,3). We found that while the crude phosphodiesterase of bovine brain cerebra was fully active, the purified enzyme was relatively inactive (4). The purified enzyme depended on a stimulatory factor(s) in snake venoms for maximal activity. We now show that an activator of phosphodiesterase is present in a crude homogenate of bovine brain cerebra. The activator, which has been prepared free of phosphodiesterase, markedly stimulates the purified enzyme.

METHODS

Phosphodiesterase was prepared and assayed as described elsewhere (4). Briefly, the enzyme was extracted with water from bovine brain cerebra, and was purified by pH and ammonium sulfate fractionation, calcium phosphate gel and DEAE-cellulose column chromatography. The purified enzyme was relatively inactive unless supplemented with its activator. To demonstrate activation, enzyme activity was measured using a two-stage procedure, modified slightly after that of Butcher and Sutherland (5). At the end of the first stage of incubation, enzymic activity was arrested by boiling. After thermal equilibration, snake venom (Crotalus atrox) was added to all assayed tubes for a second incubation. Venom was used as a source of 5'-nucleotidase, which converted 5'-AMP into adenosine and inorganic phosphate. The latter was measured colorimetrically. Unless otherwise indicated, the venom was not present in the first incubation. It should be pointed out that the venom was inactive towards cyclic AMP and that all data were corrected for a control containing no phosphodiesterase.

The activator was isolated free of phosphodiesterase at the last stage of our purification procedure. It was eluted after the enzyme on the DEAE-cellulose column. Tubes following the peak activity of phosphodiesterase were pooled. The combined eluate was lyophilized and then dialyzed against 20 mM Tris-Cl, pH 7.5. The activator was retained inside the dialysis tubing. The dialyzed sample usually contained a small amount of denatured protein, which was removed by centrifugation.

Proteins were determined by the biuret reagent containing deoxycholate or by the spectrophotometric method of Warburg and Christian (6).

RESULTS AND DISCUSSION

Table I compares the activity of a crude and a purified phosphodiesterase assayed individually to that of a mixture of these two enzymes assayed together. The activity of the mixture was more than twice the summed activities of the

TABLE I

COMPARISON OF THE ACTIVITY OF A CRUDE AND A PURIFIED PHOSPHODIESTERASE WITH THAT OF A MIXTURE OF THESE TWO.

	Fraction	Activity (mμ moles)
[a]	Crude phosphodiesterase	66
[b]	Purified phosphodiesterase	40
[c]	Crude + purified phosphodiesterase	260

Phosphodiesterase was assayed using a two-stage procedure as summarized under Methods. A homogenate of bovine brain cerebra was dialyzed extensively against 20 mM Tris-Cl, pH 7.5 and was then centrifuged at 40,000 g for 20 minutes. An aliquot of the supernatant fluid was used as a source of the crude phosphodiesterase. The purified phosphodiesterase was prepared from a DEAE-cellulose column and was relatively inactive prior to activation (4). Protein concentration in μg per 0.5 ml of the reaction mixture: crude phosphodiesterase, 110; purified phosphodiesterase, 50. Activity is expressed as mu moles of inorganic phosphate formed per 10 minutes.

individual preparations. This experiment demonstrated the synergistic effect of the mixture but did not reveal which one of the two components was the activating agent.

Our studies on the stimulation of purified phosphodiesterase by snake venom is illuminating in this respect. Table II shows the effect of the venom on the activity of a crude and a purified phosphodiesterase. The venom activated the purified but not the crude enzyme (compare [d] with [c] and [b] with [a]). Also, the activity of the mixture was markedly greater than the summed activities of the individual preparations, as was shown in Table I. Of particular interest is the fact that the activity of the mixture was equivalent to the summed activities of the crude and the purified enzyme subsequent to its activation by the venom. This experiment suggested two things. First, since the purified,

TABLE II

EFFECT OF SNAKE VENOM ON THE ACTIVITY OF CRUDE AND PURIFIED PHOSPHODIESTERASE.

Fractions	Activity (mμ moles)
[a] Crude phosphodiesterase	262
[b] Crude phosphodiesterase + venom	279
[c] Purified phosphodiesterase	80
[d] Purified phosphodiesterase + venom	454
[e] Crude + purified phosphodiesterase	658

Phosphodiesterase was assayed using a two-stage procedure. When indicated, venom was present in the first stage of incubation. Protein concentration in μg per one ml of the reaction mixture: crude phosphodiesterase, 245; purified phosphodiesterase, 100; snake venom (Crotalus atrox), 100. Activity is expressed as mu moles of inorganic phosphate formed per 10 minutes.

but not the crude enzyme, was stimulated by the venom, the purified enzyme was the component whose activity had been stimulated. In other words, the activating agent (or activator) was probably associated with the crude enzyme. Second, this activating agent was as effective as the venom in stimulating the purified enzyme.

Phosphodiesterase is a stable enzyme in our hands. Other experiments have shown that the stimulation of the purified enzyme by the venom is not due to protection of some unstable phosphodiesterase activity afforded by proteins in the venom (4). This would suggest that the increased activity of the purified enzyme in the presence of the homogenate was not due to protection of some unstable activity, but rather to a stimulation of the enzyme.

TABLE III

EFFECT OF ACTIVATOR ON THE ACTIVITY OF PURIFIED PHOSPHODIESTERASE.

Fractions	Activity (mμ moles)
[a] Purified phosphodiesterase	56
[b] Activator	0
[c] Purified phosphodiesterase + activator	315
[d] Purified phosphodiesterase + venom	306

Phosphodiesterase was assayed using a two-stage procedure. When indicated, venom was present in the first stage of incubation. The activator was a preparation which had been stored at -20° for six months prior to use. Protein concentration in μg per one ml of the reaction mixture: purified phosphodiesterase, 100; activator, 50; snake venom (Crotalus atrox), 100. Activity is expresses as mu moles of inorganic phosphate formed per 10 minutes.

From such considerations, we reasoned that the relative inactivity of the purified enzyme was due to removal of an activator during the course of enzyme purification. Indeed, we found that the activator was present originally in the crude homogenate and that it was removed from phosphodiesterase as purification proceeded. Fractions obtained early in the purification procedure were rich in the activator while those obtained at a later stage were deficient. To illustrate this point, the preparation of the activator from the DEAE-cellulose column as described under Methods may be cited. The enzyme prior to this step was fully active (4). When the activator was dissociated from the enzyme on the column, the enzyme became relatively inactive. Table III shows the effect of the activator on the activity of a purified phosphodiesterase. In this

experiment, the activator, itself inactive [b], increased the activity of the purified enzyme more than five-fold (compare [c] with [a]). The venom, as usual, caused a pronounced stimulation [d]. It is noted that the extent of stimulation by the activator and by the venom was comparable.

The activator was inactivated by trypsin, and not by DNase or RNase, indicating that it was a protein. Gel filtration using a calibrated Sephadex G-100 column gave a molecular weight of about 40,000. The activator was remarkably stable to heat, to acidic pH's and to 8M urea. No proteolytic activity could be demonstrated. Stimulation of the purified enzyme by the activator appeared specific, as several proteins of various molecular weights were unable to mimic its stimulatory effect.

Stimulation of the enzyme was directly proportional to the concentration of the activator, indicating a stoichiometric interaction between the two. Kinetic analysis showed that the increase of Vmax was coupled to a decrease of Km for cyclic AMP. Although these results do not reveal the mechanisms of stimulation, it is conceivable that the activator may be important in regulating phosphodiesterase in vivo.

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