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Weak Interactions between Folate and Osmolytes in Solution

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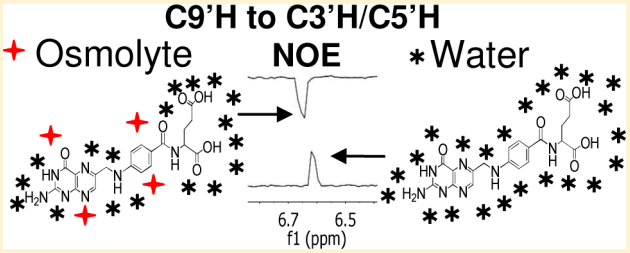
Supporting Information

ABSTRACT: Previous osmotic stress studies on the role of solvent in two structurally unrelated dihydrofolate reductases (DHFRs) found weaker binding of dihydrofolate (DHF) to either enzyme in the presence of osmolytes. To explain these unusual results, weak interactions between DHF and osmolytes were proposed, with a competition between osmolyte and DHFR for DHF. High osmolyte concentrations will inhibit binding of the cognate pair. To evaluate this hypothesis, we devised a small molecule approach. Dimerization of folate, monitored by nuclear magnetic resonance, was weakened 2−3-fold upon addition of betaine or dimethyl sulfoxide (DMSO), supporting preferential interaction of either osmolyte with the monomer (as it possesses a larger surface area). Nuclear Overhauser effect (NOE) spectroscopy experiments found a positive NOE for the interaction of the C3′/C5′ benzoyl ring protons with the C9 proton in buffer; however, a negative NOE was observed upon addition of betaine or DMSO. This change indicated a decreased tumbling rate, consistent with osmolyte interaction. Osmotic stress experiments also showed that betaine, DMSO, and sucrose preferentially interact with folate. Further, studies with the folate fragments, *p*-aminobenzoic acid and pterin 6-carboxylate, revealed interactions for both model compounds with betaine and sucrose. In contrast, DMSO was strongly excluded from the pterin ring but preferentially interacted with the *p*-aminobenzoyl moiety. These interactions are likely to be important in vivo because of the crowded conditions of the cell where weak contacts can more readily compete with specific binding interactions.



Vitamin B9, or folate, is an important cellular metabolite that is used by enzymes for diverse functions such as synthesis of deoxythymidine monophosphate used in DNA synthesis and one-carbon metabolism.[1] Enzymes in the folate cycle convert folate into its various biologically active derivatives. One such enzyme is dihydrofolate reductase (DHFR), which reduces dihydrofolate (DHF) to tetrahydrofolate (THF) using the cofactor NADPH.

There are two distinct types of DHFRs, type I and type II, which are structurally very different enzymes.[2] Type I DHFR is ubiquitous to all kingdoms of life and is well-conserved among different species.[3] The enzyme is a monomeric protein with two subdomains connected by a hinge region. A well-studied representative of type I DHFR is *Escherichia coli* DHFR (EcDHFR), which is a very efficient enzyme.[4] Conversely, type II DHFR is a homotetramer with 222 symmetry and a single active site pore in the middle of the structure.[5] R67 DHFR is an example of a type II DHFR and has been suggested to be an example of a primitive enzyme because of the constraints posed by its symmetric active site.[2] While EcDHFR is the target of trimethoprim, R67 DHFR provides resistance to this antibacterial drug.[6]

In our previous work, we examined the role of water in the binding of ligands to the two types of DHFRs.[7,8] Addition of osmolytes decreases the effective concentration of water, so release of water upon ligand binding should result in tighter binding in the presence of osmolytes. Conversely, osmolytes should decrease the affinity of a ligand for which there is water uptake upon binding. Tighter binding of NADPH to R67 DHFR occurs with an increasing osmolyte concentration,[7] which is consistent with water release upon binding. The change in binding was the same for all osmolytes used; this is consistent with preferential exclusion of the osmolytes from the protein.[9−11] Binding of DHF to R67 DHFR, however, was weaker with an increasing osmolyte concentration, which is indicative of water uptake upon ligand binding.[7] To explore this unusual result, the effect of osmolytes on binding of a ligand to EcDHFR was examined.[8] In the case of NADPH binding to EcDHFR, the affinity increased with most of the osmolytes used, the lone exception being binding studies performed with sucrose as the osmolyte, and water uptake was noted. For DHF binding to EcDHFR, "water uptake" was again noted for all osmolytes, with Δn_w values (the number of waters released or taken up upon binding of the ligand to the protein) similar to that of R67 DHFR.

Net water uptake was observed for DHF binding to both R67 DHFR and EcDHFR. A similar effect for the two dissimilar proteins suggests the decrease in affinity with osmolytes is probably not due to interaction of the proteins with the osmolytes. Additionally, because of the symmetry of the R67 DHFR tetramer, both NADPH and DHF have related binding

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sites in the active site pore. The cofactor had a net release of water upon binding to R67 DHFR, while there was "water uptake" for DHF. These results further suggest the uptake of water is not related to differences in the preferential solvation of the protein by the osmolytes. This leaves interaction of the osmolyte with the substrate, DHF, as the most likely reason for "water uptake". In our previous work, we hypothesized that "water uptake" can be explained by interaction of osmolytes with the substrate, DHF.[8] If binding of the osmolyte to DHF is tighter than that of water, then osmolytes would need to dissociate prior to binding of DHF to DHFR.

Here we test our hypothesis that osmolytes interact with folate derivatives by osmometry and nuclear magnetic resonance (NMR) approaches. Folate is used as a model for DHF because of its greater stability compared to that of DHF. Folate also displays "water uptake" upon binding to R67 DHFR in the presence of osmolytes.[7] The DHFR cofactor NADPH, or its oxidized form, $NADP^+$, is used as a control for osmolyte interactions because our previous studies indicated water release upon binding of the cofactor to DHFR. The structures of folate and NADPH are given in Figure 1. Some alternate explanations are also explored for the decrease in the affinity of folate and DHF for the two DHFR enzymes caused by osmolytes.

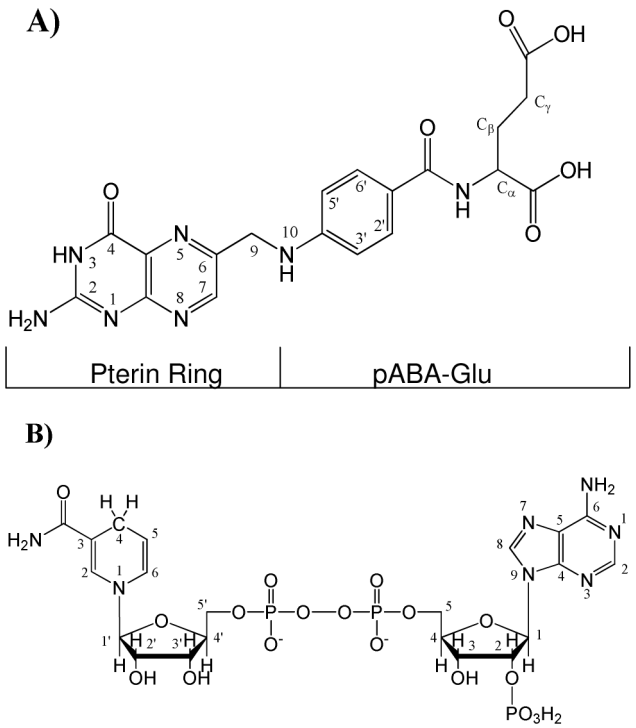

Figure 1. (A) Structure and numbering of folic acid with the pterin ring system and the *p*-aminobenzoyl-glutamate (pABA-Glu) portions of the molecule indicated. Reduction of the C7–N8 bond of folate yields dihydrofolate. (B) NADPH structure along with its numbering.

EXPERIMENTAL PROCEDURES

Materials. Betaine, sucrose, pterin-6-carboxylic acid (P6C), *p*-aminobenzoyl glutamic acid (pABA-Glu), and folate were purchased from Sigma-Aldrich. DHF was prepared from folate as described previously.[12] Glycerol, ethylene glycol, PEG400, NADPH, and $NADP^+$ were purchased from Fisher Scientific. For NMR studies, D_2O, DCl [33% (w/v) in D_2O], NaOD [40% (w/v) in D_2O], Tris-d_{11} (1 M in D_2O), DMSO-d_6, and betaine-d_{11} were purchased from Cambridge Isotope Laboratories.

Folate pK_a. The pK_a of the N3–O4 group on folate was monitored by absorbance spectroscopy according to the method of Poe.[13] A solution of 12–16 μM folate was prepared in MTA buffer (100 mM Tris, 50 mM MES, and 50 mM acetic acid, which has an ionic strength of 0.1 between pH 4.5 and 9.5[14]), and the pH was adjusted to 10.5 with 0.1 M NaOH. Spectra were recorded on a Perkin-Elmer Lambda 35 spectrometer from 220 to 500 nm with a 0.5 nm resolution at 25 °C. The solution was titrated with small volumes of 1–6 M HCl, and the pH was measured. Titrations were also performed with 12–16 μM folate in MTA buffer with 20% betaine or 20% DMSO. Duplicate experiments were performed. Titration curves were fit to eq 1:

$$A_{obs} = (A_{fh} - A_f)/(1 + 10^{-pK_a}/10^{-pH}) + A_f \quad (1)$$

where A_{obs} is the observed absorbance, A_{fh} is the absorbance of protonated folate, and A_f is the absorbance of deprotonated folate.

NMR. Dimerization of folate was monitored by NMR via a method similar to that described by Poe.[13] Stock solutions of folate were prepared in 10 mM deuterated Tris (pH 7.1) or in deuterated Tris with 20% osmolyte (deuterated betaine or deuterated DMSO). The concentration of folate was determined by absorbance (ε_{282} = 27000 M^{-1} cm^{-1}).[15] Final folate concentrations of the stock solutions were between 75 and 200 mM. NMR samples were prepared at 150 mM or, in the case of 20% DMSO, at 75 mM because of insufficient solubility. Spectra were recorded, and then the sample was diluted with buffer containing the appropriate osmolyte. This process was repeated until a folate concentration of 0.5 mM was reached. Spectra were recorded on a Varian 500 MHz NMR spectrometer with a pulse length of 3.7 μs using 64 scans from 14 to −0.5 ppm per spectrum. Spectra were processed using MestreNova version 7 (Mestrelab Research, Compostela, Spain).[16] The spectra were phase and baseline corrected, and the peaks were referenced to either the water peak or, in the case of samples with 20% DMSO, the DMSO peak.

From plots of the chemical shifts (measured in parts per million) versus concentration, the dimerization constant was calculated according to eq 2:

$$\delta_{obs} = \delta_D + (\delta_M - \delta_D)[-K_d + (K_d^2 + 8K_d[F]_{tot})^{1/2}]/(4[F]_{tot}) \quad (2)$$

where δ_{obs} is the observed chemical shift, δ_M and δ_D are the chemical shifts of pure monomer and pure dimer, respectively, K_d is the dimerization constant, and $[F]_{tot}$ is the total folate concentration.

^{1}H NOESY. Homonuclear (^{1}H) NOESY experiments were performed with folate (5 mM in D_2O) in either buffer [10 mM deuterated Tris (pH 7.1)] or buffer containing 20% (v/v) deuterated DMSO or 20% (w/v) betaine-d_{11}. The samples were adjusted with 0.1 M DCl or 0.1 M NaOD to pH 7.0. Experiments were performed on a Varian 500 MHz NMR spectrometer at 299 °K. The effect of mixing time on the NOEs was measured by altering the mixing time from 100 to 2000 ms. Spectra were recorded with 1000 t_1 × 2000 t_2 points, using eight scans per 256 increments and a delay time of 1.5 s after each scan. Data were processed using MestreNova. The spectra were phase and baseline corrected to optimize the aromatic

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proton NOEs. No other cosmetic procedures were applied. As a control, the effect of osmolytes on the NADPH (5 mM) NOEs at different mixing times was also determined.

Osmometry. Osmotic stress experiments were performed on a Wescor Vapro 5520 osmometer. Stock solutions of osmolytes were prepared in water and kept at 4 °C prior to being used. New osmolyte stock solutions were prepared every few days. Folate and $NADP^+$ stock solutions (~200 mM) were prepared fresh daily in 10 mM Tris-HCl (pH 7.0). A series of solutions at different osmolyte concentrations were prepared, and each osmolality reading was measured in triplicate. Solutions were prepared such that the osmolality ranged between 0.15 and 0.9 Osm. Then, solutions containing 100−125 mM folate with equivalent osmolyte concentrations were prepared, and the osmolality of the solutions was measured. The data were fit to eq 3 according to Harries:[17]

$$\Delta m_s^{osm}/m_s^{o} \approx n_w(m_m/55.6) \quad (3)$$

The difference in osmolality between the solution with and without folate, Δm_s^{osm}, is plotted versus the change in osmolyte molality, m_s^{o}. The slope of the plot is the product of the number of osmolyte-excluding waters, n_w, and the ratio of the osmolality of folate, m_m, to the molality of water. The number of osmolyte-excluding waters was determined for folate with betaine, DMSO, sucrose, ethylene glycol, glycerol, and PEG400. Duplicate, or triplicate, experiments were performed to obtain errors for the number of excluding waters. Similar experiments were conducted for 110−135 mM $NADP^+$ with betaine, DMSO, and sucrose.

Additional experiments were performed to improve our understanding of osmolyte−folate interactions. The number of excluding waters for folate was measured at a number of pH values up to 10.4 using betaine, DMSO, and sucrose. Also, osmotic stress experiments were performed with the model folate fragments *p*-aminobenzoyl glutamate (pABA-Glu) and pterin-6-carboxylate (P6C) at pH 10.4 with betaine, DMSO, and sucrose. The accessible surface area (ASA) was determined for the folate monomer, folate dimer, $NADP^+$, pABA-Glu, and P6C using the molecular modeling program MOE version 2009.10 (Chemical Computing Group, Ltd., Montréal, QC). A head-to-tail model of the folate dimer in which the pteridine ring of each monomer stacks with the pABA ring of the other monomer and the glutamates dangle free was proposed by Poe.[13] This dimer was built in MOE and then energy minimized.

Isothermal Titration Calorimetry. Affinities, stoichiometries, and ΔH values associated with binding of DHF to EcDHFR·$NADP^+$ were determined using isothermal titration calorimetry (ITC) at 25 °C as previously described.[18] At least two replicate titrations for each condition were performed using a VP-ITC microcalorimeter from MicroCal Inc. (Northampton, MA). Injections were separated by 240 s to allow baseline equilibration. The concentration of DHFR was 12 μM, and the concentration of $NADP^+$ was 200 μM. MTA buffer (pH 7) containing 1 mM EDTA and 5 mM β-mercaptoethanol was used. The "*c* value" ($=[P]_{total}/K_d$) ranged from 5 to 25, within the suggested range of 1−1000.[19]

Origin version 7 and SEDPHAT[20] were used for ITC data analysis. Origin fits were exported into SEDPHAT, and global fitting of replicate data sets was performed using the single-site model (A + B ↔ AB). Errors were calculated using the Monte Carlo for nonlinear regression option in the program.

The effect of either 50 or 100 μM (monomer concentration) bovine serum albumin (BSA) on DHF ternary complex formation was investigated. Fits of matched data sets were performed as described above. In addition, a global fit of all data sets was performed using the A + B + C ⇆ AB + C ⇆ AC + B (competing B and C for A) model in SEDPHAT. As a control, BSA effects on $NADP^+$ ternary complex formation were also measured, with $NADP^+$ injections into 12 μM EcDHFR saturated with 100 μM DHF. For these titrations, the *c* value ranged from 10 to 16.

RESULTS

Folate pK_a. One possibility for explaining "water uptake" in our previous studies of binding of DHF to R67 DHFR and EcDHFR is that osmolytes can alter the K_d for folate dimer formation. An increase in the dimer concentration of DHF would weaken the affinity of DHFR for the substrate as only monomeric DHF binds to DHFR.[21] As folate dimerization is pH-dependent,[13] we first determined whether the presence of osmolytes altered the N3−O4 pK_a of folate. Folate absorbance peaks increase or decrease in intensity as the protonation state of the N3−O4 amide in the pterin ring changes.[12,13,22] In the folate spectra, the intensities of the 255 and 370 nm peaks decrease and that of the 280 nm peak increases with a decrease in the pH of the solution (Figure S1 of the Supporting Information). This pattern agrees with previous folate pK_a titrations.[13,22,23] From our data, a pK_a of 7.94 ± 0.06 for folate was calculated according to eq 1. As a comparison, Poe found the pK_a to be 8.38 from a global fit of folate concentration versus chemical shift curves at three different pH values.[13] However, more recently, the pK_a for the N3−O4 amide of folate was found to be 7.98, at an ionic strength of 0.15.[23] The latter value agrees with our pK_a for folate, so we continued with similar pK_a titrations performed in MTA buffer with 20% betaine or 20% DMSO. The pK_a values under all solvent conditions are listed in Table 1. The pK_a of folate is only marginally greater (<0.2 pH unit) in the presence of osmolytes compared to that in buffer alone. As our NMR experiments are performed at pH 7.1 and if this ΔpK_a has an effect on folate dimerization, it would be predicted to increase the concentration of neutral folate species as this dimerization K_d is much lower (20 mM) than for the basic folate species (340 mM).[13]

Table 1. pK_a Values for the N3−O4 Proton of Folate in MTA Buffer, with or without 20% Betaine or 20% DMSO[a]

osmolyte	osmolality (Osm)	pK_a
none	0.20	7.94 ± 0.06
20% betaine	2.22	8.10 ± 0.06
20% DMSO	3.27	8.11 ± 0.01

[a]The spectral shift in the absorbance titration at 370 nm was used in the data analysis.

Folate Dimerization. According to Poe, when the N3−O4 amide of the pterin ring in folate is fully protonated, folate forms a dimer with a dimerization constant of 20 mM.[13] If osmolytes were to increase the concentration of the dimer in solution, this in turn could decrease the affinity of folate toward DHFR as the dimer would need to dissociate, accompanied by water uptake at the monomer surfaces, to bind to the enzyme.[21] To explore this possibility, the K_d for folate dimerization was measured using ^{1}H NMR at pH 7.1. The C7, C9, C3′/C5′, and C2′/C6′ chemical shifts all moved downfield with an increase in the folate concentration, while the NMR signals from the glutamate moiety on folate were unaffected by the concentration

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of folate. These results are consistent with earlier NMR studies.[13] The sum of the C7, C9, C3′/C5′, and C2′/C6 proton chemical shifts was fit to eq 2, giving a dimerization K_d of 80 ± 10 mM for folate at pH 7.1. Dimerization experiments were also performed in 20% betaine and 20% DMSO. The K_d for the folate monomer−dimer equilibrium increased 2-fold in the presence of betaine (2.34 Osm) to 160 ± 40 mM, and the K_d was even greater in 20% DMSO (3.23 Osm) at 200 ± 20 mM. Because the ΔpK_a shift predicts an opposite trend, it cannot be the underlying cause. Rather, if the interaction of osmolytes with folate is favored over hydration, then all equilibria will be shifted to the state with the larger surface area.[24]

To determine which protons are more sensitive to folate dimerization, the chemical shifts for each individual proton were fit to eq 2 (Figure 2). Chemical shifts of protons in the benzoyl ring, C7 in the pterin ring, and the C9 methylene shifted with an increase in folate concentration. The glutamate protons did not shift with changes in folate concentration [shown for the Cα proton (Figure 2E)]. These results are consistent with Poe's head-to-tail dimer model in which each pterin ring stacks with the pABA ring of the other monomer and the glutamate tails are free to rotate.[13] Dimerization constants for the individual folate protons are listed in Table 2. The lower K_d values for the C9 and C3′/C5′ protons suggest they are more sensitive to folate dimerization than those for the pterin ring C7 and the C2′/C6′ protons.

The effect of osmolytes on the K_d values determined for the individual folate protons was also explored (Figure 2). It is apparent that the chemical shifts change to a lesser extent with folate concentration in the presence of osmolytes. The data were fit to determine the dimerization constant for each proton (Table 2). Compared to those in buffer, the K_d for the C9 proton increased 2-fold in 20% betaine and 3-fold in 20% DMSO. The K_d of the C7 proton is little changed in DMSO compared to buffer, while it is almost 2 times greater in betaine. Additionally, the K_d from the benzoyl ring protons remains the same in betaine and buffer, but the dimer is weakened by half in the presence of DMSO. The K_d values for the C7 and C2′/C6′ protons were ∼4 and ∼2 times greater than the overall K_d for betaine and DMSO, respectively. If these protons are near the outer edges of the stacked dimer, perhaps molecular motion weakens their K_d values. Overall, the presence of osmolytes decreases the concentration of the dimer. While the shift in the folate dimerization equilibrium toward the monomer in the presence of osmolytes will not appreciably affect the binding of folate to DHFR, it does give clear evidence in favor of the interaction of osmolytes with folate.

NOE. Folate−osmolyte interactions were explored further using NOESY. A representative set of NOESY spectra for 5 mM folate is shown in Figure S2 of the Supporting Information at a mixing time of 750 ms. The most notable NOEs are between the protons on C7 with C9 and C3′/C5′, and also between the C9 proton and the C3′/C5′ protons. Most of the NOEs noted in the spectra are to be expected for a monomeric folate molecule. NOEs between just the protons within the glutamate moiety or between the two aromatic systems and their methylene bridge are also noted. Similar NOEs are present in the folate samples containing osmolytes. No new NOEs appear in osmolyte solutions. These results indicate that folate is an extended molecule in solution, consistent with the folate concentration (5 mM), which is below the folate K_d. No intermolecular NOEs between folate and water or with either osmolyte are noted in the spectra. Upon examination of the spectra, the sign of the NOE between the C9 and C3′/C5′ protons in both osmolytes is opposite to that in buffer alone (Figure 3). A change in the sign of the NOE from positive to negative is indicative of a slowing of the rotational rate of the pABA ring protons in folate, supporting osmolyte interaction.[25]

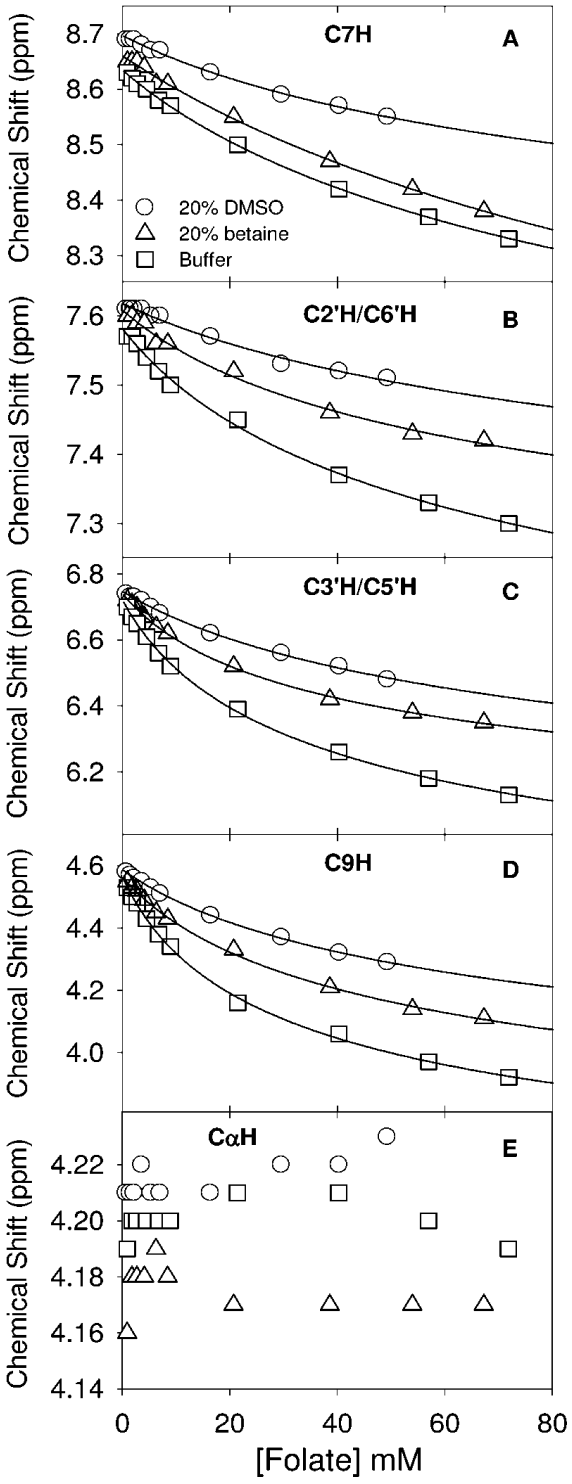

Figure 2. Effect of folate concentration on the chemical shifts in 10 mM Tris-d_{11} (□), 10 mM Tris-d_{11} with 20% deuterated betaine (△), and 10 mM Tris-d_{11} with deuterated DMSO (○). The pH was 7.1. Lines through the data show the fit to eq 2. Best fit values are listed in Table 2.

Further information for osmolyte effects on the conformation of the folate molecule can potentially be gained from NOE

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Table 2. Dimerization Constants for Folate Obtained from the Proton Chemical Shifts in 10 mM Tris-DCl Buffer (pH 7.1) or in Buffer Containing 20% Betaine or 20% DMSO

		K_d (mM)			
solvent	osmolality (Osm)	C7	C9	C3′/C5′	C2′/C6′
buffer	0.20	250 ± 20	60 ± 10	80 ± 10	150 ± 30
20% betaine	2.34	430 ± 130	110 ± 20	90 ± 10	160 ± 50
20% DMSO	3.23	260 ± 70	160 ± 10	200 ± 40	280 ± 170

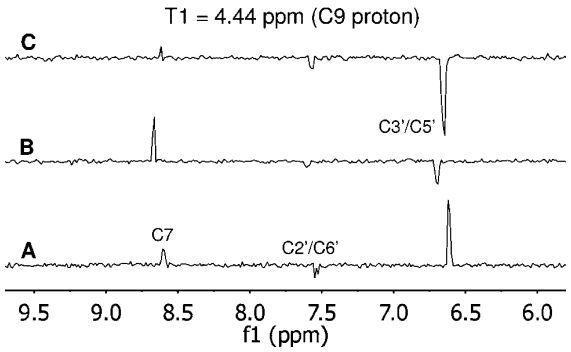

Figure 3. Stacked NOESY spectra slices for 5 mM folate in (A) 10 mM Tris-DCl buffer (pH 7.0), (B) 10 mM Tris-DCl (pH 7.0) with 20% DMSO, and (C) 10 mM Tris-DCl (pH 7.0) with 20% betaine. Comparison of the spectra shows a change in sign of the NOEs for the protons from C9 with the C3′/C5′ protons. The complete NOESY spectra for each buffer condition are given in Figure S2 of the Supporting Information.

buildup curves. In a NOE buildup curve, the NOE signal increases linearly with the mixing time of the experiment. If the nuclear spin diffuses to other protons, then the NOE signal will plateau at longer time points. NOESY spectra were recorded for folate with mixing times from 100 to 2000 ms, and the relative percent NOE was determined from the integrated area of the NOE relative to that of the cross-peak NOE (see Figure S3 of the Supporting Information). While there are differences in the rate of buildup, it is not clear whether solvation by, or interactions with, an osmolyte leads to this change. In an attempt to differentiate between the two factors, NOE buildup experiments were performed for NADPH (Figure S4 of the Supporting Information) using proton assignments for NADPH from Oppenheimer.[26] In buffer, NOEs were noted between the protons in the nicotinamide ring. Because water was released upon binding of NADPH to DHFR,[7,8] and no interactions between osmolytes and NADPH were expected, NADPH was used as a control for the osmolyte effects. However, the results in the osmolyte solutions were significantly different from those in buffer alone, with a significant loss of some NOEs. Clearly, osmolytes affect the conformation and thus the NOEs of both folate and NADPH, suggesting multiple factors, such as viscosity, bulk magnetic susceptibility, and/or complexation between the molecules and the osmolytes,[25,27] contribute to the difference between "water uptake" for folate and water release for NADPH binding to DHFR.

Osmotic Stress. Osmotic stress experiments can reveal information about the preferential solvation of molecules by osmolytes.[13,22,23] In these experiments, the difference in the osmolality of an osmolyte solution with and without folate is plotted versus the osmolality of the osmolyte. If the osmolytes interact with folate, the bulk water activity will increase relative to that in the osmolyte-only solution. While identical values would indicate no interaction, a positive slope for the plot of Δm_s^{osm} versus osmolality is proportional to the number of osmolyte-excluding waters in the folate solvation sphere, according to eq 3. Negative slopes mean that osmolytes strongly interact with folate and exclude water from folate. The difference between the osmolality of the betaine solutions with and without folate, Δm_s^{osm}, was plotted versus the osmolality of the osmolyte-only solution (see Figure S5 of the Supporting Information). There are two processes that can affect the osmotic pressure of the betaine-containing solution. First, betaine shifts the dimer equilibrium toward the folate monomer; this increases the osmotic pressure. However, the interaction of betaine with folate decreases the osmotic pressure. The net effect is monitored. The number of waters associated with folate that are not removed from the solvation sphere by added osmolyte (which equals the number of osmolyte-excluding waters) was found to be 42 ± 6 for folate at pH 7.0 for betaine. Osmotic stress experiments were performed for folate using several other osmolytes to determine if any osmolyte is more excluded than others; Table 3 lists the number of excluding waters. The larger PEG400 is significantly more excluded from the surface, perhaps as its size and mass are similar to those of folate (molecular weight of 441 for folate).

A prediction of the number of waters expected to associate with folate can be made from the accessible surface area (ASA) of the molecule. Approximating an area of 9 Å^2 for water[28] and assuming that there is only a monolayer of water surrounding the molecule, we find the predicted number of waters associated with the folate dimer is 115 (Table 3). The number of osmolyte-excluding waters determined from osmotic stress experiments is significantly lower than this value, irrespective of the osmolyte used. These results suggest that many different osmolytes may be preferentially interacting with folate.

Table 3. Numbers of Osmolyte-Excluding Waters for the Folate Dimer or NADP$^+$ in 10 mM Tris-HCl (pH 7.0)

osmolyte	folate	NADP$^+$
predicted	115	89
betaine	42 ± 6	51 ± 6
DMSO	47 ± 8	57 ± 3
sucrose	43 ± 3	−49 ± 4
ethylene glycol	37 ± 4	N/D[a]
glycerol	29 ± 7	N/D[a]
PEG400	72 ± 8	N/D[a]

[a]Not determined.

NADP$^+$ was used as a control for these experiments. In previous studies on the role of water in NADP$^+$ and DHF binding to EcDHFR,[7,8] water was released upon binding of NADP$^+$ to EcDHFR, while water was taken up upon DHF binding. If, as those experiments suggested, there is preferential exclusion of osmolytes from NADP$^+$, then perhaps a full, or nearly full, complement of waters might be associated with the NADP$^+$ molecule. The maximal number of waters associated

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with $NADP^+$ is predicted to be 89. However, the observed values for $NADP^+$ are smaller than those predicted (Table 3). In addition, a negative value for the number of excluding waters is obtained with sucrose. These results suggest that sucrose preferentially interacts with $NADP^+$. This observation agrees with our previous observation of a net uptake of water accompanying binding of $NADP^+$ to EcDHFR in the presence of sucrose.[8] Preferential interaction of sucrose with $NADP^+$ suggests that sucrose has to be released prior to binding of $NADP^+$ to EcDHFR, resulting in a decrease in binding affinity.

Our NMR studies yielded a dimerization constant of 80 mM for folate at pH 7.1. This means that the majority of the folate in the osmotic stress samples was dimeric. Because the basic form of folate has a much higher dimerization constant of 390 mM,[13] the fraction of folate monomer increases at higher pH values. Therefore, the number of excluding waters was measured at higher pH values to determine if fewer waters are associated with the folate monomer and/or the basic folate molecule (Figure 4). The number of waters associated with

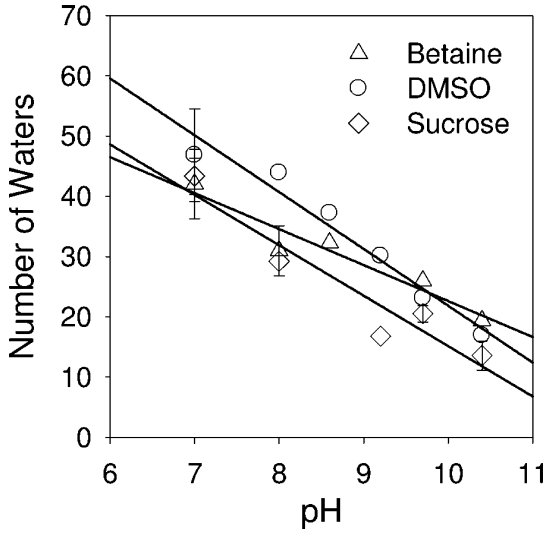

Figure 4. Effect of pH on the number of waters associated with folate that exclude the osmolytes betaine (△), DMSO (○), and sucrose (◇). In some cases, the error bars are smaller than the symbols.

folate decreases with an increase in pH from around 40 at pH 7 to 15 at pH 10.4. The number of excluding waters at pH 10.4 is much smaller than the 78 waters predicted for the folate monomer. It seems that water is loosely bound and displacement by many different osmolytes can occur.

To understand if any part of the folate molecule is more likely to interact with osmolytes, osmotic stress experiments were performed with the folate fragments *p*-aminobenzoyl glutamic acid (pABA-Glu) and pterin-6-carboxylic acid (P6C). Experiments were performed at pH 10.4 because of the insolubility of P6C at pH 7 and also to aid comparison with the results for monomeric folate. For betaine, the number of excluding waters was smaller for each of the folate fragments than it was for folate, and all were much smaller than the predicted values (Table 4). These results predict strong interaction of betaine with both the P6C and pABA-glu fragments. A somewhat different scenario is observed with DMSO, as the number of excluding waters is higher in each of the fragments than for the folate molecule itself. However, all values are smaller than expected from ASA calculations. Sucrose

Table 4. Numbers of Osmolyte-Excluding Waters Associated with the Folate Monomer and Folate Fragments at pH 10.4

	no. of excluding waters			
molecule	betaine	DMSO	sucrose	predicted
folate	23 ± 5	20 ± 4	17 ± 5	78
pABA-Glu	6 ± 3	32 ± 6	16 ± 1	53
P6C	1 ± 2	37 ± 2	−19 ± 2	41

shows very different behavior, with a negative value for interaction with P6C, indicating a very strong association of these two molecules. We also note that the sum of the number of osmolyte-excluding waters for pABA-Glu and P6C does not equal the number of osmolyte-excluding waters for folate alone. This may be due to pABA-Glu and P6C not being the exact fragments of folate.

Isothermal Titration Calorimetry in the Presence of Bovine Serum Albumin. An extension of our hypothesis that osmolytes interact with folate and impede binding to DHFR is that folate may bind to various macromolecules. Albumin, for example, binds numerous small, hydrophobic molecules[29] and has previously been identified as a low-affinity, high capacity folate binding protein.[26−28] To determine if addition of BSA weakens the binding of DHF to EcDHFR·$NADP^+$, we performed a series of ITC titrations. The K_d values obtained from global fitting of at least two separate titrations are listed in Table 5. The addition of 50 or 100 μM BSA weakens DHF binding by a factor of 2- or 3-fold, respectively. These results support a weak DHF−BSA interaction, which needs to be broken prior to binding of DHF to EcDHFR·$NADP^+$. Using SEDPHAT, we additionally performed a global fit of six data sets (with 0−100 μM BSA added) to an A + B + C ⇆ AB + C ⇆ AC + B (competing B and C for A) model. The global fit is shown in Figure S6 of the Supporting Information, and the fit values are listed in Table 5. The constant for binding of DHF to BSA is 51 μM, which is ∼20-fold tighter than the value of 1.1 mM previously obtained using equilibrium dialysis.[30] Finally, a control titration of binding of $NADP^+$ to EcDHFR·DHF did not show any difference in K_d upon addition of 100 μM BSA.

DISCUSSION

Osmotic stress studies in dihydrofolate reductase suggest that water uptake is required for the substrate, DHF, to bind.[7,8] Water uptake upon ligand binding is highly unusual,[31−33] and because water uptake occurred with two structurally different DHFR enzymes, we hypothesized that osmolytes interact with folate.[8] The notion of organic molecules interacting with folate derivatives has some precedence as crystals of methotrexate from water/organic solvent mixtures were found to have organic solvate in the crystal lattice.[34] Additionally, anthracene has been proposed to interact with folate from Fourier transform infrared studies.[35] Therefore, we devised a series of experiments to test our hypothesis about folate−osmolyte interactions.

Interaction of Osmolytes with the Glutamate Tail. Because the glutamate tail is not involved in folate dimerization, no information was gained from the folate dimerization experiments. Buildup of NOEs between the glutamate protons was not affected by the presence of either DMSO or betaine. Osmolytes did not significantly perturb the environment around these protons, and we conclude that osmolytes probably do not interact with the glutamate tail of folate. This is consistent with the Record lab finding that betaine does not interact with carboxylate group oxygens,[36] such as glutamate.[37]

Table 5. Thermodynamic Parameters Associated with Ternary Complex Formation in MTA Buffer at pH 7, 25 °C, and μ = 0.1[a]

complex	K_d (μM)	ΔG (kcal/mol)	ΔH_{obs} (kcal/mol)	$T\Delta S$ (kcal/mol)	N
DHF to EcDHFR·NADP$^+$	0.354 ± 0.014	−8.80	−8.47 ± 0.05	0.32	0.73 ± 0.01
DHF to EcDHFR·NADP$^+$ with 50 μM BSA	0.701 ± 0.052	−8.39	−8.17 ± 0.14	0.22	0.69 ± 0.01
DHF to EcDHFR·NADP$^+$ with 100 μM BSA	1.10 ± 0.008	−8.12	−8.43 ± 0.17	−0.31	0.72 ± 0.01
global fit of all DHF ternary data sets	0.355 ± 0.016 (DHFR)	−8.80 (DHFR)	−8.45 ± 0.061 (DHFR)	0.34 (DHFR)	0.72 ± 0.01 (DHFR)
	50.7 ± 6.0 (BSA)	−5.86 (BSA)	−0.51 ± 0.23 (BSA)	5.35 (BSA)	1.0 (BSA)
NADP$^+$ to EcDHFR·DHF	1.08 ± 0.04	−8.14	−9.36 ± 0.09	−1.22	0.92 ± 0.01
NADP$^+$ to EcDHFR·DHF with 100 μM BSA	1.09 ± 0.05	−8.13	−8.31 ± 0.09	−0.18	0.94 ± 0.01

[a]Global fits of two data sets to a single-site model (A + B ↔ AB) were analyzed using SEDPHAT.[20] Global fits for the DHF ITC titrations were also performed using SEDPHAT and a competitive binding model. The Gibbs energy values were calculated from the equation $\Delta G = -RT \ln K_d$, and $T\Delta S$ values were obtained from the relationship $\Delta G = \Delta H - T\Delta S$.

Interaction of Osmolytes with the Pterin Moiety. As folate has limited protons on its pteridine ring, it is difficult to discern from our NMR results if osmolytes interact with this moiety. Specifically, the only nonexchangeable proton on the pterin ring is the C7 proton. Betaine decreases the K_d associated with this pterin ring proton, suggesting that betaine interacts with the pterin ring. Additionally, in osmotic stress experiments, P6C was preferentially solvated by betaine. A recent study of interaction of betaine with small molecules found betaine favorably interacts with aromatic, amine, and amide molecules, most likely through cation−π and hydrogen bonding between the carboxylate of betaine and the nitrogen protons.[36,37] There is also the potential for anion−quadrupole interactions between the ring systems and the carboxylate of betaine.[38] However, any potential osmolyte interaction due solely to the aromaticity of the folate pterin ring would be mitigated in DHF because reduction of the C7−N8 bond destroys the aromaticity of the pterin ring.

In contrast to betaine, DMSO does not affect the K_d for the C7 proton. Also, the buildup of the NOE between the C7 and C3′/C5′ protons was not affected by DMSO. Further, the number of excluding waters (37 ± 2) describing interaction of DMSO with P6C is near the predicted value of 41. This result suggests DMSO is highly excluded from the solvent shell of P6C. However, both betaine and DMSO do preferentially solvate aromatic systems because the NOEs arising from the aromatic protons in NADPH decreased or, in the case of the nicotinamide ring, were completely eliminated, in the presence of either osmolyte. Reports showing intermolecular NOEs between the aromatic protons of adenosine and trifluoroethanol in a mixture with water,[39] or in neat DMSO,[40] indicate that osmolytes can preferentially solvate aromatic systems like adenine and nicotinamide rings. Additionally, our osmotic stress experiments suggest that sucrose interacts more strongly with P6C than water. All these data suggest that betaine and sucrose interact with the pterin ring of folate, while DMSO probably does not.

Interaction of Osmolytes with the pABA Moiety. In contrast to the data for the pterin ring, the data for the pABA moiety require more consideration. While betaine preferentially solvates pABA-Glu according to the osmotic stress experiments, it does not affect the K_d for the benzoyl protons. However, NOEs between the C3′/C5′ and C9 protons changed from positive in buffer to negative in buffer with betaine, suggesting betaine does associate with the pABA ring and changes the rotational correlation time of the protons. The change in sign of the NOE (Figure 3), along with the osmotic stress experiments, gives strong evidence of the interaction of betaine with the pABA ring.

The evidence is more consistent for the interaction of DMSO with the pABA ring. DMSO weakens the K_d determined using the benzoyl ring and C9 protons. The sign of the NOE between the C9 and C3′/C5′ was negative in 20% DMSO, while it was positive in buffer alone. Finally, the number of DMSO-excluding waters was half the predicted value. If we calculate the expected number of excluding waters for the pABA ring only, this value is 34. This is within error of the experimental value (32 ± 6). This congruency strongly supports interaction of DMSO with the pABA ring. Finally, our studies with sucrose were limited to osmotic stress measurements. From a comparison of calculated and measured solvation waters, sucrose does appear to interact with the pABA ring.

These studies find that osmolytes show interaction preferences, consistent with some degree of discrimination. For example, betaine interacts strongly with the pterin ring while DMSO appears to be excluded. Both DMSO and betaine interact with the glutamate, and both appear to be excluded from the glutamate tail. However, we note that several different types of effects may be occurring, including weak binding interactions and/or solvation effects. The increase in the folate dimerization constant by betaine and DMSO and the change in sign of the NOEs between the C9 and C3′/C5′ protons clearly indicate that there are binding interactions occurring. Also, the preferential interaction elucidated by osmotic stress experiments suggests that this interaction is stronger than water solvation. However, similar effects on the buildup of NOEs for both folate and NADPH indicate that solvation effects also occur. Additionally, it was found through difference absorbance studies that osmolytes cause hyperchromic and bathochromic (red) shifts in the absorbance spectrum of folate, NADP$^+$, and some model compounds (data not shown). This is consistent with the effects of osmolytes on the polarity, dielectric constant, and refractive index of the solution, which alters the orientation of the solvent dipoles around the ground and excited state dipoles of the molecule, in this case folate.[27] Bathochromic shifts are typical for nonpolar molecules or molecules in which the dipole of the excited state is smaller than the dipole of the ground state regardless of the solvent polarity. The difference absorbance results indicate that osmolytes do affect NADPH solvation, and perhaps, there are both specific and nonspecific interactions between osmolytes and folate occurring in solution, though the preferences of betaine for both aromatic rings and

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DMSO for the pABA ring indicate that more than bulk solution properties must be responsible for data indicating specific interactions between the osmolytes and folate. For example, the solution viscosities or dielectrics of betaine, DMSO, and sucrose do not correlate with percent NOE between the C9 and C3′/C5′ protons (see Figure S7 of the Supporting Information).

Consequences of Folate Interactions. Our model for describing effects of osmolytes on binding of DHF and folate to either R67 DHFR or EcDHFR involves a competition between the osmolyte and enzyme for free DHF. While osmolyte binding is weak, the osmolyte concentration is high. In contrast, binding of DHF to DHFR is tight, and typically, the enzyme concentration is low in the cell. In an extension of our model, we ask if it is possible that folate may bind to various macromolecules. Other examples of weak binding of folate to various proteins have been noted.[41−43] Also, folate has been crystallized in the central cavity of hemoglobin.[44] Most recently, a mass spectroscopy "allosterome" approach has searched for metabolite−protein interactions and found that folate inhibits glutamate dehydrogenase, glucose-6-phosphate dehydrogenase, and NAD kinase.[45] To test this possibility, we added 50−100 μM BSA to ITC titrations to determine if an 8.3-fold excess of BSA over DHFR could weaken the observed K_d value for the enzyme. Table 5 shows that addition of BSA does indeed weaken binding of DHF to EcDHFR by 2−3-fold. This observation indicates "soft interactions" are important and have functional consequences. Soft interactions have previously been found to occur between proteins and crowding agents.[46,47]

It has been suggested that under crowding conditions, hydrophobic patches on protein surfaces may be responsible for weak, nonspecific interactions.[47] The promiscuous binding of folate to proteins and osmolytes could be due to the hydrophobicity of the folate molecule. The log P value for DHF/folate is −3.875, while the value for NADPH is −7.774. Further, studies of protein crystals soaked in organic solvents have shown that the organic molecules can associate with small hydrophobic patches on the outer surface of proteins as well as the active site.[48] While the interactions may be weak between osmolytes and folate, the high concentration of osmolytes likely shifts the equilibrium toward the folate−osmolyte complex. Thus, the need for the folate−osmolyte complex to dissociate will weaken the binding of folate, or DHF, to DHFR.

CONCLUSIONS

We have begun to decipher why "water uptake" upon binding of DHF to either R67 DHFR or EcDHFR has been observed in our previous ITC experiments. From this study, we conclude that osmolytes interact with folate, mostly with the pterin and benzoyl ring systems. Correlating the number of osmolyte-excluding waters to the number of waters taken up when DHF binds to DHFR (~30 for betaine[7,8]) would suggest 40 waters are taken up from the folate and 10 waters are released from the protein. Further, there is discrimination in the interactions; for example, betaine interacts with P6C, while DMSO does not. While we were not able to quantify the binding strength or stoichiometry for the interaction between folate, our results suggest that the interaction is stronger than solvation by water. Future experiments will be necessary to gain more quantitative data about folate−osmolyte interactions.

As folate appears to have the capacity to interact with many different types of osmolytes and as high concentrations of molecules exist in the cellular milieu (~200 mg/mL in the eukaryotic cytoplasm and >400 mg/mL in the cytoplasm of prokaryotes),[46,49,50] it seems likely that soft interactions may also exist between folates and other enzymes in folate one-carbon metabolism. In addition, anti-folate drugs may interact with osmolytes and affect their efficacy. Thus, our studies begin to address the basic question of whether the behavior of macromolecules at infinite dilution (e.g., in vitro) accurately reflects their behavior in vivo.

ASSOCIATED CONTENT

Supporting Information

Folate pK_a titration plots and their fits, NOESY spectra of folate in buffer and buffer with 20% betaine or 20% DMSO at a mixing time of 750 ms, mixing time buildup plots for NADPH, osmotic stress plots for folate with betaine at pH 7.0, and a global fit of binding of DHF to EcDHFR in the presence of 0−100 μM BSA. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

pABA-Glu, *p*-aminobenzoyl glutamic acid; ASA, water accessible surface area; BSA, bovine serum albumin; DHF, dihydrofolate; DHFR, dihydrofolate reductase; DMSO, dimethyl sulfoxide; EcDHFR, *E. coli* chromosomal dihydrofolate reductase; ITC, isothermal titration calorimetry; MTA, 100 mM Tris, 50 mM MES, and 50 mM acetate buffer; $NADP^+$, nicotinamide adenine dinucleotide phosphate; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NOE, nuclear Overhauser effect; P6C, pterin-6-carboxylate.

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