Mechanism of the Reaction Catalyzed by the Formate-activating Enzyme from Micrococcus aerogenes*

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The formate-activating enzyme (also called tetrahydrofolate formylase (1, 2)) catalyzes the reversible synthesis of N10-formyl-tetrahydrofolate from formate, adenosine triphosphate, and tetrahydrofolate according to Reaction 1:

\[ \text{Formate} + \text{ATP} + \text{tetrahydrofolate} \rightarrow N^{10}\text{-formyldihydrofolate} + \text{ADP} + P_i \] (1)

Early studies (3) with a formate-activating enzyme from pigeon liver led to the suggestion that a phosphorylated derivative of tetrahydrofolate is produced as an intermediate in the over-all reaction. Further evidence (4, 5) for the formation of such a derivative according to the two-step mechanism represented by

\[ \text{ATP} + \text{tetrahydrofolate} \rightarrow \text{phosphoryltetrahydrofolate} + \text{ADP} \] (2)

\[ \text{Phosphoryltetrahydrofolate} + \text{formate} \rightarrow N^{10}\text{-formyldihydrofolate} + P_i \] (3)

Equations 2 and 3, came from investigations with a highly purified enzyme from Micrococcus aerogenes (6), and from experiments with the pigeon liver enzyme (7-9). On the other hand, data (10, 11) obtained with a formate-activating enzyme from Clostridium cylindrosporum have led to an alternate interpretation, namely that Reaction 1 goes via a concerted mechanism without the formation of any detectable intermediates.

The present communication provides further information defining the over-all reaction (Equation 1) catalyzed by the M. aerogenes enzyme. A two-step mechanism is presented which modifies Equations 2 and 3 to include the role of the enzyme.

EXPERIMENTAL PROCEDURE

Materials

Chemicals were obtained from the following sources: ATP, ADP, TPN, DPNH, and glucose-6-P dehydrogenase from Sigma Chemical Company; hexokinase from the Pabst Laboratories; lactic dehydrogenase from Mann Laboratories; phosphoenolpyruvate from the California Corporation for Biochemical Research; and ATP-8-C\textsuperscript{14} from Schwarz Bioresearch, Inc. We wish to thank Drs. E. L. R. Stokstad and S. A. Kuby for providing generous samples of NS-formyltetrahydrofolate and crystalline creatine kinase, respectively.

The preparation of tetrahydrofolate and N10-formyltetrahydrofolate have been described elsewhere (12, 13). ATP labeled with P\textsuperscript{32} in the terminal phosphate group was obtained by exchange between P\textsubscript{i} and ATP using a partially purified enzyme from Micrococcus lactilyticus; or by the chemical condensation of AMP and P\textsuperscript{32} promoted by dicyclohexylcarbodiimide (14); we are indebted to Mr. J. G. O'0lins for carrying out the latter preparation.

Assay Systems

The procedures used in the purification of the formate-activating enzyme from M. aerogenes have been described previously (6). Unless otherwise noted, all experiments were performed with fractions obtained from DEAE cellulose columns having specific activity values of 200 to 500 (micromoles of N10-formyl-tetrahydrofolate synthesized per mg of protein per 10 minutes). The following assay systems were used:

System A. Assay of Reaction 1 in Forward Direction—Measurement of N10-formyltetrahydrofolate synthesis was carried out by the protocol described earlier (6) except that 100 \textmu moles of KCl or NH\textsubscript{4}Cl were included in the reaction mixture.

System B. Assay of Reaction 1 in Reverse Direction—This reaction was followed by measuring either the synthesis of ATP as described previously (6), or the disappearance of N10-formyltetrahydrofolate. The latter procedure utilized a reaction mixture containing 0.5 \textmu mole of N10-formyltetrahydrofolate, 2.5 \textmu moles of ADP, 40 \textmu moles of P\textsubscript{i}, 100 \textmu moles of NH\textsubscript{4}Cl or KCl, 40 \textmu moles of MgCl\textsubscript{2}, 40 \textmu moles of malate buffer (pH 7.5), 50 \textmu moles of NaF, and 20 \textmu moles of cysteine in a total volume of 1.2 ml. The tubes were flushed with nitrogen, stoppered, and incubated at 37° for 10 to 30 minutes, depending upon the conditions of the experiment. Beginning with a zero time sample, aliquots of 0.2 ml were removed at 10 minute intervals, diluted 1:10 with 3.5% perchloric acid, and centrifuged to remove denatured protein; the optical density was measured at 355 nm with a Beckman model DU spectrophotometer against a blank in which the N10-formyltetrahydrofolate had been omitted. Acidification converts the N10-formyl derivative to N8,N10-methenyltetrahydrofolate which has an absorption maximum at 335 nm (\epsilon = 262 \times 10\textsuperscript{2} cm\textsuperscript{-1} per mole (15, 16)).

System C. Assay for ADP—The formation of ADP from ATP in the presence of tetrahydrofolate and enzyme was measured spectrophotometrically by coupling pyruvic kinase with lactate dehydrogenase. The reaction mixture contained 0.1 \textmu mole of

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tetrahydrofolate, 0.5 pmole of ATP, 20 pmoles of maleate buffer (pH 7.5), 10 pmoles of MgCl₂, 25 pmoles of NH₄Cl or KCl, 50 pmoles of NaF, 20 pmoles of cysteine, 0.5 pmole of phospho-
enolpyruvate, 0.17 pmole of DPNH, 100 pg of formate-activating
enzyme, and 5 µg of crystalline lactic dehydrogenase (containing
pyruvic kinase) in a total volume of 1.5 ml. The reaction was
started by the addition of the formate-activating enzyme and,
at various time intervals, readings were made at 340 nm using
a Beckman model DU spectrophotometer; the blank cuvette
was identical except for the omission of DPNH. For measuring
ADP formation via Reaction 1, the same reaction mixture was
used with the inclusion of 2.5 pmoles of formate. Trial experi-
ments were necessary with each batch of lactic dehydrogenase
to establish the excess amount needed for the ADP assay.

System D. Spectroscopic Measurement of Intermediate from
Tetrahydrofolate—Changes in the absorption spectrum of tetra-
hydrofolate in the presence of enzyme and ATP were determined
using a Cary recording spectrophotometer, model 11M, or a
Beckman recording spectrophotometer, model DK-2, in a reac-
tion mixture containing 40 pmoles of maleate buffer (pH 7.5),
0.2 pmole of dl-L-tetrahydrofolate, 20 pmoles of cysteine or
mercaptoethanol, 0.5 to 2.5 pmoles of ATP, 5 pmoles of formate
(where indicated), and 100 µg of formate-activating enzyme
in a total volume of 3.0 ml. In some experiments, the amount of
ATP was decreased to 0.25 pmole and 2.5 pmoles of phospho-
enolpyruvate and 125 µg of lactic dehydrogenase (containing
pyruvic kinase) were added. The blank cuvette was identical
except for the omission of tetrahydrofolate. The initial spec-
trum was taken in the absence of either ATP or enzyme and the
reaction was started by the addition of the missing component.
It should be noted that the enzyme is inactivated by dilution in
the absence of ATP and lower rates are obtained when the addi-
tion of ATP is delayed for 15 to 30 minutes.

Other Methods—ADP and ATP were separated by column
chromatography on Dowex-1 resin with formic acid-ammonium
formate buffers or by paper chromatography using isobutyric
acid-NH₃-water (66:1:33) as the solvent system. The extinction
coefficient, ε, of the adenine nucleotides at 259 mp was
taken as 15.4 × 10² cm² per µmol. The radioactivity of ATP and
other labeled compounds was located by exposing the paper
chromatograms to “no screen” x-ray film (Eastman Kodak
Company) for 4 to 10 days and the extent of labeling was de-
termined by eluting the radioactive areas, plating an aliquot of
the eluate on planchets, and counting with a thin window gas
flow counter (Nuclear-Chicago Corporation) equipped with an
automatic sample changer.

The exchange of formate into N¹⁰-formyltetrahydrofolate was
measured by incubating 1 µmole of C¹⁴-labeled formate (0.1 to
0.5 µC) with the reaction mixture described above for the dis-
appearance of N¹⁰-formyltetrahydrofolate via the reversal of Reac-
tion 1. The tubes were flushed with nitrogen, stoppered, and
incubated at 37°. After incubation for 120 minutes, 0.4 ml
samples were removed, diluted 1:1 with 10% perchloric acid,
centrifuged, and aliquots of 0.2 ml of the supernatant fluids were
plated on planchets. Two-tenths milliliter of 10% HCl was
added to the planchets to assist the volatilization of formate,
and the material remaining after evaporation was counted.
Aliquots of the above supernatant fluids were chromatographed
with a modification of the solvent system described by Green-
berg and Jaenicke (3), n-butanol-20% trichloroacetic acid-n-pro-
panol-acetone-water (40:15:20:25:25). Radioautography of

<table>
<thead>
<tr>
<th>Cation</th>
<th>N¹⁰-Formyltetrahydrofolate</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.000</td>
</tr>
<tr>
<td>NH₄⁺</td>
<td>0.010</td>
</tr>
<tr>
<td>K⁺</td>
<td>0.142</td>
</tr>
<tr>
<td>Na⁺</td>
<td>0.175</td>
</tr>
<tr>
<td>Rb⁺</td>
<td>0.105</td>
</tr>
<tr>
<td>Li⁺</td>
<td>0.175</td>
</tr>
<tr>
<td>Cs⁺</td>
<td>0.010</td>
</tr>
</tbody>
</table>

the paper chromatograms revealed that all C¹⁴ was fixed into
N¹⁰-formyltetrahydrofolate (RF = 0.45 in the above system); when
this compound was eluted from the paper chromatograms
and counted, the radioactivity agreed to within 5% with the
direct counts of the nonvolatile C¹⁴ material obtained by evapo-
ration.

The exchange of P³² into ATP was measured under conditions
described previously (6) for the formation of N¹⁰-formyltetra-
hydrofolate (Reaction 1) with 0.001 µc of P³² added to the
reaction mixture. The method of Boyer, Luchsinger, and Falcone
(17) was used to determine the radioactivity of ATP. In addi-
tion, other aliquots of the reaction mixtures were subjected to
paper chromatography, using the isobutyrlic acid-ammonia-
water system, followed by radioautography; only ATP and P₁
were labeled.

RESULTS

I. Over-all Reaction

Synthesis of N¹⁰-formyltetrahydrofolate—Data have already
been presented (6) to illustrate the absolute specificity of the
enzyme for the three reactants (formate, ATP, and tetrahydro-
folate) participating in Reaction 1, and the additional require-
ments for a reducing agent and divalent cation such as Mg²⁺ or
Mn²⁺. More recently, it has been found that the over-all reac-
tion catalyzed by avian liver preparations also requires a mono-
valent cation. As seen from Table I, the M. aerogenes enzyme
has a similar requirement. This enzyme is stimulated markedly
by addition of NH₄⁺ or K⁺, whereas Na⁺, Rb⁺, and Li⁺ are less
effective and Cs⁺ is inhibitory.

At pH 7.5, the equilibrium of Reaction 1 lies to the right. A
ΔF value of -1 to -2 kilocalories per mole was estimated for this
reaction from thermodynamic data for related reactions
(3). More recently, a value of ΔF = -2.15 kilocalories per
mole, based upon direct measurement of the equilibrium, has
been reported by Rabonowitz and Himes (16). However, the
to extent to which the over-all reaction proceeds is affected
markedly by the presence of the reaction products and by the
amount of enzyme present. For example, the over-all reaction
may be inhibited 50% (cf. Fig. 1) by the addition of 0.12 µmole

* J. Bertino, unpublished results.
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FIG. 1. Inhibition of over-all reaction by products. Conditions are described in "Experimental Procedure" under assay System A. One unit on abscissa = 1 µmole of ADP, 0.05 µmole of N¹⁰-formyltetrahydrofolate, or 20 µmoles of P₁.

FIG. 2. Effect of enzyme concentration on the synthesis of N¹⁰-formyltetrahydrofolate. Conditions are described in "Experimental Procedure" under assay System A but with the incubation period reduced to 5 minutes.

of N¹⁰-formyltetrahydrofolate, 3.8 µmoles of ADP, or 26 µmoles of P₁ to the standard assay system. The inhibition of the over-all reaction by increasing amounts of enzyme is illustrated in Fig. 2; a comparison of the effects of enzyme concentration upon the over-all reaction and on the first postulated step in the over-all reaction will be presented subsequently.

The addition of ATP and Mg²⁺ protects the enzyme from inactivation by dilution and by heat. Enzyme preparations fortified with ATP and Mg²⁺ at concentrations of 1 × 10⁻⁴ M and 1 × 10⁻³ M, respectively, retain full activity after being heated at 50° for 12 minutes, whereas preparations without ATP are rapidly inactivated under these conditions. In contrast, the addition of tetrahydrofolate or formate did not protect the enzyme, as evidenced by a 60 to 80% loss in activity under these conditions.

Experiments with labeled ATP suggest that this compound is bound to the enzyme, although it is not known whether the interaction occurs at the active site of the enzyme or at some nonspecific site. Thus, when 8-C²⁺-ATP is incubated with the enzyme in the presence of Mg²⁺ and the resulting mixture is then chromatographed on DEAE-cellulose (cf. Fig. 3) most of the radioactivity is then associated with the enzyme (Tubes 22 to 32). When the column fractions containing both formate-activating enzyme and radioactivity are deproteinized with perchloric acid and the acid-soluble material examined by paper chromatography, only labeled ATP is found. The remaining radioactivity (Tubes 34 to 42) represents a mixture of free ADP and free ATP; 95% of the total radioactivity was recovered in this experiment. In a control experiment, enzyme, ATP, and ADP were added separately to a column in the absence of Mg²⁺ and chromatographed as before. All three entities emerged at their expected positions in the profile and there was no labeling of the enzyme. With the assumptions and calculations for molecular weight and purity described in footnote 6, it may be estimated from the data in Fig. 3 that at least one, and possibly several, moles of ATP are bound to each mole of enzyme.

Disappearance of N¹⁰-Formyltetrahydrofolate—In earlier experiments (6), reversal of Reaction 1 was facilitated and also measured by "trapping" the product, ATP, via the hexokinase-glucose-6-P dehydrogenase system (Reactions 4 and 5):

\[
\text{ATP + glucose} \xrightarrow{\text{hexokinase}} \text{glucose-6-P + ADP} \tag{4}
\]

\[
\text{glucose-6-P + TPN}^+ \xrightarrow{\text{dehydrogenase}} \text{6-phosphogluconate + TPNH + H}^+ \tag{5}
\]

Reversal of Reaction 1 may be measured also by determining the disappearance of N¹⁰-formyltetrahydrofolate which can be shown to be equivalent to ATP synthesis. For example, with a purified enzyme preparation, the disappearance of 0.1 µmole of N¹⁰-formyltetrahydrofolate was paralleled by the appearance of ATP in Reaction 4. However, if Reaction 5 is not included (i.e., the glucose-6-P dehydrogenase system is omitted), the disappearance of N¹⁰-formyltetrahydrofolate may be paralleled by the appearance of radioactivity in the form of glucose.

FIG. 3. Chromatography of ATP-labeled enzyme. 8-C²⁺-ATP (0.34 µmole, 1.11 µc) was incubated with 64.5 mg of enzyme fraction (specific activity = 250) in assay System A from which formate and tetrahydrofolate were omitted. After 10 minutes of incubation, the reaction mixture was chromatographed on DEAE-cellulose as described previously (6) except that the pH of the eluting buffer was changed to 6.0.

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0.1 pmole of ATP. Even when components of Reaction 4 and 5 are added, Reaction 1 does not go to completion in the reverse direction owing to product inhibition as discussed below.

Formate-activating enzyme, ADP and $P_i$, a divalent cation, a monovalent cation, and a reducing agent are all required for the disappearance of $N^{10}$-formyltetrahydrofolate via reversal of Reaction 1. The following values were obtained for the Michaelis constants for the substrates of the reverse reaction: $8.1 \times 10^{-4}$ M for $N^{10}$-formyltetrahydrofolate; $3.3 \times 10^{-3}$ m for ADP; and $4.4 \times 10^{-3}$ m for $P_i$. $K_m$ values for the reactants in the forward direction have been given previously (6). The effectiveness of various monovalent and divalent cations and reducing agents in the reverse reaction parallels that found for the forward reaction. ADP cannot be replaced by any other nucleoside diphosphate but inorganic phosphate may be replaced by arsenate. However, arsenate is only 75% as effective as phosphate and thus its addition in the presence of phosphate does not lead to an enhanced rate of $N^{10}$-formyltetrahydrofolate breakdown (5). This suggests that the presumed arsenyl derivative is as stable as the analogous phosphoryl intermediate.

![Fig. 4. Effect of ADP concentration on the reversal of Reaction 1 in the presence of phosphate or arsenate. Assay System B was used with 40 pmol of $P_i$ or arsenate and varying amounts of ADP as indicated.](http://www.jbc.org/)

**Table II**

Amounts of enzyme required for forward and reverse reactions

Assay Systems A and B for measuring Reaction 1 in the forward and reverse directions are described in “Experimental Procedure.” The incubation time was 10 minutes for both assays.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Per cent of total reaction*</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg</td>
<td>Forward</td>
</tr>
<tr>
<td>1.85</td>
<td>94</td>
</tr>
<tr>
<td>$9.25 \times 10^{-1}$</td>
<td>69</td>
</tr>
<tr>
<td>1.85 $\times 10^{-1}$</td>
<td>100</td>
</tr>
<tr>
<td>1.85 $\times 10^{-2}$</td>
<td>100</td>
</tr>
<tr>
<td>3.70 $\times 10^{-1}$</td>
<td>68</td>
</tr>
<tr>
<td>1.85 $\times 10^{-4}$</td>
<td>34</td>
</tr>
<tr>
<td>9.25 $\times 10^{-4}$</td>
<td>16</td>
</tr>
</tbody>
</table>

* Based on 100% of forward reaction = 0.400 pmole of $N^{10}$-formyltetrahydrofolate formed; 100% of reverse reaction = 0.170 pmole of $N^{10}$-formyltetrahydrofolate disappearing.

As seen from Fig. 4, the disappearance of $N^{10}$-formyltetrahydrofolate in either phosphate or arsenate is negligible in the absence of ADP.

As shown in Table II, much larger amounts of enzyme are required to catalyze Reaction 1 in the reverse direction than in the forward direction. This disparity is not affected appreciably by the addition of enzyme systems, such as hexokinase plus glucose, or creatine kinase plus creatine, which are capable of “pulling” the reaction by removing ATP.

Even with large amounts of enzyme, the reverse reaction does not go to completion. In addition to the fact that the equilibrium of the reaction is unfavorable in this direction, the products of the reverse reaction are inhibitory (Fig. 5). For example, a 50% inhibition is observed when 0.04 pmole of tetrahydrofolate, 0.40 pmole of formate, or 0.02 pmole of ATP is added. Since the amount of tetrahydrofolate and ATP formed in the reverse reaction under the usual assay conditions can easily reach an inhibitory level, the failure to observe complete disappearance of $N^{10}$-formyltetrahydrofolate may be attributed to this effect.

It is of interest to note that $N^{10}$-formyltetrahydrofolate, like ATP, protects the enzyme from thermal inactivation. In the presence of $N^{10}$-formyltetrahydrofolate at a concentration of 0.5 to $1.0 \times 10^{-3}$ M, the enzyme retained 75 to 80% of its activity after being heated at 52° for 12 minutes. Under the same conditions, the control without $N^{10}$-formyltetrahydrofolate had only 20 to 30% of the original activity. On the other hand, if the concentration of $N^{10}$-formyltetrahydrofolate was increased 10-fold, only 5% of the enzyme activity remained after 6 minutes of heating. As a corollary to the above protective effect, the enzyme likewise stabilized $N^{10}$-formyltetrahydrofolate against air oxidation, e.g. 5% and 50% of the $N^{10}$-formyltetrahydrofolate was destroyed in air after 1 hour at room temperature in the presence and absence of enzyme, respectively.

This phenomenon is also encountered when attempting to assay Reaction 1 in reverse with crude, or even partially purified, preparations from invertebrate tissues or from avian and mammalian tissues. Even though the activity in the forward direction is appreciable, almost no reverse activity is seen; when more purified enzyme preparations are used, however, the reverse reaction may be demonstrated.
11. Examination of First Step in Postulated Mechanism

It has been suggested previously (6) that the formate-activating enzyme catalyzes the over-all reaction (Equation 1) via a sequential mechanism involving as the first step an interaction between ATP and tetrahydrofolate, according to Equation 2. Evidence in support of this formulation is obtained principally from two types of experiments in which ATP and tetrahydrofolate are mixed in the presence of the enzyme: (a) formation of a new substance having an absorption spectrum different from tetrahydrofolate or ATP and (b) formation of free ADP. Spectroscopic evidence for the synthesis of N10-formyltetrahydrofolate ($\lambda_{\text{max}}$ at 290 mp, shoulder at 300 mp) from tetrahydrofolate ($\lambda_{\text{max}}$ at 298 mp) has been documented previously in Fig. 2 of reference (6). On the other hand, when the enzyme is incubated with tetrahydrofolate and ATP in the absence of formate, the absorbancy at 298 mp (due almost entirely to tetrahydrofolate) decreases slowly and progressively and there is a slight shift in the absorption maximum to shorter wave lengths, i.e. 292-295 mp (Fig. 6). If formate is added at this time, the spectrum of the intermediate disappears rapidly and is replaced by that of N10-formyltetrahydrofolate. These changes in the absorption spectrum suggest that tetrahydrofolate is being transformed into a new compound before its conversion to N10-formyltetrahydrofolate; they are not observed when either enzyme or ATP is omitted. Like the over-all reaction, the reaction between tetrahydrofolate and ATP also requires a divalent cation, a nonenovalent cation, and a reducing agent. In addition, the amount of enzyme required for the demonstration of this reaction exceeds by approximately 100-fold the amount needed to catalyze the over-all reaction.

Experiments were carried out to ascertain whether the same amount of product, N10-formyltetrahydrofolate, was synthesized under the following conditions: (a) tetrahydrofolate and ATP were added to enzyme and, after the intermediate had accumulated, formate was added to complete the reaction; and (b) all three reactants were added at the same time to the enzyme. As seen in Table III, essentially the same amount of product is formed under both conditions in each of four separate experiments where the initial amount of tetrahydrofolate is varied. The conversion of tetrahydrofolate to the N10-formyl derivative is greater with higher concentrations of tetrahydrofolate but does not proceed to completion under the conditions used for these spectroscopic experiments because suboptimal amounts of tetrahydrofolate and ATP were present. The results in Table III indicate that tetrahydrofolate has not undergone any chemical or enzymatic destruction during the preliminary incubation period in which the intermediate is formed, and that it is still fully available for conversion to the end product, N10-formyltetrahydrofolate.

Assuming that the above change in absorption spectrum reflects the synthesis of a new compound which is an intermediate in the over-all reaction, an estimate may be made of the amount formed. Under the conditions of the experiment shown in Fig. 6, approximately 0.06 $\mu$ mole of the intermediate is formed, assuming complete conversion of one-half of the d1,L-tetrahydrofolate; whereas in a separate experiment under the same conditions, about 0.07 $\mu$ mole of ADP is formed. Thus, production of ADP and the “intermediate” from ATP and tetrahydrofolate are of the same order of magnitude, and about $10^2$ times greater than the amount of enzyme present.

**ADP Formation from ATP and Tetrahydrofolate**—The stoichiometry of the peak changes from 0.972 to 0.769 when the reaction is virtually completed. Addition of further ATP or enzyme does not cause further changes in absorbancy indicating that all of the tetrahydrofolate has reacted and that the enzyme has not become inactivated. With an extinction coefficient of $26 \times 10^3$ cm$^2$ per mole for tetrahydrofolate and correction for the fact that one-half of the d1,L-tetrahydrofolate has not reacted, the extinction coefficient of the product would be approximately equal to $16 \times 10^3$ cm$^2$ per mole. Although this calculation, and the one in footnote 4, are only approximate; it is believed that the accuracy is sufficient for the comparisons of order of magnitude made in the text.

![Fig. 6](http://example.com/fig6.png)

**Fig. 6. Changes in absorption spectrum of tetrahydrofolate in the presence of ATP and enzyme.** Conditions are described in “Experimental Procedure” under assay system D. A = control without enzyme; B = control without ATP; C = complete system. Numbers to the right of absorption curves indicate duration of incubation in minutes.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>N10-Formyltetrahydrofolate synthesized when formate was added at</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 minutes</td>
</tr>
<tr>
<td>1</td>
<td>0.069</td>
</tr>
<tr>
<td>2</td>
<td>0.062</td>
</tr>
<tr>
<td>3</td>
<td>0.087</td>
</tr>
<tr>
<td>4</td>
<td>0.111</td>
</tr>
</tbody>
</table>

*In Fig. 6C the absorbancy of the peak changes from 0.972 to 0.769 when the reaction is virtually completed. Addition of further ATP or enzyme does not cause further changes in absorbancy indicating that all of the tetrahydrofolate has reacted and that the enzyme has not become inactivated. With an extinction coefficient of $26 \times 10^3$ cm$^2$ per mole for tetrahydrofolate and correction for the fact that one-half of the d1,L-tetrahydrofolate has not reacted, the extinction coefficient of the product would be approximately equal to $16 \times 10^3$ cm$^2$ per mole. Although this calculation, and the one in footnote 4, are only approximate; it is believed that the accuracy is sufficient for the comparisons of order of magnitude made in the text.

*In the experiment described in Fig. 6, 2.2 $\times 10^{-2}$ mg of enzyme with a specific activity of 680 were used. Since the best preparations of the *Micrococcus aerogenes* enzyme have values of specific activity of about 1,000, the fraction used in this experiment could be only 50% pure, at best. If the molecular weight of the *M. aerogenes* enzyme is comparable to that of the crystalline formate-activating enzyme from *Clostridium cylindrosporum*, i.e. 210,000 (10), it may be calculated that approximately $5.4 \times 10^{-2}$ $\mu$ moles of enzyme were used.
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The enzymatic synthesis of ADP can be observed also when the _M. aerogenes_ enzyme is incubated with ATP and tetrahydrofolate in the absence of formate. ADP formation via Reaction 2 has been verified by chromatography of the reaction products on Dowex 1-formate (19). ADP can be assayed more readily and continuously by coupling the primary reaction with pyruvic kinase and lactic dehydrogenase (Reactions 6 and 7) and measuring DPNH formation spectrophotometrically at 340 nm. When assayed by the above spectrophotometric system, the requirements for the synthesis of ADP in the presence of the enzyme, tetrahydrofolate, and ATP differ from the requirements for the over-all Reaction 1 only with regard to the absence of formate and the amount of enzyme used. As shown in Fig. 7, larger amounts of enzyme are required for the formation of ADP in the absence of formate than in its presence and, in fact, at high dilutions of the enzyme, ADP formation can be demonstrated only in the presence of all three components. With larger amounts of enzyme, however, ADP formation from ATP and tetrahydrofolate exceeds that observed with the complete system. This latter effect may be referable to the inhibition of the complete system by excess enzyme (cf. Fig. 2).

At all concentrations of enzyme and at all stages of enzyme purification, measurements of the tetrahydrofolate-dependent release of ADP from ATP must be corrected for the formation of ADP in the absence of ATP alone (cf. Curve C of Fig. 7). The magnitude of this ATPase activity may be decreased by substituting in the assay system Fe**+** for Mg**+** or by substituting ascorbate for the thiol-reducing agents, cysteine or mercaptoethanol. In each instance, however, ADP formation via Reactions 1 and 2 is decreased proportionately. When the coupled assay system for ADP (Reactions 6 and 7) is not used, and the amount of ADP is estimated by ion exchange chromatography (20), the release of ADP from ATP is reduced, suggesting that the ATPase action of the enzyme is enhanced by the removal of ADP.

The tetrahydrofolate-dependent formation of ADP is inhibited completely by _p_-chloromercuribenzoate at 1.3 X 10^-4 M. If a series of enzyme dilutions are incubated with varying amounts of _p_-chloromercuribenzoate, it is seen that ADP formation is inhibited equally in Reactions 1 and 2 and that the ATPase activity is inhibited equally in these reactions. These results indicate that —SH groups are required for both Reactions 2 and 3 and not, as reported earlier (5, 7), only for the latter reaction.

Exchange Experiments—The enzyme-catalyzed incorporation of _C^14_—formate into _N^10_—formyltetrahydrofolate in the presence of _P_i and ADP has been investigated (Table IV). The conditions required for maximal exchange are the same as those noted previously for the disappearance of _N^10_—formyltetrahydrofolate via the reversal of Reaction 1, i.e. both ATP and _P_i must be present. The small amount of exchange detected in the absence of ADP corresponds approximately to the small amount of _N^10_—formyltetrahydrofolate disappearing in the absence of ADP (cf. Fig. 4). This effect may be referable to traces of bound components.

Confirming the experiments of Rabinowitz and Himes (10, 11), maximal exchange of _P_i into ATP was also observed when all components of the reaction mixture were present (Table V). There was a lesser, but still measurable, exchange upon adding only _P_i and ATP to the enzyme. This effect may be related to the inherent ATPase activity of the enzyme or, as stated above, to the presence of traces of bound components.

Earlier experiments on the site of inhibition of _p_-chloromercuribenzoate (5), which were performed only with large amounts of enzyme, indicated that considerably more ADP was synthesized in Reaction 2 as compared to the over-all Reaction 1 when the inhibitor was present at 2 X 10^-4 M.

**Figure 7.** Formation of ADP by varying amounts of enzyme in Reactions 1 and 2. Conditions are described in "Experimental Procedure" under assay System C with an incubation time of 10 minutes. _ΔA_340 = change in absorbancy at 340 nm. Curve _A_ obtained in the presence of enzyme, ATP and tetrahydrofolate; Curve _B_ with enzyme, ATP, tetrahydrofolate, and formate; and Curve _C_ with enzyme and ATP. _Curves_ _A_ and _B_ have been corrected for ADP produced from ATP (Curve _C_).

---

**Table IV**

<table>
<thead>
<tr>
<th>Amount of component</th>
<th>ADP</th>
<th>Radioactivity of <em>N^10</em>—formyltetrahydrofolate</th>
</tr>
</thead>
<tbody>
<tr>
<td>µmoles</td>
<td>µmoles</td>
<td>c. p.m.</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>50</td>
</tr>
<tr>
<td>20</td>
<td>0</td>
<td>520</td>
</tr>
<tr>
<td>20</td>
<td>0.025</td>
<td>860</td>
</tr>
<tr>
<td>20</td>
<td>0.25</td>
<td>2,400</td>
</tr>
<tr>
<td>20</td>
<td>2.5</td>
<td>2,410</td>
</tr>
</tbody>
</table>

**Table V**

<table>
<thead>
<tr>
<th>Component omitted</th>
<th>Radioactivity of ATP</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1,500</td>
</tr>
<tr>
<td>Tetrahydrofolate + formate</td>
<td>540</td>
</tr>
<tr>
<td>Formate</td>
<td>493</td>
</tr>
<tr>
<td>Enzyme</td>
<td>60</td>
</tr>
</tbody>
</table>

---

7 Earlier experiments on the site of inhibition of _p_-chloromercuribenzoate (5), which were performed only with large amounts of enzyme, indicated that considerably more ADP was synthesized in Reaction 2 as compared to the over-all Reaction 1 when the inhibitor was present at 2 X 10^-4 M.
**DISCUSSION**

Any mechanism proposed for the reaction catalyzed by the formate-activating enzyme from *M. aerogenes* must take into account the following experimental observations: (a) Reaction 1 is specific for all six components except P, which can be replaced to a large extent by arsenate; (b) only ATP and N¹⁰-formyltetrahydrofolate protect the enzyme against inactivation; (c) Reaction 1 in the forward direction is inhibited by high concentrations of enzyme, whereas the reverse reaction is favored under these conditions; (d) each of the end products of Reaction 1 in the forward direction inhibits the reaction to a different extent, the order being N¹⁰-formyltetrahydrofolate > ADP > Pi. As substrates for the reaction in reverse, these compounds may be arranged in the same order in terms of affinity for the enzyme; (e) Reaction 1 in the reverse direction is inhibited by each of the products in this order: ATP > tetrahydrofolate > formate. As substrates in the forward direction, these compounds may also be arranged in the same order in terms of affinity for the enzyme; (f) maximal rates of exchange of Pi into ATP, ADP and tetrahydrofolate protect the enzyme against inactivation; this has been observed in the present investigation; (g) the enzyme has an inherent ATPase activity; and, (h) the enzyme catalyzes a reaction between ATP and tetrahydrofolate which yields approximately equivalent amounts of ADP and an intermediate detected spectrophotometrically and assumed to be N¹⁰-phosphoryltetrahydrofolic acid. Mg²⁺, NH₄⁺, or K⁺, a reducing agent, and relatively large amounts of enzyme are required to demonstrate this reaction. Formation of the intermediate from tetrahydrofolate does not depress the amount of N¹⁰-formyltetrahydrofolate produced when formate is eventually added, thus supporting the contention that the enzymatic interaction between ATP and tetrahydrofolate is an obligatory part of the over-all reaction and not a side reaction.

Two different types of mechanisms have been proposed for the formate-activating enzyme: (a) a two-step mechanism (5) as outlined in Equations 2 and 3, and (b) a concerted mechanism (10, 20) with no recognizable intermediates. It should be noted, however, that even an apparently concerted mechanism might consist of two reactions which could not be detected because the rate of disappearance of an intermediate might exceed its rate of formation. In attempting to apply a concerted type of mechanism to the formate-activating enzyme, two sequences of events may be suggested. The first proposal involves the formation of formyl phosphate in the transition state (reference (21), Breslow's "Discussion"). The formation of formyl phosphate via the formokinase reaction has been demonstrated in *Escherichia coli* (22), but this compound would appear to be eliminated as an intermediate in the *M. aerogenes* system because of the failure of hydroxylamine at high concentrations to inhibit the over-all reaction. A second type of concerted mechanism may be envisioned involving a series of nucleophilic displacements in which ATP acts as an acceptor of OH⁻ (cf. Fig. 8).

Support for a concerted type of mechanism for the formate-activating enzyme has been derived largely from exchange experiments and from the inability to observe the accumulation of any intermediates. On the other hand, the present investigation has demonstrated that ADP and a derivative of tetrahydrofolate can be made to accumulate as a consequence of a reaction between ATP and tetrahydrofolate. These results are consistent with the mechanism outlined in Equations 8 to 11 which extends the simple two-step formulation of Equations 2 and 3 to indicate more fully the role of the enzyme.

This mechanism assumes that there is an obligatory order of the four reversible reactions: ATP must react first with the enzyme (Equation 8), tetrahydrofolate must then react with the enzyme-ATP complex (Equation 9), and formate can react only with enzyme-bound phosphoryltetrahydrofolate (Equation 10). The final step involves the dissociation of enzyme-bound N¹⁰-formyltetrahydrofolate (Equation 11). Thus, prior combination of ATP with the enzyme would be required before an adjacent site on the enzyme would be capable of binding tetrahydrofolate; in turn, the formation of phosphoryltetrahydrofolate on the enzyme would be required before the third site could bind formate. The reverse reaction is likewise governed by the same restrictions in terms of a compulsory order of events.

Reversible binding of ATP by the enzyme has also been postulated by Kabnówitz and Himes (10) as a first step in their mechanism for the formate-activating enzyme. According to the present formulation, only ATP and N¹⁰-formyltetrahydrofolate of the six components in Equation 1 could be bound without prior binding of other components or intermediates, and, hence, these two compounds would be expected to protect the enzyme against inactivation; this has been observed in the present investigation.

The effect of concentration upon the forward and reverse reactions may also be examined in light of the proposed mechanism. Large amounts of enzyme, although favoring the equilibrium in the direction of enzyme-ATP complex formation, hinder the over-all forward reaction at the final step by preventing the dissociation of the complex between N¹⁰-formyltetrahydrofolate and the enzyme. Large amounts of enzyme, therefore, would favor the reaction in the reverse direction for the same reasons. This argument assumes that inherent dissociation of enzyme-N¹⁰-formyltetrahydrofolate is greater than that of enzyme-ATP.

In addition to Reactions 8 to 11, the enzyme may catalyze Reactions 12 and 13. Evidence for the formation of ADP via Reaction 12 has been obtained in the present investigation (cf. 8).

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8 For Reaction 1, and for the analogous succinic thiokinase reaction (23), it has been shown that O¹⁸ is transferred from the acyl group to P, during the course of the reaction (10). The mechanism presented in Equations 8 to 11, and discussed earlier (5), is consistent with this finding.

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**Fig. 8. Formation of N¹⁰-formyltetrahydrofolate by a concerted mechanism.** H-N: represents the N¹⁰ atom of tetrahydrofolate.
Fig. 7) and in exchange experiments (10). The ATPase activity

$$\text{Enzyme-ATP} \rightleftharpoons \text{enzyme-P} + \text{ADP} \quad (12)$$

$$\text{Enzyme-phosphoryl-tetrahydrofolate} \rightleftharpoons \text{enzyme + phosphoryl-tetrahydrofolate} \quad (13)$$

of the formate-activating enzyme may be attributed to hydrolysis of the enzyme-P complex formed in Equation 12.

Accumulation of the proposed intermediate, phosphoryl-tetrahydrofolate, occurs when formate is absent. Evidence for Reaction 13 is provided by the fact that the amount of intermediate produced under these conditions greatly exceeds the amount of enzyme. From the extremely rapid rate of conversion of the free intermediate to N^10-formyltetrahydrofolate upon the addition of formate, it may be concluded that Reaction 13 is reversible and that Reactions 10, 11, and 13 are among the most rapid in the entire sequence. Conversely, the inability to produce the phosphorylated intermediate by the phosphorylation of N^10-formyltetrahydrofolate may be attributable to a low affinity of the enzyme-N^10-formyltetrahydrofolate complex for Pi or to an unfavorable equilibrium for Reaction 10 in reverse.

Although the present mechanism is more satisfactory than the previous proposal (Equations 2 and 3) in accounting for the role of the enzyme as detailed in items (b), (c), (g), and (h) listed above, it is still not entirely adequate for explaining all the exchange data reported in this paper and elsewhere (10).

For example, the exchange of formate into N^10-formyltetrahydrofolate and of ADP into ATP should proceed in the absence of ADP and formate, respectively. In both instances some exchange is actually observed under these conditions (cf. Table IV and Fig. 8 of reference (10)), but maximal exchange is obtained only when all components of the over-all reaction are present. The formate-N^10-formyltetrahydrofolate exchange proceeds via Reactions 11 and 10 and the reactions used previously in discussing the phosphoryl-tetrahydrofolate may be applied, i.e., the coupled reactions may not occur maximally unless the unfavorable equilibrium of Reaction 10 in reverse is shifted by removal of enzyme-phosphoryl-tetrahydrofolate via Reactions 8 and 9. Similar arguments might be invoked for the ADP-ATP exchange. On the other hand, the exchange of Pi into ATP should require all components according to the mechanism shown in Equations 8 to 11; this has been observed by Rabinowitz and Himes (10) and in the present investigation.

**SUMMARY**

1. The synthesis of N^10-formyltetrahydrofolate from formate, adenosine triphosphate (ATP), and tetrahydrofolate by a highly purified formate-activating enzyme from Micrococcus aerogenes requires a divalent cation (Mg^{2+} or Mn^{2+}), a monovalent cation (K^+ or Na^+), and a reducing agent. The reaction is inhibited by large amounts of enzyme and by the reaction products.

2. The enzyme is protected by ATP and Mg^{2+} or by N^10-formyltetrahydrofolate from inactivation by heat or by dilution; experiments with labeled ATP suggest that it is bound to the enzyme.

3. Reversal of the over-all reaction is specific for N^10-formyltetrahydrofolate and adenosine diphosphate (ADP), but orthophosphate may be replaced to the extent of 75% by arsenate. $K_a$ values for the three components of the reaction in the reverse direction are: 8.1 x 10^{-4} M for N^10-formyltetrahydrofolate; 3.3 x 10^{-3} M for ADP, and 4.4 x 10^{-2} M for orthophosphate.

4. The disappearance of N^10-formyltetrahydrofolate via reversal of the over-all reaction requires about 100 times more enzyme than the synthesis and is inhibited by the corresponding reaction products: tetrahydrofolate, formate, and ADP.

5. Spectroscopic evidence has been obtained for the formation of an intermediate when tetrahydrofolate and ATP, in the absence of formate, are incubated with large quantities of enzyme; under these conditions, an approximately equivalent amount of ADP is produced. Upon the addition of formate, N^10-formyltetrahydrofolate is produced.

6. At all stages of purification the enzyme possesses an inherent adenosine triphosphatase activity.

7. Exchange of orthophosphate-P of ATP and HC=O-OH into N^10-formyltetrahydrofolate requires the presence of all components of the over-all reaction.

8. Based on the above observations, a four-step mechanism is presented involving enzyme-bound intermediates.

**REFERENCES**

Mechanism of the Reaction Catalyzed by the Formate-activating Enzyme from 
*Micrococcus aerogenes*

H. R. Whiteley and F. M. Huennekens


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