Purification and Characterization of Glutamate Decarboxylase from *Neurospora crassa* Conidia*

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L-glutamate decarboxylase, an enzyme under the control of the asexual developmental cycle of *Neurospora crassa*, was purified to homogeneity from conidia. The purification procedure included ammonium sulfate fractionation and DEAE-Sephadex and cellulose phosphate column chromatography. The final preparation gave a single band on sodium dodecyl sulfate-polyacrylamide gels with a molecular weight of 33,200 ± 200. A single band coincident with enzyme activity was found on native 7.5% polyacrylamide gels. The molecular weight of glutamate decarboxylase was 30,500 as determined by gel permeation column chromatography at pH 6.0. The enzyme had an acidic pH optimum and showed hyperbolic kinetics at pH 5.5 with a $K_m$ for glutamic acid of 2.2 mM and a $K_m$ for pyridoxal-5'- phosphate of 0.04 $\mu$M.

*Neurospora crassa* has a simple asexual developmental cycle which consists of the differentiation and growth of aerial hyphae from vegetative hyphae, and then the formation of conidia, asexual spores, from the aerial hyphae (1). Each of these three cellular types, vegetative hyphae, aerial hyphae, and conidia, can be isolated in amounts sufficient for enzyme and protein analyses (2-4). The germination of *N. crassa* conidia, which culminates in the growth of a germ tube from the conidium, requires an exogenous carbon source (1). However, the early biochemical changes that occur during the activation of dormant conidia do not require an exogenous carbon source and are complete within a few minutes after the conidia are suspended in distilled water (1, 5-7). Apparently, the metabolites and enzymes required for the activation of dormant conidia and for the initial stages of differentiation during conidial germination are prepackaged in the conidia during conidiation.

One enzyme that is specifically stored in dormant conidia is glutamate decarboxylase (GAD, E.C. 4.1.1.15) (5). This enzyme catalyzes the $\alpha$-decarboxylation of L-glutamic acid to form $\gamma$-aminobutyric acid (GABA) and carbon dioxide. Free L-glutamic acid is an abundant amino acid in dormant conidia (6). This amino acid alone can account for up to 25% of the conidial dry weight. During the early stages of conidial germination, the endogenous pool of L-glutamic acid is metabolized (6). GAD is apparently responsible for the metabolism of L-glutamic acid during germination because there is almost a one-to-one correlation with the loss of L-glutamic acid and the transient appearance of GABA during the early stages of conidial germination (6, 8).

GAD has been found to be a developmentally regulated enzyme in many organisms. In vertebrates and invertebrates, GAD has been associated with specific neurons of the mature central nervous system (9, 10-12), and GAD has been implicated in the regulation of neural growth during insect embryogenesis (13). GAD is apparently involved in the germination of seeds (14-16) and bacterial endospores (17, 18). A mutant strain of *Bacillus megaterium* with low levels of GAD activity in its endospores required GABA for germination (19). In *Escherichia coli* GAD can be induced by growth on glutamic acid in acidic media (20). Thus, GAD is associated with differentiated cells from vertebrates, invertebrates, plants, and bacteria and appears to play a special role in the germination of some plant seeds and bacterial endospores.

GAD appears to play a role in the germination of *N. crassa* conidia. This enzyme is under the control of the asexual developmental cycle in *N. crassa*. Of the three cell types in a conidiating culture, only conidia have high levels of GAD activity. Even though GAD activity is high in dormant conidia, it does not catalyze the metabolism of glutamic acid until germination (5, 6, 8). GAD activity disappears as conidia differentiate and develop into vegetative hyphae (5). The loss of GAD activity occurs more rapidly than the synthesis of new cellular protein indicating that there is a special mechanism for inactivating GAD during germination. However, the synthesis of new cellular protein is necessary for the loss of GAD activity because cycloheximide prevents the disappearance of GAD activity during germination (5). The regulatory mechanisms that control GAD activity during conidiation and germination in *N. crassa* are not known. In order to investigate these regulatory mechanisms we purified GAD to homogeneity from dormant conidia. This paper describes the purification and initial characterization of this conidial-specific, developmentally regulated enzyme.

**EXPERIMENTAL PROCEDURES**

**Materials**—The wild type strain of *N. crassa* (FGSC 988) used in these studies was obtained from the Fungal Genetics Stock Center, Department of Microbiology, University of Kansas Medical Center (Kansas City, KS). L-[1,14C]Glutamic acid (46.0 mCi/mmol) was purchased from Du Pont-New England Nuclear. DEAE-Sephadex A50-120, Sephadex G100-120 L-glutamic acid, pyridoxal 5'-phosphate, phenylmethylsulfonyl fluoride, and pepstatin A were obtained from Sigma. Cellulose phosphate P11 was purchased from Whatman. All other chemicals were reagent grade.

**Glutamate Decarboxylase Assay**—GAD activity was assayed by...
Glutamate Decarboxylase

FIG. 1. Elution profile of GAD from a DEAE-Sephadex A-50 column. For details see under "Experimental Procedures." For this figure, GAD specific activity was normalized to the fraction with the highest specific activity. ●, GAD (relative specific activity); ■, protein.

FIG. 2. Elution profile of GAD from the cellulose phosphate P11 column. For details see under "Experimental Procedures." ●, GAD activity; ■, protein.

FIG. 3. SDS-polyacrylamide gel electrophoresis of GAD. Lane A, 2 µg of protein from the pooled fractions of the cellulose phosphate P11 column; lane B, 6 µg of protein from the pooled fractions of the DEAE-Sephadex column; lane C, 10 µg of protein from the crude extract. The standards and their molecular weights were: 1, rabbit muscle phosphorylase b, 97,000; 2, bovine serum albumin, 66,200; 3, rabbit muscle lactic dehydrogenase 36,500; 4, bovine carbonic anhydrase, 31,000; 5, porcine immunoglobulin light chain, 23,400; and 6, hen egg white lysozyme, 14,400. The gel was stained with Coomassie Blue R-250.

measuring the amount of CO₂ released from L-[1-1⁴C]glutamic acid (5). Disposable plastic tubes (12 × 75 mm) were used as assay vessels. A scintillation pad (Arthur H. Thomas Co.) with 250 µmol of KOH was inserted in the tube 6 cm above the reaction mixture. The assay mixture contained 0.9 mM pyridoxal 5'-phosphate, 0.9 mM EDTA, 45 mM sodium piperazine-N,N'-bis(2-ethanesulfonic acid), 0.9 mM 2-mercaptoethanol, and 10 µl of enzyme from different purification steps. The reaction was started by adding 30 mM L-glutamic acid and 0.055 µCi of L-[1-1⁴C]glutamic acid which had been adjusted to pH 5.5. The total assay volume was 110 µl. After 60-min incubation at 37 °C in a shaking water bath, 200 µl of 2 N H₂SO₄ was injected into each tube to stop the reaction. The CO₂ was allowed to absorb into the KOH on the scintillation pads for at least 2 h. The scintillation pads were transferred to scintillation vials containing 5-ml scintillation cocktail, and the amount of ¹⁴CO₂ was determined with a Beckman scintillation counter. A unit of GAD activity will catalyze the release of 1 µmol of CO₂ from glutamic acid per min at 37 °C. Specific activity was defined as units of GAD activity per mg of protein.

Protein Assay—Protein concentrations of the various preparations were determined by the method of Lowry et al. (16) with bovine serum albumin as the standard.

FIG. 4. Molecular weight of GAD on SDS-PAGE. The distances migrated by the standards (■) and GAD (●) on the gel shown in Fig. 3 were plotted as a function of the log of their molecular weights.

FIG. 5. Molecular weight of GAD by gel-permeation column chromatography. A Sephadex G-100-150 column (1.5 × 30 cm) was equilibrated with the standard buffer adjusted to pH 6.0. The column was calibrated with the following molecular weight standards. 1, ribonuclease, 13,690; 2, alcohol dehydrogenase, 39,860; 3, ovalbumin, 42,700; and 4, bovine serum albumin, 66,200. The curve was obtained by plotting the log of the molecular weight of standards (■) and GAD (●) as a function of their elution volumes.

TABLE I

| Amino acid residues | Minimal residue number | Residue/protein |
|---------------------|------------------------|----------------|
| nmol/100 µmol       |                        | mole/mol       |
| Asx                 | 9.10                   | 29.1           |
| Thr                 | 8.71                   | 27.9           |
| Ser                 | 8.98                   | 28.7           |
| Glx                 | 1.58                   | 5.0            |
| Pro                 | 4.89                   | 15.6           |
| Gly                 | 9.33                   | 29.8           |
| Ala                 | 10.03                  | 32.1           |
| Val                 | 9.58                   | 30.6           |
| Met                 | 2.01                   | 6.4            |
| Ile                 | 4.83                   | 15.4           |
| Leu                 | 9.51                   | 30.4           |
| Tyr                 | 3.07                   | 9.8            |
| Phe                 | 4.93                   | 15.8           |
| His                 | 1.49                   | 4.8            |
| Lys                 | 6.77                   | 21.7           |
| Arg                 | 5.24                   | 16.8           |
| Trp                 | ND*                    |                |
| Cys                 | ND                     |                |

*These values were calculated based on the frequency of histidine.

†The molecular weight of GAD was taken as 33,200 from the results of SDS-PAGE.

ND, not determined.
purification of glutamate decarboxylase from N. crassa conidia

**TABLE II**

| Purification step | Volume ml | Total protein mg | Total activity units | Specific activity units/mg | Purification x-fold | Yield % |
|-------------------|-----------|------------------|----------------------|-----------------------------|---------------------|---------|
| Crude extract     | 400       | 980              | 24                   | 0.024                       | 1                   | 100     |
| Ammonium sulfate  | 35        | 350              | 32                   | 0.091                       | 3.8                 | 130     |
| DEAE-Sephadex A-50| 9.0       | 54.0             | 8.3                  | 0.153                       | 6.4                 | 35      |
| Cellulose phosphate P11 | 2.3 | 1.00             | 2.6                  | 2.76                        | 115                 | 11      |

**TABLE III**

| Organism          | Molar weight | Subunit molecular weight(s) | Specific activity | K_{m} for Glutamic acid (mM) | Optimal pH |
|-------------------|--------------|----------------------------|-------------------|------------------------------|------------|
| N. crassa         | 33,200       | 33,200                     | 2.8               | 2.2                          | 5.0        |
| E. coli           | 300,000      | 50,000                     | 1.1               | 1.0                          | 4.4        |
| Squash            | 28           |                            | 25                | 8.3                          | 5.8        |
| Rat brain         | 110,000      | 40,000                     | 2.4               | 1.6                          | 7.3        |
| Mouse brain       | 85,000       | 44,000                     | 2.0               | 0.7                          | 7.0        |
| Locust brain      | 95,000       | 51,000                     | 1.1               | 5                            | 7.2        |

Preparation of Conidia—Conidia were obtained from cultures grown in Pyrex baking dishes (38 x 25 x 5 cm) containing 500 ml of minimal medium (2% glucose, 1.5% agar, and 2% Vogel’s (21) salts). The baking dish cultures were inoculated with conidia from a slant tube culture and incubated for 5 days at 25 °C. The conidia were lyophilized and stored at ~70 °C. The mycelial fragments containing the conidia preparation were removed with a sieve.

Crude Extract—The standard buffer used for purifying GAD contained 50 mM potassium phosphate, pH 7.2, 2 mM EDTA, 0.2 mM pyridoxal 5'-phosphate, 2 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM pepstatin A, and 10% glycerol. Approximately 15 g of lyophilized conidia were homogenized for 2 min with 0.4-0.6-mM acid-washed glass beads at 4 °C in the standard buffer in a “Bead-Beater” (Biospec product). The homogenate was centrifuged at 100,000 × g for 10 min at 4 °C, and the pellet was washed twice with the standard buffer. The supernatants were combined and centrifuged at 100,000 × g for 30 min at 4 °C to give the crude extract.

Ammonium Sulfate Fractionation—The crude extract was brought to 25% saturation with the addition of saturated ammonium sulfate. The pH was adjusted to pH 7.2 with 1 N KOH. The solution was centrifuged at 100,000 × g for 30 min at 4 °C, and the pellet was discarded. The supernatant was brought to 65% saturation with ammonium sulfate and centrifuged at 100,000 × g for 50 min at 4 °C. The pellet containing GAD was either stored at ~20 °C for use at a later time or prepared immediately for DEAE-Sephadex column chromatography.

**RESULTS**

Purification of GAD—N. crassa conidia were suspended in buffer containing 10% glycerol, 2 mM 2-mercaptoethanol, 2 mM EDTA, and 0.2 mM pyridoxal 5'-phosphate to stabilize GAD. The protease inhibitors, phenylmethylsulfonyl fluoride (1 mM) and pepstatin A (0.5 μM) were included to prevent proteolytic degradation of GAD during purification. The conidia were homogenized with glass beads, and cell debris was removed by centrifugation. In the first step of the purification, GAD was precipitated with ammonium sulfate (25-65% saturation). The ammonium sulfate pellet was dissolved in a minimal amount of standard buffer and dialyzed against this buffer before application to DEAE-Sephadex A-50 columns. GAD was eluted from the DEAE-Sephadex columns with a linear potassium phosphate-pH gradient (Fig. 1). GAD began to elute from these columns at a potassium phosphate con-
centration of 0.18 M. Only a single peak of GAD activity was detected. The fractions with GAD specific activity of 60% of the peak fraction were combined, concentrated by ammonium sulfate precipitation (70% saturation), dialyzed against 0.05 M potassium phosphate, pH 7.2, containing all of the other components in the standard buffer, and applied to a cellulose phosphate P11 column.

GAD began to elute from the cellulose phosphate P11 column at a potassium phosphate concentration of 0.3 M (Fig. 2). The protein profile from this column showed the same pattern as did GAD activity indicating that there were few contaminating proteins. The specific activity through the peak fractions was constant. The protein in the combined peak fraction was constant. The protein in the combined peak fraction were combined, concentrated by ammonium sulfate precipitation, DEAE-Sephadex column chromatography, and cellulose phosphate P11 column chromatography. Cellulose phosphate P11 column chromatography was very effective for purifying GAD. GAD apparently has a high affinity for the phosphate groups on the column. About 1 mg of GAD was obtained from 15 g of dry conidia with a 11% yield. The specific activity of purified GAD was 2.7 units per mg of protein. GAD was a relatively abundant protein constituting about 1% of the protein in the crude extract of conidia.

The molecular weight, subunit composition, specific activity, $K_m$, and pH optima of purified GADs from N. crassa and several other organisms are summarized in Table III. GAD has been purified from vertebrates (9, 10, 27), invertebrates (12), squash (28), and Escherichia coli (20, 29). The $K_m$ for glutamic acid for the N. crassa conidial enzyme is intermediate among those reported for GAD from various sources (Table III). The pH optimum for purified N. crassa GAD was in the acid range. GAD with acidic pH optima have been found in E. coli (30, 31) and in plants (15, 16, 28).

The active form of N. crassa conidial GAD is apparently a monomeric protein. SDS-PAGE under reducing conditions gave a molecular weight of 33,200 (Fig. 4), and gel-permeation column chromatography at pH 6 gave a molecular weight of 30,500 (Fig. 5). Since there is little GAD activity when this enzyme is assayed at pH 7.0 (6, and this paper), the monomeric form of N. crassa GAD is presumably the active species. In contrast, purified GADs from other organisms exist as multimeric proteins of identical (29) and nonidentical (9-12) subunits (Table III).

Purified N. crassa conidial GAD can aggregate to higher molecular weight forms. Aggregates of GAD were detected on other SDS gels, and on gel-permeation columns when these columns were run at pH 7.2 (see "Results"). GAD from bacteria (29) and rat brain (9) also form aggregates. The purification of GAD was done largely at pH 7.2. The aggregation of GAD at this pH did not apparently adversely affect purification. The high ionic strengths and lowered pH of the elution buffers for the DEAE-Sephadex and cellulose phosphate columns would presumably favor the dissociation of GAD, though this has not been tested.

We found no indication that there are additional forms of GAD in N. crassa conidia other than the 33,200-molecular weight form and its aggregates. Multiple molecular forms of GAD have been observed in mammals (9-11) and plants (15, 28). Whether these multiple forms represent distinct species of GAD or different aggregational states remains to be resolved (13).

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