SOME CHARACTERISTICS OF THE DIHYDROFOLATE SYNTHETASE FROM SERRATIA INDICA

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Summary Dihydrofolate synthetase (EC 6.3.2.12) from Serratia indica IFO 3759 require a divalent cation and a univalent cation for its activity. The divalent cation requirement was satisfied by magnesium ion, manganese ion or ferrous ion. High activity was obtained with 5 mM of magnesium ion. The effect of manganese ion was weak. The univalent cation requirement was satisfied by potassium ion, ammonium ion or rubidium ion, and high activity was obtained with 100 mM of each univalent cation. Increase in the potassium concentration lowered $K_m$ values for dihydropteroate and L-glutamate, and raised $V_{max}$ for ATP and dihydropteroate. Potassium ion had little effect on $K_m$ value for ATP. These results suggest that potassium ion may function on the affinities of dihydropteroate and L-glutamate to the enzyme.

Dihydrofolate synthetase was inhibited by the addition of reduced forms of homopteroic acid. Stronger inhibition was observed by dihydrohomopteroate than by tetrahydrohomopteroate.

Our previous paper showed that dihydrofolate synthetase in pea seedlings requires magnesium ion, ATP and potassium ion as catalysts. The reactive species of magnesium ion and ATP in transphosphorylation reaction has been shown to be a complex (i.e., MgATP). Although a number of enzymes have been shown to require potassium, ammonium or rubidium ions for their activities (1-3), no detailed studies on the potassium ion requirement were made.

Homopteroic acid, an intermediary product in the synthesis of homofolate, and its tetrahydro derivative have been reported to be a potent growth inhibitor to Streptococcus faecalis R, a folate dependent organism (4). Kisliuk et al.

1 Studies on the Biosynthesis of Folic Acid Compounds. Part X. For Part IX, see Ref. 6. A part of this report was presented at the Annual Meeting of Japanese Society of Food and Nutrition held at Tokyo on May 25, 1974.

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have reported that tetrahydrohomopteroic acid displays an inhibitory effect on the growth of the pyrimethamine-resistant strain of *Plasmodium cynomolgi* in monkeys (5).

The purification and properties of the dihydrofolate synthetase from *Serratia indica* were described in a preceding paper (6). This paper deals with studies on the activation of the dihydrofolate synthetase by magnesium ion and univalent cations. The inhibition of the dihydrofolate synthetase by reduced forms of homopteroic acid is also discussed.

**MATERIALS AND METHODS**

**Chemicals.** Disodium and Tris-salt of ATP were purchased from Sigma Chemical Company. Pteroic acid was kindly provided from the Lederle Laboratories Division, American Cyanamid Company. Homopteroate and homofolate were kindly provided by the Cancer Chemotherapy National Service Center of the U. S. Public Health Service through the courtesy of Prof. R. L. Kisliuk, Department of Biochemistry, Tufts University. L-Glutamic acid, 2-mercaptoethanol, folic acid and other chemicals were purchased from Nakarai Chemicals, Ltd., Kyoto.

**Reduction of pteroate and folate compounds.** Pteroic acid was reduced to the dihydro form by the treatment with sodium dithionite as described by FUTTERMAN (8). Homopteroic and homofolic acids were reduced to their dihydro forms by the treatment with sodium dithionite as described by FRIEDKIN et al. (9). Tetrahydrohomopteroic acid was prepared by the reduction of homopteroic acid according to the directions of KISLIUK (10).

**Purification of the dihydrofolate synthetase from *S. indica*.** The enzyme used in this studies was purified from *S. indica* by the procedure described in a preceding paper (6).

**Standard assay conditions.** Reaction mixtures contained 100 μmoles of Tris-HCL buffer (pH 9.0); 5.0 μmoles of magnesium sulfate; 5.0 μmoles of L-glutamic acid; 50 μmoles of potassium sulfate; 50 μmoles of 2-mercaptoethanol; 5.0 μmoles of ATP; 1.5 μg of enzyme and 0.05 μmole of dihydropteroic acid in a final volume of 1.0 ml. The reaction was carried out at 37°C for 30 min, then stopped by heating the whole in a boiling water bath for 1 min. After diluting the mixtures with cold water, the amount of dihydrofolate formed was determined by microbiological assay with *Lactobacillus casei* as described previously (6). Activity is expressed as pmoles of folate equivalents formed per 30 min. Tris-salts were used to eliminate the effect of potassium and sodium ions from the assay mixtures for the experiments of the effect of potassium ion.

Protein concentration was determined by the method of LOWRY et al. (11).
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RESULTS

Activation by divalent cations

The effects of Mg²⁺, Mn²⁺ and Fe²⁺ on the activity of the dihydrofolate synthetase from S. indica are shown in Fig. 1. The maximum activity was produced by 5×10⁻³ M of Mg²⁺. Higher amounts of Mg²⁺ inhibited the enzyme activity. Mn²⁺, Fe²⁺ and Ca²⁺ also activated the enzyme, but none of these was so effective as Mg²⁺ (Table 1).

![Fig. 1. Effects of divalent cations on the dihydrofolate synthetase. Divalent cations were used in the form of sulfate.](image)

Table 1. Effects of divalent cations on the dihydrofolate synthetase.
The standard assay method was used, except that the divalent cations indicated were added.

| Divalent cations | Conc. (mm) | Folate equivalent formed (pmoles) |
|-----------------|-----------|----------------------------------|
| Mg²⁺            | 5.0       | 195                              |
|                 | 1.0       | 68                               |
| Mn²⁺            | 5.0       | 69                               |
|                 | 1.0       | 61                               |
| Fe²⁺            | 5.0       | 79                               |
|                 | 1.0       | 77                               |
| Co²⁺            | 1.0       | 0                                |
| Ni²⁺            | 5.0       | 0                                |
| Zn²⁺            | 5.0       | 0                                |
| Ca²⁺            | 5.0       | 0                                |
| Cd²⁺            | 5.0       | 0                                |
| Cu²⁺            | 5.0       | 0                                |

* Used in the form of sulfate.

The effect of ATP concentration on the enzyme activity is shown in Fig. 2. From the reciprocal plots according to the method of Lineweaver and Burk (13), Michaelis constant (Km value) for ATP was determined to be 2.2×10⁻⁴ M.
Fig. 2. Effect of ATP on the dihydrofolate synthetase in the presence of 100 mM (A) and 7.5 mM (B) of K⁺. (A) The standard assay was used except that the dialyzed enzyme against Tris buffer containing 0.05 M 2-mercaptoethanol was added. (B) The standard assay was used except that the dialyzed enzyme and 7.5 mM of K⁺ were used.

**Activation by univalent cations**

The effects of K⁺, NH₄⁺ and Rb⁺ on the activity of the dihydrofolate synthetase are shown in Fig. 3. The optimum concentration for K⁺ and Rb⁺ was 50 to 100 mM. With 10 mM of K⁺, 70% of the maximum activity was obtained. The optimum concentration of NH₄⁺ was 20 to 30 mM and higher amounts of NH₄⁺ resulted in a slight inhibition. The apparent Michaelis constants (Km values) for K⁺, NH₄⁺ and Rb⁺ were determined from Fig. 3 to be about 7×10⁻³, 3.5×10⁻³ and 1.3×10⁻² M, respectively. The effect of K⁺ was partly replaceable.

Fig. 3. Effect of univalent cations on the dihydrofolate synthetase. The standard assay was used except that the dialyzed enzyme against 0.01 M Tris buffer, pH 8.0, containing 0.05 M 2-mercaptoethanol was added. Univalent cations were used in the form of sulfate.
by Tl⁺ and Cs⁺. Na⁺ and Li⁺ were ineffective. These results are summarized in Table 2.

| Additions | Optimum concen. (mm) | Km (mM) | Vmax (pmoles of folate equivalent formed) | Ionic radius α (Å) |
|-----------|----------------------|---------|----------------------------------------|-------------------|
| None      | 25                   | 3.5     | 100                                    | 1.48              |
| NH₄⁺      | 100                  | 7       | 95                                     | 1.33              |
| K⁺        | 100                  | 13      | 88                                     | 1.48              |
| Rb⁺       | 100                  | 100     | 46                                     | 1.44              |
| Tl⁺       | 100                  | 100     | 14                                     | 1.69              |
| Cs⁺       | 100                  | 100     | 0                                      | 0.95              |
| Na⁺       | 100                  | 100     | 0                                      | 0.60              |

α Data from Ref. 23.

Effect of K⁺ on the kinetic constants of the enzyme reaction

To investigate the effect of K⁺, the kinetic constants for substrates (i.e., ATP, dihydropteroate, or L-glutamate) were determined in the presence of 7.5 and 100 mM of K⁺. The standard assay was used, except that the enzyme dialyzed for 48 hr against 0.01 M Tris-HCl buffer, pH 8.0, containing 0.05 M 2-mercaptoethanol was added. Results are shown in Table 3. By decreasing K⁺ concentration from 100 to 7.5 mM as shown in Figs. 4 and 5, Km values for dihydropteroate and L-glutamate were increased and that for ATP was a little changed though Vmax for ATP was decreased. The Vmax for dihydropteroate was also decreased by decreasing K⁺ concentration to 7.5 mM, but the Vmax for L-glutamate was not changed.

Table 3. Effect of potassium ion on kinetic constants for the reaction.
The standard assay was used except that the dialyzed enzyme was used.

| Substrates       | Potassium ion (mM) | Km (mM) | Relative reaction rate α |
|-----------------|--------------------|---------|-------------------------|
| ATP             | 100                | 2.2×10⁻⁴| 1.0                     |
|                 | 7.5b               | 2.9×10⁻⁴| 0.85                    |
| Dihydropteroate | 100                | 2.5×10⁻⁷| 1.0                     |
|                 | 7.5b               | 1.3×10⁻⁶| 0.63                    |
| L-Glutamate     | 100                | 2.5×10⁻⁴| 1.0                     |
|                 | 7.5b               | 9.1×10⁻³| 0.97                    |

α Ratio of Vmax value for each substrate in the presence of 7.5 mM of potassium ion to that in the presence of 100 mM of the ion.

b The standard assay conditions were used except that dialyzed enzyme and 7.5 mM of K⁺ were added.
Fig. 4. Effect of dihydropteroate on the dihydrofolate synthetase at the presence of 100 mM (A) and 7.5 mM (B) of K+. The conditions were the same as in Fig. 2.

Fig. 5. Effect of L-glutamate on the dihydrofolate synthetase at the presence of 100 mM (A) and 7.5 mM (B) of K+. The conditions were the same as in Fig. 2.

Inhibition of the dihydrofolate synthetase by reduced forms of homopteroate

The effects of homopteroate and homofolate on the growth of L. casei under the microbial assay conditions are shown in Fig. 6. The growth was not inhibited in the presence of 10^{-6} M of homopteroate or the reduced compounds. Table 4 shows that the dihydrofolate synthetase activity was strongly inhibited by the addition of dihydrohomopteroate, but slightly by tetrahydrohomopteroate. The inhibition of the activity by tetrahydrohomopteroate may be due to the dihydro form produced by the oxidation of the tetrahydro form during the reaction in air.

DISCUSSION

MgATP is a reactive species in many reactions where ATP serves as a phosphate donor (12). Mg^{2+} was essential to the reaction catalyzed by the dihydro-
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Fig. 6. Growth inhibition of L. casei by homopteroate and homofolate.

Table 4. Inhibition of the dihydrofolate synthetase by homopteroate and the reduced compounds. The standard assay was used, except for the addition of indicated substances. For the microbial assay with L. casei, homopteroate and the reduced compounds were diluted below 10⁻⁶ M.

| Additions           | Conc. (m) | Folate equivalents formed (pmoles) | Inhibition (%) |
|---------------------|-----------|------------------------------------|---------------|
| Homopteroate        | 0         | 198                                | 0             |
|                     | 1 x 10⁻⁸  | 210                                | 0             |
|                     | 1 x 10⁻⁸  | 205                                | 0             |
| Dihydrohomopteroate | 1 x 10⁻⁷  | 192                                | 3.1           |
|                     | 1 x 10⁻⁸  | 172                                | 13.3          |
|                     | 5 x 10⁻⁸  | 158                                | 20.4          |
|                     | 1 x 10⁻⁸  | 139                                | 29.8          |
|                     | 5 x 10⁻⁸  | 86                                 | 56.7          |
|                     | 1 x 10⁻⁴  | 30                                 | 74.8          |
| Tetrahydrohomopteroate | 5 x 10⁻⁶ | 201                                | 0             |
|                     | 1 x 10⁻⁸  | 192                                | 3.0           |
|                     | 5 x 10⁻⁸  | 172                                | 13.3          |
|                     | 1 x 10⁻⁴  | 130                                | 35.4          |

Folate synthetase of S. indica. The required amount of Mg²⁺ was so excess to form MgATP (Figs. 1 and 2). MgATP, however, may be the required substrate. The requirement for Mg²⁺ was partially replaceable by Mn²⁺ or Fe³⁺. On the dihydrofolate synthetase from pea seedling (14), Mn²⁺ was more effective than Mg²⁺ in stimulating the enzyme activity, but the high concentration of Mn²⁺ (5 mM) markedly inhibited the enzyme activity. With the dihydrofolate synthetase from S. indica, however, no other divalent cations were so effective as Mg²⁺ and the high concentration of Mn²⁺ (5 mM) have not inhibited the enzyme activity, though it inhibited the enzymes from Escherichia coli (15) and pea seedlings (14).

Potassium or related univalent cations are also essential to the reaction. High activity was obtained at 100 mM of K⁺. Increase in the K⁺ concentration from
7.5 to 100 mM resulted in a marked lowering of the \( Km \) values for dihydropteroate and L-glutamate, and raised the \( V_{\text{max}} \) for ATP and dihydropteroate (Table 3). These data indicate that \( K^+ \) increases the apparent affinity of the enzyme to dihydropteroate and L-glutamate, and suggest that \( K^+ \) is required to contact for dihydropteroate and L-glutamate with the enzyme. From the kinetic investigation, it has been suggested that the conformation of the enzyme protein which requires univalent cations for its activity is changed by the ions (16–18). It has been reported that formyltetrahydrofolate synthetase was dissociated into four subunits in the absence of a univalent cation such as \( K^+ \) (19, 20). There seems to be a correlation between ionic radii of univalent cations and their abilities to activate the enzyme: \( Na^+ \), \( Li^+ \) and \( Cs^+ \) have shorter (0.7–1.0 Å) or longer (1.69 Å) ionic radii than those of effective univalent cations (1.33–1.48 Å). It has been reported that some univalent cations which have the ionic radii as \( K^+ \) (1.33 Å) are effective to the complex formation between the apopropanediol dehydratase and coenzyme \( B_{12} \), and their effects are closely related to the catalytic activity of the propanediol dehydratase reaction (22). To detect possible changes of the fourth dimensional structure of the dihydrofolate synthetase in the presence or absence of \( K^+ \), the Svedberg value was measured by the ultracentrifugal analysis, but no change of the value was detected. This result would indicate that the effect of \( K^+ \) cannot be attributable to the change in the fourth dimensional structure of the enzyme but to the microenvironmental change at the active site of the enzyme.

Homopteroic acid is an intermediary product in the synthesis of homofolic acid and it is occurred as a contaminant in some commercial preparations of homofolic acid. KISLIUK et al. (5) reported that tetrahydrohomopteroate displayed the inhibitory action against the pyrimethamine-resistant strain of \( P. \) cynomolgi in monkey. FERONE and HITCHINGS (21) reported that pyrimethamine exerts its antimalarial effect by inhibiting dihydrofolate reductase and that sulfonamides show an antimalarial activity by inhibiting the incorporation of \( p \)-amino benzoic acid into dihydrofolic acid. Since tetrahydrohomopteroic acid has a \( p \)-amino benzoic acid moiety in its molecule, it might inhibit the same site as sulfonamides do. The pteridine moiety involved in the new drug introduces an additional possibility that the enzymes in the dihydrofolate biosynthetic pathway from pteridine may be inhibited. Two examples of the enzymes in the category are; (i) the enzyme that catalyzes the pyrophosphorylation of \( 6 \)-hydroxymethyl dihydropterin (7), (ii) the enzyme that catalyzes the binding reaction of L-glutamate to dihydropteroate (15). The action site of the drug on the dihydrofolate biosynthetic pathway, however, is obscure. Dihydrohomopteroate and tetrahydrohomopteroate inhibited the dihydrofolate synthetase from \( S. \) indica (Table 4). The inhibition by tetrahydrohomopteroate may be due to the inhibition by the dihydro form produced by the oxidation during the reaction in air. When tetrahydrohomopteroate was added \textit{in vivo} to the medium of \( S. \) indica, it may be oxidized to dihydrohomopteroate in the same way as \textit{in vitro}. The inhibition of dihydrofolate synthetase
from *S. indica* by dihydrohomopteroate suggests that the enzyme is an important enzyme in the dihydrofolate biosynthetic pathway.

We have recently shown that the purified enzyme preparation of dihydrofolate synthetase can be separated into three major protein bands (active) and one minor band (inactive) by electrophoresis on polyacrylamide gel.

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