Evidence for a Single Enzyme Reducing Folate and Dihydrofolate*

SIGMUND F. ZAKRZEWSKI and CHARLES A. NICHOL

From the Department of Experimental Therapeutics, Roswell Park Memorial Institute, Buffalo, New York

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Partial purification of an enzyme from chicken liver which reduces folic acid to tetrahydrofolate was reported previously (1). This reduction has been assumed to proceed in two sequential steps,

\[ \text{folate} \rightarrow E_1 \rightarrow \text{folate-H}_2 \rightarrow E_2 \rightarrow \text{folate-H}_4 \]

each step being catalyzed by a separate enzyme, \( E_1 \) (folic acid reductase) and \( E_2 \) (dihydrofolate reductase) (2). The purification of dihydrofolic acid reductase from chicken liver (3) and from sheep liver (4) has been reported. In each case the final preparation reduced only dihydrofolate; no mention was made of the distribution in different fractions of folic acid reductase which was presumably present in the starting material.

In this laboratory all attempts to resolve the system reducing folate to folate-H\(_4\) into its components, \( E_1 \) and \( E_2 \), were unsuccessful. Moreover, evidence has accumulated that both reactions (reduction of folate and folate-H\(_2\)) are carried out by the same enzyme (5). The basis for differences between these results and those of earlier investigations (2–4) is discussed.

EXPERIMENTAL PROCEDURE

The folic acid reductase was purified from acetone powder preparations of chicken liver as described elsewhere (1). Dihydrofolate was prepared by the reduction of folate (Nutritional Biochemicals Corporation) with sodium dithionite according to the procedure of Futterman (2). The product was stored as a suspension in 0.001 M HCl. Solutions were prepared for use by diluting an aliquot of the suspension with 0.01 M NaOH containing 0.13 M mercaptoethanol. TPNH was prepared by enzymatic reduction of TPN (6) (Pabst Laboratories) by means of a procedure described previously (1). DPNH and glucose 6-phosphate dehydrogenase were obtained from Sigma Chemical Company.

The enzymatic reduction was measured by the following methods: (I) determination of the diazotizable amine formed upon spontaneous decomposition of folate-H\(_4\), (II) determination with glucose 6-phosphate dehydrogenase of TPN formed during the course of the reaction, and (III) determination of the amount of TPNH oxidized as measured by the change of absorbancy at 340 m\(\mu\). Methods I and II were described in detail elsewhere (1). DPNH and glucose 6-phosphate dehydrogenase were obtained from Sigma Chemical Company.

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When Method III was used the incubations were carried out in Beckman cuvettes at room temperature. Since, under these conditions, both TPNH and folate or dihydrofolate contribute to the absorbancy at 340 m\(\mu\), the sum of two molar extinction coefficients was used. For the reduction of folate, which requires two equivalents of TPNH, and of dihydrofolate, which requires one equivalent of TPNH, these values were 15.6 \(\times\) 10\(^3\) and 9.4 \(\times\) 10\(^3\), respectively, representing the sums of \(E_{340} = 6.2 \times 10^3\) for the oxidation of TPNH (7) (or \(2 \times 6.2 \times 10^3\) when folate was the substrate) plus \(E_{340} = 3.2 \times 10^3\) for the reduction of either folate or folate-H\(_2\). The latter value was determined from the change in the ultraviolet spectra of folate and folate-H\(_2\) upon reduction (8). The agreement between Methods II and III was demonstrated by the following experiment. Two identical samples in which folate-H\(_2\) was the substrate were incubated with TPNH and enzyme preparation at room temperature. The concentration of folate-H\(_2\) formed after 10 minutes was found to be \(2.00 \times 10^{-4}\) \(M\) when determined by Method II and \(2.35 \times 10^{-4}\) \(M\) when determined by Method III.

RESULTS

Preliminary Observations—The partially purified preparation of folic acid reductase reduced dihydrofolate much faster than folate. However, all attempts to separate fractions reducing only folate or dihydrofolate by selective adsorption on phosphate gel, chromatography on DEAE-cellulose, chromatography on carboxymethyl cellulose and paper electrophoresis were unsuccessful. Also, no enrichment of either activity could be demonstrated during the routine purification of the enzyme (Table I). In contrast, the complete separation of tetrahydrofolic acid formylase from folic acid reductase was achieved during the same procedure. The slight differences in the ratio of the activities at different stages of the enzyme purification can be explained by the variations in TPNH levels due to the presence of interfering enzymes. It is obvious that the change in concentration of TPNH will affect the reduction of folate more than that of folate-H\(_2\).

Effect of \(pH\) and Cofactors—The effect of \(pH\) on the reaction velocity with dihydrofolate or folate as substrate is shown in Fig. 1. Between \(pH\) 5.2 and 5.6 the TPNH dependent reduction of folate-H\(_2\) proceeds 20 to 60 times faster than does that of folate. With DPNH as hydrogen donor, the velocity of the reduction of folate-H\(_2\) is greatly reduced. The same effect was observed when folate was used as substrate (1, 2). At neutral \(pH\), however, reduction of folate did not proceed at a measurable

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Abbreviations: folate, folic acid (pteroylglutamic acid); folate-H\(_2\), dihydrofolic acid; folate-H\(_4\), tetrahydrofolic acid.
Inhibition of Reductase Activity by Amethopterin—Amethopterin inhibits folic acid reductase in rat liver (10) and sheep liver (11) in an irreversible manner (12). The same type of inhibition by amethopterin was also observed in the system described here when either folate or folate-H₂ was used as substrate (Fig. 4). When the inhibition is of the irreversible type, the inhibitor can be directly titrated with the enzyme and vice versa. For a constant enzyme concentration the plot of the reaction velocity versus the inhibitor concentration should produce a straight line.

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**Table I**

<table>
<thead>
<tr>
<th>Enzyme preparation</th>
<th>Relative specific activity</th>
<th>Ratio of activities</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Folate reduction</td>
<td>Folate-H₂ reduction</td>
</tr>
<tr>
<td>1. Extract of acetone powder (chicken liver)</td>
<td>1</td>
<td>10.14</td>
</tr>
<tr>
<td>2. Extract of acetone powder treated with Dowex 1-Cl</td>
<td>1</td>
<td>1.3</td>
</tr>
<tr>
<td>3. Supernatant after precipitation of protein at pH 5.5</td>
<td>1.64</td>
<td>9.8</td>
</tr>
<tr>
<td>4. Supernatant after precipitation of protein at pH 4.5</td>
<td>1.84</td>
<td>∞</td>
</tr>
<tr>
<td>5. Extract of the 40-60% acetone precipitate</td>
<td>7.35</td>
<td>12.5</td>
</tr>
</tbody>
</table>

*a* The procedures used to purify the enzyme and to assay for folic acid reductase and tetrahydrofolate formylase were described previously (1).

To determine the activity toward folate-H₂, 0.02 ml of the enzyme solution was incubated at room temperature in the medium containing 0.1 m citrate pH 6.0, 4.36 × 10⁻⁵ m folate-H₂, 9.0 × 10⁻⁵ m TPNH, and 6.5 × 10⁻⁵ m mercaptoethanol in a total volume of 1 ml. The oxidation of TPNH was followed by measurement of absorbancy at 340 nm (Method III). Readings were taken at 1-minute intervals for 6 minutes. The initial velocity was computed per minute per 0.2 ml of the enzyme solution.

To determine the activity toward folate, 0.2 ml of the enzyme solution was incubated at 37° in a medium containing 0.1 m citrate pH 5.2, 4.36 × 10⁻⁵ m folate and 3.64 × 10⁻⁴ m TPNH in a total volume of 1.0 ml. In order to obtain the initial velocity as accurately as possible, the time of incubation was varied depending on the activity of the enzyme between 3 and 6 minutes. In every case the initial velocity was computed per minute. The reaction was stopped by addition of 0.2 ml of 5 m HCl. The amount of folate-H₂ formed was calculated from the determination of diazotizable amine (Method I).

Duplicate analyses on the same preparation.

Rate even in the presence of TPNH, whereas dihydrofolate was readily reduced under these conditions.

Inhibition of Reduction of Dihydrofolate by Folate—When the reduction of folate-H₂ was allowed to proceed in the presence of folate, considerable inhibition of the reaction has been observed. This observation was possible only because of the great difference in the reaction velocities of the two substrates. An analysis of this inhibition according to Lineweaver and Burk (9) is presented in Fig. 2. The common intercept for all three lines indicates that the inhibition is competitive. Fig. 3 represents a similar graphic analysis of the reaction when folate was the substrate. The kinetic constants calculated from the data in Figs. 2 and 3 are summarized in Table II. The fact that the affinity of folate to "dihydrofolate reductase" (Kₐ) is the same as its affinity to "folic acid reductase" (Kₐ) indicates strongly that in both cases the same enzyme is involved.²

² The significance of Kₐ as true dissociation constant of folic acid reductase-folate complex was discussed elsewhere (14).
FIG. 3. Velocity of reduction of folate in relation to substrate concentration. The conditions for incubation and analytical procedure were the same as described in Fig. 2, except that folate was the substrate and 0.2 ml of enzyme solution was used for each incubation. $S =$ Folate; moles per liter. $V =$ Initial velocity; moles of product per liter per minute per 0.2 ml of enzyme solution.

**TABLE II**

Kinetic constants of folic acid reductase with folate and dihydrofolate as substrates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$V_{\text{max}}$ (mole/min/0.2 ml enzyme)</th>
<th>$K_m$ (M)</th>
<th>$K_i$ (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Folate</td>
<td>2.5</td>
<td>7.9</td>
<td>8.1†</td>
</tr>
<tr>
<td>Dihydrofolate</td>
<td>67</td>
<td>8.6</td>
<td></td>
</tr>
</tbody>
</table>

* Folate as inhibitor in the presence of dihydrofolate as substrate.
† Average of two values ($8.5 \times 10^{-7}$ and $7.6 \times 10^{-6}$) obtained at two different levels of the inhibitor.

FIG. 4. Irreversible inhibition by amethopterin of reduction of folate and dihydrofolate (Ackerman and Potter inhibition analysis). The indicated aliquots of the enzyme solution were incubated at room temperature in media containing 0.1 M citrate pH 5.2, 4.0 $\times$ 10$^{-4}$ M TPNH, and 4.36 $\times$ 10$^{-4}$ M folate or folate-H$_2$, with or without amethopterin in the total volume of 1.0 ml. Time of incubation was 5 minutes with folate and 1 minute with folate-H$_2$ as substrate. The extent of the reaction was measured in the former case by determination of diazotizable amine (Method I) (Δ O.D. at 560 mu) and in the latter case by determination of TPN with glucose 6-phosphate dehydrogenase (Method II) (Δ O.D. at 340 mu). ○ No amethopterin; ●, amethopterin, 4 $\times$ 10$^{-4}$ moles per liter.

FIG. 5. Titration of reductase activity with amethopterin. The conditions of incubation and analytical procedures were the same as described in Fig. 4, except that the amount of enzyme was constant (0.2 ml) in each case and the concentration of the amethopterin was varied as indicated in the graph. $V =$ Initial velocity; moles of product per liter per min per 0.2 ml of enzyme solution.
occur only briefly as an intermediate. The fact that folate inhibits the reduction of folate-\(\text{H}_2\) competitively implies that the same active site on a single enzyme is involved in the reduction of both substrates.

Apart from a much faster rate of reduction of folate-\(\text{H}_2\) as compared to folate, the only difference found in the behaviour of the reductase toward these two substrates was with respect to pH optima. With folate-\(\text{H}_2\) as substrate, the rate of the reaction was fastest between pH 5.2 and 5.6, whereas the reduction of folate was most rapid at pH 4.4 to 4.8 (1). Since pH optimum depends on the ionic state of the substrate as well as that of the enzyme, this difference does not necessarily indicate the existence of two enzymes.

Futterman (2) reported that two enzymes are involved in the conversion of folate to folate-\(\text{H}_4\) by preparations of chicken liver. This conclusion was based on the observation that TPNH alone was required for the reduction of folate, whereas either TPNH or DPNH could be utilized for the reduction of folate-\(\text{H}_3\). Osborn and Hueneke (3) described the partial purification of “dihydrofolate reductase” from chicken liver. This enzyme preparation was unable to reduce folate when tested at pH 7. In contrast to the observation by Futterman (2), DPNH was inactive under these conditions.

These observations, which differ from each other and from our findings, can be explained by the fact that folate-\(\text{H}_3\) is reduced so much more rapidly than folate. It is evident, from data presented in Fig. 1, that the reduction of folate-\(\text{H}_3\) can proceed easily at a concentration of the enzyme at which no reduction of folate can be detected. This is especially true when the enzymatic reactions are carried out above pH 6. When DPNH was used in place of TPNH, the rate of reduction of folate-\(\text{H}_3\) was greatly decreased, approximating the rate of reduction of folate in the presence of TPNH. In the experiments described by Futterman (2) in which the effect of DPNH and TPNH on the reduction of folate and folate-\(\text{H}_3\) was studied, folic acid reductase was coupled with a tetrahydrofolic acid formylase system and the formation of folinic acid was used as a measure of the reductase activity. In such a system the formylation of folate-\(\text{H}_3\) was probably the rate limiting reaction. Thus, the increased rate of reduction of folate-\(\text{H}_3\) in the presence of TPNH would not be observed.

A different reductase preparation which was reported to reduce only folate-\(\text{H}_4\) was purified from sheep liver by Peters and Greenberg (4). The requirement of this system for DPNH below pH 6 and TPNH above pH 6 differs from the consistent requirement for TPNH by the reductase of chicken liver.

The observation that the ratio of activities of the reductase for folate-\(\text{H}_3\) and folate remained constant during purification of the enzyme led to the question: “Does a single enzyme catalyze both reactions?” In studies attempting to answer this question, substantial evidence for the existence of a single enzyme was obtained by two different experiments in which inhibition of the activity by amethopterin and heat inactivation of the enzyme were studied. With either folate or folate-\(\text{H}_3\) exactly the same amount of drug was required to completely inhibit the enzymatic activity. If two enzymes were involved, then both must have the same binding capacity for the inhibitor. Furthermore, the rate of thermal inactivation was identical with either substrate. If two enzymes were involved, both must have the same thermal lability. Such a combination of coincidences is unlikely.

The difference in the activity of folate and dihydrofolate as substrates for folic acid reductase may account for the apparent separation of the two activities previously reported (2, 3). Although the identical behaviour and similar properties of “folic acid reductase” and “dihydrofolate reductase” support the interpretation that both activities are attributable to a single enzyme, the possibility must be considered that two enzymes very similar to each other might be present. Thus, final proof must await isolation of the enzyme as a single protein and the study of its properties in similar experiments. Dihydrofolate may indeed be the natural substrate for this reductase, since folate appears to be an artifact formed during isolation of the vitamin from the heat-labile reduced derivatives native to animal tissues and green leaves (13). The function of the enzyme, however, in the reduction of pteroylglutamate (14) and monoglutamate (synthetic) forms of the vitamin supports the suggestion that the name “folic acid reductase” be retained.

**SUMMARY**

Folic acid reductase from chicken liver reduces dihydrofolate at least twenty times faster than folate. The ratio of activities for folate and dihydrofolate remains constant during partial purification. Reduced triphosphopyridine nucleotide is required for the reduction of both substrates; reduced dipropophyridine nucleotide is much less active in this respect. Amethopterin inhibits both reactions in a manner that is practically irreversible; the amount of antagonist just sufficient to inhibit both reactions completely is the same with either folate or dihydrofolate as substrate. Folate inhibits the reduction of dihydrofolate competitively. Heating inactivates the reduction of folate and dihydrofolate.
dihydrofolate at the same rate. Thus, both folate and dihydro-
folate appear to be reduced by a single enzyme.

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