PURIFICATION AND PROPERTIES OF THE DIHYDROFOLATE SYNTHETASE FROM PEA SEEDLINGS¹

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(Received December 10, 1974)

Summary Dihydrofolate synthetase [EC 6.3.2.12] was extracted from the cell particles (mitochondrial fraction) of pea seedlings and purified about 2,000-fold by ammonium sulfate fraction, DEAE-cellulose column chromatography, Sephadex G-200 gel filtration, and hydroxylapatite column chromatography. The enzyme preparation obtained was confirmed ultracentrifugally to be in the homogeneous state. The sedimentation coefficient of this enzyme was calculated as 3.9 S. The apparent molecular weight of the enzyme was determined to be about 56,000.

Optimum pH for the reaction was 8.8. The enzymatic reaction required dihydropteroate, L-glutamate and ATP as substrates, and divalent (Mg^{2+} or Mn^{2+}) and univalent (K^+ , NH_4^+ or Rb^+) cations as cofactors. The enzyme was specific for dihydropteroic acid as the substrate. ATP was not replaceable with any other nucleotides. K_m values for dihydropteroate, L-glutamate, ATP, Mg^{2+} , and Mn^{2+} were 1.0×10^{-6} , 1.5×10^{-3} , 1.0×10^{-4} , 1.1×10^{-3} , and 6.3×10^{-5} M, respectively. The enzymatic reaction was inhibited by the addition of ADP, but not by AMP. This suggests that the product from ATP in the reaction is composed of ADP + P_i .

Thus, it is proposed that this enzyme catalyzes the following reaction:

¹ The biosynthesis of folic acid compounds in plants, VIII (for Part VII, see Reference (1)). Part of this report was presented at the Annual Meeting of the Agricultural Chemical Society of Japan at Meiji-gakuin University on April, 1969 and at the Fourth International Symposium on Pteridines at Toba, on July 23, 1969. This work was supported in part by a grant-in-aid (to K. I.) for scientific research from the Ministry of Education.

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Previous investigations (1,3) have shown that the probable intermediate in the biosynthesis of folate compounds in plants is dihydropteroic acid, which combines enzymatically with L-glutamic acid to form dihydrofolic acid, and that the enzyme (dihydrofolate synthetase), which catalyzes the condensation of dihydropteroic acid and L-glutamic acid, was partially purified from pea seedlings (3).

GRIFFIN and BROWN (2) have reported the occurrence and partial purification of the enzyme from *Escherichia coli*. The high purification of the enzyme from microorganisms and higher plants was difficult, since the enzyme is fairly labile. We described in the previous report (11) that the high enzyme activity located in the cell particles (mitochondrial fraction) of pea seedlings and the enzyme extracted from the cell particles was relatively stable compared with that extracted from the cell homogenates.

Thus, in this paper, an attempt to extract and highly purify from the cell particles was carried out and the properties of the enzyme were investigated.

MATERIALS AND METHODS

Chemicals. ATP, GTP, ITP, CTP, UTP, ADP, and AMP were purchased from the Sigma Chemical Company. Albumin from bovine serum, ovalbumin, chymotrypsinogen A from beef pancreas, and myoglobin from the sperm whale were from Schwarz/Mann. DEAE-cellulose was from the Brown Company and Sephadex G-200 was from Pharmacia Fine Chemicals. Pteric acid was the gift of Dr. G. Toennies and Dr. John A. King. Pteric acid was reduced to dihydro form with sodium dithionite by the method of FUTTERMAN (4), and to the tetrahydro form by hydrogenation in glacial acetic acid according to the directions of O'DELL *et al.* (5).

Standard assay conditions. The reaction mixtures contained Tris-HCl buffer (100 μ moles, pH 8.8), magnesium sulfate (5.0 μ moles), L-glutamic acid (5.0 μ moles); 2-mercaptoethanol (50 μ moles); ATP (1.0 μ mole), enzyme (1.2 μ g), and dihydropteroic acid (0.05 μ mole) in a final volume of 1.0 ml. The reaction was carried out at 37°C for 30 min, then was stopped by heating the entire mixture in a boiling water bath for one minute. After diluting the reaction mixture with cold water, the amount of dihydrofolate formed was determined by microbiological assay with *Lactobacillus casei* ATCC 7469 in 10 ml of an assay medium for folic acid (7). Amounts are expressed as folate equivalents. One unit of dihydrofolate synthetase catalyzes the formation of 0.1 nmole of folate equivalent per 30 min under standard assay conditions. The specific activity of dihydrofolate synthetase is expressed in terms of units per mg protein.

Determination of protein. The amount of protein was determined by the method of LOWRY *et al.* (6), using crystalline bovine serum albumin as the standard.

Germination of pea seeds. Air-dried, healthy pea seeds (*Pisum sativum* L. var. Alaska) were soaked in distilled water at 25°C for 18 hr, then were allowed to ger-

minate in a greenhouse at 20°C for 6 days on moist absorbent cotton in a vat covered with a wet filter paper.

RESULTS AND DISCUSSION

Purification of the dihydrofolate synthetase from pea seedlings

Step I. Purification of the enzyme directly extracted from whole pea seedlings was difficult because the enzyme is labile, as previously reported (3). However, the enzyme extracted from particles (mitochondrial fraction) is fairly stable (11), so we used this extraction of the enzyme. The medium used to isolate the cell particles contained 0.5 M sucrose, 0.05 M Tris-HCl buffer at pH 7.5 and 50 mM 2-mercaptoethanol. Six-day-old seedlings (100 kg) were homogenized with 100 liters of the isolation medium. This and all subsequent steps were performed at 0–5°C, except where otherwise noted. The homogenate was squeezed through cotton cloth by basket centrifugation, and the green juice (130 liters) was collected.

Step II. After the juice which had stood for more than 2 hr to remove debris was decanted, the green supernatant solution was centrifuged at $20,000 \times g$ for 30 min. The precipitate was used as the particle fraction (mitochondrial fraction). The precipitate was suspended in 10 liters of 0.01 M Tris-HCl buffer at pH 7.5 containing 0.05 M 2-mercaptoethanol for 20 min with mechanical stirring, and then it was centrifuged at $20,000 \times g$ for 30 min. The supernatant solution was used as the extract of the mitochondrial fraction.

Step III. Solid ammonium sulfate was added to the above extract to give 0.10 saturation. The mixture was equilibrated by stirring for 30 min, then it was centrifuged at $20,000 \times g$ for 30 min. Solid ammonium sulfate was then added to the supernatant solution to give 0.35 saturation. The mixture was again equilibrated by stirring for 30 min, then it was centrifuged at $20,000 \times g$ for 30 min.

Step IV. The resultant precipitate was dissolved in 2.4 liters of 0.01 M Tris-HCl buffer at pH 7.5 containing 0.05 M 2-mercaptoethanol. Then the solution was divided into six parts of 400 ml each for convenient fractionation on DEAE-cellulose. Each 400 ml portion was dialyzed against 0.01 M Tris-HCl buffer at pH 7.5 containing 0.05 M 2-mercaptoethanol. The resulting precipitate was centrifuged and discarded. Each dialysate was placed in a DEAE-cellulose column (6 × 70 cm) previously equilibrated with 0.01 M ammonium sulfate in 0.01 M Tris-HCl buffer at pH 7.5 and 0.05 M 2-mercaptoethanol. The column was washed with 2 liters of the same buffer and developed by a linear gradient elution. The mixing chamber contained 2 liters of 0.01 M ammonium sulfate in 0.01 M Tris-HCl buffer at pH 7.5 and 0.05 M 2-mercaptoethanol, and the reservoir contained 2 liters of 0.2 M ammonium sulfate in the same buffer solution. The enzymatically active elutions from the DEAE-cellulose column were combined, and then the precipitate with ammonium sulfate (0.60 saturation) was recovered and stored at 0°C.

Step V. After the precipitate had been dissolved in 100 ml of 0.01 M Tris-HCl

buffer at pH 7.5 containing 0.05 M 2-mercaptoethanol, the solution was divided into two parts of 50 ml each for convenient fractionation on a Sephadex. Each 50 ml portion was applied to a Sephadex G-200 column (6×90 cm) equilibrated with 0.8 M ammonium sulfate in 0.1 M Tris-HCl buffer at pH 7.5 and 0.05 M 2-mercaptoethanol. The same buffer solution was allowed to flow through the column. The enzymatically active eluates were combined and their 0.60 saturation precipitate with solid ammonium sulfate was dissolved in 0.01 M Tris-HCl buffer at pH 7.5 containing 0.05 M 2-mercaptoethanol.

Step VI. The solution was rechromatographed on a Sephadex G-200 column (6×90 cm) using the same conditions as in Step V. The enzymatically active eluates were combined and their 0.60 saturation precipitate with solid ammonium sulfate was collected and dialyzed against 0.01 M potassium phosphate buffer at pH 7.5 containing 0.05 M 2-mercaptoethanol.

Step VII. The dialyzed enzyme solution was applied to a hydroxylapatite column (4×15 cm) equilibrated with 0.01 M potassium phosphate buffer at pH 7.5 containing 0.05 M 2-mercaptoethanol. Elution was carried out with 0.01 M and 0.05 M potassium phosphate buffers at pH 7.5 containing 0.05 M 2-mercaptoethanol. The results are shown in Fig. 1. Enzyme fractions in tube number 80 to 96 were pooled, and the precipitate with ammonium sulfate (0.60 saturation) was recovered and dissolved in 3 ml of 0.01 M Tris-HCl buffer at pH 7.5 containing 0.05 M 2-mercaptoethanol.

Fig. 1. Elution pattern of the dihydrofolate synthetase from a hydroxylapatite column. The dihydrofolate synthetase fraction obtained by a second Sephadex G-200 treatment was applied to a hydroxylapatite column (4×15 cm) equilibrated with 0.01 M phosphate buffer at pH 7.5 containing 0.05 M 2-mercaptoethanol. Fractions of 5 ml were collected at a flow rate of 10 ml per hour.

Step VIII. The solution was chromatographed on a Sephadex G-200 column (2.5×90 cm) under the conditions used for Step V on the Sephadex G-200 column. The elution pattern is shown in Fig. 2. The enzymatically active eluates were

Fig. 2. Third chromatography of dihydrofolate synthetase on a Sephadex G-200. The dihydrofolate synthetase fraction obtained by hydroxylapatite treatment was applied to a Sephadex G-200 column (2.5×90 cm) equilibrated with 0.8 M ammonium sulfate in Tris-HCl buffer at pH 7.5 and 0.05 M 2-mercaptoethanol. Fractions of 5 ml were collected at a flow rate of 25 ml per hour.

Table 1. Summary of the purification of the dihydrofolate synthetase from pea seedlings.

Fraction	Total protein (mg)	Total activity (units ^a)	Specific activity (units ^a /mg)	Purification (ratio)	Yield (%)
I. Juice	4,128,000	477,300	0.116	1.0	100
II. Extracts of mitochondrial fraction	698,000	321,556	0.460	4.0	67.3
III. Am ₂ SO ₄ ppt. (0.1~0.35 sat.)	129,000	162,540	1.26	10.9	34.1
IV. DEAE-cellulose column chromatography	4,930	32,045	6.50	56.1	6.7
V. 1st chromatography on Sephadex G-200	727	9,742	13.4	115.5	2.0
VI. 2nd chromatography on Sephadex G-200	100	8,560	85.6	738.0	1.8
VII. Hydroxylapatite column chromatography	45	8,190	182	1569.0	1.7
VIII. 3rd chromatography on Sephadex G-200	30	6,840	228	1965.6	1.4

^a 1 unit = 0.1 nmole of folate equivalent formed per 30 min under standard assay conditions.

combined and their 0.60 saturation precipitate with ammonium sulfate was dissolved in 0.01 M Tris-HCl buffer at pH 7.5 containing 0.05 M 2-mercaptoethanol. Solid ammonium sulfate was added to the solution (0.60 saturation) and the whole was stored at 0°C as the purified enzyme precipitation. Purification steps and the yields of dihydrofolate synthetase from pea seedlings are summarized in Table 1.

Ultracentrifugal analysis and the apparent molecular weight of the enzyme

The homogeneity and sedimentation constant of the purified enzyme were

Fig. 3. Sedimentation patterns of the dihydrofolate synthetase from pea seedlings. The sample contained 1.2% enzyme, 0.1 M KCl, 0.01 MET, and 0.01 M potassium phosphate buffer at pH 7.5. The determination was carried out at 5.1°C and at 52,640 rpm.

Fig. 4. Plot of elution volume against log molecular weight for proteins on a Sephadex G-100 column (1.5 × 84 cm).

tested using sedimentation analysis with a Spinco Model E ultracentrifuge. The enzyme preparation was dialyzed overnight against 0.01 M potassium phosphate buffer at pH 7.5 in 0.1 M KCl and 0.01 M 2-mercaptoethanol at 5°C. Sedimentation patterns at a maximum speed of 52,640 rpm are shown in Fig. 3. The enzyme preparation showed a homogeneous peak. The calculated sedimentation coefficient at 20°C was about 3.9 S⁴. The apparent molecular weight of the enzyme was determined according to the method of ANDREWS (8), using a Sephadex G-100 (1.5×70 cm). Bovine serum albumin (mol. wt. 67,000), ovalbumin (mol. wt. 45,000), chymotrypsinogen A from beef pancreas (mol. wt. 25,000), and myoglobin from the sperm whale (mol. wt. 17,800) were used as the reference standards. When compared with the reference standards, the molecular weight of the dihydrofolate synthetase from pea seedlings seems to be about 56,000, as shown in Fig. 4. After the enzyme preparation had been stored at 0°C for 3 months in 0.01 M Tris-HCl buffer at pH 7.5 containing 2 M ammonium sulfate and 0.05 M 2-mercaptoethanol, it was applied to a Sephadex G-100 column. The elution pattern is shown in Fig. 5. The new protein peak which had no enzyme activity appeared in fractions 29 to 34. The apparent molecular weight was about 120,000. The main peak was active for enzyme activity, and its molecular weight was 56,000. This indicates that the non-active peak may be a dimer of the active enzyme and may be formed during storage of the enzyme. The value of the sedimentation coefficient (7.5 S), which we previously reported (10), was thought to be the value of the non-active dimer.

Fig. 5. Chromatography of purified dihydrofolate synthetase on a Sephadex G-100. The purified enzyme preparation which had been stored at 0°C for 3 months in a 0.01 M Tris-HCl buffer, pH 7.5, containing 0.05 M 2-mercaptoethanol and 2 M ammonium sulfate, was used. ○---○, O.D. at 280 nm; ○—○, activity.

⁴ In a previous report, the sedimentation coefficient was reported to be 7.5S (10). Further repeated experiments, however, indicated that it is 3.9S.

Ultraviolet absorption spectrum of the enzyme

The ultraviolet absorption spectrum of the enzyme in 0.01 M Tris-HCl buffer at pH 7.5 containing 0.005 M 2-mercaptoethanol is presented in Fig. 6. The enzyme shows a single absorption peak with a maximum at 278 nm and a minimum at 250 nm. The extinction coefficient ($E_{1\text{cm}}^{1\%}$) value of the enzyme at 280 nm was 10.8.

Fig. 6. Absorption spectrum of the dihydrofolate synthetase purified from pea seedlings. Protein concentration was 0.485 mg per ml.

Fig. 7. Relationship between the time course of the reaction and enzyme concentrations. The standard assay procedure was used, except where altered reaction time and amounts of enzyme are indicated. A, 2.4 μg ; B, 1.2 μg ; C, 0.24 μg .

Time course of the reaction

The time course of the reaction was tested at various concentrations of the enzyme. The reaction proceeded linearly and the amounts of folate equivalent formed were proportional to the amounts of enzyme preparation used, as shown in Fig. 7.

Effect of pH on enzyme activity

The effect of pH on enzyme activity is illustrated in Fig. 8. Tris-HCl and glycine-NaOH buffers were used at final concentrations of 0.1 M. Maximum activity was obtained at pH 8.8. This value is in agreement with the optimum pH obtained in a previous experiment using a crude enzyme preparation (3).

Fig. 8. Effect of pH on enzyme activity. Assay conditions were the same as those described in "MATERIALS AND METHODS," except that various buffers at the indicated pH values were used at final concentrations of 0.1 M.

Table 2. Component study for dihydrofolate synthetase (I).

The standard assay method was used except for the omission of the indicated substances. The enzyme already contained 5 μmoles of ammonium sulfate.

Omission	Folate equivalent formed (pmoles)
None	108
Dihydropteroate	0
L-Glutamate	0
ATP	0
Mg ²⁺	0
K ⁺	23
2-Mercaptoethanol	74
Enzyme	0

Table 3. Component study for dihydrofolate synthetase (II).

Omission	Addition	Folate equivalent formed (pmoles)
None	None	119
Dihydropteroate	None	0
Dihydropteroate	Pteroate	0
Dihydropteroate	Tetrahydropteroate ^a	0
ATP	None	0
ATP	ADP	0
ATP	AMP	0
ATP	GTP	0
ATP	ITP	0
ATP	CTP	0
ATP	UTP	0

^a Incubation was performed at 37°C for 30 min in an argon atmosphere.

Table 4. Divalent cation requirements for dihydrofolate synthetase.

Each metal was used in the form of its sulfate.

Divalent cations	Conc. (mM)	Folate equivalent formed (pmoles)
None	0	0
Mg ²⁺	1.0	53
	5.0 ^a	98
Mn ²⁺	0.5 ^a	124
	1.0	77
Fe ²⁺	1.0	16
Co ²⁺	1.0	6
Zn ²⁺	1.0	4
Cu ²⁺	1.0	0
Ni ²⁺	1.0	0
Ca ²⁺	1.0	0

^a Optimal concentration.

Table 5. Univalent cation requirements for dihydrofolate synthetase.

The standard assay was used except that the enzyme was dialyzed against 0.01 M Tris-HCl buffer at pH 7.5 containing 0.05 M of 2-mercaptoethanol. 100 μ moles of each metal was used in the form of the chloride.

Addition	Folate equivalent formed (pmoles)
None	0
K ⁺	108
NH ₄ ⁺	95
Rb ⁺	89
Na ⁺	8
Cs ⁺	5
Li ⁺	3

Component study of the reaction. As shown in Table 2, the reaction was dependent on dihydropteroate, L-glutamate, ATP, Mg²⁺, and the enzyme. Omission of K⁺ or 2-mercaptoethanol from the complete system resulted in a decrease in the reaction velocity. This enzyme was specific for dihydropteroic acid as the substrate. Pteric and tetrahydropteroic acids were not used as substrates. ATP was not replaceable with any other nucleotides, as shown in Table 3. GRIFFIN and BROWN (2) reported that in the partially purified enzyme from *E. coli*, ITP, GTP, and ADP could be utilized 60, 35, and 10%, respectively, as effectively as ATP. K_m values for dihydropteroate, L-glutamate, and ATP were calculated as 1.0×10^{-6} , 1.5×10^{-3} and 1.0×10^{-4} M, respectively. These K_m values are summarized in Table 6.

Divalent cation requirements. The effect of various divalent cations on the enzyme activity was investigated using their sulfate forms. The results are shown in Table 4. Mn²⁺ was more effective than Mg²⁺ in promoting enzyme activity, and this effect could be replaced by Fe²⁺ to a lesser extent. Mn²⁺ was, however, only 10 to 15% as effective as Mg²⁺ in the partially purified enzyme from *E. coli* (2). K_m values for Mg²⁺ and Mn²⁺ were calculated as 1.1×10^{-3} and 6.3×10^{-5} M, respectively, in the highly purified enzyme from pea seedlings. The K_m value of the highly purified enzyme from pea seedlings had about a 5-fold decrease in comparison with that from the partially purified enzyme from pea seedlings for Mn²⁺. This presents the possibility that the enzyme activity might require Mn²⁺ rather than Mg²⁺ as the divalent cation *in vivo*.

Univalent cation requirement. The enzyme, dialyzed for 18 hr against 0.01 M Tris-HCl buffer at pH 7.5 containing 0.01 M 2-mercaptoethanol in the cold was used for the following experiments.

The effect of various univalent cations in their chloride forms on the enzyme activity was investigated at a final concentration of 100 mM. The results are shown in Table 5. An absolute univalent cation requirement for enzyme activity was observed. K⁺ was the most effective, and was replaceable by NH₄⁺ and Rb⁺. Na⁺, Li⁺, and Cs⁺ were not effective.

Table 6. Michaelis-Menten constant (K_m) for the binding of the substrate.

Substrate	K_m
Dihydropteroate	1.0×10^{-6} M
L-Glutamate	1.5×10^{-3} M
ATP	1.0×10^{-4} M
MgSO ₄	1.1×10^{-3} M
MnSO ₄	6.3×10^{-5} M

Inhibition of the enzymatic reaction by ADP

The enzymatic reaction was inhibited by the addition of ADP, but not AMP,

Table 7. The effect of ADP and AMP on the enzymatic formation of dihydrofolate.

Addition	Conc. (mM)	Folate equivalent (pmoles)
None		118
ADP	2.5	88
	5.0	64
AMP	2.5	112
	5.0	110

as shown in Table 7. Furthermore, another experiment using the enzyme prepared from *Serratia indica* (12) detected ADP formed enzymatically from ATP using chromatographic procedures. This indicates that the product from ATP in the reaction was composed of ADP and P_i , as shown in the following equation:

The systematic name for dihydrofolate synthetase in plants should be 7,8-dihydropteroate: L-glutamate ligase (ADP) in analogy with that in *E. coli*.

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