Spectral Changes Associated with Binding of Folate Compounds to Bacteriophage T4 Dihydrofolate Reductase

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SUMMARY

The difference spectra associated with complex formation at pH 7.0 between bacteriophage T4-specific dihydrofolate reductase and the 4-amino folate analogues, aminopterin, methotrexate, and N10-formyl-aminopterin, have features which are similar to the difference spectra obtained for the free compounds in acid versus neutral solutions. These results suggest that oxidized 4-amino-folate compounds bind to T4 Hz-folate reductase in the cationic form. This similarity does not extend to the difference spectra for folic acid, Hz-folate, or Hz-aminopterin. The difference spectra of complex formation between Hz-folate and T4 Hz-folate reductase show enhanced absorption with peaks at 305 nm and 243 nm, which are characteristics of the absorption spectra of the pteridine moiety of Hz-folate. We observed no evidence for protonation of Nt of Hz-folate as a result of complex formation. The difference spectrum of the complex between T4 Hz-folate reductase and Hz-aminopterin, but not T4 Hz-folate reductase and aminopterin, is perturbed by the cofactor NADPH. This suggests that the binding site for Hz-aminopterin might not be identical with that for aminopterin.

The folate antagonists methotrexate and aminopterin have been used in the treatment of neoplasms and other diseases for a number of years (1). These agents appear to exert their chemotherapeutic effect via the very potent inhibition of the enzyme Hz-folate reductase (2-4), a type of inhibition which has been described as stoichiometric (5). While the $K_w$ or dissociation constant, $K_{diss}$ for Hz-folate tends to be between $10^{-7}$ M and $10^{-4}$ M, the $K_t$ or $K_{diss}$ for aminopterin or MTX$^1$ tends to be too small to measure accurately by such techniques as inhibition of enzymatic activity or quenching of protein fluorescence (6, 7). The large increase in binding observed when the 4-hydroxyl group of folate is replaced by a 4-amino group (aminopterin) has been the subject of much speculation. In general, two schools of thought have arisen to explain this enhanced binding: (a) hydrogen bonding (8-10), and (b) the greater basicity of the 4-amino compounds (11-13). Due to the difficulty of obtaining even a few milligrams of homogeneous Hz-folate reductase, previous evidence in support of one or the other of these concepts has relied to a large extent upon kinetic data. With the development of an affinity chromatographic method for purification of the T4 bacteriophage-specific Hz-folate reductase (14), we have been able to obtain sufficient homogeneous enzyme to study directly its interaction with certain 4-amino compounds as well as its substrate, Hz-folate, by the technique of different spectroscopy. Our results are the subject of this paper.

EXPERIMENTAL PROCEDURE

Materials—The preparation of homogeneous T4 Hz-folate reductase has been described (14). Hz-aminopterin was prepared and purified on DEAE-cellulose by the methods of Kislik and Levine (15). Its purity was confirmed on the basis of its absorption spectrum (15). The difference spectra reported with this compound were obtained within 12 hours after purification. Hz-folate was prepared by the method of Friedkin et al. (16), on the day of its use. The preparation of NFA has been described (14). Its structure (see Fig. 1) has been confirmed by the following observations. (a) The method of synthesis is analogous to that used for the quantitative conversion of folic acid to N10-formylfolic acid (17). Since the N10 position of aminopterin is "insulated" from the pterin moiety by a methylene group as it is in folate acid, its reactivity should be about the same in the two compounds. (b) The 280 nm absorption peak of aminopterin is shifted to 260 nm (Fig. 2) by formylation just as the 280 nm peak of folic acid is shifted to 260 nm by formylation. (c) Exposure of NFA to 0.1 N NaOH for a few minutes produces a compound with the same spectrum (pH 7.0) as aminopterin. Brief exposure of N10-formyl-folate to 0.1 N NaOH produces a compound with the same spectrum (pH 7.0) as aminopterin.

$^1$The abbreviations used are: MTX, methotrexate or 4-amino-4-deoxy-10-methyl-folic acid; NFA, N10-formylaminopterin or 4-amino-4-deoxy-10-formylfolic acid.
FIG. 1. Difference spectra of the T4 H$_2$-folate reductase-NFA complex versus free components, and difference spectra of NFA in acid versus neutral solution. —-T4 H$_2$-folate reductase (2.8 μM) in the presence of 5.7 μM NFA. --, free NFA, 2.7 μM, pH 2.8 versus pH 7.9. The magnitudes of the 261 nm peak of free NFA (2.7 μM) at various pH values, all versus pH 7.0, are indicated. NaOH is known to produce folic acid (17). (d) Elemental analysis of NFA (Spang Microanalytical Laboratory, Ann Arbor, Michigan) agrees with the expected empirical formula calculated for C$_{30}$N$_{20}$O$_6$.

C$_{30}$N$_{20}$O$_6$

Calculated: C 50.25, H 4.45, N 22.70

Found: C 49.89, H 4.45, N 22.40

The sources of the other materials used were as previously noted (14). Solutions of MTX, aminopterin, folate, and NFA were prepared on the day of their use. These compounds showed a single "quenching" spot (as viewed under ultraviolet light) after chromatography on Whatman No. 1 paper with 0.1 M Tris-glycine buffer, pH 9.0. No decomposition of these compounds (1.0 × 10$^{-4}$ M solutions in Buffer B) was observed after standing 6.0 hours at room temperature under ordinary laboratory lighting conditions, as determined by paper chromatography. Under similar laboratory conditions a solution of Hz-folate (2.0 × 10$^{-5}$ M) showed no alteration in its ultraviolet absorption spectrum for up to 3.0 hours if the solution (in Buffer B) contained 0.1 M mercaptoethanol. None of the experiments reported here involving Hz-folate difference spectra exceeded a time period of 90 min.

Methods—Absorption difference spectra were obtained with a Cary 15 spectrophotometer. Difference spectra were obtained with the 0 to 0.1 absorbance unit sliderwire setting. A set of four matched semi-micro rectangular quartz cuvettes of 1.0 cm path length was employed. Each cuvette occupied the same position in the cell compartments throughout these measurements. Path length differences of the cuvettes were found to be negligible.

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All initial sample and blank volumes employed 0.80 ml of enzyme solution in Buffer B or 0.80 ml of Buffer B. The same 1.0-ml pipette was used throughout these measurements for transfer of buffers and solutions, to insure as much as possible that identical volumes were placed in each cuvette. Enzyme solutions were centrifuged prior to use if turbidity was suspected. All transfers of solutions and all spectral measurements were performed at ambient temperatures (23° ± 1°). To obtain difference spectra a buffer blank cuvette (placed first in the light path) and a cuvette containing enzyme solution were placed in each compartment and a base-line established with the multipots. Identical aliquots (0.01 ml to 0.04 ml) of substrate or inhibitor were delivered to the enzyme cuvette in the sample compartment and to the buffer blank in the reference compartment. In those cases where a dihydro compound required the presence of mercaptoethanol in the buffer, an identical aliquot of the mercaptoethanol-containing buffer (see below) was added to the enzyme cuvette in the reference compartment and to the buffer cuvette in the sample compartment. Mixing was accomplished by cuvette inversion, and spectra were recorded immediately. All difference spectra involving enzyme interactions were performed at pH 7.0.

Difference spectra obtained on the 0 to 0.1 absorbance sliderwire were subject to certain instrumental errors. It proved extremely difficult to obtain a perfectly straight base-line over the entire spectral range studied, 240 to 420 nm. In order to make spectra more directly comparable, spectra were corrected for these fluctuations and presented relative to a straight base-line. Since the base-line fluctuations themselves were reproducible, this procedure seems justified. In addition, small shifts in absorbance were observed in repetitive scans. These shifts were either positive or negative and ranged up to 0.005 absorbance.
Because of this problem, difference spectra may contain errors inversely proportional to their magnitude, estimated to be about 25% for an absorbance change of 0.01.

In the Cary model 15 the slit width increases in response to increased absorption by the reference solutions. For the system employed here maximum absorption occurs in the 260 to 280 nm region. The maximum slit width did not exceed 0.20 nm except for the folate spectra, in which the maximum slit width was 0.35 nm at 280 nm. Below 240 nm the spectra became increasingly unreliable because high absorbances of the aromatic amino acids and the peptide bonds necessitated large slit widths.

Errors in the difference spectra due to fluorescence are considered to be negligible. This conclusion may be drawn from the study of Donovan (18), where the difference in extinction coefficient (of a solution of tyrosine subjected to ethylene glycol perturbation) obtained on the Cary model 15 was identical with the difference in extinction coefficient obtained with a Turner model 210 spectrophotofluorometer. In this latter instrument, light fluorosed by the sample is dispersed by the second monochromator before it reaches the detector. We, therefore, conclude (as did Donovan) that only a negligible amount of light fluorosed by the sample reaches the detector of the Cary model 15.

All difference spectra involving pH changes (involving folate compounds only) employed only two cuvettes, one in each compartment. This simplification could be made since negligible absorbance differences were noted between buffers of varying pH values. The reference pH was 7.0 (Buffer B) in all cases. All folate compounds were dissolved in Buffer B (plus 0.1 M mercaptoethanol in the case of H4-folate and H2-aminopterin). The concentration of these solutions was such that 0.09 ml of this solution in 0.80 ml of buffer gave a final concentration of approximately 3 x 10^-6 M. To obtain pH difference spectra, a 0.1 M phosphate buffer of pH 5.4 and mixtures of this buffer and 0.1 M HCl were used to cover the pH range 1.0 to 5.6. Since the addition of the folate compound stock solution altered the initial buffer pH somewhat, the pH values reported are those determined after the spectra were obtained.

RESULTS

As an initial experiment we examined the interaction of NFA, the ligand used in affinity chromatography with T4 H4-folate reductase (14) (Fig. 1). The difference spectrum obtained from the enzyme-NFA complex versus the free components (Fig. 1) indicated decreased absorption in the long wave length (greater than 350 nm) absorption region of NFA, a region of enhanced absorption in the 280 to 375 nm region and a second region of decreased absorption, forming a peak close to 260 nm. During the characterization of free NFA, the spectra of this compound were examined at pH 7.0 and in 0.1 N HCl (Fig. 2). A comparison of Figs. 1 and 2 discloses that the changes observed when NFA binds T4 H4-folate reductase are similar to the changes observed between the neutral and acid spectra of NFA. In order to make a better comparison, we obtained difference spectra of NFA at several acid pH values versus NFA at pH 7.0. As is shown in Fig. 1, the difference spectra obtained at acid versus neutral pH values bear a striking similarity to the spectrum of the T4 H4-folate reductase-NFA system.

There are some differences, however, notably the fact that the long wave length transition from plus to minus occurs at about 374 nm for the enzyme difference spectra as opposed to about 357 nm for the acid neutral system. Another difference between the two is a 3 or 4 nm shift in the large negative peak near 260 nm. Here, also, the enzyme system is shifted in the long wave length direction relative to the acid-neutral system.

To see if the spectral changes observed for NFA applied to other 4-amino folate compounds, the study was extended to the more potent inhibitors MTX and aminopterin. Figs. 3 and 4 indicate that the main features of the spectral changes observed with NFA are also present in the difference spectra of MTX and aminopterin, both with respect to the enzyme-inhibitor difference spectra and the acid-neutral system. However, in going from NFA to aminopterin to MTX there is a deepening "trough" at

![Fig. 3. Difference spectra of the T4 H4-folate reductase-MTX complex and of MTX in acid versus neutral solutions. ---, difference spectra of 3.7 \( \mu \)M MTX in the presence of 2.8 \( \mu \)M enzyme; -- --, 3.0 \( \mu \)M MTX at pH 4.8 versus pH 7.0.](http://www.jbc.org/)

![Fig. 4. Difference spectra of the T4 H4-folate reductase-aminopterin complex and of aminopterin in acid versus neutral solutions. ---, difference spectra of 3.6 \( \mu \)M aminopterin in the presence of 2.8 \( \mu \)M enzyme; -- --, 2.9 \( \mu \)M aminopterin at pH 2.8 versus pH 7.0.](http://www.jbc.org/)
about 315 to 320 nm, becoming a minor negative peak in the case of MTX. While the acid-neutral difference spectra of the three compounds show a progressive change in a shoulder at about 310 nm, there are no negative absorption changes in this region.

As is indicated in Fig. 1, the magnitude of the acid-neutral difference spectra of NFA is a sensitive function of the acid pH. Presumably these spectral changes represent the differential absorption between the protonated and unprotonated species and, therefore, should yield information on the pK of these compounds (10, 19). From the inflection point of a plot of pH versus the absolute magnitude of the peak at 260 nm, the pK values of NFA, MTX, and aminopterin were found to be 5.3 in each case. Thus, the substitutions at the 10 position do not affect the apparent pK values of these compounds. The similarity of the difference spectra of enzyme-inhibitor complex formation to those of the acid-neutral system would suggest that these inhibitors bind to T4 Hf-folate reductase in the cationic form. It was, therefore, of interest to measure the magnitudes of the absorption change associated with complex formation. As is shown in Fig. 5, these absorption changes obey Beer's law. The following molar absorptivity changes for the peak near 260 nm were calculated: NFA, 8,600; MTX, 10,000, and aminopterin, 13,000. In the acid-neutral system of the free inhibitor, these magnitudes correspond to pH values in the 4.8 to 5.5 pH range for NFA and MTX and about pH 2.8 for aminopterin.

MTX has been reported to be about 1000 times more basic than folic acid. Thus, the pK of folic acid is about 2.8 (10, 19). To see if the spectral changes observed for the 4-amino inhibitors also applied to 4-hydroxyl compounds, we investigated the spectral changes accompanying the binding of folate to T4 Hf-folate reductase and in the acid-neutral system. As is shown in Fig. 6, the main feature of the difference spectra of the enzyme-folic acid system is two peaks at about 248 and 303 nm, both of which are positive, and a small peak at 353 nm. Since the interaction of folic acid with T4 Hf-folate reductase is weak (7), the enzyme concentration was increased to 8 µM and the final folic acid concentration to about 17 µM. Even with these relatively high concentrations the absorbance changes are rather small. Since the concentration of the enzyme-folate complex is not known, we cannot determine whether the small absorbance changes are due to lack of complex formation or to small molar absorptivity changes.

Folate difference spectra at acid versus neutral pH values (Fig. 6) show that the relationship between the enzyme and acid-neutral system observed for the 4-amino compounds did not extend to this 4-hydroxyl compound. While the most prominent peak in the acid-neutral system of folic acid is a negative peak (as is the case with the 4-amino compounds) only a small portion of the enzyme-folate spectrum is negative.

The difference spectrum obtained from the system composed of T4 Hf-folate reductase and its substrate Hf-folate is shown in Fig. 7. As with folate, the acid-neutral spectra of Hf-folate (not shown) bear no resemblance to the enzyme system. The T4 Hf-folate reductase-Hf-folate difference spectra are all positive, showing a large peak near 243 nm and a smaller one at about 305 nm. There is also a shoulder at about 355 nm. Thus, the three peaks observed in the enzyme-folic acid system are also observed in the enzyme-Hf-folate system, although the relative intensities are different and the positions of the maxima are shifted slightly. Based on a Kd of 5 × 10⁻⁷ M (7) Curve 2 is estimated to reflect the difference spectrum of an enzyme-folate-Hf complex of 5.1 µM concentration. Therefore, the 243

![Fig. 5. Beer's law plots of the difference spectra produced by complex formation between T4 Hf-folate reductase and NFA (○), MTX (●), aminopterin (□) (left-hand ordinate), or Hf-folate (■) (right-hand ordinate). Magnitudes of the difference spectra were determined at 201 nm, 202 nm, 203 nm, and 243 nm for NFA, MTX, aminopterin, and Hf-folate, respectively. The concentrations of the enzyme-NFA complex and the enzyme-Hf-folate complex were determined from dissociation constants of 1.5 × 10⁻⁷ M and 5.0 × 10⁻⁷ M, respectively (7). MTX and aminopterin are stoichiometric inhibitors of T4 Hf-folate reductase (14, 20). Therefore, the concentrations of their complexes with the enzyme were equal to the concentration of the enzyme, since inhibitors were present in excess.](http://www.jbc.org/)

![Fig. 6. Difference spectra of the T4 Hf-folate reductase-folate complex and of folate in acid versus neutral solutions. Curves 1 and 2 show 8.0 µM enzyme in the presence of 8.5 and 17 µM folate, respectively. Curves 3 and 4 show the difference spectra for folate at pH 2.8 versus 7.0 and 1.5 versus 7.0, respectively. K is p-aminobenzoyleglutamate.](http://www.jbc.org/)
FIG. 7. Difference spectra of the T4 Hz-folate reductase-Hz-folate complex. Lines 1 and 2 show the difference spectra obtained for 5.9 pM enzyme in the presence of 4.1 µM Hz-folate, and 5.6 pM enzyme in the presence of 10.5 µM Hz-folate, respectively. R is p-aminobenzoylglutamate.

FIG. 8. Difference spectra of the T4 Hz-folate reductase-Hz-aminopterin complex. Scans 1 and 2 are for 5.9 µM enzyme in the presence of 2.9 µM Hz-aminopterin, and Scans 3 and 4 are for 5.7 µM enzyme in the presence of 5.7 µM Hz-aminopterin. Conditions for Scan 5, obtained within 30 min after Scan 4, were the same as conditions in Scan 4, except 10 µM NADPH was present in both reference and sample cuvettes. See text for further explanation. R is p-aminobenzoylglutamate. The inset shows the difference spectrum of Hz-aminopterin at pH 0.3 versus pH 7.0.

DISCUSSION

The greatly enhanced binding that 4-amino-4-deoxyfolate compounds display towards all Hz-folate reductases which have been examined suggests the involvement of some indispensable feature of enzyme structure. Baker has suggested (11) that the 2-amino group of folate is one of the binding sites on this compound which contributes to the stability of the enzyme-inhibitor complex. Since the amino group is basic, it is assumed...
that the enzyme group in question is acidic. Baker further suggested that when the 4-hydroxy group is replaced by a 4-amino group, the 2-amino group becomes a much stronger base, and therefore makes a much stronger interaction with the acidic function of the enzyme.

While pKₐ data indicate greater basicity for the 4-amino compounds compared to 4-hydroxyl compounds in general, they do not indicate which group is being protonated in either case. To explore this question further, Perault and Pullman determined the theoretical basicity (molecular orbital calculations) of each nitrogen atom of the pteridine moiety for a number of folate compounds (13). These calculations did indeed support the concept of a more basic 2-amino group in aminopterin as compared to folate. However, they also show that the most basic nitrogen atom in aminopterin is N₁, indicating that this is the group which is protonated at acid pH.

The marked similarity of the difference spectra of Figs. 1, 3, and 4 between the enzyme-inhibitor complex and the acid neutral system suggests that the inhibitor is undergoing similar changes in each system. This in turn would suggest that NFA, aminopterin, and MTX bind to T₄ Hz-folate reductase as the same cationic form of the inhibitor that exists in acid solutions, i.e. protonated at N₁. If this is the case, it would seem that the apparent pKₐ of the bound inhibitor has been increased to values of 7 or greater, since the magnitude of the absorbance change suggests that protonation is 50% to 100% complete for NFA and aminopterin, respectively. The reason for the apparent shift in the pKₐ of these inhibitors remains to be elucidated. However, it is clear that the cationic form of the inhibitor would permit formation of salt linkages with the enzyme, which could account for an enhancement in binding on the order of several Cal of free energy (21).

The identity of the most basic nitrogen in folic acid is a controversial point. The calculations of Perault and Pullman indicate that it is N₅ (13), while from studies of the model compound 2-amino-4-hydroxy-6-methylpteridine, Whiteley and Huehnerkoen have suggested that it is most likely N₇ (19). If folate binds to a site on Hz folate reductase identical with the site occupied by aminopterin, N₁ of folate could be expected to make an interaction with the postulated acidic function of the enzyme. However, if the most basic nitrogen atom in folic acid is N₅, the lack of correspondence of the difference spectra of the enzyme-folate system and its corresponding acid-neutral system can be rationalized on the basis of the 1000-fold lower pKₐ of folate as compared to aminopterin. Thus, with folate, the postulated acidic function would not make more than a minor interaction with this position.

It is interesting that the above mentioned lack of correspondence also applies to the 4-amino compound at the dihydro oxidation level, as indicated in Fig. 8. (This conclusion is made from the portion of the difference spectrum obtained at wave lengths greater than 300 nm which were relatively stable.) The most basic position of H₂-aminopterin, according to Perault and Pullman, is still N₁, as with aminopterin, although in the dihydro compound it is about 1.7 pKₐ units less basic (12, 22). Since the increase in the 300 nm peak of the T₄ Hz-folate reductase-H₂-aminopterin difference spectra was nearly complete when the concentration of the inhibitor was equal to that of the enzyme, a nearly stoichiometric inhibition is suggested. Thus, H₂-aminopterin makes a significantly stronger interaction with T₄ Hz-folate reductase than does the corresponding 4-OH compound, Hz-folate, but the difference spectra do not suggest that H₂-aminopterin binds to T₄ Hz-folate reductase in the cationic form.

It is possible that the difference in pKₐ between the N₁ positions of aminopterin and H₂-aminopterin spans the basicity region where the inhibitor binds in the largely protonated form in the case of aminopterin, and the largely unprotonated form in the case of H₂-aminopterin. Since H₂-aminopterin nevertheless is bound to T₄ Hz-folate reductase more strongly than H₂-folate, it would appear that binding determinants other than an ionic bond can contribute to the enhanced binding of the 4 NH₂-folate compounds.

The most striking feature of the Hz-folate difference spectra (Fig. 7) is that the two peaks (at 243 nm and near 310 nm) correspond with previously existing features of the absorption spectra of H₂-folate (19). The absorption spectrum of H₂-folate at pH 7.0 is thought to be composed of two noninteracting chromophores, p-amino-benzoyl-glutamate and the pteridine portion (19). The p-amino-benzoyl group shows a single maximum at about 285 nm, which is a minimum in the difference spectra. Thus, the observed difference spectra appear to be enhancements of the 243 nm peak and 305 nm shoulder of the Hz-folate absorption spectra, both of which contributions probably derive from the pteridine moiety. The significance of this in relation to enzymatic function, however, is not presently clear. It is possible that, in part, the Hz-folate difference spectra reflect enzyme-induced electronic rearrangements of Hz-folate which would "prepare" the substrate for hydride transfer from NADPH to carbon of Hz-folate. This would suggest an alteration producing a more electropositive carbon 6 and possibly a more basic N₅. However, if the most basic nitrogen in Hz-folate is N₅ as suggested by Perault and Pullman (13), the difference spectra support a reduction mechanism in which the N₅ position is not protonated merely by binding of the substrate to the enzyme, since the enzyme and acid-neutral systems do not display any correlation.

It is significant that the binding of NADPH failed to perturb the absorption characteristics of the T₄ Hz-folate reductase-aminopterin complex, while markedly enhancing the 305 nm peak of the enzyme-H₂-aminopterin complex, since Hz-folate, but not folate, can serve as substrate for T₄ Hz-folate reductase. One possible interpretation is that the dihydro compounds bind to the enzyme at a site which is different from but overlapping with the site occupied by the oxidized compounds. To determine the validity of this assumption will require further experimental effort.

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