Implication of a Tryptophyl Residue in the Active Site of Dihydrofolate Reductase†

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ABSTRACT: Dihydrofolate reductase from amethopterin resistant *Lactobacillus casei* has been examined with regard to the importance of tryptophan in the function of the enzyme. *N*-Bromosuccinimide titration of the enzyme denatured in 8 M urea revealed the presence of three tryptophyl residues per enzyme molecule, a value further supported by amino acid analysis following hydrolysis of native dihydrofolate reductase in the presence of *p*-toluenesulfonic acid. Spectral evidence suggests and amino acid analyses confirm that the effect of low molar ratios (~9) of *N*-bromosuccinimide to enzyme is to cause the selective oxidation of only one tryptophyl residue per enzyme molecule. The extent of oxidation of the sensitive tryptophyl residue is well correlated with both the extent of inactivation of the enzyme and the loss in the NADPH-dependent conversion of free enzyme (form I) into a stable enzyme–NADPH complex (form II). Results of the *N*-bromosuccinimide treatment of form II were interpreted to indicate that the subdued reactivity of the enzyme–coenzyme complex delayed both the oxidation of the bound NADPH and the inactivation of the enzyme. A tenfold excess of $NADP^+$ also protected against inactivation of the enzyme by *N*-bromosuccinimide. In related experiments, the presence of folate compounds was found to have little or no protective effect on the *N*-bromosuccinimide titration of tryptophan in the enzyme or on the inactivation of the enzyme. The overall experimental findings serve to implicate the sensitive tryptophyl residue in the active site of dihydrofolate reductase and, more specifically, in the pyridine nucleotide binding region of the enzyme.

Dihydrofolate reductase catalyzes the NADPH-dependent reduction of L-7,8-dihydrofolate to *l*,L-5,6,7,8-tetrahydrofolate. The significance of this enzyme lies both in the fact that dihydrofolate reductase catalyzes a strategic reaction in the sequence of metabolic events leading to the utilization of tetrahydrofolate in its role as the chief biochemical carrier and transfer agent for one-carbon units and in the fact that this enzyme is widely regarded to be the main target for the action of amethopterin, a folate antagonist highly useful in the treatment of certain cancers, psoriasis, malaria, and other diseases (Huennekens, 1968; Blakley, 1969). Although the purification and properties of dihydrofolate reductases from a wide variety of mammalian (Soucek *et al.*, 1972; Nakamura and Littlefield, 1972; Jarabak and Bachur, 1971), avian (Huennekens *et al.*, 1971), protozoan (Ferone *et al.*, 1969; Gutteridge and Trigg, 1971), bacterial (Gundersen *et al.*, 1972; Poe *et al.*, 1972; D'Souza *et al.*, 1972; Sirotnak and Salser, 1971), and other sources (Nagelschmidt and Jaenicke, 1972; Erickson and Mathews, 1971; Jaffe *et al.*, 1972; Whiteley *et al.*, 1972; Chello *et al.*, 1972; Gauldie and Hillcoat, 1972) have been reported, the available quantities of purified enzyme have restricted structure–function relationship studies to those dihydrofolate reductases obtained from chicken liver (Freisheim and Huennekens, 1969; Huennekens *et al.*, 1971), amethopterin-resistant *Streptococcus faecium* (D'Souza and Freisheim, 1972; D'Souza *et al.*, 1972; Freisheim and D'Souza, 1971; Warwick *et al.*, 1972), amethopterin-resistant *Escherichia coli* (Greenfield *et al.*, 1972, 1973; Poe *et al.*, 1927; Williams *et al.*, 1973a,b), bacteriophage T4 infected *E. coli* (Erickson and Mathews, 1972, 1973), and amethopterin-resistant *Lactobacillus casei* (Dunlap *et al.*, 1971a,b; Gundersen *et al.*, 1972; Liu and Dunlap, 1973).

In a preliminary study with dihydrofolate reductase from an amethopterin-resistant mutant of *S. faecium*, Gleisner and Blakley (1973) reported that the iodoacetate-dependent modification of two methionine residues in this enzyme resulted in a loss of 85% of the enzymic activity and further suggested that one of the methionine residues modified is located in the vicinity of the "folate" binding site of this dihydrofolate reductase. In earlier investigations *N*-bromosuccinimide oxidation of certain tryptophyl residues in dihydrofolate reductases from chicken liver (Freisheim and Huennekens, 1969) and from amethopterin-resistant *S. faecium* (Warwick *et al.*, 1972) resulted in the total loss of enzymic activity. *N*-Bromosuccinimide and diethyl pyrocarbonate treatment of dihydrofolate reductase from an amethopterin-resistant mutant of *E. coli* suggested that tryptophan and histidine residues play important roles in the active site of this enzyme (Greenfield *et al.*, 1973). Further, D'Souza and Freisheim (1971; Freisheim and D'Souza, 1972) found that dramatic alterations in the aromatic side chain Cotton effect region of the circular dichroic spectrum of the *S. faecium* enzyme were generated by enzymic binding of certain pyridine nucleotide or folate analogs. Such changes were not observed, however, if enzyme was employed in which only two of the nine tryptophyl residues present had been oxidized with *N*-bromosuccinimide (Warwick *et al.*, 1972). Greenfield *et al.* (1972) working with the *E. coli* dihydrofolate reductase found that the enzymic binding of folate, dihydrofolate, and amethopterin resulted in large Cotton effects in the circular dichroic spectrum of the enzyme, but in contrast to the work of Freisheim and coworkers, the binding of NADPH elicited only very slight changes in the circular dichroic spectrum of dihydrofolate reductase from this source. Contrasting observations with respect to the structural and mechanistic aspects of dihydrofolate reductases are not unexpected in light of the fact that substantial variations in turnover number, kinetic properties, amino acid composition, molecular weight, etc. (Huennekens *et al.*, 1971), are found when dihydrofolate reductases from different sources are

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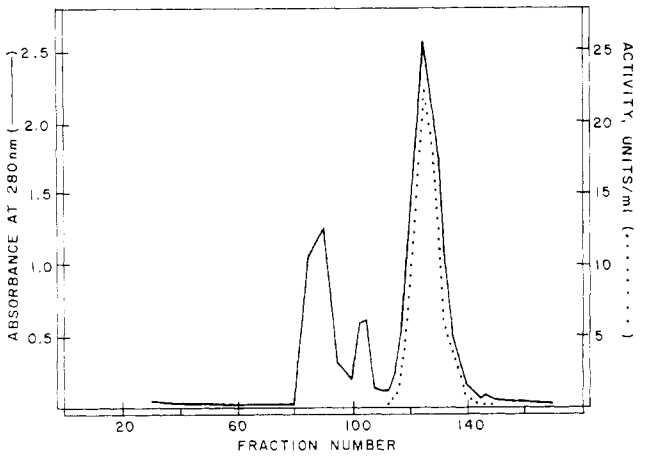

FIGURE 1: Gel filtration of Sephadex G-50 (Fine) for the purification of dihydrofolate reductase. Fraction volume, 6.5 ml; protein (—); enzyme activity (·····); see Experimental Section for details.

compared. Finally, spectrofluorometric studies have shown that the binding of NADPH to dihydrofolate reductases, obtained from a wide variety of sources (Perkins and Bertino, 1966; Huennekens *et al.*, 1971; Gundersen and Dunlap, 1971; Levison *et al.*, 1971; Gundersen *et al.*, 1972; Erickson and Mathews, 1973; Williams *et al.*, 1973a,b), elicits quenching of protein fluorescence concomitant with an increase in the fluorescence in the region of 440–445 nm, an effect attributed to enzyme-bound NADPH. Such fluorescence, termed NADPH energy transfer fluorescence (Velick, 1958; Udenfriend, 1962), is suggestive of the involvement of tryptophan in the binding of the NADPH to dihydrofolate reductases.

An amethopterin-resistant strain of *L. casei* was earlier found to be an excellent source for the purification of dihydrofolate reductase on a large scale (100–500 mg) (Gundersen *et al.*, 1972). Homogeneous dihydrofolate reductase from this source was shown to have a molecular weight in the vicinity of 16,000 and exhibited two forms (I and II) which were resolved from one another by both gel electrophoresis and ion exchange chromatography (Dunlap *et al.*, 1971a). Further work has established that these two forms differ only by the presence of an equimolar amount of tightly, but noncovalently bound NADPH in form II (Dunlap *et al.*, 1971a; Huennekens *et al.*, 1971; Gundersen *et al.*, 1972). The metabolic significance of dihydrofolate reductase coupled with the ease of availability, the size, and the unusual NADPH binding property of the enzyme from amethopterin-resistant *L. casei* made this enzyme a strong candidate for structural and mechanistic investigations. The initial goal of these studies was to identify amino acid side chains of dihydrofolate reductase which are critically required for enzyme activity and/or for the tight binding of NADPH to the enzyme.

Experimental Section

Materials

Materials were obtained as follows: amethopterin from Nutritional Biochemical Co., Bromophenol Blue and Aniline Blue Black from Matheson, Coleman & Bell Co., NAD^+, $NADP^+$, NADH, NADPH, and ribonuclease from Sigma Chemical Co., carboxymethyl-Sephadex C-50 and Sephadex G-50 (fine) from Pharmacia Fine Chemicals, *N*-bromosuccinimide from Columbia Organic Chemicals, and folic acid from Calbiochem. *N*-Bromosuccinimide was recrystallized three times from water before use. Dihydrofolate was prepared according to the procedure of Blakley (1960) and stored in serum bottles which were evacuated prior to sealing (Caldwell *et al.*, 1973). Reduced nicotinamide hypoxanthine dinucleotide phosphate[1] (NHDPH), prepared as described by Kaplan *et al.* (1956) and Tischler and Fisher (1973), was a generous gift of Dr. Ronald R. Fisher of this department. All other chemicals were of reagent grade.

Methods

Assays. Dihydrofolate reductase activity was measured as described previously (Gundersen *et al.*, 1972) and assays were performed on a Beckman Acta III recording spectrophotometer equipped with an automatic sampling system. One unit of dihydrofolate reductase is defined as that amount of enzyme catalyzing the reduction of 1 μmol of dihydrofolate/min at 25°. Protein concentration was measured by the biuret method (Gornall *et al.*, 1949). The concentration of pure dihydrofolate reductase was determined by employing a molar extinction coefficient of 26,400 M^{-1} cm^{-1} at 278 nm.

Purification of Dihydrofolate Reductase. The purification procedure for dihydrofolate reductase from amethopterin-resistant *L. casei* described by Gundersen *et al.* (1972) was shortened and improved. All purification steps were performed at 0–5°. Following sonication of a suspension of 250 g of amethopterin-resistant *L. casei* and fractionation of the cell-free extract with ammonium sulfate, the pellet (100 g) resulting from the 35–65% ammonium sulfate fraction was taken up in 50 mM Tris-HCl buffer (pH 7.3), containing 50 mM KCl, 10 mM 2-mercaptoethanol, and 1 mM EDTA. This intensely yellow solution was dialyzed initially for 24 hr against 6 l. of the preceding buffer followed by a second 24-hr dialysis against 6 l. of 50 mM Tris-HCl buffer (pH 6.5), containing 10 mM KCl, 10 mM 2-mercaptoethanol, and 1 mM EDTA before loading the dialysate (235 ml; specific activity of dihydrofolate reductase = 0.37 unit/mg of protein) on a carboxymethyl-Sephadex column (5 × 80 cm) which had been equilibrated and packed in the latter buffer. On washing the column, many of the contaminating proteins and all of the yellow color washed directly through the column but most of the dihydrofolate reductase activity was strongly retained. When the absorbance of the effluent at 280 nm from this column decreased to 0.10, the column was eluted with a linear gradient consisting of 750 ml of 50 mM Tris-HCl buffer, 50 mM KCl, 10 mM 2-mercaptoethanol, and 1 mM EDTA (pH 6.5) in the mixing chamber and 750 ml of 50 mM Tris-HCl, 700 mM KCl, 10 mM 2-metcaptoethanol, and 1 mM EDTA (pH 7.7) in the reservoir. Dihydrofolate reductase was eluted late in this gradient as a large, fairly broad peak, which, when pooled, gave a volume of about 500 ml containing 2300 units of enzyme with a specific activity of about 0.95.

The pool from the carboxymethyl-Sephadex step was treated with solid ammonium sulfate until 95% saturation was attained. The precipitate (7 g) was collected by centrifugation and dissolved in a minimum volume of 0.1 M potassium phosphate buffer (pH 6.5). After filtering through Whatman No. 1 paper, the 45 ml of concentrated enzyme solution was chromatographed on a Sephadex G-50 (fine) column (5 × 90 cm) which was packed, equilibrated, and eluted with 0.1 M potassium phosphate buffer (pH 6.5) (see Figure 1). The fractions (110–145) having dihydrofolate reductase activity were analyzed by electrophoresis on polyacrylamide gels (see below). The fractions corresponding to the gels in which a

[1] Reduced nicotinamide hypoxanthine dinucleotide phosphate is abbreviated NHDPH.

single protein band was observed were pooled (fractions 121–137) to give 100 ml containing 13 units/ml of dihydrofolate reductase with a specific activity of 19 units/mg, a value slightly higher than that reported by Gundersen *et al.* (1972) for homogeneous enzyme. Dihydrofolate reductase purified by the preceding procedure was employed in all the experiments described herein.

Titration of Tryptophan in Dihydrofolate Reductase with N-Bromosuccinimide. The oxidation of tryptophyl residues in dihydrofolate reductase was studied under a variety of conditions as a function of incremental additions of an aqueous *N*-bromosuccinimide solution (10^{-3} M). Successive 5–10-μl aliquots of *N*-bromosuccinimide solutions were added to 2.5 ml of dihydrofolate reductase ($1–3 \times 10^{-5}$ M in 0.1 M potassium phosphate buffer, pH 6.5) in a 1-cm path-length quartz cuvet and to a blank cuvet containing 2.5 ml of buffer. After stirring for 5 min at 25° following each addition, the absorption spectrum of the enzyme solution was recorded from 450 to 240 nm with a Beckman Acta III recording spectrophotometer. The number of tryptophyl residues oxidized (n) per enzyme molecule was calculated from eq 1 (Witkop, 1961; Ramachandran and Witkop, 1967) after corrections for turbidity

$$n = \frac{(1.31)(\Delta 278)_c}{(5500)(\text{molarity of enzyme})_c} \quad (1)$$

and dilution were applied to $\Delta 278$ (the decrease in absorbance at 278 nm) and the molarity of the enzyme, respectively. The total number of tryptophyl residues in dihydrofolate reductase was estimated by *N*-bromosuccinimide titration of the enzyme in 0.1 M potassium phosphate buffer (pH 6.5), containing 8 M urea. The effect of *N*-bromosuccinimide on the catalytic activity of dihydrofolate reductase was measured by assaying aliquots withdrawn from comparable titration reaction mixtures incubated at 0–5°. The effect of *N*-bromosuccinimide on the *activity* of dihydrofolate reductase incubated with various additives, including NADPH, NADH, NHDPH, $NADP^+$, folic acid, and 7,8-dihydrofolate, was measured as indicated above. The *titration* of tryptophan in dihydrofolate reductase was studied in the presence of folic acid and amethopterin. However, similar experiments could not be performed on dihydrofolate reductase incubated with reduced pyridine nucleotides or reduced folates, such as 7,8-dihydrofolate and 5,6,7,8-tetrahydrofolate, because the oxidation of the reduced additive itself results in spectrophotometric changes which obscured the spectral alterations accompanying tryptophan titration in the enzyme. The *N*-bromosuccinimide-dependent oxidation of reduced pyridine nucleotides (NADH, NADPH, NHDPH) in the presence and absence of enzyme was detected by following the changes in absorption spectra of these nucleotides, especially the decrease in the 340-nm peak, as a function of the molar ratio of oxidant present (Summers, 1972).

Electrophoresis and staining of dihydrofolate reductase in 7.5% polyacrylamide gels were performed according to the procedure of Dunlap *et al.* (1971a). In order to monitor the extent of the NADPH-dependent conversion of form I to form II, form I enzyme was incubated for 10 min at 5° with a 2.5 molar excess of NADPH, followed by electrophoresis and staining with Aniline Blue Black (see Figure 4). Following destaining, gels were scanned at 600 nm with a Gilford Model 240 spectrophotometer operating on an absorbance scale of 3.0. The extent of conversion of form I to form II was determined by comparing the areas on the densitometer tracings under the peaks corresponding to form I and form II.

Amino Acid Analysis. Native dihydrofolate reductase in 0.1 M potassium phosphate buffer (pH 6.5) was incubated with an 8-fold molar excess of *N*-bromosuccinimide for 30 min at 25°. Following dialysis against glass distilled water and lyophilization of the modified enzyme, both the *N*-bromosuccinimide treated sample and a sample of the native enzyme were subjected to amino acid analysis following hydrolysis in the presence of *p*-toluenesulfonic acid (Liu and Chang, 1971) in order to minimize the destruction of tryptophan. The amino acid analyses were performed by Dr. James H. Freisheim, Department of Biological Chemistry, University of Cincinnati.

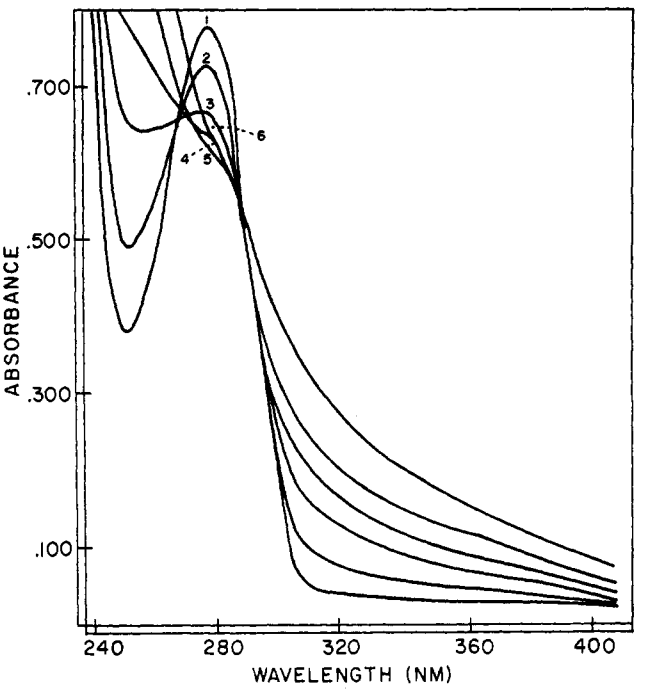

FIGURE 2: Effect of *N*-bromosuccinimide titration on the spectrum of native dihydrofolate reductase. Dihydrofolate reductase concentration = 2.9×10^{-5} M. Curves 1–6 obtained as a function of the molar ratio of *N*-bromosuccinimide to enzyme as follows: curve 1, 0; curve 2, 2.9; curve 3, 5.8; curve 4, 7.5; curve 5, 9.2; curve 6, 12.7.

Results

Titration of Denatured Dihydrofolate Reductase. Titration of form I (2.73×10^{-5} M) in 0.1 M potassium phosphate buffer (pH 6.5), containing 8 M urea with *N*-bromosuccinimide resulted in a maximum decrease in absorbance at 278 nm which was achieved at a molar ratio of *N*-bromosuccinimide to enzyme of 12.3. When the corrected maximum $\Delta 278$ value and the corrected enzyme molarity from this experiment were used in eq 1, an estimate of 2.8 tryptophyl residues/dihydrofolate reductase molecule was obtained. This value is in complete agreement with the previous estimates of total tryptophan content in the enzyme provided by other techniques (Gundersen *et al.*, 1972).

Titration of Native Dihydrofolate Reductase and Correlation with Inactivation. Comparable *N*-bromosuccinimide titrations were performed on native dihydrofolate reductase. In Figure 2 the alteration of the absorption spectrum of the enzyme is displayed as a function of increasing the molar ratio of *N*-bromosuccinimide to enzyme. The maximum decrease at 278 nm occurs at a molar ratio of *N*-bromosuccinimide to dihydrofolate reductase of 9.2 and corresponds to a value of 1.25 tryptophyl residues titrated per native enzyme molecule. The subsequent increase in absorbance on further additions of *N*-

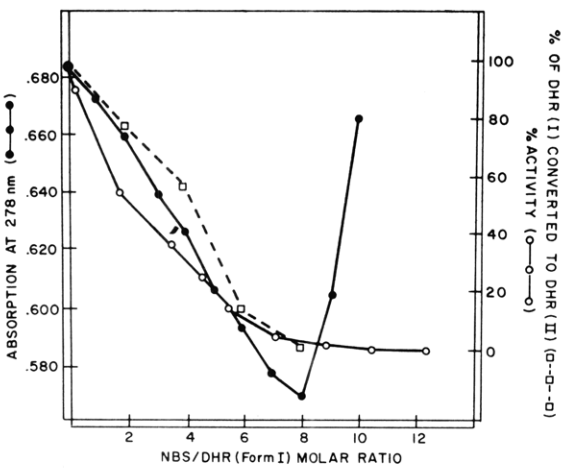

FIGURE 3: Effect of *N*-bromosuccinimide on dihydrofolate reductase. The loss in absorbance at 278 nm plotted against the molar ratio of *N*-bromosuccinimide to form I of dihydrofolate reductase illustrates the titration of tryptophyl residues in the native protein (see Experimental Section). The titration of tryptophan in dihydrofolate reductase by *N*-bromosuccinimide is correlated both with the loss of enzymatic activity (see Experimental Section) and the loss of the NADPH-dependent conversion of form I to form II. To perform the latter experiment five samples of dihydrofolate reductase, form I (2 ml, 7.95×10^{-5} M in 0.1 M potassium phosphate buffer, pH 6.5), were treated with various aliquots of *N*-bromosuccinimide (1.57×10^{-2} M in water) and were allowed to react for about 10 min in the cold. Excess oxidizing agent was removed by dialysis of each of the samples separately against 1 l. of 0.1 M potassium phosphate buffer (pH 6.5) in the cold room. Prior to electrophoresis on 7.5% polyacrylamide gels, 50 μl of each sample ($\sim 8 \times 10^{-5}$ M) was added to the sucrose solution (50 μl; see Gundersen *et al.* (1972) for further details) overlaying the polyacrylamide gel (6×60 mm). 4 μl of NADPH (2.66×10^{-2} M) in 0.5 Tris-HCl buffer was then added to each sample (sufficient to give a ratio of NADPH:enzyme of 2.5), mixed, and allowed to interact with enzyme in the cold for about 10 min. The gels were then run at 3 mA/gel for 2.5 hr and were stained for protein with Amido Black.

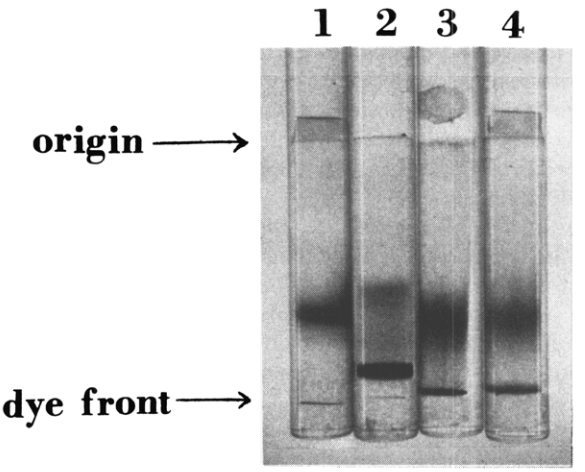

FIGURE 4: Electrophoresis of dihydrofolate reductase samples. Electrophoreses of dihydrofolate reductase in 7.5% polyacrylamide gels (6×60 mm) were run at 3 mA/gel for 2 hr and stained with Amido Black. Each gel was loaded with 37 μg of protein (2.3×10^{-9} mol). Gel 1 was loaded with pure form I only in 0.1 M potassium phosphate buffer (pH 6.5). Gel 2 was loaded with pure form I plus 5.6×10^{-9} mol of NADPH; the solution was mixed, and allowed to stand in the cold for about 10 min prior to electrophoresis. The enzyme shown in gels 3 and 4 was treated in 0.1 M potassium phosphate buffer (pH 6.5), with a 9-fold molar excess of *N*-bromosuccinimide for 30 min, and the excess *N*-bromosuccinimide was removed by dialysis. Gel 3 was loaded with *N*-bromosuccinimide-treated form I only, while gel 4 was loaded with *N*-bromosuccinimide-treated form I and 5.6×10^{-9} mol of NADPH; the solution was mixed, and allowed to stand for about 10 min in the cold before initiating electrophoresis.

bromosuccinimide is due to the increasing turbidity of the solution. The results from a similar titration of dihydrofolate reductase are plotted in Figure 3 and illustrate the relationship between the absorption at 278 nm and the molar ratio of *N*-bromosuccinimide to enzyme. Thus, increasing ratios of *N*-bromosuccinimide to enzyme lead to an almost linear decrease at 278 nm. The $\Delta 278$ value at the minimum in this curve occurs at a molar ratio of oxidant to enzyme of 8 and corresponds to the titration of 1.11 tryptophyl residues per enzyme. Activity analyses (see Figure 3) performed on aliquots of dihydrofolate reductase undergoing titration with *N*-bromosuccinimide illustrate the fact that the loss of enzymic activity closely parallels the extent of tryptophan modification. Thus, at a molar ratio of *N*-bromosuccinimide to enzyme of 8, the dihydrofolate reductase activity is less than 1% of its original value.

Electrophoretic Behavior of Modified Dihydrofolate Reductase. To further investigate the effect of *N*-bromosuccinimide oxidation of dihydrofolate reductase, the unique property of the NADPH-dependent conversion of form I to form II together with the characteristic migration of both enzyme forms on electrophoresis was employed as a convenient analytical tool. In Figure 4, the electrophoretic behavior of native form I and form II is compared with that of form I, following oxidation with *N*-bromosuccinimide. The oxidized form I enzyme (gel 3) shows a single, rather diffuse protein band migrating with about the same R_F value as that of native form I (gel 1). The fact that other bands are not observed in gel 3, nor following electrophoresis of the oxidized enzyme in polyacrylamide gels containing sodium dodecyl sulfate, decreased the possibility that *N*-bromosuccinimide might have caused cleavage of peptide bonds in the protein (Burstein and Patchornik, 1972) in addition to the oxidation of tryptophan. Furthermore, the pattern of protein migration in gel 4 when compared to that of form II in gel 2 indicates that the usual NADPH-dependent conversion of form I to form II is no longer observed when *N*-bromosuccinimide-treated form I (following dialysis to remove excess oxidant) is employed. It is of interest as well that the coenzyme analog, reduced nicotinamide hypoxanthine dinucleotide (NHDPH), also caused conversion of form I to a form II species but failed to alter the electrophoretic pattern of enzyme inactivated by *N*-bromosuccinimide.

To explore the observation further, an experiment was performed in which aliquots of form I enzyme were oxidized to various stages by increasing molar ratios of *N*-bromosuccinimide to enzyme (see Methods and legend to Figure 3). Following dialysis, each sample was treated with a 2.5 molar excess of NADPH in an attempt to convert any remaining form I to form II prior to electrophoresis on polyacrylamide gels. Following electrophoresis, staining, and destaining, of the polyacrylamide gels, densitometer tracings of each gel were recorded and analyzed by comparing the area under the peak corresponding to form I with the area under the peak representing form II of dihydrofolate reductase. Qualitatively, it was noted that the area under the peaks corresponding to form II became progressively smaller as the molar ratios of *N*-bromosuccinimide to enzyme increased. This effect was also quantitatively observed when comparing ratios of form II peak area to the total peak area of form I and form II (the per cent conversion of form I to form II) as a function of increasing the molar ratio of *N*-bromosuccinimide to enzyme in the sample. The per cent conversion of form I to form II

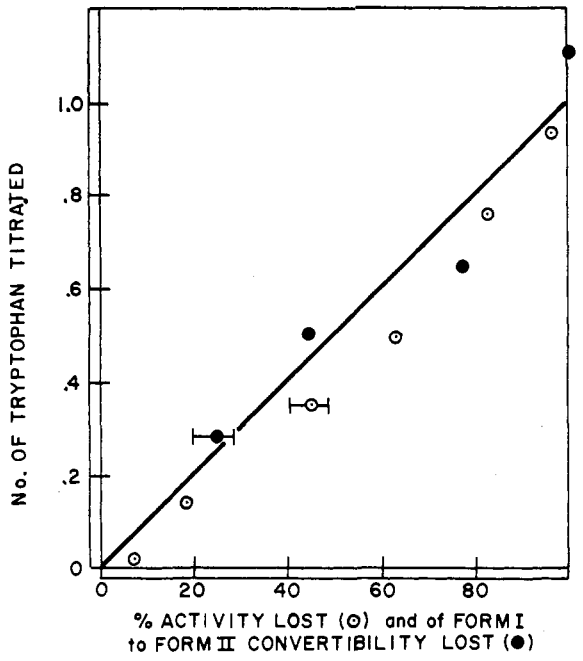

FIGURE 5: Correlation of tryptophan titration with inactivation and loss of convertibility of dihydrofolate reductase. This figure represents a linear correlation diagram of the data illustrated in Figure 3.

derived from this experiment is plotted in Figure 3 to illustrate the fact that the extent of titration of one tryptophyl residue in dihydrofolate reductase from amethopterin-resistant *L. casei* corresponds closely to both the loss of enzymic activity and the loss in the ability of form I to undergo the NADPH-dependent conversion to form II.

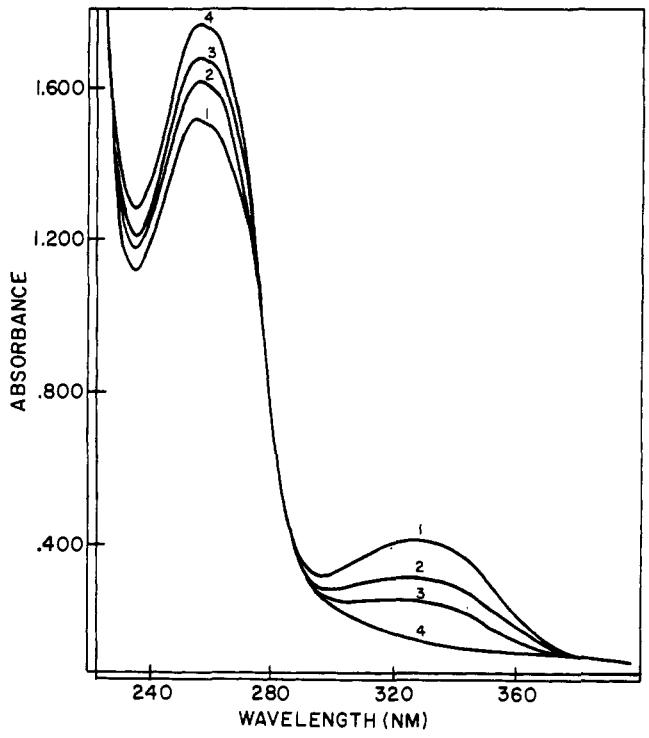

FIGURE 6: Effect of *N*-bromosuccinimide on spectrum of form II dihydrofolate reductase. Form II enzyme was present at 5.67 × 10^{-5} M. Curves 1, 2, 3, and 4 correspond to molar ratios of *N*-bromosuccinimide to form II of 0, 1.65, 2.5, and 4.1. respectively. See text for further details.

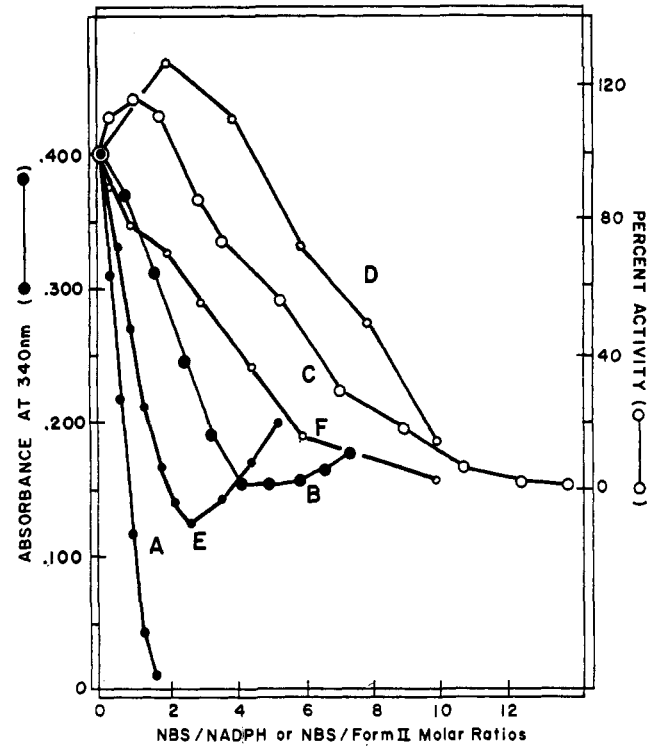

FIGURE 7: The effect of *N*-bromosuccinimide on the oxidation and inactivation of pyridine nucleotide–dihydrofolate reductase complexes. Curve A represents the oxidation (loss in absorbance at 340 nm) of a 6.5 × 10^{-5} M solution of NADPH as a function of increasing concentrations of *N*-bromosuccinimide. Similarly, curve B is the oxidation of the NADPH in form II enzyme present at 5.6 × 10^{-5} M (equimolar to the enzyme). Curve C represents the *N*-bromosuccinimide dependent inactivation of form II enzyme at 4.8 × 10^{-5} M; curve D describes the inactivation of dihydrofolate reductase at 2.85 × 10^{-4} M, in the presence of a 10-fold excess of $NADP^+$. Curve E shows the oxidation (loss in absorbance at 340 mm) of a 6.1 × 10^{-5} M solution of NADH in presence of equimolar dihydrofolate reductase; curve F represents the inactivation of this binary mixture by *N*-bromosuccinimide.

In order to view the results of the tryptophan titration, the loss of enzymic activity, and the loss of convertibility of form I to form II from a different perspective, the data have been plotted in Figure 5 in terms of a linear correlation diagram in which the solid line represents the *N*-bromosuccinimide-dependent titration of one tryptophyl residue in dihydrofolate reductase. The disposition of the data points in this plot confirms the postulate that the three experimental parameters are well correlated and closely interrelated to one another.

Amino Acid Analysis of N-Bromosuccinimide-Treated Enzyme. Although *N*-bromosuccinimide is useful in determining the number of tryptophyl residues in enzymes and their relationship, if any, to enzymic activity, this reagent is notoriously nonspecific and is known to also react with the side chains of cysteine, tyrosine, histidine, and methionine residues (Means and Feeney, 1971) and to cause peptide-bond cleavage when present at high molar ratios. However, complications in the interpretation of the *N*-bromosuccinimide titration and inactivation data encountered in earlier studies (Freisheim and Huennekens, 1969; Warwick *et al.*, 1972) arising from the possible oxidation of cysteinyl side chains do not encumber this investigation since, unlike dihydrofolate reductases from many sources, the enzyme isolated from amethopterin-resistant *L. casei* was shown to contain no cysteine (Dunlap *et al.*, 1971b). To reduce the possibility that some mechanism other than the oxidation of one tryptophyl

TABLE I: Effect of *N*-Bromosuccinimide on Oxidizable Amino Acids in Dihydrofolate Reductase.

Amino Acid Residue Determined[a]	Native Dihydrofolate Reductase	*N*-Bromosuccinimide-Oxidized Dihydrofolate Reductase	Theoretical Value[b]
Histidine	6.6	6.2	7
Tyrosine	4.7	4.8	5
Methionine	1.7	1.6	2
Tryptophan	2.8	1.9	3

[a] Dihydrofolate reductase from amethopterin-resistant *L. casei* contains no cysteinyl residues. [b] See Dunlap *et al.* (1971b).

residue in the enzyme results in both the loss of enzymic activity and the failure of NADPH to convert form I to form II, a total amino acid analysis was performed on both native dihydrofolate reductase and enzyme in which one tryptophan per enzyme was oxidized with *N*-bromosuccinimide. In order to quantitate tryptophan, the samples of enzyme were hydrolyzed for 24 hr in the presence of *p*-toluenesulfonic acid prior to amino acid analysis (Liu and Chang, 1971). The amino acid analysis data are reported (Table I) in terms of those residues susceptible to oxidation by *N*-bromosuccinimide. The results indicate that in the range of molar ratios of *N*-bromosuccinimide to enzyme employed in these studies, the reagent modifies only one tryptophan per enzyme molecule and has little effect on other amino acid residues.

Titration of Form II with N-Bromosuccinimide. Form II dihydrofolate reductase, which was artificially prepared by incubating equimolar amounts of form I and NADPH, was treated in the usual manner with increasing concentrations of *N*-bromosuccinimide. The effect of increasing the molar ratio of *N*-bromosuccinimide to form II is illustrated in a series of absorption spectra provided in Figure 6. The spectrum of untreated form II is characterized by maxima at 340 nm (ϵ 7200 M^{-1} cm^{-1}; Gundersen *et al.*, 1972) and 260 nm in contrast to the single maximum at 278 nm observed with form I (see Figure 1). As the molar ratio of oxidant to form II is increased in increments to 4.1, the absorbance at 340 nm decreases and finally coincides with the base line while the absorbance at 260 nm increases proportionally. The changes in absorption of form II in the vicinity of 278 nm as a function of the molar ratio of *N*-bromosuccinimide to enzyme obscure spectral alterations arising from the titration of tryptophyl residues in form II and make the use of eq 1 unreliable.

However, the treatment of free NADPH and form II with small molar ratios of *N*-bromosuccinimide produces changes in the absorption spectra of both NADPH and form II which are reminiscent of the spectral changes observed when NADPH is oxidized either chemically or enzymatically to $NADP^+$. Summers (1972) has reported and we have confirmed (curve A in Figure 7) that *N*-bromosuccinimide reacts almost stoichiometrically with reduced pyridine nucleotides to give oxidized products typified by the absence of a chromophore absorbing maximally at 340 nm. In contrast, when the oxidation of form II is studied as a function of *N*-bromosuccinimide concentration, the oxidation of the NADPH in the form II complex, as measured by the decrease in absorbance at 340 nm (curve B in Figure 7), requires a fourfold excess of oxidant to complete the destruction of this chromophore. The possibility that the protection of the NADPH in form II from oxidation by *N*-bromosuccinimide may have resulted from a nonspecific interaction between the reduced coenzyme and dihydrofolate reductase was evaluated by studying separately the oxidation of NADH and NADPH, both free in solution and in the presence of equimolar ribonuclease. Ribonuclease was selected for these experiments because it has little or no affinity for reduced pyridine nucleotides and because low molar ratios of *N*-bromosuccinimide have little effect on the absorbance of this protein since tryptophan is absent in ribonuclease. The decrease in absorption at 340 nm of NADH or NADPH in the presence of ribonuclease was recorded as a function of increasing oxidant concentrations, and the data obtained from these experiments strongly resemble the curve (see curve A in Figure 7) resulting from the oxidation of NADPH in the absence of any added ribonuclease. To provide further evidence that the extent of enzymatic protection of the reduced coenzyme from *N*-bromosuccinimide oxidation depends on the degree of interaction of the coenzyme and enzyme, the oxidation of NADH and NHDPH was carried out separately in the presence of equimolar dihydrofolate reductase as a function of *N*-bromosuccinimide concentration. On the one hand, dihydrofolate reductase provides only moderate protection from *N*-bromosuccinimide oxidation to NADH (curve E in Figure 7), which is only a poor coenzyme for dihydrofolate reductase and which is incapable of converting form I to a form II species. On the other hand, NHDPH, which is an effective coenzyme in this system (unpublished results, Liu and Dunlap) and which interacts with dihydrofolate reductase to form a form II species, is protected from *N*-bromosuccinimide oxidation by equimolar enzyme to essentially the same extent as NADPH (curve B in Figure 7).

From further examination of Figure 7 it is evident that the inactivation of form II by *N*-bromosuccinimide does not become significant until most of the reduced pyridine nucleotide, NADPH, has been oxidized and is not complete until a molar ratio of oxidant to form II of about 12 is attained. These data suggest that the presence of NADPH in form II protects the sensitive tryptophyl residue from oxidation and, thus, delays the onset of inactivation of the enzyme. Similar results are also obtained with the enzyme–NHDPH complex. In comparison, the *N*-bromosuccinimide-dependent inactivation of dihydrofolate reductase incubated with equimolar NADH (curve F in Figure 7) is very similar to the inactivation of native enzyme alone (see Figure 3) and, thus, NADH affords little or no protection from inactivation to the enzyme. Figure 7 (curve D) shows that the oxidized coenzymatic product, $NADP^+$, when present in a tenfold excess with respect to dihydrofolate reductase, provides considerable protection from *N*-bromosuccinimide inactivation to the enzyme.

Effect of N-Bromosuccinimide on Dihydrofolate Reductase in the Presence of Selected Folates. Dihydrofolate reductase (9×10^{-6} M) incubated in the presence of 9×10^{-6} or 2.2×10^{-5} M folic acid was titrated with increasing molar ratios of *N*-bromosuccinimide. The results of these experiments were essentially identical with those carried out with native dihydrofolate reductase alone (see Figure 3) and indicate that the presence of folic acid, at least at low concentrations, has no effect on the extent of the loss of activity or the titration of tryptophan in dihydrofolate reductase. Similar experiments were attempted in the presence of 7,8-dihydrofolate or amethopterin. Spectral changes resulting from the *N*-bromosuccinimide-dependent oxidation of 7,8-dihydrofolate in the presence of dihydrofolate reductase are complicated and

prevented the assessment of the extent of tryptophan oxidation in these experiments. In agreement with Summers (1972), we found that 7,8-dihydrofolate was oxidized to a folic acid like species by an equimolar concentration of *N*-bromosuccinimide. From analysis of the spectral data alone, it is impossible to determine whether or not an interaction between 7,8-dihydrofolate and the enzyme exists which would result in protection of either the substrate or the dihydrofolate reductase from the oxidizing agent. However, in contrast to the *N*-bromosuccinimide-dependent inactivation of form II, the curve describing the inactivation of dihydrofolate reductase in the presence of equimolar 7,8-dihydrofolate is only slightly displaced to the right of the corresponding curve for the inactivation of native enzyme (see Figure 3). Further, total inactivation of dihydrofolate reductase incubated with an equimolar concentration of 7,8-dihydrofolate occurs at only a tenfold excess of oxidizing agent, reflecting little or no mutual protection between the enzyme and its substrate. Finally, *N*-bromosuccinimide titrations were also performed on dihydrofolate reductase in the presence of equimolar concentrations of amethopterin. As indicated by the decrease in absorbance at 278 nm, the *N*-bromosuccinimide-dependent titration of tryptophan in dihydrofolate reductase incubated in the presence of amethopterin (which is known to be tightly bound by the enzyme) does not differ substantially from the corresponding titration of tryptophan in native enzyme alone.

Discussion

The results of previous studies of dihydrofolate reductases from chicken liver (Freisheim and Huennekens, 1969), amethopterin-resistant *S. faecium* (D'Souza *et al.*, 1972; D'Souza and Freisheim, 1972; Warwick *et al.*, 1972), and amethopterin-resistant *E. coli* (Greenfield *et al.*, 1973) have suggested that tryptophyl residues play important, though as yet only poorly characterized, roles crucial to the activity of dihydrofolate reductase. In this investigation we have shown, by both spectrophotometric and amino acid analyses, that one of the three tryptophyl residues present in dihydrofolate reductase from amethopterin resistant *L. casei* is easily oxidized by *N*-bromosuccinimide in the native enzyme. The oxidation of this tryptophan residue correlates both with the inactivation of the enzyme and the loss of the NADPH-dependent conversion of form I to form II. These results are consistent with the hypothesis that the sensitive tryptophan residue resides in the active-site region of dihydrofolate reductase. The fact that the extent of oxidation of one tryptophyl residue in the enzyme is closely related to the degree of loss of the NADPH-dependent conversion of form I to form II may be interpreted to implicate this residue in the binding of pyridine nucleotides to dihydrofolate reductase. When this possibility was further evaluated by *N*-bromosuccinimide titration and activity studies of dihydrofolate reductase incubated in the presence of a variety of pyridine nucleotides and folate compounds, the data obtained provided further support for the localization and involvement of the sensitive tryptophan residue in the pyridine nucleotide binding site. That is, the presence of folate compounds neither significantly altered the extent of the *N*-bromosuccinimide-dependent titration of tryptophan in the enzyme nor the degree to which the oxidant caused inactivation of the enzyme. On the other hand, NADPH and NHDPH, present separately at equimolar concentrations with respect to the enzyme, both protected the enzyme from inactivation by *N*-bromosuccinimide and, in addition, were themselves protected, by virtue of their specific interaction with dihydrofolate reductase, from oxidation by *N*-bromosuccinimide. Finally, even though $NADP^+$ has a relatively weak binding to dihydrofolate reductase, a tenfold excess of this oxidized coenzyme is sufficient to afford substantial protection from inactivation to the enzyme.

The results of earlier studies of the *N*-bromosuccinimide-dependent inactivation of dihydrofolate reductase from chicken liver (Freisheim and Huennkens, 1969) performed in the presence of NADPH or 7,8-dihydrofolate suggested that either the coenzyme or the substrate was capable of protecting the enzyme from inactivation. However, Summers (1972) showed that both NADPH and 7,8-dihydrofolate were readily oxidized by *N*-bromosuccinimide, thus necessitating a reevaluation of the previous experimental data. In a comparable investigation with the glutamate dehydrogenase–NADH system, Summers (1972) studied the effect of *N*-bromosuccinimide on both the extent of inactivation of the enzyme and the degree of oxidation of the NADH bound to the enzyme. These experiments showed that the *N*-bromosuccinimide-dependent inactivation of the glutamate dehydrogenase–NADH complex was delayed in comparison to the inactivation of the enzyme in the absence of NADH, an effect which was attributed solely to the competition of the NADH and the enzyme for the available oxidant. The author concluded that these results represented only an *apparent* protection of the enzyme by the NADH and did not reflect an altered reactivity of the enzyme–NADH complex to the oxidant. On the contrary, studies of the effect of *N*-bromosuccinimide on both the inactivation of the dihydrofolate reductase–NADPH complex and the oxidation of the NADPH in this complex were interpreted earlier in this paper to indicate that mutual protection from oxidation resulted from enzyme–NADPH complex formation. That this "protective" effect was, in fact, due to an altered reactivity of the enzyme-reduced pyridine nucleotide complex is borne out by the results of similar experiments with dihydrofolate reductase carried out in the presence of NADPH, NHDPH, and NADH. If the delay in the inactivation of the enzyme–NADPH system was just the product of simple competition for oxidant between the enzyme and the reduced pyridine nucleotide, then the *apparent* protection observed should not be a function of the structure of the reduced pyridine nucleotides employed. However, experimentally NADPH and NHDPH afford substantially more protection from enzyme inactivation than does NADH. These results indicate that the protective effect is related both to the extent of complex formation and the reactivity of the enzyme–reduced pyridine nucleotide complex. Furthermore, the fact that $NADP^+$, which is not oxidized by low molar ratios of *N*-bromosuccinimide, also provides protection from inactivation to the enzyme lends further credence to the idea that a dihydrofolate reductase–pyridine nucleotide complex will exhibit an altered reactivity to oxidant with respect to the reactivity of the enzyme alone. A similar argument may be used to explain the extent to which reduced pyridine nucleotides are themselves protected from oxidation by the presence of dihydrofolate reductase.

In conclusion, although the results of these studies implicate a tryptophyl residue in the active site, and specifically in the pyridine nucleotide binding region of dihydrofolate reductase, they do not serve to indicate whether the tryptophyl residue is involved in catalysis, maintenance of tertiary structure, or in some other role in the enzyme. Experiments are now in progress in this laboratory to delineate the role of this crucial tryptophyl residue and to identify its position in the primary structure of the enzyme.

Acknowledgments

We express our gratitude to Dr. Frank M. Huennekens, Dr. James H. Freisheim, and Dr. Ronald R. Fisher for their helpful discussions.

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