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Calculations by Complete Neglect of Differential Overlap (CNDO/2) on Dihydrofolic Acid: Role of N(5) in Reduction by Dihydrofolate Reductase

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SUMMARY

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CNDO/2 calculations on 7,8-dihydropteroylamide, a model of 7,8-dihydrofolic acid, have been made to examine the conformational and electronic properties of the molecule. The quantum mechanical calculations indicate that while the extended forms of the neutral molecule, its N(1)-cation, and N(5)-cation were favored with respect to folded forms, bent conformations are probably energetically accessible. Calculated binding energies for the protonated cations suggested that protonation at N(5) is strongly favored over N(1) by about 16.4 kcal/mole. This first site of protonation and the conformational preference agree with those observed in solution for 7,8-dihydrofolic acid. The lowest unoccupied molecular orbital (LUMO) of the N(5)-cation exhibited a much higher wave function magnitude at C(6) than did the LUMO of the N(1)-cation or the neutral molecule. Thus, according to frontier orbital theory, the N(5)-cation is especially well suited for hydride transfer to C(6). This latter result rationalizes the observed ultraviolet difference spectra of dihydrofolate reductase upon binding of dihydrofolate, indicative of protonation of dihydrofolate at N(5) upon binding. The protonation-upon-binding argument also may explain why dihydrofolate is so potently inhibited by analogues more basic than dihydrofolate in its pteridinyl moiety.

INTRODUCTION

The enzyme dihydrofolate reductase (5,6,7,8-tetrahydrofolate:$NADP^+$ oxidoreductase, EC 1.5.1.3) is an important drug target in parasitic and bacterial infections (1) and in cancer chemotherapy (2). Quantum mechanical calculations on folic acid coenzymes by Pullman and co-workers (3-6) and by Neely (7, 8) have provided insight into the role of various moieties of folates in their interaction with dihydrofolate reductase.

However, these calculations, which were at the simple Hückel molecular orbital level of approximation, did not explicitly account for the important effect of the σ-electrons. Furthermore, there is new experimental evidence to be explained, specifically the preferred protonation at N(5) of dihydrofolate (9), and the spectrophotometric evidence that folates are protonated when bound to dihydrofolate reductase (10-12).

We therefore undertook all-valence-electron calculations by the CNDO/2[1]

[1] The abbreviations used are: CNDO/2, complete neglect of differential overlap self-consistent field; LUMO, lowest unoccupied molecular orbital.

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method (13).[2] We specifically addressed the questions of preferred conformation of dihydrofolate (a problem not accessible to Hückel molecular orbital theory, which does not explicitly include molecular geometry effects) and orbital effects on proton affinity and reactivity.

We present here a summary of CNDO/2 (12) calculations on 7,8-dihydropteroylamide, a model for dihydrofolate, which suggest that although the extended conformer of dihydrofolate [the form found in solution (14)] is energetically favored, folded conformations are probably energetically accessible. The calculations also point to preferred protonation at N(5), and the lowest unoccupied molecular orbital of this cation rationalizes transfer of hydride to C(6) by the enzyme complex. Supposing protonation of dihydrofolate before or during binding to its reductase rationalizes the avidity of the strongly basic analogues (like the antineoplastic agent aminopterin) for the same enzyme.

CALCULATIONS

Since we could not find a suitable crystal structure, we generated a standard geometry for the 7,8-dihydropteridine ring by suitably modifying the crystal structure of a 3,4-dihydropteridine (15). Standard bond lengths and angles (13) were used to build up the rest of the molecule.

Calculations were done for the more stable (6) keto form of 7,8-dihydropteroylamide, with the structure given in Fig. 1. The three rings were assumed to be planar, with the bond lengths and bond angles shown. Standard hydrogens were assumed. Inclusion of the flexible L-glutamate moiety of 7,8-dihydrofolate would immensely complicate the problem of determining preferred conformation. However, this portion of 7,8-dihydrofolate appears to be more important for molecule transport than for receptor binding (16) and is not involved in oxidoreduction. We have therefore followed other authors (3–8) in ignoring the L-glutamate moiety. Structures were generated and calculations performed with the Merck molecular modeling system (17).

With three major rotational degrees of freedom in this large molecule, full search of conformational space is a nontrivial computational problem. In an effort to define the bounds of the problem, we chose two extreme conformations for comparison: one fully extended (Fig. 1, $\phi_1 = \phi_2 = \phi_3 = 180°$, with ϕ_4 as shown) and one folded ($\phi_1 = 90°$, $\phi_2 = 0°$, and $\phi_3 = 90°$, with ϕ_4 as shown). [The numbering shown for 7,8-dihydropteroylamide is the same as for folic acid (18).] The CNDO/2 calculations indicate that the extended form is favored by 6.4 kcal/mole for the free base, by 5.2 kcal/mole for the N(1)-protonated, and by 5.3 kcal/mole for the N(5)-protonated 7,8-dihydropteroylamide. The extended form of dihydrofolate predominates in solution (14).

Although N(1) has a greater calculated negative charge (−0.34) than N(5) (−0.09) in neutral 7,8-dihydropteroylamide, the computed binding energy of the 5-protonated cation is much greater (16.4 kcal/mole) than that of the 1-protonated cation. Dihydrofolate in solution has a greater proton affinity at N(5): N(5) pK′ = 3.84 and N(1) pK′ = 1.38 (9).

The CNDO/2-calculated LUMO coefficients for the extended N(5)-cation, the free base, and the N(1)-protonated cation of 7,8-dihydropteroylamide are shown in Fig. 2. The LUMO of the N(5)-cation possesses by far the largest amplitude at C(6). Since frontier orbital theory predicts (19) that nucleophilic addition should be governed by the highest occupied molecular orbital of the nucleophile and the LUMO of the electron-deficient substrate, protonation at N(5) favors hydride transfer to C(6). The LUMO coefficients for the N(5)- and N(1)-cations and for the free base are virtually unchanged upon passing from the extended to the folded conformations.

DISCUSSION

The quantum mechanical calculations on 7,8-dihydropteroylamide summarized here should be directly applicable to dihydrofolate (7,8-dihydropteroyl-L-glutamate), since the glutamate portion is re-

[2] The program was modified by J. D. Andose to accommodate larger molecules. Standard Pople-Santry parametrization was used.

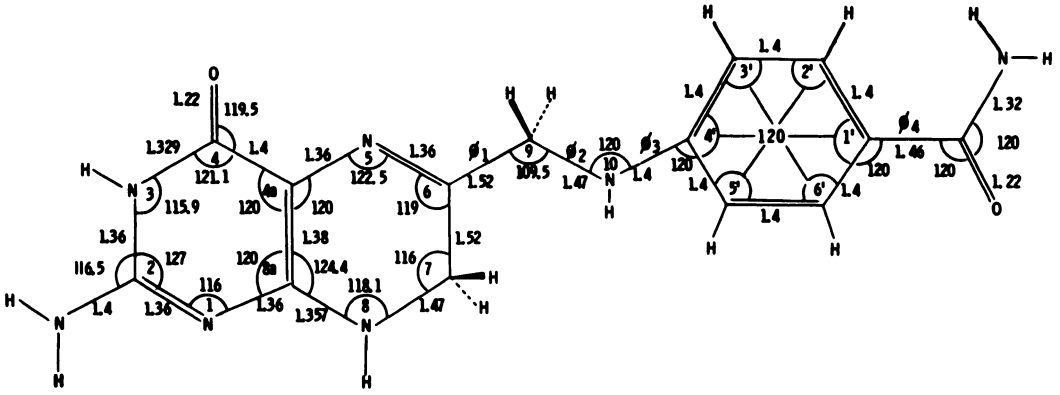

FIG. 1. *Numbering system for 7,8-dihydropteroylamide*

ϕ_1–ϕ_4 dihedral angles are discussed in the text. The bond lengths, in angstroms, and bond angles used in the calculations are shown.

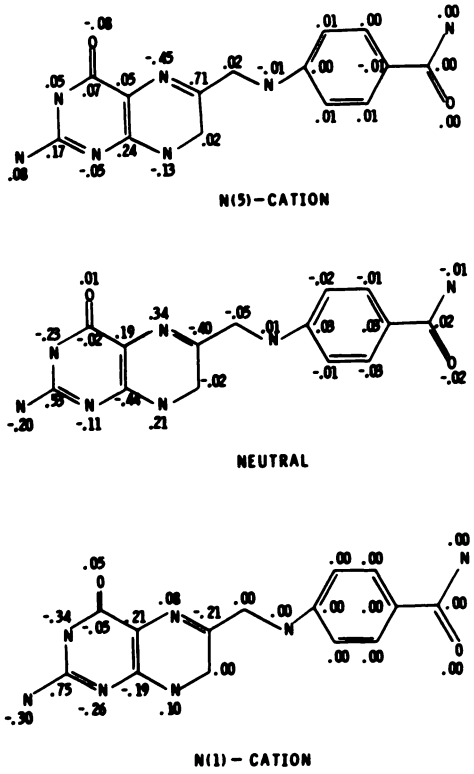

FIG. 2. *Coefficients of wave function for lowest unoccupied molecular orbital for N(5)-cation, neutral form, and N(1)-cation of 7,8-dihydropteroylamide.*

mote from the pteroyl ring in most conformations. The two rings and the glutamate are electrically insulated from each other by saturated carbon "spacers"; thus the values of ultraviolet absorbance of the two ring systems in dihydrofolate are independent of one another and of the glutamate moiety [see Huennekens (20) for a discussion]. Furthermore, our limited calculations suggest that conformation has only a small effect on the frontier orbitals and on the partial charges of the neutral form, the N(1)-cation, and the N(5)-cation.

The conformation of dihydrofolate has not previously been considered theoretically. Since there are three rotatable bonds, full search of conformational space for these large molecules was not attempted. Our preliminary calculations suggest that the extended form is more stable than one extreme folded conformation, in agreement with experiment: the extended form is observed in solution (14). Nevertheless, the calculated 6-kcal difference in energy is small enough that folded conformations must be considered when the molecule is bound to reductase.

While N(5) is not the site of highest net charge by CNDO calculation, it is the site of protonation for the calculated most stable cation. Analogously, Perault and Pullman (4) found that π-electron charge densities did not correlate with preferred sites of protonation of folates, while a more complex function did. Using a self-consistent-field approach, these authors predicted 5-protonation to be favored for dihydrofolate, in agreement both with our all-valence-electron calculations and with experiment (9).

A priori, N(1) might be expected to be

the preferred protonation site for all folates, since it is part of a strongly basic guanidine system (consisting of N_1, N_3, C_2, and C_2-amino), although the basicity would be somewhat lessened by the adjacent C_4-carbonyl. Indeed, N(1) is experimentally the most basic site in folic acid (see discussion in ref. 9) and in aminopterin (9), although Pullman and Pullman (6) calculated that N(8) in folic acid was most basic. Despite the well-known hazard of attempting to correlate basicity, a highly solvation-dependent effect (21), with CNDO calculations *in vacuo* ("isolated molecule"), N(5) actually does appear to be the most basic center of dihydrofolate (9).

There is strong spectrophotometric evidence (10–12) that folates bound to dihydrofolate reductase exist in a partially protonated state. The difference spectrum generated upon binding of dihydrofolate to *Escherichia coli* MB 1428 dihydrofolate reductase (22) resembles the absorbance change noted upon N(5) protonation of dihydrofolate (9), suggesting that dihydrofolate is protonated at N(5) when bound to reductase. The N(5)-cation of dihydrofolate would be expected to be favorably disposed toward reduction by hydride transfer to C(6), since the LUMO is highly localized at that atom (50% probability density centered there). Furthermore, this localization appears to be independent of exact conformation of the substrate when bound to the enzyme: the LUMO coefficients in the pteridine ring were virtually identical for the folded and extended conformations of N(5)-protonated dihydrofolate. Conversely, hydride transfer to C(6) is much less favored for the dihydrofolate free base [coefficient of 0.40, probability density of 16% at C(6)] or for the N(1)-protonated cation [highest coefficient (0.75) at guanidinium C(2) vs. 0.21 at C(6)]. Finally, the N(5)-cation possesses a LUMO which is bonding in character ($E_{LUMO} = -0.0317$ hartree), so that it is predicted to be a powerful electron acceptor. The free base has a normal antibonding LUMO ($E_{LUMO} = 0.0900$).

Dihydrofolate must accept the formal equivalent of hydride at C(6) during enzymatic reduction by dihydrofolate reductase, although the mechanism does not necessarily involve free hydride ion. We suggest that N(5) protonation of dihydrofolate accompanies its binding to dihydrofolate reductase, and that this in turn enhances hydride attack at C(6). This is in agreement with Perault and Pullman (4, 6), who argued from simple Hückel SCF-calculated π-electron charge densities that reduction of dihydrofolate involved N(5) protonation, followed by hydride transfer to electropositive C(6).

In agreement with the "basicity" hypothesis of Pullman and co-workers (3–6) and the ultraviolet data (10–12), we suggest that dihydrofolate reductase preferentially binds substrates in their protonated form. The high affinity of the enzyme for antineoplastic agents like aminopterin (4-amino-4-deoxyfolate) and methotrexate [*N*(10)-methylaminopterin] may be explained on this basis. Aminopterin and methotrexate have more basic nitrogens at N(1) (pK′ = 5.50 and 5.71, respectively) than does dihydrofolate at N(5) (pK′ = 3.84) and thus are more easily protonated for tight binding to the enzyme. This probably requires that there be a different orientation of inhibitor and substrate on the enzyme.

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