A NOVEL CHEMOTAXIS REGULATING ENZYME THAT SPLITS FOLIC ACID INTO 6-HYDROXYMETHYLPTERIN AND *P*-AMINOBENZOYLGLUTAMIC ACID

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1. Introduction

After starvation, amoebae of the cellular slime molds secrete acrasin [1] by which they attract each other and form an aggregate that differentiates into stalk cells and spores. In a favourable environment spores germinate yielding amoebae which feed on bacteria and multiply. Vegetative amoebae are also sensitive to chemotactic stimuli [4,6], which differ from acrasins. They are attracted to bacteria, which apparently secrete chemoattractants for amoebae [2,3]. Folic acid was reported a chemoattractant for vegetative amoebae of the cellular slime molds [4]. Folic acid is also, although less chemotactically active in the aggregative stage except in *Dictyostelium discoideum* [5,7]. The function of folic acid in the cellular slime molds is thought to be a signal for food detection [4,7]. The inactivation of the chemotactic signal is regulated by enzymatic degradation. One way to inactivate folic acid is by folic acid deaminase, as studied in *D. discoideum* and *Polysphondylium violaceum* [8,9]. We examined the chemotactic activity of the deaminated folic acid in several species of the cellular slime molds and discovered that in some species the chemotactic activity is not lost by deamination. This indicates that another chemotactic regulator enzyme exists. Folic acid metabolism has been studied in several organisms [10–16]. Degradation of folic acid in bacteria (*Pseudomonas*) results in deaminated folic acid, pteroic acid, deaminated pteroic acid and lumazine-6-carboxylic acid [10]. In *Flavobacterium* folic acid is mainly degraded to pteroic acid [13]. In the rat the C_9–N_{10} bond of folic acid is cleaved [11]. Malignant cells secrete a folate catabolite into the growth medium which has been identified as 6-hydroxymethylpterin and cleavage of the C_9–N_{10} bond of folic acid has also been demonstrated in malignant cells [12].

Here we show that cleavage of the C_9–N_{10} bond is essential for inactivation of the chemotactic activity of folic acid in some species of the cellular slime molds; deamination of folic acid had no effect on its chemotactic attraction of these species. The reaction products were identified as 6HMP and *p* ABAglu.

Abbreviations: HPLC, high performance liquid chromatography; DAFA, 2-hydroxy-2-deamino folic acid; P6COOH, pterin-6-carboxylic acid; 6HMP, 6-hydroxymethylpterin; 6HML, 6-hydroxymethyl-lumazine; *p*ABAglu, *para*-aminobenzoylglutamic acid; L6COOH, lumazine-6-carboxylic acid; FAS, folic acid splitting enzyme (C_9–N_{10} bond cleavage); $k' = (t_X - t_O)/t_O$; t_X is the retention time of a compound; t_O corresponds with the void volume

2. Materials and methods

D. discoideum NC-4(H), *D. minutum* V_3, *D. lacteum* and *P. violaceum*, all kindly supplied by K. B. Raper, were grown in association with *Escherichia coli* B/r on a solid medium [6,17]. The cells were harvested, freed of bacteria by centrifugation [17] and resuspended in 10 mM phosphate buffer (pH 6.0) at $5 \cdot 10^6$ cells/ml. Starvation was induced by shaking [18].

The chemotactic activity of compounds was determined by the small population assay [19]. Folic acid (Sigma) was deaminated by the method in [20] and purified by HPLC on a Partisil PXS 10/25 SAX Whatman column (fig.1) using an Altex pump, model 100A and a Latek UV III 1203 detector. Oxidative degradation of folic acid and DAFA with $KMnO_4$

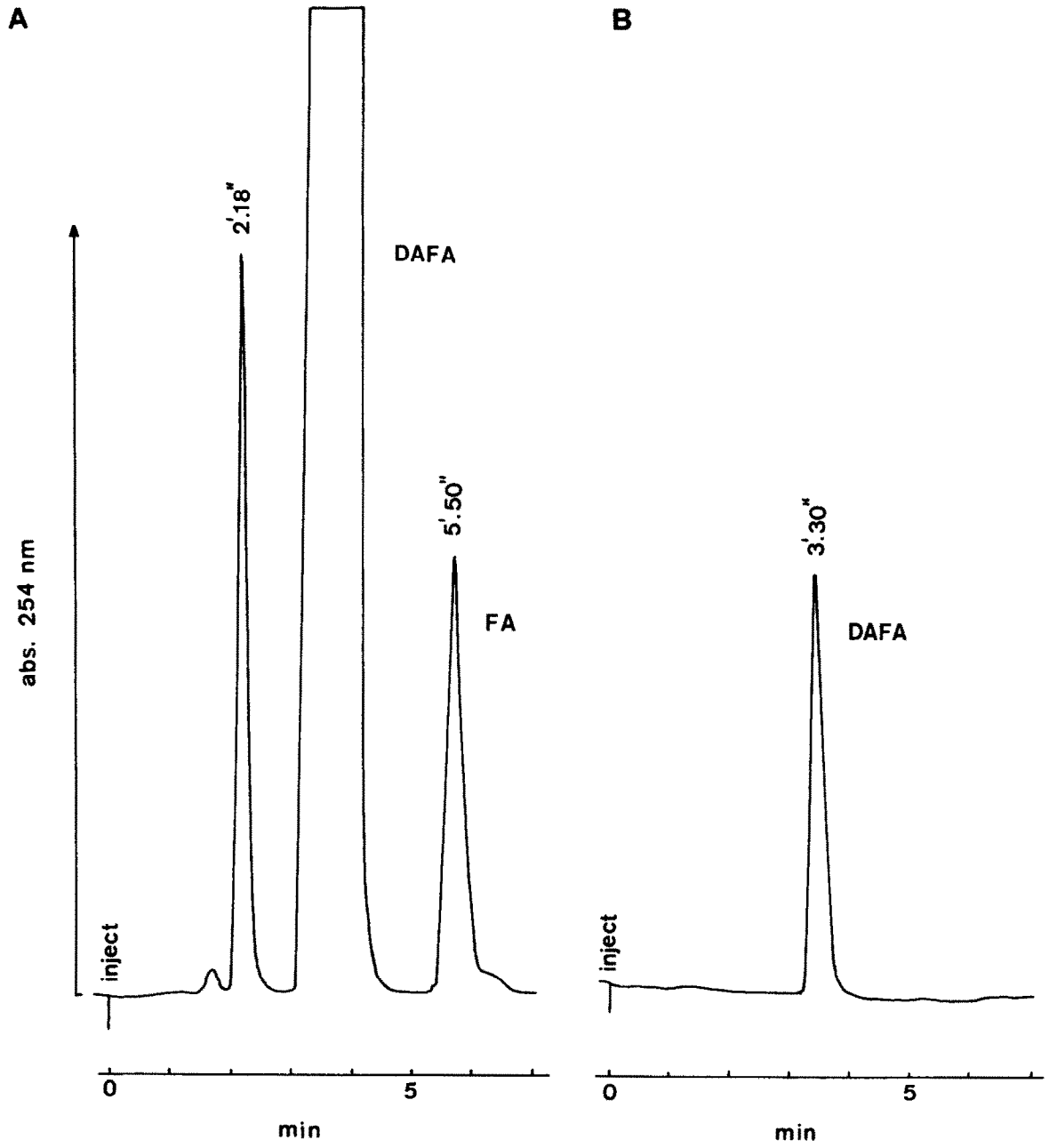

Fig.1(A) Chromatogram of DAFA prepared by the method in [20]. Column, Partisil PXS 10/25 SAX Whatman; eluent, 50 mM sodium phosphate buffer (pH 7.0); UV, 254 nm, 2.048 AUFS (absorption unit full scale); flow rate, 2 ml/min. (B) 10 μl of the collected DAFA peak was reinjected under the same conditions; UV, 0.064 AUFS.

was done as in [21]. By using an excess of folic acid partial oxidative degradation occurs. The reaction products were separated by HPLC and indentified by UV absorption spectra at different pH values [15,24, 25]. Diazotizable amines were detected as in [22,23].

3. Results and discussion

Folic acid induces chemotaxis in vegetative amoebae of all species tested [5,7]. In the small population assay [19], *D. minutum* had a threshold of 10^{-7}–10^{-8} M in the vegetative stage and 10^{-6}–10^{-7} M in the aggregative stage. Purified DAFA, which was free of folic acid attracted vegetative cells of *D. minutum* with the same threshold as folic acid. To test for the inactivation of the chemoattractant, folic acid was added to a cell suspension of *D. minutum* (5 . 10^6 cells/ml) and of *D. lacteum* (5 . 10^6 cells/ml) to final conc. 10^{-6} M, and the suspension was shaken for 30 min after which the cells were centrifuged. The supernatant of *D. minutum* cells was chemotactically active for *D. lacteum* but not for *D. minutum*. The supernatant of *D. lacteum* cells was chemotactically active for *D. minutum* but not for *D. lacteum*. Apparently two types of folic acid-degrading enzymes exist. *D. lacteum* was tested for folic acid deaminase activity [8] and a high (800 pmol . 10^6 cells^{-1} . min^{-1}) deaminase activity was measured (fig.2). The reaction products of folic acid degradation by *D. minutum* cells were examined by HPLC. Two columns were used, a Partisil SAX (anion-exchanger) and a Partisil SCX (cation-exchanger). Fig.3 demonstrates the retention behaviour of the degradation products of folic acid obtained by partial oxidative degradation with $KMnO_4$. Complete degradation results in pABAglu (t = 6 h) and P6COOH (t = 3 h 59 min). The peak at 1 h 42 min was collected and treated with $KMnO_4$ and reinjected into the column. This resulted in a peak shift from 1 h 42 min to 3 h 59 min, the P6COOH peak. The peak at 1 h 42 min could be either pterin-6-aldehyde or 6HMP. UV spectra showed that it was not pterin-6-aldehyde but 6HMP [24,25]. Additionally [7,9,3′,5-^{3}H]folic acid was degraded by the FAS

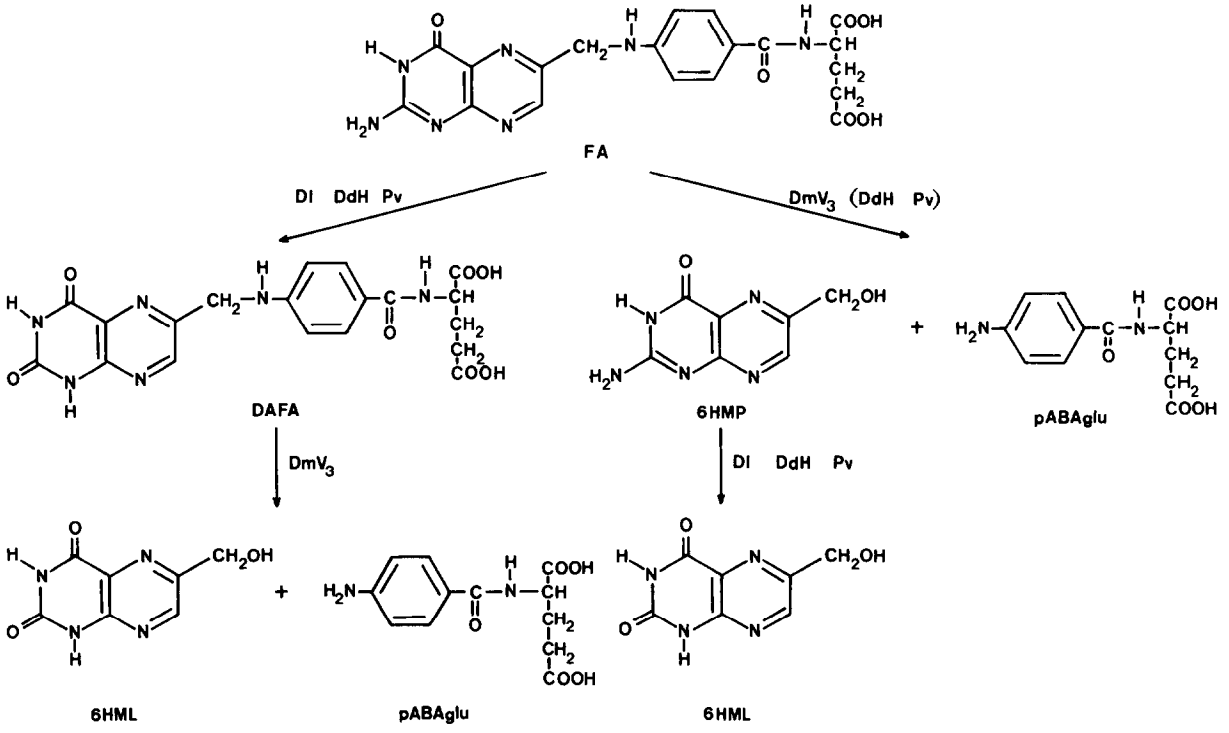

Fig.2. Folic acid degradation in the cellular slime molds. DdH and Pv are in parentheses because the deamination activity of *D. discoideum* NC-4 and *P. violaceum*, respectively, is much higher (>100 times) than the FAS activity. DI, *D. lactam*; DmV_3, *D. minutum* V_3.

from *D. minutum* cells and the reaction mixture was chromatographed on a SP-Sephadex cation-exchanger eluted with 0.01 M $HCOONH_4$, (pH 2.5). Tritiated water should be present if the aldehyde was formed,

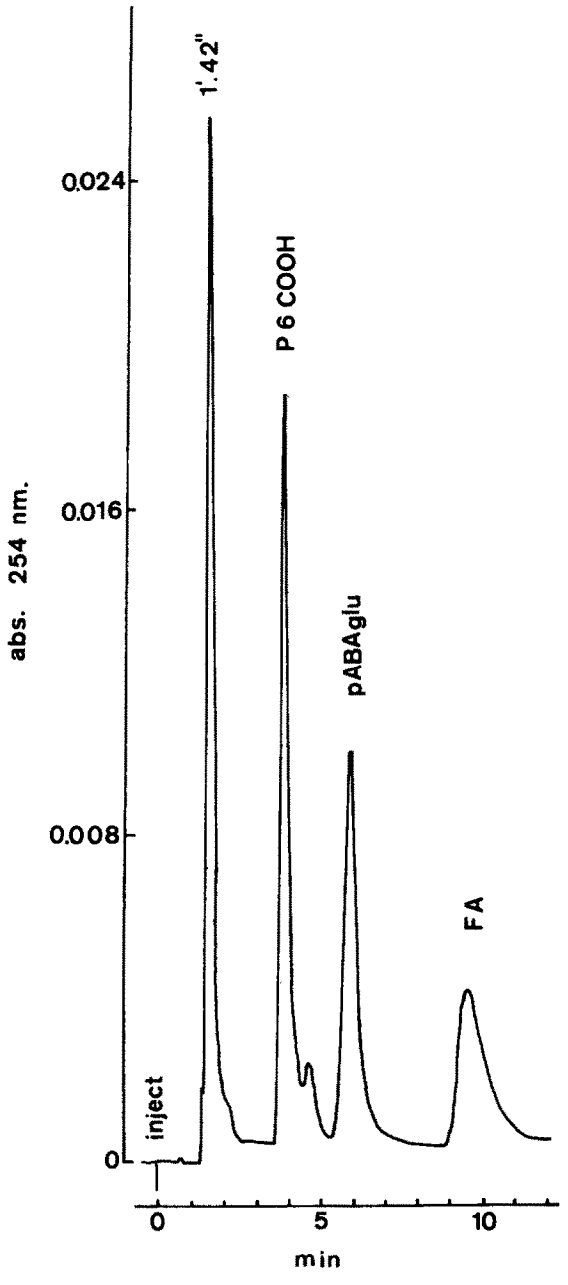

Fig.3. Chromatogram of partial oxidative degradation of folic acid (FA) with $KMnO_4$. Column, Partisil PXS 10/25 SAX Whatman; eluent 50 mM KH_2PO_4, 20% methanol; UV, 254 nm, 0.032 AUFS; flow rate, 2 ml/min.

because it would release a ^3H atom from the C-9 position and this was not found in the void volume.

The mixture of reaction products contained a diazotizable amine detected as in [22,23]. After the separation of the reaction products on an anion-exchanger the same procedure was repeated on a Partisil PXS 10/25 SCX cation exchanger with 10 mM $HCOONH_4$ (pH 3.0) as eluent. The same peak shift was observed (folic acid $k' = 4.41$; pABAglu $k' = 3.28$; P6COOH $k' = 1.60$; 6HMP $k' = 2.57$).

DAFA, which is very sensitive to light, was split by FAS of *D. minutum* (fig.2). The products were pABAglu and two other compounds of which one peak (I), L6COOH, may be derived from the other (II), probably 6HML. Peak II shifts to L6COOH when treated with $KMnO_4$ (retention on Partisil SAX, eluent 50 mM KH_2PO_4, 20% methanol: DAFA 5 h 12 min; pABAglu 6 h 01 min; L6COOH 2 h 18 min and peak II 1 h 30 min).

D. discoideum and *P. violaceum* also secrete FAS (fig.2). In these species, however, deamination occurred faster than splitting of folic acid as detected with HPLC. FAS from *D. discoideum* was inactivated within a few days, while FAS from *D. minutum* is stable for months when stored at −20°C. The FAS activity of *D. lacteum* was very weak. *E. coli* B/r does not excrete FAS activity when 10^9 cells/ml were shaken in 10 mM phosphate buffer (pH 6.0) for 5 h, centrifuged for 5 min at 15 000 × *g* and the supernatant was tested for enzyme activity.

The degradation of the chemotactic signal, folic acid, in the cellular slime molds is not achieved uniformly. One way is the deamination at the C2 position [8,9] and the other way is the cleavage of the C_9–N_{10} bond, resulting in 6HMP and pABAglu. The degradation of folic acid by different species of the cellular slime molds is summarized in fig.2. Substrate specificity and other characteristics of FAS in the different species should be examined.

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