Reaction of Dihydrofolate Reductase with Dansyl Chloride. Chemical Modification of a Sensitive Lysine Residue and Fluorometric Studies of the Dansylated Enzyme†

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ABSTRACT: Dihydrofolate reductase from amethopterin-resistant *Lactobacillus casei* is virtually completely and irreversibly inactivated by relatively low concentrations of dansyl chloride. The complete inactivation can be correlated with the dansylation of a single lysine residue and ca. 90% quenching of protein fluorescence. This quenching phenomenon appears to be due, at least in part, to energy transfer from one or more excited state tryptophan residues to the covalently attached dansyl moiety. Under identical conditions lysine is not modified when the ternary complex of enzyme–NADPH–amethopterin is dansylated. The unreactive dansyl hydroxide protects the enzyme against dansyl chloride dependent inactivation and fluorescence studies indicate a single ligand binding site (K_D = 1×10^{-4} M). It is suggested that the dimethylamino-naphthyl moiety of dansyl chloride is directed to a hydrophobic region at or near the active center of the enzyme where a particularly susceptible lysine residue reacts to form a covalent bond with the reagent.

Dihydrofolate reductase (EC 1.5.1.3) catalyzes the NADPH-dependent reduction of L-7,8-dihydrofolate to *l*,L-5,6,7,8-tetrahydrofolate, the coenzyme carrier involved in a variety of one-carbon transfer reactions. The enzyme from most mammalian and bacterial sources exists as a single polypeptide chain in the molecular weight range of 15 000–30 000 (Blakley, 1969; Huennekens et al., 1971). The reductase is also the intracellular target site or molecular receptor for an important class of folate antagonist drugs, such as amethopterin (Methotrexate); this compound is currently being employed in the treatment of certain neoplastic diseases, psoriasis, and other disorders.

Compared with other larger pyridine nucleotide dependent dehydrogenases, this enzyme is uncomplicated with regard to quaternary structure, which should facilitate structure-function studies. Thus, in the absence of subunit–subunit interactions, the manner in which substrates and inhibitors bind to the catalytic site of this enzyme should be elucidated with considerably more ease. The present investigation was undertaken in order to assess the possible functional role of lysine residues in dihydrofolate reductase. Examination of the structural features of both dihydrofolate and NADPH suggested the possibility that one or more basic amino acid side chains on the enzyme could form one or more salt bridges with anionic sites on either or both of these substrates. The results of a recent communication (Vehar and Freisheim, 1976) suggest the involvement of essential arginine residues in dinucleotide coenzyme binding by dihydrofolate reductase.

Experimental Procedure

Materials

Dihydrofolate reductase was isolated and purified from an amethopterin-resistant strain of *Lactobacillus casei* by a modification (Liu and Dunlap, 1974) of the procedure of Gundersen et al. (1972). The enzyme is isolated as two electrophoretic forms and only form I, which does not contain bound NADPH, was used in these studies. NADPH was obtained from P-L Biochemicals; Dns-Cl[1] was obtained from Sigma; naphthalene-labeled [^{3}H]Dns-Cl (14.4 Ci/mmol) was obtained from Amersham/Searle. Dihydrofolate was prepared by the reduction of folic acid (Cyclo Chemicals) with sodium dithionite according to the method of Futterman (1957), as modified by Blakley (1960). The dihydrofolate was lyophilized and stored in argon-flushed, evacuated Thunberg tubes. Tryptophan (MCB) was recrystallized twice from water and once from ethanol for the fluorescence studies.

Methods

Enzyme activity was measured spectrophotometrically by following the decrease in absorbance at 340 nm using a Beckman Model DU spectrophotometer equipped with a Gilford Model 208 auxiliary offset control unit, a Gilford Model 210 automatic cuvette positioner, and a Sargent Model SRLG recorder. The assay mixture, in a volume of 1.2 ml, contained 50 mM KPO_4 buffer, pH 7.0, 40 μM dihydrofolate, and 50 μM NADPH. The assays were performed at 25 °C with an enzyme concentration of approximately 1×10^{-7} M.

Amino acid analyses were performed employing a Durrum Model D-500 amino acid analyzer. Protein hydrolyses were done in 6 N HCl in evacuated, sealed tubes for 20 h at 110 °C following the general procedures of Moore and Stein (1963). Dns-enzyme samples were protected from light as much as possible due to the sensitivity of the Dns bond to ultraviolet light (D'Souza et al., 1970).

Absorbance spectra were recorded on a Cary Model 15 spectrophotometer using a 0–1.0 absorbance slide wire.

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[1] Abbreviations used are: Dns-Cl, dansyl chloride, 8-dimethylamino-1-naphthalenesulfonyl chloride; Dns-OH, dansyl hydroxide; Dns-, dansyl or dansylated; NAD, nicotinamide adenine dinucleotide; NADPH, reduced NAD phosphate; TLC, thin-layer chromatography.

Modification of dihydrofolate reductase by Dns-Cl was performed at 28 °C in 0.1 M KPO_4 buffer, pH 7.0, unless otherwise specified. The reaction was initiated by the addition of 0.06 ml of Dns-Cl in acetone (2 mg/ml) to a 1.0-ml solution of enzyme (5×10^{-5} M). The mixture was mechanically stirred during the course of the reaction. Aliquots (5–10 μl) were removed from the reaction at various time intervals and assayed for enzyme activity. The fractional activity remaining is expressed as V/V_0, where V is the enzyme activity of the Dns-modified reductase and V_0 is the activity of an unreacted control. After intervals of 15–20 min, when the disappearance of yellow color from the reaction mixture indicated that hydrolysis of the Dns-Cl to the unreactive Dns-OH had occurred, further additions of 0.02 ml of the Dns-Cl acetone solution were made as needed. The initial 0.06-ml addition of Dns-Cl represents a molar excess of reagent to enzyme of ca. 9:1. Each 0.02-ml addition represents an excess of Dns-Cl to the initial enzyme concentration of ca. 3. Due to the hydrolysis of Dns-Cl in aqueous solution, Gray (1972) has suggested that labeling is dependent on the absolute concentration of Dns-Cl and not on the ratio of Dns-Cl to free amino groups. In these terms the initial concentration of Dns-Cl at time zero is 4.2×10^{-4} M and each addition of 0.02 ml produces a concentration of 1.4×10^{-5} M.

Incorporation of Radioactivity. To a 2.0-ml solution of dihydrofolate reductase (0.85 mg/ml) in 0.1 M KPO_4, pH 7.0, was added [^{3}H]Dns-Cl in 15-μl increments (0.12 μmol of Dns-Cl). Following incubation at 28 °C for 12 min after each addition, 0.1-ml aliquots were removed and precipitated with 1 ml of 10% trichloroacetic acid, washed with water, and dissolved in 50 μl of 0.1 N NaOH. To each sample was added 10 ml of scintillant (3a70B, Research Products International) and radioactivity measured with a Beckman Model LS-335 liquid scintillation spectrometer. Separate aliquots were taken to determine enzyme activity and protein concentration during the radiolabeling procedure.

Fluorometric studies were performed on a Baird-Atomic Fluorispec Model SF-100 fluorescence spectrofluorometer with an Engelhard Hanovia 150-W power supply and a Bausch and Lomb Omnigraphic 2000 recorder. Both entrance and exit slits for the monochromators were set at 6 nm. Temperature control was maintained at 25 °C using a circulating water bath. The fluorescence spectra were measured at right angles to the exciting light in 1-cm quartz cuvettes. No attempt was made to deaerate the solutions. The light source and detector response were calibrated by the method of Argauer and White (1964), with emission spectra correction obtained using compounds whose corrected emission spectra are commonly reported in the literature. Excitation spectra correction were obtained using the absorption spectra of fluorescent compounds as the actual fluorescence excitation intensities (Argauer and White, 1964). All spectra shown are corrected and presented in units of relative quanta vs. wavelength. Unless otherwise noted, the absorbance in the cuvettes was not allowed to exceed 0.25. Self-absorption and inner filter effects were corrected as suggested by Kirby (1971) using the equation:

$$I_c = I_0 \operatorname{antiln} \frac{A_1 + A_2}{2} \qquad (1)$$

where I_0 and I_c are the observed and corrected fluorescence intensities and A_1 and A_2 are the absorbancies of the solution at the wavelengths of excitation and emission, respectively. Fluorescence quantum yields, Q, were obtained by exciting the sample of unknown Q and a standard of known Q at the same wavelength and were calculated as follows:

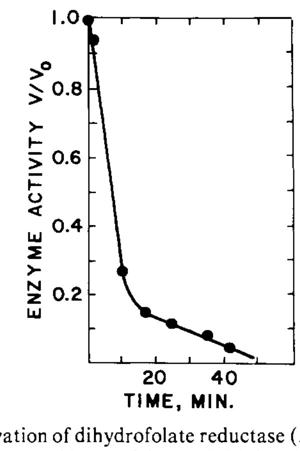

FIGURE 1: Inactivation of dihydrofolate reductase (5×10^{-5} M in 0.1 M KPO_4, pH 7.0) by Dns-Cl at 28 °C. The initial molar ratio of Dns-Cl to enzyme was 9:1. After 29 min an additional threefold molar excess of Dns-Cl was added. Enzyme activity, V/V_0, is expressed as a fraction of an untreated control.

$$Q_{\text{unknown}} = Q_{\text{standard}} \frac{F_{\text{unknown}} A_{\text{standard}}}{F_{\text{standard}} A_{\text{unknown}}} \qquad (2)$$

where F is the relative fluorescence as determined by weighing the area beneath the corrected fluorescence emission spectrum and A is the absorbance of the solutions at the exciting wavelength.

Results

Effect of Dansyl Chloride on Enzyme Activity. A pH of 7.0 was chosen for the inactivation studies since this value is at the pH optimum of the enzyme (pH 6.8–7.0) and is also a pH at which amino groups in general should be relatively unreactive with Dns-Cl (Gray, 1972). The effect of Dns-Cl at pH 7.0 on the enzyme activity is shown in Figure 1. The reagent causes a rapid inactivation of the enzyme, while the control sample retained more than 95% of its original activity throughout the course of the experiment. Dialysis of the reaction mixture to remove Dns-OH and acetone resulted in the precipitation of the Dns–enzyme. The problem of solubility of dansylated proteins has been reported previously (e.g., Chen, 1970). It was subsequently found that the Dns-reductase could be maintained in solution and separated from reaction products by gel filtration of the reaction mixture on a Bio-Gel P-2 column (1.0×26 cm) equilibrated with 0.1 M KPO_4 buffer, pH 7.0, containing 25% glycerol.

Ultraviolet Absorption Spectra of the Dansylated Enzyme. The uv absorption spectra of the native and modified enzymes are compared in Figure 2. The Dns–enzyme exhibits an absorption maximum in the region of 340–350 nm, due to the bound Dns moiety.

Fluorescence Emission Spectra of Native and Dansyl Dihydrofolate Reductases. The fluorescence emission spectra of native and Dns-dihydrofolate reductases are shown in Figure 3. The native enzyme, upon excitation at 295 nm, has an emission maximum at 334 nm. Using tryptophan as a standard with a quantum yield of 0.13 (Chen, 1967a), a quantum yield of 0.11 was obtained for the reductase. The protein fluorescence is markedly reduced upon dansylation of the enzyme (Figure 3). The fluorescence from the Dns group of Dns-dihydrofolate reductase interferes with the fluorescence of the protein above 420 nm. In order to obtain the quantum yield for the protein fluorescence in the modified enzyme, the fluorescence at 350 nm was extrapolated to a zero value at 480 nm, the wavelength where the fluorescence of the native enzyme

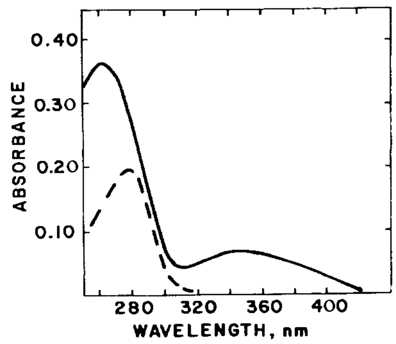

FIGURE 2: Comparison of the ultraviolet spectra of Dns-modified (—) and native (- - -) dihydrofolate reductases (7×10^{-6} M). Enzyme was inactivated as described under Methods and passed through a Bio-Gel P-2 column (1 × 26 cm) to remove free Dns-OH.

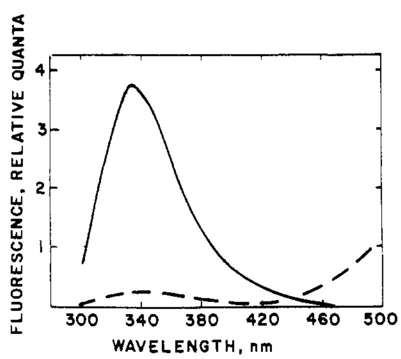

FIGURE 3: Fluorescence emission spectra of native (—) and Dns-modified (- - -) dihydrofolate reductase (7×10^{-6} M) on excitation at 295 nm. The native enzyme was in 0.1 M potassium phosphate buffer, pH 7.0; the Dns-enzyme was in the same buffer containing 25% glycerol.

approached zero. A quantum yield of less then 0.01 is thus obtained for the protein fluorescence of the Dns-reductase. Thus, modification of *L. casei* dihydrofolate reductase with dansyl chloride results in a greater than 90% reduction of the protein fluorescence.

Substrate Protection against Inactivation by Dansyl Chloride. The results of incubation of the enzyme with either of the substrates, NADPH or dihydrofolate, prior to treatment with Dns-Cl, are indicated in Table I. At molar ratios of NADPH or dihydrofolate to enzyme of ca. 1:1 significant protection of the enzyme occurs, and at molar ratios of ca. 2:1 the protection is even greater. Both NADPH and dihydrofolate afford essentially the same degree of protection against inactivation of the enzyme by Dns-Cl.

Amino Acid Analysis of Dns-Dihydrofolate Reductase. In order to identify the modified residue(s) responsible for the inactivation of dihydrofolate reductase by Dns-Cl, amino acid analyses were performed on native, Dns-Cl-inactivated, and inhibitor-protected samples. For the inhibitor protection studies, the ternary complex formed by amethopterin, a protein inhibitor of the enzyme, and NADPH were employed. The inactivated enzyme had ca. 5% activity remaining following Dns-Cl modification. Dns-Cl treatment of the native enzyme and of the inhibitor-protected enzyme was identical, but no enzymatic determination of the extent of protection could be obtained due to the potent stoichiometric inhibition of the enzyme by amethopterin (Werkheiser, 1961). Table II shows the results of the amino acid analyses. In both the inhibitor-protected and unprotected enzyme samples, a minor loss of 0.2–0.4 tyrosine residues occurred, possibly due to losses during hydrolysis or the formation of *O*-dansyltyrosine in small amounts. In the unprotected sample, a loss of 0.9 residue of lysine occurred, whereas no loss of lysine was observed in the inhibitor-protected enzyme.

Incorporation of [^{3}H]Dns Groups into Dihydrofolate Reductase. Due to the large variation in molar absorptivity of the Dns group when covalently bound to proteins (Chen, 1968, 1970), a more direct determination of the number of Dns groups incorporated into the enzyme was obtained employing [^{3}H]Dns-Cl. The progressive incorporation of [^{3}H]Dns groups

TABLE I: Substrate Protection against Inactivation of Dihydrofolate Reductase by Dns-Cl.[a]

Molar Ratio NADPH/Enz	Enzyme Act. V/V_0	Molar Ratio Dihydrofolate/Enz	Enzyme Act. V/V_0
0	0.22	0	0.22
1.2	0.62	1.3	0.61
2.0	0.76	2.0	0.86

[a] The enzyme (5.4×10^{-5} M) was incubated at 28 °C with either NADPH or dihydrofolate for 5 min in a total volume of 0.25 ml prior to the addition of an 8.2-fold excess of Dns-Cl in acetone. The results shown were obtained following incubation for 50 min. Neither dihydrofolate nor NADPH reacted with Dns-Cl under the experimental conditions described. Either substrate alone was reacted with Dns-Cl, as above, spotted on an Eastman 6064 cellulose TLC sheet, and developed with 0.2 M ammonium acetate. No difference was detected in the R_f for either substrate prior to or following incubation with Dns-Cl. In addition no Dns fluorescent material was associated with either substrate on the TLC plate.

TABLE II: Amino Acid Analysis of Native and Dns-Modified Dihydrofolate Reductases.[a]

Amino Acid	Native Enzyme	Enzyme–Inhibitor Ternary Complex + Dns-Cl	Dns-Enz
Asp	20.1	20.2	19.7
Thr	15.4	15.4	15.3
Ser	5.3	5.1	5.0
Glu	14.8	14.9	14.7
Pro	8.8	8.7	8.6
Gly	11.0	11.0	10.9
Ala	16.0	16.0	16.0
Val	11.4	11.3	11.6
Ile	4.2	4.1	4.1
Leu	12.9	13.1	12.9
Tyr	5.8	5.6	5.4
Phe	8.5	8.2	8.2
His	6.7	6.6	6.7
Lys	9.0	9.0	8.1
Arg	8.0	8.2	8.1

[a] Each sample contained 4.6×10^{-5} mmol of dihydrofolate reductase. Inhibitor protection was obtained by incubating the enzyme for 5 min at 25 °C with 8.4×10^{-5} mmol of NADPH and 9.0×10^{-5} mmol of amethopterin prior to the addition of Dns-Cl. An eightfold excess of Dns-Cl was added at time zero, followed by a twofold excess at 30 min, to the substrate-protected and Dns-enzyme samples. The native enzyme was inactivated to the extent of 95% with Dns-Cl after 45 min of incubation. Then 0.10 ml of 0.05 M Tris-HCl, pH 7.5, was added to react with any remaining Dns-Cl. The enzyme was precipitated by the addition of ethanol to a concentration of 50% and centrifuged, and the samples were analyzed for amino acid content, as described in the Methods section. Each value is an average of two determinations, based on 16 alanine residues.

TABLE III: Protection against Dns-Cl Inactivation of Dihydrofolate Reductase by Dns-OH.[a]

Time (min)	Enzyme Activity, V/V_0 (Enz + Dns-OH) + Dns-Cl	Enzyme + Dns-Cl
0.5	1.0	1.0
9	0.83	0.44
22	0.60	0.18

[a] The enzyme (1.3×10^{-5} mM) was incubated at 25 °C with 6.5×10^{-4} mM Dns-OH for 5 min. Dns-Cl (1.04×10^{-4} mM) was then added to this sample and an identical amount to a sample of enzyme alone. The maximum concentration of Dns-OH (1.6×10^{-6} M) in the assay mixture did not inhibit the enzyme.

TABLE IV: Effect of Dns-OH on Tryptophan and Dihydrofolate Reductase Fluorescence.[a]

Concentration of Dns-OH ($\times 10^{-6}$ M)	Fluorescence: Tryptophan[b]	Fluorescence: Enzyme[c]	$F_{enzyme}/F_{tryptophan}$
0	17.33	15.55	0.897
5.3	17.16	14.81	0.863
10.6	17.03	14.20	0.834
15.8	16.87	13.77	0.816
26.0	17.05	12.78	0.750
36.1	16.75	11.98	0.715
50.8	17.06	11.58	0.679
60.4	16.90	10.86	0.643

[a] Dns-OH was added in small volumes (5–10 μl) to 2.0 ml of the solution to be analyzed. The values have been corrected for dilutions and absorption effects. The measurements were made in 0.1 M potassium phosphate buffer, pH 7.0. [b] The tryptophan solution had an absorbance at 280 nm of 0.014. Excitation was at 280 nm and emission was monitored at 350 nm. [c] Dihydrofolate reductase was present at a concentration of 4.6×10^{-7} M, which had an absorbance at 280 nm of 0.014. Excitation was at 280 nm and emission monitored at 334 nm.

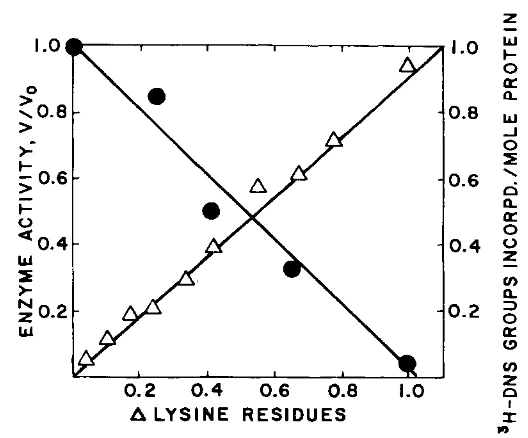

FIGURE 4: Inactivation of dihydrofolate reductase by Dns-Cl. Degree of inactivation was followed vs. loss of lysine residues/mol of protein (●) and by incorporation of [^{3}H]Dns groups (Δ) into the enzyme. Enzyme (5×10^{-5} M) was reacted with [^{3}H]Dns-Cl (see Methods), aliquots were taken at various time intervals and the reaction was terminated by the addition of a 300-fold molar excess of Tris-HCl, pH 7.5, to enzyme.

into the protein and the loss of lysine residues were investigated at various stages of enzyme inactivation. As indicated in Figure 4, the incorporation of a single [^{3}H]Dns group into dihydrofolate reductase correlates with the loss of one lysine residue and virtually complete enzyme inactivation.

Protection against Dns-Cl Inactivation of Dihydrofolate Reductase by Dns-OH. The possibility that Dns-Cl was being directed to the active site of dihydrofolate reductase via the hydrophobic dimethylaminonaphthalene ring was investigated employing the unreactive acid, Dns-OH. Inactivation of the enzyme by Dns-Cl was performed in the presence of a 50-fold molar excess of Dns-OH to enzyme. On addition of a sixfold molar excess of Dns-Cl to enzyme, substantial protection of the enzyme occurred (Table III). Furthermore, the reductase is inhibited 50% by Dns-OH at a concentration of ca. 8×10^{-4} M; this inhibition is completely reversible upon dilution of the enzyme–Dns-OH solution with assay buffer at all concentrations of Dns-OH examined (2×10^{-4} to 1.2×10^{-3} M). The data suggest that Dns-OH is binding at the same site on the enzyme as the Dns group from Dns-Cl.

Binding of Dns-OH to Dihydrofolate Reductase. The marked substrate protection against inactivation by Dns-Cl suggested that the Dns group might be binding at or near the active site of the enzyme. The quenching of protein fluorescence was used to further investigate this possibility. Increasing concentrations of Dns-OH were added to a solution of the enzyme and the protein fluorescence was measured. A complicating feature of this type of analysis is the dependence of fluorescence on the absorption in the cuvette at the exciting wavelength. To circumvent this problem, a tryptophan solution was prepared with the same absorbance at 280 nm as that of the protein solution. Identical amounts of Dns-OH were added to each cuvette, the solutions were excited at 280 nm, and the decrease in the tryptophan fluorescence was used as a standard to obtain the decrease in dihydrofolate reductase fluorescence due solely to Dns-OH binding. The results are shown in Table IV. As Klotz (1947) first established, and later Weber and Young (1964), interaction of a protein with small molecules can be described by two constants: N, the total number of binding sites on the protein molecule, and K, the dissociation constant. In order to determine both constants the data were substituted into eq 3:

$$\frac{P}{XD} = \frac{1}{N} + \frac{K}{(1-X)D} \tag{3}$$

where N and K are defined above; P is the total protein concentration; D is the total Dns-OH concentration; and X the fraction of Dns-OH bound, is obtained from eq 4,

$$f = \frac{F_{obsd} - F_f}{F_{Dns\text{-}E} - F_f};\ [E\cdot D] = f[P];\ X = \frac{[E\cdot D]}{D} \tag{4}$$

where f is the fraction of enzyme bound Dns-OH; F_{obsd} is the observed fluorescence of the enzyme in the presence of varying amounts of Dns-OH; $F_{DNS\text{-}E}$ is the fluorescence of the inactivated Dns–enzyme, for which a quenching of 95% was assumed (cf. Figure 3); F_f is the fluorescence of the native enzyme in the absence of Dns-OH; P is the total protein concentration; E·D is the enzyme-bound ligand concentration; and D is the total Dns-OH concentration. A plot of P/XD vs. $1/(1-X)D$ produces a line with an intercept on the abscissa equal to $-1/K$ and an intercept on the ordinate equal to $1/N$. Results of such an analysis of the data in Table IV are shown in Figure 5, and show that *L. casei* dihydrofolate reductase has a single Dns-OH binding site with a dissociation constant of 1×10^{-4} M.

Energy Transfer in Dansylated Dihydrofolate Reductase.

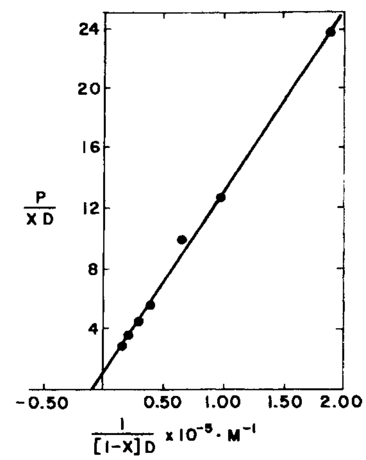

FIGURE 5: Determination of the number of binding sites and the dissociation constant for the Dns-OH-dihydrofolate reductase complex using the data in Table IV. For further details consult the text.

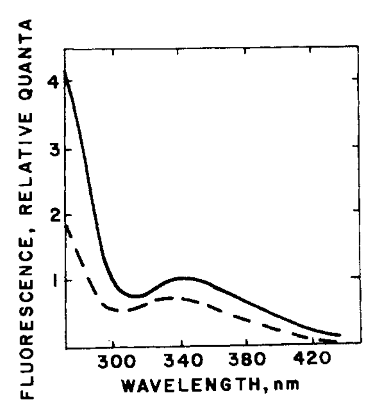

FIGURE 6: Excitation spectra of Dns-dihydrofolate reductase (4×10^{-7} M) in 0.1 M potassium phosphate buffer, pH 7.0 (—), and in the same buffer containing 7 M urea (- - -). The fluorescence emission was maintained at 540 nm.

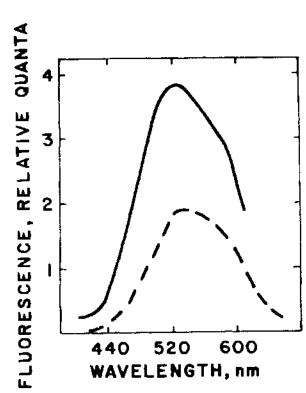

FIGURE 7: Fluorescence emission spectra of Dns-dihydrofolate reductase (7×10^{-6} M) upon excitation at 295 (—) and 365 nm (- - -) in 0.1 M potassium phosphate buffer, pH 7.0, containing 25% glycerol.

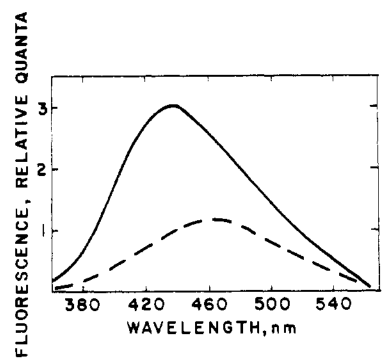

FIGURE 8: Fluorescence emission spectra of free (- - -) and enzyme-bound (—) NADPH on excitation at 340 nm. Each sample contained 3.8×10^{-7} M NADPH in 0.1 M potassium phosphate buffer, pH 7.0. For the enzyme-bound NADPH, dihydrofolate reductase was added to give a final concentration of 2.8×10^{-6} M.

The quenching of the enzyme fluorescence by modification with Dns-Cl is due, at least in part, to energy transfer from the excited-state tryptophan residues to the covalently attached ligand. In addition to spatial and steric requirements, the emission band of the energy donor molecule must overlap the absorption band of the energy acceptor molecule in order for transfer to occur. This prerequisite is satisfied in this system, since the protein fluorescence has a maximum at 334 nm and the dansyl absorption maximum is approximately 340 nm. Energy transfer was observed by varying the exciting wavelength while maintaining the dansyl fluorescence emission at 540 nm (Figure 6). The excitation spectrum of the Dns-enzyme shows a large Dns fluorescence intensity at wavelengths below 300 nm. Disruption of the tertiary structure of the enzyme should remove the Dns moiety from the vicinity of the tryptophan residue(s) and abolish energy transfer, since the necessary spatial and steric requirements would no longer be satisfied. The excitation spectrum of the Dns-enzyme in 7 M urea is also indicated in Figure 6. There is a pronounced decrease in the Dns fluorescence at excitation wavelengths below 300 nm. To ensure that the results were not due to solvent effects, the excitation spectra of E-Dns-Lys in both buffers were determined, and only very slight differences were observed.

Fluorescence Emission Spectrum of Dns-Dihydrofolate Reductase. Emission spectra of the Dns-enzyme are shown in Figure 7. The lower curve was observed upon direct excitation of the dansyl group at 365 nm. The upper curve was obtained by excitation at 295 nm, where observed emission is due to energy transfer from tryptophan to dansyl. The quantum yields reported by Chen (1967b) for Dns-tryptophan in ethanol and in water were used in an attempt to obtain a quantum yield for the dansyl moiety of the modified enzyme. The samples were excited at 340 nm, and a quantum yield of ca. 0.1 was obtained for the Dns group, with an emission maximum at ca. 540 nm.

Binding of NADPH to Native and Dns-Dihydrofolate Reductases. The fluorescence emission of NADPH, when excited at 340 nm, increases 2.3-fold upon binding to the native enzyme, and the fluorescence maximum shifts from 464 nm for the free dinucleotide to 436 nm for the bound form, as indicated in Figure 8. The well-known phenomenon of energy transfer from a protein to a bound pyridine nucleotide (Udenfriend, 1962) was used to investigate the binding of NADPH to the native and Dns-modified reductases. The wavelength of excitation was varied while maintaining the NADPH fluorescence emission at 470 nm. A large increase in the fluorescence is observed on excitation below 300 nm upon binding the dinucleotide to the enzyme (Figure 9). The insert to Figure 9 represents the difference between the free and bound forms of the pyridine nucleotide. The result of the addition of NADPH to Dns-dihydrofolate reductase under identical conditions showed no increase in this wavelength region, indicating that binding of the dinucleotide to the modified enzyme does not occur.

Binding of Dihydrofolate to Native and Dns-Dihydrofolate Reductases. The fluorescence emission of dihydrofolate, when excited at 325 nm, increases between 350 and 420 nm in the

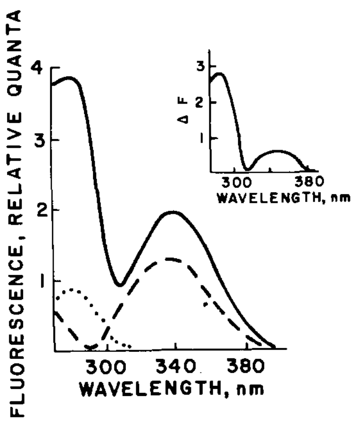

FIGURE 9: Excitation spectra of free (- - -) and enzyme-bound (—) NADPH with emission set at 470 nm. Each sample contained 2.3×10^{-7} M NADPH in 0.1 M potassium phosphate buffer, pH 7.0. Dihydrofolate reductase was added to give a final concentration of 1.7×10^{-6} M. The excitation spectrum of the native enzyme with emission at 470 nm is also shown (····). The inset shows the difference excitation spectrum of the [enzyme-NADPH] complex − [NADPH + enzyme].

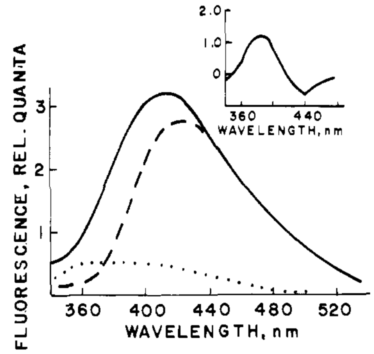

FIGURE 10: Emission spectra of dihydrofolate in buffer (- - -) and in the presence of dihydrofolate reductase (—) upon excitation at 325 nm. Emission of enzyme alone is also shown (····). Dihydrofolate was present at a concentration of 2.7×10^{-6} M; the enzyme concentration was 2.6×10^{-6} M; the buffer was 0.1 M potassium phosphate, pH 7.0. The insert represents the difference in emission of dihydrofolate in the presence and absence of enzyme, corrected for enzyme emission in this region.

presence of equimolar concentrations of dihydrofolate reductase (Figure 10). The insert to Figure 10 represents the differences between the free and bound forms of the substrate, with a maximum increase of 1.2 fluorescence units at ca. 385 nm. The result of the addition of dihydrofolate to the dansylated enzyme suggests that binding no longer occurs. The fluorescence due to the dansyl moiety prevents interpretation of the emission spectra above 440 nm, but does not interfere in the region of the emission spectra which changes upon the binding of dihydrofolate to the enzyme. There is no change in the emission spectrum of dihydrofolate below 400 nm, indicating a lack of binding of dihydrofolate to Dns-dihydrofolate reductase.

Similarly, characteristic circular dichroic ellipticity bands generated upon the formation of dihydrofolate reductase–NADPH, -dihydrofolate, or -amethopterin binary complexes[2] are not observed when these ligands are added to solutions of the Dns-enzyme. In addition, the circular dichroic spectra of native and Dns-reductases are virtually identical in the wavelength region 400–220 nm, suggesting that no gross conformational alterations have occurred following dansylation.

Discussion

Dihydrofolate reductase from amethopterin-resistant *L. casei* is rapidly and irreversibly inactivated at pH 7.0 by relatively low concentrations of dansyl chloride. Results of radiolabeling studies with [^{3}H]Dns-Cl and amino acid analyses indicate the involvement of a single lysine of a total of nine such residues. The ϵ-amino group of this residue may well have an abnormally low pK_a, as evidenced by its reactivity at pH 7.0. Such a lowered pK_a for the reactive ϵ-amino moiety would be consistent with its location in a relatively hydrophobic environment, as suggested by the fluorescence data (cf. Figure 3).

There are several possible subsites on the enzyme for the involvement of a functional lysine residue. The negatively charged glutamate portion of the folate molecule, the negatively charged pyrophosphate grouping, or the 2′-phosphate on the adenosine ribose of NADPH are possible anionic sites which could form a salt bridge with a lysine residue. In lactate dehydrogenase the positively charged guanidinium group of arginine-101 interacts with the pyrophosphate grouping of NAD based on x-ray crystallographic and sequence data (Adams et al., 1973).

The native enzyme has a fluorescence emission maximum at 334 nm upon excitation at 295 nm, which is in agreement with that determined by Otting and Huennekens (1972), who reported a maximum of 335 nm for the *L. casei* reductase. This is a markedly lower emission maximum than previous reports for dihydrofolate reductase from other sources: the L1210 enzyme emission maximum occurring at 345–350 nm (Perkins and Bertino, 1966), *E. coli* and bacteriophage T4 maxima at 350 nm (Erickson and Mathews, 1973), amethopterin-resistant *E. coli* maximum at 350 nm (Williams et al., 1973a), and amethopterin-resistant *S. faecium* with a maximum at 345 nm (Warwick and Freisheim, 1975).

Dansylation of the enzyme produces a dramatic decrease in the protein fluorescence. The relative quantum yield decreases from 0.11 for the native reductase to less than 0.01 for the dansylated enzyme (Figure 3). It appears common among dihydrofolate reductases from other sources that binding of substrates or inhibitors to the enzyme quenches the protein fluorescence (Huennekens et al., 1970; Williams et al., 1973a; Williams et al., 1973b; Perkins and Bertino, 1966; Erickson and Mathews, 1973). Both substrates of the *L. casei* dihydrofolate reductase also quench protein fluorescence. For example, a twofold excess of NADPH to enzyme results in a 90% quenching of protein fluorescence.[2] The quenching observed upon dansylation of the enzyme or in the Dns-OH–enzyme complex may be related to that observed on substrate or inhibitor binding. Direct excitation of the covalently attached Dns moiety on the enzyme at 365 nm produces an emission with a maximum between 530 and 540 nm. Excitation at 295 nm produces a Dns fluorescence emission maximum between 520 and 530 nm, presumably via enzyme tryptophan → Dns energy transfer (cf. Figure 7). The blue shift in Dns fluorescence and increased intensity observed upon excitation at the lower wavelength may again indicate that the Dns group is in a relatively hydrophobic environment, possibly in the vicinity of tryptophan residues.

Tryptophan residues have been implicated in the active centers of dihydrofolate reductases from *L. casei* (Liu and Dunlap, 1974), as well as from chicken liver (Freisheim and Huennekens, 1969) and amethopterin-resistant strains of *S. faecium* (Warwick et al., 1972) and *E. coli* (Greenfield, 1974). Transfer of electronic excitation energy from one or more

[2] Unpublished observations from this laboratory.

tryptophan residues to the attached dansyl moiety was, a priori, a distinct possibility. Maintaining dansyl fluorescence at 540 nm and examining the excitation spectra at wavelengths below 300 nm indicate that such an energy transfer occurs, whereas the effect is greatly reduced when the tertiary structure of the dansylated enzyme is disrupted (cf. Figure 6).

The emission spectrum of free NADPH with excitation at 340 nm has a wavelength maximum at 465 nm. The relative quantum yield of the reduced dinucleotide is increased 2.3-fold upon binding to the *L. casei* dihydrofolate reductase. The observed blue shift of the enzyme-bound NADPH of ca. 30 nm is one of the largest reported in the literature (cf. Figure 8) and provides additional evidence for a relatively hydrophobic binding domain.

The transfer of electronic excitation energy from enzyme tryptophan residues to bound pyridine nucleotide is another well-studied fluorescence characteristic exhibited by the *L. casei* dihydrofolate reductase (Figure 9). The fluorescence enhancement of NADPH following enzyme binding upon excitation below 300 nm was used to determine if this substrate could bind to the dansylated reductase. Similar studies with the Dns-enzyme indicate that the modified enzyme does not bind NADPH, or that it does so with a greatly reduced affinity. Similarity, although dihydrofolate fluorescence is enhanced upon binding to the native reductase (cf. Figure 10), no such effect occurs between dihydrofolate and the dansylated enzyme. These data indicate that the site of dansylation may be located in a region on the protein molecule which overlaps the binding domains for both folate and dinucleotide substrates. This notion is supported by the observation that either substrate protects about equally well against the enzyme inactivation produced by Dns-Cl. Although protein tryptophan to dansyl energy transfer appears to occur, the bulky dimethylaminonaphthalene moiety may sterically hinder the binding of either substrate. It could be argued that the attachment of the Dns moiety at a location distant from the active site might cause a conformational change in the enzyme that alters the active site. Alternatively, substrate binding could result in a conformational change distant to the active site which buries the reactive lysine and renders it unavailable for reaction.

Chen (1967b) has suggested that the dansyl group has a special affinity for hydrophobic sites on proteins due to the naphthalene nucleus. The apparent hydrophobic nature of the active center of dihydrofolate reductase is in keeping with the aromatic nature of the substrates. The similarity of the structure of dansyl chloride with the adenine moiety of NADPH, as well as with the pteridine portion of folate, suggests that the naphthalene nucleus of the reagent might be directed to the active center of the enzyme. It has been shown that Dns-OH protects the reductase against inactivation by Dns-Cl, implying that the inactivation occurs through the binding of the reagent to the enzyme. In addition, Dns-OH inhibits enzyme activity and binds to a single site on the enzyme. These observations suggest that the dimethylaminonaphthyl moiety may be directed to a hydrophobic region at or near the active center of dihydrofolate reductase followed by reaction of the sulfonyl chloride of the reagent with the susceptible lysine residue.

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