Dihydrofolate Reductase from a Methotrexate-resistant
Escherichia coli

BINDING OF PYRIDINE NUCLEOTIDES AS MONITORED BY ULTRAVIOLET DIFFERENCE SPECTROSCOPY

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MARTIN POE, NORMA J. GREENFIELD, AND MYRA N. WILLIAMS

From the Department of Biophysics and Pharmacology, Merck Institute for Therapeutic Research, Rahway, New Jersey 07065

SUMMARY

Upon binding of pyridine nucleotides to Escherichia coli MB 1428 dihydrofolate reductase, absorption bands of both enzyme and pyridine nucleotide are changed. Titration of enzyme with TPNH confirms the presence of two TPNH binding sites. Similar titrations show two binding sites for TPNH on a 1:1 complex of enzyme with methotrexate, two sites for TPN+ on both enzyme and a 1:1 enzyme-methotrexate complex, and at least one DPNH binding site on the enzyme-methotrexate complex. Equilibrium ultrafiltration measurements confirm the presence of two TPN+ binding sites on both enzyme and 1:1 enzyme-methotrexate and demonstrate the presence of two DPNH binding sites on 1:1 enzyme-methotrexate. Titrations and comparison of 2:1 with 1:1 pyridine nucleotide-enzyme difference spectra reveal that most of the absorbance changes associated with binding occur upon binding at the higher affinity site. Dissociation constants for TPN+ and DPNH were calculated from these titrations: $E\times(TPN^+)\times_{2}, 80 \pm 40 \mu M$ and $5 \pm 3 \mu M$; $E\times$MTX\times (TPN^+)\times_{1}, 1.7 \pm 0.0 \mu M$ and $0.06 \pm 0.03 \mu M$; $E\times$MTX\times (DPNH)\times_{2}, 100 \pm 50 \mu M$ and $9 \pm 5 \mu M$. Absorbance changes at 280 and 290 nm suggest the involvement of at least 1 tryptophan residue in the binding of TPNH, and possibly TPN+, at the higher affinity site. Absorbance changes upon binding of DPNH, TPNH, and TPN+ to the enzyme-methotrexate complex indicate that all three cofactors are bound identically. The binding of pyridine nucleotides to the enzyme-methotrexate complex changes the absorption bands of methotrexate toward an even more acidic spectrum.

A previous communication (1) described a study by ultraviolet difference spectroscopy of the interaction of three folates with dihydrofolate reductase (5,6,7,tetrahydrofolate:TPN+ oxidoreductase, EC 1.5.1.3) which had previously been purified from the methotrexate-resistant strain MB 1428 of Escherichia coli B (2, 3). This article examines the stoichiometry, binding affinities, and spectral characteristics of the interactions of TPNH, TPN+, and DPNH with the enzyme and the interaction of these three pyridine nucleotides with a 1:1 enzyme-methotrexate complex.

Parallel studies on E. coli MB 1428 dihydrofolate reductase by fluorescence (4) and circular dichroism (5) have demonstrated that triphosphopyridine nucleotides are tightly bound. Equilibrium ultrafiltration studies have shown that this enzyme, with a molecular weight near 17,600, has two TPNH binding sites (4) in addition to the folate binding site, and all three sites have high affinity for their ligands. These ultraviolet difference studies confirm that there are two high affinity triphosphopyridine nucleotide binding sites on the enzyme and on the enzyme-methotrexate complex.

EXPERIMENTAL PROCEDURE

Materials

The reductase was isolated and purified as described previously (2, 3). Folic acid dihydrate was purchased from Calbiochem, methotrexate from Nutritional Biochemicals. TPNH, TPN+, and DPNH were purchased from P-L Biochemicals, Inc., Milwaukee. All other chemicals were the highest grade commercially available. Buffer B is 0.05 M NaCl-0.05 M Tris-HCl, pH 7.20, at 23°C.

A 1:1 complex of enzyme with methotrexate was prepared by mixing solid methotrexate with an enzyme solution which was then chromatographed on a column (1.5 X 36 cm) of Sephadex G-25 which was previously equilibrated with Buffer B. Excess methotrexate was separated from the enzyme-methotrexate complex by this procedure.

Methods

Absorption and Difference Spectra—Absolute absorption spectra were obtained with a Cary 15 spectrophotometer. Difference spectra were run on the Cary 15 or on an Aminco DW-2 (American Instruments Co., Silver Spring, Maryland) using the split-compartment cell of Yankeelov (6). With the Aminco DW-2 set in the split-beam mode, difference spectra were measured from 240 to about 400 nm at a scan rate of 1 nm per s and bandpass of 3 nm on either the 0.02 or 0.05 absorbance scale. Difference spectra were also run with a bandpass set at 1.2, and 4 nm for $E\times(TPNH)\times_{2}$; there were no apparent differences between the difference spectra. All other difference spectra were run with a 3-nm bandpass in order to have a more reproducible base line.

Difference Spectra Titrations—Titrations were done on the Aminco DW-2 in the dual wavelength mode. Methotrexate and dihydrofolate reductase were standardized spectrophotometrically.
in Buffer B using the extinction coefficients quoted in Ref. 5. For TPNH and DPNH in Buffer B, \(e(340\text{ nm}) = 6.22 \text{ cm}^{-1}\text{ mM}^{-1}\) (7) and for TPN+ in Buffer B, \(e(260\text{ nm}) = 18.0 \text{ cm}^{-1}\text{ mM}^{-1}\) (8).

Calculation of Binding Constants—It was clear from parallel studies (4, 5) that the dissociation constants for the binding of TPNH to the enzyme and enzyme-methotrexate complex were much smaller than the enzyme concentrations required to give successful difference spectra titrations. Therefore, binding constants for these titrations were not calculated. Binding constants from TPN+ and DPNH titrations were calculated according to the procedure described by Williams et al. (4) using the formalism of Deranleau (9, 10). For these calculations it was assumed that the total difference spectrum observed could be attributed to the first or higher affinity site, and that the two sites were independent and noninteracting. The estimates of uncertainty for the calculated dissociation constants (Table I) were made by comparing calculated titration curves for given constants with the experimental titration curve. Those constants that gave better than poor fits to the experimental curve, as judged by eye, were all included within the error limits quoted.

Binding Stoichiometry by Equilibrium Ultrafiltration—The binding ratios for TPN+ and DPNH to 1:1 reductase-methotrexate complex were measured by an ultrafiltration technique described earlier (4).

RESULTS

Absorption Spectra

The absolute absorption spectra of *E. coli* MB 1428 dihydrofolate reductase and mixtures of the reductase with equimolar and twice equimolar TPNH in Buffer B are given in Fig. 1. Spectra such as these were used to determine initial values of \(A_m - A_s\) for difference spectra titrations.

Ultrafiltration

The stoichiometry of binding of TPN+ to reductase and of TPN+ and DPNH to 1:1 reductase-methotrexate was measured by equilibrium ultrafiltration. In three measurements, 11.5 \(\mu\text{M}\) enzyme plus an 8-fold excess of TPN+ had 1.6 \(\pm\) 0.2 moles of TPN+ bound per mole of protein. For four measurements of binding to 1:1 enzyme-methotrexate at the same enzyme and TPN+ concentrations, there were 1.8 \(\pm\) 0.2 moles of TPN+ bound per mole of protein. In three experiments, 19.0 \(\mu\text{M}\) 1:1 enzyme-methotrexate in Buffer B plus 113.4 \(\mu\text{M}\) DPNH bound 1.5 \(\pm\) 0.15 moles of DPNH per mole of protein.

These measurements demonstrate the presence of more than one binding site for TPN+ and DPNH on the enzyme-methotrexate complex, and for TPN+ on the enzyme. The binding ratios measured are consistent with the binding ratios calculated for the dissociation constants listed in Table I.

Difference Spectra

Enzyme plus TPNH—Presented graphically in Fig. 2 are the difference spectra obtained on the Aminco DW-2 for TPNH binding to the enzyme. Before mixing, the enzyme compartments in the sample and reference cuvettes contained 1.00 ml of enzyme at a concentration of 1.0 \(\mu\text{M}\) in Buffer B at 23°, while the other compartment contained 1.00 ml of either 11.0 or 22.0 \(\mu\text{M}\) TPNH in the same buffer. The spectra presented in Fig. 2 have been replotted to correct for the small differences in absorbance noted between the sample and reference cuvettes before mixing the sample cuvette's contents. At this enzyme concentration, virtually all of the TPNH is bound (4). Of course, unbound enzyme and TPNH are automatically cancelled.

Table I

* Escherichia coli MB 1428 dihydrofolate reductase: pyridine nucleotide dissociation constants

| Species          | Ligand | Dissociation constants \(\mu\text{M}\) |
|------------------|--------|--------------------------------------|
| Enzyme           | TPNH   | 0.02 \(\pm\) 0.01 \(\pm\) 0.6 \(\pm\) 0.1* |
|                  | TPN+   | 5 \(\pm\) 3 \(\pm\) 80 \(\pm\) 40 |
|                  | DPNH   | 5 * Too weak to measure. |
| 1:1 enzyme-      | TPN+   | 0.06 \(\pm\) 0.03 \(\pm\) 1.7 \(\pm\) 0.9 |
| methotrexate     | DPNH   | 9 \(\pm\) 5 \(\pm\) 100 \(\pm\) 50 |

* From Ref. 4.

* Too weak to measure.

* Too tight to measure.

![Fig. 1](image1)

![Fig. 2](image2)

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**TABLE I**

*Escherichia coli* MB 1428 dihydrofolate reductase: pyridine nucleotide dissociation constants

Measurements were taken at 23° in 0.05 \(\mu\text{M}\) Tris-HCl (pH 7.2)-0.05 \(\mu\text{M}\) NaCl.
out in the split-compartment cell technique, so that the difference spectra presented are really only the difference in absorbance between the amount of complexed enzyme-TPNH and a corresponding amount of free enzyme and TPNH. Dividing ΔA by 5.50 μM and 0.88 cm converts the observed ΔA values presented in Fig. 2 to Δv values.

There are a number of interesting features in the difference spectra presented in Fig. 2. First, the difference spectra for E-TPNH and E-(TPNH)₂ are quite similar. The E-(TPNH)₂ spectrum does have slightly more intense bands, except for the long wavelength band where the apparent diminution is within experimental error. Titrations monitored by both fluorescence (4) and ultraviolet difference spectroscopy (see below) demonstrate that in Buffer B the dissociation constant for the first site is significantly smaller than that of the second site, so that the first site is almost completely filled when equimolar TPNH is added. Thus, most of the difference spectrum noted upon binding of excess TPNH is due to binding at the first site.

Enzyme plus TPN⁺ and DPNH—The difference spectra produced by binding of TPN⁺ and DPNH are portrayed in Fig. 3. The enzyme also has two binding sites for TPN⁺, and possibly for DPNH as well, but binding is weaker than for TPNH. These difference spectra were taken with sufficient TPN⁺ to largely saturate the two binding sites, while the DPNH certainly did not saturate even the first binding site.

Neither TPN⁺ nor DPNH gave enzymatic difference spectra as intense or detailed as seen with TPNH or the folates. Furthermore, the principal features of the difference spectra are in spectral regions where the complexes have high absolute absorbance. These facts combine to make difference spectrum titrations difficult.

Enzyme-Methotrexate plus TPNH—The difference spectrum resulting from the binding of 1 and 2 moles of TPNH to 1 mole of a 1:1 enzyme-methotrexate complex in Buffer B at 23° are graphically presented in Fig. 4. The experimental protocol was essentially identical with that used for the enzyme plus TPNH difference spectra described above.

As noted for the binding of TPNH to the reductase alone, the binding of 2 eq of TPNH to an enzyme-methotrexate complex gave essentially the same difference spectrum as noted with 1 eq, although a little more intense. The titrations presented in Refs. 4 and 5 and in the following section demonstrate that the first TPNH site on the enzyme-methotrexate has significantly higher affinity for TPNH than the second site. But, in contrast to the case with the fluorescence (4) and circular dichroism (5) properties of the enzyme-methotrexate complex, where addition of 1 mole of TPNH completes the changes observed, there is a small additional change in absorbance produced by TPNH binding at the second site.

There are a large number of overlapping bands in the difference spectra plotted in Fig. 4, many more than seen in Fig. 2 for TPNH binding to uncomplexed enzyme. This probably means that TPNH binding affects the absorption bands of bound methotrexate, since neither TPNH nor the enzyme has absorption bands at some of the wavelengths where difference spectra bands are seen in Fig. 4. It is noteworthy that it is the TPNH binding at the site with higher affinity which is responsible for the altered methotrexate bands. This is in agreement with the circular dichroism results, where there are large changes in the enzyme-methotrexate circular dichroism spectrum upon TPNH addition up to equimolar TPNH but no further changes upon addition of more TPNH (5). The bands at 280 and 290 nm seen in the enzyme plus TPNH difference spectrum (see Fig. 2) are present in the enzyme-methotrexate plus TPNH difference spectrum.

Enzyme-Methotrexate plus TPN⁺—The difference spectra generated upon mixing equimolar and twice equimolar (triangles) TPN⁺ with a solution of 1:1 enzyme-methotrexate are presented graphically in Fig. 5. There was clearly a significant intensification of the same difference spectrum in comparing twice equimolar with equimolar TPN⁺. This intensification is attributable to a closer equivalence of the binding affinities of the first and second pyridine nucleotide sites on the enzyme-methotrexate for TPN⁺. The second site, while having no significant intrinsic difference spectrum, prevents saturation of the first site with TPN⁺ when only 1 eq of TPN⁺ has been added by binding part of the TPN⁺.

The difference spectrum observed with TPN⁺ binding to enzyme-methotrexate is remarkably similar to that noted for TPNH binding. This is in contrast to the case for TPN⁺ and

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**Fig. 3.** Difference spectra obtained upon mixing excess TPN⁺ and DPNH with the enzyme. After mixing, the enzyme was 13.9 μM, while TPN⁺ was 42.2 μM and DPNH 46.0 μM, in 0.05 M NaCl-0.05 M Tris-HCl, pH 7.20, and at 23°. Light path 0.88 cm. Difference spectra were obtained by the split-compartment cell technique, which cancels out any contribution to the difference spectrum by uncomplexed ligand.

**Fig. 4.** Difference spectrum for binding of TPNH to a 1:1 enzyme-methotrexate complex. The open circles are for 1 eq of TPNH, the filled circles for 2 eq. For the difference spectrum, after mixing, the enzyme was 5.5 μM in 0.05 M Tris-HCl, pH 7.2, and 0.05 M NaCl at 23°. Light path 0.88 cm.
TPNH binding to enzyme alone, where the respective difference spectra bear little resemblance to one another. The principal changes in the difference spectra for binding to enzyme-methotrexate when comparing the TPN⁺ spectrum to the TPNH spectrum are that the small negative band at 263 nm in the TPNH spectrum disappears, the positive band near 275 nm is about twice as intense, and the negative band at 326 nm shifts to 318 nm. The absorption band at 340 nm in TPNH, which corresponds to the reduced nicotinamide ring, is near 260 nm in TPN⁺. The small changes in the difference spectra noted in the comparison of TPN⁺ and TPNH are probably attributable to this changed absorption band. With this interpretation, the conclusion is reached that the ultraviolet absorption bands of enzyme, methotrexate, and pyridine nucleotide are affected identically upon binding of TPN⁺ and TPNH to the enzyme-methotrexate complex.

Since neither enzyme nor TPN⁺ has absorption bands above 300 nm, the spectra in Fig. 5 confirm the suggestion that the difference spectrum generated between 300 and 400 nm may be attributed to changes in the absorption bands of bound methotrexate. Further, these ultraviolet absorption bands, which are associated with the pterin ring system of methotrexate, are affected in the same way by both TPN⁺ and TPNH binding.

**Enzyme-Methotrexate plus DPNH**—The difference spectra obtained by adding equimolar, twice equimolar (dots), and 14 times equimolar (triangles) DPNH to a 7.5 μM enzyme-methotrexate complex are reproduced in Fig. 6. The three difference spectra appear to be the same, although higher levels of DPNH produce more intense spectra. Therefore, all of the difference spectrum may be attributed to binding at one site. The difference spectrum is superimposable upon the difference spectrum noted for TPNH binding to enzyme-methotrexate. Thus, the binding of DPNH appears to be identical with that of TPNH insofar as the binding affects ultraviolet absorption bands, although the binding of DPNH is weaker.

**Difference Spectra Titrations**—The changes in absorbance that are seen upon binding of triphosphopyridine nucleotides to *E. coli* MD 1428 dihydrofolate reductase and its 1:1 methotrexate complex are sufficiently large that titrations of the change were feasible. The Aminco DW-2 in the dual wavelength mode was used for all these titrations; in this mode, the spectrophotometer alternatively samples the absorbance at two wavelengths in the same cuvette and reads the difference in absorbance \( A_{\lambda_1} - A_{\lambda_2} \) directly. This has the advantage that dilution corrections for the measure and reference wavelengths must be identical.

**TPNH**—Presented graphically in the left-hand side of Fig. 7 are the results of a typical difference spectrum titration of enzyme with TPNH. For the wavelength pair chosen, there is
no change in $A_{350.5} - A_{305}$ for TPNH additions to Buffer B. The plot of dilution-corrected $A_{350.5} - A_{305}$ versus $\Delta V$ given in Fig. 7 shows that the change in $A_{350.5} - A_{305}$ was not complete until 29.4 nmoles of TPNH had been added to the 14.7 nmoles of enzyme. This is the point labeled 1:1. At TPNH levels beyond the 2:1 point, there is no significant change in dilution-corrected $A_{350.5} - A_{305}$ with TPNH additions. This plot demonstrates that the titration is completed by addition of 2 eq of TPNH and confirms the presence of two TPNH binding sites. The curve in the data plot between the 1:1 and 2:1 levels of TPNH results from competition between the two sites for available TPNH rather than from incomplete binding.

When an enzyme-methotrexate solution at the same concentration was used for titration with the same TPNH solution, the results depicted on the right-hand side of Fig. 7 were obtained. The lower initial $A_{350.5} - A_{305}$ is due to the higher absorbance of methotrexate at 305 nm compared to 280.5 nm. Just as with the addition of TPNH to the enzyme alone, the TPNH titration did not reach an end point until 29.4 nmoles of TPNH had been added (the point labeled 2:1). The bulk of the absorbance change was over by the addition of 1:1 TPNH. It is interesting to note that the plot of $A_{350.5} - A_{305}$ versus TPNH added is linear up to a 1:1:1 complex of enzyme-TPNH-methotrexate, and then again linear to the point where the 1:2:1 complex is obtained. This suggests that the first TPNH site has much higher affinity for TPNH than the second site, and that both sites are very tight. This ratio and the relative value of binding constants are in agreement with previous circular dichroism (5) and fluorescence (4) titrations of enzyme-methotrexate with TPNH. Therefore, the small changes in dilution-corrected $A_{350.5} - A_{305}$ between 1:1 and 2:1 TPNH must be attributable to a small change in absorption spectra due to binding at the second site.

$TPN^+ - TPN^+$ does not have a near-ultraviolet absorption band, so that a wavelength pair above 300 nm for dual wavelength operation cannot be chosen to give a zero $A_{322} - A_{354}$ for TPN$^+$ additions to buffer alone. However, above 300 nm the changes in $A_{322} - A_{354}$ with TPN$^+$ addition are not large, and titration data may easily be corrected for it if desired.

To avoid the high absorbance of concentrated enzyme solutions below 300 nm, $\lambda_1$ was chosen to be 305 nm and $\lambda_2$ to be 350 nm. In the experiments whose results are summarized in the left-hand side of Fig. 8, aliquots of 3.37 mM TPN$^+$ in Buffer B were added to 1 ml of 74.8 $\mu$m enzyme, also in Buffer B. The dilution-corrected $A_{350} - A_{305}$ increased monotonically with TPN$^+$ and approached an asymptote whose slope corresponded to the $A_{350} - A_{305}$ noted for TPN$^+$ addition to Buffer B alone. It appeared that the titration was mostly but not wholly completed by the addition of 150 $\mu$m TPN$^+$ (the 2:1 point). Calculations indicate that the dissociation constants for the two sites are 5 and 80 $\mu$m.

When TPN$^+$ was used to titrate a 1:1 enzyme-methotrexate complex at 8.05 $\mu$m, the data replotted for the right-hand side of Fig. 8 was obtained. For this titration, $\lambda_1$ was selected to be 368 nm and $\lambda_2$ as 390 nm; the data presented have been corrected for the $A_{368} - A_{390}$ for TPN$^+$ addition to buffer alone so that the dotted line was the corrected data asymptote at high TPN$^+$ levels. $\Delta(A_{350} - A_{305})$ was defined as $A_{350} - A_{305}$ at any given TPN$^+$ level minus the initial $A_{350} - A_{305}$. As with titration of the enzyme alone, the titration with TPN$^+$ was mostly completed by the addition of 2 eq of TPN$^+$. Calculations gave dissociation constants of 0.06 and 1.7 $\mu$m.

$DPNH Difference$ spectra titrations of the reductase with DPNH were not as simple to interpret as those with TPNH and TPN$^+$. The small changes noted upon DPNH binding to the enzyme are in spectral regions with high absorbance. In a titration with $\lambda_1 = 280.5$ nm and $\lambda_2 = 305$ nm, additions of DPNH up to a concentration of 300 $\mu$m for a 39 $\mu$m enzyme solution did not reach an end point or even an asymptote. For this wavelength pair, addition of DPNH to Buffer B gave no measurable $A_{322} - A_{354}$. At the higher DPNH concentrations, the absorbance became so high as to possibly degrade the performance of the spectrophotometer. Thus, no estimate of the binding stoichiometry or affinity was possible. When DPNH was used to titrate a 7.5 $\mu$m solution of 1:1 enzyme-methotrexate, the data replotted for Fig. 9 were gotten. The original plot of $A_{322} - A_{354}$ versus added DPNH was corrected as described above for the initial $A_{322} - A_{354}$ and the $A_{350} - A_{305}$ noted upon DPNH addition to buffer alone; the dotted line is the corrected asymptote of the replotted data at

![Fig. 8. Left, difference spectrum titrations of reductase with TPN$^+$.](image-url)

**Fig. 8. Left**, difference spectrum titrations of reductase with TPN$^+$. Aliquots of 3.37 mM TPN$^+$ in 0.05 M NaCl-0.05 M Tris-HCl, pH 7.20, were added to 1.00 ml of 74.8 $\mu$m enzyme in the same buffer. $\Delta V$ was the cumulative total of added TPN$^+$, and $A_{350} - A_{305}$ was the dilution-corrected absorbance at 305 nm minus the absorbance at 350 nm for the enzyme solution, $T$, at 23°. **Right,** difference spectrum titration of 1:1 reductase-methotrexate with TPN$^+$. The enzyme was 8.05 $\mu$m and TPN$^+$ 904 $\mu$m, both in 0.05 M Tris-HCl (pH 7.2)-0.05 M NaCl. $\Delta (A_{350} - A_{305})$ was the dilution-corrected difference in absorbance between 368 and 390 nm, corrected for the initial $A_{350} - A_{305}$ and for the $A_{350} - A_{305}$ seen in additions of TPN$^+$ to buffer alone.
high DPNH levels. This titration fails to prove the presence of two binding sites for DPNH on the enzyme-methotrexate complex. Calculations assuming the presence of two binding sites gave dissociation constants of 9 and 100 μM.

**DISCUSSION**

There is clear evidence from the ultraviolet difference titrations with TPNH and TPN⁺ that there are two tight binding sites for triphosphopyridine nucleotides on E. coli MB 1428 dihydrolate reductase, in addition to the folate site. The second site is seen primarily through its ability to compete with the first site for added TPNH, since the TPNH in this site does not appear to affect significantly the circular dichroism (5), fluorescence (4), or ultraviolet absorbance of either the enzyme or this TPNH molecule. How these properties fail to be affected by such tenacious binding is an interesting question.

The binding constants calculated from the titration curves presented in this article are summarized in Table 1. These constants are in the same range as the binding constants for pyridine nucleotides published by Colowick et al. (11). The dissociation constants for TPN⁺ are 2 orders of magnitude smaller for the 1:1 enzyme-methotrexate complex than for enzyme alone. If similar ratios between these constants are in the same range as the binding constants for pyridine nucleotides published by Colowick et al. (11), then the enzyme-methotrexate complex might be explained by the presence of two tight binding sites for triphosphopyridine nucleotides on E. coli MB 1428 dihydrolate reductase. The enzyme was 7.5 μM and DPNH 969 μM, both in the buffer of Fig. 3. (A - Δ) was the dilution-corrected difference in absorbance between 322 and 345 nm, corrected for the A sub 322 - A sub 345 seen in additions of DPNH to buffer alone, and for the initial A sub 322 - A sub 345.

The dissociation constants for TPNH and DPNH in Table 1 were calculated under the assumption that all the observed difference spectra could be attributed to binding at the tighter site. This assumption is supported by the existence of isoosbestic points, and by the spectral homology for various concentrations of pyridine nucleotides, as shown in Figs. 5 and 6. It is noteworthy that calculations using one binding site and one band consistent failed to give reasonable fits to the experimental data for TPNH and TPN⁺ titrations and did not give as good a fit to the DPNH titration data.

The difference spectra for E-TPNH, given in Fig. 2, have three interesting regions. The near-ultraviolet region between 310 and 400 nm clearly is different from the 340-nm absorption band of TPNH, which demonstrates that the 340-nm band does not merely undergo a hyperchromism. Hyperchromism is herein defined according to Tinoco (12) as a proportional change in absorbance at all wavelengths without a shift in wavelength. The E-TPNH₂ difference spectrum is also different from the first derivative of the 340-nm absorption band, which shows that the band does not just shift slightly with no change in extinction (13, 14). The fact that the difference spectrum maximum is above 340 nm at roughly 355 nm, while the isoosbestic point is at about 325 nm, suggests that the 340-nm band undergoes a shift to a longer wavelength and a hyperchromism, just as noted by Fisher et al. (13) for mitochondrial malate dehydrogenase plus DPNH. The E-TPNH₃ difference spectrum between 270 and 310 nm exhibits a pair of maxima at approximately 280 and 290 nm. Similar features are seen in the difference spectrum upon binding of L-glutamate by glutamate dehydrogenase (15), in the binding of phenylalanine by rabbit muscle pyruvate kinase (16), in the binding of reduced coenzyme by a number of dehydrogenases (13, 17), and in the solvent perturbation spectra of numerous proteins (18) due to red-shifted aromatic residues, especially tyrosine and tryptophan.

Fisher and Cross (16) interpret the features seen in binding of glutamate by glutamate dehydrogenase as a demonstration of the involvement of a tryptophan residue in the binding process. The solvent perturbation spectrum of tryptophan has three maxima at 290, 283, and 273 nm (18); the breadth of the difference spectrum band at 280 nm might suggest that it is two unresolved bands. Spectra run at a full width bandwidth of 1 nm failed to resolve any components in this band, however. Tyrosine may also exhibit similar spectral features upon solvent perturbation (18), but such features are typically lower in extinction than for tryptophan and have maxima at lower wavelengths and the bands are spaced differently (19), being at 255, 278, and 274 nm. The reductase contains 4 tyrosine and 5 tryptophan residues. The extinction coefficient for solvent perturbation of tryptophan depends appreciably on the exact conditions used (14, 18), but the features observed in the 270- to 310-nm region could be accounted for by a change in the local electronic environment of as few as 1 tryptophan residue. Thus, we propose that the binding of the 1st mole of TPNH to the reductase alters the local environment of at least 1 tryptophan residue. Williams (16) has measured the protection of enzyme tryptophan residues by TPNH against N-bromosuccinimide oxidation. Unfortunately, TPNH is chemically modified by N-bromosuccinimide so that interpretation of the protection experiment was ambiguous. The E-TPNH₄ difference spectrum below 270 nm is difficult to interpret because there are three absorption bands contributing in this region (20, 21). The strong negative band centered at about 257 nm is seen in the "denaturation" of intramolecularly folded DPNH by guanidine, methanol, and urea (21). Lee et al. (22) report that chicken muscle and lobster tail lactate dehydrogenase and yeast alcohol dehydrogenase bind DPN⁺ in the open or destacked form of the coenzyme.

The difference spectra seen upon the binding of TPN⁺ and DPNH to the reductase in the presence of excess cofactor (see Fig. 3) are quite different from the corresponding spectra for TPNH. The difference spectrum of DPNH exhibits a pair of bands in the 270 to 310 nm region as does the TPNH difference spectrum, but the bands are at slightly longer wavelengths and the longer wavelength band for the TPN⁺ difference spectrum is appreciably broader. Therefore, the suggestion that the environment of at least 1 tryptophan residue is altered upon binding of TPNH may probably be extended to TPN⁺ but with less assurance. The difference spectra bands seen with DPNH are opposite in sign to those observed with TPNH and TPN⁺.

1 M. N. Williams, manuscript in preparation.
principal features are a shoulder at roughly 285 nm and a broad band centered at about 274 nm. Difference spectra bands with the same sign in the same spectral region have been observed by Herskovitz and Laskowski (23) for solvent perturbation of bovine serum albumin, and Kronman and Robbins (18) state that negative bands may be seen with solvent perturbation of N-acetyl tyrosine and tryptophan ethyl esters by H2O. The breadth of the features in the DPNH difference spectrum preclude any identification of the bands with specific residues.

There is a possible physiological role for the second pyridine nucleotide site. The E. coli dihydrofolate reductase will actively catalyze the transfer of hydrogen from TPNH to acetylpyridine-TPN1 in the presence of catalytic amounts of tetrahydrofolate.2 In the cell, this transhydrogenase activity could be utilized to transfer reducing equivalents from triphosphopyridine nucleotides to diphosphopyridine nucleotides or vice versa. The dihydrofolate reductase isolated from Lactobacillus casei (24) also catalyzes the transhydrogenase reaction, but no direct evidence for two distinct pyridine nucleotide sites has as yet been reported for this enzyme.

The ability of TPNH and TPN+ in the tighter binding site (the site which predominantly affects the ultraviolet absorbance of the enzyme and ligand) to affect the absorption bands of bound methotrexate suggests that the first site is proximal to the methotrexate site.

The alteration in the absorption bands of enzyme-bound methotrexate upon binding of TPNH, TPN+, and DPNH is not as simply interpreted as the change in the methotrexate absorption spectrum upon binding to enzyme. This is because some of the bands in the difference spectra of Figs. 4, 5, and 6 almost certainly correspond to altered absorptions of enzyme and pyridine nucleotide, particularly for the bands between 240 and 300 nm. However, the bands between 300 and 400 nm in the three figures, most of which probably correspond to methotrexate absorption bands, roughly approximate the difference in absorbance between strongly acidified methotrexate and methotrexate at pH 1.5. This, it appears, that methotrexate in the enzyme-methotrexate-pyridine nucleotide complexes, where methotrexate is even more tightly bound to enzyme than in the binary complex (3), has an absorption spectrum like that of strongly acidified methotrexate. This was confirmed by measuring the difference spectrum for methotrexate binding to an enzyme-(TPNH)2 complex. The qualitative correlation between degree of acidification and strength of binding for folates noted earlier (1) therefore extends to reductase which has bound pyridine nucleotides.

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