Effect of N-Bromosuccinimide Modification on Dihydrofolate Reductase from a Methotrexate-resistant Strain of Escherichia coli

ACTIVITY, SPECTROPHOTOMETRIC, FLUORESCENCE, AND CIRCULAR DICHROISM STUDIES*

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SUMMARY

When dihydrofolate reductase from a methotrexate-resistant strain of Escherichia coli B, MB 1428, is treated with approximately a 5 mol ratio of N-bromosuccinimide (NBS) to enzyme at pH 7.2 and assayed at the same pH, there is a 40% loss of activity due to the modification of 1 histidine residue and possibly 1 methionine residue before oxidation of tryptophan occurs. The initial modification is accompanied by a shift of the pH for maximal enzymatic activity from pH 7.2 to pH 5.5. Upon further treatment with N-bromosuccinimide, the activity is gradually reduced from 60 to 0% as tryptophan residues become oxidized. An NBS to enzyme mole ratio of approximately 20 results in 90% inactivation of the enzyme. When the enzyme is titrated with NBS in 6 M guanidine HCl, 5 mol of tryptophan react per mol of enzyme, a result in agreement with the total tryptophan content as determined by magnetic circular dichroism.

The 40% NBS-inactivated sample possesses full binding capacity for methotrexate and reduced triphosphopyridine nucleotide, and the K_a values for dihydrofolate and TPNH are the same as for the native enzyme. After 90% inactivation, only half of the enzyme molecules bind methotrexate, and the dissociation constant for methotrexate is 40 nm as compared to 4 nm for native enzyme in solutions of 0.1 M ionic strength, pH 7.2. Also, TPNH is not bound as tightly to the modified enzyme-methotrexate complex as to the unmodified enzyme-methotrexate complex. Circular dichroism studies indicate the 90% NBS-inactivated enzyme has the same α helix content as the native enzyme but less β structure, while the 40% inactivated enzyme is essentially the same as the native enzyme.

Protection experiments were complicated by the fact that NBS reacts with the substrates and cofactors of the enzyme. Although protection of specific residues was not determined, it was clear that TPNH was partially protected from NBS reaction when bound to the enzyme, and the enzyme was not inactivated by NBS until the TPNH had reacted.

Dihydrofolate reductase is an enzyme of considerable pharmacological interest since it is the target site for a number of chemotherapeutic agents (1). These include the immunosuppressive drugs, methotrexate (2) and aminopterin (3); the antimalarial drug, pyrimethamine (4); and the antibiotic, trimethoprim (5). The enzyme used in the present study is isolated from a methotrexate-resistant strain of Escherichia coli B which yields elevated levels of the enzyme as compared to the sensitive parent strain. The enzyme is composed of one polypeptide chain of molecular weight 17,600, and its primary structure has recently been determined (6).

Previous circular dichroism, fluorescence, and ultraviolet difference spectroscopy studies performed in this laboratory (7-11) implicated tryptophan in the binding of substrates, cofactors, and several inhibitors to dihydrofolate reductase. Circular dichroism bands of the enzyme due to tryptophyl residues were sharpened slightly by the binding of triphosphopyridine nucleotide (7). Also, ultraviolet difference spectra observed when pyridine nucleotides were bound to the enzyme indicated an alteration in the environment of tryptophan (10, 11). The partial quenching of the enzyme emission fluorescence on binding of folate analogs and TPNH suggested the perturbation of tryptophyl residues (8, 9). Since chemical modifications have proven to be excellent probes of the structure-function relationship of biological macromolecules (12), it was felt that a tryptophan reagent might provide valuable information on the role of tryptophan in the catalytic activity of this enzyme.

Several researchers have utilized N-bromosuccinimide to modify dihydrofolate reductase from chicken liver (13) and methotrexate-resistant strains of Streptococcus faecium (14) and Lactobacillus casei (15). In the first of these studies (13), Freisheim and Huennekens reported an initial 2- to 3-fold increase in catalytic activity upon NBS titration which was attributed to oxidation of protein sulphydryl groups. Further additions of NBS resulted in complete loss of activity corresponding to oxidation of 1 mol of tryptophan per mol of enzyme. They also observed apparent protection by both dihydrofolate and TPNH against activity loss. In the second study, Warwick

* The abbreviations used are: NBS, N-bromosuccinimide; MCD, magnetic circular dichroism; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); p-HMB, p-hydroxymercurobenzoate; DTT, dithiothreitol.
et al. (14) indicated complete loss of activity upon NBS titration was concomitant with the oxidation of 2 m eq of tryptophan. In addition to noting apparent substrate and cofactor protection, they found that the tryptophans contribute equally to the aromatic side chain Cotton effect in the 200- and 290-nm regions of the circular dichroism spectrum of the native enzyme. Liu and Dunlop (15) reported that the oxidation of 1 of the 3 tryptophyl residues in \textit{L. casei} resulted in complete loss of enzymatic activity. They observed mutual protection of TPNH and enzyme against NBS oxidation.

The current study explores the NBS treatment of dihydrofolate reductase from a methotrexate-resistant strain of \textit{E. coli} B and provides further evidence for the species specificity of this enzyme. This work examines the effect of the modification on the activity pH profile, the binding capacity for substrates, and the conformation of the modified enzyme through activity, fluorescence, and circular dichroism studies. The study clearly indicates the complicated nature of substrate protection experiments, and, as also observed by Summers (16), NBS reacts with many of the substrates, cofactors, and inhibitors of dihydrofolate reductase.

\section*{Materials and Methods}

\textit{N}-Bromosuccinimide was purchased from Aldrich Chemical Co. and was recrystallized at least twice from distilled water before use. Unless the experiments were performed on 2 successive days, the material was freshly recrystallized. All solutions of NBS were maintained in the dark.

Triphosphopyridine nucleotide was purchased from F-L Biochemicals, and methotrexate (amethopterin) from Nutritional Biochemical Co. Tri(hydroxy)aminomethane was enzyme grade (ultrapure) from Sigma-Chemical. p-Hydroxymercurationbenzoate was obtained from Sigma. Guanidine HCl, ultrapure, was purchased from Schwan-Mann. All other chemicals were reagent grade.

Dihydrofolate was prepared by the dithionite method of Putnam (17) as modified by Blakely (18) from folic acid purchased from Cyclo Chemical Co. The dihydrofolate was stored at $-20^\circ$ in 5 mM HCl and 50 mM 2-mercaptoethanol.

The concentrations of TPNH, methotrexate, and dihydrofolate were determined spectrophotometrically by means of a molar extinction of $e_{235} = 6200$ for TPNH at pH 7.2 (19), $e_{235} = 22,300$ and $e_{221} = 22,100$ for methotrexate at pH 13 (20), and $e_{235} = 28,000$ for dihydrofolate at pH 7.2 (21).

Standard buffers used were as follows: Buffer A, 0.05 M Tris-HCl, pH 7.2, at 23$^\circ$; Buffer B, 0.05 M Tris-HCl, 0.05 M NaCl, pH 7.2, at 23$^\circ$; Buffer C, 0.05 M Tris-HCl, 0.30 M NaCl, pH 7.2, at 23$^\circ$; and Buffer D, 0.01 M acetic acid, 0.10 M NaCl, pH 5.5, at 23$^\circ$.

\textbf{Enzyme Preparation—}Dihydrofolate reductase was isolated from \textit{E. coli} B (strain MB 1428) by the procedure of Poe et al. (22) as modified by Williams et al. (8) with a methotrexate-aminoethyl-Sepharose affinity resin.

The protein content was determined by the microbiur method of Goa (23) with lysozyme and chymotrypsin as standards and by methotrexate titrations as monitored by quenching of enzyme fluorescence (8).

\textbf{Enzymatic Assay—}The procedure for the assays was that of Poe et al. (22) in Buffer A at 25$^\circ$. The assays were performed on a Gilford 2000 multiple sample absorbance spectrophotometer or a Cary 14 spectrophotometer. One unit of enzyme is defined as the amount of enzyme which reduces 1 nmol of substrate per min by means of a difference molar extinction coefficient of 11,650 at 340 nm for the conversion of dihydrofolate and TPNH to tetrahydrofolate and TPN$^+$ (24). The standard assay buffer contained approximately 90 mM TPNH and 100 mM dihydrofolate.

The pH profile of the enzymatic rate for the native and NBS-modified enzyme was determined in 0.01 M buffers of sodium acetate, sodium phosphate, and Tris HCl, containing 0.1 M NaCl.

The Michaelis constants of the native and NBS-modified enzyme for TPNH were measured in Buffer B and 100 mM dihydrofolate, and the $K_m$ for dihydrofolate was determined in Buffer B and 90 mM TPNH. The slope and intercept of the reciprocal plot of velocity versus substrate concentration were used to evaluate the $K_m$.

\textit{N}-Bromosuccinimide Modification—Small aliquots (2 to 10 $\mu$l) of $5 \times 10^{-4}$ to $10^{-3}$ M NBS were added serially to solutions of 1 to 20 $\mu$l of dihydrofolate reductase (1.5 to $3 \times 10^{-3}$ M) in either Buffer A or Buffer C, pH 7.2. Additions were made slowly into the vortex as the solution was stirred on an air-driven magnetic stirrer. This procedure minimized formation of turbidity in the solution. After stirring for 2 min, the spectrum from 400 nm to 240 nm was recorded on a Cary 14 spectrophotometer. A control was run to verify that the alterations in the spectrum and enzymatic activity were complete within 2 min. At each point of the titration, small aliquots of enzyme (1 to 2 $\mu$l) were withdrawn for determination of enzymatic activity in Buffer A, pH 7.2, or Buffer B, pH 5.5.

The NBS titrations were also performed in the presence of a 1:1.5 ratio of TPNH to enzyme and a 5-fold excess of folate to enzyme in order to establish if any of these compounds protected the enzyme from modification. Appropriate controls were run to determine the effect of NBS on these ligands as well as its effect on methotrexate.

The oxidation of tryptophan by NBS results in the formation of a new chromophore with a $323 \text{ nm}$ absorption peak at 280 nm and a concomitant loss of absorption at 280 nm (25). The total moles of tryptophan oxidized per mol of enzyme can be calculated with the following equation (26, 27):

$$\text{Moles of tryptophan oxidized per mol of enzyme} = \frac{\Delta A_{323} \text{ nm}}{\text{A}_{323} \text{nm} (1.31)}$$

\textbf{Determination of Tryptophan Content—}The tryptophan content of \textit{E. coli} B dihydrofolate reductase (MB 1428) was determined by magnetic circular dichroism studies in solutions of 0.1 M NaCl, pH 7.2, and by NBS protein titrations in 0 M guanidine HCl. The first method utilizes the MCD band of tryptophan at 291 nm (28). Measurements were performed by Dr. Jesse True on an instrument at Princeton University, similar to that described by Jasperson and Schnatterly (29). All MCD experiments were conducted at room temperature in a 0.2-cm pathlength cell. The MCD spectra of dihydrofolate reductase, lysozyme, and tryptophan were recorded, and the magnitude of the reductase 291-nm MCD band was correlated with that of the standards to yield the tryptophan content. The ultraviolet absorbance of the samples at 280 nm ranged from 3 to 6 absorbance units (1-cm pathlength). The ultraviolet spectra of 1:10 dilutions were recorded on a Cary 15 spectrophotometer. The extinction coefficients used for lysozyme, tryptophan, and dihydrofolate reductase were 3.83 X 10$^4$ at 280 nm (26, 27) and 5.71 X 10$^4$ at 279 nm (28), and 4.0 X 10$^4$ at 282 nm (7), respectively.

The second method consisted of an NBS titration of the denatured protein in a buffer of 6 M guanidine HCl, 0.05 M Tris-HCl, pH 6.5. NBS addition was continued until there was no further loss of absorption at 280 nm. The enzyme concentration was determined by the microbiur method of Goa (23) and the total moles of tryptophan per mol of enzyme were calculated with the use of the equation shown above.

\textbf{Determination and Masking of Reactive Sulphhydryl Groups—}5'S-Dithiobis(2-nitrobenzoic acid), a reagent described by Ellman (31, 32), was used to establish the number of reactive sulphhydryl groups. Aliquots of native, NBS-modified enzyme solutions (1 to 2 X 10$^{-4}$ M in buffer B), and buffer were added to sufficient solid guanidine HCl and ethylenediaminetetraacetate to prepare 1-mL enzyme and buffer samples in 6 M guanidine HCl, 10$^{-4}$ M EDTA, pH 6.5. Then 50 $\mu$l of 4 X 10$^{-4}$ M DTNB in 0.05 M Tris-HCl, 0.05 M NaCl, pH 8.0, were added to 0.8 $\mu$l of each of the above enzyme samples and to 0.8 $\mu$l of the corresponding buffer. The absorbance at 412 nm was monitored until the maximum change occurred. The number of reactive thiol groups were calculated from the change of absorbance of 412 nm with the use of glutathione as a DTNB standard under the same conditions.

While dihydrofolate reductase has 2 cysteines (6), in the absence of denaturing reagents only one of these is specifically modified by either DTNB or $p$-hydroxymercurationbenzoate. The latter
were determined by the method of Kurganov (36) as applied by Williams et al. (8). The dissociation constants for TPNH and modified enzyme were calculated by the procedure of Williams et al. (9). The "difference" technique of Kurganov (36) is particularly valuable since it does not require a preliminary evaluation of the concentration of binding sites. It yields both the dissociation constant, $K_d$, and the concentration of binding sites when there is only one binding site.

**RESULTS**

**N-Bromosuccinimide Modification of Native Dihydrofolate Reductase**—As shown in Fig. 1 when the enzyme is titrated with NBS there is a 40% loss of activity at pH 7.2 before any substantial loss of absorbance at 280 nm occurs. At a 42-fold excess of NBS, all enzymatic activity is lost and the decrease in optical density corresponds to the oxidation of 3.6 mol of tryptophan per mol of enzyme. Further additions of NBS cause a large increase in turbidity. The initial consumption of NBS with no oxidation of tryptophan suggests the modification of other amino acid residues, which results in inactivation of the enzyme.

Several researchers have shown that cysteine and cystine are easily oxidized by NBS (13, 17, 37, 38). The total number of reactive sulfhydryl groups on the unmodified and modified dihydrofolate reductase were found by titration with DTNB in solutions of 6 M guanidine HCl, 10$^{-3}$ M EDTA. Table 1 indicates that in the 40% inactivated enzyme, one cysteine has been modified.

**Fluorescence Measurements**—The fluorescence studies were conducted on an Amino-Bowman spectrophotofluorometer equipped with a 150-watt Hanovia xenon light source and an RCA 1P28 photomultiplier. The sensitivity was maintained between 50 and 100%. NBS-modified enzyme was prepared as described under "Materials and Methods." Excess NBS reagent was removed either by chromatography on a Sephadex G-25 column previously equilibrated in 0.1 M acetic acid. The respective peak cuts were titrated with p-HMB by the procedure of Boyer (33).

**Circular Dichroism Studies**—A Cary 60 recording spectropolarimeter equipped with a 1.5 cm light path was employed for detection of residual methionine sulfoxide.
oxidized by NBS. In the 90% NBS-inactivated enzyme, 1.4 mol of cysteine per mol of enzyme have been oxidized. This analysis assumes that the conditions developed for stoichiometric sulphydryl reactions for the native enzyme are also applicable for the NBS-modified enzyme.

When the DTNB titrations are performed in Buffer C (in the absence of detergent), only the readily accessible sulphydryl group reacts on the native enzyme, and no sulphydryl groups react in the 40% and 90% NBS-inactivated enzyme solutions. These results suggest that the easily accessible sulphydryl group is oxidized by NBS. If this sulphydryl group is first masked by p-HAB, the excess p-HAB stripped, and the enzyme is then titrated with NBS, the progress of the reaction is exactly the same as observed with native enzyme (i.e., 40% loss of activity at pH 7.2 before substantial loss of absorbance at 280 nm). Thus, the easily reacted sulphydryl group is not responsible for the NBS inactivation.

This conclusion was further confirmed by incubating the unmodified and the 40% and 90% NBS-modified enzyme solutions in 10 mM DT, 6 mM guanidine-HCl, pH 8, for 72 hours. While all of the samples now demonstrated 2 mol of cysteine reacting with p-HAB per mol of enzyme, the samples exhibited no change in enzymatic activity at either pH 5.5 or pH 7.2 as a result of sulphydryl reduction. Hence, cysteine oxidation may be eliminated as contributing to the 40% NBS inactivation of the enzyme, and it cannot be the sole cause of the 90% inactivation. While the exact oxidation state of the cysteines was not determined, it is not likely they were oxidized to a state above sulfinic acid since they were reduced by DTDT treatment and higher states would not be.

To examine possible modification of other residues, the 40% inactivated enzyme (no tryptophan oxidation) and the 90% inactivated enzyme (≈2 mol of tryptophan oxidized per mol of enzyme) were subjected to acid and alkaline hydrolysis and subsequent amino acid analysis. The results of the amino acid analyses are shown in Table II. There are two important conclusions derived from these analyses. First, the only residue in the acid hydrolysates which is consistently low in either the 40% or 90% NBS inactivated sample is histidine. Secondly, as revealed by alkaline hydrolysates, 1 to 2 mol of methionine sulfoxide are formed during the titration. The methionine sulfoxide is not reduced by the DTDT treatment described above, but, it is reduced by 2-mercaptoethanol. However, the reduction does not restore either the activity of the enzyme or the histidine.

Another possible cause of activity loss would be cleavage of the peptide chain which can result from NBS modification (37, 39, 40). Hence, the 40% and 90% inactivated samples were subjected to end group analysis as described under “Materials and Methods.” The analysis of both samples showed only methionine as an NBS terminus, the same as for native enzyme; thus it appears that under the mild conditions of reaction no cleavage occurred. The intensity of the methionine end group was the same as that found with native enzyme, hence it is unlikely that cleavage occurred exposing a second methionyl residue.

**Determination of Tryptophan Content**—The total tryptophan content was determined by magnetic circular dichroism and NBS titration in 6 mM guanidine HCl, pH 7.2. MCD results indicate there are 4.94 mol of tryptophan per mol of enzyme with L-tryptophan as a standard and 5.00 tryptophans with lysozyme as a standard. NBS titration of denatured dihydrofolate reductase in 6 mM guanidine HCl yields 4.9 mol of tryptophan oxidized per mol of enzyme. These results are in agreement with the number of tryptophans recently reported in the sequence of the enzyme (6).

**pH Activity Profile**—The activity of the enzyme modified with NBS at pH 7.2 was determined as a function of pH in the buffers described in the legend of Fig. 2. As shown in this figure, as the enzyme is titrated with NBS, the pH for maximal activity is gradually shifted from pH 7.2 to pH 5.5. The enzyme samples used in these experiments were stripped of excess NBS by dialy-

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### Table II

<table>
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<th>Amino acid</th>
<th>Theory</th>
<th>Average</th>
<th>Native</th>
<th>40% Inactivated</th>
<th>90% Inactivated</th>
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<tr>
<td>Histidine</td>
<td>5</td>
<td>4.8 ± 0.2</td>
<td>4.0 ± 0.3</td>
<td>4.3 ± 0.3</td>
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<tr>
<td>Methionine</td>
<td>5</td>
<td>4.2 ± 0.3</td>
<td>4.1 ± 0.3</td>
<td>3.9 ± 0.2</td>
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<tr>
<td>Methionine sulfoxide</td>
<td>0</td>
<td>0</td>
<td>-1</td>
<td>-1</td>
<td>-1</td>
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<tr>
<td>Tyrosine</td>
<td>4</td>
<td>4.0 ± 0.1</td>
<td>4.0 ± 0.2</td>
<td>3.7 ± 0.5</td>
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<tr>
<td>Lysine</td>
<td>7</td>
<td>7.0 ± 0.1</td>
<td>6.9 ± 0.2</td>
<td>7.0 ± 0.5</td>
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<tr>
<td>Arginine</td>
<td>8</td>
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<td>7.9 ± 0.2</td>
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<tr>
<td>Cysteine</td>
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<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
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<td>Tryptophan</td>
<td>5</td>
<td>4.1 ± 0.5</td>
<td>4.2 ± 0.6</td>
<td>1.9 ± 0.4</td>
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</table>

* As reported in Ref. 6.
* Determined by alkaline hydrolysis. All other amino acids were determined by acid hydrolysis. The methionine and tryptophan results were not corrected for partial destruction by acid and alkaline hydrolyses, respectively. Also, methionine sulfoxide is reduced to methionine by acid hydrolysis.

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![Fig. 2](http://www.jbc.org/)
Fig. 3. NBS titration of dihydrofolate reductase in 0.05 M Tris-HCl, 0.30 M NaCl, pH 7.2, as assayed at pH 5.5 in 0.01 M acetate, 0.10 M NaCl. Inset, change in absorbance of enzyme at 280 nm as titrated with NBS and corresponding moles of tryptophan oxidized (TRP<sub>ox</sub>) per mol of enzyme. Enzyme concentration, 1.71 x 10<sup>-8</sup> M.

sis, and the activities were normalized to the same protein concentration as determined by the microburet procedure described under "Materials and Methods."

As indicated in Fig. 2, when the enzyme is titrated with NBS at pH 7.2 and assayed at pH 5.5, there is an initial increase in activity of the partially modified enzyme. Fig. 3 shows the complete NBS titration as assayed at pH 5.5. The partially modified enzyme exhibits approximately twice the activity of native enzyme, but as tryptophan oxidation occurs, the enzyme is completely inactivated. It is apparent from this figure that the initial modification is not complete before tryptophan oxidation is initiated since the activity maximum at pH 5.5 is not achieved until 0.3 mol of tryptophan per mol of enzyme has been oxidized.

**TPNH Protection Studies**—It was of interest to determine if the substrates, cofactors, or inhibitors could specifically protect the enzyme against inactivation. These experiments were complicated by the fact that, contrary to results published by Warwick et al. (14), NBS does react with these compounds, with the reaction often accompanied by large ultraviolet spectral perturbations.

The curves in Fig. 4 are typical spectra for the titration of TPNH with NBS. The initial decrease of absorbance at 340 nm and increase at 260 nm would suggest the conversion of TPNH to TPN<sup>+</sup>. The formation of one species is further supported by the existence of an isosbestic point at 290 nm up to a 1:1 molar ratio of NBS to TPNH. But, in contrast with the effect of NBS on DPNH reported recently by Summers (16), there are further changes beyond a 1:1 molar ratio which are accompanied by a decrease in absorbance at 260 nm. Since the total dilution between Curve 0 and Curve 4 is only 2%, the observed changes are not due to dilution and must be attributed to the formation of a new species. Similar changes, though of smaller magnitude, were observed when the study was repeated with DPNH.<sup>a</sup>

Even though TPNH reacts with NBS, it was felt that TPNH might still provide some protection of the enzyme from NBS modification. Since there are two TPNH-binding sites on this strain of dihydrofolate reductase (9), the NBS titrations were performed under ionic strength conditions such that at a 1:1 molar ratio of TPNH to enzyme, essentially the first site was saturated, and at a 2:1 ratio both sites were occupied (9). The results of this study are shown in Fig. 5. Fig. 5A indicates that the molar ratio of NBS to enzyme required to inactivate the enzyme increases as the concentration of TPNH increases. But, once the activity loss begins, the rate of inactivation as a function of NBS concentration is very similar in each case.

Since most of the absorbance changes at 340 nm are due to the reaction of TPNH with NBS, the rate of the reaction of free TPNH and enzyme-bound TPNH may be compared. Figure 5B illustrates that the molar ratio of NBS to TPNH for saturation of changes at 340 nm is about 3-fold larger for bound TPNH than for free TPNH. Thus, TPNH bound to the enzyme is protected from reaction with NBS. However, this apparent protection may be due to the fact that there are more reactive sites on the enzyme-TPNH complex than on TPNH.

The initial increase and subsequent loss of absorbance at 280 nm exhibited in Fig. 5C corresponds to the reaction of enzyme-bound TPNH with NBS. A comparison of Figs. 5A and 5C indicates that there is little loss of activity upon NBS titration of the enzyme-TPNH complex until the TPNH reacts with NBS. A fluorescence study<sup>4</sup> of the binding of NBS-treated TPNH to the enzyme as compared to TPNH binding indicated NBS-treated TPNH is bound more weakly than TPNH, with the high

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<sup>a</sup> Unpublished results.
Fig. 5. TPNH protection against NBS modification of dihydrofolate reductase (DHFR). In all cases enzyme concentration was $1.72 \times 10^{-4}$ M in 1 ml of the same buffer as Fig. 4. A, loss of activity as function of NBS concentration with following ratios of TPNH to enzyme: $\bullet$, $r = 0$; $O-O$, $r = 1$; $X-X$, $r = 2$. B, change of TPNH absorbance at 340 nm as function of NBS concentration. $\bullet$, free TPNH, $3.44 \times 10^{-4}$ M; $O-O$, $r = 0$; $O-O$, $r = 1$; $X-X$, $r = 2$. C, change of absorbance at 280 nm as function of NBS concentration with following ratios of TPNH to enzyme: $\bullet$, $r = 0$; $O-O$, $r = 1$; $X-X$, $r = 2$.

Table III

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Enzyme concentration</th>
<th>Ratio of total TPNH to enzyme</th>
<th>Ratio of bound TPNH to enzyme</th>
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<tbody>
<tr>
<td>Native</td>
<td>$10^{-4}$ M</td>
<td>1.13</td>
<td>3.7</td>
</tr>
<tr>
<td>40% NBS inactivated</td>
<td>1.23</td>
<td>3.3</td>
<td>2.0 ± 1</td>
</tr>
<tr>
<td>90% NBS inactivated</td>
<td>0.96</td>
<td>3.9</td>
<td>1.3 ± 1</td>
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</table>

affinity site having a dissociation constant of greater than $10^{-5}$ M.

Folate Protection Studies—Although folate, dihydrofolate, and methotrexate provide apparent protection to the enzyme against NBS modification, they are not suitable for unequivocal protection studies since they react with NBS, in some cases with no accompanying ultraviolet spectral changes.$^6$

Molar Extinction Coefficient—The extinction coefficients ascertained by the microbiuret technique were $4.2 \times 10^{4}$ M$^{-1}$ cm$^{-1}$ for the 40% sample and $3.2 \times 10^{4}$ M$^{-1}$ cm$^{-1}$ for the 90% inactivated sample. The molar extinction coefficient for the native enzyme was reported previously (7) as $4.0 \pm 0.4 \times 10^{4}$ M$^{-1}$ cm$^{-1}$. These extinction coefficients were used in the following sections for determining the concentrations of the enzyme.

Binding of TPNH to N-Bromosuccinimide-modified Enzyme by Ultrafiltration—The average number of moles of TPNH bound per mol of NBS-modified enzyme was determined by ultrafiltration. The results are shown in Table III. The 40% inactivated sample binds 2 mol of TPNH per mol of enzyme, the same as the native enzyme. But the 90% inactivated sample has less TPNH-binding capacity with the molar ratio becoming $1.3 \pm 0.1$.

Fluorescence Studies—As reported previously (8), the fluorescence emission spectrum (Fig. 6, Curve B) of dihydrofolate reductase from a methotrexate-resistant strain of E. coli is typical of that of a tryptophan-containing protein. When excited at 290 nm, there is a strong emission maximum at 347 nm. The$^6$ M. Poe, unpublished NMR results.

Fig. 6. Fluorescence emission spectra of native and NBS-modified dihydrofolate reductase. Activation wavelength was 290 nm. The enzyme concentration for each case was determined by the microbiuret technique described under "Materials and Methods" to be $1.5 \times 10^{-4}$ M in same buffer as in Fig. 4. Curve A, 40% NBS-enzyme; Curve B, native enzyme; Curve C, 90% NBS-enzyme.

effect of NBS modification on the fluorescence emission spectrum of the enzyme is shown in Fig. 6. The concentration of all samples was $1.5 \times 10^{-4}$ M in 0.10 M ionic strength buffer with the use of the molar extinction coefficients determined by microbiuret. The fluorescence intensity of the 40% NBS-inactivated sample is about 5% greater than that of the native sample. Since the extinction coefficients have a 10% error, the fluorescence yields of the native and 40% inactivated sample are approximately equal. But a significant difference is seen in the 90% NBS-inactivated sample, Curve C, as compared to the other two. There is a definite loss of fluorescence intensity which corresponds to the oxidation of tryptophan.

$^6$ M. Poe, unpublished NMR results.
FIG. 7. Methotrexate (MTX) titration of NBS-modified dihydrofolate reductase as monitored by fluorescence quenching. The activation wavelength was 290 nm, and the emission intensity was recorded at 350 nm in same buffer as in Fig. 4. The concentration of the enzyme was determined by microbiuret as described in the text. •, 40% inactivated enzyme, 2.78 x 10^{-9} mol in 2.0 ml of buffer; ○, 90% inactivated enzyme, 3.64 x 10^{-9} mol in 2.0 ml of buffer.

TABLE IV

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Enzyme concentration</th>
<th>$K_D$</th>
<th>Concentration of methotrexate binding site $\mu$M</th>
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<tr>
<td>Native</td>
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<td>4 ± 2 x 10^{-6}</td>
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</tr>
<tr>
<td>40% NBS inactivated</td>
<td>1.39 x 10^{-6}</td>
<td>0 ± 3 x 10^{-4}</td>
<td>1.31 x 10^{-6}</td>
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<tr>
<td>90% NBS inactivated</td>
<td>1.82 x 10^{-6}</td>
<td>4 ± 2 x 10^{-4}</td>
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</tbody>
</table>

* Calculated with molar extinction coefficients determined by microbiuret as described under "Materials and Methods."  
* Predicted from Kurganov's difference technique for analysis of ligand-enzyme association data (36).  
* As published in Ref. 8.

The binding of methotrexate to the 40% and 90% NBS-inactivated dihydrofolate reductase was monitored by enzyme emission fluorescence. The titrations shown in Fig. 7 were performed as described by Williams et al. (8) and analyzed by the "difference" technique of Kurganov (36). The $K_D$ values, the concentration of methotrexate-binding sites, and the enzyme concentrations are tabulated in Table IV. This table reveals that the 40% inactivated enzyme is virtually identical with the native enzyme in its capacity to bind methotrexate, but the 90% inactivated enzyme has a dissociation constant which is almost an order of magnitude weaker than that of the native enzyme and only half of the enzyme in solution binds methotrexate.

The enhancement of TPNH fluorescence was used to study the binding of TPNH to the 40% and 90% NBS-inactivated samples. The procedure and method of analysis were described in detail for native dihydrofolate reductase by Williams et al. (9). As reported in that study, E. coli dihydrofolate reductase, strain MB 1428, binds two molar equivalents of TPNH with dissociation constants of $K_1 = 0.02 \pm 0.1 \mu M$ and $K_2 = 0.6 \pm 0.1 \mu M$ in buffer of 0.10 M ionic strength, pH 7.2. The 40% NBS-inactivated sample is the same as the native enzyme in its fluorescence behavior and its affinity for TPNH in the presence and absence of methotrexate. However, the results shown in Fig. 8, Curve B, for the binding of TPNH to the 90% inactivated enzyme indicate that TPNH is bound more weakly than to native enzyme since the titration has not reached saturation at 20 mmol TPNH. It is not possible to determine the dissociation constant for the modified enzyme from these data since this would require knowing whether all the enzyme in solution still binds two molar equivalents of TPNH and the relative contribution of both sites to the fluorescence enhancement. In addition, the curvature in the TPNH titration of the 90% inactivated enzyme-methotrexate complex (Curve C) suggests that the dissociation constant for binding of TPNH to the 1:1 enzyme-methotrexate complex may be larger than for the native enzyme where binding was essentially stoichiometric. This will be illustrated more clearly in the circular dichroism experiments described in the following section.

Circular Dichroism Studies—Circular dichroism enables one to monitor conformational changes as well as determine dissociation constants. Experiments similar to those described by Greenfield et al. (7) were performed on the 40% and 90% NBS-inactivated enzyme. The far ultraviolet circular dichroism of the 40% inactivated sample is identical with that of the native enzyme with a peak of negative ellipticity at 220 nm. The calculated $\alpha$ helical content (-40%) and $\beta$ structure (50 to 60%) are the same as observed for the native enzyme (7); therefore, the modification of the histidine, methionine, and cysteine residues does not result in a large change of the enzyme conformation. In contrast, the circular dichroism band of the 90% NBS-
inactivated sample is shifted to 205 nm and the calculated $\beta$ structure is about 10% less than that of the unmodified enzyme although the $\alpha$ helical content appears to remain the same.\footnote{With the use of compounds synthesized by Meir Wilchek, E. H. Strickland, University of California, Los Angeles, has shown that the far ultraviolet circular dichroism band of tryptophan shifts from 225 nm to 210 nm upon tryptophan oxidation with little change in its intensity (private communication). However, this band represents a very low percentage of the total far ultraviolet circular dichroism of the enzyme and is unlikely to be responsible for the apparent loss of $\beta$ structure.} Hence the 90% NBS-inactivated enzyme apparently contains more random coil than the native enzyme. The $\alpha$ helical content and $\beta$ structure were estimated by comparison of the ellipticity to the graphs of Greenfield and Fasman (41).

As monitored by circular dichroism, the binding of methotrexate to the 40% inactivated enzyme and the binding of TPNH to the 1:1 methotrexate to modified enzyme complex are identical with those observed with native enzyme. However, there are distinct differences when these experiments are performed with the 90% inactivated enzyme. Fig. 9 shows these results. Extrapolation of the early and late points of the titration indicates that approximately 50% of the enzyme in solution binds methotrexate (upper curve); this is in agreement with the fluorescence studies. Also, TPNH is no longer bound as tightly to modified enzyme.\footnote{Fig. 4. The variation of the ellipticity at 305 nm of 90% inactivated dihydrofolate reductase as a function of added methotrexate (MTX) (O---O) and at 365 nm for 1:1 modified enzyme-methotrexate as a function of added TPNH (0- - -0). Solution contained 1.95 \times 10^{-8} \text{ mol of enzyme in 0.85 ml of the same buffer}}

The dependence of the rate of reduction of dihydrofolate on substrate and cofactor concentration appears to obey Michaelis Menten kinetics for the native and 40% NBS-inactivated enzyme. Within experimental error, the $K_m$ values for the 40% NBS-inactivated sample are the same as those for the native enzyme (Table V). The $K_m$ values for the 90% inactivated enzyme were not determined since only part of the sample binds methotrexate and TPNH, and the corresponding dissociation constants differ from those for the native enzyme.

**DISCUSSION**

As reported in the present study, when dihydrofolate reductase is treated with NBS at pH 7.2 and assayed at the same pH, there is a 40% loss of activity before any change occurs in either the fluorescence spectrum or the ultraviolet absorption spectrum. This phenomenon is indicative of initial NBS reaction with side chains of amino acids other than tryptophan. Amino acid and end group analyses suggest that NBS is modifying one histidine, as well as partially oxidizing methionine and cysteine, with no peptide bond cleavage. Since NBS-treated enzyme is identical with native enzyme in its reactivity toward NBS, the oxidation of the easily reacted sulfhydryl group does not result in inactivation. Moreover, the 40% activity loss is not reversed by DTI which does reduce the oxidized cysteine. Unfortunately, the denaturing conditions which were required for reduction of the methionine sulfoxide prevent a definitive elimination of its involvement. Since the activity of the native and modified enzyme is the same before and after sulfoxide reversal, it is unlikely that formation of methionine sulfoxide inactivates the enzyme. However, an argument might be made that the histidine modification affects the efficiency of refolding of the enzyme. Thus, selective modification of methionine would be required to examine its role.

It is highly unusual for a histidine residue to be more reactive than tryptophan in a protein when treated with NBS. In model peptides of tryptophan, tyrosine, and histidine, the reactivity of NBS for these amino acids is Trp > Tyr > His in 50% aqueous acetic acid (42). However, as the pH approaches neutrality several studies report increased side chain reactivity of tyrosine and histidine relative to tryptophan as monitored by amino acid analysis and increased consumption of NBS (27, 43). Brand and Shaltiel (44, 45) have observed rapid attack on histidine residues by NBS at neutral pH. The NBS reactivity of one histidine residue in dihydrofolate reductase from a methotrexate-resistant strain of *E. coli* \( \text{H} \) provides additional evidence for the species specificity of dihydrofolate reductase since other NBS studies on different sources of the same enzyme reflect no modification of histidine and report initial activation when assayed at pH 6.5 and pH 7.5 due to oxidation of sulfhydril groups (13, 14). Amino acid analyses in the present study indicate tyrosine has not been modified, and this is further verified by the absence of ultraviolet spectral changes characteristic of the reaction of tyrosine with NBS (42).

When dihydrofolate reductase from *E. coli* \( \text{H} \), strain MB 1428, is titrated with NBS, there is no loss of capacity to bind the inhibitor, methotrexate, or the cofactor, TPNH, until tryptophan oxidation begins, even though there is a 40% loss of activity at pH 7.2. As the enzyme is modified, the pH for maximal activity is shifted to lower pH (from pH 7.2 to pH 5.5) suggesting the alteration of pK of 1 or more residues in the active site of the enzyme. Since the shift preceding tryptophan oxidation is not accompanied by any change in the $K_m$ values or loss of capacity to bind substrates, the residues involved may mediate proton transport.
transfer. It is possible that the oxidation of a histidine residue results in a shift of its pK to lower pH, or that its modification may have long range effects on other residues. Since the fluorescence and circular dichroism behavior is identical with that of the native enzyme, it is unlikely that the 40% activity loss is due to a large conformational change. Elucidation of the role of histidine may be obtained from protein NMR experiments now in progress.

Further additions of NBS to the enzyme result in oxidation of tryptophans and a concomitant loss of activity. However, it is impossible to determine the number of required tryptophans from these data since the tryptophans appear to overlap in their NBS reactivity and only some of these may be required for enzymatic activity. Also, the activity results at pH 5.9 suggest histidine oxidation is not complete before tryptophan oxidation is initiated; thus the activity loss due solely to tryptophan oxidation cannot be distinguished. The population of the 90% inactivated enzyme is clearly heterogeneous since only half of this enzyme binds methotrexate and more than half of the enzyme still binds 2 mol of TPNH.

Substrate protection experiments would be a logical approach for isolating the involvement of tryptophan and histidine in catalysis, but the current work extends the study of Summers (16) in indicating the complicated nature of the protection experiments. It is clear that TPNIU protects the enzyme from inactivation and that the enzyme provides “apparent” protection to TPNIU. But the results presented here suggest that once TPNIU reacts with NBS, it is no longer tightly bound to the enzyme and hence affords little protection at a 1:1 and 2:1 molar ratio.

The data reported in this manuscript implicate both histidine and tryptophan as critical residues in dihydrofolate reductase. Although the histidine loss does not affect the binding of substrate or cofactor, its modification does result in decreased enzymatic activity at pH 7.2. A similar result was also obtained in this laboratory upon treatment of the enzyme with ethylenediamine hydride (46), a reagent which reacts preferentially with histidine. While other publications from this laboratory (7-11) reported perturbation of tryptophyl residues upon binding of substrate or cofactor, the present study extends this involvement to suggest that 1 or more of the tryptophyl residues are required for maintaining the conformational integrity of the enzyme. It is possible that the perturbation of tryptophan upon binding of substrate or cofactor may be due to long range interactions such as a conformational change induced by binding instead of direct interaction. Such a situation has recently been reported for lactate dehydrogenase (47).

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