Purification and Properties of Thymidylate Synthetase from Calf Thymus*

DAVID M. GREENBERG, RAVINDRA NATH, AND GLADYS K. HUMPHREYS

From the Department of Biochemistry, University of California School of Medicine, San Francisco 22, California

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The enzyme, thymidylate synthetase, catalyzes the methylation of deoxuryridilic acid to thymidylate, with formaldehyde as the source of the methyl group, in the presence of tetrahydrofolate. Some of the properties of this enzyme system in crude soluble protein extracts of rat thymus have previously been reported (1). Reports have also appeared of this enzyme reaction in Escherichia coli (2, 3).

The present communication describes the purification of thymidylate synthetase from calf thymus, the kinetic properties of the reaction, and some observations on the reaction mechanism.

EXPERIMENTAL PROCEDURE

Materials

Protamine sulfate was obtained from Nutritional Biochemicals Corporation, DEAE-cellulose from Eastman Kodak Company or Brown Corporation, and folic acid from the Lederle Laboratories Division of the American Cyanamide Company and Mann Research Laboratories, Inc.

Analytical Methods

Incubation Procedure—Incubations were performed for 1 hour, unless indicated otherwise, at 37° in a Dubnoff shaking metabolic incubator under nitrogen. The reaction vessels, 20-ml beakers, contained the following components in a total volume of 1.8 ml: 0.3 to 1.0 ml of enzyme solution (6 g of protein per liter), 0.05 M Veronal buffer, pH 7.6, 1.25 mM formaldehyde, 1.5 mM dUMP, and 1.25 mM folate-H4. Two tenth milliliters of 4 N HClO4 was added to terminate the enzyme activity. The thymidylic acid was determined by the method of Roberts and Friedkin (6), somewhat modified to remove folic acid degradation products. In this method, bromination of the thymine results in an alkali-unstable product, that decomposes with the formation of acetal (from the C—5—CH3 group) in alkaline solution. The acetal coupled to o-aminobenzaldehyde yields a 3-hydroxyquinaldine derivative whose fluorescence can be measured with the Farrand spectrofluorometer.

To perform the analysis, 30 µl of a 1:2 dilution of saturated bromine water were added to 1-ml aliquots of the test samples and these were allowed to stand at room temperature for 20 minutes. The alkalinity was then adjusted to 0.15 N in each sample by addition of 0.15 ml of 4 N NaOH (0.01 M EDTA). To remove folate-H4 decomposition products (which also form interfering fluorescing material) the alkaline solutions were filtered with suction through a 1.25-cm bed of Dowex 1 (Cl-) in a sintered glass funnel (2-cm inside diameter, 1.3 cm high, medium porosity). The acetal was recovered quantitatively in 5-ml volumetric flasks by washing first with 1 ml and then with 0.5 ml of 0.15 N NaOH (0.01 M EDTA). The final concentration of NaOH is critical for the coupling reaction and the concentrations of the HClO4 and NaOH requires careful adjustment.

The coupling with o-aminobenzaldehyde was carried out by adding 60 µl of the latter to each flask and then heating the stoppered flasks in a water bath at 73° for 30 minutes.

The enzyme was centrifuged down, and the supernatant liquids were decanted into 5-ml test tubes. 10-ml centrifuge tubes, the protein was centrifuged down, and the supernatant liquids were decanted into 5-ml test tubes. The thymidylate synthetase was determined by the method of Roberts and Friedkin (6), somewhat modified to remove folic acid degradation products. In this method, bromination of the thymine results in an alkali-unstable product, that decomposes with the formation of acetal (from the C—5—CH3 group) in alkaline solution. The acetal coupled to o-aminobenzaldehyde yields a 3-hydroxyquinoline derivative whose fluorescence can be measured with the Farrand spectrofluorometer.

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Because folate-H4, folate-H3, and their degradation products fluoresce at the same wave lengths, it was necessary to remove these compounds from the incubation mixture to avoid high blank correction. Moreover, nonenzymatic chemical changes of folate-H4 take place in the presence of the substrate, dUMP, and give false thymine values. This interference was eliminated by passage of the brominated samples in alkaline medium through Dowex 1 (Cl-). Nonenzyme blanks were run routinely to correct for any residual interference. The experiments were run in pairs, one sample with dUMP, the other without dUMP, to correct for any fluorescence from the enzyme, etc.

The abbreviation used is: EDTA, ethylenediaminetetraacetic acid.

The volume of o-aminobenzaldehyde added varies with each preparation of amine. Sixty microliters are used if the absorbance at 260 nm is 0.54 for 0.1 ml diluted to 50 ml. The volume must be adjusted so that this constant amount of amine is used for each assay. The proportionality of the method to thymine content is greatly influenced by the concentration of the amine as well as the degree of fluorescence. A balance between optimal concentration for the reaction and the minimal level for quenching of the fluorescence is attained by trial.
flasks were cooled, and 2 drops of bromomethyl blue indicator were added to each: 0.1 M sodium phosphate buffer, pH 7.6, was then added followed by 0.1 N HCl to adjust the pH to 6.9. The flasks were made up to volume with additional phosphate buffer.

Three standards containing 5, 10, and 20 μg of thymidine per flask, respectively, and a blank were run with each assay series.

Protein Determination—In crude enzyme preparations this was determined by the biuret method (7). With purer preparations the ultraviolet absorption method was used (7) and the proteins were calculated from the ratios at 280 nm and 260 nm.

Heat treatment. The enzyme solution at this temperature for 5 minutes, with continued agitation. The protein precipitate was centrifuged down and the enzyme in a hot water bath to 70°C, while the contents were kept agitated for approximately 3 minutes, and keeping the enzyme solution at this temperature for 5 minutes, with continued agitation. The protein precipitate was centrifuged down and a second ammonium sulfate fractionation was carried out on the supernatant liquid as already described.

For the column chromatography procedure, the DEAE-cellulose was first purified by stirring with 1 N alkali for ½ hour and then washing with water by decantation until neutrality was restored. The DEAE-cellulose column (2.5 X 40 cm) was equilibrated with 0.02 M glycine in buffer, pH 7.6; 200 to 260 mg of protein solution, mixed with an equal volume of the glycine-buffer mixture were introduced into the column. After two washings with 10 ml each of glycine solution, gradient elution was performed with 500 ml of the glycine buffer in the mixing chamber and a solution containing 0.02 M KH₂PO₄, 0.02 M KH₂PO₄ and 0.02 M glycine at pH 7.2 in a separatory funnel connected with the mixing chamber. Samples were collected in a Gilson fraction collector (cooled to 5°C) with an ultraviolet absorption attachment to give a record of the elution of protein from the column. The samples were stored in a refrigerator overnight and assayed the next day.

The red colored enzyme solution from the column fractionation was next treated with protamine sulfate to remove nucleic acid impurities. Aqueous protamine sulfate (10 mg per ml) was added until a permanent turbidity developed. The solution was centrifuged to remove the particulate material. This process was repeated until no further precipitate formed. Two treatments with protamine sulfate usually sufficed. This treatment decreased the ratios of absorbancy at 290:280 μm from 1.4 to 0.9. A summary of the degree of enrichment and the enzyme recovery at the different stages of the purification procedure is given in Table I.

Attempts at purification with low temperature ethanol fractionation, calcium phosphate gel, alumina gel C₇, and electrophoresis on a Porath column, with Geon type 427 resin as the solid phase, proved ineffective. The column electrophoresis runs yielded four protein peaks, but no significant increase in specific activity was observed in any of the peaks. The enzyme was distributed between all of the four protein peaks. A sedimentation test of the purified enzyme in the Spinco analytical centrifuge showed the migration of only a single boundary. However, this boundary spread and became highly diffuse as it moved. A salt anomaly may have been involved in the boundary spreading as well as the heterogeneity of the protein. The high salt concentration required to precipitate the enzyme and the characteristics of the sedimentation in the ultracentrifuge suggest that thymidylate synthetase has quite a low molecular weight.

Properties of Purified Enzyme

Effect of Enzyme Concentration—The increase in thymidylate synthesized was shown to be proportional to the enzyme concentration up to 10 mg of protein per incubation vessel (Fig. 1). The experimental points obtained all fall very well on a straight line. The amount of thymine-methyl synthesized in this test was 38.5 μmole per mg of enzyme protein per hour.

Effect of pH—The effect of pH on the reaction was measured over the pH range of 5.7 to 9.0, with 0.05 M Veronal and phosphate buffers. The plot of enzyme activity against pH is given in Fig. 2. The curve shows that the enzyme has a very sharp optimal peak at pH 7.6.

Effect of Substrate Concentration—The influence of increasing dUMP concentration (folate-H₄ maintained constant) and of increasing concentration of folate-H₄ (at constant dUMP) on the reaction velocities were determined. Kₘ values were estimated from extrapolations of the straight lines of double reciprocal plots to the intercepts on the -1/S axis. The figures obtained were 2.4 X 10⁻⁴ M for dUMP and 0.05 X 10⁻⁴ M for folate-H₄, respectively.

 Stoichiometry of Reaction—To obtain synthesis of thymidylate
FIG. 1. Test of proportionality between enzyme concentration and thymine-methyl formed. See text for experimental procedure.

FIG. 2. Effect of pH on activity of thymidylate synthetase. Phosphate buffer pH 5.7 to 6.8; Veronal buffer, pH 7.2 to 9.0.

requires only formaldehyde, dUMP, folate-H₄, and enzyme. Because the formaldehyde is reduced to a methyl group, a source of 2 atoms of hydrogen per molecular equivalent of methyl is required. It has been suggested by us (1, 8) and by Friedkin (9) that tetrahydrofolate serves as the hydrogen donor. It would be gratifying to establish this by demonstrating a proportionality between the thymine formed and the folate-H₄ oxidized. This is extremely difficult to do because of the degradation of the folate compounds during incubation. It is possible, however, to demonstrate that there is a one-to-one correspondence between the initial concentration of the folate-H₄ at low concentrations of the compound and the amount of thymidylate synthesized. The results of such experiments are summarized in Table II. It is to be seen that up to a concentration of 47 μmoles per liter of folate-H₄, in which it may be presumed that the effective isomer has been completely utilized for thymidylate synthesis, the molar ratio of folate-H₄ present to thymine synthesized is very close to the expected value of 1. This confirms a previously reported result with a crude enzyme preparation (1). At higher values of folate-H₄, the molar ratio of folate-H₄ to thymine is progressively increased, as would be expected, because the reaction then no longer results in the complete utilization of the folate-H₄.

McDougall and Blakley (10) determined the formation of folate-H₂ in the same reaction by the appearance of the absorption spectrum of the latter upon running the reaction to completion. A similar reductive function for folate-H₄ has been observed in the enzymatic hydroxylation of phenylalanine to tyrosine (11).

**Study of Mechanism of Reaction**

In our previous studies with crude enzyme preparations, it was observed that folate-H₄ could be used in catalytic amounts and that the reaction could be made to continue by supplying DPNH as the hydrogen donor (1, 12). TPN or TPNH was observed to inhibit the reaction. Similar results have been reported by McDougall and Blakley (13). Inhibition by the triphosphopyridine nucleotides was also found to occur with the purified enzyme. The inhibition was over 90% at a concentration of 1.2 mM.

Although DPNH did not inhibit the utilization of folate-H₄ in the presence of the purified enzyme, it was found not to reduce the folate-H₄, presumably formed, and thus allow the reaction to progress. Continuation of the reaction could not be promoted by the addition of purified sheep liver dihydrofolate reductase with either DPNH or TPNH.

This finding was puzzling inasmuch as dihydrofolate reductase readily catalyzes the reduction of chemically prepared folate-H₂ to folate-H₄ by TPNH.

Possible explanations for these results are: (a) the product of the oxidation of folate-H₄ in the thymidylate synthetase reaction is an isomeric form of dihydrofolate not attacked by dihydrofolate reductase, or (b) that a specific dihydrofolate reductase which utilizes DPNH at physiological pH values is present in thymus tissue that is required for the continuous operation of this reaction cycle. It was noted above that TPN strongly inhibited the synthesis of thymidylate.

The following experiments, designed to test the reason for noncycling of the reaction, were performed.

The reaction product of the oxidation of folate-H₄ did not react with either DPNH or TPNH upon addition of purified sheep liver dihydrofolate reductase (Table III). However, when some of the 50 to 60% saturation of (NH₄)₂SO₄ fraction of thymus homogenate was added with DPNH, the reduced

<table>
<thead>
<tr>
<th>Folate-H₄</th>
<th>Thymine formed</th>
<th>Ratio folate-H₄ to thymine</th>
</tr>
</thead>
<tbody>
<tr>
<td>24.9 μM</td>
<td>26.8</td>
<td>0.90</td>
</tr>
<tr>
<td>47.7 μM</td>
<td>48.3</td>
<td>1.00</td>
</tr>
<tr>
<td>70.5 μM</td>
<td>66.2</td>
<td>1.07</td>
</tr>
<tr>
<td>94.5 μM</td>
<td>84.0</td>
<td>1.12</td>
</tr>
<tr>
<td>118.5 μM</td>
<td>106.0</td>
<td>1.19</td>
</tr>
<tr>
<td>137.0 μM</td>
<td>115.0</td>
<td>1.19</td>
</tr>
<tr>
<td>39.0 μM</td>
<td>34.0</td>
<td>1.03</td>
</tr>
</tbody>
</table>

* Enzymatically active folate-H₄ isomer.
† Average of four determinations in another experiment.
were 0.25 and 0.46 mM, respectively. These figures represent
synthetase, & Verona1 buffer in a total volume of 1.8 ml in
umes of solution containing 1.6 mM of formaldehyde; 0.4-ml
ml of 0.005
DPNH (in Verona1 buffer), 1.6 m&r folate-Hz (suspension in 2
was a conversion to 1.55
hour at 37" under nitrogen of the following mixture: 3.4 mM
folate concentration in the incubation mixture was 1.6
The calculated absorbancy value at 340 rnrp for complete oxida-
7.6. The observed change in absorbancy obtained was 0.196.
After incubating for 30 minutes, the mixture was cooled in an
ice bath at 0" and 2-ml aliquots were used to test for "isomer-
activity.
This test was performed by adding to each 2 ml of the in-
cubate, 0.2 ml of purified sheep liver dihydrofolate reductase
(0.2 g per liter), 0.2 ml of the different (NH4)2SO4 fractions of
thymus homogenate, and 0.05 ml of 0.2 m TPNH made up to
a 3-ml volume with 0.05 veronal buffer, pH 7.6. Similar
mixtures were prepared from the control incubations without
dUMP. The results obtained with these incubations are re-
corded in Table IV.
It was pointed out above that the highest dihydrofolate re-
ductase activity was present in the 60 to 70% (NH4)2SO4 frac-
tion, although the 50 to 60% fraction was also approximately
60% as active. The above results appear to indicate the pres-
cence of an "isomerase" which is sharply confined to the 50 to
60% saturation of (NH4)2SO4 fraction.
A similar experiment was attempted with DPNH as the re-
ducing agent, but without addition of extraneous dihydrofolate
reductase, because this was contained in the thymus homogenate
fractions. In this test, as expected, there were no significant
differences in the oxidation rates of DPNH between the test
samples incubated with dUMP and those incubated without
dUMP.

Tests for Other Coenzymes—Work with other methylating
enzyme systems leads to the inference that a riboflavin compo-
pyridine nucleotide was oxidized; the amount oxidized was
increased with the quantity of crude extract added (Table III).
In another experiment, performed with increasing concentra-
tions of folate-H4 and addition of the 50 to 60% saturation of
(NH4)2SO4 fraction (Fig. 3), the presence of DPNH significantly
increased the yield of thymine-methyl above that with folate-H4
alone, up to comparatively high concentrations of folate-H4.
This result does not distinguish between whether the homoge-
nate contains an isomerase that converts the material (presuma-
ibly the 5, 8-folate-H4) in the incubation mixture to the enzymati-
cally reducible 7, 8-folate-H2 (14, 15) or a DPNH-mediated
folate-H2 reductase active in this enzyme system.
Utilization of Enzymatically-formed Folate-H2, by Thymidylate
Synthetase—To determine whether enzymatically reduced
folate-H4 was a substrate for thymus gland thymidylate syn-
thetase, folate-H2 was reduced with crude enzyme preparation
from thymus with DPNH as the hydrogen donor. This mate-
rial was then used in an incubation with thymidylate synthetase
and formaldehyde. The enzymatically formed folate-H2 was
utilized substantially for thymine synthesis in this experiment.
A large scale incubation (8-ml volume) was carried out for 1
hour at 37° under nitrogen of the following mixture: 3.4 mM
DPNH (in Veronal buffer), 1.6 mM folate-H2 (suspension in 2
ml of 0.005 M HCl), 1.9 g per liter of enzyme protein (60 to 70%
saturation of (NH4)2SO4 fraction), 0.05 M Veronal buffer, pH
7.6. The observed change in absorbancy obtained was 0.196.
The calculated absorbancy value at 340 mnr for complete oxida-
tion of DPNH was 0.201. The per cent reduction to folate-H4
alone, up to comparatively high concentrations of folate-H4.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Test incubation</th>
<th>( \text{Thymidine} ) formed</th>
<th>( \Delta \text{Pyridine nucleotide} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dihydrofolate reductase + DPNH</td>
<td>8</td>
<td>167</td>
<td></td>
</tr>
<tr>
<td>Dihydrofolate reductase + TPNH</td>
<td>5</td>
<td>190</td>
<td></td>
</tr>
<tr>
<td>Thymus extract, 3 mg, + DPNH</td>
<td>41</td>
<td>151</td>
<td></td>
</tr>
<tr>
<td>Thymus extract, 5 mg, + DPNH</td>
<td>71</td>
<td>157</td>
<td></td>
</tr>
<tr>
<td>Thymus extract, 10 mg, + DPNH</td>
<td>127</td>
<td>163</td>
<td></td>
</tr>
<tr>
<td>Thymus extract, 5 mg, + TPNH</td>
<td>0</td>
<td>153</td>
<td></td>
</tr>
</tbody>
</table>

* Thymus extract was fraction precipitated by 50 to 70% sat-
 ration of (NH4)2SO4.
† DPNH or TPNH concentration 0.2 mM.
\( \text{Dihydrofolate reductase} + \text{DPNH} \) or DPNH-mediated
folate-H2 reductase active in this enzyme system.

Effect of crude thymus extract* on reduction of dihydrofolate
Preincubation with dUMP, formaldehyde, and folate-H4, 2.8
mM each, (active isomer) and 2 mg of enzyme protein in 0.05 M
Veronal buffer, pH 7.6, in 1.8-ml volume.

Effect of DPNH on the synthesis of thymine methyl with increasing concentrations of folate-H4. Incubation mediums
were prepared containing 1.25 mM dUMP, varying and equivalent
concentrations of folate-H4, and formaldehyde (to form folate-
H2-formaldehyde complex), in 0.05 M Veronal buffer, pH 7.6.
Duplicate portions of 1.8 ml volume of each mixture, one with and
the other without DPNH, were incubated with 20 mg of purified
thymidylate synthetase and 2 mg of 50 to 70% ammonium sulfate
saturation fraction of thymus homogenate (source of thymus di-
hydrofolate reductase) for 1 hour under N2 at 37°. Assay for
thymine-methyl was performed in the usual manner: O, with
DPNH; O, without DPNH.

![Fig. 3. Effect of DPNH on the synthesis of thymine methyl with increasing concentrations of folate-H4.](http://www.jbc.org/)
that the several possible forms of dihydrofolate do not tauto-

tation of DPNH (50 to 60% saturation) is one found to be poor in dihydrofolate reductase; the latter comes down in the 60 to 70% saturation fraction. Further work leading to a more complete separation of the several enzyme activities and on the identification of the different isomers of dihydrofolate, if they exist, will be required before the true explanation of the resistance of enzymatically formed dihydrofolate can be determined.

Test for the possible requirements of a riboflavin and vitamin B12 coenzyme in the thymidylate synthetase reaction gave negative results. However, these cannot be accepted as conclusive until the degree of purity of the enzyme is better known and results with coenzyme dissociation experiments are performed.

SUMMARY

1. The enzyme, thymidylate synthetase, has been purified over 200-fold from thymus gland homogenates. The required components for reaction with the purified enzyme preparation are deoxyuridylic, formylmethylic, and tetrahydrofolic acid. There is strong evidence that tetrahydrofolic acid is the hydrogen donor for reduction of formylmethylic to thymine-methyl.

2. Attempts to utilize tetrahydrofolic catalytically by adding purified liver dihydrofolic reductase and a reduced pyridine nucleotide were unsuccessful. The addition of crude thymus homogenate induced the reduction of the enzyme reaction product of tetrahydrofolic by reduced dipiphosphopurine nucleotide in the presence of liver dihydrofolic reductase, but not by reduced triphosphopurine nucleotide.

3. Evidence has been obtained of the possible presence of an "isomerase" in thymus extracts that converts the tetrahydrofolic oxidation product to 7,8-dihydrofolate, the form that is presumed to be the substrate for dihydrofolic reductase.

REFERENCES


DISCUSSION

From its solubility and sedimentation characteristics, thymus thymidylate synthetase appears to be a comparatively low molecular weight protein. The results of column electrophoresis of the enzyme preparation suggests that the enzyme protein either consists of several protein species or is complexed with other proteins to yield at least four separate components of differing electrical mobility.

The ability of folate-H4 to serve as a hydrogen donor for the reduction of formaldehyde to methyl, seems indisputable, because no other obvious source of hydrogen is present in the required components of the incubation mixture. The conclusion is supported by the one-to-one proportionality between thymine-methyl formed and folate-H4 concentrations at low levels of the latter (Table II). A similar reductive function of folate-H4 has been discovered in the hydroxylation of phenylalanine to tyrosine (11).

An interesting observation is the inability of purified liver dihydrofolic reductase to catalyze the hydroxylation of the oxidation product, presumably folate-H2, formed from folate-H4 during thymine-methyl synthesis. McDougall and Blakley (10), however, observed no difficulty in catalyzing the reduction of folate-H2, which is generated in the same manner by reaction with thymidylate synthetase isolated from Streptococcus faecalis B, with a dihydrofolic reductase from the same organism in the presence of TPNH. Perault and Pullman (17) have calculated the electrical charge distribution of the several forms of folate-H2 and have concluded that the 5,8-folate-H2 would be strongly resistant to reduction, because of its unusually low ionization potential and its extremely pronounced electron donor capacities. Inasmuch as C-6 is an asymmetric carbon, 5,6-dihydrofolic acid is an improbable form of the compound, because 100% of the dihydrofolic acid in the dihydrofolic reductase system (14). From this and chemical evidence (15) it has been argued that the 7,8-dihydrofolic acid is the substrate for dihydrofolic reductase. The results reported in this article suggest that the several possible forms of dihydrofolic acid do not tautomerize readily, and that an "isomerase" may be required to catalyze the interconversion of the different isomers.

Suggestive evidence for this are the data reported in Table IV. The ammonium sulfate fraction which catalyzes the utilization


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