CONTROL OF FOLATE DEAMINASE ACTIVITY OF *DICTYOSTELIUM DISCOIDEUM* BY CYCLIC AMP

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1. Introduction

The cellular slime mold *Dictyostelium discoideum* grows as single amoeboid cells, aggregates after growth, and proceeds through a well-marked developmental cycle [1]. During the preaggregative state following growth, cyclic AMP applied as pulses of low concentration can stimulate development [2]. A cyclic AMP signaling and relay system exists, consisting of several proteins whose amounts and activities are controlled by cyclic AMP [3]. Among other proteins secreted by *D. discoideum* at this time is folate deaminase. The enzyme probably acts during growth to convert the chemoattractant folic acid into a non-chemoattractant [4]. We report here that added cyclic AMP enhances the production of folate deaminase during the preaggregative state. The enzyme has a K_M for folate of $5–6 \times 10^{-6}$ M and is mainly extracellular. Because folate deaminase inactivates folic acid and is partly controlled by cyclic AMP, it may be a component of a complex network for intercellular communication between differentiating cells.

2. Methods

2.1. *Growth and starvation*

Strain A X 2, clone 214, of *D. discoideum* was grown axenically in rich broth containing 1.8% maltose [5]. Exponential phase cells were harvested at $4–6 \times 10^6$ cells/ml and washed 3 times in 0.017 M phosphate buffer (pH 6.0 at 4°C) by centrifugation. Stationary phase cells were incubated 2–3 days longer, until they had been at $1–2 \times 10^7$ cells/ml for 24–36 h, then harvested and washed. For starvation cells were resuspended in phosphate buffer at 1×10^7 cells/ml (time t_o) and shaken at 150 rev./min, 23°C, under fluorescent light in a large flask for adequate aeration. Samples were removed at hourly intervals up to 8 h. Some flasks of starved cells were given cyclic AMP (Serva, Heidelberg) at 6–8 min intervals, starting at t_o. Each addition of cyclic AMP contributed 2×10^{-8} in the flask. Cells from all flasks were regularly observed microscopically for the ability to elongate and form streams on glass slides, as an indicator of development.

2.2. *Folate deaminase acitivity*

Enzymatic activity was assessed by following the initial rate of change of absorbance of a folic acid (pteroylglutamic acid) (Merck, Darmstadt) solution at 325 nm in a Zeiss PM6 spectrophotometer with recorder. Samples were measured in 100 μM folic acid, 0.10 M imidazole–HCl (pH 7.3 at 23°C) with respect to a reference cuvette containing a similar solution without enzyme. One unit of folate deaminase activity is defined as 1.0 nmol folic acid converted to product per minute as measured by this assay. For the determination of K_m the assay was modified by using lower substrate concentrations.

2.3. *Optical spectra*

The difference spectrum for light absorbance of folic acid and its enzymatic product was determined in a Kontron Uvikon 820 recording spectrophotome-

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ter. The product was diluted from an enzymatic reaction gone to completion in the dark (no further change in A_{325} for a duplicate reaction). Under the conditions used the enzyme preparation itself contributed <0.01 A units at wavelengths above 250 nm.

2.4. *Cell fractionation*

The supernatant fluid of centrifuged cultures was collected directly. Cells were washed 3 times as above, then resuspended in the 0.10 M imidazole buffer and broken by sonication for 5 s in a Branson sonifier (model B-12, small tip, setting 4 on output control). In some experiments cells were lysed by 3 cycles of freezing in liquid nitrogen and thawing. The lysate was centrifuged at 10 000 × g for 10 min. The supernatant fluid was assayed for folate deaminase activity, and a sample was centrifuged at 280 000 × g for 60 min and the supernatant fluid assayed. The broken-cell pellet was resuspended and assayed without washing. The ultracentrifuged pellet was not asayed.

3. Results

Folate deaminase probably replaces the amino group of folate at the 2-position of the pterin ring with a hydroxyl group; hence the name folate deaminase. The difference spectrum of folic acid and its enzymatic product of pH 7.3 is shown in fig.1. The wavelength of the maximum positive difference between the enzymatic product and folic acid is 325 nm. This wavelength was chosen for the routine measurement of initial rates to assess the activity in cell extracts. The difference spectrum is as expected from the spectra at neutral pH of folic acid and 2-deaminofolic acid [*N*-(2-hydroxy-4-hydroxy-pteridin-6-ylmethyl)-*p*-aminobenzoylglutamic acid] [4].

The distribution of folate deaminase, extracellular and intracellular, was assessed by cell fractionation studies on exponentially growing cells, stationary phase cells, and starved cells. The results in table 1 indicate that folate deaminase is essentially an extracellular enzyme. No activity is found in the soluble cytoplasmic fraction of sonicated cells. A highly purified plasma membrane fraction had relatively little activity, ~10–15% of the total activity in the culture (not shown). The enzymatic activity is present mainly in the medium of growing cells. It is also secreted and

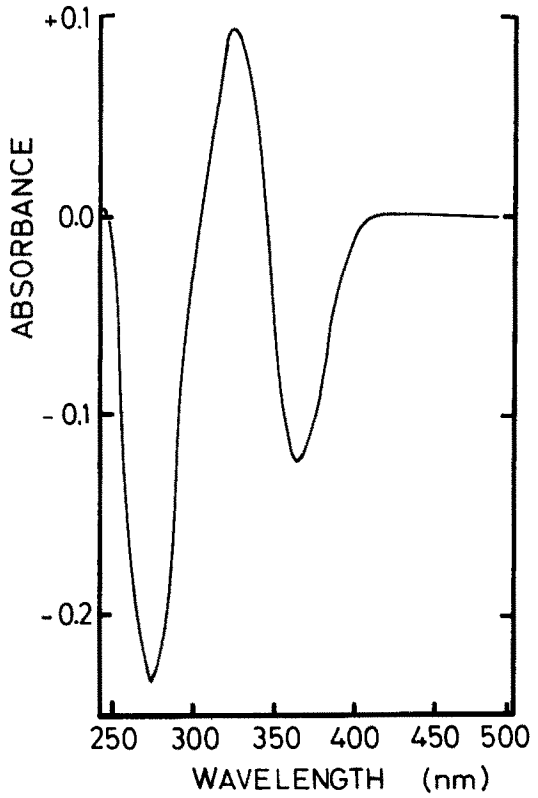

Fig.1. Difference spectrum of folic acid after incubation with folate deaminase, compared with unreacted folic acid. Both samples were diluted to 40 μM in 0.10 M imidazole–HCl (pH 7.3 at 23°C).

can be recovered in the low-speed supernatant fluid of cells which have been washed and resuspended in buffer for starvation. Similar experiments with cells broken by cycles of freezing and thawing confirm that >80% of the total activity in the culture is extracellular and that the soluble cytoplasm has <1%. These results contrast with those in [4] report that cytoplasmic extracts of *D. discoideum* inactivated the chemoattractant activity of folic acid. However, cellular slime molds may have other ways of inactivating folic acid as a chemoattractant besides deamination [6].

The K_m of folate deaminase for folate is 5–6 × 10^{-6} M, as estimated from the double-reciprocal plot of fig.2. The K_m is 5–25-times higher than the concentration of folic acid needed for effective stimulation of early differentiation [7,8]. Thus folate deaminase is poised to control ambient levels of folate and to respond to surges of folate concentration in the

Table 1
Folate deaminase activity in medium and subcellular fractions of *D. discoideum* A × 2

	Folate deaminase activity (units/10^7 cells)	Percent of total activity
1. Exponential phase cells		
Medium after removing cells	16.2	93.4%
Broken-cell pellet (unwashed)	0.89	5.1%
10 000 × *g* supernatant fluid	0.26	1.5%
280 000 × *g* supernatant fluid	0.00	0.0%
2. Stationary phase cells		
Medium after removing cells	11.0	83.2%
Broken-cell pellet (unwashed)	1.76	13.3%
10 000 × *g* supernatant fluid	0.46	3.5%
280 000 × *g* supernatant fluid	0.00	0.0%
3. After 4 h starvation of exponential phase cells		
Buffer after removing cells	4.44	82.4%
Broken-cell pellet (unwashed)	0.69	12.8%
10 000 × *g* supernatant fluid	0.26	4.8%
280 000 × *g* supernatant fluid	0.00	0.0%

Cells were harvested in exponential phase at 4.2×10^6 cells/ml and after 1 day in stationary phase at 1.4×10^7 cells/ml. Some exponential phase cells were washed and resuspended in phosphate buffer at 8.4×10^6 cells/ml for starvation for 4 h. Cells were broken by sonication

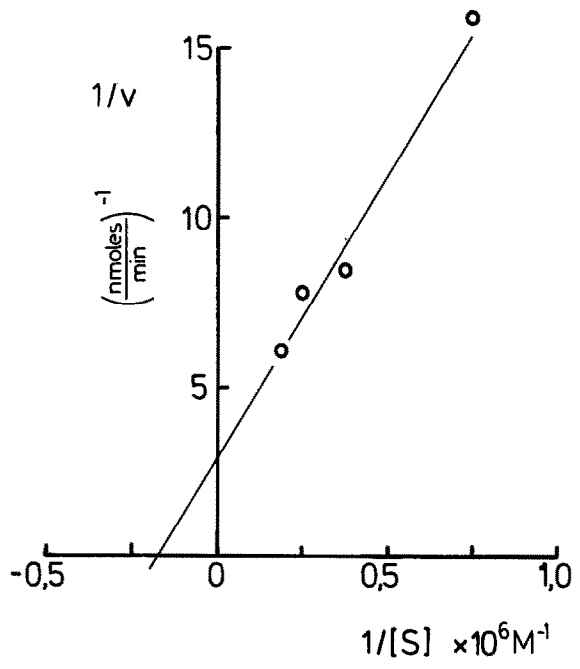

Fig.2. Double reciprocal plot to determine Michaelis constant (K_m). A diluted sample of supernatant fluid from a starved cell culture (t_4) was used. Initial rates at each substrate concentration are averages of ⩾3 measurements. The regression line has a correlation coefficient of 0.990.

extracellular fluid. Furthermore, other experiments not reported here in detail show a broad pH profile for folate deaminase, optimum around pH 6.0–6.5, which is the range of pH of the medium needed for fastest growth and early development [1,9].

Applied pulses of cyclic AMP increase the activity of folate deaminase in the extracellular fluid of preaggregative *D. discoideum*. Fig.3 shows that folate deaminase activity increases and maintains a relatively constant level after ~4 h starvation by suspension in buffer. Pulsed cultures have reproducibly 40–50% more folate deaminase activity than unpulsed cultures, and like unpulsed cells maintain this level throughout starvation. Cyclic AMP is released spontaneously from cells under both con-

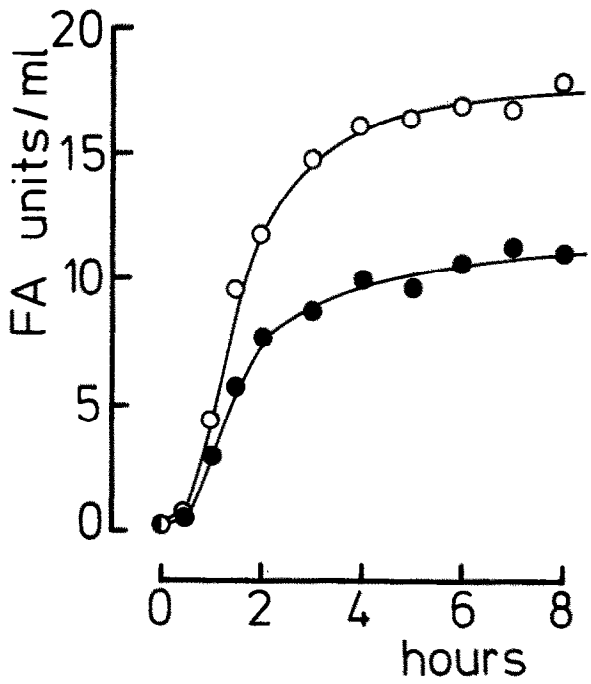

Fig.3. Folate deaminase (FD) activity during starvation of *D. discoideum* A × 2 cells taken from exponential phase. At t_0 cells were suspended in phosphate buffer at 1×10^7 cells/ml. At intervals samples were centrifuged to remove cells. The supernatant fluid was assayed: (●——●) control culture, no additions; (○——○) culture pulsed with cyclic AMP, 2×10^{-8} M, every 6–8 min.

ditions, but applied pulses of cyclic AMP as low as 10^{-9} M have been shown to stimulate development [10].

4. Discussion

From the evidence presented folate deaminase appears to be at least partially under the control of cyclic AMP during early development of *D. discoideum*. A period of 5–10 h starvation following growth is needed for *D. discoideum* to undergo differentiation and aggregate for multicellular development [1]. During this time cyclic AMP controls the activity of cellular functions involved in differentiation, including the expression of cell surface proteins and internal cyclic GMP concentrations, as shown by studies using applied pulses of cyclic AMP [11,12]. Proteins which constitute the cyclic AMP signaling system are controlled by cyclic AMP [3]. Since applied cyclic AMP pulses also enhance the activity of folate deaminase, this enzyme may likewise participate in the cellular interactions that lead to normal differentiation in preaggregative cells.

Folate deaminase acts to lower the concentration of folate in the extracellular fluid. The product, apparently identical to 2-deaminofolate, is a non-chemoattractant [4]. Thus the role of the enzyme during growth may be to enhance the response of *D. discoideum* amoeboid cells to folic acid excreted by their natural food source, bacteria [13]. The enzymatic product as well as authentic 2-deaminofolate bind very poorly to folate receptors on the cell surface (R. v. D. unpublished). Since these receptors are present on starving preaggregative cells as well as growing cells, folate signals may be important not only during growth but also during early development.

Like cyclic AMP, folic acid applied at intervals >2 min stimulates differentiation of preaggregative cells, but not if the intervals are <2 min or if folic acid is applied continously [8]. Extracellular folate deaminase probably acts during the preaggregative state to lower the ambient concentration of folate in the extracellular fluid, allowing the cells to perceive changes in folate concentration better. Thus there appear to be at least 2 systems, involving extracellular small molecules as information carriers, by which cell differentiation can be controlled:

(i) The well-studied cyclic AMP system, consisting of cyclic AMP receptors, adenylate cyclase, phosphodiesterase, and phosphodiesterase inhibitor;
(ii) The folic acid system with folate receptors, extracellular folate deaminase, and perhaps other components.

These systems mutually interact. Cyclic AMP is shown here to enhance folate deaminase activity, whereas folic acid applied in pulses enhances extracellular phosphodiesterase activity [7]. Furthermore, each small molecule increases the extracellular activity of its own inactivating enzyme ([7], R. L. B., R. v. D., unpublished). Thus the signaling systems which operate during early development interact in a reciprocal fashion.

We are characterizing the molecular and enzymatic properties of folate deaminase. Preliminary results indicate at least 2 isozymes which are glycoproteins. Also, we are investigating further the role of folate deaminase in *D. discoideum* development.

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