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Crowders Steal Dihydrofolate Reductase Ligands through Quinary Interactions

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Abstract

Dihydrofolate reductase (DHFR) reduces dihydrofolate (DHF) to tetrahydrofolate using NADPH as a cofactor. Due to its role in one carbon metabolism, chromosomal DHFR is the target of the antibacterial drug, trimethoprim. Resistance to trimethoprim has resulted in a type II DHFR that is not structurally related to the chromosomal enzyme target. Because of its metabolic significance, understanding DHFR kinetics and ligand binding behavior in more cell-like conditions, where the total macromolecule concentration can be as great as 300 mg/mL, is important. The progress-curve kinetics and ligand binding properties of the drug target (chromosomal *E. coli* DHFR) and the drug resistant (R67 DHFR) enzymes were studied in the presence of macromolecular cosolutes. There were varied effects on NADPH oxidation and binding to the two DHFRs, with some cosolutes increasing affinity and others weakening binding. However, DHF binding and reduction in both DHFRs decreased in the presence of all cosolutes. The decreased binding of ligands is mostly attributed to weak associations with the macromolecules, as opposed to crowder effects on the DHFRs. Computer simulations found weak, transient interactions for both ligands with several proteins. The net charge of protein cosolutes correlated with effects on NADP⁺ binding, with near neutral and positively charged proteins having more detrimental effects on

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Supporting Information:

The supporting information contains: structures of some of the typical functional groups found on osmolytes and crowders; examples of progress curve kinetic traces; tables of the kinetic parameters obtained for R67 DHFR; plots of the k_{cat} s versus crowder osmolality; 3D plot of the Michaelis-Menten kinetics for R67 DHFR in buffer; table of the k_{cat} s and K_M s from the Michaelis-Menten kinetics; tables of the thermodynamic parameters obtained from ITC for ligand binding to R67 DHFR; tables containing the parameters for EcDHFR kinetics; an example of the ITC thermogram and fit for DHF binding to the EcDHFR-NADP⁺ binary complex; raw ITC thermograms for the titration of folate into macromolecule cosolutes; entropy-enthalpy compensation plots for ligand binding to DHFRs in the presence of crowders; tables of the thermodynamic parameters for ligand binding to EcDHFR; results and a table for DHF binding to apo-EcDHFR; a figure of the $\ln(1/K_d)$ versus osmolality for DHF binding to apo-EcDHFR; methods and results for stopped-flow kinetic analysis of the effects of crowders on ligand and the inhibitor methotrexate binding to EcDHFR; a representative stopped-flow kinetic trace; the k_{app} from stopped flow versus ligand concentration; a table of the stopped-flow kinetic parameters; a plot of the k_{app} versus methotrexate concentration; a table of the forward and reverse association constants obtained from stopped-flow for methotrexate; secondary structure analysis in the presence of crowders as measured by circular dichroism; DSC thermograms and a table of their fits for DHFRs in the presence of crowders; a figure of fraction of apparent R67 DHFR dimer versus pH in the presence of selected crowders; heatmaps of the interaction energies from the MD simulations of ligands in the presence of protein crowders; a table of the interactions found in MD simulations between crowders and ligands; figures showing the movement of the center of mass of folate during the time course of select simulations; a plot of the top 10 root-mean square fluctuations of the quasi-harmonic modes of EcDHFR simulated in the presence of lysozyme along with structures showing the flexibility of loops in the EcDHFR structure over the course of the simulations; figures of the slope in $\ln(1/K_d)$, or $\ln(k_{cat}/K_M)$, versus osmolality plots versus cosolute molecular volume and viscosity; tables of the slopes of $\ln(1/K_d)$, or $\ln(k_{cat}/K_M)$, versus osmolality plots; figures of the slopes of $\ln(1/K_d)$, or $\ln(k_{cat}/K_M)$, versus osmolality plots versus the net negative or positive charge on the protein crowders.

binding. For DHF binding, effects correlated more with the size of binding pockets on the protein crowders. These non-specific interactions between DHFR ligands and proteins predict that the *in vivo* efficiency of DHFRs may be much lower than expected from their *in vitro* rates.

Introduction:

Dihydrofolate reductases (DHFRs) are enzymes that convert dihydrofolate (DHF) to tetrahydrofolate using nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor. The chromosomal form is a highly efficient enzyme and has been targeted by anti-cancer as well as antibiotic drugs.^{1, 2} Drugs that target the bacterial forms of the enzyme have led to several drug resistant mechanisms, including production of a genetically and structurally unrelated plasmid form of the enzyme.^{3–6} One of these type II DHFRs is plasmid encoded R67 DHFR, which is a homotetrameric enzyme with a central pore that contains a single active site.^{7–9} Symmetry related residues on all four monomers form the active site residues, meaning both NADPH and DHF bind to residues on opposite sides of the pore. The symmetric active site, with residues unoptimized for substrate and cofactor binding, suggests that R67 DHFR is a primitive enzyme not yet fully evolved.¹⁰

We have taken advantage of the differences in sequence and structure between the chromosomal DHFR and R67 DHFR (see Figure 1 and Table S1 in the supplement for a comparison of the enzyme properties) to explore the role of water in ligand binding to the two enzymes using osmotic stress studies.^{11, 12} Our previous studies found small molecule osmolytes interact with free DHF in solution,^{13, 14} hindering binding of ligand to the structurally unrelated *E. coli* chromosomal DHFR (EcDHFR), R67 DHFR and the *E. coli* homologue of pteridine-reductase, FolM.^{11, 12, 15} Binding is weakened in the presence of osmolytes because their weak interactions with free DHF shift the binding equilibrium from the bound state to the unbound state. In contrast, cofactor (NADPH or NADP⁺) binding was tightened in the presence of osmolytes, indicating the release of hydrating waters.^{11, 12}

Small molecule osmolytes and large macromolecules typically used in crowding studies share similar chemical functional groups (i.e. charges, hydrogen bond donors and acceptors, hydrophobic moieties, etc., see Supplemental Figure S1). We therefore hypothesized that large macromolecules would similarly interact with free DHF in solution. Interestingly, Aumiller et al. have also shown weak or soft interactions between crowders and substrates of horseradish peroxidase.¹⁷ Both PEG8000 and dextran-10 increase the K_M for two horse radish peroxidase substrates with different hydrophobicities. This was attributed to the interaction between the crowders and the two substrates. Similarly, ATP, because of its amphipathic chemical nature, acts as a hydrotrope by weakly interacting with proteins, stabilizing them against aggregation,¹⁸ and molecular dynamics (MD) simulations on a bacterial cytoplasm indicated many other charged metabolites may form non-specific electrostatic interactions with proteins in the cell.¹⁹ Together, these data indicate that small, biologically relevant molecules, such as DHF, might interact with macromolecular cosolutes in a way that is similar to the DHF interaction with small molecule osmolytes.

The primary force that macromolecules are expected to exert at high concentration is volume exclusion effects due to the reduced space for the enzyme and ligand.^{20–22} The reduced

available volume favors more compact states, such as protein-protein or ligand-protein complexes. Additionally, the inaccessible space will also increase the effective concentration of the enzyme and ligand, which will further drive the equilibrium towards the bound state. However, more recent research indicates that there may be weak, transient (or soft) interactions between macromolecules that can have additional effects in addition to volume exclusion.^{23–27} These weak interactions are similar to the preferential interactions that occur with small molecules osmolytes.^{14, 28–32}

Thus we study the potential for DHF to interact with macromolecules by examining their effects on binding and DHFR activity. Using two structurally unrelated DHFRs, EcDHFR and R67 DHFR, having different sequences and active site structures and volumes,¹⁰ will allow us to parse out any effects of the cosolutes on the proteins themselves. For example, similar trends on DHF binding for both EcDHFR and R67 DHFR can mostly be related to the effect of the cosolutes on DHF, not the enzymes. Additionally, because R67 DHFR contains a single symmetric active site, and as NADPH and DHF bind to symmetry related residues, we can also use NADPH/NADP⁺ binding as a second method of eliminating cosolvent effects on R67 DHFR. For our studies, we picked Ficoll-70, PVP-55 and PEGs and dextrans of different sizes. We have also selected five proteins: hen egg-white lysozyme, ovalbumin, the rhamnose synthesis protein, dTDP-4-keto-6-deoxy-D-hexulose 3,5-epimerase (RmlC), hemoglobin and casein. While these proteins are not meant to be mimics of the *E. coli* cytosol, the first four proteins have known structures, which can help tease apart molecular properties controlling the changes in ligand binding in the presence of the macromolecules. We hypothesize that each macromolecule will impact the binding of ligands depending upon the chemical identity of both the cosolute and ligand.

Methods and Materials:

Chemicals

PVP-55 (average molecular weight 58,000 Da), PEG3350, and PEG8000 were purchased from Fisher. Methotrexate, lysozyme (14,300 Da, UniProt ID Q6LEL2), bovine hemoglobin (64,500 Da, UniProt ID P02070), dextran-10, dextran-40, dextran-70 (10,000 Da, 40,000 Da and 70,000 Da average molecular weights, respectively) and Ficoll 400 (400,000 Da) were purchased from Sigma. Ficoll-70 (70,000 Da average molecular weight) was purchased from VWR. NADPH, and NADP⁺ were purchased from Enzo Life Sciences. DHF was prepared using previously published protocols.³³

Protein Purification

E. coli chromosomal DHFR (UniProt ID P0ABQ4) and R67 DHFR (UniProt ID P00383) were expressed and purified according to previously published procedures.^{11, 12} The proteins chosen as crowders in this study were selected as large quantities are commercially available, or because we already had high expressing clones. Casein (UniProt ID P02666) was purified from commercially available dry, defatted milk.³⁴ Ovalbumin (UniProt ID P01012) was isolated from fresh eggs.^{35, 36} RmlC (UniProt ID O27818) was purified according to previous reports.^{37, 38} The purified proteins were dialyzed against deionized

water, lyophilized and stored at -20°C . All proteins were assessed by SDS PAGE and found to be $>90\%$ pure.

Progress Curve Kinetics

Progress curve analysis was performed as per Gekko et al.³⁹ Experiments were performed at 30°C by following the change in absorbance at 340 nm on a Perkin Elmer λ 3B spectrometer until all the limiting reagent was consumed (5–10 minutes). The molar extinction coefficient for the reduction of DHF by DHFR is $12,300\text{ M}^{-1}\text{ cm}^{-1}$.⁴⁰ Ligand concentrations were determined with an extinction coefficient of $28,000\text{ M}^{-1}\text{ cm}^{-1}$ at 282 nm for DHF,³³ while $6230\text{ M}^{-1}\text{ cm}^{-1}$ at 340 nm was used for NADPH.⁴¹ DHF reduction kinetics were studied by pre-incubating NADPH (90 μM) and EcDHFR (3–9 nM) in MTA buffer (100 mM Tris, 50 mM MES, 50 mM acetic acid), 1 mM EDTA, 5 mM β -mercaptoethanol (BME), pH 7.0 for two minutes prior to starting the reaction with DHF (7–12 μM).⁴² For NADPH kinetics, EcDHFR (3–20 nM) was pre-incubated with a saturating amount of DHF (140–200 μM) and the reaction was initiated with NADPH (7–12 μM). The reduction of DHF (10–45 μM) by R67 DHFR (0.25–1 μM) was monitored in MTA buffer, pH 7.0 with a saturating concentration of NADPH (100 μM). Kinetic parameters for NADPH (10–25 μM) with R67 DHFR (0.2–2 μM) in the presence of DHF (300–400 μM) were determined at 360 nm ($\epsilon_{360} = 6,000\text{ M}^{-1}\text{ cm}^{-1}$). MATLAB (v. r2018a, MathWorks, Inc.) was used to fit the kinetics data to the integrated Michaelis-Menten equation.³⁹ At least two kinetics traces were acquired for each cosolute concentration. The osmolalities of the macromolecule solutions were measured using a Vapro 5520 from Wescor.

Michaelis-Menten Kinetics

As a double check of the progress curve data, initial rate Michaelis-Menten kinetics were performed at 30°C at one concentration of the non-protein crowders. The initial rates at 340 nm were determined for R67 DHFR (40–80 nM) over a range of DHF concentrations (3–140 μM) at a constant concentration of NADPH. This experiment was repeated at five different concentrations of NADPH (3–70 μM). The data were fit globally in SAS 9.4 to a bi-substrate model to obtain a single k_{cat} for the reaction, as well the K_{M} s for both NADPH and DHF.⁴³ Similar experiments were performed for EcDHFR (0.8–2 nM) in buffer and in 20% (w/w) PEG3350 using 3–75 μM DHF and 2–55 μM NADPH concentration ranges. The stock solution of EcDHFR used in the kinetics assays was stabilized by the addition of 2.5 mg/mL BSA, so that the final concentration of BSA in the kinetics experiment was less than 4 μM .

Isothermal Titration Calorimetry

The binding of ligands to both DHFRs was monitored by isothermal titration calorimetry (ITC) similar to our previous studies of osmolyte effects on ligand binding.^{11, 12, 15} All titrations were performed at 25°C on a MicroCal VP-ITC. NADP^{+} (0.5–1 mM) was titrated into apo EcDHFR (9–35 μM) in the MTA poly-buffer containing 1 mM EDTA, 5 mM BME, pH 7.0. The peaks were integrated by the automated analysis program NITPIC,⁴⁴ and the data processed by the program SEDPHAT.^{45, 46} At least two data sets were analyzed globally in SEDPHAT to the $A + B \rightarrow AB$ model. Similar titrations were also performed for DHF (450–600 μM) binding to apo EcDHFR (25–35 μM) as well as DHF (150–650 μM) binding to the binary complex of EcDHFR (8–16 μM) saturated with NADP^{+} (900 μM).

Binding of ligands to R67 DHFR was also monitored. Typically, NADP⁺ (5–17 mM) was titrated into 100–200 μ M R67 DHFR. For ternary complex formation, binding of DHF (1.3–2.2 mM) was titrated into 100–200 μ M R67 DHFR in the presence of 900 μ M NADP⁺.

Circular Dichroism

The effect of macromolecules on the secondary structure of R67 DHFR and EcDHFR were measured by circular dichroism (CD). The spectra were measured on an AVIV model 202 CD spectrometer at 25 °C in a 1 mm cuvette with 10–14 μ M DHFR in 45 mM Na₂HPO₄, pH 7.0 buffer. Blanks were measured for each macromolecule in buffer and used to subtract from the CD spectra of the DHFR in macromolecule solution.

Differential Scanning Calorimetry.

The thermal stabilities of the DHFRs were measured on a MicroCal VP-DSC. For EcDHFR, 25–35 μ M of the enzyme in 45 mM Na₂HPO₄, 1 mM EDTA, 5 mM β -mercaptoethanol, pH 7.0 buffer was scanned from 25–80 °C at a scan rate of 1 °C/min. For R67 DHFR, 45–60 μ M tetramer in 45 mM Na₂HPO₄, pH 8.0 buffer was scanned from 35–85 °C. A pH of 8 for R67 DHFR ensures that there is minimal dimer present in solution.⁴⁷ The effects of macromolecules on the thermal stabilities of the two DHFRs were measured by adding in 10% (w/w) of PEGs, dextrans, or Ficoll-70.

Computer Simulations:

To determine where folate may interact on protein surfaces, docking and MD studies were performed. DHF and NADP⁺ were docked onto lysozyme (PDB ID 5B1F), RmlC (PDB ID 1EP0)³⁷ and ovalbumin (PDB ID 1OVA)⁴⁸ using Autodock Vina.⁴⁹ Docking was performed with rigid protein residues and flexible ligands. At least three docking grids were used in separate searches. One encapsulated the entire protein, while the other grids encompassed as much of the protein as possible while excluding the active site.

Four folate-lysozyme complexes, three NADP⁺-lysozyme complexes as well as three folate-RmlC, two NADP⁺-RmlC complexes and three folate-ovalbumin, two NADP⁺-ovalbumin complexes were prepared using the AMBER simulation package.⁵⁰ For each of these complexes, a full independent MD simulation was performed (to rule out bias from a single MD trajectory). AMBER's *ff14SB* force-field was used for proteins and the parameters for folate and NADP⁺ developed in a previous study were used.⁵¹ For simulation system preparation, each of the protein-ligand complex was solvated with a 12 Å water shell and water was modeled using the TIP3P model. (The water box was selected to allow for ligand molecules to interact only with water in case of weak binding, without interacting with the nearest image of the protein.) The system was neutralized through the addition of chloride (Cl⁻) counterions for lysozyme or sodium (Na⁺) ions for RmlC and ovalbumin. An additional 20 Na⁺ and 20 Cl⁻ ions were added to provide some salt to shield electrostatic interactions due to charges associated with the ligand. This corresponds to ~0.06 M NaCl for lysozyme simulations and ~0.13 M NaCl for RmlC and ovalbumin trajectories. After these pre-processing steps, the system was equilibrated as per the protocol described by Agarwal.⁵²

Production runs of ~1 microsecond were performed under constant volume and energy (NVE) conditions using 2 fs time-steps for folate and 1 fs time steps for NADP⁺. All simulations were performed using NVIDIA graphics cards and AMBER's pmemd.cuda MD simulation engine.^{53, 54}

Energy of interaction:

Interaction energies for individual atom pairs in the protein-folate trajectories were calculated using a sum of the electrostatic and van der Waals contributions. This approach was developed for protein-protein interactions,⁵² and has also been used for interactions of small molecules with proteins. Overall energies were calculated by averaging the total interaction energies for typically 10,000–12,000 snapshots, sampled for the 1 microsecond trajectories. MATLAB was used to plot the energies, allowing identification of pairs of atoms with good interaction energies. Then AMBER's *ptraj* analysis program was used to calculate interesting distance profiles from the 10,000–12,000 conformational snapshots stored during MD simulations.

Results:

R67 DHFR

Progress Curve Analysis.—To test if addition of macromolecular cosolutes affects ligand binding to DHFRs, we first used progress curve analysis of enzyme activity (sample shown in Figure S2). Results for the reduction of a limiting DHF concentration and a saturating NADPH concentration by R67 DHFR are given in Figure 2 and Table S2. We plot our data versus osmolality instead of macromolecule concentration because high cosolute concentrations reduce the concentration of water, lowering the water activity of the solution. The change in binding with osmolality can give information on the number of waters interacting with the ligands.^{55, 56} The magnitude and sign of the change in the k_{cat}/K_M value was dependent upon the macromolecule. Parsegian et al. argue that plots of a change in ΔG should be linear with respect to osmolality.⁵⁷ Thus we fit our data to a straight line. Any apparent deviation from linearity can be considered to be within error based on the size of the error bars of some of the data points. Lysozyme is the only macromolecule that increases the efficiency of R67 DHFR reduction of DHF. Ovalbumin had compensatory increases in both k_{cat} and K_M . The rest of the macromolecules decreased the k_{cat}/K_M for R67 DHFR reduction of DHF. Small molecule osmolytes similarly decreased the activity of R67 DHFR by increasing the K_M without perturbing the k_{cat} .^{11, 58} With the macromolecules, however, both k_{cat} and K_M were affected (Figure S3). There was no overarching trend in k_{cat} , with some macromolecules causing a decrease in k_{cat} , while others increased k_{cat} or left it unperturbed. All macromolecules that decrease enzyme efficiency increased the K_M to some extent.

We additionally attempted to perform progress curve kinetics for NADPH oxidation by R67 DHFR. Progress curve kinetics required high concentrations (300–400 μM) DHF to ensure saturating concentrations of substrate. The high concentrations of DHF caused a slope in the baseline that lead to unreliable fits of the data. Therefore, we did not use the progress curve data for further analysis.

To obtain some of the kinetic parameters for NADPH oxidation by R67 DHFR, we performed Michaelis-Menten kinetics with varying DHF concentrations and at least five different NADPH concentrations (Figure S4 and Table S3).⁴³ Non-linear, global fitting of the data allows us to obtain one k_{cat} for the reaction as well as two K_{M} s. The experiments were performed in buffer, and one concentration of non-protein crowder. The amount of cosolute needed to perform this type of experiment made doing them with protein crowders prohibitive. While there are only two osmolality points for our Michaelis-Menten kinetic data, the general trend was a decrease in k_{cat} under crowding conditions (Figure S5), with the exceptions of PEG3350, PEG8000 and Ficoll-70. The $k_{\text{cat}}/K_{\text{M}}$, likewise, decreased for most of the crowders, with the PEGs being the only crowders that increased $k_{\text{cat}}/K_{\text{M}}$ (Figure 3). These results were consistent with the DHF progress curve data (Figure S4 and Table S3).

ITC—To measure ligand binding directly, the effects of macromolecules on ligand binding to R67 DHFR were explored by ITC (Figure 4). An example ITC thermogram and fit are given in Supplemental Figure S6. Our results for NADP⁺ binding in buffer gave a K_{d} of $98 \pm 9 \mu\text{M}$, similar to previous values.⁵⁹ Exploring the results of NADP⁺ binding to apo-R67 DHFR finds weaker affinity upon addition of most of the macromolecules (Table S4). PEG3350 is the lone exception. At first glance, the ITC data apparently give different results from the progress curve data; however that is only because the latter take into account the effects of the cosolutes on k_{cat} , in addition to K_{M} . When K_{M} and K_{d} values are compared, the effects are mostly similar. Previous studies showed that small molecule osmolytes all increase the affinity of NADPH for R67 DHFR and all osmolytes have the same effect.¹¹ In contrast, the change in K_{d} is dependent upon the identity of the crowder.

DHF binding to the binary complex of R67 DHFR and NADP⁺ was also explored (Figure 4 and Table S5). The control titration of DHF binding to R67 DHFR-NADP⁺ had a K_{d} of $4.5 \pm 2.7 \mu\text{M}$, which is close to previous values.^{11, 59} Effects of macromolecules were then tested. Both Ficoll-70 and ovalbumin had little effect on the affinity of R67 DHFR for DHF. The rest of the macromolecules, to varying degrees, weakened R67 DHFR's affinity for DHF. Hemoglobin had the largest effect, weakening the affinity 5.5-fold at 50 mg/mL, which is not surprising as folate binds weakly ($K_{\text{d}} \sim 1 \text{ mM}$) to the central cavity between the four tetramers.⁶⁰ Titration of folate into the crowders themselves yielded no heat of binding (Figure S7), either because folate does not bind tightly enough for binding to be measured by ITC or the enthalpy of binding is zero.

In addition to the effects on K_{d} , the crowders also can affect other thermodynamic parameters. Enthalpy-entropy compensation plots were constructed for ligand binding in the presence of crowders and show very good linear correlations (Figure S8). These plots indicate the crowders are having a uniform effect on ligand binding, perhaps through water-effects or more traditional effects on the ligand and protein.^{61–64}

***E. coli* chromosomal DHFR**

Progress Curve Analysis.—Macromolecule effects on EcDHFR reduction of DHF were also explored by progress curve kinetics (Table S6 and Figure 2). In buffer alone, the

reduction of DHF by EcDHFR in the presence of a saturating concentration of NADPH yielded a k_{cat} of $32.5 \pm 3.0 \text{ s}^{-1}$ and a K_{M} of $1.6 \pm 0.3 \text{ }\mu\text{M}$. These kinetic values are similar to those previously reported by Michaelis-Menten studies and by progress curve analysis.^{39, 65, 66} All the macromolecules tested decreased the activity of EcDHFR, though the magnitude of the effect was dependent upon the macromolecular identity. The K_{M} of DHF remains relatively constant at 1.6 to 2.0 μM over the range of concentrations used for most of the macromolecules. The exceptions lie with PEG3350, PVP-55 and casein, which all increased the K_{M} at least 2-fold at their highest concentrations (300, 300 and 15 mg/mL respectively). The k_{cat} decreases linearly for all macromolecules; specifically k_{cat} decreased 3-fold at 300 mg/mL of PEGs, dextrans, PVP-55 and Ficoll-70 (Figure S5). Lysozyme and ovalbumin decreased the k_{cat} about 60% at 50 mg/mL, while 50 mg/mL RmIC caused the k_{cat} to decrease 3-fold. Addition of casein did not alter the k_{cat} over the range of concentrations used.

Figure 3 and Table S7 show the effects of crowders on the oxidation of NADPH by EcDHFR in the presence of DHF. In buffer alone, a k_{cat} and NADPH K_{M} of $30.7 \pm 6.2 \text{ s}^{-1}$ and $5.2 \pm 1.1 \text{ }\mu\text{M}$, were measured respectively. These values are higher than those typically obtained for the cofactor using steady state kinetics.^{66, 67} However, previous studies suggest these differences may be due to product inhibition and/or some contribution from a reverse reaction.^{39, 68} When macromolecular cosolutes were added, a range of effects were noted. Most cosolutes decreased the activity of EcDHFR. The effects were primarily on k_{cat} (Table S7 and Figure S5), which decreased as much as 3-fold in the presence of 300 mg/mL PVP-55. The K_{M} increased in the presence of most macromolecules, while it stayed constant for addition of ovalbumin, though the changes were linear in the presence of all the cosolutes. Dextran-10 and dextran-70 increased the K_{M} linearly, while the K_{M} for dextran-40 remained constant, within error.

Michaelis-Menten kinetics for EcDHFR were performed in buffer and 20% PEG3350 to cross-check the progress curve data (Table S3). The k_{cat} s and K_{M} s correlated well with the data obtained from progress curves.

Isothermal Titration Calorimetry—Effects on the binding affinity of NADP^+ for apo-EcDHFR were measured by ITC (Figure 4 and Table S8). Initial experiments in buffer alone yielded a K_{d} of $5.0 \pm 0.4 \text{ }\mu\text{M}$, which compares well to previously published values.¹² The PEGs, dextrans 40 and 70, and Ficoll-70 only slightly increased the affinity of NADP^+ for EcDHFR. Several macromolecules used, dextran-10, PVP-55 and ovalbumin, had no effect on affinity over the concentration ranges used (up to 150 mg/mL with PVP-55). Rather addition of proteins had the most detrimental effects, with lysozyme having the largest effect, decreasing the affinity of NADP^+ 5-fold at 50 mg/mL. The myriad of effects noted with the macromolecules on NADP^+ binding to apo-EcDHFR was similar to our previous results with osmolytes, where, for example, N,N,N-trimethylglycine (glycine betaine) and dimethylsulfoxide caused NADP^+ to bind tighter, while sucrose decreased cofactor affinity.¹²

The effects on DHF binding to the binary complex of EcDHFR and NADP^+ were also explored (Figure 5 and Table S9). Binding of DHF in buffer yielded a K_{d} of $260 \pm 20 \text{ nM}$,

which agrees with our previous results.¹² Unlike the case for variable effects on NADP⁺ binding by the macromolecules, DHF bound more weakly in the presence of all the macromolecules tested. The weaker affinity of DHF concurs with our previous results with small molecule osmolytes.¹² The macromolecules decreased the affinity of DHF to varying degrees with casein and RmLC having the largest effects, while the effects on K_d were marginal in the presence of ovalbumin and dextran-10.

DHF binding to apo-EcDHFR was also explored for a limited set of cosolutes, and this information is provided in the supplement (Figure S9 and Table S10).

Stopped-Flow.—Stopped-flow experiments were also performed for DHF and NADPH binding to EcDHFR (see the supplement for the methods and a more detailed results section and Figure S10 and S11, as well as Table S11). Dextran cosolutes decreased the rate of association for NADPH by at most 20%, while the PEGs and Ficoll-70 had no effect (Figure S11). Lysozyme, at 2.5 mg/mL, which is 40-fold lower than the 100 mg/mL concentration of the other crowders, decreased the association rate by 40%. The dissociation rates decreased by 30–40% in the presence of PEGs and lysozyme, but were unaffected by the presence of other crowders. For DHF, the cosolutes decreased the k_{on} by 30–50%, though this was significantly less than the 6-fold decrease in k_{on} noted in the presence of the osmolyte betaine.¹² The k_{off} was unaffected by the crowders, except the PEGs which decrease the dissociation rate by ~35%. Using equation 1 from Fierke et al.,⁶⁹ the K_d s for DHF were calculated from the stopped-flow kinetics in buffer, PEG8000, dextran-70 and Ficoll-70, and compared to those determined by ITC, indicating both methods monitor the same effect. Additionally, the K_d calculated for NADPH was similar to that determined in previous ITC experiments.¹² Overall, the crowders have less of an effect on the kinetics of ligands binding to EcDHFR than did betaine.

We have also looked at binding of the anti-cancer drug, methotrexate to EcDHFR by stopped-flow (see supplemental section for details). Methotrexate binds to EcDHFR with pM affinity.⁷⁰ Despite it binding tighter than DHF or NADPH, crowders also decrease the association rate of methotrexate by 25–50% (Figure S12 and Table S12). This suggests that methotrexate interacts with the crowders, slowing down its association with EcDHFR. We have previously found that interactions with osmolytes also weaken the binding of methotrexate to FoIM, a pteridine reductase that can catalyze the DHFR reaction.¹⁵

Effects on structure and stability of both DHFRs.—CD spectra of both DHFRs in the presence of some cosolutes find no significant changes to the secondary structure of the proteins in PEG3350, PEG8000 or PVP-55 (Figure S13).

The thermal stabilities of EcDHFR and R67 DHFR in the absence of ligands were also measured to determine whether the cosolutes might be perturbing the binding equilibrium due to alterations in the native and denatured states. R67 DHFR is a tetramer at pH 8 and its thermal denaturation is partially reversible.⁷¹ Fitting the data for R67 DHFR in buffer yielded two transitions, with melting temperatures (T_m s) of 62.7 ± 0.1 and 65.8 ± 0.1 °C (Figure S14, Table S13). Similar values were previously obtained.⁷¹ Addition of dextrans and Ficoll-70 either had no effect, or mildly increased the thermal stability of R67 DHFR

(Figure S14). The absence of an effect of cosolutes on the thermal stability of R67 DHFR might be due to the positive change in volume upon unfolding.⁷² Volume exclusion by the macromolecules would favor the folded state compared to the larger volume of the unfolded state. The PEGs, however, decreased both T_m s by approximately 4 °C.

For EcDHFR, all macromolecules studied decreased the stability of the enzyme (Figure S14 and Table S13). Thermal denaturation of EcDHFR has been described as a three-state process, with native state going to an intermediate state, and the intermediate state unfolding to the denatured state. Our T_m for EcDHFR in buffer was 57 °C, which is 8 °C higher than previously published values for the transition from the native to an intermediate structure.^{73, 74} Therefore our DSC thermogram most likely corresponds with the intermediate to disordered structural transition, which has a T_m of 59 °C.⁷⁴ Our data showed no evidence of a thermal transition around 49 °C; perhaps we primarily see a single transition as a lower concentration of EcDHFR was used in our study. Alternately, our use of a higher concentration buffer could play a role. The addition of 10% (w/w) dextrans decreased the T_m to 55 °C, while PEGs further destabilized the enzyme with T_m s 4–6 °C lower than in buffer alone.

Computer Simulations.—To determine how the DHF analog, folate, and NADP⁺ behave in the presence of just the crowder proteins, we performed a total of 17 unique ~1 μ s molecular dynamics simulations. These simulations explored only the interactions of folate/NADP⁺ with lysozyme, ovalbumin, or RmlC. The starting points for these simulations were based on docking of the ligands to crowdors (see methods). During the simulations with folate, the pterin and p-aminobenzoate (pABA) rings tended to associate with the surface of the crowdors, while the glutamate moiety would move around in solution. For NADP⁺ simulations, different parts of the ligand would associate with the crowder protein surfaces. The interaction energies (see methods section for details) between the ligands and the residues of the crowder proteins were calculated and plotted as heat maps. Folate would either find a site on the protein to interact with for most of the simulation or sample many different sites over the simulation, while NADP⁺ tended to have more transient (<100 ns) interactions with the proteins. The shorter interaction times with the crowder proteins indicate that NADP⁺ associations with the cosolutes are weaker than for folate. Plots of some of the more stable interactions and the more transient simulations are shown in Figures 6, S15 and S16. The other simulations fell somewhere between these two extremes (Table S14). A representative simulation where folate moves around the surface of lysozyme until it finds a site to interact with is shown in Figure S17. Simulations of trimethoprim binding to chromosomal DHFR from *Mycobacterium tuberculosis* similarly found the ligand diffusing along the surface of the protein until it found the active site.⁷⁵ Some of the sites with which the ligands interacted showed up in multiple simulations, while the ligands did not associate with other surfaces of the protein at all (Figures S17-S19). Therefore, the simulations seem to indicate that there is not necessarily one well-defined site or orientation of interaction between each ligand-protein pair, but that the sites are more similar to the “fuzzy” sites seen in the protein-protein interactions between an intrinsically disordered protein and its binding partner.^{76, 77}

To determine what type of interactions might be occurring, we also looked at the distances over the course of the simulation for the center of masses of the interacting residue-ligand pairs. Figure 7 shows a few of the interactions occurring in Figure 6A. The majority of the interactions were between the pterin and/or pABA rings and the protein. This would support van der Waals and π - π stacking interactions. There were also a significant number of electrostatic interactions between positively charged protein residues and the glutamate tail of folate. For NADP⁺ interactions, both the phosphate and the aromatic rings on NADP⁺ associate with the surfaces of the crowders, though these associations are very short lived.

Effect of crowding on EcDHFR dynamics: How lysozyme interacts with EcDHFR was also explored by performing MD simulations of EcDHFR in the presence of several lysozyme molecules (see supplement for experimental details). Eight independent EcDHFR-lysozyme systems were used consisting of a single EcDHFR system surrounded by 6 to 8 lysozyme molecules. The dynamics of EcDHFR were not markedly altered by the presence of these lysozyme molecules. Also, the most flexible regions of the EcDHFR Met20 and β F- β G loops show similar flexibilities in the apo form as well as in presence of the crowders (Figure S20). In one of the eight simulations, the β G- β H loop became more dynamic (Figure S21).

Discussion:

Macromolecular cosolutes have similar effects on ligand binding to both EcDHFR and R67 DHFR

The use of two structurally different DHFRs allows us to separate any effects of the macromolecular cosolutes on the ligand versus the protein. Similar effects noted for both EcDHFR and R67 DHFR likely indicate that the effects are on the ligand, rather than the protein (assuming no coincidence). If there are divergent effects (i.e., increased binding for one DHFR, while weaker binding to the other), this would indicate that there are additional effects on at least one of the DHFRs as well.

The consistent decrease in DHF binding or activity with both DHFRs is in stark contrast to multivariate effects on NADPH or NADP⁺ (NADP(H⁺)) binding. The decrease in DHF binding to both EcDHFR and R67 DHFR is consistent with the detrimental effects arising from interactions between DHF and the cosolutes. It is unlikely that cosolute interactions with the DHFRs would cause weaker binding of DHF, when the same cosolutes have variable effects on NADP(H⁺) binding.

To some degree, the binding of NADP(H⁺) to both DHFRs is the more interesting case. There are variable effects both by kinetics and by ITC, though they are more pronounced when measuring the enzyme activity. When looking at the ITC results, only lysozyme, ovalbumin, RmlC and casein gave similar decreases in binding affinity. The degree to which the affinity decreased was similar for lysozyme, casein and RmlC, but the effects of ovalbumin on NADP⁺ binding to EcDHFR were less pronounced than for R67 DHFR, perhaps because of the tighter binding affinity of EcDHFR for NADP⁺. For the non-protein cosolutes, only PEG3350 similarly increased the affinity of NADP⁺ to both DHFRs. For the

rest of the cosolutes, they decreased the affinity of NADP⁺ for R67 DHFR, but increased the affinity for EcDHFR.

Macromolecular cosolutes also had negative effects on NADPH oxidation by EcDHFR as decreases in k_{cat}/K_M were noted. Since the DHF data suggest interactions between the crowders and DHF with minimal interactions between the cosolutes and DHFRs, the varied effects seen with NADP(H⁺) most likely also come from interactions between the cosolutes and cofactor.

How typical are the effects of macromolecule cosolutes on DHFRs?

Using ligand binding and k_{cat}/K_M values for DHF and NADPH, we compared the effects of cosolutes on EcDHFR to those measured by Acosta et al.⁷⁸ Three of the macromolecules used in our study, Ficoll-70, Ficoll-400 and PVP-55, were also used by Acosta et al.⁷⁸ who found these three crowders increased the specific activity of EcDHFR at concentrations below 100 mg/mL. Our results find that for Ficoll-70 and PVP-55, the k_{cat} values for both NADPH and DHF decreased fairly linearly with increased concentration, even for those samples that contained less than 100 mg/mL of the macromolecule. The k_{cat} s of the 50 mg/mL Ficoll-400 samples were within error of buffer, but the DHF K_M did increase, causing the k_{cat}/K_M to decrease. The NADPH K_M s for Ficoll-70 and PVP-55 were within error of the buffer value, while for Ficoll-400, they increased with macromolecule concentration. Therefore, our results do not find an increase in activity of EcDHFR at lower crowder concentrations. One potential explanation for the different results is that we measured the k_{cat} and K_M values for the reaction, while Acosta et al.⁷⁸ measured the specific activity of EcDHFR under saturating concentrations of both DHF and NADPH at 25 °C. Additionally, a different buffer system was used.

Other groups have found that non-charged crowders have greater effects than charged crowders on V_{max} .^{79, 80} In our studies this is true for DHF reduction by R67 DHFR and NADPH oxidation by EcDHFR, where k_{cat} was unchanged, or increased, in the presence of charged crowders. However, overall, charged crowders tended to decrease ligand binding to DHFRs more significantly than uncharged crowders.

The supplement of Acosta et al. provides a very thorough list of the literature describing the effects of crowders on the activity of enzymes,⁷⁸ as cosolutes can have a myriad of effects on both the k_{cat} and K_M .⁸¹ The differential effects of macromolecules on enzyme activity are mirrored by what happens *in vivo*. A majority of enzymes that had their catalytic rates measured *in vivo* by determining the changes in metabolite concentrations using mass spectrometry showed a decrease in their apparent catalytic rates *in vivo* compared to *in vitro*.⁸² Indeed, several enzymes that use folate-based substrates/cofactors (dihydropteroate synthase, methionine synthase, 7,8-dihydro-6-hydroxymethylpterin-pyrophosphate kinase, GTP cyclohydrolase I, methylenetetrahydrofolate reductases, folyl-polyglutamate synthase, 5,10-methenyltetrahydrofolate cyclohydrolase, serine hydroxymethyl transferase) had decreased enzyme rates *in vivo*, while methylenetetrahydrofolate dehydrogenase and thymidylate synthase both showed increased activity.⁸² Based on most of the measured folate cycle enzymes showing decreased catalytic rates by 1.5 to 75-fold (for serine hydroxymethyltransferase and dihydropteroate synthase, respectively), we might expect the

in vivo activity of EcDHFR to decrease somewhere in this range as well. Therefore, the 3 to 4-fold decreased EcDHFR k_{cat} in the presence of some cosolutes at 300 mg/mL, which is close to cellular macromolecule concentrations,⁸³ may yield a more realistic activity for EcDHFR *in vivo*. That relatively low concentrations (50 mg/mL or less) of macromolecule cosolutes could weaken ligand binding at least two-fold indicates that in the much more crowded cellular milieu (~300 mg/mL), these effects could be even more detrimental. The potential weaker binding *in vivo* may explain why most metabolites are present in *E. coli* at concentration above their K_{M} s.⁸⁴ Though, Rivas and Minton caution that care should be taken to directly compare what occurs in the more complicated environment inside the cell, with *in vitro* studies in the presence of macromolecules.²¹

What controls these macromolecule-induced effects?

We next ask whether there is one particular property of the macromolecules that modulates the effect on ligand binding? We address this issue as NADP⁺ and DHF binding to either EcDHFR or R67 DHFR were dependent on crowder osmolality (Figures 4 and 5). The change in ligand binding is not dependent upon the size of the crowder (i.e., dextran-70 and Ficoll-70 have similar sizes but different effects). Thus volume exclusion most likely is not the primary factor in the detrimental effects of macromolecules on DHF binding to two DHFRs.

One factor that could cause disparate effects on ligand binding is that the macromolecular cosolutes might be interacting with the proteins themselves. Indeed, one study showed through broadening NMR peaks that both chromosomal DHFR and NADPH interact with ribosomes *in vitro*.⁸⁵ Addition of 0.5 μM ribosome was enough to decrease the k_{cat} and increase the K_{M} both by 20-fold. Our DSC data also indicate that some of our cosolutes interact with at least EcDHFR, as the T_{m} decreases in the presence of the macromolecules.

Further, the effects of macromolecular cosolutes on the k_{cat} s of both EcDHFR and R67 DHFR indicate that there are some effects of the cosolutes on the enzymes themselves. The general trend for both DHFRs is a decrease in k_{cat} upon cosolute addition. For EcDHFR, this might be due to a 7.8 mL/mol activation volume for k_{cat} , as measured by high hydrostatic pressure.³⁹ The positive activation volume indicates that the protein-ligand complex expands in the rate limiting step. Steric hindrance in the presence of crowders would disfavor the more expanded state for EcDHFR in the catalytic step, and potentially decrease the k_{cat} of the reaction, which measures release of tetrahydrofolate.⁶⁹ For R67 DHFR, the larger crowders may decrease k_{cat} by preventing access of the ligands to the R67 DHFR active site pore. For example, the crowders may interact with the disordered N-termini (residues 1–20) of R67 DHFR and interfere with access to the active site. Additionally, the effective concentrations of the ligands for both enzymes will decrease due to their interactions with the cosolutes. A lower effective ligand concentration will decrease the amount of protein that has ligand bound and the amount of enzymatic reactions that can occur at any point in time. Nijhout and Reed have speculated that weak non-competitive inhibition by folate of various enzymes in the folate pathway supports folate homeostasis.^{86, 87}

However, cosolute-DHFR interactions are unlikely to be the primary factor in the effects seen on ligand binding in our studies. As the cofactor and substrate both bind to symmetry

related residues on opposite sides of the active site pore of R67 DHFR,⁸ one would expect macromolecular cosolutes to have the same effects on NADP(H⁺) and DHF binding. In other words, if a cosolute were to affect the binding of DHF in a negative way by interacting with R67 DHFR, then through the symmetry relatedness of the NADPH and DHF binding sites, the same cosolute should also decrease NADPH binding. This is clearly not the case with NADP⁺ and DHF binding in the presence of PEG3350 (Figure 4 and 5). Similar differences in the binding of NADPH and DHF to R67 DHFR in the presence of small molecule osmolytes were used to determine that the osmolytes did not directly affect the ligand binding site on R67 DHFR.¹¹

A second reason that the macromolecules interacting with the DHFRs likely do not play a major role in the effects on ligand binding is that DHF binding to both EcDHFR and R67 DHFR show similar decreases in binding affinity, or k_{cat}/K_M . It is unlikely that all the cosolutes would interact in similarly detrimental ways with two structurally unrelated enzymes.

Third, the macromolecule cosolutes did not significantly affect the secondary structure of either EcDHFR or R67 DHFR (Figure S13), nor was the thermal stability of R67 DHFR perturbed (Figure S14). Additionally, the E1 to E2 equilibrium of EcDHFR was unaffected by the presence of cosolutes (see stopped flow section in the supplement). Also, the dynamics of EcDHFR were not significantly different in our simulations in the absence and presence of lysozyme. Finally, the structures of chromosomal DHFRs are the same when determined by either NMR or crystallography.^{16, 88, 89} Similarly, crystallography and NMR yield the same structures for R67 DHFR.^{9, 90} Proteins are typically crystallized in the presence of high concentrations of different solutes, such as PEGs. Therefore, it is unlikely that interactions between the macromolecular cosolutes and the DHFRs are the primary determinant of the changes in ligand binding.

If the primary factor is not volume exclusion effects or interactions of the cosolutes with the DHFRs, then this leaves effects of the cosolutes on the ligands themselves. Interactions between the macromolecule cosolutes and the ligands of DHFR are not unreasonable as we have previously shown that small molecule osmolytes will interact with free DHF in solution, and have only minimal interactions with NADP⁺, if at all.^{11–15} The decrease in affinity of DHF for both EcDHFR and R67 DHFR could arise due to the interaction of the free DHF with the cosolutes, causing a shift in the binding equilibrium away from the protein-ligand complex to the free species.

There are indications that ligands may have “soft” or “quinary” interactions” with macromolecules. Aumiller et al.^{17, 91} found that PEG interactions with the substrates of horseradish peroxidase increased with the hydrophobicity of the ligand, as measured by its logD. Likewise, when dextran was used as a crowder, less pronounced effects were noted on the k_{cat} and K_M of both the hydrophobic and hydrophilic ligands.^{17, 92} The more subdued effects of dextran compared to PEG were hypothesized to come about from the more hydrophobic nature of PEG compared to dextran. Similarly, Zotter et al.⁹³ found that small, hydrophobic molecules diffused somewhat slower in cells than expected. It was hypothesized that the molecules were interacting with other hydrophobic molecules in the

cell, thus attenuating their diffusion.¹⁸ Though, charged small molecules may associate non-specifically with proteins through electrostatic interactions.¹⁹

DHF and NADP(H⁺) also likely form soft interactions with the crowders, similar to those noted for small molecule osmolytes.^{11–15} The variable nature of the effects of the macromolecular cosolutes on NADP(H⁺) binding to both EcDHFR and R67 DHFR suggests that the chemical makeup of the cosolutes plays a role in their effects. The decrease in the rates of DHF association with apo-EcDHFR in the presence of cosolutes also gives evidence of soft interactions. A similar decrease in the k_{on} for DHF-EcDHFR association in buffer containing 20% (w/w) glycine betaine was attributed to weak interactions between the unbound ligand and glycine betaine^{12, 14} Perhaps in the case of the macromolecular cosolutes, a similar phenomenon occurs, where the free DHF is associating with the cosolutes, and this weak association needs to be released for the ligand to bind to EcDHFR.

Our MD simulations of DHF with lysozyme, ovalbumin or RmlC frequently showed stacking interactions between the pterin ring and aromatic amino acids, while the glutamate tail was more dynamic. In our computational analyses, docking and MD simulations predicted that both DHF and NADP⁺ tended to interact with the active sites of lysozyme and RmlC. Perhaps the ligand binding pockets of lysozyme and RmlC are hydrophobic enough to interact with DHF and NADP⁺, and this weak binding could factor into the effects of these two enzymes on ligand binding to DHFRs, in addition to any weak, non-specific interactions. Though, folate, even when interacting with the crowder, still moves around the protein surface quite a bit (Figures S17-S19). These may be “fuzzy” interactions, similar to those proposed for intrinsically disordered proteins.^{76, 77} We also note that computer simulations of trimethoprim docked on the surface of *Mycobacterium tuberculosis* DHFR show it diffuses on the protein surface as a way to reach the active site.⁷⁵

The evidence from the association rates, the kinetics and binding affinities of both DHF and NADP(H⁺) with two different DHFRs indicates that the free ligands are likely interacting with most of the cosolutes, and thus the DHFR binding equilibrium is shifted towards the free species. Weak interaction between DHFR substrates and other proteins may explain why several folate cycle enzymes were found to have lower *in vivo* activities relative to *in vitro* conditions.⁸²

What controls the interactions with ligands?

If the primary means of macromolecules affecting ligand binding to DHFR is through interactions of the cosolutes with the ligands, then we asked which aspect(s) of the cosolutes leads to these interactions? The effects of the crowders were not related to the molecular volume of the cosolutes or viscosity of the solution (Figures S22 and S23). We have explored whether size (radius of gyration, hydrodynamic radius or molecular weight), the electrostatic potential, net charge of the cosolutes, the scores for docking or the interaction energy from MD simulations correlate with the change in binding (Figure S24). We have also added data from our previous experiments on the effects of bovine serum albumin (BSA) on NADPH and DHF binding to EcDHFR.¹³ Neither the molecular weight nor the radius of gyration of the cosolutes correlated with any of our data. None of the non-protein cosolute properties correlated with their effects on ligand binding to DHFRs. Only protein

crowders had properties that correlated well with their effects. It should be noted that the correlations we obtained with our limited data set may change if/when more crowders are considered.

The effects on NADP(H⁺) binding linearly correlate with the net charge of the proteins. More negatively charged protein crowders have less of an effect on NADP⁺ binding than those with near zero, or positive charges. Charge-charge interactions of NADP(H⁺) with crowder proteins concurs with the results of MD simulations of the cytoplasm of *Mycoplasma genitalium* that showed highly charged metabolites, like NAD⁺, form non-specific electrostatic interactions with proteins inside the cell.¹⁹ Though, because cytosolic proteins in both bacteria and eukaryotes tend to have pIs below 7,^{94, 95} the interactions of NADP(H⁺) with other cytosolic proteins may still occur, but will be less prevalent at physiological pHs. The slopes of these correlation plots were all between -1 and -2, indicating that the effect is the same no matter which DHFR is being used, and likely reveals the interaction of NADP(H⁺) with the cosolutes. The potential for charge-charge interactions also means that the effects of the crowders would likely be modulated by the choice of buffer, pH and ionic strength used as higher ionic strengths would shield the interactions.

DHF binding to either R67 DHFR or EcDHFR linearly correlates best with the total surface area of the active sites of the protein cosolutes ($R^2 > 0.874$). The correlation with the R67 DHFR kinetics is not as good as the EcDHFR kinetic and ITC data. This corresponds with our MD data that suggests DHF may weakly bind to the active sites of lysozyme, ovalbumin and RmlC. Hydrophobic molecules, like DHF, will avoid being solvated by water by interacting with hydrophobic patches on the crowder proteins. The weak, non-specific interactions of small molecules with proteins is reminiscent of ATP, which acts as a hydrotrope, weakly interacting with the surface of proteins keeping them soluble and reducing aggregation.¹⁸ Another study found that there is a finite number of binding site structures found in nature, and proteins are able to accommodate a wide variety of ligands with different chemical structures.⁹⁶ Several of the folate cycle enzymes bind DHF as well, and they may bind free DHF inside the cell. Folate binding is also promiscuous, binding to the central cavity in hemoglobin and to serum albumin,^{60, 97} and the anti-folate drug, methotrexate, binds to as many as 17 different proteins.⁹⁸⁻¹⁰⁰

Very little work has been done looking at the effects of crowders on small molecules,¹⁷ and whether small molecules, just like proteins,²³ can also form quinary interactions with macromolecules. This work is a first step in trying to uncover what type of interactions may drive the formation of quinary complexes between small ligands and macromolecules. While lysozyme, ovalbumin, RmlC, casein and hemoglobin are not *E. coli* cytosolic proteins, the results presented here provide some information about how DHF and NADP(H⁺) may behave in the cell. By examining the effects of individual protein crowders on ligand binding to DHFRs, the properties that are most likely to drive interactions with ligands/drugs in the cell can be uncovered.

Conclusion:

Macromolecule cosolutes perturb the binding of DHF and NADP(H⁺) to two structurally unrelated DHFRs. The macromolecules appear primarily to affect binding by interacting with the ligands, not with the DHFRs. This pattern is similar to that previously noted with small molecule osmolytes,^{11, 12, 15} and concurs with the prevailing notion that in addition to volume exclusion effects under crowding conditions, weak interactions can also be impactful. Of the protein properties explored in this study, the net charge and the surface areas of the binding pockets of the protein cosolutes best correlated with the change in ligand binding versus osmolality, with the exception of DHF binding to EcDHFR. NADP(H⁺) appears to be repelled by the negative charges on the protein cosolutes. DHF weakly binds to the active sites of lysozyme and RmlC in MD simulations as well as to known sites present on hemoglobin and BSA. These quinary interactions between NADP(H⁺) and DHF with protein macromolecules are likely present *in vivo*, leading to lower activity of DHFR. Though, as seen by the variety of crowder effects on the two DHFRs, the heterogeneous local environment will impact how the DHFRs behave *in vivo*. Based on our stopped-flow studies showing the association rate of methotrexate decreases in the presence of crowders, these data imply that the binding of drugs targeting either of the two DHFRs may similarly bind more weakly in the cell compared to *in vitro*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

BME	β-mercaptoethanol
BSA	bovine serum albumin
DHF	dihydrofolate
DHFR	dihydrofolate reductase
EcDHFR	<i>E. coli</i> chromosomal DHFR
EDTA	N,N,N',N'-ethylenediaminetetraacetic acid
ITC	isothermal titration calorimetry
MD	molecular dynamics
MTA buffer	100 mM Tris, 50 mM MES, 50 mM acetic acid buffer

pABA	p-aminobenzoate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NADP⁺	oxidized nicotinamide adenine dinucleotide phosphate
NADP(H⁺)	either, or both, NADPH and NADP ⁺
PEG	polyethylene glycol
PVP	polyvinylpyrrolidone
T_m	protein melting temperature

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Figure 1. Comparison of the protein structures for the two unrelated DHFRs. A) EcDHFR (PDB ID 1RA2)¹⁶ and B) R67 DHFR (PDB ID 2RK1)⁸ with each monomer in the tetramer colored separately. The positions of the bound ligands NADP⁺ (green) and folate bound to EcDHFR or DHF bound to R67 DHFR (magenta) are shown in the structures. The p-aminobenzoyl-glutamate tail of DHF is disordered in the R67 DHFR structure.

Figure 2.

The effects of macromolecules on k_{cat}/K_M ($M^{-1} s^{-1}$) measured by progress curves for the reduction of DHF by both DHFRs with saturating concentrations of NADPH. Panels A-K show data for R67 DHFR while panels L-V show data for EcDHFR. Crowder additions for R67 DHFR include panel A) which has PEG3350 added (●, fit is a solid black line), B) PEG8000 (■, red line), C) PVP-55 (▲, blue line), D) dextran-10 (○, dashed black line), E) dextran-40 (□, light green line), F) dextran-70 (△, gold line), G) Ficoll-70 (★, purple line), H) lysozyme (★, magenta line), I) ovalbumin (●, cyan line), J) RmlC (▽, dark cyan line) or K) casein (◆, green line). Data for EcDHFR are shown for addition of L) PEG3350, M) PEG8000, N) PVP-55, O) dextran-10, P) dextran-40, Q) dextran-70, R) Ficoll-70, S) lysozyme, T) ovalbumin, U) RmlC, and V) casein.

PEG8000, N) PVP-55, O) dextran-10, P) dextran-40, Q) dextran-70, R) Ficoll-70, S) lysozyme, T) ovalbumin, U) RmlC and V) casein. Buffer controls are shown as grey circles (●). The typical R^2 value is >0.9 . However, there are a few R^2 values that are lower, likely due to either a fewer numbers of points or a less steep slope so that the assay error/noise is more evident.

Figure 3.

Macromolecules alter the k_{cat}/K_M ($M^{-1} s^{-1}$) as measured by progress curves for the oxidation of NADPH by both DHFRs in the presence of saturating DHF. Data are shown for R67 DHFR using 5 concentrations of NADPH and DHF in A) PEG3350 (●), B) PEG8000 (■), C) PVP-55 (▲), D) dextran-10 (○), E) dextran-40 (□), F) dextran-70 (△) and G) Ficoll-70 (★). Progress curve data for EcDHFR in the presence of limiting NADPH and saturating DHF in panel H) for PEG3350, I) PEG8000, J) PVP-55, K) dextran-10, L)

dextran-40, M) dextran-70, N) Ficoll-70, O) lysozyme (★), P) ovalbumin (●), Q) RmlC (▽) or R) casein (◆). Buffer controls are shown as grey circles (●).

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Figure 4.

ITC experiments that measure the effect of macromolecules on the $K_a (=1/K_d, M^{-1})$ for $NADP^+$ binding to apo-R67 DHFR in the presence of A) PEG3350 (●), B) PEG8000 (■), C) PVP-55 (▲), D) dextran-10 (○), E) dextran-40 (□), F) dextran-70 (△), G) Ficoll-70 (★), H) lysozyme (✱), I) ovalbumin (●), J) RmlC (▽), K) casein (◆) or L) hemoglobin (▼). $NADP^+$ binding to apo-EcDHFR was also performed in the presence of M) PEG3350, N) PEG8000, O) PVP-55, P) dextran-10, Q) dextran-40, R) dextran-70, S) Ficoll-70, T) lysozyme, U) ovalbumin, V) RmlC or W) casein. Buffer controls are shown as grey circles

(●). The fit lines are the same colors as in Figure 2, except hemoglobin, which is fit with a brown line. (●). The typical R^2 value is >0.9 . However, there are a few R^2 values that are lower, likely due to either a fewer numbers of points as ITC requires use of high concentrations of protein or a less steep slope so that the assay error/noise is more evident.

Figure 5.

ITC experiments that measure the effect of macromolecules on K_a ($=1/K_d$, M^{-1}) of DHF binding to both DHFRs. Data for formation of the R67 DHFR-NADP⁺-DHF complex are shown in the presence of A) PEG3350 (●), B) PEG8000 (■), C) PVP-55 (▲), D) dextran-10 (○), E) dextran-40 (□), F) dextran-70 (△), G) Ficoll-70 (★), H) lysozyme (★), I) ovalbumin (●), J) RmlC (▽), K) casein (◆) or L) hemoglobin (▼). DHF binding to the EcDHFR-NADP⁺ binary complex was also performed in the presence of M) PEG3350, N) PEG8000, O) PVP-55, P) dextran-10, Q) dextran-40, R) dextran-70, S) Ficoll-70, T) lysozyme, U)

ovalbumin, V) RmlC or W) casein. Buffer controls are shown as grey circles (●). The color of the fit lines is the same as in Figure 2.

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Figure 6.

Representative heat maps of the interaction energies (sum of electrostatic and van der Waals terms) between the ligands of DHFR and lysozyme from MD simulations. A) A simulation that shows folate interacting with a region near the active site on lysozyme over the course of most of the simulation. Residues involved include Lys33, Phe34, Glu35, Ser36, Asn37, Asn39, Ala42 and Gln57. B) NADP⁺ associations with lysozyme are more ephemeral.

Figure 7.

Distance plots showing the center of masses for different moieties of the folate molecule (the glutamate moiety, black line; the pterin ring, red line; and the pABA ring, blue line) with residues on lysozyme. A) Electrostatic interactions occur between the glutamate of folate and positively charged residues (the R-group amine of lysine 33 with the α -carboxylate oxygens of the glutamate tail, green line). B) There are π - π type interactions between folate and lysozyme (C δ of the phenyl ring of Phe34 with the pABA ring, green line). C) Van der Waals interactions (with Asn37 C β with the pABA ring, green line) also occur mostly

between the aromatic rings of folate, while the glutamate moiety is moving around in the solution.

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