Dissociation Constants for Dihydrofolic Acid and Dihydrobiopterin and Implications for Mechanistic Models for Dihydrofolate Reductase[†]

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ABSTRACT: The dissociation constants (pK_a) for the pteridine ring system of dihydrofolate (H_2folate) have been redetermined, and those for dihydrobiopterin (H_2biopterin) have been determined. Determination of the pK_a for N^5 of H_2folate is complicated by the low solubility and instability of H_2folate at pH 2–4, and other complicating factors. The initial rate of absorbance change due to degradation is a maximum at pH 2.5, and the products depend on the oxygen concentration: under aerobic conditions, (*p*-aminobenzoyl)glutamic acid and 7,8-dihydropterin-6-carboxaldehyde are major products. H_2Biopterin is much more soluble and more stable at low pH. For protonation of N^5, the pK_a is 2.56 ± 0.01 for H_2biopterin and 2.59 ± 0.03 for H_2folic acid. Spectrophotometric determination of the pK_a for the N^3–O^4 amide group of H_2folate is subject to serious errors when a wavelength between 220 and 235 nm is used. These errors arise from the pH-dependent absorbance of mercaptoethanol often present in the preparation. The amide group has a pK_a of 10.41 ± 0.04 in H_2biopterin and 10.85 ± 0.04 in H_2folate. The redetermined value for the pK_a of N^5 of H_2folate has implications for mechanistic models for dihydrofolate reductase, and revised kinetic constants have been calculated for one model.

Dihydrofolate reductase (5,6,7,8-tetrahydrofolate:NADP$^+$ oxidoreductase, EC 1.5.1.3, DHFR)[1] is a target enzyme for clinically important drugs such as the antitumor agent methotrexate, the antibacterial trimethoprim, and the antimalarial pyrimethamine. In the course of the chemical step in the enzyme-catalyzed reaction, that is, the reduction of 7,8-dihydrofolate (H_2folate) to 5,6,7,8-tetrahydrofolate (H_4folate), a hydride ion is transferred from NADPH to C^6 of H_2folate and a proton is added to N^5. The sequence of hydride and proton transfers and the immediate donor of the proton are still matters of speculation.

X-ray crystallography has shown that the active-site pocket of the enzyme is lined with hydrophobic residues and the only ionizable side chain in the cavity is a conserved aspartate (bacterial enzymes) or glutamate (eukaryotic enzymes) [reviewed by Blakley (1984)]. The active-site carboxyl group is widely thought to participate in catalysis by facilitating proton addition to N^5 of H_2folate, which in turn facilitates subsequent hydride addition at C^6 (Gready, 1985), and one mechanism by which this is proposed to occur is by way of initial O^4 protonation (Gready, 1985; Taira et al., 1987; Morrison & Stone, 1988; Oefner et al., 1988; Uchimaru et al., 1989). The evidence in favor of obligatory preprotonation of H_2folate at N^5 comes from pH profiles of k_{cat} and $k_{cat}/K_{m(dihydrofolate)}$ for DHFR from *Escherichia coli* and for mutants of this enzyme in which the active-site aspartate (Asp^{27}) is replaced by Asn or Ser, with a major decrease in activity at neutral pH (Howell et al., 1986). However, at pH 4.5, the activity of both mutants is increased about 20-fold, and the Ser^{27} mutant becomes almost as active as the wild-type enzyme. Preprotonation of H_2folate either before or after binding to mutant enzyme was proposed (Howell et al., 1987), and data were fitted to the corresponding rate equation, but with use of the pK_a of 3.8 for N^5. Since some of our results were inconsistent with this value of the pK_a for H_2folate, new pK_a values for H_2folate have been determined and compared with those for H_2biopterin which is relatively stable even at low pH. The corrected value of the pK_a for protonation of N^5 of H_2folate (2.59) must be taken into account in mechanistic models for DHFR. In addition, the corrected UV spectra for H_2folate at low pH must be used in evaluation of difference spectra obtained when H_2folate binds to DHFR.

MATERIALS AND METHODS

Dihydrofolic acid was prepared as previously described (Blakley, 1960) and stored as a suspension in 0.001 N HCl containing 100 mM 2-mercaptoethanol at −70 °C. It is completely stable under these conditions for many months as judged by enzymatic assay, HPLC, and ^{1}H NMR. H_2Biopterin was obtained from Schircks Laboratories (Jona, Switzerland). Both compounds were in a high state of purity as indicated by the absence of any impurities detectable by NMR or HPLC.

Absorption Spectra. Crystals of dihydrofolic acid from a thawed suspension were washed 3 times in cold 0.001 N HCl by centrifugation to remove 2-mercaptoethanol. To prepare

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[1] Abbreviations: H_2folate, 7,8-dihydrofolic acid; H_2biopterin, 7,8-dihydrobiopterin; NMR, nuclear magnetic resonance; TMS, tetramethylsilane; DHFR, dihydrofolate reductase; UV, ultraviolet; HPLC, high-performance liquid chromatography.

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a stock solution, a portion of the mercaptoethanol-free suspension was diluted with ice-cold 50 mM Tris-HCl, pH 7.3, to give approximately 3 mM H_2folate and kept at 0 °C until use the same day. HPLC indicated that there was no degradation during this period under these conditions, but at 20–25 °C temperatures significant degradation occurred in 1 h. Spectra were determined with a Beckman DU 70 spectrophotometer interfaced with an Apple 2GS computer. Cuvettes were thermostatted, and the temperature was determined by thermistor. To prepare solutions for absorbance measurements during investigation of H_2folate degradation, 1.5–2.8 mL of water in a standard cuvette was brought to temperature (20 °C unless otherwise indicated) in the instrument. A volume of the stock H_2folate solution sufficient to give a final concentration of 30 μM was thoroughly mixed with the water, before addition of 0.2 M buffer or HCl at the appropriate concentration to bring the final volume to 3.0 mL and the pH to the desired value. The solution was thoroughly mixed, and absorbance readings were immediately commenced. When spectra or rates of absorbance change were determined under anaerobic conditions, solutions under a layer of paraffin were deoxygenated by bubbling humidified high-purity nitrogen through the solution.

Determination of pK_a *Values from NMR Spectra.* Proton NMR spectra were obtained on a General Electric GN-500 NMR spectrometer operated at 500.114 MHz with quadrature detection and deuterium lock. H_2Biopterin spectra were acquired by using a 10 mM solution in 50 mM phosphate buffer in D_2O, and 2 mM sodium 2,2-dimethyl-2-silapentane-5-sulfonate (0.013 ppm relative to TMS). The pH was adjusted by addition of NaOD and DCl (ca. 1 M) solutions and measured with a glass electrode without correction for deuterium (pH*). Accumulation of 32 scans for each pH was performed at 23 °C (ambient temperature) in 8K (real) data sets using an acquisition time of 680 ms, a 3.0-s delay between scans, and a flip angle of 36°. Presaturation of the residual water resonance was achieved by continuous-wave decoupling at the water frequency for 2 s during the interpulse delay. For spectra of H_2folate, a 5 mM solution in 50 mM phosphate buffer was employed using 2 mM internal 3-(trimethylsilyl)propionic acid (0.000 ppm relative to TMS) as a reference standard. At each pH, 32 scans were acquired at 25 °C (using a variable-temperature controller) in 32K (real) data sets using an acquisition time of 2.72 s, a 1.36-s delay between scans, and a pulse width of 15 μs.

Spectrophotometric Determination of pK_a *Values.* A wavelength was chosen for each pK_a determination at which the absorbance change during the ionization was a maximum. It was found important to avoid local high concentrations of acid or alkali, exposure to elevated temperature (≥20 °C), or to a pH far from neutrality for more than 1 min. This precludes serial addition of small volumes of concentrated acid or alkali to make successive adjustments of the pH of the same solution. Instead, for each point on the titration curve, the selected pH value was obtained by mixing a freshly diluted solution of H_2folate with an approximately equal volume of buffer at the appropriate pH, and measuring the absorbance at the selected wavelength within 40 s. During this time period, the maximum amount of degradation (as measured by HPLC) at 20 °C and various pH values was the following: pH 1.12, 9%; 2.5, 5%; 7.0, 3%; 12.2, 6%. The pH of the mixture was determined within 2 min from mixing at a glass electrode recently standardized with fresh standard buffers (Fisher) with a pH close to the range of the pK_a. For mixtures in which the desired pH was achieved by addition of HCl at a final concentration of 0.1 N or higher, H_0 values obtained from Paul and Long (1957) were used.

Absorbance or NMR chemical shift values (y) at various pHs or H_0 values were computer-fitted by using nonlinear least-squares techniques to the equation:

$$y = \frac{c_1H^+ + c_2K_a}{H^+ + K_a}$$

where c_1 is the value of y for the protonated species and c_2 is the value of y for the unprotonated species. pK_a values were sufficiently well separated that the data for each pK_a could be fitted separately.

Fitting of Data to the Model for Mutant E. coli DHFR Activity. The data of Howell et al. (1987) were fitted to the model for enzyme activity that they had proposed. The values of the parameters were obtained by simultaneously computer fitting the data to the equations given by Howell et al. for k_{cat} and k_{cat}/K_m using nonlinear least-squares techniques, and assuming that the standard deviation in k_{cat} or k_{cat}/K_m was 5% of the measured value.

Identification of the Products of Aerobic H_2*Folate Degradation.* Solutions of 30 μM H_2folate were adjusted to pH 2.5 by addition of HCl with vigorous stirring and incubated at 35 °C until the absorbance change was complete, when the solution was neutralized with NaOH. The residue from a freeze-dried sample was dissolved in cold water and examined by HPLC at room temperature on a Beckman reversed-phase C18 Ultrasphere ODS 5-μm (10 × 250 mm) column with detection at 254 nm and a flow rate of 1 mL/min. Two systems were used. In system 1, solvent A was water, and solvent B was 2% acetic acid in 80% methanol. A linear gradient was used from 0.1% B at time zero to 40% B at 40 min. In system 2, solvent A was 5 mM tetrabutylammonium phosphate, pH 7.16, solvent B was 5 mM tetrabutylammonium phosphate, pH 7.16, in 80% methanol, and the gradient was 0–40% B over 30 min followed by isocratic 40% B.

A 3-L preparation was concentrated by rotary evaporation to about 10 mL, a small amount of precipitate removed by centrifugation, and the supernatant applied to a DEAE-cellulose column (Whatman DE52) (2.8 × 45 cm). Overlapping peaks of two yellow, UV-absorbing compounds were eluted with 50 mM Tris-HCl, pH 7.0. Further elution with 0.5 M NaCl in 50 mM Tris-HCl, pH 7.3, produced a colorless, UV-absorbing peak. The overlapping yellow peaks were further separated by preparative HPLC, and all three compounds were examined by analytical HPLC and spectrophotometry.

Enzymatic Assay of H_2*Folate.* The amount of H_2folate in a sample was determined from the total absorbance change at 340 nm during its enzymatic reduction, based on $\Delta\epsilon$ = 12 800 (Appleman et al., 1989). The reaction mixture contained 45 nM recombinant human DHFR, 120 μM NADPH, and buffer consisting of 25 mM MES, 25 mM acetate, 50 mM Tris, 100 mM NaCl, and 0.02% azide, pH 7.65. The assay was carried out at 20 °C in a volume of 1.00 mL, and amounts of H_2folate in the range 3–60 nmol were determined. The reduction of H_2folate was complete in less than 5 min. This assay is subject to some error due to enzymatic reduction of some degradation products, and thus gives a maximum value for H_2folate concentration.

RESULTS

Solubility of H_2*Folate at Low pH.* When solutions of H_2folate at high concentrations (50–800 μM) were maintained at pH 2–3 and 15–25 °C, they showed a slow decrease in absorbance at the maximum but an increase at other wave-

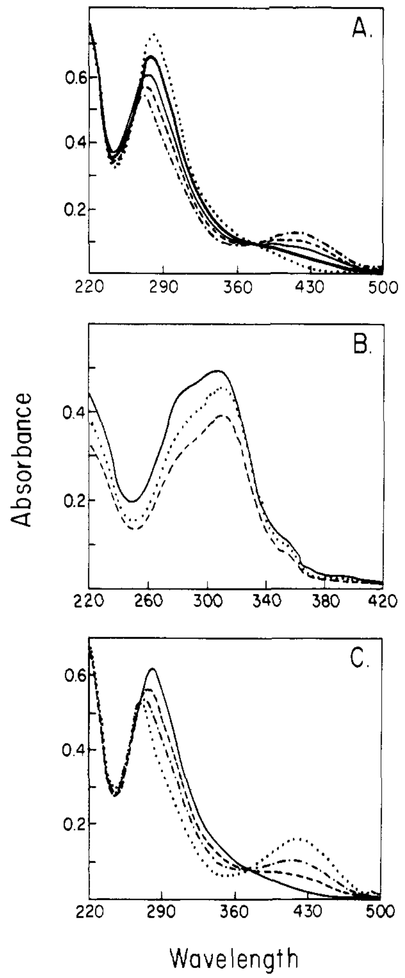

FIGURE 1: Ultraviolet spectra of 30 μM H_2folate at pH 2.49, and changes in spectra due to degradation. Spectra were recorded as described under Materials and Methods at the temperatures indicated. Components were added as indicated. (A) 15 °C. Dilute HCl (10 μL of 1 N HCl and 1.48 mL of water) was added to 1.52 mL of 60 μM H_2folate. Spectra were recorded at 0, 25, 50, 75, and 120 min. (B) 15 °C. H_2Folate (30 μL of 3 mM) was added to 2.97 mL of dilute HCl (10 μL of 1 N HCl and 2.96 mL of water). Spectra were recorded at 0, 16, and 66 min. (C) 35 °C. Mixing as in (A). Spectra recorded at 0, 8, 20, and 90 min. In each panel, the absorbance at 280 nm decreases with time.

lengths at which the absorbance was initially low. This was due to slow precipitation of material from solution to form a fine suspension. That the material precipitating at 20 °C is H_2folate rather than a decomposition product was shown by the fact that when the precipitated material was recovered by centrifugation in the cold and redissolved in Tris-HCl, pH 7.3, the UV spectrum was the same as that for H_2folate, HPLC showed an absence of decomposition products, and the material was 100% H_2folate as determined by enzymatic assay. Although precise determination of the solubility in the low-pH range was not performed, it was found necessary to use a final concentration no greater than 30 μM at pH 1.5–3.5 to avoid such precipitation at 15 °C. If concentrated H_2folate is added last to buffer at low pH, partial precipitation of H_2folate occurs even at projected final H_2folate concentrations below 50 μM.

Changes in H_2Folate at Acid pH. When UV absorbance spectra for 30 μM H_2folate were recorded at pH 1.5–3.5, the spectrum obtained was dependent on the order of mixing (Figure 1). Figure 1A shows the initial spectrum obtained at 15 °C when 30 μM H_2folate at pH 2.49 was prepared by mixing equal volumes of cold 60 μM H_2folate (pH 7.3) and cold 0.2 M buffer, pH 2.49. Also shown are the spectral changes occurring over a period of 2 h. Figure 1B shows a spectrum consistently obtained when concentrated H_2folate (30 μL of 3 mM) was mixed with dilute HCl (10 μL of 1 N HCl mixed with 2.95 mL of water, giving a final pH of 2.43). This spectrum has a maximum at 310 nm, and some precipitation occurred during mixing and subsequently. While the decrease in absorbance corresponds to loss of material from solution, the difference between the spectrum and that in Figure 1A suggests the occurrence of another phenomenon, possibly self-association. The spectral changes occurring under these conditions did not correspond to degradation since on adjustment of the pH to 7 the spectrum was indistinguishable from that of unchanged H_2folate, and enzymatic assay, as well as HPLC, indicated over 90–100% H_2folate remaining. If 30 μL of 3 mM H_2folate was diluted into 6 M guanidine hydrochloride (adjusted with HCl to pH 2.47) or 8 M urea (adjusted with HCl to pH 2.48) at 15 °C, the spectra obtained closely resembled the initial spectrum in Figure 1A. However, dilution into 3.5 M ammonium sulfate (adjusted to pH 2.44) resulted in a spectrum like that in Figure 1B.

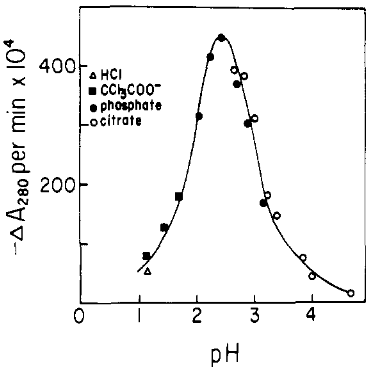

FIGURE 2: Effect of pH on the rate of absorbance change at 280 nm in solutions of H_2folate at 35 °C. After thermal equilibration of 1.50 mL of 60 μM H_2folate in the cuvette, 1.50 mL of 0.2 M buffer or HCl at 35 °C was added, the solution was mixed, and absorbance change over the first minute was recorded. Solutions added were (△) HCl, (●) sodium phosphate, (○) sodium citrate, (■) sodium trichloroacetate.

When the solutions were prepared as in Figure 1A, spectral changes were similar at 15 and at 35 °C (Figure 1C) and corresponded to an irreversible process. Thus, the spectrum after neutralization was different from that of H_2folate, and enzymatic assay and HPLC indicated 30–44% loss of H_2folate after 2 h at 15 °C and 72–86% loss after 70 min at 35 °C. Investigation of the rate of the spectrophotometric change at pH 2.5 gave a $t_{1/2}$ at 15 °C of 71 min; at 25 °C, 28 min; and at 35 °C, 17 min. The initial rate of the absorbance change is dependent on pH (Figure 2) with a maximum rate at about pH 2.5.

Under anaerobic conditions, a different spectral change occurred at pH 2.5, resulting in an even greater loss of absorbance at 280 nm, a decrease in the absorbance maximum, and a shift to 254 nm, with no appearance of a maximum at 420 nm. The rate of irreversible degradation of H_2folate was slower under anaerobic conditions with 42% loss in 70 min at 35 °C, as indicated by HPLC and enzymatic assay.

Products of Acid Degradation of H_2Folate. The final product formed aerobically from H_2folate at 35 °C at acid pH has a spectrum characterized by decreased absorption at 280 nm, a shift of the maximum to 270 nm, and the appearance of a new absorption peak with a maximum at about 420 nm (Figure 1C). The long-wavelength absorption peak suggests that the products formed might be the same as those previously described for decomposition of H_2folate at neutral pH, (*p*-aminobenzoyl)-L-glutamate and 7,8-dihydropterin-6-

Table I: Effect of Ionic Strength on pK_a Values

compound	dissociating group	method^a	temp^b	measured pK_a	I^c	calculated pK_a $I = 0$	calculated pK_a $I = 0.1$	Z^d
H_2biopterin	N^1	S(258)	20	-0.36 ± 0.05^e				
	N^5	S(231)	20	2.56 ± 0.01	0.015	2.52	2.61	$+1 \rightarrow 0$
	N^5	^{1}H NMR	23	2.55 ± 0.02^f	0.0425	2.49	2.57	$+1 \rightarrow 0$
	N^3–O_4	S(231)	20	10.41 ± 0.04	0.061	10.48	10.40	$0 \rightarrow -1$
	N^3–O_4	^{1}H NMR	23	10.36 ± 0.04^f	0.158	10.46	10.37	$0 \rightarrow -1$
H_2folate	N^1	S(258)	20	0.28 ± 0.02^e				
	N^5	S(228)	20	2.59 ± 0.03	0.0154	2.54	2.65	$+1 \rightarrow 0$
	N^3–O^4	S(228)	20	10.81 ± 0.01	0.0625	10.89	10.80	$0 \rightarrow -1$
	N^3–O^4	^{1}H NMR	25	10.71 ± 0.04^f	0.1798	10.82	10.73	$0 \rightarrow -1$
	N^3–O^4	^{1}H NMR	10	10.97 ± 0.04^f	0.1854	11.08	10.99	$0 \rightarrow -1$

[a] S = spectrophotometric, with wavelength in parentheses. ^{1}H NMR refers to mean chemical shift of the C^7 protons. [b] Temperature in degree centigrade. [c] Ionic strength due to all components including H_2biopterin or H_2folate at the measured pK_a. [d] Change in charge on the pteridine ring due to the proton dissociation. [e] The ionic strength was too high for calculation of pK_a at $I = 0$ or 0.1. [f] Observed readings from the glass electrode are not corrected for use of D_2O as solvent, or for differences in constants for D^+ dissociation versus H^+ dissociation from pteridines or buffer.

carboxaldehyde (Hillcoat et al., 1967; Whiteley et al., 1968). The latter is reported to have absorption maxima at pH 7.0 at 268 and 422 nm (Whiteley et al., 1968). This identification is consistent with the fact that oxidation is required to produce the carboxaldehyde and no absorbance maximum appears at 420 nm during anaerobic degradation at pH 2.5.

Examination of the products of aerobic degradation of H_2folate by HPLC in two systems indicated the presence of three compounds in addition to residual H_2folate. One of these had an elution time similar to that of (*p*-aminobenzoyl)-glutamate in both systems, and the others had elution times characteristic of unconjugated pterins. Chromatographic separation of these compounds on DEAE-cellulose followed by preparative HPLC indicated the formation of a large amount of a colorless UV-absorbing compound that was identified as (*p*-aminobenzoyl)glutamate on the basis of HPLC and UV spectra at pH 1, 7, and 13. One of the putative pterins had the UV spectral characteristics of 7,8-dihydropterin-6-carboxaldehyde. The other had the following absorbance maxima and relative absorbance at the maxima (in parentheses): pH 1.22, 276 (1.00) and 304 nm (0.75); pH 7.63, 274 (0.97) and 308 nm (0.66); pH 12.4, 276 nm (0.97) and 304 nm (inflexion, 0.52). On the basis of spectral changes, the compound was found to have two ionizations with pK_a values of 1.99 ± 0.03 and 9.4 ± 0.06. Although the UV spectra do not correspond to any common pterin, the properties are consistent with an unreduced pterin.

Anaerobic products were also examined by HPLC. The proportion of (*p*-aminobenzoyl)glutamate formed was quite small, so that under these conditions relatively little loss of the side chain seemed to be occurring. In confirmation of the spectral changes during anaerobic degradation, HPLC showed that the pterin products were clearly different from those formed aerobically.

Ultraviolet Absorption Spectra of H_2Folate and H_2Biopterin. Spectra of H_2folate unchanged by precipitation, irreversible degradation, or other reactions causing anomalies were obtained by mixing dilute H_2folate with a buffer at the desired pH at 15 °C and scanning over the range 220–400 nm within 2 min of preparation of the solution (Figure 3). Although the lower pK_a values are too close together to obtain spectra of all the pure ionic forms, the spectra at the various pH values correspond approximately to the following forms (ignoring carboxyl ionizations): 12.3, monoanion; 7.66, neutral; 1.39, monocation; H_0 −0.51, dication. The spectrum of H_2biopterin did not change with time at low pH, and spectra for this compound at various pH values are shown in Figure 4 and again correspond approximately to the various ionic species.

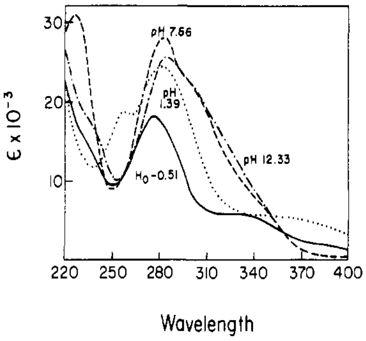

FIGURE 3: Absorbance spectra of 31 μM H_2folate at various pH values. Spectra were recorded at 15 °C in the following solutions: pH 12.33, 0.1 N NaOH; pH 7.66, 50 mM Tris-HCl; pH 1.39, 0.05 N HCl; H_0 −0.51, 1.6 N HCl. Ordinate values are molar extinction coefficients. Concentrations were determined by estimating the concentration of the stock solution using $\epsilon_{282} = 28.6 \times 10^3$ at pH 7.2 (Blakley, 1960).

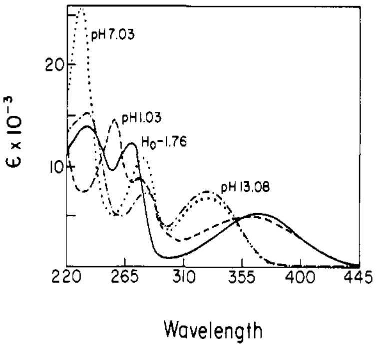

FIGURE 4: Absorbance spectra of H_2biopterin at various pH values. Spectra were recorded at 20 °C in the following solutions: pH 13.08, 0.1 N NaOH; pH 7.0, 25 mM potassium phosphate buffer; pH 1.03, 0.15 N HCl; H_0 −1.76, 5 N HCl. Solutions were prepared by mixing as described under Materials and Methods. Ordinate values are molar extinction coefficients. Concentrations were determined by estimating the concentration of the stock solution using published extinction coefficients for pH 7.4 (Pfleiderer, 1985).

Determination of pK_a Values for H_2Biopterin and H_2Folate. Three pK_a values corresponding to dissociation of protons from N^1, N^5 and the N^3–O^4 group were determined spectrophotometrically for H_2biopterin and H_2folate (Figure 5). Wavelengths for determination of pK_a values were chosen to give a maximum or close to maximum absorbance change with change in ionization state. Values of pK_a were found to be similar for the two compounds (Table I) as expected from the similarity of their structures. Determination of these pK_a values for dihydrobiopterin and of the N^3–O^4 pK_a for H_2folate by proton NMR (Figure 6) gave values in agreement with the spectrophotometric ones (Table I). The uncorrected glass

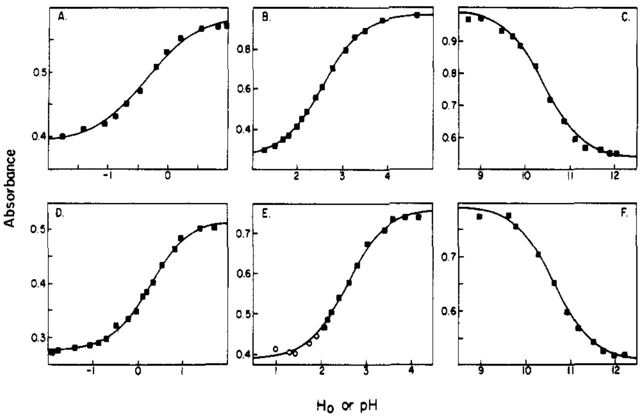

FIGURE 5: Determination of pK_a values of H_2biopterin and H_2folate from absorbance measurements. (A) Absorbance of 41 μM H_2biopterin measured at 258 nm in HCl of various concentrations; (B and C) absorbance of 40 μM H_2biopterin measured at 231 nm in 0.1 M sodium phosphate buffer; (D) absorbance of 30 μM H_2folate measured at 258 nm in HCl of various concentrations; (E and F) absorbance of 30 μM H_2folate measured at 228 nm in 0.02 M sodium phosphate buffer, except for open symbols (panel E) for which HCl solutions were used. Lines are best-fit curves obtained as described under Materials and Methods.

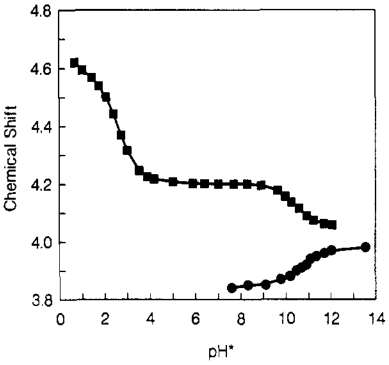

FIGURE 6: Determination of pK_a values of H_2biopterin and H_2folate from ^{1}H NMR at 23 °C. Upper curve, 7-CH_2 of H_2biopterin (dd). The chemical shifts are an average of the signals for H-7 and H-7′, which constitute an AB system. Lower curve, 7-CH_2 (s) of H_2folate.

electrode meter readings determined in D_2O (pD values) must be corrected to the corresponding pH values. However, pK_a values obtained from the NMR chemical shifts must also be corrected for the effect of D_2O on the ionization of the group studied. These two corrections approximately cancel, since the agreement with the spectrophotometric results is close.

Effect of 2-Mercaptoethanol on H_2Folate Spectra. Since H_2folate preparations are frequently stored in 2-mercaptoethanol and stock solutions are frequently prepared with this reagent present, care must be taken to avoid effects of this compound on absorbance spectra and on spectrophotometric determination of pK_a values. 2-Mercaptoethanol absorbs in the ultraviolet below 300 nm and has and absorbance maximum at <220 nm. When a 2 mM solution was examined at 228 nm or at 236 nm, the absorbance increased as the pH was increased with a pK_a of 9.07 ± 0.02. Attempts to determine the N^3–O^4 pK_a for H_2folate in the presence of 85 μM 2-mercaptoethanol from the absorbance *decrease* at 228 or 236 nm with increasing pH gave high values because of changes in absorbance due to mercaptoethanol over the pH range 8–10. In the presence of 200 μM mercaptoethanol, the absorbance at the indicated wavelengths *increased* with increased pH, and the apparent pK_a values were 9.53 ± 0.04 and 9.62 ± 0.02 at 22, and 236 nm, respectively. Thus, the absorbance change observed was largely that of 2-mercaptoethanol. However, absorbances at 325 nm (where mercaptoethanol does not absorb) from the same solutions gave a pK_a of 10.84 ± 0.11 which is close to the value obtained at lower wavelength in the absence of mercaptoethanol (Table I).

Effect of Ionic Strength on pK_a Values. The observed pK_a value for an ionizing species is identical with the thermodynamic pK_a only at zero ionic strength but in practical determinations can be corrected for the ionic strength whether or not this remains constant during the titration (Ellis & Morrison, 1982). The measured pK_a values in Table I were corrected to zero ionic strength (I = 0), and values were also calculated for I = 0.1 by the computational method of Poncelet et al. (1985) with the results shown in Table I. It may be seen that the corrections are relatively small.

DISCUSSION

It has long been known that strong acids convert H_2folate to a degradation product with an absorbance maximum at about 420 nm (Wright et al., 1958; Mathews & Huennekens, 1963a,b). Examination of the decomposition products from H_2folate at neutral pH indicated that they were (*p*-aminobenzoyl)glutamic acid and a pterin that was thought to be 7,8-dihydropterin-6-carboxaldehyde (Hillcoat et al., 1967). The identity of the pterin was later decisively established by Whiteley et al. (1968), but it was not investigated whether the same products were formed at low pH. Our results indicate that there is extensive loss of the (*p*-aminobenzoyl)glutamate side chain and that under aerobic conditions 7,8-dihydropterin-6-carboxaldehyde is also a major product.

In addition to irreversible breakdown of H_2folate, at least one other process occurs at acid pH and leads to marked changes in the UV absorption spectrum. This occurs if concentrated H_2folate (~1 mM) is added to a dilute HCl solution at pH 2.5 (Figure 1B). Repeat spectra show a progressive change occurring with a maximum at 310 nm developing, but only a slight increase in absorbance at 420 nm, indicating little formation of 7,8-dihydropterin-6-carboxaldehyde. After neutralization of such solutions, the UV spectrum is indistinguishable from that of H_2folate, and enzymatic assay and HPLC indicate recovery of 90–93% of H_2folate. Published NMR evidence indicates that there is dimerization of folate (Poe, 1973; Lam & Kotowycz, 1972) and H_2folate (Poe, 1973) in neutral solution at concentrations in the range 1–100 mM. The dimerization of H_2folate is readily reversible with a dissociation constant of 38.5 ± 1.5 mM at neutral pH (Poe, 1973). Poe did not study the extent of H_2folate dimerization below neutral pH because of the low solubility of H_2folate in this pH range. However, the loss of the charge from the glutamate carboxyl groups that is responsible for the decrease in solubility should also greatly favor dimerization, so that dimerization is possible at the low concentrations used in our studies.

Further evidence that the spectra of the type shown in Figure 1B are due to association was provided by the observation that these spectra were not observed at the same pH in the presence of chaotropic agents such as 6 M guanidine hydrochloride or 8 M urea. That the effect of these agents was not due to ionic strength effects was shown by the fact that 3.5 M ammonium sulfate did not abolish the anomalous spectrum.

Determination of the Amide Dissociation Constants. There is a large discrepancy between the value we obtained spectrophotometrically for the N^3–O^4 amide group of H_2folate (10.81 ± 0.01) and confirmed by NMR and the value (9.54 ± 0.02) reported by Poe (1973). The UV spectra that we obtained for H_2folate at pH 7.66 and 12.33 (Figure 3) agree closely with those reported by Poe (1977), but the latter do not include the range 220–240 nm. The titration curve reported by Poe (1973) indicates an absorbance *increase* at 236 nm as the pH is increased from 7 to 12, whereas we observed an absorbance *decrease* at this wavelength with an isosbestic point at 240 nm (Figure 3). Our pK_a value was obtained with the use of fresh solutions made up in a series of buffers with pH values over the desired range, and we used absorbance measurements at 228 nm where the molar extinction change is high (10 600 cm^{-1}). We found it critical for this determination to remove 2-mercaptoethanol which is usually included in stock solutions of H_2folate and suspensions of dihydrofolic acid, but which absorbs strongly in this region of the spectrum and also titrates over this pH range (pK_a 9.07 ± 0.02) with increasing absorbance as the pH is increased. If the absorbance was measured at 325 nm where 2-mercaptoethanol does not absorb, we obtained a pK_a of 10.59 ± 0.05 for H_2folate even in the presence of mercaptoethanol. This is in reasonable agreement with the other estimates considering the small absorbance change (Figure 3). It should be noted that spectral changes that occur when 7,8-dihydrobiopterin is adjusted from pH 7.0 to 13.08 (Figure 4) are very similar to those for H_2folate. Measurements of the pH dependence of absorbance at 231 nm gave a pK_a of 10.41 ± 0.04 for H_2biopterin. When the changes in NMR chemical shift of the C^7 protons of H_2folate and H_2biopterin were determined as a function of pH in the range of 7–12 (Figure 6), the pK_a values obtained confirmed the spectrophotometric result (Table I).

Determination of Cationic Dissociation Constants. The spectra reported by Poe (1977) for H_2folate at pH 0.8 and H_0 −1.4 are fairly similar to those we obtained at pH 1.39 and H_0 −0.51, respectively, but with some differences, in part due to differences in states of ionization resulting from the different pH values selected. Thus, the molar extinction coefficients at the maxima are different, and the maximum at 258 nm in the pH 1.39 spectrum (Figure 3) is only a shoulder in the pH 0.8 spectrum reported by Poe. The major difference, however, is the pH 2.6 spectrum reported by Poe. In this spectrum, the absorbance in the 274–282-nm range is lower than at either pH 0.8 or H_0 −1.4 while in the 310–330-nm region it has fallen much less than at pH 0.8, resulting in a very broad maximum. When we recorded spectra by mixing cold 60 μM H_2folate with an equal volume of cold buffer at the appropriate pH, we obtained no spectra of this type at any pH (e.g., Figure 1A). Instead, there was a continuous transition from the spectrum shown for pH 7.66 to that shown for pH 1.39, with a pK_a of 2.56 ± 0.03.

By using the change in absorbance at 314 nm over the range H_0 −0.5 to pH 5.0, we obtained data that fitted to two pK_a values much better than to a single value. The values obtained were 2.74 ± 0.03 and 0.48 ± 0.12. A better estimate of the higher pK_a was obtained by measuring the absorbance change at 228 nm and gave a value of 2.59 ± 0.03. The lower dissociation constant was obtained more accurately from the absorbance change at 258 nm, which gave a value of 0.28 ± 0.02. A very similar value was obtained by using the absorbance change at 280 nm. The absorbance changes occurring through the ionization with pK_a 2.59 are remarkably similar to those occurring in the UV spectrum of H_2biopterin as the dissociation with pK_a 2.56 ± 0.01 occurs. As each of these groups becomes protonated, there is a large decrease in absorbance over the range 220–240 nm, a considerable increase in absorbance over the range 255–260 nm, an absorbance decrease over the range 275–340 nm, and an increase in wavelengths >350 nm. This general similarity of the spectral changes indicates that the dissociating group is in the pterin structure, and on grounds previously suggested for H_4folate (Kallen & Jencks, 1966), N^5 is the most likely group. The association of the N^5 protonation with a pK_a at 2.6 is unequivocal on the basis of ^{15}N NMR studies of [5-^{15}N]H_2biopterin (Selinsky et al., 1989). It is also consistent with the larger pH titration shift observed for H-7,7′, H-11, and H-9 (data not shown) for the pK_a at 2.6 than at 10.4.

As the pH is dropped below pH 1, there are two groups of H_2folate that can become protonated: N^{10} in the (*p*-aminobenzoyl)glutamic acid moiety and N^1 in the dihydropterin ring. From the behavior of (*p*-aminobenzoyl)glutamate, protonation of N^{10} can be expected to lead to a major decrease in absorbance at 275–285 nm and increased absorbance at 220 nm (Kallen & Jencks, 1966). Since the difficulties of the system prevent performance of potentiometric titration of H_2folate in this pH range, it is not feasible to determine whether one or two groups are protonating as the solution is acidified from pH 1.0 to H_0 −0.51. Protonation with an apparent pK_a of 0.28 ± 0.02 results in spectral changes that to some extent resemble those occurring when H_2biopterin is protonated with pK_a −0.36 ± 0.05. In both cases, there is an absorbance increase in the long-wavelength region (>350 nm), but it is much greater in the case of H_2folate. The two protonated compounds have similar absorbance maxima (H_2folate, 275 nm; H_2biopterin, 269 nm), but the millimolar extinction coefficients are quite different (18.1 and 12.3 cm^{-1}, respectively). In fact, there is a good deal more absorbance by protonated H_2folate over the range 250–350 nm than by H_2biopterin but not as much as would be obtained by adding the molar extinction coefficient of unprotonated (*p*-aminobenzoyl)glutamic acid and the extinction of protonated H_2biopterin. In the spectrum of the latter at H_0 −1.76, there is a profound minimum at 299 nm which is absent from the H_2folate spectrum. The spectra also differ in the short-wavelength region (220–250 nm) with considerably more absorbance by H_2folate in the 220–235-nm region. These differences seem best explained by considering that the N^{10} group is largely if not completely unprotonated down to pH 0. The published pK_a for *N*-methyl-(*p*-aminobenzoyl)glutamic acid is 2.9 (Osborn et al., 1960), but the corresponding pK_a for N^{10} of H_2folate must drop below −0.51. Kallen and Jencks (1966) give a value of −1.25 for the pK_a of N^{10} in H_4folate and interpret the lower value as compared with *N*-methyl-(*p*-aminobenzoyl)glutamate as due to the positive charges on N^1 and N^5 in the pteridine ring. Accordingly, the pK_a of 0.28 for H_2folate must be due to protonation of N^1.

Effect of the Corrected N^5 pK_a on Modeling of the Mechanism of Dihydrofolate Reductase. Howell et al. (1987) reported the pH dependence of k_{cat} and k_{cat}/K_m for two mutants of *E. coli* DHFR. In one mutant, D27S, the active-site aspartate (Asp^{27}) was replaced with serine. In the other, D27S + T113E, an additional mutation provided an alternative active-site carboxyl by converting Thr^{113} to glutamate. The authors proposed a model for DHFR action in which either protonated or unprotonated H_2folate can bind to either protonated or unprotonated enzyme. Complexes, except that of unprotonated H_2folate with unprotonated enzyme, were assumed to be productive. The pK_a for H_2folate was taken as 3.8 as reported by Poe (1977). Curves fitting the data reasonably well were obtained with appropriate values of kinetic constants (Figure 7). However, substitution of the correct pK_a for H_2folate (2.59) results in a very poor fit to the data for the D27S mutant when combined with the values for the kinetic parameters chosen by Howell et al. (Figure 7A,C). This clearly illustrates the effect of the correction in the pK_a

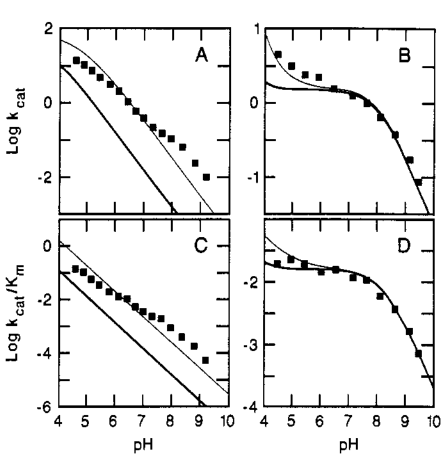

FIGURE 7: Effect of the correction of N^5 pK_a for H_2folate on model fitting for dihydrofolate reductase. Data for the D27S and D27S + T113E mutants of *E. coli* DHFR from Howell et al. (1987) were simulated for the mechanistic model assumed by those authors, using the same values of kinetic parameters that they used. Panels A and C are for the D27S mutant; panels B and D for the D27S + T113E mutant. Heavy lines were computed with the pK_a of 2.59 for H_2folate. Light lines were obtained with the pK_a of 3.8 (Poe, 1977).

Table II: Recalculated Kinetic Parameters for Two *E. coli* Mutant DHFRs[a]

kinetic parameter[b]	D27S DHFR A	D27S DHFR B	D27S + T113E DHFR A	D27S + T113E DHFR B
k_1 (s^{-1})	8.0	8.0	NC[c]	68.9
k_2 (s^{-1})			1.63	1.04
k_3/K_s[d]			0.75	0.097
K_x (μM)	452	494	106	104
K_y (μM)	4.57	0.288	NC[c]	0.037
K_t (μM)			130	136
pK_a			8.14	8.15

[a] Values are based on the model of Howell et al. (1987) and use for the pK_a for N^5 of H_2folate either (A) the literature value of 3.8 (Poe, 1977) or (B) the redetermined value of 2.59. [b] Values computed as described under Materials and Methods. The meaning of the parameters is explained in the text. [c] NC, not calculated. The best-fit value of K_y is so large that essentially no binding occurs with this value and the portion of the pathway involving $E \cdot H_2folateH^+$ may be treated as nonexistent. [d] Only the ratio can be obtained from these data. The value of K_s is large compared with K_t and K_x.

for H_2folate on modeling of the DHFR reaction.

The model of Howell et al. can, however, give a good fit to their data if the correct pK_a is used together with the computed best-fit values of the remaining eight parameters. The parameters used in their model are as follows, where E represents enzyme, H_2folate the unprotonated ligand, EH the protonated enzyme, and $H_2folate^+$ the ligand protonated at N^5. K_a and K_b are acid dissociation constants for the enzyme and ligand, respectively. k_1, k_2, and k_3 are catalytic rate constants for the formation of product from $E \cdot H_2folateH^+$, $EH \cdot H_2folate$, and $EH \cdot H_2folateH^+$, respectively. K_s, K_t, K_x, and K_y are dissociation constants for the complexes $EH \cdot H_2folateH^+$, $EH \cdot H_2folate$, $E \cdot H_2folate$, and $E \cdot H_2folateH^+$, respectively. It may be seen from Table II that use of the correct pK for H_2folate produces a major change in K_y and smaller changes in other parameters. It should be noted that even when the pK_b of 3.8 is used we obtained quite different values for some parameters from those published by Howell et al. (1987). The overall fit obtained with both pK_b values was about the same for both mutants as shown by Howell et al.

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