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Characterization of the Folic Acid C_9-N_{10}-Cleaving Enzyme of *Dictyostelium minutum* V3

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Folic acid is a chemoattractant for the slime mold *Dictyostelium minutum* V3. The activity of extracellular folic acid is regulated by a folic acid C_9-N_{10} splitting enzyme (FAS). The products were identified as pterin-6-aldehyde and *p*-aminobenzoylglutamic acid. The enzyme was stabilized by EDTA. For the extracellular enzyme, the K_m was 10^{-7} M, and the optimal pH was 4.0. During starvation, FAS activity was mainly secreted into the medium; after 3 h, a plateau was reached. The membrane-bound activity was constant, but only 12% of the extracellular activity at 3 h. Intracellular activity also increased up to 3 h to a level of 23% of the extracellular FAS. The substrate recognition of FAS was found to be based on 4-O or N_3 or both, N_5 or N_8 or both, N_{10}, and the *p*-aminobenzoic acid moiety, whereas 2-NH_2, N_1, and the glutamic acid moiety were not recognized. Other slime mold species were found to secrete FAS with 20-fold or more reduced activity than *D. minutum* V3.

Free-living vegetative cells of the cellular slime molds feed on bacteria, which are detected by the cells via chemotaxis. The chemoattractant involved in this process is thought to be folic acid (8, 12). This compound is secreted by bacteria and is a chemoattractant for all species of the cellular slime molds so far tested (13; unpublished observations).

When cells are deprived of a food source, they start to secrete an often species-specific attractant (acrasin), that mediates cell aggregation. Several acrasins have been identified: cyclic AMP in *Dictyostelium discoideum* and related species (1, 8, 10), a pterin derivative in *Dictyostelium lacteum* (16a), a peptide in *Polysphondylium violaceum* (19), and a folic acid derivative in *Dictyostelium minutum* (5a).

Aggregative cells of all species are less sensitive to folic acid than vegetative cells, except *D. minutum*, which is equally sensitive in both stages. Generally, the activity of an attractant outside the cell is regulated by a degrading enzyme. In all species, folic acid is mainly inactivated via deamination at C_2 (3, 6, 14, 18). In contrast, *D. minutum* degrades folic acid by cleavage of the C_9-N_{10} bond (7). This exceptional behavior of *D. minutum* towards folic acid is thought to originate from the fact that folic acid is a derivative of the acrasin of this species and not only a mediator in the detection of bacteria (5a). Thus, the folic acid splitting enzyme (FAS) may be the acrasindegrading enzyme or acrasinase in *D. minutum*.

In this study, the products of the enzymatic conversion were identified, and an assay procedure was developed. Several biological and biochemical properties were determined.

MATERIALS AND METHODS

Chemicals. Pterin, pterin-6-carboxylic acid, *p*-aminobenzoylglutamic acid, 4-amino folic acid, and 4-amino-10-methyl folic acid (methotrexate) were purchased from Sigma Chemical Co., St. Louis, Mo.; folic acid and 5,6,7,8-tetrahydrofolic acid were from Fluka AG; pteroic acid was from Lederle Laboratories, Pearl River, N.Y. 2,4-Diamino-5-methyl-6[(3,4,5-trimethoxyanilino) methyl]quinazoline (trimetrexate) was a gift from J. R. Bertino, Yale University School of Medicine, New Haven, Conn., and 6-hydroxymethylpterin was generously supplied by T. Sugimoto, Nagoya University, Nagoya, Japan.

Folic acid was deaminated enzymatically to 2-deamino-2-hydroxyfolic acid as reported before (17). Pterin-6-aldehyde was prepared by partial alkaline permanganate oxidation of folic acid (4) and purified by high-performance liquid chromatography (HPLC) system A. The identity of the product was checked by its UV spectra at pH 2.0 and 6.0 (4). Further oxidation of the aldehyde yielded pterin-6-carboxylic acid as determined by HPLC systems A and B. [2-^{14}C]folic acid and [7,9,3′,5′-^{3}H]folic acid were purchased from Amersham Radiochemical Centre, Amersham, England.

HPLC. The HPLC equipment consisted of a Beckman 100 A pump and a Laboratory Data Control UV III 1203 monitor set at 254 nm. The columns used were an anion exchanger, Partisil PXS 10/25 SAX, and a reversed-phase Lichrosorb 10 RP18. The flow rate was

1 ml/min. The mobile phase for system A consisted of 15 mM Na_2HPO_4-KH_2PO_4, pH 6.0, and 10% methanol (for reversed phase). The mobile phase for system B consisted of 0.1 M NH_3 solution, 0.2 M NaCl, 20% (vol/vol) 1-propanol, and 10% acetonitrile, adjusted with acetic acid to pH 5.3 (for anion exchange).

Cultures. All cellular slime mold species were grown on 0.1% lactose-peptone agar in association with *Escherichia coli* B/r, except *D. discoideum* NC-4(H), which was cultivated on SM agar (9). Cells were harvested in the late log phase and washed three times with 1% standard salt solution (5) by centrifugation at 150 × *g* for 4 min at 5°C. *D. minutum* V3 was supplied by K. B. Raper, and strains 39 and 71 were supplied by G. Gerisch.

Enzyme isolation. Cells at a density of 10^7 per ml were shaken in 10 mM phosphate buffer, pH 6.0, on a rotary shaker at 150 rpm. After 3 h, the suspension was centrifuged at 150 × *g* during 4 min, and the supernatant was centrifuged at 8,000 × *g* for 10 min. The resulting supernatant was used immediately or stored at −20°C. Enzyme activity is given in units, defined as 1 pmol of folic acid cleaved per min. The extracellular medium of 10^7 cells per ml is referred to as 10^7 cell equivalents per ml. The protein content in the medium was 70 μg per 10^7 cell equivalents. *E. coli* did not secrete measurable hydrolase activity.

Hydrolase assay procedure. [2-^{14}C]folic acid was present in the samples at a concentration of 2×10^{-7} M, which corresponds to 2,000 cpm per 100-μl sample. The assay buffer composition was 10 mM sodium-potassium phosphate buffer, pH 6.0, 1 mM EDTA, and 1 mM dithiothreitol (DTT) to prevent oxidation of the products of the enzymatic reaction. Incubation was terminated by adding 0.5 ml of ice-cold DOWEX (AG1X2) anion-exchange resin suspension. The DOWEX resin was previously equilibrated in 10 mM NH_4-CH_3COO, pH 5.0–1 mM DTT–20% ethanol, and 1 volume of this buffer was added to the settled particles before use in the assay. The samples were mixed and centrifuged at 8,000 × *g* for 1 min. Of the supernatant containing the ^{14}C-labeled product, 300 μl was taken and added to 1 ml of water and 1.5 ml of Instagel scintillation liquid (Packard Instrument Co., Inc., Rockville, Md.) and counted for 5 min. The blank value was about 1% of the total counts per minute present in a sample.

RESULTS

The products of the enzymatic degradation of [2-^{14}C]- and [7,9,3′,5′-^{3}H]folic acid were identified by using HPLC. Figure 1A demonstrates the comigration of the [2-^{14}C]labeled product with pterin-6-aldehyde, whereas Fig. 1B shows both tritiated products, which comigrated with pterin-6-aldehyde and *p*-aminobenzoylglutamic acid. In both cases, not all of the radioactivity present in folic acid was found in these two products. This was due to nonenzymatic oxidation of pterin-6-aldehyde to pterin-6-carboxylic acid. In the presence of 1 mM DTT, not more than 2% of the aldehyde was oxidized. Since [^{3}H]folic acid was labeled at C_9, which corresponds to the carboxyaldehyde group in the product ^3H_2O was released during the enzymatic conversion (Fig. 1B). Furthermore, the possibility was checked that pterin-6-aldehyde was not the product of folic acid hydrolysis but 6-hydroxymethylpterin, which would yield the alde-

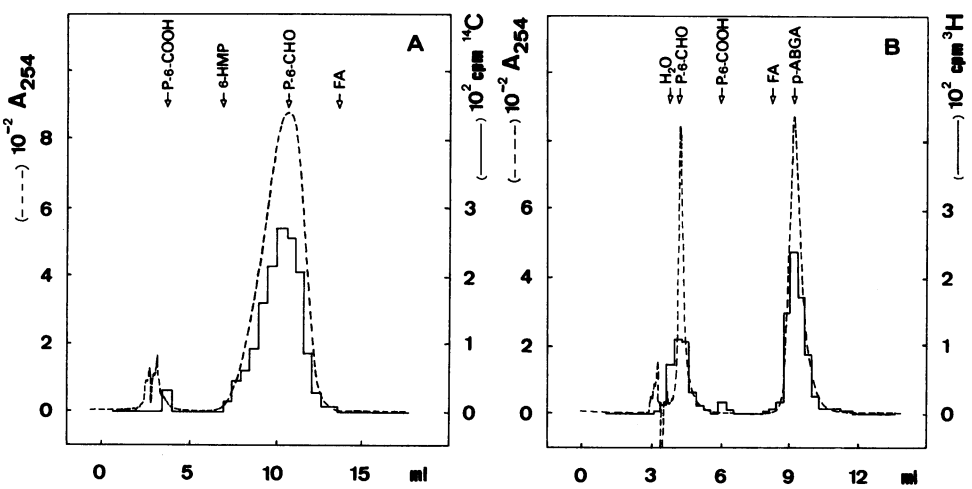

FIG. 1. Identification of the products of folic acid C_9-N_{10} cleavage. (A) [2-^{14}C]folic acid was incubated with FAS and coinjected with pterin-6-aldehyde on HPLC system A. Radioactivity was measured in 0.5-ml fractions. (B) [7,9,3′,5′-^{3}H]folic acid was incubated with FAS and coinjected with pterin-6-aldehyde and *p*-aminobenzoylglutamic acid on HPLC system B. Radioactivity was determined in 0.5 ml fractions. Arrows indicate retention volumes of related compounds. Abbreviations: P-6-COOH, pterin-6-carboxylic acid; 6 HMP, 6-hydroxymethylpterin; P-6-CHO, pterin-6-aldehyde; FA, folic acid; p-ABGA, *p*-aminobenzoylglutamic acid.

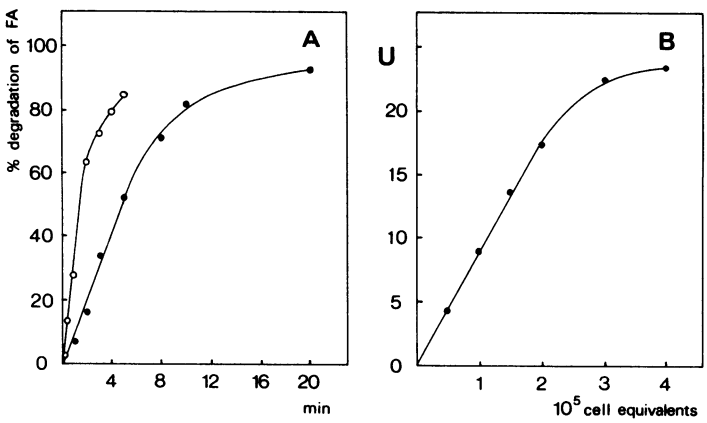

FIG. 2. Linearity of the assay procedure with time and enzyme concentration. (A) Extracellular FAS, isolated as described in the text, at a concentration of 10^5 cell equivalents per sample, was incubated at 20°C with [^{14}C]folic acid. Symbols: ○, 2×10^{-7} M folic acid; ●, 10^{-6} M folic acid. (B) Several concentrations of the same enzyme preparation were incubated with 10^{-6} M for 1 min. No more than 23% of the substrate was degraded.

hyde by enzymatic or chemical oxidation. The presence of 5×10^{-4} M 6-hydroxymethylpterin during incubation did not affect the formation of [2-^{14}C]pterin-6-aldehyde from 10^{-7} M [2-^{14}C]folic acid, and 6-hydroxymethylpterin was not oxidized by the enzyme preparation. This proves that pterin-6-aldehyde is the product of folic acid C_9-N_{10} cleavage.

Figure 2A shows that the enzymatic conversion is linear with time up to 50% degradation at folic acid concentrations of 2×10^{-7} M and 10^{-6} M. The degradation velocity was also linear with the enzyme concentration up to the equivalent of 2×10^6 cells per ml (Fig. 2B). Incubation of the enzyme at 20°C resulted in a significant decrease in enzymatic activity (half-life, 30 min). This effect was not seen at 0°C. The addition of 1 mM EDTA stabilized the enzyme at 20°C.

The enzyme showed maximal activity at pH 4.0 (Fig. 3), and the velocity at 0°C was 20% of the rate at 20°C. For four enzyme preparations, linear Eadie-Hofstee plots were obtained with $K_m = 10^{-7}$ M and $V_{max} = 10 \pm 2$ U per 10^5 cell equivalents. The substrate specificity was studied by two approaches: (i) inhibition of [^{14}C]folic acid degradation by derivatives of folic acid, and (ii) degradation velocity of the derivatives measured with HPLC systems A and B. The results of both inhibition and degradation (Table 1) indicate that N_1, 2-NH_2, and the glutamic acid group were ineffective, whereas C_4-O or N_3 or both, N_5 or N_8 or both, N_{10}, and the *p*-aminobenzoic acid moiety were essential for binding to the enzyme and subsequent degradation. The FAS was mainly secreted into the starvation medium; only a low activity of 1.5 U per 10^5 cells, constant during starvation, was present on the cell surface. This membrane-bound activity was obtained by subtracting the extracellular activity from the total activity present in the medium and on the cell surface (Fig. 4). Measuring the membrane-bound FAS directly (with washed cells) gave the same results. The extracellular activity reached a plateau of 12 U after 3 h of starvation (Fig. 4). Soluble cytoplasmic activity also increased during starvation to a

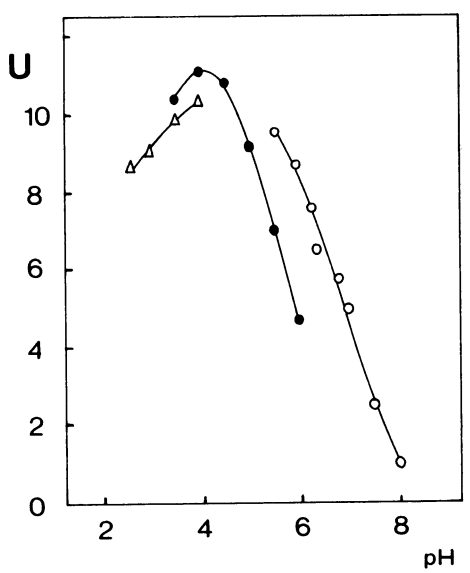

FIG. 3. pH optimum of the catalytic activity. FAS activities at 10^5 cell equivalents per sample and 10^{-6} M folic acid were measured at different pH values. Symbols: △, 20 mM formic acid, adjusted with NaOH; ●, 10 mM acetic acid, adjusted with NaOH; and ○, 10 mM phosphoric acid adjusted with NaOH. DTT (1 mM) and EDTA (1 mM) were added to all buffers. The enzyme was stable over the entire pH range as indicated by preincubation of the enzyme at these pH values.

TABLE 1. Substrate specificity of FAS

Analog	K_I (folic acid)/ K_I (analog)[a]	Relative degradation rate[b]
Folic acid	1.0	1.0
2-Deamino-2-hydroxy folic acid	2.1	2.0
Pteroic acid	0.6	0.8
5,6,7,8-Tetrahydro-folic acid	0.15	0.1
4-Aminofolic acid	0.04	0.1
4-Amino-10-methyl folic acid	0.004	0.01
Trimetrexate	$<10^{-4}$	$<10^{-3}$
Pterin	$<10^{-4}$	0

[a] Inhibition constants (K_I) were obtained by inhibition of degradation of [2-^{14}C]folic acid and were related to the K_I of folic acid.

[b] Degradation was measured by HPLC. The concentration of the analog was 10^{-4} M. The degradation rate has been related to that of folic acid.

level of 23% of the extracellular activity after 3 h of secretion. This intracellular activity showed the same pH optimum as the extracellular enzyme. Reconstitution of the 48,000 × *g* pellet and supernatant of the homogenate yielded only 65% of the activity of the supernatant alone. The extracellular enzyme also was slightly inhibited by the particulate fraction of the homogenate.

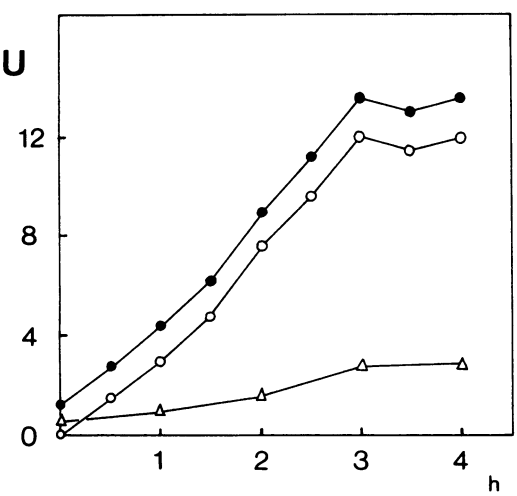

FIG. 4. Activity and localization of FAS during tarvation. Cells were shaken in 10 mM phosphate ouffer, pH 6.0, as described in the text. At the indicated times, samples were taken and incubated. Symbols: ●, total enzyme activity (extracellular plus membrane bound) determined by using intact cells plus medium; ○, extracellular activity obtained by centrifugation of the cell suspension for 3 min at 150 × *g* and of the supernatant for 1 min at 8,000 × *g* (the resulting supernatant was assayed); △, soluble cytoplasmic FAS activity. Cells were washed by centrifugation at 150 × *g* for 3 min, suspended, sonified at 50 W for 5 s with a Branson B12 sonifier with microtip, and centrifuged in buffer in the presence of 1 mM EDTA at 0°C for 20 min at 48,000 × *g*. The supernatant was assayed.

TABLE 2. Folic acid-cleaving activity secreted by several cellular slime mold species

Species or strain	Activity[a] (U per 10^6 cell equivalents)
D. minutum V3	100
D. minutum 39	5.8
D. minutum 71	1.2
D. discoideum NC 4(H)	5.4
D. rosarium	0.8
D. mucoroides	0.3
D. purpureum	1.2
D. mexicanum	3.9
D. lacteum	<0.1
P. violaceum	2.8
P. pallidum	<0.1

[a] The enzyme was isolated as described in the text.

Table 2 shows the activity of FAS in several cellular slime mold species after 3 h of starvation. *D. minutum* V3 clearly secreted more than two other *D. minutum* strains, 39 and 71, and other species. *D. lacteum* and *Polysphondylium pallidum* secreted no detectable amounts of FAS activity.

DISCUSSION

In *D. minutum* V3, the chemotactic activity of the acrasin and of folic acid are regulated by a C_9-N_{10}-cleaving enzyme. This enzyme was reported previously to yield 6-hydroxymethylpterin and not pterin-6-aldehyde, a conclusion based on the absence of ^3H_2O in the product of enzymatic [7,9,3′,5′-^{3}H]folic acid cleavage (7). This may have been due on the one hand to a large amount of ^3H_2O already present in the [^{3}H]folic acid preparation, possibly as a result of (photo) oxidation, and on the other hand to a relatively low amount of ^{3}H released from C_9 due to oxidation to the aldehyde (15%). The present report clearly shows that the aldehyde is the reaction product and that 6-hydroxymethylpterin is not an intermediate. In other organisms, C_9-N_{10} cleavage of folic acid has been reported, but the first reaction product containing the pterin moiety has not been identified (11, 15, 16). On HPLC system A, pterin-6-aldehyde yielded a broad asymmetric peak, which is thought to be due to oxidation of the aldehyde during chromatography. The oxidation product, pterin-6-carboxylic acid, was less retarded on system A and should cause fronting of the aldehyde peak.

The acid pH optimum of FAS might suggest that it is a lysosomal enzyme, released into the medium by broken cells or exocytosis. However, the activity outside the cells is relatively

high, and it is the only folic acid-degrading enzyme in *D. minutum* V3. The latter observations indicate an extracellular function. The inhibiting action of the particulate fraction of the homogenate may not be due to the presence of a specific inhibitor, but to the high concentration of fragments of organelles. An inhibition of the same type was observed with the folic acid deaminase in *D. discoideum* (6).

The FAS of *D. minutum* V3 yielded linear Eadie-Hofstee plots with a low K_m of 10^{-7} M. In contrast, the folic acid deaminase in *D. discoideum* was reported to consist of at least three isozymes differing in stability (3), and several K_m values were observed: 5×10^{-6} M and 1×10^{-7} M (6), and 3×10^{-5} M and 2×10^{-6} M, the first of which probably is a pterin deaminase (18). The secretion of FAS activity was fast, shortly after removal of the food source for the cells, but decreased during prolonged starvation. After 3 h, a plateau was reached. This pattern was also reported for folic acid deaminase in *D. discoideum* (6, 18), in which folic acid has a higher chemotactic activity in the postvegetative stage than after a longer starvation period. However, the secretion of the acrasinase of this species, a cAMP phosphodiesterase, was slow during early starvation and increased sharply after several hours (2). This may correspond to the low chemotactic activity of cAMP for postvegetative amoebae, which is 100-fold enhanced in aggregation-competent cells (5). Folic acid in *D. minutum* V3 was equally active for postvegetative and preaggregative cells, and the acrasin increased only 3- to 10-fold in activity. (5a). The observation above may indicate that FAS activity is already essential in the postvegetative stage and thus is secreted during early starvation.

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