MOLECULAR PHARMACOLOGY, 20:154–158

Models for the Binding of Methotrexate to *Escherichia coli* Dihydrofolate Reductase

Direct Effect of Carboxylate of Aspartic Acid 27 upon Ultraviolet Spectrum of Methotrexate

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Received October 23, 1980; Accepted March 30, 1981

SUMMARY

SCHLEGEL, H. B., M. POE, AND K. HOOGSTEEN. Models for the binding of methotrexate to *Escherichia coli* dihydrofolate reductase: direct effect of carboxylate of aspartic acid 27 upon ultraviolet spectrum of methotrexate. *Mol. Pharmacol.* **20:** 154–158 (1981).

Ab initio molecular orbital calculations were performed on neutral and protonated 2,4-diamino-6-methylpteridine. The computations were repeated for the N-1-protonated molecule in a highly simplified model of the methotrexate binding site of *Escherichia coli* dihydrofolate reductase. The strengths of the ionic bond to Asp 27, the hydrogen bond to Thr 113, and the interaction with the peptide bonds between Ile 5 and Ala 6 and between Ala 6 and Ala 7 were calculated to be 100, 10, 5, and 1 kcal/mole, respectively, in the absence of solvent. These strengths suggest that the ionic bond is the most important component of the binding of protonated methotrexate. The energy calculations also provide a semiquantitative explanation of the hitherto unexplained shift to longer wavelength of the lowest-frequency UV absorbance band of N-1-protonated methotrexate upon binding to dihydrofolate reductase. This shift is due to a direct effect of the electrostatic field of the carboxylate ion of Asp 27 upon the molecular orbitals of the methotrexate.

INTRODUCTION

The enzyme dihydrofolate reductase (5,6,7,8-tetrahydrofolate: $NADP^+$ oxidoreductase, EC 1.5.1.3) is a drug target of considerable therapeutic importance in cancer chemotherapy (1). The most widely used dihydrofolate reductase inhibitor in cancer chemotherapy is methotrexate, which is *N*-10-methyl-4-amino-4-deoxy-folic acid (see Fig. 1). The high-resolution crystal structure of the binary methotrexate complex of the dihydrofolate reductase from *Escherichia coli* has been solved by Matthews *et al* (2). The *E. coli* reductase has a central, eight-stranded β-sheet as the main feature of its polypeptide backbone, with methotrexate bound in a 15-A deep cavity with its pteridine ring buried in a primarily hydrophobic pocket. Matthews *et al.* (2) found three important features of the enzyme which were responsible for tight binding of the 2,4-diaminopyrimidine moiety of methotrexate to dihydrofolate reductase: an ionic linkage of the carboxylate ion of Asp 27 to protonated N-1; a hydrogen bond of the Thr 113 hydroxyl to the C-2 amino group; and a π-π^* interaction of the peptide bond between Ala 6 and Ala 7 and N-1, C-2 and its amino group, and N-3 (the π-π^* interaction was proposed on the basis of the geometry of the interaction). The features of the 2,4-diaminopyrimidine moiety of methotrexate, i.e., N-1, C-2 with its amino group, N-3, and C-4 with its amino group, are found in all dihydrofolate reductase inhibitors that are very tightly bound (3).

Our earlier calculations on dihydrofolate by the complete neglect of differential overlap, self-consistent field method [CNDO/2; see Gund *et al.* (4)] have now been extended to a series of *ab initio* molecular orbital calculations on a highly simplified model of the methotrexate binding site of *E. coli* dihydrofolate reductase containing 2,4-diamino-6-methylpteridine in order to assess the relative importance of the various interactions. These calculations correctly account for the pH dependence of the longest-wavelength UV absorbance band of methotrexate, and suggest that the ionic interaction between the carboxylate ion of Asp 27 and N-1 of protonated methotrexate is the most important.

When methotrexate is bound to dihydrofolate reductase, calorimetric data (5) and UV-difference spectrophotometric (6–9) evidence indicate that it is protonated at N-1 when enzyme-bound. The UV difference spectrum has three noteworthy components: (a) a component

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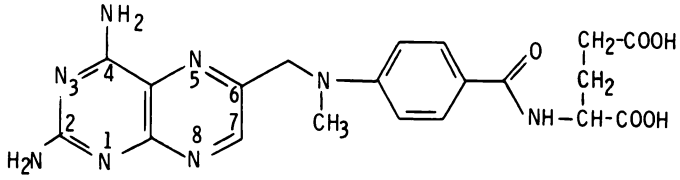

FIG. 1. *Structure of methotrexate*

strongly resembling that observed on protonation of methotrexate, reflecting an increased proportion of protonation on binding; (b) several narrow bands corresponding to the alteration of the environment of one or more tryptophan residues of the enzyme upon methotrexate binding; and (c) a shift to longer wavelength by 10–40 nm of the longest-wavelength UV absorption band. This latter component, hitherto unexplained, is shown in our calculations to be attributable to a direct effect of the electrostatic field of the carboxylate ion of Asp 27 upon the molecular orbitals of the pteridine ring.

CALCULATIONS

Our model for the methotrexate binding site in dihydrofolate reductase is based on the crystal structure of the binary methotrexate complex of *E. coli* dihydrofolate reductase (2). This model is given schematically in Fig. 2 and in a stereo pair in Fig. 3. These coordinates were given to us by Drs. J. T. Bolin, D. A. Matthews, and J. Kraut, of the University of California at San Diego. In the model, methotrexate is represented by 2,4-diamino-6-methylpteridine, Asp 27 by formate, Thr 113 by water, and the peptide bonds between Ile 5 and Ala 6 and between Ala 6 and Ala 7 both by formamide. Each nonhydrogen atom of the pteridine in the model is at the same position as the corresponding atom of methotrexate in the binary methotrexate complex of *E. coli* dihydrofolate reductase. Similarly, the coordinates of the formate are obtained from the carboxyl group on the side chain of Asp 27. The locations of the formamide carbon, oxygen, and nitrogen atoms are taken from the appropriate atoms in the Ile 5—Ala 6 and Ala 6—Ala 7 peptide bonds in the enzyme complex. The oxygen atom of the water modeling Thr 113 is at the same position as the hydroxyl oxygen atom of Thr 113 in the enzyme complex, with one O—H bond directed along the Cβ—Oγ bond axis. The hydrogen atoms in the model are attached with standard bond lengths and angles[1] unless otherwise noted. The 2-amino and 4-amino groups were chosen to be planar, the rotation of the 6-methyl group was fixed arbitrarily, and the free hydrogen atom in the Thr 113 model was oriented in two different ways so that it could either accept or donate a hydrogen bond in its interaction with the 2-amino group.

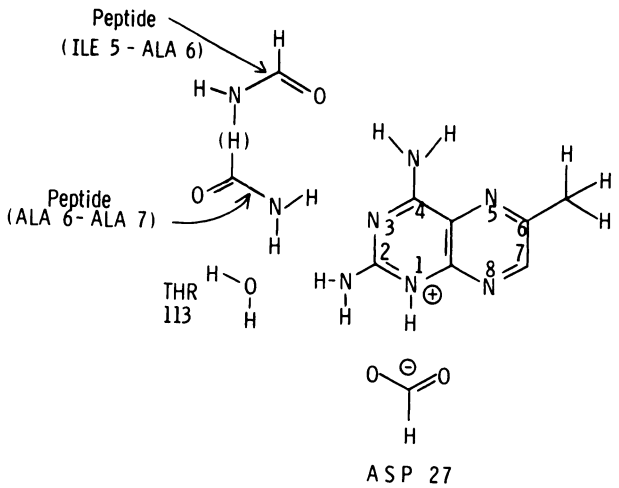

FIG. 2. *Schematic model for binding site*

2,4-Diamino-6-methylpteridine is numbered like the corresponding atoms of the methotrexate. The peptide bonds between Ile 5 and Ala 6 and between Ala 6 and Ala 7 are represented by formamides, the Thr 113 hydroxyl by water, and the Asp 27 carboxyl by formate. The hydrogen atom representing the α-carbon of the Ala 6 was nearly at the same position as the formamides representing both peptide bonds; this is indicated by the atom in *parentheses* (*H*).

Calculations were performed with the Gaussian 76 series of *ab initio* molecular orbital programs (10) using the STO-3G basis set (11). Isolated 2,4-diamino-6-methyl pteridine was computed in the neutral form and protonated, in turn, at N-1, N-3, N-5, and N-8 to determine the preferred site of protonation. The supermolecule approach was used to calculate the interaction energies between the pteridine ring (both neutral and N-1 protonated), and each of the features of the enzyme model was taken one at a time. No counterpoise corrections were made, and the total interaction was approximated as the sum of the individual interactions. Even with these simplifications, only minimal basis set calculations were practical. Such basis sets including STO-3G basis sets have a number of shortcomings. For example, the relative proton affinities computed for neutral nitrogen compounds and carboxylate are wrong. Hence, the position of the hydrogen atom in the methotrexate-Asp 27 interaction cannot be determined from our model by geometry optimization. Electrostatic interactions and hydrogen bonds are predicted fairly well when minimal basis sets are used, but polarization and charge transfer may be underestimated. Hydrophobic effects are not handled correctly in these calculations, since this would require the explicit consideration of solvent entropy. With these reservations in mind, we proceed to a cautious analysis of our calculations.

The proton affinities for methotrexate have been calculated. Although the absolute magnitudes are overestimated, the relative values are reliable at the minimal basis set level. The order of the proton affinities is N-1 (290.3 kcal/mole) > N-3 (284.4 kcal/mole) > N-8 (273.4) > N-5 (255.8), indicating that protonation at N-1 is preferred.[2] The most basic nitrogen atom of methotrexate, which has a pK_a of 5.71 in water at 25°, has been shown to be N-1 (12).

[1] Standard parameters for hydrogen atoms: R(C—H) = 1.09 A except for amide and carboxyl groups, where R(C—H) = 1.07 A and R(N—H) = 1.00 A; for CH_3 groups, where HCH = 109.5°; and for NH_2 groups, where CHN = 120°. Other hydrogen atoms are placed on the bisector of the associated three-heavy-atom angle.

[2] Our calculations on the relative proton affinities of 2,4-diamino-6-methylpteridine give the same order of proton affinities as earlier calculations by the complete neglect of differential overlap, self-consistent field method (CNDO/2, P. Gund, private communication).

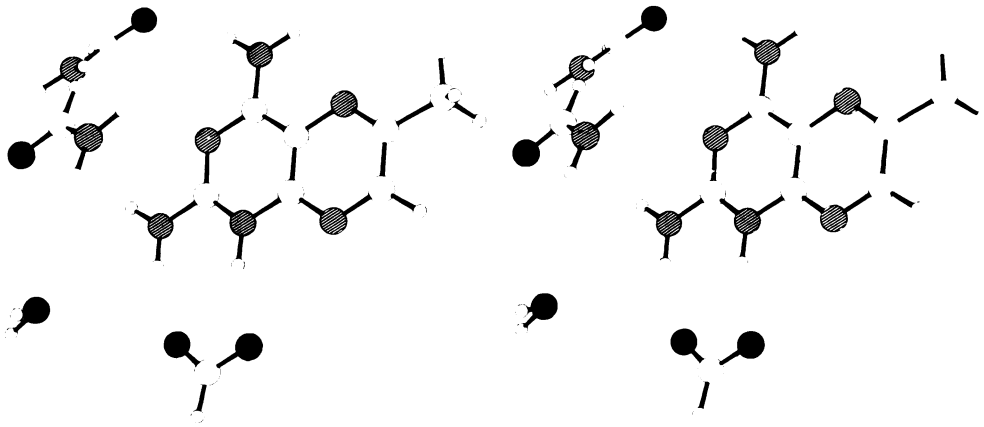

FIG. 3. *Stereoscopic pair of the model for the binding of methotrexate to the active site of dihydrofolate reductase showing the relative orientation of 2,4-diamino-6-methylpteridine representing methotrexate, two formamides representing peptide bonds between Ile 5—Ala 6 and Ala 6—Ala 7, water representing the Thr 113 hydroxyl, and formate representing the Asp 27 carboxyl*

The total energies for the various fragments used to model the enzyme active site are presented in Table 1. The interactions of these fragments with the neutral and N-1 protonated pteridine are presented in Table 2. For neutral methotrexate, the interactions are small, as expected from the fact that methotrexate is protonated when bound to *E. coli* dihydrofolate reductase (13). The net positive charge on protonated methotrexate strengthens each of the interactions, especially the interaction with Asp 27. The calculations indicate that the carbonyl oxygen atom of the Ile 5–Ala 6 peptide bond forms a distorted hydrogen bond with the amino group at C-4. The peptide bond between Ala 6 and Ala 7 does not interact strongly with the pteridine ring. Possibly calculations with an extended basis set would enhance this π-π^* effect. The interaction with Asp 27 is by far the largest and is the result of bringing two oppositely charged molecules into close proximity. The Thr 113 can act as either a hydrogen donor or acceptor in the bond with the amino group at C-2. Calculations on both geometries indicate that Thr 113 behaves as a proton acceptor. These interaction energies are relative to monomers in the gas phase, since a detailed calculation of the effect of solvent is beyond the scope of our study. Solvation of the methotrexate and active-site models will reduce the calculated energies substantially, with the ionic interaction being affected most, perhaps by as much as one order of magnitude. Nevertheless, the role of Asp 27 in the binding of methotrexate should still dominate.

TABLE 1

Total energies for fragments in enzyme model

Feature modeled[a]	Total energy
	atomic units
Neutral methotrexate	−588.89051
N-1-H^+-methotrexate	−589.35309
Ala 5—Ala 6 peptide bond	−166.67118
Ala 6—Ala 7 peptide bond	−166.68017
Asp 27 carboxyl group	−185.43201
Thr 113 hydroxyl group[b]	−74.96590
Solvent water molecule[b]	−74.96590

[a] See text for details on structures used for models.
[b] STO-3G optimized geometry used for water.

TABLE 2

Total energies of models for interaction of enzyme fragments with neutral and N-1-protonated methotrexate[a]

Enzyme fragment	Total energy[b]		Interaction energy[b]	
	Neutral	N-1-protonated	Neutral	N-1-protonated
	atomic units		*kcal/mole*	
Ile 5—Ala 6 bond	−755.56557	−756.03179	2.4	4.7
Ala 6—Ala 7 bond	−755.57139	−756.03181	−0.4	0.9
Asp 27 carboxyl	−774.32397	−774.94498	0.9	100.3
Thr 113 hydroxyl	−663.85759	−664.33477	0.7	9.9

[a] See text for details of structures.
[b] A negative value indicates a repulsive interaction.

The molecular orbitals obtained in the preceding calculation can be interpreted to provide additional information about the nature of the methotrexate-dihydrofolate reductase interaction. The difference in the orbital energies between the HOMO[3] and the LUMO can be related to the lowest-energy electronic transition, which in the case of the methotrexate is a π-π^* transition of the pteridine ring. The orbital energy differences $\Delta\epsilon$, herein defined as the difference in energy between the HOMO and LUMO, i.e., ϵ(HOMO) − ϵ(LUMO), are summarized in Table 3. A comparison of $\Delta\epsilon$ for neutral and N-1 protonated 2,4-diamino-6-methylpteridine ($\Delta\epsilon$ = 0.4070 and $\Delta\epsilon$ = 0.4302 atomic unit, respectively), shows that protonation leads to an increase in $\Delta\epsilon$, in agreement with the shift to shorter wavelength of the lowest-energy UV absorbance band. For the protonated pteridine plus the Asp 27 carboxyl group, the increase in $\Delta\epsilon$ compared with the neutral pteridine is much less. Interactions with the other fragments of the enzyme model affect $\Delta\epsilon$ only slightly. The combined effect of all four components is estimated to yield $\Delta\epsilon$ = 0.4145 atomic unit.

Several additional computations have been performed to assess the influence of solvent water molecules. The largest effect relevant to our calculations would occur when water is hydrogen-bonded to N-1, since this is the site where interactions with the enzyme has its greatest

[3] The abbreviations used are: HOMO, highest occupied molecular orbital; LUMO, lowest unoccupied molecular orbital; atomic unit, 627.51 kcal/mole.

TABLE 3

Effect of model enzyme interactions on the HOMO-LUMO orbital energy difference

Structure	Symbol	ϵ(LUMO)-ϵ(HOMO)
		atomic units
2,4-Diamino-6-methylpteridine (MTX)	—	0.4070
N-1-H^+ 2,4-diamino-6-methylpteridine (MTX 1)	$\Delta\epsilon_0$	0.4302
MTX 1 plus Ile 5—Ala 6 peptide bond	$\Delta\epsilon_1$	0.4306
MTX 1 plus Ala 6—Ala 7 peptide bond	$\Delta\epsilon_2$	0.4294
MTX 1 plus Asp 27 carboxyl group	$\Delta\epsilon_3$	0.4162
MTX 1 plus Thr 113 hydroxyl group	$\Delta\epsilon_4$	0.4289
MTX 1 plus all four enzyme fragments[a]	$\Delta\epsilon_5$	0.4145

[a] Total effect of interaction with enzyme fragments estimated as the sum of the individual interactions: $\Delta\epsilon_5 - \Delta\epsilon_0 = [\Delta\epsilon_1 - \Delta\epsilon_0] + [\Delta\epsilon_2 - \Delta\epsilon_0] + [\Delta\epsilon_3 - \Delta\epsilon_0] + [\Delta\epsilon_4 - \Delta\epsilon_0]$, where $\Delta\epsilon_0$ is the HOMO-LUMO energy difference for the protonated methotrexate model in the absence of any interactions.

effect. However, a water molecule appropriately placed near N-1 has little effect on $\Delta\epsilon$ of either the protonated or unprotonated pteridines ($\Delta\epsilon = 0.4302$ and $\Delta\epsilon = 0.4080$ atomic unit, respectively). In summary, the calculations indicate that the shift in the wavelength of the lowest UV band should be less for enzyme-bound, protonated pteridines than for the protonated pteridine in solution.

DISCUSSION

The *ab initio* quantum chemical calculations on 2,4-diamino-6-methylpteridine summarized here should be directly applicable to the 2,4-diaminopteridine portion of methothrexate, because the 2,4-diaminopteridine is electrically insulated from the *p*-aminobenzoyl-L-glutamate moiety of methotrexate by a saturated carbon "spacer," which would make the electronic properties of the 2,4-diaminopteridine independent of the rest of the molecule [see Gund *et al.* (4) for an analogous calculation]. Our model for the portions of dihydrofolate reductase that we believe are most important in binding the 2,4-diaminopteridine moiety of methotrexate also involves portions of the enzyme that are separated from the rest of the enzyme by saturated carbon spacers, although these saturated carbon atoms are replaced by hydrogen atoms for our calculations. The replacement of the remainder of the 2,4-diaminopteridine binding site in dihydrofolate reductase by vacuum probably is not too bad an approximation for the effects of the rest of the site, since the interior of the enzyme is a nonpolar or low dielectric medium; the rest of the site is also more remote from the 2,4-diaminopyrimidine portion of the methotrexate binding site.

Our calculations of the strengths of interactions between dihydrofolate reductase and methotrexate, summarized in Table 3, indicate that the ionic interaction between the carboxylate of Asp 27 and protonated N-1 is by far the most important. This result is at variance with the conclusion of Hood and Roberts (9), who have asserted that only one-third of the difference in binding

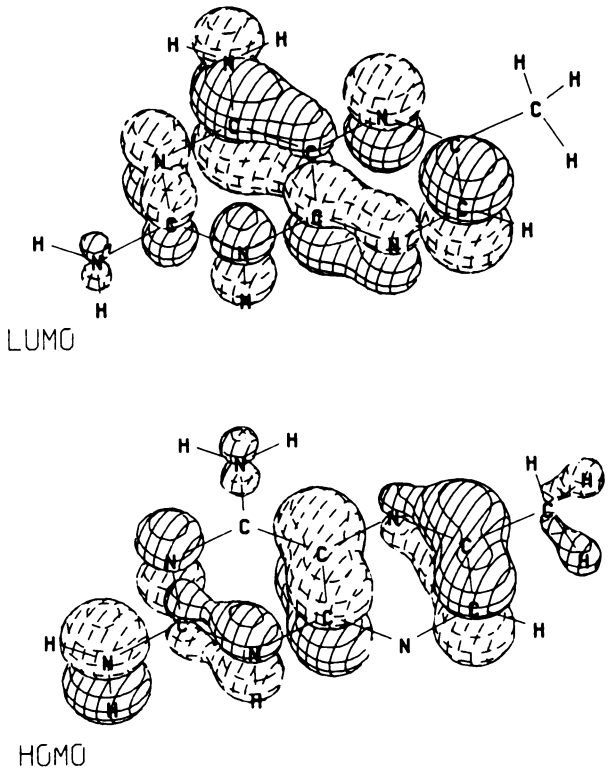

FIG. 4. *The HOMOs and LUMOs of N-1-protonated 2,4-diamino-6-methylpteridine*

Solid and *dashed 3-dimensional contours* are drawn for orbital amplitudes of ±0.05 electron.

energy between folate and methotrexate to *Lactobacillus casei* dihydrofolate reductase arises from the difference in charge state. Hood and Roberts (9) measured the association constants for folate, neutral methotrexate, and protonated methotrexate to *L. casei* dihydrofolate reductase, and found them to be 9.8×10^4 M^{-1}, 1.75×10^7

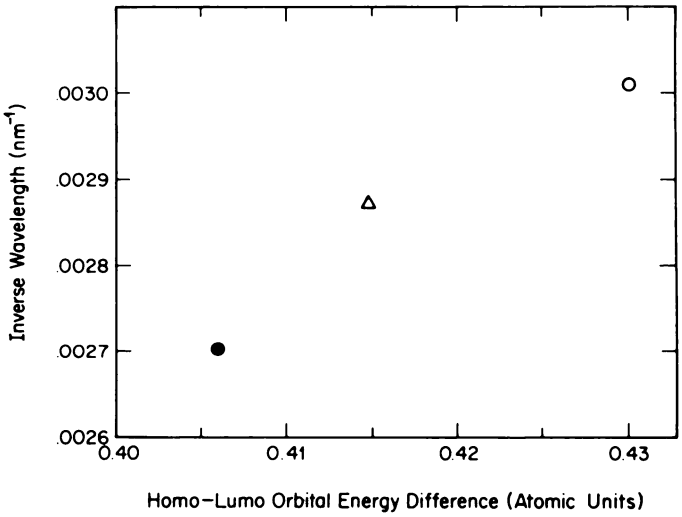

FIG. 5. *Comparison of calculated orbital energy differences and observed UV spectrum maxima*

●, Neutral methotrexate in solution (12); ○, N-1-protonated methotrexate in solution (12); △, N-1-protonated methotrexate bound to *Escherichia coli* dihydrofolate reductase (6).

M^{-1}, and $2.75 \times 10^{+10}$ M^{-1} at pH 7.5, respectively. They asserted that the relative values of these association constants means that only one-third of the difference in binding to dihydrofolate reductase between folic acid and methotrexate, which is mostly protonated when bound, arises from the difference in charge state. However, this conclusion implicitly assumes that neutral methotrexate and protonated methotrexate are bound in the same conformation on the enzyme. This implicit assumption is probably incorrect, since both folic acid (14, 15) and dihydrofolic acid (16) appear to be bound in a different conformation than protonated methotrexate on dihydrofolate reductase. What is more likely to be correct is that protonated methotrexate is bound in a conformation that is less favorable for binding than folate or neutral methotrexate except for the ionic interaction, but that the strength of the ionic interaction overcomes the other unfavorable aspects of its binding conformation. It has long been believed, on the basis of quantum calculations by Perault and Pullman (17) and Collin and Pullman (18) and by analysis of structure-activity relationships for inhibitors by Hitchings and Burchall (3) and Baker (19), that the increased basicity of N-1 and the 2-amino group in 2,4-diaminopteridines as compared with that of 2-amino-4-oxopteridines is largely responsible for their substantially greater affinity for dihydrofolate reductase. Laser raman studies by Saperstein *et al.* (20) showed that methotrexate was protonated at N-1 when bound to dihydrofolate reductase. Our *ab initio* calculations also support this view. It should be noted that there are many assumptions and simplifications necessary to make these calculations possible. It is probably true that our assumptions and simplifications lead our calculations to somewhat overstate the importance of the ionic bond, relative to what a more elaborate calculation would indicate. We believe that a more elaborate calculation would confirm the essential features of our results, and in particular would confirm the primacy of the ionic interaction.

The difference between the UV absorbance of N-1-protonated and neutral methotrexate above 300 nm seems to match the change in the UV absorbance of methotrexate upon binding to dihydrofolate reductase. However, the change in UV absorbance upon enzyme binding must be shifted 10 nm (6) to 40 nm (9) to shorter wavelength to match the difference in UV absorbance between N-1-protonated and neutral methotrexate. The calculations given here provide an explanation for this previously unexplained shift. In the *ab initio* calculations, the net effect of introducing a proton is to increase the HOMO versus LUMO separation for N-1-protonated methotrexate as compared with that of neutral methotrexate. However, introducing the Asp 27 carboxylate has a direct electrostatic effect on N-1 and the 2-amino group, decreasing the HOMO versus LUMO separation ($\Delta\epsilon$). The HOMO of the protonated pteridine has a larger coefficient on the 2-amino group than does the LUMO (see Fig. 4), so its energy will be raised more by the negatively charged carboxylate. This decreases the HOMO versus LUMO separation ($\Delta\epsilon$). The carboxylate oxygen atoms can also hydrogen-bond to N-1 and the 2-amino group and indirectly affect orbitals with large densities on these atoms. The formamide and water molecules have much smaller effects, since they are not formally charged and do not interact as strongly. The results of a plot of inverse of the wavelength of maximal UV absorbance (λ_{max}) for the longest-wavelength absorbance band of neutral, N-1-protonated, and enzyme-bound methotrexate against our calculated $\Delta\epsilon$ between HOMO and LUMO for neutral, N-1-protonated, and enzyme model-bound 2,4-diamino-6-methylpteridine, respectively, are given in Fig. 5. This correlation between theoretical and experimental results for the UV data gives us confidence that the assumptions and simplifications in our model are not too severe to prevent us from drawing useful conclusions.

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