Structure−Activity Relationships for the 8-Alkylpterins: A New Class of Mechanism-Based Substrates for Dihydrofolate Reductase (DHFR)†

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ABSTRACT: The substrate activity with both human and chicken dihydrofolate reductases (DHFR) has been examined for a series of 8-alkylpterins, 6-methyl-8-alkylpterins, and 7-methyl-8-propylpterin. All the 8-alkylpterins exhibited substrate activity with $V_{max}/[E]_o$ values ranging from 1.0 to 5.4 and 2.6 to 14.8 s^{-1} for chicken and human DHFRs, respectively, with activity varying in the order 8-methyl > 8-allyl > 8-isopropyl ≥ 8-ethyl ≥ 8-propyl for both enzymes. K_m values were found to range from 6.2 to 47 and 14 to 261 μM for chicken and human DHFRs, respectively, with the strength of binding varying in the order 8-propyl > 8-isopropyl > 8-allyl > 8-methyl > 8-ethyl for both enzymes. Addition of a 6-methyl substituent affected the activity of the 8-alkylpterins significantly. While 6,8-dimethylpterin was a much better substrate than 8-methylpterin, the 6-methyl-8-propyl, 6-methyl-8-allyl, and 6-methyl-8-isopropyl compounds showed no substrate activity and 6-methyl-8-ethylpterin showed very weak activity with chicken enzyme only. 7-Methyl-8-propylpterin showed no substrate activity. Thermodynamic dissociation constants (K_d) for the compounds in binary complex with both human and chicken DHFRs ranged from 23 to 351 and 15 to 127 μM, respectively. Trends for the K_ds were consistent with the kinetic data in suggesting stronger binding for 8-alkylpterins with larger 8-substituents. Comparison of K_d values with corresponding K_m values suggested both strong cooperativity (6,8-dimethylpterin) and antagonism (6-methyl-8-isopropylpterin) with NADPH in binding to DHFR. K_d values of 20 and 10 μM for the ternary complexes of 7-methyl-8-propylpterin with human and chicken enzyme, respectively, suggest modest inhibitory activity. Application of molecular graphics modeling of ligands in the DHFR binding site has provided insight in interpreting the structure−activity relationships. The finding that different binding orientations are possible for ligands with small (8-methyl) or larger (e.g., 8-propyl) 8-substituents helps to explain the 6-methyl substituent effect and the transition from weak binding and high activity to tight binding and low activity as a function of ring-substituent pattern.

Dihydrofolate reductase (DHFR)[1] is an ubiquitous enzyme present in all dividing cells which catalyzes the NADPH-mediated reduction of 7,8-dihydrofolate to 5,6,7,8-tetrahydrofolate. DHFR from some sources also catalyzes reduction of folate to 5,6,7,8-tetrahydrofolate using 2 equiv of NADPH (Blakley, 1984). Extensive studies of the chicken, human, and bacterial enzymes including structural (e.g., Oefner et al., 1988; Davies et al., 1990; Bystroff et al., 1990; McTigue et al., 1992) and kinetic (e.g., Fierke et al., 1987; Howell et al., 1987; Beard et al., 1989; Thillet et al., 1990; Appleman et al., 1990) investigations have been reported and are also summarized in a number of reviews (Blakley, 1984; Freisheim & Matthews, 1984; Kraut & Matthews, 1987; Morrison, 1991). Interest in the physical−chemical properties of the enzyme has been further heightened by its importance as a target for a number of potent inhibitors in clinical use as antibacterial and antimalarial (Hitchings & Baccanari, 1984) and anticancer (Tattersall, 1984) drugs.

The 8-alkylpterins (Scheme 1) are a new class of mechanism-based substrates of DHFR currently under study within our laboratory which have been designed (Gready, 1990; Gready et al., 1993) as analogues of the natural substrate folate, but with increased basicity so as to readily form an N^3-protonated cation at physiological pH. This cation is expected to mimic the N^8-protonated cation of folate which has been proposed (Huennekens & Scrimgeour, 1964; Gready, 1985) to be the enzymically active form of this substrate. The substrate activity of the 8-alkylpterins was established (Thibault et al., 1989) when 8-methylpterin (**8**) was shown to undergo a rapid enzyme-catalyzed reaction with NADPH. Full reaction-course studies suggested that the reaction involved an initial relatively rapid reduction of 8-methylpterin to 8-methyl-7,8-dihydropterin which was followed by a slower and incomplete reduction of 8-methyl-7,8-dihydropterin to 8-methyl-5,6,7,8-tetrahydropterin, and this mechanism has subsequently been confirmed by UV/vis spectral difference and HPLC techniques (Gready, 1990; Wong & Gready, 1992, and unpublished results). The magnitude of the activity was found to be pH dependent with optimum activity at approximately pH 5.8−6.0 for chicken, bovine, and human DHFRs. The K_m value was found to vary substantially with enzyme source and pH. At the optimum pH, specific activities for the reduction of 8-methylpterin (**8**) were reported to be 130% (chicken), 65% (bovine), and 180% (human) those for dihydrofolate reduction, although

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[1] Abbreviations: DHFR, dihydrofolate reductase; K_m, Michaelis constant; V_{max}, initial reaction velocity at saturating substrate concentration; $[E]_o$, total active enzyme concentration; K_d, thermodynamic dissociation constant; NADPH, nicotinamide adenine dinucleotide phosphate (reduced form); SCX, strong cation exchange; WCX, weak cation exchange; RP, reverse phase.

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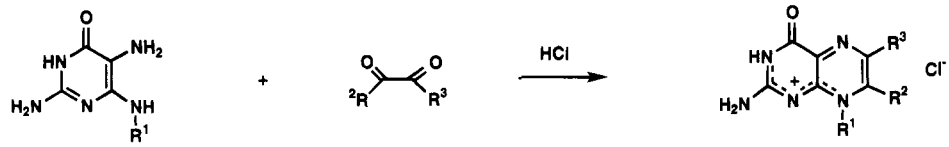
Scheme 1



	R^1
1	$-CH_3$
2	$-CH_2CH_3$
3	$-CH_2CH_2CH_3$
4	$-CH(CH_3)_2$
5	$-CH_2-CH=CH_2$

	R^1	R^2
6	-H	-H
7	$-CH_3$	-H

	R^1	R^2	R^3
8	$-CH_3$	H	H
9	$-CH_2CH_3$	H	H
10	$-CH_2CH_2CH_3$	H	H
11	$-CH(CH_3)_2$	H	H
12	$-CH_2-CH=CH_2$	H	H
13	$-CH_3$	H	$-CH_3$
14	$-CH_2CH_3$	H	$-CH_3$
15	$-CH_2CH_2CH_3$	H	$-CH_3$
16	$-CH(CH_3)_2$	H	$-CH_3$
17	$-CH_2-CH=CH_2$	H	$-CH_3$
18	$-CH_2CH_2CH_3$	$-CH_3$	H

it should be noted that product release is the rate-determining step for dihydrofolate reduction except at high pHs (Fierke et al., 1987; Beard et al., 1989).

A second preliminary report (Gready, 1990) on the substrate activity of the 8-alkylpterins investigated the pH dependence for the K_m and V_{max} values for a small series [8-methylpterin (**8**), 8-ethylpterin (**9**), 6,8-dimethylpterin (**13**), and 8-isopropylpterin (**11**)] of compounds and confirmed the earlier findings and established a dependence also on substituent pattern. The K_m values at pH 5.8 varied in the order 6,8-dimethylpterin (**13**) < 8-isopropylpterin (**11**) < 8-methylpterin (**8**) < 8-ethylpterin (**9**) with all values being lower with chicken DHFR compared with human DHFR. Estimates of the $V_{max}/[E]_o$ values made using theoretical reaction extinction coefficients indicated reactivity at pH 5.8 in the order 8-methylpterin (**8**) > 6,8-dimethylpterin (**13**) > 8-isopropylpterin (**4**) > 8-ethylpterin (**9**).

In this study we have extended investigations of the substrate activity of the 8-alkylpterins by consideration of a series of 11 compounds, as shown in Scheme 1, including comparison of kinetic (K_m and $V_{max}/[E]_o$) and thermodynamic (K_d) parameters. Also, molecular modeling of the 8-alkylpterins in the active site of DHFR has been undertaken in an attempt to rationalize trends in the kinetic and thermodynamic data.

MATERIALS AND METHODS

Instruments and Reagents. Perkin-Elmer LS 50 and Shimadzu UV 160 spectrometers were used for fluorimetric binding and UV/vis spectral kinetic studies, respectively. The UV/vis and NMR spectral data were recorded using Cary 3 and Bruker AS200 spectrometers. All ^1H NMR spectra were recorded in D_2O with spectra referenced to water at δ 4.76. pK_a values were determined by spectrophotometric analysis (Albert & Serjeant, 1984). Kinetic assays were performed in MES/TRIZMA/NaCl/ethanolamine constant ionic strength pH buffer (Ellis & Morrison, 1982) with I = 0.15 M, and thermodynamic dissociation, K_d, determinations were performed in the same buffer but with I = 0.20 M. Recombinant human DHFR was a gift from Prof. J. H. Freisheim, chicken liver DHFR was from Sigma, and NADPH and $NADP^+$ were from Boehringer. Enzyme concentrations were determined by methotrexate titration (Williams et al., 1979). Pyruvic aldehyde (40% water solution) and glyoxal trimeric dihydrate were from Aldrich and Fluka, respectively. Pyrimidine starting materials for 8-alkylpterin synthesis were prepared as previously outlined (Ivery & Gready, 1992). Reverse-phase silica was prepared as reported (Kuhler & Lindsten, 1983) or purchased from Aldrich. Amberlite CG-50 WCX resin was from ICN Chemicals. The purity of the 8-alkylpterins was determined using SCX HPLC as described previously (Wong & Gready, 1992; Ivery & Gready, 1992).

Kinetic Studies. Initial rate enzyme assays for all 8-alkylpterins were performed at pH 5.8 and 30 °C with NADPH at a saturating concentration of 60 μM and the 8-alkylpterin as the variable substrate in a total reaction volume of 1 mL (Thibault et al., 1989). Reactions were started by the addition of 6 μL of NADPH and then enzyme (2−4 μL) to prewarmed cuvettes containing buffer, substrate, and water, and the initial rate of reaction was recorded by following the decrease in the absorbance at 410 nm, which is due solely to the reduction of the 8-alkylpterin. Correction for nonenzymic reduction of the pterin was made by use of a blank cell containing all other components except enzyme based on the earlier studies for 8-methylpterin (Thibault et al., 1989). For 8-alkylpterins and 6,8-dimethylpterin, enzyme concentrations of 3.9−7.8 nM were used. For 6-methyl-8-propyl-, 6-methyl-8-isopropyl, and 6-methyl-8-allylpterin, enzyme concentrations up to 207 nM enzyme for both chicken and human DHFRs were used, and no significant reduction in absorbance at 410 nm was observed. For 6-methyl-8-ethylpterin, enzyme concentrations up to 207 nM were used and no rate was observed with human enzyme, but a small but measurable rate was observed with chicken DHFR at 207 nM. The initial rate data were fitted to standard equations for Michaelis−Menten enzyme kinetics using the GraFit (Leatherbarrow, 1992) nonlinear regression software to obtain V_{max} and K_m values. V_{max} values were converted to absolute turnover values per second ($V_{max}/[E]_o$) using the extinction coefficient at 410 nm for the particular 8-alkylpterin substrate at the reaction pH (5.8) and the active enzyme concentration. The pH dependence of the reduction of 8-propylpterin was examined using buffers from pH 5.2 to 7.5.

Thermodynamic Dissociation Constants (K_d). K_d values were determined by a fluorimetric titration procedure as outlined previously (Birdsall et al., 1983; Ivery & Gready, 1995) by following the quenching of the enzyme fluorescence (excitation, 280 nm; emission, 320 nm) on addition of ligand in a total volume of 300 μL with enzyme concentrations in the range 0.12−0.50 μM. Data were analyzed by the procedures of Birdsall et al. (1983) using the nonlinear

regression program GraFit (Leatherbarrow, 1992). For determinations in the presence of cofactor, molar ratios of 10:1 for NADPH/DHFR were used with enzyme concentrations also in the range 0.12−0.5 μM.

Molecular Modeling. X-ray coordinates for chicken DHFR·biopterin·$NADP^+$ ternary complex (1DR1) from the Brookhaven Protein Data Bank were displayed using InsightII from Biosym Technologies (Version 2.3.0, 1993) on an SGI Personal Iris 4D/35GT.

Preparation of 8-Alkylpterins

The 8-alkylpterins were prepared using a modification of the procedure of Brown and Jacobsen (1961) and Pfleiderer et al. (1968, 1971).

8-Methylpterin Hydrochloride (8). To a solution of 0.20 g (1.29 mmol) of pyrimidine (**1**) in 5 mL of methanol with 5 drops of 11 M methanolic HCl was added 0.10 g (0.50 mmol) of glyoxal trimeric dihydrate (**6**), and the mixture was refluxed for 30 min. After standing in the freezer overnight, the mixture was filtered to obtain 0.19 g (55%) of olive green solid, which was recrystallized from methanol containing 2 drops 11 M methanolic HCl to give 0.038 g of greenish-yellow solid. This solid was purified using RP silica column chromatography eluting with 0.01 M HCl to give 0.028 g (8%, purity by SCX HPLC > 99.5%) of 8-methylpterin hydrochloride (**8**) after freeze-drying as a yellow solid. pK_a 5.35 ± 0.06. λ_{max} (0.01 M HCl)/nm 389 (log ϵ 4.01), 276 sh (4.07), 262 (4.16) [pK_a 5.32 ± 0.07, λ_{max} 386 (4.01), 276 (4.06), 260 (4.15) (Pfleiderer et al., 1968)]; (pH 8 phosphate buffer) 400 (3.97), 328 sh (3.48), 266 (4.28) [λ_{max} 400 (4.00), 330 sh (3.56), 265 (4.30) (Pfleiderer et al., 1968)]. ^{1}H NMR (0.01 M DCl/D_2O) δ 8.66 (d, 1H, 6-*H* or 7-*H*), δ 8.55 (d, 1H, 6-*H* or 7-*H*), δ 4.12 (s, 3H, 8-CH_3). Anal. Calcd: $C_7H_8N_5OCl$ 0.07 HCl (216.18) requires: C, 38.89; H, 3.76; N, 32.40; Cl, 17.55. Found: C, 39.12; H, 3.63; N, 32.12; Cl, 17.48.

8-Ethylpterin Hydrochloride (9). A solution of 0.25 g (1.23 mmol) of pyrimidine (**2**) with 0.12 g (0.57 mmol) of **6** was treated as for **8**; 0.209 g (75%) of green solid was recovered and recrystallized as for **8** to give 0.092 g of olive-green solid. 0.04 g was purified as for **8** to give 0.027 g (10%, purity by SCX HPLC >99.5%) of 8-ethylpterin hydrochloride (**9**) as a bright yellow solid. pK_a 5.43 ± 0.06. λ_{max} (0.01 M HCl)/nm 389 (log ϵ 4.04), 274 sh (4.08), 260 (4.16); (pH 8 phosphate buffer) 402 (4.00), 329 sh (3.49), 265 (4.29). ^{1}H NMR (0.01 M DCl/D_2O) δ 8.70 (d, 1H, 6-*H* or 7-*H*), δ 8.57 (d, 1H, 6-*H* or 7-*H*), δ 4.64 (qrt, 2H, N-CH_2), δ 1.53 (t, 3H, N-CH_2CH_3). Anal. Calcd: $C_8H_{10}N_5OCl$ 0.05 HCl (233.08) requires: C, 41.23; H, 4.52; N, 30.05; Cl, 15.97. Found: C, 41.20; H, 4.56; N, 29.92; Cl, 15.81.

8-Propylpterin Hydrochloride (10). A solution of 0.21 g (0.95 mmol) of pyrimidine (**3**) with 0.20 g (0.95 mmol) of **6** was treated as for **8**. After standing in the freezer for 2 h, the mixture was filtered to give a first crop of 0.094 g of green solid and a second crop of 0.041 g after further standing (total yield 0.135 g, 59%). The two crops were recrystallized separately from methanol and ethanol, both with methanolic HCl, to yield 0.012 (purity by SCX HPLC 99.3%) and 0.015 g (purity by SCX HPLC 99.3%), respectively. The two solids were combined and purified as for **8** to obtain 0.022 g (10%, purity by SCX HPLC >99.5%) of 8-propylpterin hydrochloride (**10**) as a yellow solid. pK_a 5.42 ± 0.02. λ_{max} (0.01 M HCl)/nm 390 (log ϵ 4.02), 276 sh (4.06), 261 (4.14); (pH 8 phosphate buffer) 403 (3.99), 330 sh (3.49), 265 (4.27). ^{1}H NMR (0.01 M DCl/D_2O) δ 8.70 (d, 1H, 6-*H* or 7-*H*), δ 8.58 (d, 1H, 6-*H* or 7-*H*), δ 4.56 (t, 2H, N-CH_2), δ 1.95 (m, 2H, N-CH_2-CH_2), δ 0.96 (t, 3H, N-CH_2-CH_2-CH_3). Anal. Calcd: $C_9H_{12}N_5OCl$ 0.1 H_2O 0.05 HCl (245.31) requires: C, 44.07; H, 5.03; N, 28.55; Cl, 15.18. Found: C, 44.05; H, 4.97; N, 28.43; Cl, 15.03.

8-Isopropylpterin Hydrochloride (11). A solution of 0.69 g (3.14 mmol) of pyrimidine (**4**) with 0.60 g (2.86 mmol) of **6** was treated as for **8**; 0.309 g (41%) of greenish-yellow solid was recovered as for **8** and recrystallized from ethanol containing a few drops of 11 M methanolic HCl to give 0.184 g of greenish-yellow solid. This solid was further purified as for **8** to give 0.164 g (21%, purity by SCX HPLC 99.4%) of 8-isopropylpterin hydrochloride (**11**) as a bright yellow solid. pK_a 5.43 ± 0.02. λ_{max} (0.01 M HCl)/nm 390 (log ϵ 4.02), 277 sh (4.04), 261 (4.11) [pK_a 5.29 ± 0.02, λ_{max} 390 (4.03), 276 (4.06), 261 (4.18) (Pfleiderer et al., 1971)]; (pH 8 phosphate buffer) 403 (4.01), 330 sh (3.47), 265 (4.29) [402 (4.00), 320 (3.46), 265 (4.29) (Pfleiderer et al., 1971)]. ^{1}H NMR (0.01 M DCl/D_2O) δ 8.76 (d, 1H, 6-*H* or 7-*H*), δ 8.63 (d, 1H, 6-*H* or 7-*H*), δ 5.72 (m, 1H, N-C*H*), δ 1.56 (d, 6H, N-CH(CH_3)$_2$). Anal. Calcd: $C_9H_{12}N_5OCl$ 0.9 H_2O (257.90) requires: C, 41.92; H, 5.39; N, 27.16; Cl, 13.75. Found: C, 42.00; H, 5.52; N, 26.95; Cl, 14.01.

8-Allylpterin Hydrochloride (12). A solution of 0.63 g (3.48 mmol) of pyrimidine (**5**) with 0.35 g (1.66 mmol) of **6** was treated as for **8**. After standing in the freezer for 2 h, the mixture was filtered to obtain 0.40 g (47%) of green solid which was recrystallized as for **8** to give 0.068 g of mustard colored solid. This solid was purified as for **8** to give 0.062 g (8%, purity by SCX HPLC > 99.5%) of 8-allylpterin hydrochloride (**12**) as a yellow solid. pK_a 5.39 ± 0.04. λ_{max} (0.01 M HCl)/nm 391 (log ϵ 3.99), 280 sh (4.02), 261 (4.13); (pH 8 phosphate buffer) 405 (3.96), 325 (3.50), 266 (4.24). ^{1}H NMR (0.01 M DCl/D_2O) δ 8.69 (d, 1H, 6-*H* or 7-*H*), δ 8.59 (d, 1H, 6-*H* or 7-*H*), δ 6.07 (m, 1H, N-CH_2-C*H*), δ 5.46 (m, 2H, N-CH_2-CH=CH_2), δ 5.19 (dd, 2H, N-CH_2). Anal. Calcd: $C_9H_{10}N_5OCl$ 0.85 H_2O 0.065 HCl (257.67) requires: C, 42.00; H, 4.61; N, 27.21; Cl, 14.44. Found: C, 41.66; H, 4.21; N, 26.86; Cl, 14.37.

Preparation of 6-Methyl-8-alkylpterins and 7-Methyl-8-propylpterin.

The preparation of 6-methyl-8-propylpterin (**15**) and 7-methyl-8-propylpterin (**18**) has been described previously (Ivery & Gready, 1992). Similar procedures were used for the preparation of the remaining 6-methyl-8-alkylpterins.

6,8-Dimethylpterin Hydrochloride (13). A suspension of 0.36 g (2.30 mmol) of pyrimidine (**1**) in 20 mL of water was cooled in ice, and a solution of 0.45 mL (2.50 mmol) of pyruvic aldehyde (**7**) (40% water solution) and 0.54 g (5.2 mmol) of sodium bisulfite in 5 mL of water was added slowly. The pH of the solution was adjusted to 4 and the mixture stirred in ice for 1 h and then at room temperature for 17 h. The mixture was applied to a WCX column and eluted with a 0.01 M sodium bisulfite solution. A rapidly eluting yellow fraction was collected, and 3 drops of 11 M HCl was added. After standing in the refrigerator overnight, the solution was filtered to give 0.136 g of a greenish-yellow solid. The solid was dissolved in 0.1 M HCl and the solution stirred under vacuum (water jet pump) for 2 h to destroy bisulfite. After freeze-drying, 0.085 g (16%) of yellow solid

Table 1: Kinetic Parameters for 8-Alkylpterins at pH 5.8 and 30 °C with NADPH at Saturating Concentration (60 μM) for Both Human and Chicken DHFRs

	chicken DHFR			human DHFR		
compound	K_m (μM)	$V_{max}/[E]_o$ (s^{-1})	$(V_{max}/[E]_o)/K_m$ (μM^{-1} s^{-1})	K_m (μM)	$V_{max}/[E]_o$ (s^{-1})	$(V_{max}/[E]_o)/K_m$ (μM^{-1} s^{-1})
8 (8-methyl)	25 ± 3	5.4 ± 0.3	0.22 ± 0.04	212 ± 47	14.8 ± 0.5	0.07 ± 0.02
9 (8-ethyl)	47 ± 6	1.1 ± 0.1	0.022 ± 0.004	261 ± 46	2.6 ± 0.1	0.010 ± 0.002
10 (8-propyl)	6.2 ± 0.5	1.0 ± 0.1	0.16 ± 0.02	14 ± 1	2.9 ± 0.1	0.21 ± 0.02
11 (8-isopropyl)	7.8 ± 0.6	1.1 ± 0.1	0.14 ± 0.02	21 ± 1	3.7 ± 0.1	0.18 ± 0.02
12 (8-allyl)	14 ± 1	3.1 ± 0.1	0.23 ± 0.02	64 ± 6	12.1 ± 0.2	0.19 ± 0.02
13 (6,8-dimethyl)	3.7 ± 0.4	6.4 ± 0.1	0.58 ± 0.07	9.8 ± 0.6	17.3 ± 0.3	1.8 ± 0.2
14 (6-methyl-8-ethyl)	125 ± 18	0.13 ± 0.01	0.0011 ± 0.0002	*a*	*a*	*a*
15 (6-methyl-8-propyl)	*a*	*a*	*a*	*a*	*a*	*a*
16 (6-methyl-8-isopropyl)	*a*	*a*	*a*	*a*	*a*	*a*
17 (6-methyl-8-allyl)	*a*	*a*	*a*	*a*	*a*	*a*
18 (7-methyl-8-propyl)	*a*	*a*	*a*	*a*	*a*	*a*

[a] No activity.

was recovered which was purified using RP silica column chromatography as for **8** to give 0.08 g (15%, purity by SCX HPLC >99.8%) of 6,8-dimethylpterin hydrochloride (**13**). pK_a 5.60 ± 0.02. λ_{max} (0.01 M HCl)/nm 398 (log ϵ 4.01), 281 (4.11), 262 (4.11); (pH 8 phosphate buffer) 410 (3.98), 322 (3.43), 268 (4.32). ^{1}H NMR (0.01 M DCl/D_2O) δ 8.65 (s, 1H, 7-*H*), δ 4.11 (s, 3H, 8-C*H*$_3$), δ 2.62 (s, 3H, 6-C*H*$_3$). Anal. Calcd: $C_8H_{10}N_5OCl$ 0.1 H_2O 0.05 HCl (231.28) requires: C, 41.55; H, 4.47; N, 30.28; Cl, 16.10. Found: C, 41.43; H, 4.59; N, 30.12; Cl, 16.21.

*6-Methyl-8-ethylpterin Hydrochloride (**14**).* A suspension of 0.48 g (2.8 mmol) of pyrimidine (**2**) was treated with a solution of 0.5 mL (2.8 mmol) of **7** and 0.75 g (7.3 mmol) of sodium bisulfite and reacted as for **13**. The reaction mixture was filtered to give 0.25 g of green solid which was washed three times with 2 mL of ethanol. After dissolving the solid in 0.1 M HCl, the solution was stirred under vacuum for 4 h to remove bisulfite and then freeze dried to give 0.15 g (22%) of yellowish-green solid. The solid was purified as for **8** to give 0.136 g (20%, purity by SCX HPLC 99.3%) of 6-methyl-8-ethylpterin hydrochloride (**14**). pK_a 5.70 ± 0.06. λ_{max} (0.01 M HCl)/nm 399 (log ϵ 4.02), 281 (4.11), 263 (4.15); (pH 8 phosphate buffer) 411 (3.99), 323 (3.39), 268 (4.34). ^{1}H NMR (0.01 M DCl/D_2O) δ 8.70 (s, 1H, 7-*H*), δ 4.64 (qrt, 2H, N-C*H*$_2$), δ 2.64 (s, 3H, 6-C*H*$_3$), δ 1.51 (t, 3H, N-CH$_2$-C*H*$_3$). Anal. Calcd: $C_9H_{12}N_5OCl$ 0.6 H_2O 0.22 HCl (260.51) requires: C, 41.49; H, 5.19; N, 26.88; Cl, 16.60. Found: C, 41.21; H, 4.84; N, 26.64; Cl, 16.31.

*6-Methyl-8-isopropylpterin Hydrochloride (**16**).* A 10 mL suspension of 0.25 g (1.37 mmol) of pyrimidine (**4**) was treated with a 2 mL solution of 0.25 mL (1.39 mmol) of **7** and 0.40 g (3.9 mmol) of sodium bisulfite and reacted as for **13** except for 48 h. Product was recovered, washed and treated to remove bisulfite as for **14** and freeze dried to give a greenish-yellow solid. The solid was purified as for **8** to give 0.083 g (24%, purity by SCX HPLC >99.5%) of 6-methyl-8-isopropylpterin hydrochloride (**16**) as a yellow solid. pK_a 5.71 ± 0.02. λ_{max} (0.01 M HCl)/nm 399 (log ϵ 4.07), 281 (4.17), 260 (4.18); (pH 8 phosphate buffer) 412 (4.04), 319 (3.42), 266 (4.41). ^{1}H NMR (0.01 M DCl/D_2O) δ 8.73 (s, 1H, 7-*H*), δ 5.73 (m, 1H, N-C*H*), δ 2.66 (s, 3H, 6-C*H*$_3$), δ 1.56 (d, 6H, N-CH(C*H*$_3$)$_2$). Anal. Calcd: $C_{10}H_{14}N_5$-OCl 1.5 H_2O 0.35 HCl (295.49) requires: C, 40.65; H, 5.92; N, 23.70; Cl, 16.20. Found: C, 40.52; H, 5.85; N, 23.54; Cl, 16.34.

*6-Methyl-8-allylpterin Hydrochloride (**17**).* A suspension of 0.5 g (2.7 mmol) of pyrimidine (**5**) was treated with a solution of 0.5 mL (2.77 mmol) of **7** and 0.75 g (7.3 mmol) of sodium bisulfite and reacted as for **13**. Product was recovered, washed, and dried as for **14** to give 0.19 g of greenish-yellow solid; 0.1 g was treated to remove bisulfite as for **14** and further purified by RP silica as for **8** to give 0.042 g (6%, purity by SCX HPLC >99.5%) of 6-methyl-8-allylpterin hydrochloride (**17**) as a yellow solid. pK_a 5.56 ± 0.06. λ_{max} (0.01 M HCl)/nm 401 (log ϵ 4.03), 279 (4.15), 261 (4.20); (pH 8 phosphate buffer) 412 (3.98), 319 (3.50), 267 (4.33). ^{1}H NMR (0.01 M DCl/D_2O) δ 8.65 (s, 1H, 7-*H*), δ 6.05 (m, 1H, N-CH$_2$-C*H*), δ 5.46 (m, 2H, N-CH$_2$-CH=C*H*$_2$), δ 5.19 (d, 2H, N-C*H*$_2$), δ 2.65 (s, 3H, 6-C*H*$_3$). Anal. Calcd: $C_{10}H_{12}N_5OCl$ 0.75 HCl 0.25 H_2O (285.54) requires: C, 42.06; H, 4.68; N, 24.53; Cl, 21.72. Found: C, 42.18; H, 4.47; N, 24.28; Cl, 21.81.

RESULTS

Compound Preparation. The preparation of the 8-alkylpterins shown in Scheme 1 followed two basic approaches. 8-Alkylpterins with no other pyrazine-ring substituents were synthesized by a direct Gabriel–Colman condensation of the 2,5-diamino-6-alkylaminopyrimidin-4(3*H*)-one with glyoxal (Brown & Jacobson, 1961; Pfleiderer et al., 1968, 1971). The 6-methyl-8-substituted pterins and 7-methyl-8-propylpterin were prepared by a modification of this Gabriel–Colman condensation using pyruvic aldehyde and a procedure reported previously (Ivery & Gready, 1992) which allowed separation of 6- and 7-methyl isomers from the reaction mixture. To ensure purity suitable for enzymic tests, compounds were purified finally with reverse-phase silica column chromatography (Kuhler & Lindsten, 1983) resulting in usually bright yellow compounds greater than 99.5% pure by SCX HPLC (Wong & Gready, 1992; Ivery & Gready, 1992).

Kinetic Studies. As the large number of compounds precluded determination of full pH profiles, the activity was measured at pH 5.8 which was expected to be close to the optimum for both K_m and $V_{max}/[E]_o$ values on the basis of previous results (Gready, 1990). Table 1 summarizes the kinetic data determined for the 8-alkylpterins, 6-methyl-8-alkylpterins, and 7-methyl-8-propylpterin with both human and chicken enzymes.

The K_m values for the 8-alkylpterins with chicken enzyme ranged from 6.2 μM for 8-propylpterin (**10**) to 47 μM for 8-ethylpterin (**9**), while the $V_{max}/[E]_o$ values ranged from 5.4 s^{-1} for 8-methylpterin (**8**) to 1.0 s^{-1} for 8-propylpterin (**10**). Summarizing the data for the different 8-substituted-pterins, 8-methylpterin (**8**) is the second weakest binding compound

but has the highest reactivity, 8-ethylpterin (**9**) has the weakest binding and reactivity one-fifth that of 8-methylpterin (**8**), 8-propylpterin (**10**) has the tightest binding but reactivity similar to that of 8-ethylpterin (**9**), 8-isopropylpterin (**11**) has very similar activity to 8-propylpterin (**10**), and 8-allylpterin (**12**) has reactivity about 3 times higher than that of 8-propyl- and 8-isopropylpterins but significantly weaker binding.

For human enzyme, Table 1 shows that in general the K_m values for each 8-alkylpterin have increased from ~2 to 8 times while the $V_{max}/[E]_o$ values have increased from ~2.5 to 4 times compared with those for chicken DHFR. The largest increases are for 8-methyl- (**8**), 8-ethyl- (**9**), and 8-allylpterins (**12**) with K_m values increased 8.5, 5.5, and 4.6 times, respectively, and $V_{max}/[E]_o$ values increased 2.7, 2.6, and 3.9 times, respectively. The $(V_{max}/[E]_o)/K_m$ values indicate 8-methyl- and 8-ethylpterins have significantly greater activity with chicken enzyme, while 8-allylpterin is slightly more active with chicken DHFR. For 8-propyl- (**10**) and 8-isopropylpterins (**11**) the K_m values with human enzyme are increased by 2.3 and 2.8 times, respectively, and the $V_{max}/[E]_o$ values by 2.9 and 3.3, respectively, but the $(V_{max}/[E]_o)/K_m$ values indicate marginally stronger activity with human enzyme.

The data in Table 1 show that addition of a 6-methyl substituent greatly affects the activity of a compound. Addition of a 6-methyl group to 8-methylpterin (**8**) greatly increases activity with 6,8-dimethylpterin (**13**) showing the lowest K_m and the highest $V_{max}/[E]_o$ values with both enzymes of any compound in the study. The K_m and $V_{max}/[E]_o$ values for **13** are greater with human enzyme (both ~2.7 times) compared with chicken DHFR with resultant $(V_{max}/[E]_o)/K_m$ ratios indicating significantly more activity with human enzyme. However, addition of a methyl group to 8-isopropylpterin (**11**) to form 6-methyl-8-isopropylpterin (**16**) completely abolishes substrate activity with both enzymes, while 6-methyl-8-propyl- (**15**) and 6-methyl-8-allylpterins (**17**) show activity so low with both enzymes that it is not possible to determine their kinetic parameters using our spectrophotometric assay or to be certain even that the observed rates are due to enzyme-catalyzed reduction of the substrate. Similarly for 7-methyl-8-propylpterin no activity was observed. For 6-methyl-8-ethylpterin (**14**) kinetic parameters were unmeasurable with human enzyme but just measurable with chicken enzyme. However, compared with 8-ethylpterin (**9**) the activity of **14** was greatly reduced with $V_{max}/[E]_o$ and K_m values $^1/_8$th and 2.7 times those for **9** resulting in a $(V_{max}/[E]_o)/K_m$ value $^1/_{20}$th that of **9**.

Thermodynamic Studies. The thermodynamics of binding was examined by measuring the dissociation constants K_d for all compounds in binary complex with both enzymes at pH 5.8 (Table 2). The K_d values for 8-alkylpterins with chicken DHFR varied from 127 μM for 8-methylpterin (**8**) to 15 μM for 8-propylpterin (**10**), with compounds with larger 8-substituents showing the lowest K_d values in general. For human DHFR the K_d values are significantly higher than those for chicken enzyme but exhibit the same trend with strength of binding in the order 8-propyl- > 8-isopropyl- ≈ 8-allyl- > 8-ethyl- > 8-methylpterin.

The binding data for the 6-methyl substituted compounds show that 6-methyl-8-ethyl- (**14**), 6-methyl-8-propyl- (**15**), 6-methyl-8-isopropyl- (**16**), and 6-methyl-8-allylpterin (**17**) bind very weakly to human DHFR with K_d values in the range ~200−~350 μM. For chicken enzyme the binding of these compounds is significantly stronger with K_d values in the range ~60−~100 μM. Surprisingly, 6,8-dimethylpterin (**13**) also binds relatively weakly to both enzymes with K_d values similar to those of the other 6-methyl-substituted compounds and significantly greater than those for all the 8-substituted compounds except 8-methyl- and 8-ethylpterins. The binding constants for 7-methyl-8-propylpterin (**18**) indicate reasonably good binding to both enzymes but are atypical as the value with human enzyme is lower than that for chicken DHFR.

Table 2: Thermodynamic Dissociation Constants K_d (μM) for 8-Alkylpterins in Binary Complex with Chicken and Human DHFRs at pH 5.8

compound	chicken DHFR	human DHFR
8 (8-methyl)	127 ± 14	293 ± 42
9 (8-ethyl)	97 ± 8	178 ± 21
10 (8-propyl)	15 ± 1	23 ± 1
11 (8-isopropyl)	38 ± 3	88 ± 3
12 (8-allyl)	30 ± 2	105 ± 7
13 (6,8-dimethyl)	65 ± 5	128 ± 11
14 (6-methyl-8-ethyl)	103 ± 16	199 ± 16
15 (6-methyl-8-propyl)	96 ± 9	351 ± 86
16 (6-methyl-8-isopropyl)	83 ± 9	312 ± 41
17 (6-methyl-8-allyl)	64 ± 8	290 ± 70
18 (7-methyl-8-propyl)	66 ± 8	28 ± 2

The similarity of the binary complex data for 6,8-dimethylpterin (**13**) (a strong substrate) and 6-methyl-8-isopropylpterin (**16**) and 7-methyl-8-propylpterin (**18**) (no substrate activity) suggested that the latter compounds might bind tightly to DHFR in the ternary complex but in an unproductive mode. To test this possibility, the K_d values for **18** and **16** were measured in ternary complex with NADPH and chicken DHFR at pH 5.8 and found to be 20 ± 2 μM and >500 μM, respectively. The K_d for the ternary complex of **18** with human enzyme at pH 5.8 was also measured and found to be 10 ± 1 μM. These values suggest that the binding of 6-methyl-8-isopropylpterin (**16**) to DHFR is greatly weakened by the presence of NADPH but that of 7-methyl-8-propylpterin (**18**) was enhanced.

pH Dependence of Thermodynamic Dissociation Constants (K_d). To study the pH dependence of the binding of 8-alkylpterins to DHFR, apparent K_d values for 8-propylpterin were measured at pHs ranging from 4.15 to 7.5 with chicken DHFR. The K_d value at pH 5.8 for this ligand·enzyme complex was the lowest, and, hence, this system was the best available for measuring binding over a wider pH range. The results as plotted in Figure 1 show that from pH ~4.2 to 5.8 the K_d remains relatively constant at between 10 and 15 μM. Above pH 5.9 the apparent K_d rises rapidly to ~40 μM at pH 6.3 but then goes through an inflection to reach only ~100 by pH 7.4.

pH Dependence of Kinetic Parameters. The pH dependence of the kinetics for the reduction of 8-propylpterin with chicken DHFR were investigated between pH 5.2 and 7.5. As shown in Figure 2, the results show that while $V_{max}/[E]_o$ was pH independent, the apparent K_m values were strongly pH dependent. The K_m plot shows strong similarities with the plot of apparent K_d in Figure 1, i.e., an approximately flat region at low pH followed by a rapid increase above pH 5.9.

Molecular Modeling of the Binding of 8-Alkylpterins to DHFR. As a start to rationalizing the structure−activity data, molecular modeling was undertaken to investigate possible binding orientations of these pterin ligands in the active site

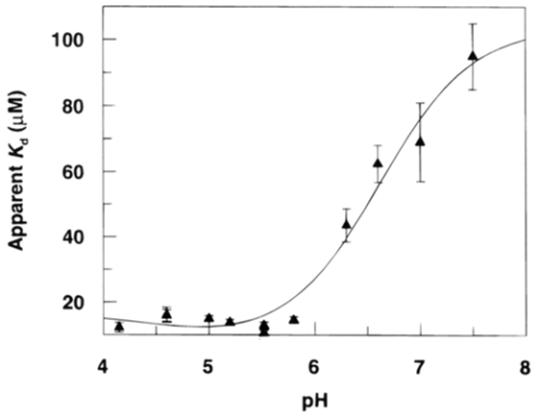


FIGURE 1: Plot of apparent K_d (μM) versus pH for 8-propylpterin (**10**) in binary complex with chicken DHFR. The data have been fitted to a model involving formation of three enzyme ligand complexes, $EHLH^+$, E^-LH^+, and E^-L (Ivery & Gready, 1994) with the ligand pK_a fixed at its experimental value of 5.42.

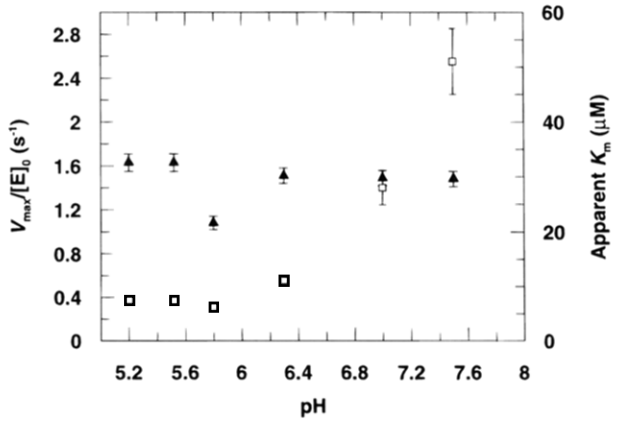


FIGURE 2: Plot of K_m (μM) [□] and $V_{max}/[E]_o$ (s^{-1}) [▲] versus pH for the chicken DHFR-catalyzed reduction of 8-propylpterin (**10**) using NADPH obtained from initial rate assays monitored at 410 nm.

of DHFR. As no crystal structures for DHFR complexes of 8-alkylpterins or the related inhibitors, the 8-alkyl-N^5-deazapterins are available, the structure for a DHFR•substrate ternary complex, chicken DHFR•biopterin•$NADP^+$ (McTigue et al., 1992), was used with overlaying of the pterin rings of substrate and particular 8-alkylpterins to determine the gross position of the 8-alkylpterin in the active site.

Figure 3A shows a view of the active site of the chicken DHFR•biopterin•$NADP^+$ structure (McTigue et al., 1992) edited to display only pertinent residues. The proposed salt bridge interaction (Kraut & Matthews, 1987) between the conserved active-site carboxylate group (Glu 30) and the pyrimidine N3(*H*) and 2-amino groups is shown. As previous (Gready, 1990; Jeong & Gready, 1994) and current studies of the pH dependence for the binding of the 8-alkylpterins suggest stronger binding for the deprotonated enzyme•protonated ligand complex, this interaction has been maintained in the modeling. The shape of the binding pocket for the substrate is further defined by a number of hydrophobic residues: Phe 34 makes contact with one face of the pteridine ring (the other faces cofactor); the backbone carbonyl oxygen atom of Ile 7, which is part of β-sheet A, is positioned ~3.3 Å directly below N^8; the methyl group carbon atoms of Leu 22 are ~5 Å directly above N^5, and the nicotinamide ring of $NADP^+$ cofactor is positioned below the pyrazine ring with the active C4 carbon atom ~3.5 Å from both C6 and C7.

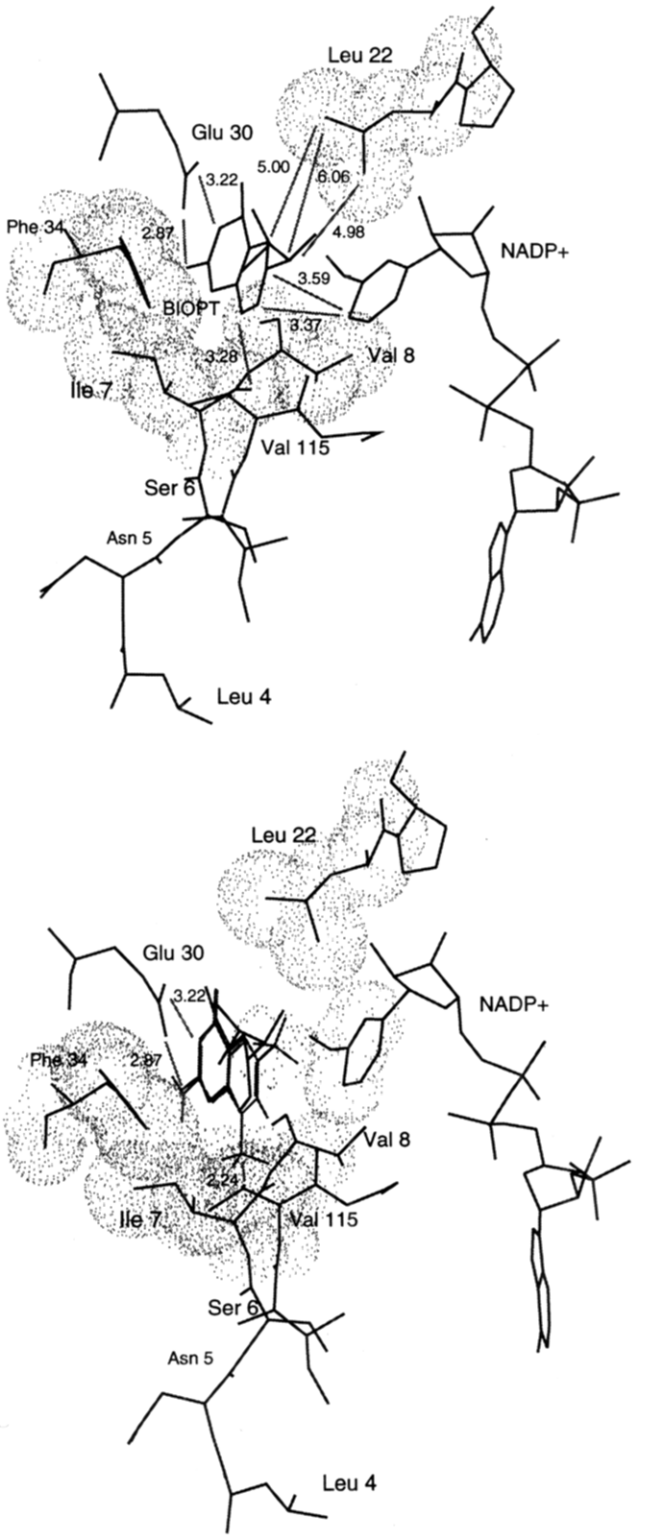


FIGURE 3: Edited view of the active site of chicken DHFR based on the X-ray structure of the enzyme•biopterin•$NADP^+$ ternary complex (McTigue et al., 1992) showing bound ligands, key active-site groups, and the conserved carboxylate group, Glu 30. (A, top) Bound biopterin and $NADP^+$ as in the the X-ray results; (B, bottom) with 6,8-dimethylpterin (**13**) overlaid onto the biopterin ring. The dotted regions indicate van der Waals surfaces and the dotted lines hydrogen-bond or other interaction distances.

In Figure 3B 6,8-dimethylpterin is shown directly overlaid on to the biopterin ring in the active site view of Figure 3A. In this orientation the 8-methyl group carbon of the ligand is only ~2 Å from the carbonyl oxygen of Ile 7, and thus the van der Waals surfaces for the 8-methyl and Ile 7 groups show very unfavorable contacts. As the Ile 7 contact is with

a backbone atom within an element of secondary structure, accommodation of the ligand more likely involves reorientation of the ligand rather than protein movement in this region. It is apparent from Figure 3 that there is free space above the biopterin pyrazine ring between N^5 and Leu 22, space normally occupied by the folate side chain of the natural substrate (Davies et al., 1990). Repositioning of the ligand into this space provides one option for removing the unfavorable contacts with Ile 7.

Figure 4A shows a possible new orientation of 6,8-dimethylpterin in the active site which utilizes this space. In this orientation the 8-methyl group can be comfortably accommodated with a contact distance with Ile 7 of ~4.5 Å, and the 6-methyl group is now positioned to make favorable contact with the methyl group carbon atoms of Leu 22 at a distance of ~3.5 Å. This latter interaction may contribute to the significantly greater enzyme affinity of 6,8-dimethylpterin compared with 8-methylpterin. This reorientation has repositioned the C7 carbon atom closer to where C6 of the natural substrates is normally positioned, and, hence, while C7 is still within range (~4 Å) for hydride transfer from the nicotinamide ring, the C6 carbon of the ligand is now ~5 Å from the cofactor C4 atom. This may be part of the reason why the intial reduction at C7 occurs much more rapidly than the second reduction at C6 (Thibault et al., 1989; Gready, 1990, and unpublished results).

To investigate how larger 8-substituents might be accommodated, 6-methyl-8-propylpterin was modeled into the active site as shown in Figure 4B. The 8-propyl group can be accommodated if the ligand is positioned even higher in the active site and tilted. However, this repositioning results in the hydrogen bonds between the active-site carboxylate and the 2-amino and N3(*H*) group protons being severely distorted. As it is expected that this interaction contributes substantially to binding and taking into account the results indicating 8-propylpterin is one of the stronger binding ligands, it may be that the ligand does not adopt this orientation or that the carboxylate group reorients to restore its interaction with ligand. Also, in this orientation the C7 carbon atom of the ligand is over 5 Å from C4 of the nicotinamide ring. This is too far to account for the observed substrate activity for 8-propylpterin, and thus another repositioning of the nicotinamide ring would be required. Depending on the degree to which the pterin ring is tilted away from Leu 22, this reorientation places the 6-methyl group in acceptable or too close proximity to Leu 22. The apparent difficulty in simultaneously accommodating both the larger 8-substituent and the 6-methyl group is consistent with the poor binding and loss of activity for 6-methyl-substituted compounds with larger 8-substituents.

In Figure 5 an alternative mode of binding for compounds with larger 8-substituents is presented. In this mode the ligand has been rotated ~90° relative to the biopterin substrate along an axis bisecting the ring (N3−C2 and C6−C7 distances). Maintenance of the ligand interaction with the active-site carboxylate group requires a simple ~80° rotation of this group about the dihedral angle Oϵ−Cδ−Cγ−Cβ and a concurrent rotation of ~40° about the dihedral angle Cδ−Cγ−Cβ−Cα. This reorientation positions the 8-substituent in the plane of the cofactor nicotinamide ring and extending into a pocket below it. This pocket is very hydrophobic in nature, and, hence, this placement could be expected to give significant favorable contributions to binding. An optimum fit would require the nicotinamide ring to shift up in the active site to a position on top of the 8-alkyl side chain, which may also result in favorable hydrophobic contacts. This repositioning of the nicotinamide ring is not, however, ideal for reaction and could explain

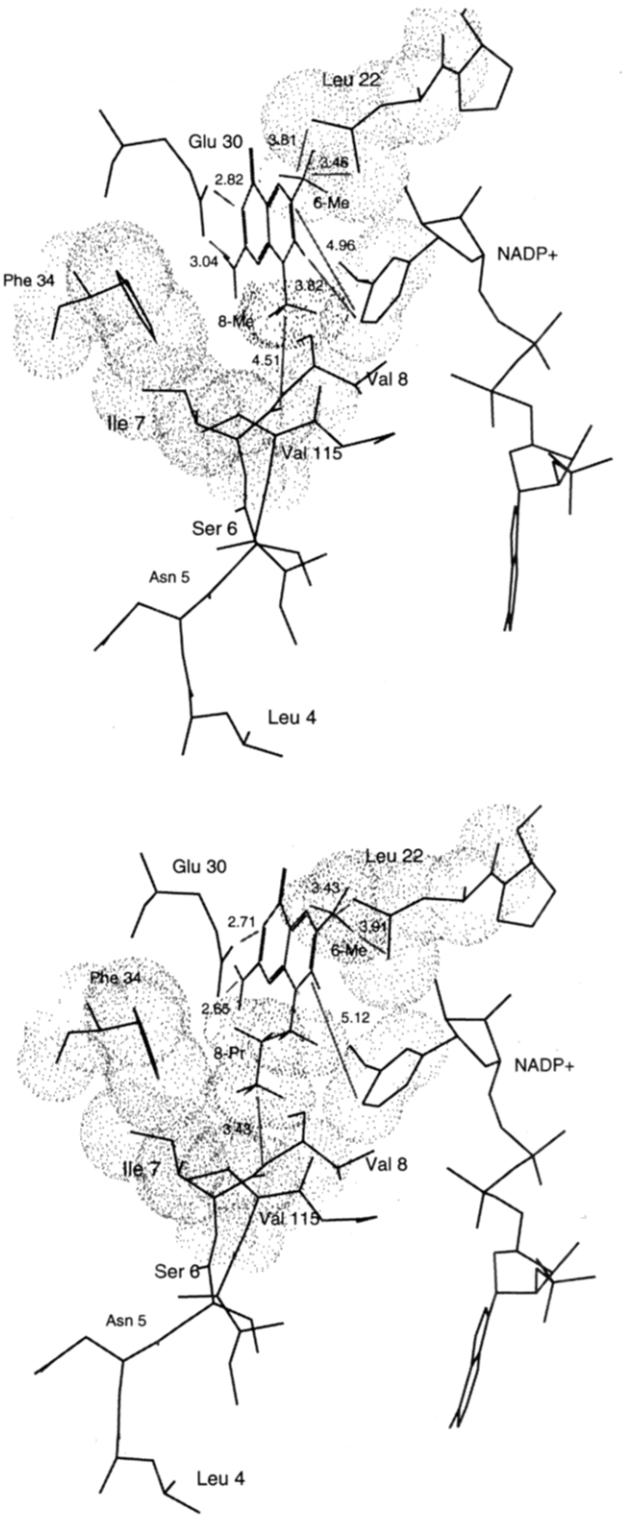


FIGURE 4: Edited view of the active site of chicken DHFR based on the X-ray structure of the enzyme•biopterin•$NADP^+$ ternary complex (McTigue et al., 1992) showing bound ligands, key active-site groups, and the conserved carboxylate group, Glu 30. Panels A (top) and B (bottom) show 6,8-dimethylpterin (**13**) and 6-methyl-8-propylpterin (**15**), respectively, modeled into the active site in hypothetical binding orientations which minimize unfavorable contact between the 8-methyl group and Ile 7 (cf. Figure 3A for **13** and see text). The biopterin molecule is not displayed. The dotted regions indicate van der Waals surfaces and the dotted lines hydrogen-bond or other interaction distances.

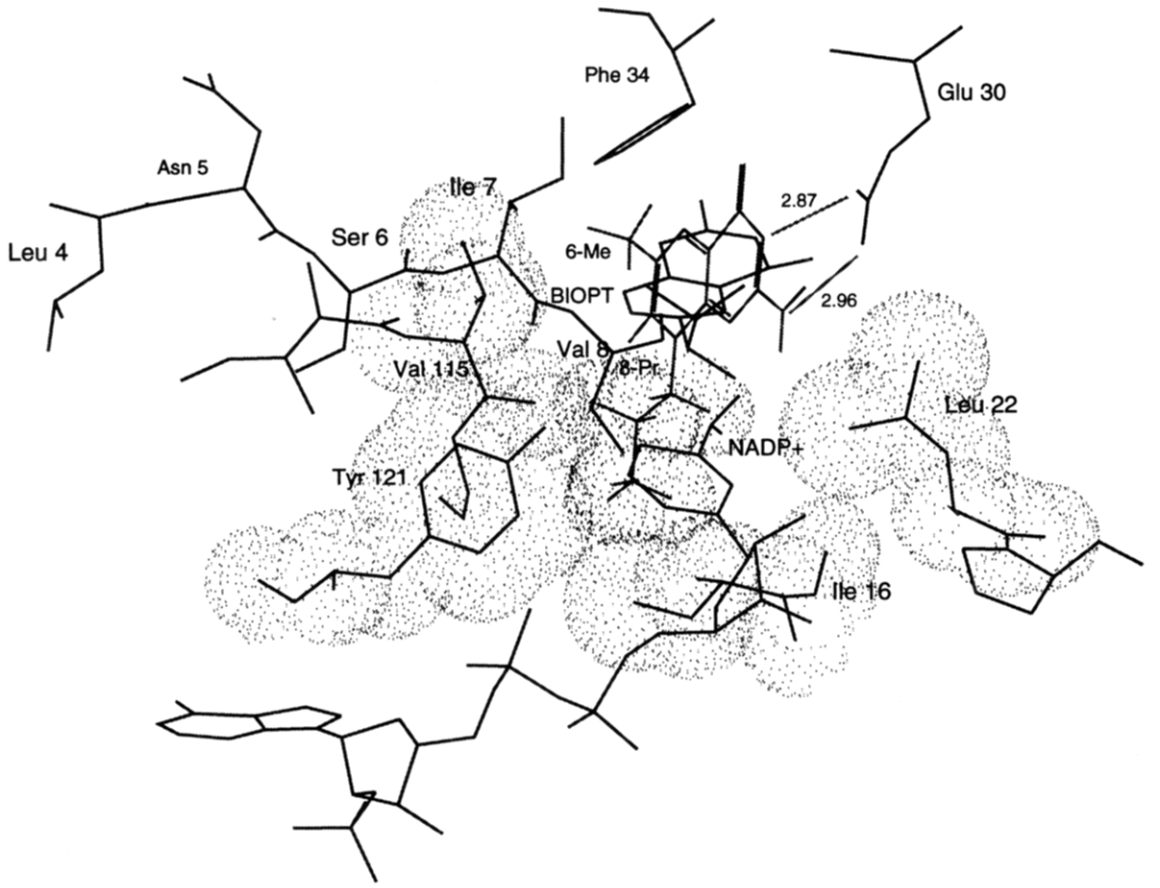


FIGURE 5: Edited view of the active site of chicken DHFR based on the X-ray structure of the enzyme·biopterin·$NADP^+$ ternary complex (McTigue et al., 1992) showing bound ligands, key active-site groups, and the conserved carboxylate group, Glu 30. 6-Methyl-8-propylpterin (**15**) is shown in a hypothetical orientation (see text) in which the pterin ring has been rotated ~90° compared with the biopterin position (not shown) along an axis bisecting the N3−C2 and C6−C7 distances. Two dihedral angles (see text) of Glu 30 have been modified to maintain the interaction of the carboxylate group with the N3 and 2-amino groups of the pterin ring. The dotted regions indicate van der Waals surfaces and the dotted lines hydrogen-bond or other interaction distances.

the low reactivity of ligands with larger 8-substituents. This lifting of the nicotinamide ring would position it too close to the 6-methyl group, which might explain the complete loss of activity of the 6-methyl compounds with large 8-substituents, especially as the K_d data for binary and ternary complexes with 6-methyl-8-isopropylpterin indicate negative cooperativity between ligand and NADPH in binding to the enzyme. However, this argument is not consistent with the K_d data for 7-methyl-8-propylpterin which show positive cooperativity as this lifting of the nicotinamide ring would impact even more on a 7-methyl group.

DISCUSSION

Spectrophotometric assays of nicotinamide-dependent enzymes are usually performed at 340 nm, the peak of a strong band in the reduced form of the cofactor which is absent in the oxidized form. While standard assays for dihydrofolate reductase with folate and dihydrofolate substrates and NADPH cofactor are also performed by monitoring the decrease of absorption at 340 nm (Blakley, 1984) and our earlier work on 8-alkylpterin activity (Thibault et al., 1989; Gready, 1990) also used this wavelength, the fact that folate and pterin substrates and products also absorb at 340 nm makes accurate estimation of the reaction extinction coefficient difficult. For the 8-alkylpterins it is also difficult to measure the reaction extinction coefficient directly using a complete-reaction assay because of the slow second-step reaction (Thibault et al., 1989) and the low activity of some of the present substrates. However, as 8-alkylpterins have a strong absorption band at long wavelength (~410 nm) but both NADPH and the dihydropterin product do not absorb at this wavelength, assay at ~410 nm allows the extinction coefficient of the substrate at this wavelength to be used as the reaction extinction coefficient. This method has also been used in related work (Jeong & Gready, 1995).

The kinetic results in Table 1 indicate that the rate and affinity of the substrate activity of the 8-alkylpterins for both enzymes depend on the size of the 8-substituent. For the small substituent (methyl), binding is relatively weak but good activity is observed, while for the larger substituents (propyl and isopropyl) binding is relatively strong but activity is low. The ethyl substituent shows characteristics of both small and large substituents in that it both binds weakly but also has low activity, while the allyl substituent shows the opposite pattern of binding relatively well as for the longer substituent but showing activity closer to that of 8-methylpterin. For compounds with a 6-methyl substituent in addition, there is also a grouping of activity depending on the size of the 8-substituent. For the small 8-methyl substituent the addition of a 6-methyl group greatly increases substrate affinity and somewhat increases activity, while for the larger substituents activity is completely or almost completely (8-ethyl) abolished as is the case also for the one 7-methyl compound tested (7-methyl-8-propylpterin).

In comparison with the K_m values, the K_d values for each compound in binary complexes with both enzymes were

2.2−5.1 times greater. These results suggest that ligand affinity for DHFR is increased by the presence of NADPH (cooperative binding). This type of cooperativity in binding between ligand and NADPH in DHFR ternary complexes has been observed previously for the related inhibitors, the 8-alkyl-N^5-deazapterins (Ivery & Gready, 1994) and also has been reported for other DHFR ligands (Blakley, 1984). Another interesting feature of the K_m and binary complex K_d results which suggests a dependence on ligand structure in forming active ternary complexes, possibly due to the way NADPH might be accommodated differently in the active site with particular ligands, was the similar K_d values for 6,8-dimethylpterin, an excellent substrate, and 6-methyl-8-isopropylpterin and 7-methyl-8-propylpterin, which were inactive as substrates. The K_d values for the two nonproductive ternary complexes which can be measured, i.e., with 6-methyl-8-isopropylpterin and 7-methyl-8-propylpterin, showed quite different results. The high K_d value for 6-methyl-8-isopropylpterin with chicken DHFR indicates that both ligand and cofactor cannot be easily fitted into the active site, while the low K_d values for 7-methyl-8-propylpterin with both DHFRs indicated both ligand and cofactor can be accommodated in the active site, with a binding cooperativity factor of ~3. This behavior contrasts with the very strong substrate affinity of 6,8-dimethylpterin (**13**) (K_m 3.7 μM) which indicates that both substrate and cofactor can be accommodated in the active site in a tightly bound active ternary complex.

Comparison of the ternary K_d values for 7-methyl-8-propylpterin with those for the structurally related 7-methyl-8-propyl-N^5-deazapterin (Ivery & Gready, 1994) indicates that the deazapterin binds approximately 4 times more tightly than the pterin to both enzymes. Differences in solvation, especially of the pterin and deazapterin rings of the respective ligands (Cummins & Gready, 1993), and different relative orientations of N^5-deaza and parent-ring forms in the active site may contribute to this differential in binding. However, the strength of binding of N^5-deazafolate to chicken DHFR has been reported previously to be 34 times greater than the binding of folate (Stone et al., 1984). The inability of the 8-alkyl-N^5-deazapterins to form a hydrogen bond from N^8 to the backbone carbonyl group of Ile 7, as proposed for an N^8-protonated form of N^5-deazafolate on the basis of the X-ray structure results (Davies et al., 1990), is a likely reason for the much greater differential in binding between N^5-deazafolate and folate compared with our results for 8-alkyl-N^5-deazapterins and 8-alkylpterins.

The binary K_d results for 8-propylpterin in Figure 1 show substantial pH dependence as has been found for other DHFR pterin-analogue ligands. The binding of ligands to DHFR has previously been described by a model for the enzyme−ligand complex which formed from the interaction of the conserved enzyme carboxylate group in the active site with either protonated or neutral ligand, and binding equations were derived for different ionic forms of ligand and also enzyme (Stone & Morrison, 1983). These equations express the measured or apparent K_d as a function of the pK_as of the ionizable groups on the enzyme and ligand, a pH-independent K_d for one of the enzyme−ligand complexes in the model, and the "acid dissociation constants" for the other enzyme−ligand complexes in the model. This treatment has been used extensively for studying the pH dependence of ligand binding to DHFR and in particular to obtain estimates of the pK_a of the enzyme carboxylate group, which have generally ranged between 5.5 and 6.5 (e.g., Stone & Morrison, 1983; Thillet et al., 1990; Jeong & Gready, 1994, 1995).

As part of recent studies of the pH dependence of the binding of 8-alkyl-N^5-deazapterins to DHFR we derived modified versions of Morrison's equations which express the apparent K_d as a function of the pK_as for the ionizable groups of the enzyme and ligand and pH-independent K_d values for each of the enzyme−ligand complexes involved in the model (Ivery & Gready, 1994; and unpublished results). The advantage of this approach is that it obviates the need for definition of acid dissociation constants ("pK_as") for enzyme−ligand complexes, which is a confusing description. These "pK_a" values do not represent the inherent acidity of these complexes, i.e., physical processes, but merely the ratios of the different ionic forms of the complexes. The studies for the 8-alkyl-N^5-deazapterins in complex with human and chicken DHFRs (Ivery & Gready, 1994) indicated that binding was strongest for a complex between deprotonated enzyme and protonated ligand (E^-LH^+) with minimum K_d values obtained at approximately pH 6.6−7.0, which is close to the pK_a of these ligands. Weaker binding was also observed for binary complexes of protonated enzyme and protonated ligand ($EHLH^+$) and for binary and ternary complexes of deprotonated enzyme and neutral ligand (E^-L), with the extent of formation of these complexes being strongly dependent on the pyridine-ring substituents.

It is clear from the K_d data for 8-propylpterin in Figure 1 that strongest binding occurs at or below pH 5.8, which is near the pK_a of ligand (5.42). In common with the results for 8-alkyl-N^5-deazapterins, this suggests that the tightest binding between enzyme and ligand involves protonated ligand with deprotonated enzyme. That the apparent K_d values do not increase as the pH is lowered below 5.8 suggests that protonated enzyme is also capable of binding protonated ligand. The rapid rise and then plateauing of the apparent K_d as the pH is raised suggests that binding does occur between deprotonated enzyme and neutral ligand but that this binding is much weaker than for the deprotonated enzyme-protonated ligand complex. While similar pH dependence in binding has been found for binary complexes of 8-alkyl-N^5-deazapterins, it should be noted that ternary complexes show a greater or lesser increase in K_d values at low pH suggesting poorer or minimal binding for protonated enzyme-protonated ligand complexes (Ivery & Gready, 1994).

On the basis of this interpretation, we have fitted the data in Figure 1 to a model involving formation of $EHLH^+$, E^-LH^+, and E^-L complexes (Ivery & Gready, 1994). The fitted curve is shown in Figure 1 and gives good agreement with the data. For this fit the pK_a for 8-propylpterin was fixed at 5.42 resulting in estimates of the enzyme pK_a of 5.0 ± 0.6 and pH-independent K_ds for the $EHLH^+$, E^-LH^+, and E^-L complexes of 17 ± 13, 6.5 ± 0.9, and 104 ± 6 μM, respectively. The low pK_a value for the enzyme and large error in the K_d value for the $EHLH^+$ complex result from the very similar values for this complex and for the E^-LH^+ complex, which makes estimation of the values difficult.

There are both similarities and differences between the K_d and K_m plots in Figures 1 and 2. In the high pH range the absence of a plateau in the K_m plot suggests that if any deprotonated enzyme−neutral ligand complex forms, it is not reactive. The shape of this plot in this region is similar to that found in other studies with 8-alkylpterin substrates (Gready, 1990; Jeong & Gready, 1995). But at low pH there is no evidence for an increase in K_m as found in the other

studies. This finding might be interpreted to suggest that a protonated enzyme-protonated ligand ternary complex forms and is reactive, behavior not previously reported for a DHFR substrate. However, the quality of the data in the low pH region must make this assessment tentative.

The molecular modeling studies have allowed some general insights into how these pterin ligands with substituents in the 8- and 6-positions might be accommodated within the active site to maintain the interaction with the conserved carboxylate group and to minimize steric interactions and maximize hydrophobic interactions with other active-site groups and the cofactor nicotinamide ring. They have thus provided a start to interpreting the structure−activity relationships. The general conclusions are that the active site provides considerable possibilities for different orientations of the pterin ring and pyrazine-ring substituents in agreement with very early modeling studies which attempted to predict how the pterin ring of substrate might bind before a structure for a substrate complex had been solved (Kraut & Matthews, 1987). The 8-alkylpterin series of ligands are substantially different from conventional oxidized pterin and dihydropterin substrates of DHFR, which differ in the structure of the 6-substituent only, and they thus provide a new and sensitive probe of the capacity of DHFR to form reactive ternary complexes with ligand, and possibly cofactor, oriented differently in the active site from the orientations of usual DHFR substrates. Further computational and docking studies are being undertaken to discriminate quantitatively between possible binding orientations for 8-alkylpterin and 8-alkyl-N^5-deazapterin ligands on the basis of the energetics of binding. It is expected these studies will allow a more confident interpretation of the structure−activity relationships leading to further predictions which may be tested by experiment.

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