The Structure of Dihydrofolate Reductase

I. INACTIVATION OF BACTERIAL DIHYDROFOLATE REDUCTASE CONCOMITANT WITH MODIFICATION OF A METHIONINE RESIDUE AT THE ACTIVE SITE*

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Carboxymethylation by iodoacetate of dihydrofolate reductase from the amethopterin-resistant mutant Streptococcus faecium var. Durans strain A leads to a loss of enzymic activity. Amino acid analysis showed that methionine is the only amino acid residue significantly affected by iodoacetate under the experimental conditions, and this was confirmed by the use of [1-14C]iodoacetate and ion exchange chromatography of the products obtained by acid hydrolysis of the modified enzyme. During loss of 90% of the activity a total of about 2 of the 7 methionine residues present in the enzymes are carboxymethylated. Over this range of activity loss the decrease is proportional to the number of methionine residues modified.

Fluorescence-quenching experiments demonstrated that dissociation constants for complexes of inhibitors with the carboxymethylated enzyme were 20 to 30 times greater than dissociation constants for corresponding complexes with native enzyme. Similarly, equilibrium dialysis studies showed that dihydrofolate binding to the modified enzyme was decreased 10-fold compared with binding to the native enzyme. These data suggest that iodoacetate modifies one or more methionine residues at the binding site for dihydrofolate and inhibitors. In accordance with this view it was shown that enzyme can be protected from inactivation by the folate analogue aminopterin and to a lesser extent by folate and dihydrofolate. Enzyme carboxymethylated in the presence of aminopterin, and subsequently freed of the latter, was found to bind inhibitors and dihydrofolate as tightly as the native enzyme.

It is concluded that the loss of enzyme activity is caused by carboxymethylation of at least 1 methionine residue which is at or near the binding site of dihydrofolate.

Dihydrofolate reductase catalyzes the reduction of dihydrofolate with NADPH as the reducing substrate. This enzyme is of special interest because several of its inhibitors have been used in the treatment of neoplastic disease, malaria, bacterial infections, psoriasis, and other disorders (1). Although these inhibitors are toxic for all replicating cells, some inhibitors can effectively discriminate between reductases from different cells, for example between the bacterial and mammalian reductases (2).

The inhibitor discriminating most effectively between bacterial and mammalian reductases is trimethoprim, which in recent years has been used to combat bacterial infections in man. Although bacteria develop resistance to such inhibitors (3), the emergence of resistance to trimethoprim is delayed when the inhibitor is used in combination with sulfamethoxazole (4). The latter drug, which blocks bacterial synthesis of dihydrofolate (5), also increases the effectiveness of trimethoprim (6). Preliminary evidence has also been reported for a cysteine residue at the active site of the reductase from the E. coli mutant (15) and of that from a resistant bacterial strain are relevant to the mechanism of resistance, which frequently involves increased bacterial levels of reductase.

In previous studies on bacterial reductase the presence of tryptophan at the active site has been postulated for the reductase from an amethopterin-resistant strain of Streptococcus faecium (8-10), an amethopterin-resistant mutant of Escherichia coli (11, 12), an amethopterin-resistant mutant of Lactobacillus casei (13), and wild L. casei (14). It has been suggested that the important tryptophan residue in the active site of resistance which frequently involves increased bacterial levels of reductase.

Comparative structural studies on dihydrofolate reductase from bacterial and mammalian sources should reveal the basis for specific inhibition of the bacterial enzyme by trimethoprim and might permit the prediction and synthesis of more selective inhibitors. Furthermore, studies on the reductase from a resistant bacterial strain are relevant to the mechanism of resistance, which frequently involves increased bacterial levels of reductase.

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methionine residue at the binding site for inhibitors and dihydrofolate results in loss of activity.

**EXPERIMENTAL PROCEDURE**

**Materials**

Dihydrofolate reductase was prepared from the amethopterin-resistant mutant *S. faecium var. Durans* strain A, as previously described (19), with some modifications so that approximately 10 kg of cell paste, prepared commerically by the Grain Processing Co., Muscatine, Iowa, could be processed at one time. Folic acid, NADPH, NADP, and iodoacetate were purchased from Sigma. Iodoacetate was recrystallized from boiling petroleum ether-cyclotron ether (20). Dihydrofolate was prepared as previously (21). [1-¹⁴C]Iodoacetate (New England Nuclear, 12.09 mCi per mmol) was recrystallized (as above) in the presence of the pure carrier acid to give material with approximately 240,000 counts per min per μmol. [5,5-³H]Dihydrofolate acid was prepared (as above) from [²H₅]Folic acid (Schwarz-Mann, 15 Ci per mmol) in the presence of carrier folic acid to give material with approximately 700,000 counts per min per μmol. L-¹⁴C]Aspartic acid (208 Ci per mmol) and L-¹⁴C]phenylalanine (464 Ci per mmol) were obtained from New England Nuclear. 1-Carboxymethyl, 3-carboxymethyl, and 1,3-dicarboxymethyl histidines were kindly provided by Dr. Jeanette of this Department who synthesized them by procedures to be published. Methionine, carboxymethylsulfonium iodide as prepared as described by Gundlach et al. (22) was converted to S-carboxymethylhomocysteine by S + HCl hydrolysis. O-Carboxymethyltyrosine was synthesized as described by Korman and Clarke (23). After incubation of the N-acetyltyrosine with the magnesium bromoacetate the pH of the solution was lowered to 1.0 with 5 N HCl and the solution lyophilized. The dry material was then triturated with ethyl ether and the remaining solid hydrolyzed with 5 N NaOH in a sealed, evacuated tube for 15 hours at 110°. The pH of the hydrolysate was adjusted to 2.5 with HCl and a sample applied directly to the analyzer. Aminopterin obtained from the International Chemical and Nuclear Co. was purified by the method of Loo (24). DTPC, a analogue of trimethoprim, was obtained through the courtesy of Dr. W. E. Scott of Hoffmann-La Roche, Inc. NCS tissue solubilizer was purchased from Amersham-Searle.

**Methods**

**Enzyme Assay**—Enzyme activity was measured spectrophotometrically (19) with a Cary model 14 recording spectrophotometer at 37°. The assay mixture, in a volume of 3.0 ml, contained 50 mM potassium phosphate buffer (pH 7.0), 50 μM NADPH, 50 μM dihydrofolate, and 10 to 60 μM reductase. In kinetic studies Δυ + 0.1 absorbance scale and 5-cm path length cells were used. The enzyme concentration was 3 to 30 μg. Buffers and temperature were the same as in the assay.

**Carboxymethylation**—A solution of dihydrofolate reductase (2 mg per ml) in citric acid-Na₂HPO₄ buffer, pH 6.3 (25), was made 0.2 M in iodoacetate and allowed to react at 0° in the dark. When aminopterin was present as a protective agent the concentration was 1 mM unless stated otherwise. Aliquots were removed at intervals and assayed for enzymic activity as described above. The concentration of iodoacetate in the assay (< 1 mg HCl per ml) had no effect on enzymic activity. Samples (0.2 to 1.0 ml) were used for further studies were passed through a Sephadex column (G-25, 1 x 55 cm). The columns were equilibrated and eluted with 0.05 M potassium phosphate buffer, pH 7.0, at 4° in the dark. Larger samples were passed through proportionately larger columns. In the case of the protection studies, the aminopterin and iodoacetate were removed by passage through a column of similar size which was equilibrated and eluted with 0.01 M Tris-HCl, pH 5.5.

**¹³C Carboxymethyl Group Content**—The concentration of protein modified as above but with [¹³C]iodoacetate was determined by amino acid analysis (JEOL, 6AH analyzer) based on a content of 10 phenylalanine residues (18). Radioactivity was measured on a Beckman LS-10C liquid scintillation spectrophotometer. A volume of 0.1 to 1.0 ml of the aqueous sample was added to 10 ml of Bray's scintillation solution (26) and counted with the use of the 3 plus 4°C fixed with the use of the ISO SSB, which allowed the detection of counting to remain constant even though some quenching occurred.

**Analysis of ¹⁴C Carboxymethyl Amino Acids and their Degradation Products**—A 6 N HCl hydrolysate of the carboxymethylated protein was analyzed by the isotopic method of Goren et al. (20). A JEOL 6AH amino acid analyzer was operated normally except that the long column was run at 36° for the first 90 min and the temperature then raised to 53.5°. The portion of the flow from the column (approximately half) which is not used for reaction with ninhydrin and not allowed to waste, was collected and fractionated in the sample changer of the Beckman liquid scintillation spectrophotometer and the radioactivity recorded each minute. [²H]Aspartic acid and [²H]phenylalanine were added to samples to provide reference positions on the amino acid analysis chart and on the radioactivity versus time plot. The extent of carboxymethylation of methionine was checked by amino acid analysis after performic acid oxidation, which converts methionine but not S-carboxymethylmethionine to methionine sulfone (27, 28). After oxidation the samples were diluted 10-fold and lyophilized. Poor recoveries of the sulfone were obtained, and destruction of tyrosine was observed when the oxidation mixtures were dried by rotary evaporation.

**Equilibrium Dialysis**—Cells (Interscience, Philadelphia, Pa.) holding 0.4 ml on each side of a Sartorius SM 1213 membrane were used. To one chamber was added 0.2 ml of 0.05 M potassium phosphate buffer, pH 7.5, containing enzyme (0.1 to 1 nm) and [²H]dihydrofolate (0.05 to 2 μM), and to the other chamber an identical solution was added except for the absence of enzyme. The cells, which also contained in each chamber three glass beads (1-mm diameter, Walter Stirn, Inc., Port Washington, N.Y.), were placed on the rotating drum in the dark in a bath at 5° for 22 hours. During this time essentially no loss in enzymic activity was observed under these conditions, and the decomposition of dihydrofolate was insignificant as measured by the change in absorbance at 420 nm (29). Samples (0.05 ml) from each chamber were transferred to vials containing 0.3 ml of NCS solubilizer. 10.0 ml of Bray's solution was added, and the radioactivity determined. The dissociation constants, Kₐ₅₀, and the number of binding sites, n., were determined as described by Yamada et al. (30).

**Circular Dichroism**—Silica cells with a light path of 0.5 cm were used in Cary model 60 spectropolarimeter with a circular dichroism attachment. Protein concentrations (about 2 mg per ml) were determined by amino acid analysis. The ellipticity (θ) values were corrected for the solvent blank. The α helix content was calculated by the method of Chen and Yang (32), which used globular proteins as reference.

**RESULTS**

**Inactivation by Iodoacetate**—During treatment of dihydrofolate reductase with iodoacetate at pH 6.3 and 0° over a period of 20 hours, enzymic activity decreases, whereas there is no
accomplished with a decrease in activity to 10 to 15% of that of the native enzyme.

Protection by Substrates—The results of protection studies (Fig. 3) also suggest that at least one of the methionine residues which are carboxymethylated by iodoacetate is at the dihydrofolate-binding site. Aminopterin is a very potent in-

loss under these conditions in the absence of iodoacetate. Amino acid analysis following performic acid oxidation of the native enzyme showed that it contains no cysteine or cystine residues, and amino acid analysis of the enzyme after treatment with iodoacetate indicated that none of the 5 histidine residues were modified under the experimental conditions. When the protein was alkylated with \([1^{14}C]\)iodoacetate, the modified protein hydrolyzed with acid, and the hydrolysis products separated on an amino acid analyzer in which a portion of the stream was directed through a scintillation counter flow cell, only one major radioactive peak was observed (Fig 1) This peak, which contained 58% of the radioactivity, eluted in the position of \(S\)-carboxymethylhomocysteine. Two minor peaks (Peaks 1 and 2, Fig. 1) which eluted before aspartic acid correspond in position and amount to other anticipated degradation products of carboxymethylmethionine (20, 22), so that the data indicate carboxymethylation of methionine residues, a reaction which can occur over a wide pH range (33). The modification of tyrosine, lysine, or histidine can be ruled out because the products are acid-stable and would have been observed in the analysis of the hydrolysis products (20). Carboxymethylation of a carboxyl group, which has been observed in the case of ribonuclease T1 (34), results in the formation of \([^{14}C]glycolic\) acid as the only labeled product after acid hydrolysis. Although one of the minor peaks in Fig. 1 corresponds in position to \([^{14}C]glycolic\) acid the latter is also formed by the decomposition of \(S\)-carboxymethylmethionine. Since the ratio of label in \([^{14}C]glycolic\) acid (Peak 2, Fig. 1) to label in \([^{14}C]carboxymethylhomocysteine\) was as predicted for formation by decomposition of \([^{14}C]carboxymethylmethionine\) (20), the data provide no evidence for carboxymethylhomocysteine. Fig. 2 shows the proportionality between activity loss and the total number of carboxymethyl groups incorporated. Extrapolation of the linear portion of the plot to zero activity shows that this corresponds to the incorporation of about two carboxymethyl groups. It can be seen that additional residues become carboxymethylated before activity reaches zero.

The number of carboxymethylmethione residues formed was also estimated by determining the number of unmodified methionine residues after conversion to the sulfone with performic acid. The results (Fig. 2) agree well with determinations of total carboxymethyl groups incorporated based on radioactivity and further rule out the possibility that residues other than methionine were carboxymethylated.

In preparing modified enzyme for further studies carboxymethylation of about 2 methionine residues per molecule was

Fig. 1. Ion exchange chromatography of the products obtained by acid hydrolysis of \([^{14}C]carboxymethylated\) enzyme. A sample of labeled protein (0.6 mg) was hydrolyzed for 24 hours at 110° in 6 N HCl and applied to the column after removal of HCl. The arrows indicate the positions where authentic carboxymethyl derivatives of histidine and tyrosine are eluted. Peaks 1 and 2 are decomposition products of \(S-[^{14}C]-\) carboxymethylmethionine (20). \([^{14}C]-\) Aspartic acid and \([^{14}C]phenylalanine\) were added as markers.

![Fig. 1. Ion exchange chromatography of the products obtained by acid hydrolysis of \([^{14}C]carboxymethylated\) enzyme.](http://www.jbc.org/)

![Fig. 2. Activity of dihydrofolate reductase as a function of the number of carboxymethyl groups incorporated.](http://www.jbc.org/)

![Fig. 3. Protection against loss of activity by various ligands.](http://www.jbc.org/)
hibitor which presumably binds at the site for dihydrofolate, since the inhibitor is an analogue of folate, an alternate substrate. The presence of aminopterin during alkylation almost completely prevented the loss in activity over an 18-hour period although about 0.8 labeled carboxymethyl group was incorporated during this interval. Examination of the hydrolysis products of the labeled enzyme showed that only methionine was carboxymethylated, and amino acid analysis following performic acid oxidation confirmed the loss of approximately 0.8 residue of methionine. The degree of protection provided by folate and dihydrofolate is less than that with aminopterin. However, whereas the dissociation constant of aminopterin from the binary complex is about 0.1 μM (Table I) so that the concentration used for protection is 5000 times the dissociation constant, equilibrium dialysis studies indicated that the binary dissociation constant for dihydrofolate is about 0.13 mM (see below), so that the protective concentration was only 7.6 times the latter. Although the dissociation of folate is not known it has a Michaelis constant similar to that for dihydrofolate (35), so that the protecting concentration was probably low compared with the dissociation constant, perhaps 10 to 20 times the latter. When it is also considered that complete degradation of the dihydrofolate was observed during the alkylation period it is not surprising that dihydrofolate was only able to afford rather poor protection and that even folate in the concentrations employed was only partially protective. NADPH, the reducing substrate, offers some protection, but NADP does not. A 2-fold increase in the concentrations of the NADP and NADPH did not significantly change the extent of protection. The possible significance of this protection is discussed later.

Binding of Substrates and Inhibitors to Native and Alkylated Reductase—Further evidence that the modified methionine(s) associated with loss of activity is located at the dihydrofolate-binding site was provided by fluorescence-quenching experiments. Fig. 4 shows the fluorescence emission spectra of the enzyme and of various enzyme complexes when they were excited at 290 nm, the excitation wavelength which produced greatest emission at 340 nm, the emission maximum for the protein tryptophans. This emission is decreased in the aminopterin-reductase complex and in the NADPH-reductase complex, but the latter also shows an emission maximum at 450 nm due to transfer of energy from tryptophan residues to bound NADPH. This energy transfer fluorescence is decreased when aminopterin or other inhibitors combine to form ternary complexes. Similar results have been obtained with the dihydrofolate reductases of E. coli (36), bacteriophage T4 (36), and L1210R murine lymphoma (37, 38).

Carboxymethylation of the enzyme had essentially no effect on the dissociation constant of the enzyme-NADPH binary complex (Table I) and on the magnitude of quenching of enzyme fluorescence by NADPH (Fig. 5). By contrast, binding of inhibitors was greatly affected by alkylation of methionine residues. In comparison with native enzyme and enzyme alkylated in the presence of aminopterin, enzyme alkylated in the absence of protective ligands showed two differences in behavior when it was titrated with aminopterin (Figs. 6 and 7). There is about a 20-fold higher dissociation constant for the enzyme-aminopterin complex (Table I), and the fluorescence of the alkylated enzyme-aminopterin complex is much greater

![Fig. 4. Emission spectra of dihydrofolate reductase and of various enzyme-ligand complexes when excited at 290 nm. Left panel: E, free enzyme (2.1 μM); E-aminopterin, 2.1 μM enzyme in the presence of 3.0 pM aminopterin; E-NADPH, 2.1 μM enzyme in the presence of 5.0 μM NADPH. Right panel: E-NADPH, 2.1 μM enzyme in the presence of 20.2 μM NADPH; E-NADPH-I, 2.1 μM enzyme, 20.2 μM NADPH, and 3.0 μM DTPCP. The buffer in all cases was 0.1 M potassium phosphate, pH 7.0).](http://www.jbc.org/)

![Table I](http://www.jbc.org/)

**Table I**

<table>
<thead>
<tr>
<th>Dissociation</th>
<th>$K_{eq}$ (M)</th>
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<tbody>
<tr>
<td></td>
<td>With native enzyme</td>
</tr>
<tr>
<td>E-NADPH + E+</td>
<td>7.25 x 10^{-7}</td>
</tr>
<tr>
<td>NADPH</td>
<td>9.54 x 10^{-8}</td>
</tr>
<tr>
<td>E-Aminopterin + E+</td>
<td>2.04 x 10^{-6}</td>
</tr>
</tbody>
</table>

![Fig. 5. Titration of the native enzyme and its carboxymethylated derivatives with NADPH. ▲, 1.18 μM native enzyme; ○, 1.15 μM enzyme which was carboxymethylated in the presence of aminopterin or 1.15 μM enzyme which was carboxymethylated in the absence of protecting agents. Other conditions were as described under “Methods.”](http://www.jbc.org/)
FIG. 6. Titration of the native enzyme and its carboxymethylated derivatives with aminopterin. O, native enzyme; •, enzyme alkylated in the presence of aminopterin; A, enzyme alkylated in the absence of protecting agents. The curve for the titration of modified enzyme (A) is expanded in Fig. 7. Conditions were the same as in Fig. 5.

FIG. 7. Fit of data for titration with inhibitors of enzyme carboxymethylated in the absence of protecting agents. Data from Figs. 6 and 9 shown on an expanded scale to indicate the fit to least squares curves computed by a nonlinear regression program for the theoretical equation. A, with aminopterin; A, with DTPCP in presence of saturating NADPH.

than the corresponding complex of the native enzyme. The latter observation suggests that there is a different orientation of tryptophan residues with respect to bound aminopterin in the complex of the alkylated enzyme compared with the orientation in the complex of the native enzyme. It should be noted that the least squares curves computed from the theoretical equation fit the data closely throughout the titration range. The closeness of the fit for the carboxymethylated enzyme is seen more clearly in Fig. 7.

In Fig. 8 are shown the circular dichroic spectra in the near ultraviolet for the native enzyme and its carboxymethylated forms. The molecular ellipticity in the aromatic region of the spectrum of the enzyme modified in absence of protective ligands is significantly different from the corresponding values for the native enzyme and that alkylated in presence of aminopterin. This confirms that carboxymethylation of methionine(s) changes the environment of aromatic residues, some of which may be at the aminopterin-binding site. Carboxymethylation caused essentially no change in the circular dichroic spectra of the enzyme in the far ultraviolet. An α helical content of about 15% was calculated, which is similar to the value for reductase from a methotrexate-resistant strain of E. coli B (11).

Modification of methionine residues also affects the binding of inhibitors in enzyme-NADPH-inhibitor ternary complexes. Aminopterin was unsuitable for these experiments because dissociation of aminopterin from the ternary complex was too small to measure (data not shown). This was true both for native enzyme and its alkylated forms. An inhibitor with a suitable dissociation constant for studying the ternary complex is DTPCP, an analogue of trimethoprim. Enzyme carboxymethylated while protected forms a ternary complex with this inhibitor and NADPH which is similar in fluorescence emission and dissociation to that formed by the native enzyme, but the enzyme carboxymethylated in absence of protective agents forms a ternary complex with a 30-fold increased constant for inhibitor dissociation (Table I) and considerably decreased quenching of the energy transfer fluorescence (Figs. 7 and 9). This again suggests that carboxymethylation of one
or more specific methionine residues changes the environment of the tryptophan near the inhibitor-binding site.

**Equilibrium Dialysis Studies**—The binding of dihydrofolate by native and carboxymethylated enzyme was measured by equilibrium dialysis (Table II). The results show that the complex of dihydrofolate with enzyme carboxymethylated in absence of pteridine ligands has a 10-fold higher dissociation constant than the similar complex with the native enzyme and that both modified and native enzyme have one binding site for dihydrofolate. Enzyme protected by aminopterin during carboxylation was unchanged with regard to binding of dihydrofolate. This is again consistent with the view that carboxymethylation modifies 1 or more methionine residues at or near the binding site for dihydrofolate.

**Kinetic Studies** To further compare the native enzyme and its carboxymethylated derivatives, kinetic constants were determined (Table III). By the use of Cleland's **Sequen program** (39) data were analyzed on an IBM-365 computer to obtain the best values of the kinetic constants and their standard errors. In comparison with native enzyme, the carboxymethylated forms of the enzyme show no great changes in their Michaelis constants or the dissociation constants for NADPH. There are some small differences, and in particular the Michaelis constant for dihydrofolate is doubled in enzyme with carboxymethylated in absence of protective agents, but this is a minor change compared with the increase in the dissociation constant for inhibitor release from the enzyme-NADPH-inhibitor complex described above.

**Discussion**

The evidence presented above demonstrates that when a total of 2 methionine residues of dihydrofolate reductase are carboxymethylated there is a substantial loss of catalytic activity. The protection studies and binding studies suggest that at least one of the carboxymethylated methionine residues is at the binding site of inhibitors and dihydrofolate.

One possible explanation for the loss of activity is that carboxymethylation causes extensive structural alterations in the enzyme. This seems unlikely because there was essentially no change in the circular dichroic spectrum of the enzyme in the far ultraviolet, and more importantly, both substrate-binding sites appear to be intact since the carboxymethylated enzyme is still able to form binary complexes with NADPH, dihydrofolate, and aminopterin and ternary complexes with NADPH and inhibitors.

Another explanation is that the carboxylation of methionine causes loss of activity because the residue(s) is directly involved in the binding of substrates, more specifically in the binding of dihydrofolate. This is an attractive possibility because the fluorescence-binding data show that after carboxymethylation the enzyme forms a binary complex with aminopterin and a ternary complex with NADPH and another inhibitor with much higher dissociation constants than the corresponding complexes of the native enzyme (Table I). Since there was no change in the constant for dissociation of NADPH from its binary complex the binding site of this substrate is not disturbed by alkylation. Dihydrofolate produces no significant changes in enzyme fluorescence or circular dichroism when it binds to enzyme or to the enzyme-NADP complex, but binding of dihydrofolate to native and modified enzyme was measured by equilibrium dialysis and was found to be decreased 10-fold in the modified enzyme. These data together with the data on inhibitor binding suggest that at least one methionine residue is at or near the binding site for inhibitors and dihydrofolate. However, they do not establish that the residue is actually involved in binding of these ligands. Decreased binding may be caused by steric hinderance or charge repulsion resulting from the introduction of the carboxymethyl group or by reaction of other residues at the binding site. Indeed the latter possibility is suggested by the fluorescence and circular dichroism data. Thus carboxymethylation changes the extent of quenching of protein fluorescence by aminopterin in the binary complex and by DTPCP in the ternary complex. The large change in the molar ellipticity in the aromatic region of the circular dichroic spectrum also suggests that alkylation alters the environment of aromatic residues in its vicinity.

Another possible explanation for the loss of activity is that carboxylation blocks a methionine which participates in the mechanism of catalysis so that alkylation completely inactivates the enzyme. If this were the case, then the small amount of enzyme activity after alkylation must be due to residual enzyme having this methionine unmodified, although other susceptible methionines might be partially or completely modified. The kinetics studies appear to be consistent with this since they show little change in the kinetic parameters (other than $V_{max}$), whereas a large increase in the Michaelis constant for dihydrofolate would be predicted from the binding studies on dihydrofolate and inhibitors. Unfortunately, greatly decreased binding of dihydrofolate to the carboxymethylated enzyme-NADPH complex would also result in ap-

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**Table II**

*Equilibrium dialysis measurements of binding of dihydrofolate by native enzyme, enzyme which had been alkylated in presence of aminopterin, and enzyme alkylated in absence of protecting agents*

<table>
<thead>
<tr>
<th>Enzyme treatment</th>
<th>No. of binding sites</th>
<th>$K_{diss}$ ($\mu$M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native enzyme $^a$</td>
<td>1.02 ± 0.1</td>
<td>0.130 ± 0.11</td>
</tr>
<tr>
<td>Enzyme carboxymethylated with aminopterin protection $^a$</td>
<td>0.91 ± 0.07</td>
<td>0.116 ± 0.06</td>
</tr>
<tr>
<td>Enzyme carboxymethylated without protection $^a$</td>
<td>1.03 ± 0.28</td>
<td>1.36 ± 0.29</td>
</tr>
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</table>

*Enzyme concentration 87.14 $\mu$M; dihydrofolate (688,000 cpm per $\mu$mol) at concentrations in the range 0.03 to 0.3 $\mu$M.

*Enzyme concentration 92.66 $\mu$M; dihydrofolate (727,000 cpm per $\mu$mol) at concentrations in the range 0.06 to 0.8 $\mu$M.

*Enzyme concentrations 1.08 mM; dihydrofolate (78,000 cpm per $\mu$mol) at concentrations in the range 0.3 to 3 mM.

**Table III**

*Kinetic constants for native reductase, and for enzyme alkylated in presence of aminopterin, in presence of NADPH, or in absence of protecting agents*

<table>
<thead>
<tr>
<th>Kinetic constant</th>
<th>Native enzyme</th>
<th>With aminopterin protection</th>
<th>With NADPH protection</th>
<th>With no protection</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{max}$ ($\mu$mol/min/mg)</td>
<td>37.8 ± 0.8</td>
<td>33.4 ± 0.8</td>
<td>19.9 ± 0.8</td>
<td>6.5 ± 0.2</td>
</tr>
<tr>
<td>$K_{NADPH}$ ($\mu$M)</td>
<td>4.9 ± 0.3</td>
<td>3.5 ± 0.3</td>
<td>4.1 ± 0.5</td>
<td>2.1 ± 0.2</td>
</tr>
<tr>
<td>$K_{NADPH}$ ($\mu$M)</td>
<td>2.3 ± 0.3</td>
<td>3.2 ± 0.7</td>
<td>1.6 ± 0.4</td>
<td>1.6 ± 0.3</td>
</tr>
<tr>
<td>$K_{DHF}$ ($\mu$M)</td>
<td>7.5 ± 0.4</td>
<td>6.0 ± 0.5</td>
<td>15.9 ± 1.3</td>
<td>15.6 ± 0.9</td>
</tr>
</tbody>
</table>

$^a$ Unpublished results.
parently inactive modified enzyme at all attainable concentrations of dihydrofolate. The ternary dissociation constant for dihydrofolate is not measurable directly (because the reaction proceeds), but if it is increased 30-fold for the carboxymethylated enzyme compared with native enzyme, as in the case of the inhibitor DTPCP, then the modified enzyme would indeed appear to be inactive. The inactivity of the modified enzyme is, therefore, not conclusive.

In studies on other enzymes a structural role has generally been assigned to methionine residues (see Table IV). In most cases the loss of activity which results from methionine modification is attributed to structural alterations or decreased binding of substrates (or both). In only one case (pig heart isocitrate dehydrogenase, Ref. 47), was there no evidence of a structural change or decreased binding of substrates, but the lack of change in the binding of substrates was based only on kinetics. Although no definite conclusion is possible at present it seems likely that in dihydrofolate reductase at least one methionine residue also plays a role in structure maintenance or substrate binding or both.

The partial protection against inactivation provided by NADPH (at a concentration 400 to 2000 times the binary dissociation constant) deserves some comment since alkylation does not affect NADPH binding. The protection may result from conformation changes that are induced at the dihydrofolate-binding site when NADPH binds and that partially shield the methionine which must be unmodified for activity. Evidence for such a conformation change is provided by the greatly decreased (more than 100-fold) dissociation constant for aminopterin release from the ternary complex (E-NADPH-aminopterin) as compared with the E-aminopterin complex. Since the enzymic mechanism is ordered with NADPH binding first (52), binding of NADPH must also greatly increase the tightness of dihydrofolate binding. Such an interpretation is consistent with lack of protection by NADP at a concentration 180 times the $K_{\text{diss}}$ value calculated from published data (52), since NADP would not produce the conformation change favorable for dihydrofolate binding.

**Acknowledgments**—Our thanks are due to Dr. D. L. Peterson for helpful advice regarding the fluorescence and circular dichroism experiments, to Wong ChorKin for carrying out the nonlinear regression calculations on the fluorescence quenching data, to Frances Reifenstahl for preparation of the reduc-tase, and to Paul Lennette for providing the carboxymethyl derivatives of histidine.

**Table IV**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Methionines modified</th>
<th>Type of modification</th>
<th>Activity of original</th>
<th>Increase in $K_m$</th>
<th>Evidence for conformational change</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypsin</td>
<td>2</td>
<td>Alkylation</td>
<td>%</td>
<td>0</td>
<td>Physical properties of denatured enzyme</td>
<td>40</td>
</tr>
<tr>
<td>Trypsin</td>
<td>2</td>
<td>Oxidation</td>
<td>ATEE, a 62</td>
<td>~0</td>
<td>Decreased thermal stability, lower affinity for benzamidine, decreased rate of acylation, and deacylation of essential Ser Tyr</td>
<td>40</td>
</tr>
<tr>
<td>Ribonuclease</td>
<td>4</td>
<td>Oxidation</td>
<td>13</td>
<td></td>
<td>Spectrophotometric titration of Tyr</td>
<td>11</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>2</td>
<td>Oxidation</td>
<td>5</td>
<td></td>
<td>Increased susceptibility to trypsin, increased levorotation, decreased thermal stability</td>
<td>42</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>2</td>
<td>Oxidation</td>
<td>20–100 for peptides, 25 for acetyltirosine ethyl ester, 0.25 for acetyltirosinamide</td>
<td>3.5 ×</td>
<td>None</td>
<td>12</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>1</td>
<td>Alkylation</td>
<td>20 in standard assay, but little change in $V_{max}$</td>
<td>10 ×</td>
<td>None</td>
<td>44</td>
</tr>
<tr>
<td>β-Galactosidase</td>
<td>1</td>
<td>Alkylation</td>
<td>0</td>
<td></td>
<td>Decreased NADPH binding by fluorescence titration</td>
<td>46</td>
</tr>
<tr>
<td>β-Galactosidase</td>
<td>1</td>
<td>Met replacement by norleucine</td>
<td>38</td>
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<td>None</td>
<td>45</td>
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<tr>
<td>Malate dehydrogenase</td>
<td>4</td>
<td>Alkylation</td>
<td>0</td>
<td></td>
<td>None</td>
<td>45</td>
</tr>
<tr>
<td>Pig heart isocitrate dehydrogenase</td>
<td>1</td>
<td>Alkylation</td>
<td>0</td>
<td></td>
<td>None</td>
<td>47</td>
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<tr>
<td>Glutamate dehydrogenase</td>
<td>2</td>
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<td>10</td>
<td></td>
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<tr>
<td>Azotobacter vinelandii isocitrate dehydrogenase</td>
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<td>Alkylation</td>
<td>1</td>
<td></td>
<td>None</td>
<td>49</td>
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<tr>
<td>Chymotrypsinogen</td>
<td>1</td>
<td>Oxidation</td>
<td>700–800 for p-nitrophenyl esters</td>
<td>20 for peptide activity</td>
<td>Increase in rate of reaction with DFP d</td>
<td>50</td>
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<tr>
<td>Carboxypeptidase</td>
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<td>Alkylation</td>
<td></td>
<td></td>
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<td>51</td>
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</table>

a ATEE = N-acetyl-L-tyrosine ethyl ester.
b BAEE = α-N-benzoyl-L-arginine ethyl ester.
c BAPNA = α-N-benzoyl-L-arginine p-nitroanilide.
d DFP = diisopropyl fluorophosphate.
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