Purification and Properties of the Formate-activating Enzyme from Erythrocytes*

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The demonstration by Lowy and Williams (2) that rabbit erythrocytes can incorporate C\textsuperscript{4}-formate into the C-2 position of purines suggests by analogy with the well studied avian liver system (3) that N\textsuperscript{15},N\textsuperscript{10}-formyltetrahydrofolate is the actual "l-carbon" donor. The synthesis of this compound is carried out by the formate activating enzyme (also called tetrahydrofolate formylase (4, 5)) as shown in Equation 1.

\[
\text{Formate + ATP + tetrahydrofolate} \rightarrow \text{N}^{10}\text{-formyltetrahydrofolate + ADP + P}_i \quad (1)
\]

This enzyme has been purified previously from avian liver (6, 7) and Micrococcus aerogenes (8) and crystallized from Clostridium cylindrosporum (9, 10).

This study was undertaken to identify and characterize the formate-activating enzyme in human erythrocytes. During this investigation, two other tetrahydrofolate-dependent enzyme activities were also detected in the erythrocyte: N\textsuperscript{5},N\textsuperscript{10}-methylene-tetrahydrofolate dehydrogenase and serine hydroxymethylase. Formate-activating enzyme activity has also been measured in erythrocytes of avian and various animal species.

EXPERIMENTAL PROCEDURE

Materials—Blood was collected in heparin, and hemolysates were prepared in the following way. The blood was centrifuged at 900 \( \times \) g for 5 minutes, and the plasma anduffy coat layer were discarded. The erythrocytes were washed twice with 0.15 M KCl and lysed with 4 volumes of 0.005 M potassium phosphate buffer, pH 8.0. The hemolysate was centrifuged at 2000 \( \times \) g for 10 minutes, and the supernatant fraction was used for assays. All steps were carried out at 4°C.

L-(-)-Tetrahydrofolate acid was obtained from Nutritional Biochemicals Corporation. ADP, ATP, DPN, and TPN were obtained from Sigma Chemical Company.

Methods—The formate-activating enzyme was assayed by measuring the N\textsuperscript{10}-formyltetrahydrofolate formed in the reaction shown in Equation 1. The assay mixture contained 50 \( \mu \)moles of sodium formate, 5 \( \mu \)moles of potassium-ATP, 1 \( \mu \)mole of L-(-)-tetrahydrofolate, 50 \( \mu \)moles of Tris chloride buffer, pH 7.5, 10 \( \mu \)moles of mercaptoethanol, 20 \( \mu \)moles of MgCl\textsubscript{2}, and the enzyme in a total volume of 1.0 ml. After incubation at 37°C for 10 minutes, 0.5 ml of 10% trichloroacetic acid was added and the denatured protein was removed by centrifugation. Acidification converted the product, N\textsuperscript{10}-formyltetrahydrofolate, to N\textsuperscript{5},N\textsuperscript{10}-methylene-tetrahydrofolate (11, 12), which was estimated by light absorption at 355 nm (\( \epsilon = 22 \times 10^6 \) cm\(^2\) per mole). The blank contained all components except formate.

N\textsuperscript{5},N\textsuperscript{10}-Methylene-tetrahydrofolate dehydrogenase was assayed by the TPN-linked oxidation of N\textsuperscript{5},N\textsuperscript{10}-methylene-tetrahydrofolate to N\textsuperscript{5},N\textsuperscript{10}-methylenetetrahydrofolate (cf. Equation 2).

\[
N^5,N^{10}\text{-Methylenetetrahydrofolate} + \text{TPN}^+ \rightarrow (N^5,N^{10}\text{-methylene-tetrahydrofolate})^+ + \text{TPNH} \quad (2)
\]

Mixtures containing 1.0 \( \mu \)mole of N\textsuperscript{5}-tetrahydrofolate, 5.0 \( \mu \)moles of HCHO, 100 \( \mu \)moles of phosphate buffer, pH 7.5, and 10 \( \mu \)moles of mercaptoethanol in a volume of 0.5 ml were allowed to stand at room temperature for 5 minutes to permit the chemical synthesis of N\textsuperscript{5},N\textsuperscript{10}-methylene-tetrahydrofolate (13, 14). TPN (0.6 \( \mu \)mole) and the enzyme were added to a final volume of 1.0 ml, and the reaction mixture was incubated at 37°C for 30 minutes. Trichloroacetic acid (0.5 ml) was added, and the denatured protein was removed by centrifugation. The amount of N\textsuperscript{5},N\textsuperscript{10}-methylenetetrahydrofolate formed was determined by light absorption at 355 nm.

Serine hydroxymethylase activity was measured indirectly by coupling with the reaction shown in Equation 2 and measuring the N\textsuperscript{5},N\textsuperscript{10}-methylenetetrahydrofolate formed as in the above assay (Equation 3).

\[
\text{Serine + TPN}^+ + \text{tetrahydrofolate} \rightarrow \text{glycine + TPNH} + (N^5,N^{10}\text{-methylenetetrahydrofolate})^+ \quad (3)
\]

The assay was identical with the assay for N\textsuperscript{5},N\textsuperscript{10}-methylene-tetrahydrofolate dehydrogenase, except that L-serine replaced HCHO as a substrate.

Cyclohydrolase was measured according to the procedure of Rabinowitz and Pricer (12), and adenylate kinase by the method of Colowick (15).

All enzyme activities are expressed as millimicromoles of product formed per hour. Specific activity is defined as millimicromoles of product per hour per mg of hemoglobin or per mg of protein. Protein was determined by light absorption at 290 and 280 nm (16). Hemoglobin was determined spectrophotometrically at 540 nm in a solution containing 0.04% NH\textsubscript{4}OH.

In experiments in which changes in the entire absorption...
spectrum were followed, a Beckman recording spectrophotometer, model DK-2, was employed.

**RESULTS**

**Measurements of Formate-activating Enzyme Activity in Erythrocyte Lysates**—In Table I is shown a component study for the formate-activating enzyme from human erythrocytes. The reaction is dependent upon tetrahydrofolate, formate, ATP, and enzyme. Optimal activity is obtained when certain monovalent cations and divalent cations are present. The amount of product, N5,N10-formyltetrahydrofolate, formed was linear with time, up to 30 minutes, and with enzyme concentration. Incubation of the enzyme for 5 minutes at 55° completely destroys the activity. The activity in crude lysates, prepared with phosphate buffer, declines slowly (50% in 8 hours at 4°); the activity of lysates prepared in Tris buffer or distilled water decays at even faster rates.

Although leukocytes contain appreciable formate-activating enzyme activity (17), erythrocytes prepared with or without theuffy coat revealed no difference in the level of formate-activating enzyme activity. Furthermore, even if complete lysis of the contaminating leukocytes is assumed, the amount of formate-activating enzyme activity contributed by the leukocytes is calculated to be less than 5%.

**Purification of Enzyme from Human Erythrocytes**—All purification steps were carried out at 4°. Centrifugations were performed at 100,000 × g for 15 minutes with a high speed head in an International refrigerated centrifuge, model PR-2, unless otherwise specified. Heparinized blood (240 ml) was centrifuged at 900 × g for 5 minutes. After the cells were washed again with 0.15 M KCl and centrifuged at 900 × g for 5 minutes. The plasma and buffy coats were removed, and the erythrocytes were washed with 0.15 M KCl and centrifuged at 900 × g for 5 minutes. After the cells were washed again with 0.15 M KCl, 30 ml of packed erythrocytes were lysed with 4 volumes of 0.001 M phosphate buffer, pH 8.0. The hemolysate was centrifuged, and 300 ml of the supernatant solution were removed. This is referred to as crude lysate. Solid ammonium sulfate (58.8 g) was then added slowly, and the mixture was stirred for 30 minutes. After centrifugation, the precipitate obtained was washed three times with 80 ml of a 33% saturated solution of ammonium sulfate and dissolved in 40 ml of 0.003 M phosphate buffer, pH 7.5. Any insoluble material was removed by centrifugation (0 to 33% saturated fraction). After dialysis against water for 3 hours, 35 ml of this enzyme solution were refractionated with a saturated solution of ammonium sulfate, pH 8.0, as follows: 25 ml of saturated ammonium sulfate solution were added, and the precipitate was collected by centrifugation (0 to 35% saturated ammonium sulfate). A 35 to 55% saturated ammonium sulfate fraction was then obtained by the further addition of 20 ml of saturated ammonium sulfate. The precipitate obtained by centrifugation was washed with a solution of 55% saturated ammonium sulfate, pH 8.0. Both of these fractions contain appreciable enzyme activity when reconstituted with 0.05 M Tris buffer, pH 8.0, although the specific activity of the 35 to 55% fraction is considerably higher than that of the 0 to 35% fraction. The 35 to 55% fraction represents a 1.10-fold purification. Approximately 1% of the total protein in this fraction is hemoglobin.

The enzyme preparation is stable for at least 30 days if stored at -10° as an ammonium sulfate precipitate, but when reconstituted with buffer, the fractions lose activity rapidly, whether stored at -10° or 4°. Attempts to prevent this loss of activity by storage in the presence of serum albumin or mercaptoethanol were unsuccessful.

A summary of the purification procedure is shown in Table II. The purified enzyme is contaminated with N5,N10-methylene-tetrahydrofolate dehydrogenase and serine hydroxymethylase activities but does not contain cyclohydrolase or adenylate kinase activity.

**Identification of N10-Formyltetrahydrofolate as Reaction Product**—Since acidification converts N10-formyltetrahydrofolate to N5,N10-methylene tetrahydrofolate, the assay described in "Experimental Procedure" does not distinguish between these two compounds. In the spectrophotometric experiment shown in Fig. 1, Curve A, represents the spectrum of tetrahydrofolate (λmax at 298 mp) in the presence of all reactants except ATP. When 0.1 μmole of ATP is added, the spectrum changes to Curve B. Curve C is obtained by correcting Curve B for the presence of the unchanged isomer of α-tetrahydrofolate and represents the spectrum of the product, N10-formyltetrahydrofolate (λmax at 200 mp, shoulder at 300 mp). Since the purified enzyme does not contain appreciable cyclohydrolase activity, N10-formyltetrahydrofolate must be the product of the reaction catalyzed by erythrocyte formate-activating enzyme. After acidification with perchloric acid, Curve C was recorded, representing the absorption spectrum of N5,N10-methylene tetrahydrofolate (λmax at 350 mp).

**Metal Ion Requirements**—The requirements for a divalent and monovalent ion are shown in Table III. Mg++ is the most effective divalent cation, but NH4+ or K+ ions are also required for optimal activity. At a concentration of 50 mM, Na+ is inhibitory. These metal ion requirements are similar to those

<table>
<thead>
<tr>
<th>Component omitted</th>
<th>N10-Formyltetrahydrofolate formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>48</td>
</tr>
<tr>
<td>Formate</td>
<td>1</td>
</tr>
<tr>
<td>ATP</td>
<td>0</td>
</tr>
<tr>
<td>Tetrahydrofolate</td>
<td>0</td>
</tr>
<tr>
<td>Mg++</td>
<td>9</td>
</tr>
<tr>
<td>NH4+</td>
<td>13</td>
</tr>
<tr>
<td>Enzyme</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein</th>
<th>Specific activity</th>
<th>Total activity</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Lysate</td>
<td>16,000</td>
<td>36.5</td>
<td>580,000</td>
<td>100</td>
</tr>
<tr>
<td>2. 0 to 33% saturated ammonium sulfate</td>
<td>1,400</td>
<td>200</td>
<td>106,000</td>
<td>70</td>
</tr>
<tr>
<td>3. 35 to 55% saturated ammonium sulfate, pH 8.0</td>
<td>26.4</td>
<td>4100</td>
<td>107,000</td>
<td>19</td>
</tr>
</tbody>
</table>
then deproteinized with 0.1 ml of 2.5 N perchloric acid, the de-
the spectrum after the reaction was completed (10 minutes).
the nonreactive isomer of tetrahydrofolate. The mixtures were
Curve B' is obtained after subtracting the absorption caused by
was added to both cuvettes to start the reaction. Curve B shows
formate-activating enzyme in a total volume of 1.0 ml. The blank
cuvette was identical except for the omission of tetrahydrofolate.
After an initial spectrum (Curve A) was taken, 0.10 @mole of ATP
recorded. Curves B' and C were corrected for volume changes.

for the formate-activating enzyme from chicken liver\(^1\) and
leukocytes (17).\footnote{G. Ozols, J. R. Bertino, M. J. Osborn, and F. M. Huennekens, unpublished observations.}

\textbf{pH Optimum.} As in avian liver and in leukocytes, the formate-
activating enzyme has an optimum at pH 7.5 and is inactive
at an acid pH.

\textbf{Inhibition by Products of Reaction—}The formation of \(N^\alpha\)-
formyltetrahydrofolate is inhibited 50\% by ADP and P\(_1\) at
final concentrations of \(3 \times 10^{-4}\) and \(4 \times 10^{-5}\) m, respectively.
\textbf{K\(_m\) Values—}The effect of concentration of ATP, formate, and
tetrahydrofolate on the rate of the reaction was studied. By
using the method of Lineweaver and Burk (18), the following K\(_m\)
values were obtained: 2.1 \(\times 10^{-3}\) m for formate, 1.5 \(\times 10^{-4}\) m
for ATP, and 1.1 \(\times 10^{-4}\) m for tetrahydrofolate.

\textbf{Studies of Formate-activating Enzyme Activity as Function of
Red Blood Cell Age—}In order to determine whether enzyme
activity was higher in young erythrocytes, blood samples from
patients with elevated reticulocyte counts were assayed for
formate-activating enzyme activity. Table IV demonstrates
that in patients with elevated reticulocyte counts, the level of
erythrocyte formate-activating enzyme activity was several
fold higher than normal.

\textbf{Enzyme Levels in Animals—}The level of formate-activating

\begin{table}
\centering
\caption{Metal ion requirements for formate-activating enzyme}
\begin{tabular}{|c|c|c|}
\hline
Metal ion & Concentration & \(N^\alpha\)-Formyltetrahydrofolate formed \tabularnewline
\hline
\hline
A. Divalent ions: & & \\
None & & 4.0 \\
Mg\(^{++}\) & 1 & 6.0 \\
Mg\(^{++}\) & 10 & 52.0 \\
Mg\(^{++}\) & 50 & 55.0 \\
Mn\(^{++}\) & 20 & 19.0 \\
Ca\(^{++}\) & 20 & 5.5 \\
Zn\(^{++}\) & 10 & 0.5 \\
\hline
B. Monovalent ions: & & \\
None & & 22.5 \\
Na\(^+\) & 50 & 15.5 \\
K\(^+\) & 50 & 49.0 \\
Na\(^+\) & 10 & 47.0 \\
NH\(_4\)\(^+\) & 50 & 54.0 \\
NH\(_4\)\(^+\) & 100 & 56.0 \\
\hline
\end{tabular}
\end{table}

\begin{table}
\centering
\caption{Increased levels of formate-activating enzyme
associated with reticulocytosis}
The standard assay was used, as described in "Experimental
Procedure." Values are for Patients No. 1 and 2 with pernicios
anemia 10 and 7 days after vitamin B\(_12\) therapy, respectively,
a patient with sickle cell anemia in a hemolytic crisis, and a pa-
tient with congenital nonspherocytic hemolytic anemia.

\begin{tabular}{|c|c|c|}
\hline
Condition & Reticulocytes & Formate-activating enzyme activity \tabularnewline
\hline
Normal (15 patients) & 0.5-1.5 & 35 (19-49) \\
Pernicious anemia, responding:
Patient No. 1 & 16.8 & 458 \\
Patient No. 2 & 6.4 & 116 \\
Sickle cell anemia & 10 \(\%\) & 260 \\
Hemolytic anemia & 15.0 & 259 \\
\hline
\end{tabular}
\end{table}

\begin{table}
\centering
\caption{Levels of erythrocyte formate-activating enzyme activity
in various animal species}
Numbers in parentheses indicate range of values. The assay
was as described in "Experimental Procedure."

\begin{tabular}{|c|c|c|c|}
\hline
Species & No. of samples & Reticulocyte count & Formate-activating enzyme activity \tabularnewline
\hline
Chicken & 3 (pooled) & Nucleated & 30 \\
& & RBC & 282 \\
Mouse & 4 & 3.1 (1.5-5.0) & 22 \\
Guinea pig & 10 & 2.0 (0.6-8.1) & 40 \\
Rat & 3 & 2.0 (1.5-2.4) & 58 \\
Rabbit & 3 & 1.9 (1.2-2.7) & 55 \\
Pig & 4 & 1.5 (0.4-2.5) & 53 \\
Dog & 3 & 0.6 (0.50-1.1) & 100 \\
Man & 15 & 0.8 (0.5-1.3) & 120 \\
\hline
\end{tabular}

\textsuperscript{*} Data summarized by Rodnan, Ebaugh, and Spivey-Fox (19).
\textsuperscript{\dag} Red blood cell life span of hamster (20).

\textbf{Experimental Procedure.} Values were obtained: 2.1 \(\times 10^{-3}\) m for formate, 1.5 \(\times 10^{-4}\) m for ATP, and 1.1 \(\times 10^{-4}\) m for tetrahydrofolate.

\textbf{Metal ion requirements for formate-activating enzyme}
In Part A, the divalent ions were varied as indicated. In
Part B, the erythrocytes were washed twice with 0.15 m NaCl,
instead of 0.16 m KCl, and lysed with 4 volumes of distilled water.
The monovalent ions were then varied as indicated.

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fold higher than normal.

\textbf{Enzyme Levels in Animals—} The level of formate-activating
enzyme activity in erythrocytes of several animal species was determined. As can be seen in Table V, both the reticulocyte and formate-activating enzyme activity increase as the red cell life span decreases. Thus the highest level of formate-activating enzyme was found in the mouse, which has the shortest erythrocyte life span among the animals studied.

N5,N10-Methylene tetrahydrofolate Dehydrogenase — Table VI is a component study of this enzyme performed with a crude erythrocyte lysate from a normal subject as the enzyme source. As observed for the formate-activating enzyme, both fractions obtained with saturated ammonium sulfate (0 to 35%, 35 to 55%) have appreciable N5,N10-methylene tetrahydrofolate dehydrogenase activity, with most of the activity in the 35 to 55% fraction. The pH optimum for the partially purified enzyme is 7.5 and is similar to that observed with the leukocyte enzyme.2

Serine Hydroxymethylase — The activity of this enzyme could not be detected in crude lysates, but some activity was detected in the 35 to 55% saturated ammonium sulfate fraction. Table VII gives the relative activities of this enzyme as compared with the other enzymes measured in the 35 to 55% saturated ammonium sulfate fraction.

DISCUSSION

The participation of tetrahydrofolate enzyme systems in purine synthesis has been well established (21). Our data demonstrate that significant levels of formate-activating enzyme activity are present in erythrocytes, i.e., approximately 0.8 μmole per hour per mg of nonhemoglobin protein, comparable to levels found in leukocytes (approximately 0.5 μmole per hour per mg). Whether the limited capacity of mature erythrocytes for purine synthesis demonstrated in vitro (2) functions in vivo would depend upon the availability to the erythrocytes of the purine precursor, 5-amino-1-ribosyl-4-imidazolecarboxamide. Studies in man by Vilenkina (22) and McGeer, McGeer, and Griffin (23) indicate that 4-amino-5-imidazolecarboxamide is constantly excreted in urine (0.98 mg per day). This compound, when ingested, readily enters body substrate pools, and only a small portion is excreted in the urine (24). These data suggest that this purine precursor would be available in vivo to the erythrocyte.

The properties of the formate-activating enzyme from human erythrocytes are similar to those of its counterpart in other tissues. The Kₘ value for formate is similar to that obtained for C. cylindrosporum (25). The Kₘ values for ATP and tetrahydrofolate are of an order of magnitude less than the reported values for M. aerogenes (8) or C. cylindrosporum (25).

Formate-activating enzyme activity, like certain other erythrocyte enzymes (26-28), is higher in erythrocytes containing a high proportion of reticulocytes. Additional studies of formate-activating enzyme activity in erythrocytes of various animal species show that enzyme activity decreases as the erythrocyte life span increases. These observations indicate that the formate-activating enzyme activity is at a high level at the reticulocyte stage and subsequently falls to lower levels as red cells mature. A similar study of glucose-6-P dehydrogenase activity in erythrocytes of several animal species showed increased activity in mouse erythrocytes, but no consistent correlation between erythrocyte life span and enzyme activity was observed (27).

A study in vivo of some glycolytic enzyme activities as a function of erythrocyte age, with the use of differential agglutination, demonstrated that the activities of two enzymes in particular decrease: glucose-6-P dehydrogenase and glyceraldehyde-3-P dehydrogenase. The diminution of these enzyme activities has been suggested as possibly limiting the length of the life span (29). The role of formate-activating enzyme activity, if any, in erythrocyte senescence remains to be determined.

SUMMARY

Three tetrahydrofolate-dependent enzymes have been detected in human erythrocytes: the formate-activating enzyme, N5,N10-methylene tetrahydrofolate dehydrogenase, and serine hydroxymethylase. The formate-activating enzyme has been purified 110-fold from human erythrocytes. The pH optimum is 7.5, and both a monovalent and a divalent cation are required for optimal activity. N10-Formyltetrahydrofolate has been shown to be a product of the reaction. Kₘ values of 2.1 × 10⁻⁴ M for formate, 1.5 × 10⁻⁴ M for ATP, and 1.1 × 10⁻⁴ M for tetrahydrofolate have been determined. Formate-activating enzyme activity has been found to be higher in young erythrocytes and in the erythrocytes of animals with shorter red cell life spans.

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