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Theoretical Studies on the Activation of the Pterin Cofactor in the Catalytic Mechanism of Dihydrofolate Reductase†

Jill E. Gready
Department of Biochemistry, University of Sydney, Sydney, N.S.W. 2006, Australia
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ABSTRACT: Two mechanisms for facilitating hydride ion transfer from NADPH involving preprotonation of the pteridine rings of the dihydrofolate reductase substrates folate and dihydrofolate have been investigated by ab initio quantum mechanical methods. Protonation energies and effective solution pK_as have been calculated for four protonated forms, three of which are nonpreferred in aqueous solution and therefore not directly accessible to experimental study. The pattern and degree of redistribution of the positive charge over the component rings of the N-heterobicyclic π-system in these protonated forms have been analyzed in terms of changes in the electron populations of the ring atoms and total ring charges. The effects of such changes in promoting hydride ion transfer to C7 in folate and C6 in dihydrofolate have been evaluated by considering the extent of development of partial carbonium ion character at these carbon atoms and also the degree of electron deficiency in the pyrazine ring as a whole. The results illustrate that perturbations due, for instance, to protonation may be propagated by π-electron coupling effects over medium-range distances of 4–6 Å across the pteridine ring. The two mechanisms have been assessed in terms of the calculated absolute and relative pK_as of the protonated species taking into account experimental information regarding possible stabilization of these forms in the enzyme active site and also the effectiveness of the various protonations in assisting the hydride ion transfer step. Judged against these criteria, the theoretical results favor the generally proposed mechanism involving preprotonation of N8 in folate and N5 in dihydrofolate. However, some support was also found for the alternative novel mechanism involving O4-protonation of both folate and dihydrofolate.

The enzyme dihydrofolate reductase (DHFR) has been the subject of intensive investigation for over 25 years, the continuing interest being prompted mostly by its importance as the biological target for a large class of drugs—the "antifolates" (Blakley, 1969, 1981; Hitchings & Smith, 1979; Gready, 1980; Freisheim & Matthews, 1984). DHFR[1] (tetrahydrofolate:NADP$^+$ oxidoreductase, EC 1.5.1.3) catalyzes two reactions:

$$\text{folate} + 2\text{NADPH} + 2\text{H}^+ \rightarrow 5,6,7,8\text{-tetrahydrofolate} + 2\text{NADP}^+ \quad (1)$$

$$7,8\text{-dihydrofolate} + \text{NADPH} + \text{H}^+ \rightarrow \text{H}_4\text{folate} + \text{NADP}^+ \quad (2)$$

However, in mammalian cells at physiological pH the maximal rate of reaction 1 is, at most, a few percent that of reaction 2 while for some bacterial DHFRs reaction 1 is undetectable (Blakley, 1969). Most of the vertebrate enzymes have two pH optima for H_2folate reduction at ca. pH 4–5 and pH 7–8, while most bacterial DHFRs have a single acidic pH optimum (Blakley, 1969; Freisheim & Matthews, 1984). For folate reduction, a single pH optimum between ca. pH 4 and pH 5 has been reported for DHFRs from bacterial (Nixon & Blakley, 1968; Baccanari et al., 1975) and vertebrate (Mathews & Huennekens, 1963; Gupta et al., 1977; Smith et al., 1979) sources: at these low pH values folate-reducing activity may be 10–20% that for H_2folate. K_m values for H_2folate and NADPH are typically in the micromolar range while the enzyme turnover numbers are typically low (4–30 s^{-1}) (Blakley, 1969; Freisheim & Matthews, 1984), thus suggesting slow catalysis rather than poor substrate-binding affinity; although the K_m values for folate reduction are typically higher than

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[1] Abbreviations: pterin, 2-aminopteridin-4(3*H*)-one; DHFR, dihydrofolate reductase; folate, 6-[[[*p*-(L-glutamocarbonyl)phenyl]-amino]methyl]pterin ≡ pteroylglutamate; H_2folate, 7,8-dihydrofolate; H_4folate, 5,6,7,8-tetrahydrofolate.

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those for H_2folate reduction (Blakley, 1969), similar conclusions apply.

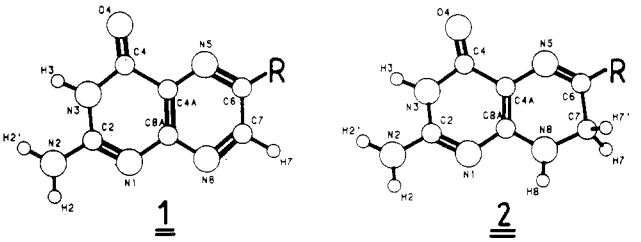
The intermediate one-step reduction product of reaction 1 has not been observed. However, ^1H NMR experiments on H_4folate produced enzymatically from folate with [4-^2H]-NADPH have shown that the deuterium labels are retained quantitatively at C6 and C7 and that the stereospecificity of the hydride ion transfer (4-*pro-R*) at C6 and C7 (C7-*si* face of the pteridine ring) is the same (Charlton et al., 1979). Enzymic H_2folate reduction has the same stereochemistry (Fontecilla-Camps et al., 1979; Charlton et al., 1979) as folate reduction, involving hydride ion transfer to C6 [see Gready (1980)]. The implications of these results are that there is no major difference in the orientation of the pteridine rings of folate and H_2folate when bound to DHFR in contrast to the ring orientation of DHFR-bound inhibitor methotrexate (Bolin et al., 1982) and also that in reaction 1 hydride ion transfer takes place initially at C7 ($\rightarrow H_2$folate) and then at C6 ($\rightarrow H_4$folate). The alternative to the latter assumption would require an enzyme-bound 5,6-dihydrofolate intermediate as the 5,6-dihydro tautomer would rapidly rearrange in solution to the more stable 7,8-dihydro form (Gready, 1984a).

Proposed mechanisms for these enzymic reactions have focussed on activation of the pterin ring of the folate and H_2folate substrates rather than activation of NADPH, although the latter is now receiving some attention (Filman et al., 1982). The mechanism generally proposed in the literature is the proton–hydride or preprotonation mechanism (Huennekens & Scrimgeour, 1964). This mechanism, which is in accord with usual chemical arguments for promotion of such reactions, involves preprotonation of the nitrogen of the (formal) C=N double bond followed by nucleophilic attack by hydride ion on the adjacent carbon atom, that is, protonation of N8 preceding C7-reduction and protonation of N5 preceding C6-reduction.

However, definitive experimental support for this mechanism is still lacking while a number of experimental results suggest alternative mechanisms to consider. Model building of DHFR–substrate complexes based on the X-ray crystal structures of inhibitor complexes (Bolin et al., 1982) and a DHFR–biopterin–NADP$^+$ complex [referred to in Filman et al. (1982) and Freisheim & Matthews (1984)] indicated that most of the strong interactions with the pteridine moiety are with the pyrimidine ring while the pyrazine ring is relatively accessible to solvent: particular interest is focussed on the carboxylate side chain of a conserved acidic residue [aspartate in bacterial and glutamate in vertebrate DHFRs (Lai et al., 1982)], which is close to the O4–N3–N2 region of the pyrimidine ring. Further evidence for strong interactions with the pyrimidine ring influencing enzyme and ligand pK_as comes from ^{13}C NMR experiments on enzyme–substrate (Birdsall et al., 1982) and inhibitor (Cocco et al., 1982) ternary complexes while kinetic experiments suggested that a group with a pK value of ~6.6 must be protonated for both inhibitor binding and enzyme activity (Williams & Morrison, 1981).

However, numerous NMR, UV spectral difference, and calorimetric experiments have failed to show that either folate or H_2folate is *measurably* bound to the enzyme in a protonated form in either binary or dead-end ternary complexes (Gready, 1980). One further problem for the preprotonation mechanism is that the solution pK_as for pteridine ring protonations in folate (2.35) and H_2folate (3.84) are low (Poe, 1977). Also, N1 not N8 is the preferred pteridine-ring protonation site in oxidized pterins and folate although experiments with multiply methylated pterin derivatives indicate N8-protonation is energetically accessible (Rokos & Pfleiderer, 1971).

Chart I



Thus, the experimental results have suggested that enzyme interactions with the pyrimidine ring may play the key role in substrate binding and catalysis. It is also possible that because of the requirement for both folate and H_2folate reduction, a nonobvious pterin-ring activation mechanism may have arisen (Gready, 1980).

In the present paper we have addressed these possibilities by considering properties of the heterobicyclic pterin ring that allow transmission of perturbations from one ring to the other via the coupled π-electron system. Specifically, we have studied two types of mechanism involving protonation of folate and H_2folate in either the pyrimidine or pyrazine rings and assessed their ability to facilitate hydride ion transfer to the pyrazine ring by two main criteria—relative protonation energies and electron depletion at C6 or C7 and total pyrazine ring charges. In addition, we have considered qualitatively features of the X-ray structure description of the enzyme active site that could stabilize the various protonated forms, i.e., increase their effective pK_as, and the possible identity of the proton donor. As most of the protonated species are nonpreferred in solution, we have exploited the chief advantage of theoretical methods for this problem, namely, that they allow study of molecular properties that are inaccessible or not readily accessible by experiment. These methods comprise ab initio quantum-mechanical calculations at the SCF/3-21G (Binkley et al., 1980) and SCF/STO-3G (Hehre et al., 1969) levels with fully optimized STO-3G basis-set geometries.

Computational Methods

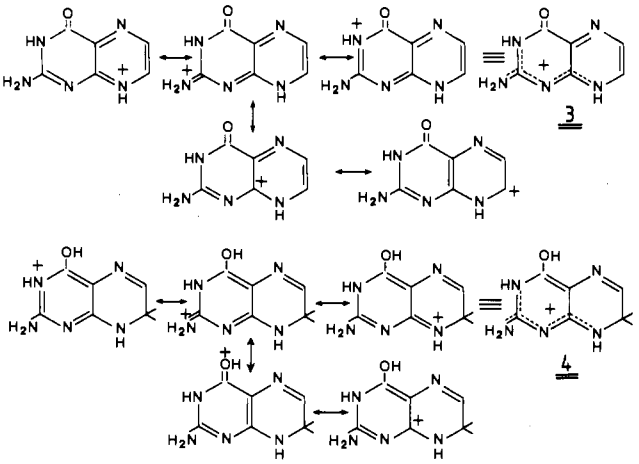
Calculations for both neutral and protonated species were performed on pteridine ring fragments of the two substrates folate (**1**) and H_2folate (**2**) (Chart I). The natural folate substrates are 6-substituted pterins where R ∝ [*p*-(methyleneamino)benzoyl]-L-glutamate. Full geometry optimizations with the STO-3G basis set for R ∝ H species were completed by using two ab initio SCF gradient programs—TEXAS (Pulay, 1979) and GAUSSIAN-80 (Singh & Kollman, 1982), implemented for Cyber-730 and VAX 11/780 machines, respectively: the geometries are reported elsewhere (Gready, 1984a, 1985). A newly developed procedure (Gready, 1984b) for improving the starting guess geometry was used in obtaining the charged-molecule geometries. Single-point SCF/STO-3G and 3-21G calculations were done with a Cyber version of GAUSSIAN-76 (Pople, 1978). Because of the large size of these molecules and, hence, the inherent high cost of the calculatons, the effect of the 6-R substituent was checked by single-point SCF/STO-3G calculations on 6-methyl and 6-(methylamino) derivatives (Gready, 1985). 6-R STO-3G-optimized geometries with methyl group geometric parameters taken from STO-3G-optimized 6-methylpteridine (unpublished results) or with standard methylamino group parameters (Pople, 1977) were used. All the reported molecules contain a planar heavy-atom pteridine ring with in-plane 2-NH_2 and 4-OH pyrimidine ring substituents: the C4–O4, C2–N2, and C8a–N8 (for trivalent N8) bond lengths are shorter than single

Table I: Calculated Protonation Energies and pK_as for Pterin and 7,8-Dihydropterin

		ΔE (kcal/mol)			pK_a (6-CH_3)	
	site	6-H[a]	6-CH_3[b]	6-CH_2NH_2[b]	calcd[c]	exptl
pterin	N1	−243	−245	−245	2.5	2.31[d]
	N5	−232	−238		1.4	
	N8	−242	−244		2.3	(1.69)[e]
	O4	−226	−228		>0	
7,8-dihydro-	N1	−236	−237		1.2	(1.27)[f]
pterin	N5	−250	−257	−254	4.2	4.17[g]
	O4	−251	−253		3.7	

[a] SCF/3-21G results with STO-3G-optimized geometries for neutral and protonated molecules. [b] 6-H 3-21G results corrected for 6-substitution by calculations at the SCF/STO-3G level. [c] Obtained from a linear correlation graph relating calculated protonated energies to experimental pK_as compiled from these and other theoretical pteridine results (Gready, 1985). [d] Braun & Pfleiderer (1973). [e] Estimate from 3,N^2,N^2-trimethylpterin results (Rokos & Pfleiderer, 1971). [f] Estimate from sepiapterin results (Pfleiderer, 1979). [g] Pfleiderer & Zondler (1966).

Scheme I



bonds with considerable effective π-bond orders (Gready, 1984b).

Results

3-21G energy and effective solution pK_a results for protonation of N8 and O4 in pterin and N5 and O4 in 7,8-dihydropterin are given in Table I together with reference theoretical and experimental results for other protonation sites. These results indicate that N8-protonation of pterin is relatively favorable compared with the preferred N1-protonation, a finding supported by experimental evidence: in the sterically blocked compound 3,N^2,N^2-trimethylpterin and its 6,7-dimethyl derivatives the monocations are a mixture of N1- and N8-protonated forms (Rokos & Pfleidere, 1971). However, O4-protonation of pterin is very disfavored compared with protonation of other sites and has a very low calculated pK_a. For 7,8-dihydropterin the results indicate O4-protonation is relatively favorable compared with the preferred N5-protonation. Due to the usual substituent proximity effects, N5-protonations of both pterin and 7,8-dihydropterin are enhanced by the 6-substitutions relative to other protonation sites in the pyrazine and pyrimidine rings.

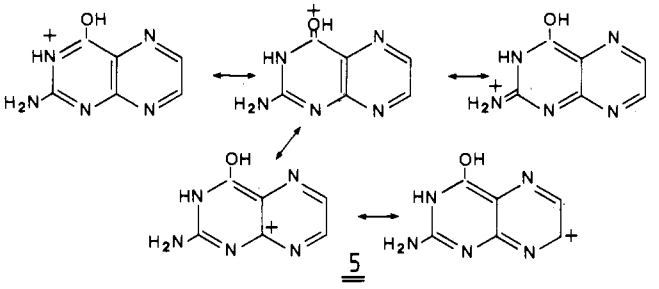
The relatively high stabilities of N^+8 pterin and O^+4 7,8-dihydropterin may be understood in terms of the substantial resonance contribution by the extended-guanidinium substructure involving the N3–C2(N2)–C1–C8a–N8 group of the pyrimidine and pyrazine rings (**3** and **4**) (Scheme I). The effectiveness of this resonance in stabilizing the N8-protonated form is even more pronounced if account is taken of the concomitant unfavorable disruption of the pyrazine ring resonance: the N8(H) tautomer of pterin is 22 kcal/mol higher in energy than the most stable N3(H) tautomer (Gready, 1985).

Table II: Electron Populations on Pyrazine Ring Carbon Atoms[a]

	total	π	Δtotal[b]	$\Delta\pi$[b]
for C7				
pterin	5.95	0.92		
N^+8 pterin	5.83	0.87	−0.12	−0.05
O^+4 pterin	5.91	0.84	−0.04	−0.08
for C6				
7,8-dihydropterin	5.92	0.98		
N^+5 7,8-dihydropterin	5.74	0.72	−0.17	−0.26
O^+4 7,8-dihydropterin	5.86	0.85	−0.05	−0.13

[a] 3-21G results for 6-H molecules. [b] Negative sign implies loss of electrons; i.e., nucleus is more positively charged.

Scheme II



The 3-21G electron population results for protonation of pterin and 7,8-dihydropterin are given in Table II; the effects of 6-substitution at the SCF/STO-3G level are minimal (unpublished results). π-Electron depletions at the relevant carbon atoms are of particular interest in the promotion of hydride ion attack as chemical arguments predict such attack should occur from the out-of-plane direction.

The results for pterin indicate that electron depletions at C7 due to protonation on N8 or O4 are roughly comparable but generally small. N8-Protonation is not an especially good means for effecting electron depletion at the adjacent carbon atom C7 because the π-system offers many possibilities for charge delocalization over both component rings (**3**) particularly within the extended-guanidinium resonance. The resonance contributors to charge delocalization in O4-protonated pterin, including a structure that places a formal positive charge on C7, are shown in **5** (Scheme II).

The results for 7,8-dihydropterin show that N5-protonation produces a large localized charge depletion at C6, particularly of π-electrons, which is consistent with the N5=C6 double bond being essentially isolated from the π-system (Gready, 1984a), and hence offering little opportunity for charge delocalization. However, the 7,8-dihydropterin results also show that O4-protonation causes a relatively large π-electron depletion at C6, an effect not predicted by formal resonance structures (**4**).

The overall effects of these protonations on the pteridine ring are shown in Figure 1 as changes in the total- and π-electron populations. Both N8- and O4-protonations of pterin cause electron depletions, especially of π-electrons, in the C2(N2)–N3 region of the pyrimidine ring as suggested by the resonance structures **3** and **5** (Figure 1a,b). Similarly, N5- and O4-protonations of 7,8-dihydropterin cause some electron depletion, especially of π-electrons, in the C2(N2)–N3 region (Figure 1c,d), although the magnitude of the effect is less than that for the protonated pterins. For O4-protonation, these π-electron depletions are predicted by the resonance structures in **4**, but for N5-protonation there are no formal resonance

Table III: Net Total Charge and π-Electron Populations of the Component Pyrimidine and Pyrazine Rings

	pyrimidine				pyrazine			
	total[a]	π[b]	Δtotal[c]	$\Delta\pi$[d]	total[a]	π[b]	Δtotal[c]	$\Delta\pi$[d]
pterin	+0.248	−0.038			−0.248	+0.038		
N^+8 pterin	+0.668	−0.306	0.420	−0.268	+0.332	+0.306	0.580	+0.268
O^+4 pterin	+0.998	+0.067	0.750	+0.105	+0.002	−0.067	0.250	−0.105
7,8-dihydropterin	+0.172	+0.083			−0.172	−0.111		
N^+5 7,8-dihydropterin	+0.460	+0.011	0.288	−0.072	+0.540	−0.020	0.712	+0.091
O^+4 7,8-dihydropterin	+0.893	+0.274	0.721	+0.191	+0.107	−0.281	0.279	−0.170

[a] Total net charge calculated as the sum of nuclear and electronic contributions. [b] Excess (+) or deficit (−) of π-electrons compared with the theoretical values of nine π-electrons for the pyrimidine ring and five for the pyrazine ring. Values for 7,8-dihydropterin are approximate only due to destruction of exact π-symmetry by the C7-H_2 group. [c] Proportions of the unit of positive charge arising from protonation that reside on the two rings. [d] Change in the ring π-electrons; decrease (−) and increase (+).

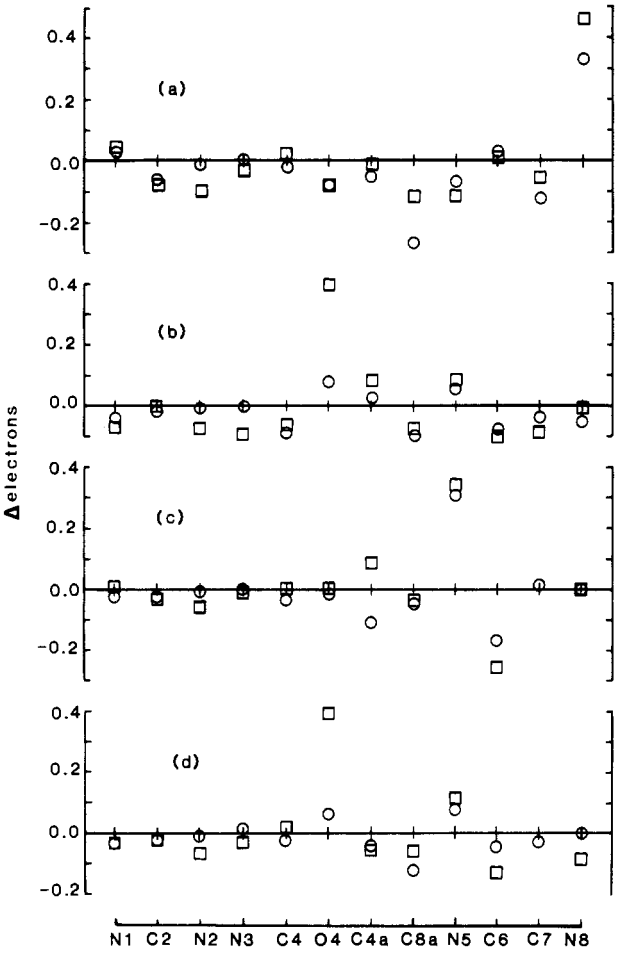


FIGURE 1: Changes in the total- (O) and π- (□) electron populations of the pteridine ring heavy atoms following protonation: (a) N^+8 pterin; (b) O^+4 pterin; (c) N^+5 7,8-dihydropterin; (d) O^+4 7,8-dihydropterin.

structures predicting charge redistribution to the pyrimidine ring.

The extent of redistribution of the positive charge over both rings may be gauged by calculating the net charges and numbers of π-electrons in the pyrimidine and pyrazine rings; these results are shown in Table III. The contributions of the bridging atoms C4a and C8a have been divided equally between the two rings. In the neutral species, the pyrimidine ring is seen to bear a net positive charge and have a slight deficit of π-electrons in pterin but an excess in 7,8-dihydropterin: charge balance ensures the converse for the pyrazine rings. Thus, the pyrazine ring is electron rich and therefore intrinsically relatively resistant to attack by the negatively charged hydride ion. Protonation of pterin on N8 causes a flow of ~0.27 π-electrons from the pyrimidine to the pyrazine ring with the *additional* positive charge being distributed 40:60

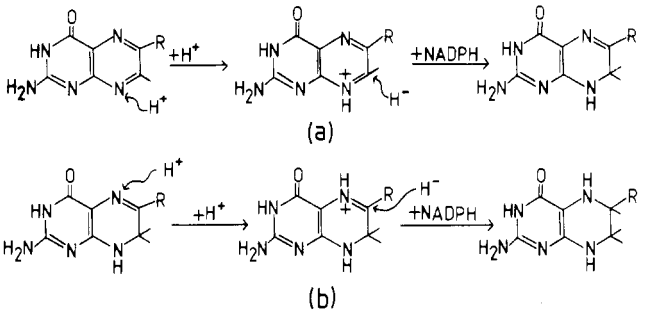


FIGURE 2: Mechanism I. N8- or N5-protonation preceding hydride ion transfer: (a) folate → H_2folate; (b) H_2folate → H_4folate.

between the pyrimidine and pyrazine rings: the *net* positive charge on the pyrazine ring is only 0.33. N5-Protonation of 7,8-dihydropterin on the other hand causes only a small flow of π-electrons to the pyrazine ring with a distribution ratio of 30:70 for the additional positive charge: however, the net positive charge on the pyrazine ring is still only 0.54. O4-Protonations of pterin and 7,8-dihydropterin cause ~0.1 and 0.2 π-electron flows to the pyrimidine ring with ~75% of the extra charge residing on that ring: the net positive charges on the pyrazine rings are very small—~0 and 0.1, respectively.

In general summary of Tables II and III, although the results for π-electron depletions at specific pyrazine ring carbon atoms due to pyrimidine ring (O4) protonations are of the same magnitude as those for the pyrazine ring (N8 and N5) protonations, the *net* positive charges on the pyrazine rings are considerably less.

Discussion

The implications of the theoretical results for the enzymic mechanism are discussed in the context of the experimental information summarized in the introduction and according to two main criteria. The first, the likelihood of the proposed intermediate protonated species, is assessed in terms of the predicted relative and absolute solution pK_as, the possibility that these pK_as could be effectively increased by stabilization of the protonated forms in the environment of the enzyme active site, and the possible source of the proton. The second criterion, the extent to which such protonations could be predicted to promote hydride ion transfer, is assessed in terms of the electron populations at the relevant carbon atoms and the net pyrazine ring charges. Both the proposed mechanisms are considered with the recognition that the proportion of the enzyme ternary complex in the active protonated form might be small: for example, if the pK_a for a given protonation of the enzyme-bound pteridine ring were 5.0, then only ca. 1% of the substrate would be bound in the protonated form at neutral pH.

We consider first the traditional mechanism depicted in Figure 2, i.e., N8-protonation of folate and N5-protonation

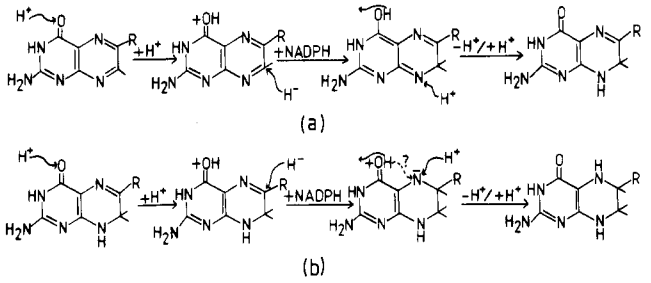


FIGURE 3: Mechanism II. O4-Protonation preceding hydride ion transfer: (a) folate → H_2folate; (b) H_2folate → H_4folate.

of H_2folate. With the allowance suggested above, the theoretical results predict that the effective solution pK_as of N8 and N5 would need to be increased by at least 3 and 1 unit, respectively, in the enzyme-bound substrates. Stabilizations of this magnitude might be achieved by an interaction of an ionized carboxylate side chain [i.e., the conserved Asp or Glu (Lai et al., 1982)] with the left-hand side of the pyrimidine ring: we recall from Table III that a large proportion of the positive charge in the protonated species resides on the pyrimidine ring. It is possible that the N8-protonated form of folate could be stabilized additionally by H-bonding with enzyme groups located near N8 (Filman et al., 1982; Freisheim & Matthews, 1984). Regarding the source of the proton, it has been suggested that the active site aspartate residue in *Lactobacillus casei* DHFR could have an elevated pK_a (Birdsall et al., 1982) and might deliver a proton to N5 in H_2folate mediated by apparently conserved water molecules (Freisheim & Matthews, 1984). An alternative mechanism consistent with the X-ray structure data is that the proton is abstracted from solvent and conveyed to N5 in H_2folate via a network of bound water molecules (Filman et al., 1982). For N8-protonation of folate, however, the X-ray structure picture provides no indication of a direct path for proton transfer from either enzyme side chains or bound water molecules (Freisheim & Matthews, 1984). The electron population results for the N8- and N5-protonated forms provide positive indications for enhanced hydride ion transfer to the pyrazine rings in terms of both localised carbonium ion character at C7 or C6 and also greatly increased electron deficiency of the pyrazine ring as a whole.

The second mechanism we consider is shown in Figure 3: this involves preprotonation of both folate and H_2folate on O4. This mechanism was studied for two reasons. First, as suggested previously (Gready, 1980), the requirement for catalysis of both folate and H_2folate reduction may have necessitated an indirect pteridine-activation mechanism as direct enzyme-assisted protonation of both N8 in folate and N5 in H_2folate poses active site design problems: in invoking the same preprotonation procedure for both folate and H_2folate reduction this difficulty is overcome. Second, a dual role for the conserved acidic residue in the pyrimidine ring binding site as both proton donor and stabilizer of the protonated pteridine is found, assuming, of course, that this residue is indeed protonated in the active enzyme complexes (Birdsall et al., 1982).

The theoretical results indicate that the O4-protonated folate is relatively unstable that a predicted pK_a < 0: while this may seem damning, it should be remembered that the pK_a of DHFR-bound inhibitor methotrexate is increased by more than 5 units compared with the solution value (from ~5.5 to >10.5) (Cocco et al., 1981). However, the predicted solution pK_a for O4-protonation of H_2folate is comparable with that for N5. As for mechanism I one could suppose direct or water-mediated transfer of the proton from O4 to N5 following hydride ion transfer to H_2folate. But for folate reduction, mechanism II merely postpones the problem of the ultimate source of the proton for N8.

Compared with the carbon electron population and pyrazine ring charge results for N8- and N5-protonations, the O4-protonation results, in general, suggest a poorer mechanism for facilitating the hydride ion transfer although the $\Delta\pi$-electron results considered alone indicate substantial local π-electron depletion.

The order of relative pK_as and the population results for both mechanisms I and II are consistent with the observed relative rates of enzymic reduction of folate and H_2folate and the lower pH maximum for folate reduction. As noted in the introduction, one of the aims of the present work was to assess the degree to which perturbations to the bicyclic system resulting from protonation might be transmitted from one ring to the other, primarily by π-electron coupling effects, and the implications of such effects for the enzymic mechanism. Given the dimensions of the pteridine ring, medium-range effects of 4–6 Å seemed possible. Previous work (Gready, 1984a) on varying substituent, tautomer, reduction, and protonation patterns on pteridine ring geometry had demonstrated that such variation could not be understood in terms of localized changes but involved closely coupled changes over both component rings. In mechanism I, positive charge delocalization from the pyrazine to pyrimidine ring is suggested to offer a means for stabilization of the bound protonated forms, hence aiding catalysis. In mechanism II, on the other hand, the positive charge delocalization from the pyrimidine to pyrazine ring is suggested as a way of causing specific electron depletions at C7 or C6, hence also aiding catalysis.

CONCLUSIONS

Of the two pterin activation mechanisms considered in this work the theoretical results generally favor the standard N8/N5-protonation mechanism (Huennekens & Scrimgeour, 1964). In this case it is suggested that the primary function of the conserved acidic residue located in the pyrimidine ring binding region of the DHFR active site (Bolin et al., 1982) is to stabilize the protonated forms of the substrates by charge–partial charge interactions, and thereby increase the effective pK_as. However, some positive support for the alternative novel mechanism involving O4-protonation of both substrates was also found.

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Registry No. **3**, 51584-41-9; **4**, 93675-19-5; folic acid, 59-30-3; dihydrofolic acid, 4033-27-6; dihydrofolate reductase, 9002-03-3; pterin, 2236-60-4; 7,8-dihydropterin, 17838-80-1.

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Differential Modulation by Spermidine of Reactions Catalyzed by Type 1 Prokaryotic and Eukaryotic Topoisomerases†

Kalkunte S. Srivenugopal and David R. Morris*
Department of Biochemistry, University of Washington, Seattle, Washington 98195
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ABSTRACT: In the absence of DNA aggregation, spermidine inhibited the relaxation of negatively supercoiled DNA by *Escherichia coli* topoisomerase I at concentrations of the polyamine normally found intracellularly. Spermidine also curtailed the cleavage of negatively supercoiled $ColE_1$ DNA by the enzyme in the absence of Mg^{2+}. On the contrary, knotting of M13 single-stranded DNA circles catalyzed by topoisomerase I was stimulated by the polyamine. Relaxation of supercoiled DNA by eukaryotic type 1 topoisomerases, such as calf thymus topoisomerase I and wheat germ topoisomerase, was significantly stimulated by spermidine in the same range of concentrations that inhibited the prokaryotic enzyme. In reactions catalyzed by S_1 nuclease, the polyamine enhanced the digestion of single-stranded DNA and inhibited the nicking of negatively supercoiled DNA. These results suggest that spermidine modifies the supercoiled duplex substrate in these reactions by modulating the degree of single strandedness.

The natural trivalent cation spermidine is a universal component of all living cells and is present at millimolar intracellular concentrations (Cohen, 1971; Tabor & Tabor, 1976; Morris & Marton, 1981). Numerous studies involving microbial mutants and enzyme inhibitors have suggested that all cells must possess appropriate levels of polyamines, such as spermidine, for optimal growth and differentiation [reviewed in Morris (1981), Pegg & McCann (1982), Heby & Jänne (1981), and Cohn et al. (1978)]. In vitro, polyamines affect a myriad of biochemical processes, including stabilization and renaturation of DNA (Liquori et al., 1967; Christiansen & Baldwin, 1977) and the synthesis of all major macromolecules in the cell, but their sites of action in vivo have not been specifically defined. Spermidine is regularly included as a component of several in vitro reactions of nucleic acid metabolism, because of its positive influence on reactions catalyzed by enzymes like DNA and RNA polymerases, DNA ligase, DNA gyrase, and others (Fisher & Korn, 1979; Gumport, 1970; Gellert et al., 1976).

Topoisomerases, which are ubiquitous both in prokaryotes and in eukaryotes, alter the topological conformation of the

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