Interaction of 1,N^6-Ethenoadenine Derivatives of Triphosphopyridine and Reduced Triphosphopyridine Nucleotides with Dihydrofolate Reductase from Amethopterin-Resistant L1210 Cells†

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ABSTRACT: The 1,N^6-ethenoadenine derivatives of triphosphopyridine and reduced triphosphopyridine nucleotides (TPN and TPNH) (ε-TPN and ε-TPNH) have been synthesized and used as fluorescent probes to examine the pyridine nucleotide binding site of L1210 dihydrofolate reductase. ε-TPNH (K_m = 16.7 μM) was able to replace TPNH (K_m = 3.8 μM) in the enzyme-catalyzed reduction of dihydrofolate, and both ε-TPN and ε-TPNH formed binary complexes with the enzyme that were stable to polyacrylamide gel electrophoresis. The fluorescence of ε-TPN was enhanced and the emission maximum shifted from 415 to 405 nm when the nucleotide was bound to the enzyme. The ethenoadenine moiety in ε-TPNH behaved similarly, but the fluorescence changes were complicated by concurrent effects of binding upon the dihydronicotinamide fluorophore. Fluorescence enhancement titrations yielded values of 1.8 and 0.59 μM, respectively, for the dissociation constants of the enzyme–ε-TPN and enzyme–ε-TPNH complexes. Titration experiments based upon quenching of enzyme fluorescence gave similar values, viz., 2.1 and 0.53 μM, for the dissociation constants of these complexes. Fluorimetric titration of the enzyme–TPNH complex with ε-TPN (or of the enzyme–TPN complex with ε-TPNH) failed to reveal the presence of a second pyridine nucleotide binding site. The fluorescence enhancement of enzyme-bound ε-TPN or dihydrofolate was quenched when amethopterin or ε-TPN, respectively, was added to form a ternary complex. These results provide information concerning the nature of the pyridine nucleotide binding site and its spatial relationship to the dihydrofolate/amethopterin binding site.

Dihydrofolate reductases (EC 1.5.1.3), which catalyze the TPNH[1]-dependent reduction of dihydrofolate to tetrahydrofolate, have been the subject of numerous investigations (reviewed in Huennekens et al., 1971; Blakley, 1969) not only because they represent the presumed target site for folate antagonists used in cancer chemotherapy, but also because they are among the smallest known pyridinoproteins (mol wt ≃ 20 000). These enzymes bind substrates and substrate analogues rather tightly (K_D values in the range of 10^{-5}–10^{-9} M), and the resulting complexes have been examined by a variety of techniques (reviewed in Huennekens et al., 1976), including the use of fluorescent probes. Both substrates, dihydrofolate and TPNH, have an intrinsic fluorescence, and nonfluorescent analogues of the folate substrate (e.g., amethopterin) can be made fluorescent by covalent attachment of fluorescein, via a spacer, to one of the carboxyl groups (Gapski et al., 1975). The facile 1,N^6-etheno modification of adenine nucleotides, first introduced by Leonard and his colleagues (Barrio et al., 1972a,b; Secrist et al., 1972), provides a means for introducing a fluorescent label into TPN (ε-TPN), or a second fluorophore into TPNH (ε-TPNH). These compounds, ε-TPN and ε-TPNH, have been utilized in the present investigation as fluorescent probes to examine the pyridine nucleotide binding site on a homogeneous dihydrofolate reductase from an amethopterin-resistant subline of cultured L1210 cells.

Experimental Procedure

Materials. The following were obtained from the indicated commercial sources: TPN and TPNH (P-L Biochemicals); [carbonyl-^{14}C]TPN (37 mC_i/mmol) (Amersham); glucose 6-phosphate and glucose-6-phosphate dehydrogenase (Sigma); Aquasol (New England Nuclear); anion exchanger AG 1-X8 (Bio-Rad Laboratories); DEAE-cellulose (DE-52) (Whatman).

Chloroacetaldehyde diethyl acetal (Aldrich) was converted to the free aldehyde by the procedure of Secrist et al. (1972). Cellulose-PEI (J. T. Baker) was washed with water prior to use. Dialysis tubing (van Waters and Rogers) was treated as described previously (Neef and Huennekens, 1975). Norit-A (Matheson Coleman & Bell) was purified by the method of Dunlap et al. (1971). Dihydrofolate was prepared by the method of Blakley (1960). Amethopterin was a gift from Dr. Florence White, National Cancer Institute, National Institutes of Health.

Dihydrofolate reductase was purified to homogeneity from an amethopterin-resistant strain of cultured murine leukemia cells (L1210/R6) by the procedure described previously (Neef and Huennekens, 1975). The enzyme (ϵ_{mM} = 29.2 at 278 nm) has a molecular weight of 20 000 and a specific activity of 23 μmol of dihydrofolate reduced per min per mg of protein when assayed in the absence of activating ions (Neef and Huennekens, 1975).

ε-TPN was prepared by the following procedure based upon that used by Barrio et al. (1972b) for ε-DPN. TPN (200 mg; 0.25 mmol) was dissolved in 10 ml of aqueous chloroacetaldehyde (ca. 1.4 M) and the pH was adjusted to 4.4 using NH_4OH. The solution (shielded from light) was stirred at 37 °C for 16 h, and the pH was maintained at 4.4. The mixture was decolorized with 250 mg of Norit-A and passed through a 0.22-μm Millipore filter. The effluent was adjusted to pH 7.0

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[1] Abbreviations used are TPN, triphosphopyridine nucleotide; TPNH, reduced TPN; DEAE, diethylaminoethyl; TLC, thin-layer chromatography.

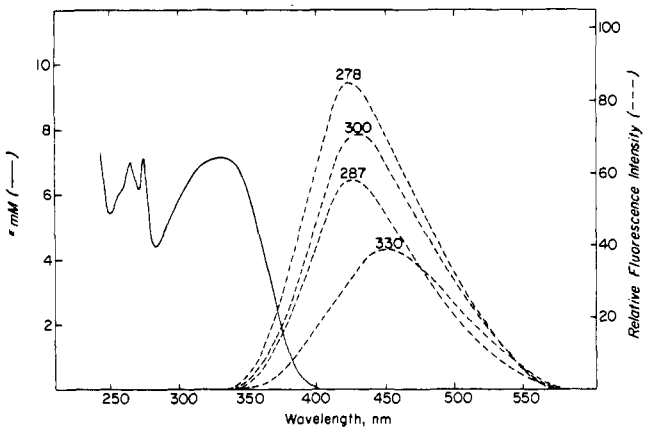

FIGURE 1: Absorbance and fluorescence spectra of ϵ-TPNH (9.0×10^{-5} M) at pH 8.0. Absorbance spectrum (—); fluorescence spectra (- - -) obtained by excitation at the indicated wavelengths.

and applied to a 3.4 × 21 cm column of AG 1-X8 anion exchanger (formate form). The column was washed with 500 ml of water and then eluted with a linear gradient of formic acid (water → 3 N formic acid; 1 l. of each). Nine-milliliter fractions were collected and monitored for absorbance at 265 nm. The contents of tubes 158–200, which contained the ϵ-TPN, were pooled and lyophilized to dryness. The material was dissolved in a minimum volume of water, precipitated with ethanol, recovered by centrifugation, washed with ethanol and ether, and dried in vacuo over $CaCl_2$. Yield, 119 mg (60%). R_f 0.65 (fluorescent) on TLC. At pH 7.5, λ_{max} at 265 nm (ϵ_{mM} = 9.7) with shoulders at 258, 274, and 295 nm. Cyanide adduct of ϵ-TPN, λ_{max} at 314 nm (ϵ_{mM} = 6.6). pK_a 4.2 (by both spectrophotometric and fluorimetric titration). The material was stable when stored as a solid in vacuo over $CaCl_2$.

ϵ-TPNH was prepared by the following procedure based upon that used by Kornberg and Horecker (1955) for TPNH. ϵ-TPN (12.4 mg; 0.015 mmol) was dissolved in 5.9 ml of 0.012 M glycylglycine buffer, pH 7.5, containing 0.03 M $MgCl_2$ and 0.0125 M glucose 6-phosphate. One-tenth milliliter of glucose-6-phosphate dehydrogenase (25 units/ml) was added, and the solution (shielded from light) was stirred at room temperature for 2 h; the pH was maintained at 7.5. After adjustment to pH 8.0, the solution was chromatographed on a 2.9 × 9 cm column of DEAE-cellulose that had been washed previously with 0.1 M glycylglycine, pH 8.0, followed by water, and then equilibrated with 0.01 M glycylglycine, pH 8.0. The column was eluted with a linear gradient of NaCl (0.01 M glycylglycine, pH 8.0 → 0.01 M glycylglycine, pH 8.0, containing 1.5% NaCl). The contents of tubes 265–300 were pooled and lyophilized to dryness. The material was dissolved in 0.5 ml of 0.1 M $(NH_4)_2CO_3$ and the solution was passed through a 1.4 × 100 cm column of Sephadex G-10 to remove NaCl; 0.1 M $(NH_4)_2CO_3$ was used as the eluant. The fractions containing ϵ-TPNH (ca. 30 ml) were stored frozen or lyophilized to dryness and processed further as described above for ϵ-TPN. Yield, 8 mg (65%). R_f 0.55 (fluorescent) on TLC. At pH 8.0, λ_{max} at 265 nm (ϵ_{mM} = 7.0), 274 nm (ϵ_{mM} = 7.1), and 332 nm (ϵ_{mM} = 7.1), with a shoulder at 258 nm. pK_a 4.1 (spectrophotometric titration). ϵ-TPNH, which is considerably less stable than ϵ-TPN, was stored frozen (−20 °C) in sealed ampules.

Methods. Thin-layer chromatography was performed on cellulose-PEI with 1 M LiCl as the solvent. The procedure for polyacrylamide gel electrophoresis of enzyme–ligand complexes has been described previously (Dunlap et al., 1971; Neef and Huennekens, 1975). Absorbance spectra were determined on a Cary recording spectrophotometer, Model 14. Extinction coefficients were obtained from absorbance measurements on samples of ϵ-TPN and ϵ-TPNH labeled with [carbonyl-^{14}C]TPN of known specific activity.

Fluorescence measurements were made with a Turner spectrofluorometer, Model 210 "Spectro," equipped with an XBO 75 W high-pressure xenon lamp and automatic correction for backscattering. Excitation and emission bandwidths were set at 10 nm. Quartz cuvets (3 ml; 1-cm light path) were used, and all measurements were made at ambient temperature. Data are expressed in terms of relative fluorescence intensity. For the determination of dissociation constants (via enhancement of ligand fluorescence or quenching of protein fluorescence), data were analyzed by a method based upon that of Winer and Schwert (1959) in which $1/\Delta f$ is plotted vs. $1/C$, according to eq 1

$$\frac{1}{\Delta f} = \frac{K_D}{\Delta F}\left(\frac{1}{C}\right) + \frac{1}{\Delta F} \quad (1)$$

where Δf is the observed change in fluorescence, ΔF is the maximum change in fluorescence (obtained by extrapolation of the double-reciprocal plot), C is the concentration of ligand added, and K_D is the dissociation constant of the enzyme–ligand complex.

Results and Discussion

Preparation and Properties of ϵ-TPN and ϵ-TPNH. Procedures have been described previously by Leonard and his colleagues (Barrio et al., 1972b; Greenfield et al., 1975) for the synthesis of ϵ-DPN (via treatment of DPN with chloroacetaldehyde) and ϵ-DPNH (via enzymatic reduction of ϵ-DPN). These derivatives were characterized with respect to elemental composition, melting point, absorbance and fluorescence spectra, and pK_a values (Barrio et al., 1972a,b; Greenfield, 1975). Lee and Everse (1973) utilized analogous procedures for the preparation of ϵ-TPN and ϵ-TPNH, but detailed chemical and physical properties of these latter compounds were not reported.

In the present investigation, ϵ-TPN was also obtained from the reaction of TPN with chloroacetaldehyde, according to the published procedure for ϵ-DPN (Barrio et al., 1972b). The yield was improved by decreasing the time of incubation to 16 h, raising the temperature to 37 °C, and maintaining the pH continuously at 4.4. The product was isolated by anion-exchange chromatography and ethanol precipitation. Enzymatic reduction of ϵ-TPN via the glucose-6-phosphate dehydrogenase system afforded ϵ-TPNH, which was isolated by chromatography on DEAE-cellulose and passage through Sephadex G-10. Purity of both products was verified by thin-layer chromatography and by absorbance spectra (see Experimental Procedure). Absorbance maxima and extinction coefficients for ϵ-TPN (and for its cyanide adduct) were nearly identical to those reported previously for ϵ-DPN (Barrio et al., 1972b). The absorbance spectrum of ϵ-TPNH at pH 8.0 was similar to that reported by Greenfield et al. (1975) for ϵ-DPNH; extinction coefficients for the absorbance maxima of ϵ-TPN and ϵ-TPNH have been summarized under Experimental Procedure.

The fluorescence emission maximum of ϵ-TPN (at pH 7.5), centered at 415 nm, did not vary with excitation from 265 to 330 nm. Fluorescence characteristics of ϵ-TPNH, conversely, depended markedly upon the excitation wavelength (Figure 1). Excitation at 330 nm (where ϵ-adenine has virtually no absorbance) led to a maximum at 455 nm, which is close to the

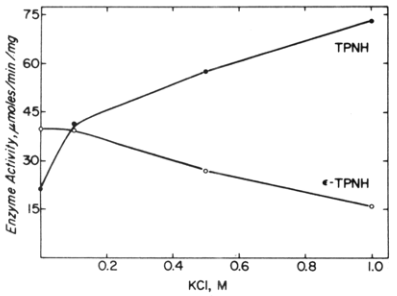

FIGURE 2: Activity of L1210 dihydrofolate reductase as a function of KCl concentration. Enzyme activity at pH 7.0 and 37 °C was determined by the previously described assay (Neef and Huennekens, 1975); ϵ-TPNH or TPNH was present at 19.5 μM and the KCl concentration was varied as indicated. For determination of activity in the presence of ϵ-TPNH, a differential extinction coefficient ($\Delta\epsilon_{mM}$) of 12.6 was used.

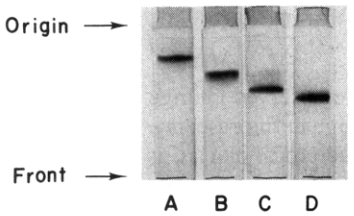

FIGURE 3: Polyacrylamide gel electrophoresis of L1210 dihydrofolate reductase (A), of its binary complexes with ϵ-TPN (B) or ϵ-TPNH (C), and of the ternary complex containing ϵ-TPNH and amethopterin (D). Gels were stained for protein using amido black. R_f values, from left to right, are 0.21, 0.33, 0.43, and 0.48.

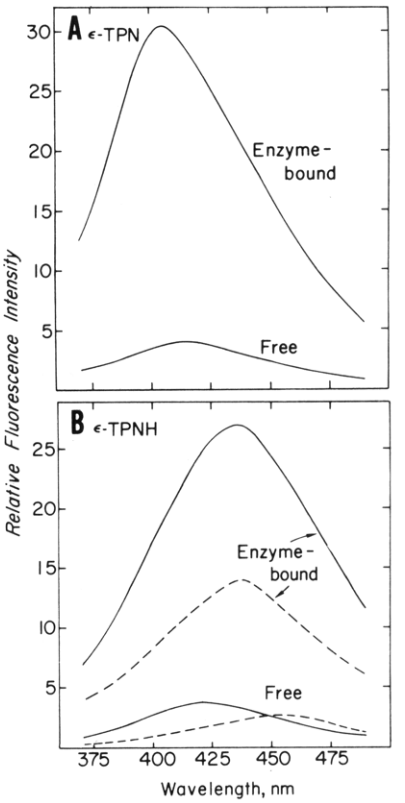

FIGURE 4: Fluorescence spectra of free and enzyme-bound ϵ-TPN (A) and ϵ-TPNH (B). L1210 dihydrofolate reductase (6.0 μM) was admixed with a fivefold molar excess of the ϵ derivative and dialyzed against 0.05 M Tris-Cl buffer (pH 7.5 for ϵ-TPN and pH 8.0 for ϵ-TPNH), according to the method previously described (Otting and Huennekens, 1972; Neef and Huennekens, 1975). Solid lines, excitation at 287 nm; dashed lines; excitation at 330 nm.

reported value of 460 nm for the dihydronicotinamide structure (Kaplan, 1960). Alternatively, excitation at 287 nm (where absorbance of dihydronicotinamide is low) resulted in emission at 422 nm, due largely to the ϵ-adenine group whose maximum is at 415 nm (Secrist et al., 1972). At the two other excitation wavelengths (287 and 300 nm) shown in Figure 1, mixed fluorescence spectra were observed. Fluorescence intensities, which also varied with excitation wavelength, were more difficult to interpret because of intramolecular interaction between the two chromophoric centers of the pyridine nucleotide (Oppenheimer et al., 1971; Luisi et al., 1975; Gruber and Leonard, 1975).

ϵ-TPNH as a Coenzyme for the Enzymatic Reduction of Dihydrofolate. ϵ-TPNH was able to substitute for TPNH as a coenzyme for the L1210 dihydrofolate reductase. A double-reciprocal plot (not shown) of the activity vs. coenzyme concentration data yielded apparent K_m values of 3.8 and 16.7 μM and V_{max} values of 32.8 and 143 μmol $min^{-1}mg^{-1}$, respectively, for TPNH and ϵ-TPNH. This somewhat unusual result, i.e., higher activity with a coenzyme analogue than with the coenzyme itself, may be due to an enhanced rate of transfer of the hydride ion (caused by closer positioning of the dihydronicotinamide moiety to the pyrazine ring) or to the more facile release of the product, ϵ-TPN, from the enzyme. Since the TPNH-dependent activity of the L1210 enzyme is also known to be increased several-fold in the presence of KCl (Reyes and Huennekens, 1967; Perkins et al., 1967), it was of interest to compare the effects of this activator upon the enzyme system when TPNH or ϵ-TPNH served as the coenzyme. As shown in Figure 2, activity of the TPNH-dependent reduction of dihydrofolate increased with increasing concentrations of KCl, but the ϵ-TPNH-dependent activity, surprisingly, decreased under these conditions.

Complex Formation Between ϵ-TPN or ϵ-TPNH and the Enzyme. Further evidence that the 1,N^6-ethenoadenine modification did not appreciably impair the ability of TPN and TPNH to interact with the L1210 dihydrofolate reductase was obtained through the use of polyacrylamide gel electrophoresis to study complex formation. The electrophoretic behavior of the binary complexes containing ϵ-TPN and ϵ-TPNH, respectively, and the ternary complex containing ϵ-TPNH and amethopterin is shown in Figure 3. R_f values given in the legend to Figure 3 may be compared with those of the enzyme–TPN (0.40), enzyme–TPNH (0.45), and enzyme–TPNH–amethopterin (0.52) complexes (Neef and Huennekens, 1975). Each complex migrated ahead of the free enzyme (R_f 0.21) but slightly behind its counterpart with the unmodified pyridine nucleotides.

Interaction of ϵ-TPN with the L1210 dihydrofolate reductase caused the fluorescence emission maximum of the nucleotide to shift from 415 to 405 nm (Figure 4A). There was also an enhancement of the fluorescence intensity which, under the conditions of this experiment (equimolar concentrations of enzyme and ϵ-TPN), was about sevenfold with respect to the fluorescence of free ϵ-TPN. This blue shift of the maximum can be attributed to transfer of the fluorophore from an aqueous to a less polar environment (Yguerabide, 1972), while the increased intensity appears to be due to a combination of this factor and a conformational change of the dinucleotide from a closed to a more open form (Barrio et al., 1972b; Luisi et al., 1975).

Similar experiments were carried out with ϵ-TPNH, but the results were more complex. When excitation occurred at 330 nm, interaction of ϵ-TPNH with the enzyme caused the fluorescence emission maximum of the former to shift from 455

to 437 nm (Figure 4B). This is similar to the blue shift (460 → 445 nm) seen when TPNH binds to the L1210 enzyme (Perkins and Bertino, 1966). Alternatively, excitation at 287 nm resulted in a shift of the fluorescence maximum from 422 (free ϵ-TPNH) to 435 nm (bound ϵ-TPNH). This red shift indicated that, contrary to the observation with free ϵ-TPNH, the dihydronicotinamide group was also being excited under these conditions (via energy transfer from the protein), and that its fluorescence was being superimposed upon that of the ϵ-adenine moiety. These results suggested that the dihydronicotinamide group would be the more preferable reporter for measuring interaction of ϵ-TPNH with the enzyme.

Dissociation Constants for the Enzyme-ϵ-TPN and Enzyme-ϵ-TPNH Complexes. Titration of the enzyme with ϵ-TPN, measured by the increase in fluorescence of the latter at 405 nm, is shown in Figure 5. Analysis of these data by means of a double-reciprocal plot, according to eq 1 (see Experimental Procedure), gave a value of 1.8 μM for the dissociation constant of the enzyme-ϵ-TPN complex. A similar titration experiment (not shown) was performed with ϵ-TPNH; in this instance, excitation was at 330 nm and emission at 437 nm. A value of 0.59 μM was obtained for the dissociation constant of the enzyme-ϵ-TPNH complex.

The fluorescence of tryptophan residues on the L1210 dihydrofolate reductase is quenched by the addition of substrates and inhibitors, and this property has been exploited previously by Perkins and Bertino (1966) to determine the dissociation constants for various binary and ternary complexes. This technique has also been used in the present investigation[2] to obtain, via an independent measurement, values for the dissociation constants of the enzyme-ϵ-TPN and enzyme-ϵ-TPNH complexes. Analysis of these data, according to eq 1, is shown in Figure 6; for comparison, data for the respective complexes with the unmodified pyridine nucleotides are also included. Dissociation constants for the ϵ-TPN, ϵ-TPNH, TPN, and TPNH complexes were 2.1, 0.53, 1.3, and 0.41 μM, respectively. Perkins and Bertino (1966) have previously reported values of 0.9 and 0.05 μM for the TPN and TPNH complexes with their preparation of the L1210 dihydrofolate reductase.

Investigation of the Possible Existence of Two Pyridine Nucleotide Binding Sites on the Enzyme. It has been suggested previously by this laboratory (Huennekens et al., 1971) that dihydrofolate reductases might contain two pyridine nucleotide binding sites because of the ability of the enzyme to catalyze the transhydrogenation reaction shown in eq 2.

$$\text{TPNH} + \text{acetylpyridine-TPN} \rightleftharpoons \text{TPN} + \text{acetylpyridine-TPNH} \quad (2)$$

This reaction requires the presence of catalytic amounts of dihydrofolate or tetrahydrofolate, and labeling experiments (F. Otting, unpublished observations) have shown that the folate compound serves as an intermediate carrier of the hydride ion during its passage from one pyridine nucleotide to the other. The rate of the TPNH-dependent reduction of acetylpyridine-TPN, although slower than that of the normal reaction (i.e., the TPNH-dependent reduction of dihydrofolate), is still sufficiently rapid to raise the question of whether it could be sustained by an enzyme with only one pyridine nucleotide binding site. If this were the case, TPNH would have to occupy the site, transfer its hydride ion to dihydrofolate, and then be replaced by acetylpyridine-TPN that would accept the hydride ion. Later Williams et al. (1973) presented evidence (based upon the equilibrium dialysis studies) for the existence of two pyridine nucleotide binding sites on the *E. coli* enzyme. In order to examine this problem further, the experiment illustrated in Figure 7 was performed. The L1210 dihydrofolate reductase was first titrated with TPNH, using fluorescence enhancement of the latter at 445 nm (Perkins and Bertino, 1966) as the measure of binding. The titration was continued well beyond the 1:1 equivalence point (intersection of the dotted lines) in order to insure essentially complete occupancy of the TPNH site. Then, at the point indicated by the arrow, the enzyme-TPNH complex was titrated with ϵ-TPN in an effort to detect a separate binding site for the oxidized pyridine nucleotide. There was, however, no response from the latter probe, except for that which could be attributed to its inherent fluorescence in the absence of enzyme. In a separate experiment (data not shown), the order of addition of the oxidized and reduced ligands was reversed. ϵ-TPNH, however, exhibited no enhanced fluorescence (at 435 nm) when it was added after saturation of the enzyme with nonfluorescent TPN. These experiments, of course, do not rule out the possibility of a second binding site having a much weaker affinity for the pyridine nucleotide, or of a type of binding that does not alter the fluorescence of the ligand.

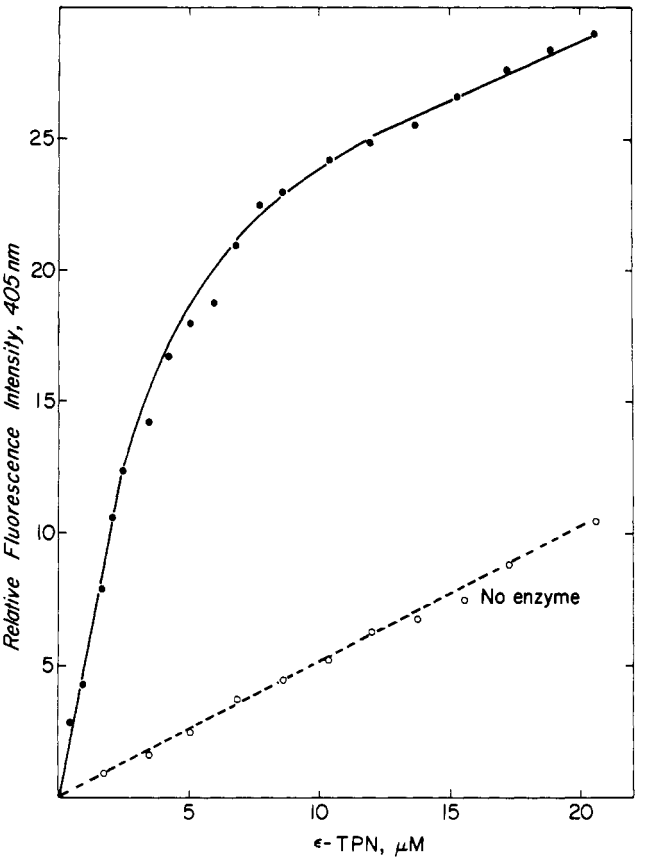

FIGURE 5: Titration of L1210 dihydrofolate reductases with ϵ-TPN (●—●). Enzyme concentration, 4.4 μM. Excitation at 287 nm, fluorescence emission at 405 nm. Increases in relative fluorescence intensity (corrected for fluorescence of the free enzyme at 405 nm) were noted after addition of the indicated amounts of ϵ-TPN. Control (O- - -O), fluorescence of free ϵ-TPN in the absence of enzyme.

Ternary Complexes Containing Enzyme, ϵ-TPN, and Amethopterin or Dihydrofolate. The fluorescent ϵ-pyridine nucleotides are also useful for studying ternary complexes containing the enzyme and appropriate combinations of ligands

[2] The fluorescence emission maximum of the L1210 enzyme used in this study occurred at 330 nm, rather than at 345–350 nm as reported by Perkins and Bertino (1966).

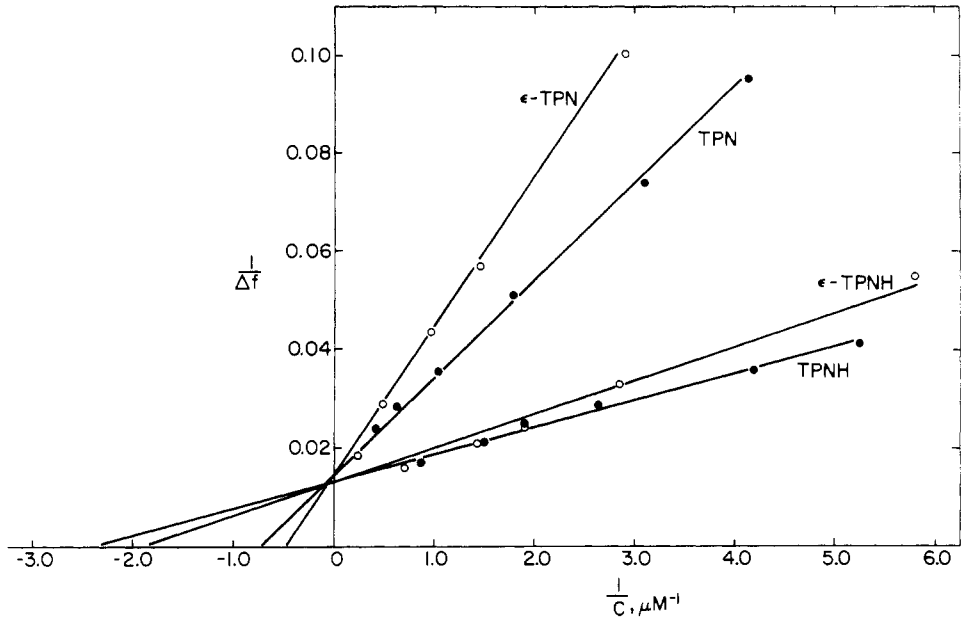

FIGURE 6: Interaction of L1210 dihydrofolate reductase with pyridine nucleotides. Enzyme concentration, 4.4 μM. Excitation at 287 nm, fluorescence emission at 330 nm. After each addition of the indicated pyridine nucleotide, the decrease in relative fluorescence intensity of the protein (Δf) was noted. Results are plotted as $1/\Delta f$ vs. $1/C$, where C is the concentration of pyridine nucleotide. Lines were fitted to data by computer-assisted linear regression analysis.

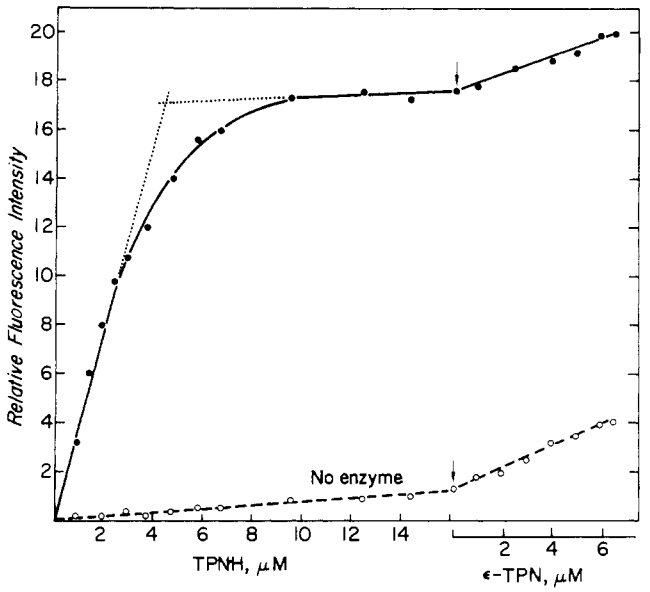

FIGURE 7: Titration of pyridine nucleotide-binding sites on L1210 dihydrofolate reductase. Enzyme concentration, 4.4 μM. Excitation at 287 nm. Changes in relative fluorescence intensity at 445 nm (corrected for the initial fluorescence of the free enzyme at this wavelength) were noted (●—●) after the indicated additions of TPNH. At the point marked with an arrow, increments of ε-TPN were added and further changes in fluorescence intensity were followed at 405 nm. Control (O- -O), fluorescence of free TPNH and ε-TPN at 445 and 405 nm, respectively.

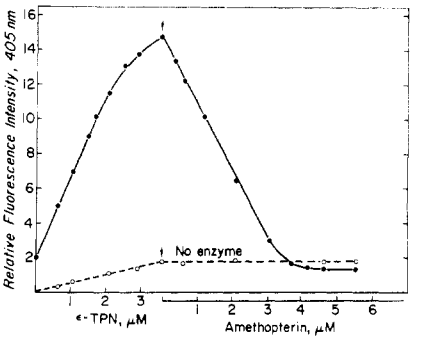

FIGURE 8: Ternary complex formation involving L1210 dihydrofolate reductase, ε-TPN, and amethopterin. Enzyme concentration, 3.6 μM. Excitation at 287 nm, fluorescence emission at 405 nm. Increases (●—●) in relative fluorescence intensity (not corrected for the initial fluorescence of the free enzyme at this wavelength) were noted after addition of the indicated increments of ε-TPN (up to an equimolar amount). At the point marked with an arrow, increments of amethopterin were added, and further changes in fluorescence were recorded. Control (O- - -O), fluorescence of free ε-TPN and amethopterin at 405 nm.

that are catalytically inactive. In the experiments described below, ε-TPN rather than ε-TPNH was used, since it binds readily to the enzyme (cf. Figures 3-6) but contains only a single fluorophore.

The sequential formation of a complex of the enzyme with ε-TPN and amethopterin is shown in Figure 8. In the initial step, ε-TPN was added to the enzyme until the molar equivalence point (indicated by the arrow) had been reached. Subsequent titration of the binary complex with amethopterin completely reversed the original fluorescence enhancement. When a molar equivalent of the drug had been added, the fluorescence was slightly less than the original value; this small increment is due to quenching of a portion of the enzyme fluorescence. For comparison, the results of a control titration (enzyme omitted) are shown as the dashed line in Figure 8.

In the above experiment, the decreased fluorescence seen in the second part of the titration cannot be due to an amethopterin-induced release of ε-TPN, since previous studies (Neef and Huennekens, 1975) using gel electrophoresis and absorbance spectra have established that the L1210 dihydrofolate reductase forms very stable ternary complexes when the enzyme is admixed with pairs of substrates or substrate analogues. Rather, the binding of amethopterin to the enzyme leads to the quenching of the enhanced fluorescence of previously-bound ε-TPN. In this connection, it should be noted that addition of amethopterin also quenches the fluorescence of the dihydronicotinamide moiety of TPNH (Perkins and Bertino, 1966). These results might be explained by assuming

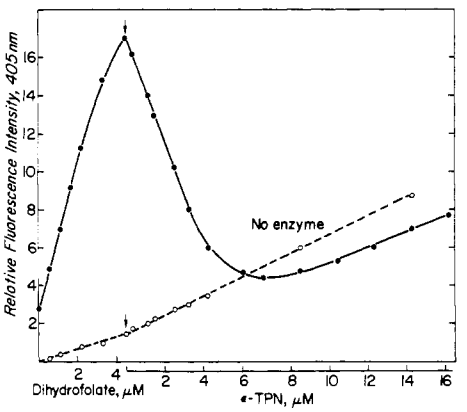

FIGURE 9: Ternary complex formation involving L1210 dihydrofolate reductase, dihydrofolate, and ϵ-TPN. Enzyme concentration, 4.5 μM. Excitation at 281 nm, fluorescence emission at 405 nm. Increases (●—●) in relative fluorescence intensity (not corrected for the initial fluorescence of the free enzyme at this wavelength) were noted after addition of the indicated increments of dihydrofolate (up to an equimolar amount). At the point marked with an arrow, increments of ϵ-TPN were added, and further changes in fluorescence were recorded. Control (O- - -O), fluorescence of free dihydrofolate and ϵ-TPN at 405 nm.

that binding of amethopterin: (a) brings portions of the molecule into close proximity with both ends of the pyridine nucleotide, (b) induces a conformational change that allows residues on the protein to interact with the pyridine nucleotide, or (c) forces the pyridine nucleotide into a closed, self-quenching conformation.

Formation of a ternary complex involving the enzyme, ϵ-TPN, and dihydrofolate has also been studied with fluorimetric techniques; in this instance, however, both ligands can serve as fluorescence probes. When excited at 281 nm, dihydrofolate (at pH 7.5) fluoresces at 430 nm; binding of this substrate to dihydrofolate reductases results in an enhancement of the fluorescence and a shift of the maximum to shorter wavelengths (Huennekens et al., 1970). In the experiment shown in Figure 9, the enzyme was first titrated with an equimolar amount of dihydrofolate and interaction was monitored via the increased fluorescence at 405 nm. At this point (arrow), titration of the enzyme–dihydrofolate complex with ϵ-TPN reversed the previous fluorescence enhancement and, in addition, there was no fluorescence enhancement from the bound ϵ-TPN. The experimental curve (solid line) may be contrasted with the control in which enzyme was omitted (dashed line). The results shown in Figure 9 again cannot be due to displacement of either ligand by the other (since one would still be bound), but, rather, is a consequence of the fact that in the ternary complex neither ligand exhibits the fluorescence enhancement seen in the respective binary complexes.

The results of the above experiments (Figures 8 and 9) provide evidence that, at the active site of the enzyme, both ends of the bound pyridine nucleotide and at least the pyrazine end of the bound folate compound appear to lie in close proximity to one another. Information about the relative positioning of the glutamate end of the folate compound may become available later as a result of similar experiments using amethopterin labeled on its carboxyl end with fluorescein (Gapski et al., 1975).

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