Cyclic Nucleotide Phosphodiesterase in Dictyostelium discoideum: Interconversion of Two Enzyme Forms

Abstract. *An extracellular cyclic nucleotide phosphodiesterase was isolated from either growing cultures or aggregating amoebas of* Dictyostelium discoideum. *The enzyme is released in a form with a low Michaelis constant (15 micromolar) and spontaneously undergoes a slow conversion to a less active form with a high Michaelis constant (2 millimolar). Inactivation was prevented or reversed by use of Cleland's reagent, dithiothreitol. The two enzyme forms may be part of a mechanism for control of concentration of cyclic adenosine monophosphate.*

Amoebas of the order Acrasiales form multicellular aggregates capable of morphogenesis and differentiation. The chemotactic factor or factors that draw the amoebas together into aggregates have been termed acrasin (*1*). Adenosine 3′,5′-monophosphate (cyclic AMP) and other cyclic nucleotides are chemotactic agents for *Dictyostelium discoideum* (*2*, *3*).

An acrasin-destroying enzyme strengthens the directional information provided by the chemotactic factor. The enzyme does this by enhancing gradients of acrasin and by destroying acrasin that has already been sensed (*4*). Acrasinase probably caused much of the difficulty in isolating and identifying acrasin (*5*). Where cyclic nucleotides, such as cyclic AMP, are chemotactic hormones, cyclic nucleotide phosphodiesterase is the acrasinase. There is evidence, however, that other chemotactic systems exist in *D. discoideum* (*6*).

An extracellular cyclic nucleotide phosphodiesterase was isolated from *D. discoideum* by Chang (*7*). Chassy *et al.* (*8*) have shown that the enzyme is not specific for cyclic AMP but hydrolyzes other cyclic nucleotides as well. With other substrates, the values for the Michaelis constant (K_m) and maximum velocity (V_{max}) are similar to those reported for cyclic AMP; the K_m for cyclic AMP is 2 m*M*. Pannbacker and Bravard (*9*) reported that the phosphodiesterase elaborated by aggregating amoebas has a K_m of 15 μM, 100 times lower than the value previously found. The results reported below indicate that these two divergent values correspond to two kinetically distinct forms of the same enzyme. The forms can be interconverted by simple chemical means.

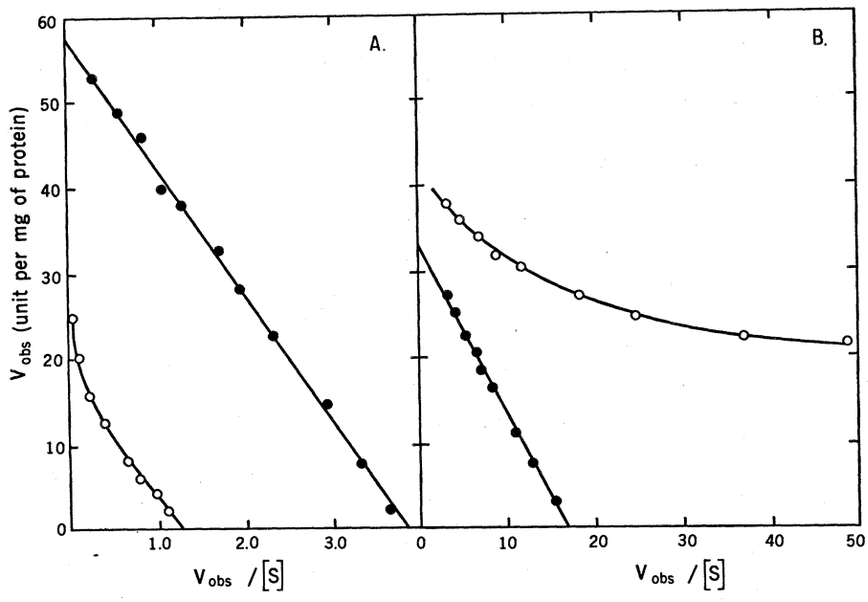

Fig. 1. Eadie graphs for data obtained with three preparations of the enzyme. The concentrations of substrate [*S*], cyclic AMP, are micromolar in (A) and millimolar in (B). V_{obs} is the observed velocity, and a unit is 1 μmole of 5′AMP produced per minute. (A) The data obtained with the enzyme form with low K_m are shown by solid circles. The data obtained with a mixture of enzyme forms are shown by open circles. (B) The solid circles again show a purified preparation of enzyme, in this case, the form with high K_m. The open circles show data from the same mixed preparation shown by the open circles in (A). The assay for radioactive 5′AMP was used.

Crude enzyme was prepared by the methods of Chang (*7*) or of Pannbacker and Bravard (*10*), or by release of the enzyme from washed amoebas suspended in 1 m*M* cyclic AMP (*11*). In the last procedure, a cell suspension was stirred at room temperature for 4 hours, and amoebas were removed by centrifugation (1000*g*, 4°C, 5 minutes). The cell-free supernatant was clarified by centrifugation at 40,000*g* for 2 hours.

Protein assays were those described by Warburg and Christian (*12*) or Lowry *et al.* (*13*). Three methods of determining enzyme activity were employed (*14*). In one method, the reaction rate was followed spectrophotometrically by absorbance at 265 nm by coupling alkaline phosphatase and adenosine deaminase to production of adenosine 5′-monophosphate (5′AMP). In the second method, used when cyclic AMP concentrations were high enough to interfere with absorbance readings at 265 nm, oxidation of reduced nicotinamide adenine dinucleotide (NADH) was followed by absorbance at 340 nm. This assay requires adenosine triphosphate, NADH, phosphoenolpyruvic acid, adenylate kinase, pyruvate kinase, and lactic dehydrogenase. Finally, when particular compounds or conditions interfere with the assays described above, radioactive 5′AMP, which was produced by enzymatic hydrolysis of radioactive cyclic AMP, was isolated by paper chromatography, and the radioactivity was determined in a liquid scintillation counter (*15*).

Enzyme activity is expressed as units per milligram of protein; a unit is 1 μmole of 5′AMP produced per minute at 30°C in tris(hydroxymethyl)-aminomethane-hydrochloride (tris-HCl) buffer, *p*H 7.4.

Purification of the crude enzyme preparation (obtained by any of the three methods) was begun by adding solid ammonium sulfate to 90 percent saturation, centrifuging (20,000*g*, 30 minutes), dissolving the precipitated protein in 0.01*M* tris-HCl at *p*H 7.4, and dialyzing against the same buffer. All purification procedures were done at 4°C unless stated otherwise. Some extraneous protein and nucleic acid were removed from the preparation by adding 1*M* magnesium chloride to a final concentration of 0.1*M*, stirring at

room temperature for 20 minutes, and discarding the precipitate (40,000*g*, 20 minutes). The supernatant was dialyzed for 48 hours against several changes of buffer that contained 0.01*M* tris-HCl and 0.2 m*M* Cleland's reagent, dithiothreitol (DTT) at *p*H 8.2.

The resulting solution was freed from any precipitate by centrifugation and passed through a 2.5 by 40 cm column of DE-52 (*16*) equilibrated with the same buffer. The activity usually occurred in the first protein-containing fractions; however, when DTT was omitted, three activity peaks were observed. Further purification was obtained by passage of the concentrated (*17*) active fractions from the DE-52 column through a 1.5 by 90 cm agarose bead gel-filtration column (*18*) equilibrated with buffer that contained 0.1*M* tris-HCl and 0.2 m*M* DTT at *p*H 7.4. When DTT was omitted, gel filtration produced two activity peaks. As judged by gel-filtration estimation (*19*), these peaks have molecular weights of 132,000 and 65,000.

The purity of these isolated fractions, as judged by disc-gel electrophoresis (*20*) and isoelectric focusing in gels (*21*), varied from 15 percent to more than 90 percent, depending on the method of obtaining the material; in some cases, isoelectric focusing was necessary to complete purification. The final preparation could be stored with little loss of activity at least 4 weeks at 4°C or for many months at −20°C if 0.2 m*M* DTT was included in all buffers.

For the component of lower molecular weight, the K_m is 15 μM and the V_{max} is 57.3 unit/mg (Fig. 1A); this component represents the freshly secreted enzyme. For the species of higher molecular weight, the K_m is 2 m*M* and the V_{max} is 33.7 unit/mg (Fig. 1B); this species resembles the form of the enzyme found in liquid cultures. Graphing the data in the method of Eadie (*22*) shows that a mixture of the two forms, obtained by storing at 4°C in the absence of DTT, has nonlinear kinetic properties (Fig. 1, A and B). As determined by chromatographic separation, approximately 30 percent of the enzyme in this mixture is the form with low K_m, and 70 percent is the form with high K_m.

In order to observe the conversion from low to high K_m, a purified preparation of enzyme of low molecular weight was stored at 4°C in the absence of DTT. With 10 m*M* cyclic AMP as substrate, activity decreased by 20 percent in 10 days (Fig. 2). In contrast, when 0.1 m*M* cyclic AMP was the substrate, the activity decreased much more, about 65 percent. The activities with 10 m*M* and 0.1 m*M* cyclic AMP were essentially the same for the enzyme form with low K_m but were quite different for the form with high K_m. Eadie graphs done on days 1 and 10 confirmed that a conversion from low K_m to high K_m took place during storage.

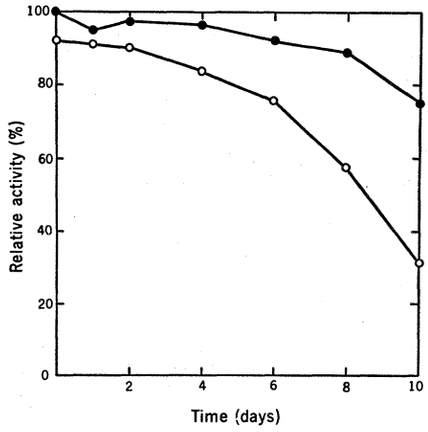

Fig. 2. Phosphodiesterase activity at two concentrations of cyclic AMP as a function of storage time. The data shown by the solid circles are for 10 m*M* cyclic AMP and the open circles are data for 0.1 m*M* cyclic AMP. Samples of purified enzyme of low molecular weight were stored at 4°C in 0.1*M* tris-HCl, *p*H 7.4. The coupled assay monitored by absorbance at 340 nm was used. All activities are set relative to the zero-time value observed with 10 m*M* cyclic AMP.

When DTT was present, the enzyme was stabilized, and simplified elution patterns were observed in column chromatography. To test the possibility that DTT stabilizes the enzyme by converting it to a single active species, I treated a preparation containing both forms of enzyme with 5 m*M* DTT (Fig. 3). At the start, the enzyme was three times more active with 10 m*M* cyclic AMP than with 0.1 m*M* cyclic AMP. After 21 hours, controls without DTT had lost about 20 percent of their activity with either substrate concentration, but samples with DTT were more active. For samples with DTT, the activity with 0.1 m*M* cyclic AMP rose to that observed with 10 m*M* cyclic AMP, a result that indicates a complete conversion to the form with low K_m.

I conclude that the two distinct kinetic profiles previously reported (*8*, *9*) represent easily interconverted states of the same enzyme. Aside from DTT, no sulfhydryl-containing compounds that were tested converted enzyme preparations into the form with low K_m. None of the common sulfhydryl inhibitors affected the activity of the enzyme. The slow emergence of the less active form and the 2:1 ratio of molecular weights suggest that air oxidation of thiol groups results in formation of a dimer.

Sulfhydryl compounds can inhibit phosphodiesterase activity, and DTT is itself an inhibitor of the enzyme (*10*). In this report, I found that when the sulfhydryl compound was diluted to a concentration at which it was not inhibitory, the enzyme preparation had undergone an activation. It is somewhat paradoxical that a kinetic inhibitor has the ability to activate the enzyme. A physiological role for re-

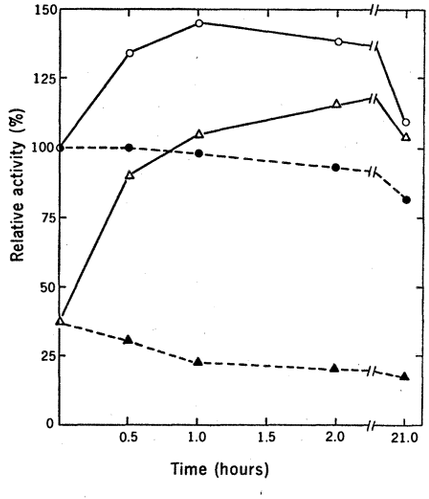

Fig. 3. Phosphodiesterase activity at two concentrations of cyclic AMP as a function of storage time in the presence or absence of DTT. An enzyme preparation containing a mixture of the two enzyme forms was used. Open circles are data for enzyme that was stored with 5 m*M* DTT, diluted 1:500, and assayed with 10 m*M* cyclic AMP. The solid circles are data for enzyme that was stored without DTT and assayed with 10 m*M* substrate. Similarly, the open triangles are data for enzyme that was treated with 5 m*M* DTT, diluted, and assayed with 0.1 m*M* cyclic AMP. The solid triangles are data for untreated enzyme that was assayed with 0.1 m*M* cyclic AMP. Both spectrophotometric assays were used.

activation of the enzyme is not yet established, and reactivation may be an artifact of laboratory manipulation.

Inactivation of the enzyme correlates well with facts about aggregation and differentiation in the cellular slime molds. During the aggregation process, the organism must be able to hydrolyze chemotactic cyclic AMP. Attractant cyclic AMP, if unhydrolyzed, would degrade the signal-to-noise ratio of the chemotactic system. Later, gradual inactivation of the enzyme allows higher concentrations of cyclic AMP to exist. These concentrations trigger phases of differentiation (*3, 23*). Concentrations of cyclic AMP higher than 1 m*M* are inhibitory to differentiation, but the residual activity of the enzyme form with high K_m would be sufficient to prevent the concentration of cyclic AMP from rising to inhibitory levels (*3*). The 5′AMP produced by hydrolysis stimulates the rate of differentiation (*24*).

Thus, cyclic nucleotide phosphodiesterase in the slime mold appears to have the novel property of possessing two kinetically distinct forms for two physiologically distinct roles. More generally, one might ask whether the two forms of the phosphodiesterase derived from brain tissue (*14, 25*) have two functions and are interconvertable, and whether the interconversion is under hormonal control.

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15. The radioassay used tritiated cyclic AMP of high specific activity from Schwarz Bio-Research. This was repurified before use in the same solvent system used for separating 5′AMP from substrate cyclic AMP. This solvent system contained saturated ammonium sulfate, 1*M* sodium acetate, and isopropyl alcohol (80 : 18 : 2; by volume). The R_F values (descending chromatography on Whatman 3MM paper) were 0.42 for 5′AMP and 0.19 for cyclic AMP.
16. A Whatman grade of diethylaminoethyl cellulose.
17. Diaflo ultrafiltration cell with UM-20 membrane, Amicon Corp.
18. Bio-Gel A-1.5, 200–400 mesh, BioRoad Corp.
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26. I thank L. Love for crude enzyme preparations and for many helpful suggestions.

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Uricotelism and Low Evaporative Water Loss in a South American Frog

Abstract. *A South American anuran* (Phyllomedusa sauvagii) *produced large amounts of semisolid urate when it was maintained on a diet of insects. Rates of evaporative water loss in* Phyllomedusa sauvagii *were only about 5 to 10 percent of those other anurans tested and were similar to those of lizards of comparable size.*

The ability of some anuran amphibians to survive in arid and semiarid regions has prompted numerous studies of their physiology and behavior. The success of the more terrestrial species is apparently not attributable to major physiological differences related to water, electrolyte, and nitrogen metabolism (*1*). Terrestrial forms generally lose water by evaporation from the skin at high rates when they are exposed to dry air, and they differ from more aquatic species primarily by greater tolerance to large water losses and by avoidance of dehydration by behavioral means. Most anurans excrete nitrogen in the form of ammonia and urea, with the more terrestrial species being ureotelic. When water is not available, these animals can store large amounts of urea in their body fluids. (*2*), but they are unable to excrete nitrogen without considerable expenditure of water. However, an African anuran, *Chiromantis xerampelina,* has recently been found by Loveridge (*3*) to have a relatively impermeable skin and to excrete uric acid. This frog tolerates long periods of exposure to dry air. This discovery by Loveridge is of major significance since it demonstrates physiological mechanisms hitherto unknown and unexpected in amphibians.

While studying a variety of anurans in Argentina, we became aware of *Phyllomedusa sauvagii,* a species that occurs in semiarid regions and appears to be exclusively arboreal. We collected these animals in January 1971 near Ojo de Agua in the province of Santiago del Estero. They were then shipped by air to California where they were kept in a terrarium that contained moist soil and was provided with perches. The animals were offered a variety of insects, but only a few individuals fed voluntarily. After about 6 weeks we began to force-feed the frogs with mealworms (larval *Tenebrio molitor*).

While handling these frogs we observed them to void large masses of white and yellow material enveloped in mucus. After drying this material to constant weight at 100°C, 5 to 10 mg of each sample was dissolved in about 1 ml of 3*N* NaOH and then diluted to 50 ml with distilled water. An additional dilution was made to give a final concentration of 10.0 μg/ml. The ultraviolet absorption spectra of these samples were compared to those of similarly treated uric acid standards. The absorption spectra of all samples were identical to that of uric acid, with maximum absorbance at 293 nm. The absorbances of the yellow and white material were, respectively, 97 and 81 percent of that of uric acid. Incubation of samples and uric acid with uricase (E.C. 1.7.3.3) in borate buffer (*p*H 8.5) abolished absorption at 293 nm.

To determine the relation between food intake and urate production, four *P. sauvagii* were kept without water in individual gallon jars and were fed known amounts of mealworms. Each jar was sealed except for a small hole in the lid and contained a perch made from a length of glass tubing. Animals were in a windowless room at 26°C and 30 percent relative humidity and were kept on a 12-hour photoperiod. Initially, frogs were allowed to hydrate by sitting on a wet synthetic sponge and were weighed after their bladders were emptied. Then, they were force-fed about 20 mg of fresh mealworms per gram of body weight every 2 days. Animals were checked