CYCLIC AMP PHOSPHODIESTERASE INTERACTION WITH ITS INHIBITOR OF THE SLIME MOLD, DICTYOSTELIUM DISCOIDEUM.

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SUMMARY

Amoebae of the cellular slime mold, Dictyostelium discoideum, release an inhibitor of their cAMP-phosphodiesterase into the culture medium at the end of growth. In this paper evidence is provided (1) that the excreted PD that reacts with the inhibitor is not identical with the bulk of soluble phosphodiesterase present in cell homogenates, (2) that the extracellular PD that reacts with the inhibitor has a K_m of 4 x 10^{-6} M, (3) that kinetic data point to a noncompetitive inhibition by a tight-binding inhibitor, (4) that trypsin inactivates the inhibitor.

Dictyostelium cells grow as single amoebae with a generation time of 3 hours when supplied with food bacteria. After exhaustion of bacteria, the amoebae aggregate and eventually differentiate into either spores or stalk cells of a multicellular fruiting body. It has been shown that cAMP acts as a chemotactic factor guiding cell movement during aggregation, and obviously also controls other morphogenetic events (1,2).

The activity of an extracellular cAMP-hydrolyzing PD is regulated during development by a non-dialyzable inhibitor, which appears in the culture medium at the end of the growth phase (3).

Abbreviations: cAMP cyclic 3',5'-adenosine phosphate
TP thymidine-5'-phosphoric acid-4-nitrophenylester
PD phosphodiesterase
ePD extracellular phosphodiesterase
iPD intracellular phosphodiesterase

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Inhibitor, because it is more heat-stable than the PD, can be prepared from heated culture supernatants; PD is most easily obtained from cultures of inhibitorless mutants. This paper deals with various properties of the PD and the inhibitor, and with the kinetics of their interaction. It will appear that the PD the inhibitor interacts with has a much lower K_m than a cAMP-phosphodiesterase described previously from the same source (4,5).

METHODS

Cultures of Dictyostelium discoideum. To obtain PD and its inhibitor, the inhibitorless mutant aggr 50, and the PD-deficient mutant ga 86 were cultivated on suspensions of Escherichia coli B/r (6), and harvested at time of either maximal PD or inhibitor activity. The cells were washed twice with 0.017 M phosphate buffer pH 6.0. The culture medium was freed from particulate matter by centrifugation for 15 min at 5,000 xg.

Preparation and partial purification of ePD. A crude preparation was obtained by harvesting the culture medium of aggr 50 4 hrs after concumption of bacteria, and subsequent dialysis against 0.01 M triethanolamine buffer pH 7.4. For purification, non-dialyzed medium was concentrated 10-fold on PM 10 membranes (Amicon, Waltham, Mass.) and the concentrated solution brought to 60 per cent saturation with ammonium sulfate. The precipitate was dissolved and dialyzed against 0.005 M triethanolamine buffer pH 7.0, and the PD was further purified on DEAE cellulose by elution with NaCl in 0.005 M triethanolamine buffer pH 7.0 (fig.2).

Preparation of soluble iPD. A suspension of approx. 8×10^8 washed aggr 50 cells/ml in a pH 7.5 buffer containing 0.05 M Tris and 0.01 M glutathione was homogenized with a Branson S 75 sonifier (50 sec, lowest power switch setting). After centrifugation for 30 min at 20,000 g and 2 hrs at 100,000 g, the supernatant was tested for iPD.

Preparation and partial purification of inhibitor. A crude inhibitor solution was prepared from culture medium of ga 86, harvested within 2 hrs after the time of complete consumption of bacteria. The medium was dialyzed against 0.01 M triethanolamine pH 7.4 and then heated 10 min at 80°C. Purified inhibitor was obtained from heated, undialyzed medium by ammonium sulfate precipitation, DEAE-cellulose chromatography and Sephadex G75 gel filtration (Malchow et al., in preparation).

PD assays. If not stated otherwise, hydrolysis of cAMP was measured as described previously (3) with alkaline phosphatase (EC 3.1.3.1) and adenosine deaminase (EC 3.5.4.4) at 265 nm (7). The reaction rate in this assay is a linear function of PD activ ity up to a rate of 3.5 nmoles cAMP/ml×min. All assays have been done within this range. With TP as substrate, PD activity was measured at 405 nm according to Ostrowski et al. (8). Hydrolysis of cGMP was determined by paper chromatography using 3H-labelled cGMP (Amersham, specific activity 4.4 Ci/mmol) as described by Okabayashi et al. (9). cIMP hydrolyzing activity was tested by descending paper chromatography in 95% ethanol/1 M ammonium acetate pH 7.5, 70 : 30 v/v, after incubation of 25 mM cIMP with PD.

pH dependence of PDs. iPD and crude, dialyzed ePD were prepared from one aggr 50 culture which was harvested 5 hrs after consumption of bacteria. Hydrolysis of TP was measured in 0.05 M or 0.08 M cacodylate buffer (pH 3.0 to 8.0) or sodium acetate buffer (pH 2.5 to 5.0), to which 10 mM Mg^{2+} and 10 mM substrate were added. After 30 or 60 min at 35°C the reaction was stopped by 10-fold dilution with 0.1 N NaOH. In the same buffer, supplemented with 7 mM Mg^{2+} and 50 µM substrate, the pH-dependence of cAMP-hydrolysis was measured by incubation for 15 min at 35°C.

The reaction was stopped by heating for 2 min in a boiling water bath and the product, 5'-AMP, determined by phosphatase and deaminase as described above.

Inhibitor assay. Interaction with PD was tested in 0.01 M triethanolamine buffer pH 7.4 (3), by incubation for 15 min at 35°C, if not stated otherwise, and subsequent PD assay. One inhibitor unit was defined as that quantity which in a total volume of 300 µl inhibits PD of an activity of 2 nMoles cAMP/min (35°C) to one half.

N-acetylglucosaminidase was assayed according to Loomis (10) using 2.4×10^{-3} M p-nitrophenyl-N-acetylglucosamine as substrate

Protein was determined according to Lowry et al. (11).

Enzyme treatment of the inhibitor. 1 ml crude inhibitor solution was incubated at 35°C with 165 µl trypsin 10 mg/ml (2 x cryst., Boehringer, Mannheim) or with 200 µl RNase A 1 mg/ml (protease free, Serva, Heidelberg). The reaction with trypsin was terminated by addition of equivalent amounts of soybean inhibitor (Worthington, Freehold, N.J.).

RESULTS

pH dependence and substrate specificity of extracellular (ePD)

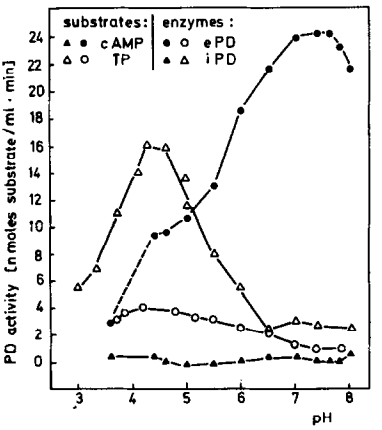

Fig. 1 pH dependence of hydrolysis of cAMP and TP by the soluble fraction of cell homogenate (iPD), and by a crude enzyme preparation from culture medium (ePD).

and soluble, intracellular phosphodiesterases (iPD). D.discoideum cells contain phosphodiesterase which reacts with TP as substrate at an optimum pH of 4.0 to 4.5. In view of the finding that other hydrolases of probably lysosomal origin are excreted into the culture supernatant (10, 14, 15), we tested if the cAMP-converting ePD is identical with the intracellular acid PD. Fig. 1 shows that the supernatant fraction of a cell sonicate prepared in presence of glutathione contained virtually no cAMP-hydrolyzing activity, and that the activity in the culture supernatant had an optimum at pH 7.4. TP-hydrolysis was high with the sonicated homogenate fraction and low with culture medium. In both cases, the pH-optimum for the TP-splitting activity was about 4.3 (fig.1). These results indicate that the extracellular cAMP-hydrolyzing PD is essentially inactive against TP as substrate, and that the small activities of TP-splitting PD, present in the culture supernatant, do not significantly contribute to cAMP-hydrolysis.

With the ePD, the ratio of V_{max} for hydrolysis of cGMP and cAMP was 0.09. cIMP was split at a rate < 2 per cent of the rate for cAMP.

Partial purification of ePD. DEAE cellulose chromatography of ammonium sulfate precipitated culture medium yielded one main

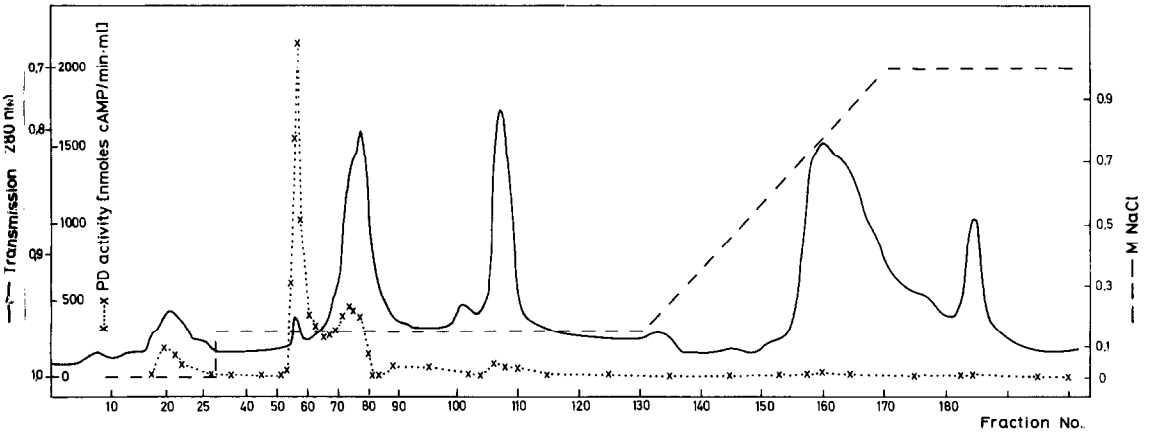

Fig. 2 DEAE cellulose chromatography of ammonium sulfate precipitated ePD.

PD-peak (fig.2). The enzyme in the peak fraction No.57 was 160-fold purified compared with dialyzed culture medium and had a specific activity of 130 µM cAMP/mg protein x min (35°C).

K_m of free and partially inhibited ePD. From the double reciprocal plots of fig.3 an apparent K_m of 4 x 10^{-6}M was calculated for the free partially purified enzyme, and the same value has been obtained with a crude ePD preparation, suggesting the absence of a non-dialyzable effector that changes the K_m. Preincubation of the enzyme with inhibitor decreased V_{max} and left the K_m unchanged under conditions of low substrate concentrations. At higher cAMP concentrations, however, a downward curvature appeared, characteristic for a tight-binding inhibitor (fig.3) (12,13). The apparent K_m for cGMP was 5 x 10^{-5}M with the free, partially purified ePD.

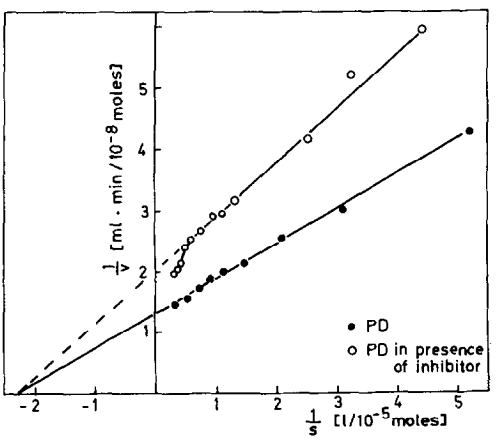

Fig. 3 Double reciprocal plots of initial velocity versus cAMP concentration for partially purified ePD, without, and after preincubation with partially purified inhibitor.

Inhibitor-enzyme interaction. Fig. 4 shows the time dependence of inhibitor action at different temperatures. At the concentrations used, the reaction was almost complete after incubation for 15 min at 35°C. In fig.5 the activity of ePD is plotted as a function of inhibitor concentration. To check specificity of inhi-

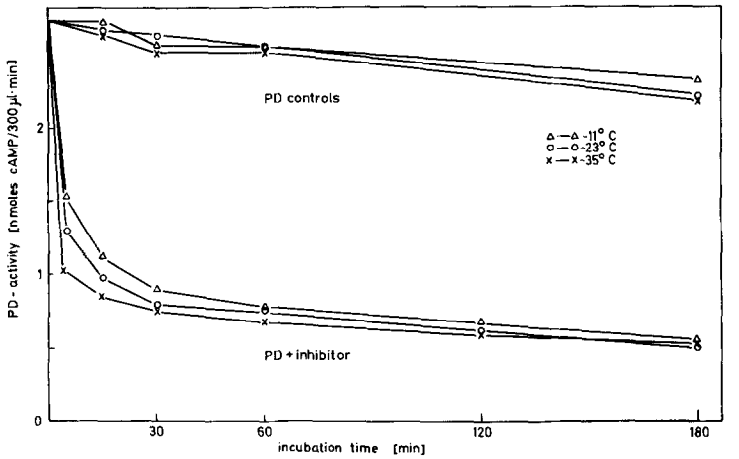

Fig. 4 Inactivation of ePD, as a function of length of incubation with inhibitor at different temperatures, measured in crude preparations.

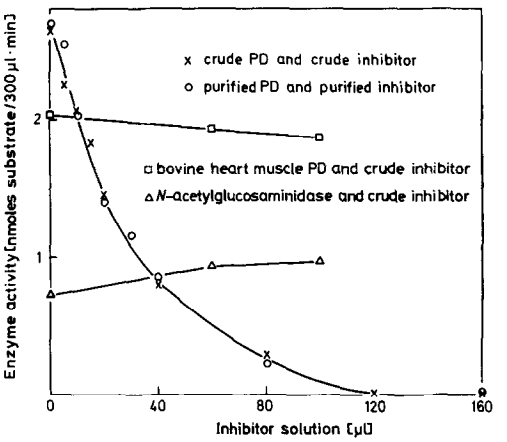

Fig. 5 Activity of ePD, bovine heart muscle PD, and N-acetylglucosaminidase from D.discoideum as a function of inhibitor concentration. The solution of partially purified inhibitor was adjusted to the same number of units/ml as the crude preparation.

bitor action, cAMP-PD from bovine heart muscle and N-acetylglucosaminidase (10), an enzyme which is also released into the culture medium of Dictyostelium cells (14), were tested under the same conditions, and no significant inhibition was found for either (fig.5).

Effects of degrading enzymes on the inhibitor. Trypsin inactivates the PD inhibitor to 64 and 96 per cent within 30 and 60 min, respectively, whereas RNase does not.

DISCUSSION

The time dependence of inhibitor/PD interaction (fig.4) and unpublished results on purification of the PD/inhibitor complex indicate that this interaction is stoichiometric, rather than an enzymatic degradation of the PD. Partially purified inhibitor and PD interact with each other as well as unpurified samples do (fig.5). Thus it is improbable that a third factor, especially an enzyme, is necessary to mediate the reaction.

Double reciprocal plots of initial velocity as a function of substrate concentration (fig.3), indicate that the inhibitor is a tight-binding one, and the inhibition is noncompetitive (12). The PD to which the inhibitor binds has a pH-optimum different from that of lysosomal hydrolases (fig.1) (15). Its K_m of 4 x 10^{-6} M also distinguishes the target enzyme of the inhibitor from an ePD found by other authors (4,5), who reported a K_m of 2 x 10^{-3} M. It remains open if both K_m values represent modifications of one PD, or characterize two different enzymes.

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