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Getting Heavy: An Exploration into the Effects of D2O and High Hydrostatic Pressure on R67 Dihydrofolate Reductase

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To the Graduate Council:

I am submitting herewith a thesis written by Mary Jane Timson entitled "Getting Heavy: An Exploration into the Effects of D2O and High Hydrostatic Pressure on R67 Dihydrofolate Reductase." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biochemistry and Cellular and Molecular Biology.

Elizabeth E. Howell, Major Professor

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Getting Heavy: An Exploration into the Effects of D_2O and High Hydrostatic Pressure on R67 Dihydrofolate Reductase

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ABSTRACT

Chromosomal dihydrofolate reductase (DHFR) enzymatically reduces dihydrofolate (DHF) to tetrahydrofolate (THF) using NADPH as a cofactor. R67 DHFR is an R-plasmid encoded enzyme that confers resistance to trimethoprim (TMP), an antibacterial drug. It shares no structural homology with TMP targeted, chromosomal DHFRs.

Previous osmolyte studies in our lab have indicated that DHF binding to R67 DHFR is accompanied by water uptake and NADPH binding is accompanied by water release. These data suggest that water plays a role in balancing the binding affinity. This may happen as R67 DHFR has a generalized binding surface and may need differential water effects to accommodate both ligands. To further examine this hypothesis, we collect binding and steady state kinetic data using hydrostatic pressure. Increasing hydrostatic pressure hydrates molecules and can essentially test the effect of increasing water concentration upon binding. Hydrostatic pressure can also affect the volume of the active site as well.

An activation volume, defined as the change in molar volume associated with the ternary ENADPH-DHF complex going to the transition state, can be determined from a plot of the natural log of k_{cat} vs pressure. The slope of this line is equal to $-\Delta[\text{delta}]\ V/R_pT$. A small slope giving an activation volume of -1.03 ± 0.9 cm_3/mol is observed until 200 Mpa. A second slope describing the effect of pressure from 200 Mpa to 500 Mpa on the activation volume was equal to 8.06 ± 0.8 cm_3/mol. Positive activation volumes indicate that the rate-limiting step described accompanies a protein volume increase.

As water reorganization may be playing a role in binding of both substrate and cofactor, studies using isothermal titration calorimetry in both H_2O and D_2O were utilized to determine the enthalpy of solvent reorganization. The observed enthalpy of the interaction between protein and substrate can be broken up into $\Delta[\text{delta}]H_i$ (enthalpy of the interaction) and $\Delta[\text{delta}]H_s$ (enthalpy of solvent reorganization). Since the enthalpy of a hydrogen bond in D_2O is approximately 10% greater than in H_2O, the $\Delta[\text{delta}]H_s$ can be estimated.

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Chapter I

Introduction

Dihydrofolate Reductase

Dihydrofolate Reductase (DHFR) is an enzyme that catalyzes the reduction of dihydrofolate (DHF) to tetrahydrofolate (THF) using NADPH as a reducing agent. THF is an important precursor of several metabolites, including methionine and purine nucleotides. Inhibition of DHFR activity has been utilized as a method of blocking the cell cycle by stopping DNA synthesis. Targeting this enzyme for inhibition can be beneficial in medical therapies for cancer as well as bacterial infection (1) (2).

Escherichia coli chromosomal DHFR (*Ec*DHFR) has been well studied as a model target to inhibit bacterial infections among humans using antibiotics such as trimethoprim (TMP). The antibiotics work as competitive inhibitors of DHF preventing the enzyme from catalyzing its reaction. When high levels of inhibition are accomplished, the bacterial cells can no longer replicate and they die. Bacterial cells have developed one type of resistance to the antibiotic drug TMP by expressing R67 DHFR, a type II R-plasmid DHFR.

R67 Dihydrofolate Reductase

R67 Dihydrofolate Reductase catalyzes the same reaction as the chromosomal *Ec*DHFR, but with a lower rate and higher K_ms for the substrates (3), (29 s^{-1} k_{cat}, K_m DHF 1.2 µM, and K_m NADPH 0.94 µM for *Ec*DHFR (4) and 1.3 s^{-1}, 5.8 µM, and 3.0 µM for R67 DHFR (5)). The rate determining step for *Ec*DHFR is product release and for R67 DHFR it is the chemical step, or hydride transfer. However simple this reaction may be, it is the difference in K_i for TMP that allows for the enzyme to confer antibiotic

resistance to TMP. As the binding constant for TMP to R67 DHFR is 0.15 mM (6), the concentrations to block the enzyme would be too high to be administered to an infected host. With the cell able to express R67 DHFR, the synthesis of THF takes place in the presence of the antibiotic. The ability of R67 DHFR to carry out the same enzymatic process is quite remarkable for many reasons. Dihydrofolate reductase in *E. coli* has very different binding sites for both ligands when compared to R67 dihydrofolate reductase. The large difference in the structure and active site of both chromosomal and R67 DHFR leads to many questions about how the reaction is carried out.

Structural Differences

R67 DHFR is a 34 kDa homotetramer with "222" symmetry, as when the molecule is rotated 180° on the x, y, and z axes the structure remains the same. The active site pore is in the middle of the tetramer and is open to the environment. There are no loops around the active site to close or open providing a more hydrophobic environment for the substrates. The ligands bind to related binding sites and the binding of 2 NADPH molecules, 2 DHF or 1 of each can occur with the latter being the productive complex (7). In contrast, the chromosomal *Ec*DHFR is an 18 kDa monomer with a Met20 active site loop (residues 9-24), which connects to the nearby FG loop (residues 119-132), and GH loop (residues 142-150). Ligand binding has been shown to be accompanied by movement of the Met20 loop. A side by side representation of the chromosomal *E. coli* DHFR and R67 DHFR is shown in Figure 1. The difference in structure of the two proteins is remarkable. If the structure and binding sites of each ligand are different between EcDHFR and R67 DHFR, how is the same reaction facilitated?

Ternary Structure of R67 DHFR

The symmetry of the protein provided complications in solving the crystal structure of the ternary complex. However, these complications were overcome and a crystal structure of the R67

DHFR:$NADP^+$:DHF complex was determined and resolved to 1.26 Å (8). The active site of R67 DHFR allows stacking between the pteridine ring of substrate and the nicotinamide ring of cofactor. There is little to no movement between the rings when the reaction occurs (as modeled by molecular dynamics (9)) and the positioning of the rings in the crystal structures is easy to determine. However, the electron density for the tail of DHF was unable to be determined and therefore the tail is proposed to be disordered in the ternary crystal structure. The electron density for bound $NADP^+$ was clear and provided a good model of the cofactor in the active site. The crystal structure showed a multiple layered stacking motif involving the indole rings of Trp38 residues and a hydrogen bonded network formed between Gln67 and Tyr69. These contacts form a "club sandwich motif" and stack the nicotinamide and pteridine rings of NADPH and DHF respectively in the endo conformation (Figure 2). This is interesting as the rings of each ligand for *Ec*DHFR are found edge to edge, consistent with an exo conformation in the transition state (10). The endo conformation has been hypothesized to be more efficient for hydride transfer (11). The use of the endo conformation may allow R67 to catalyze the DHFR reaction despite the structural differences between R67 and *Ec*DHFR.

The residues mentioned above and described in Figure 2 are important for ligand binding according to the crystal structure data. It has been noted that hydrogen bonding between the nicotinamide and pteridine rings and the back bone of Ile68 residues is also important to position the rings in the active site as shown in Figure 3. The active site is open to solvent and does not contain a hydrophobic loop to keep water molecules out. Water molecules can be seen within the active site of R67 DHFR. It is hypothesized that protonation of the N5 atom on DHF occurs via a water molecule in solution (8).

Why is R67 DHFR interesting?

In many ways R67 DHFR is a relatively simple enzyme. It is promiscuous and each ligand binds to related binding sites with different interactions. A question that arises is where did this enzyme come from? Is it a primitive enzyme that has not evolved? The findings within the Howell Laboratory seem to be consistent with R67 DHFR as a primitive enzyme as the rate limiting step is the chemical process, the binding sites are not specific for each molecule, and the protein can accept many mutations without losing activity. The study of this enzyme should prove helpful to our understanding of how an enzyme can work in a less evolved fashion and may become important to understanding how an enzyme can facilitate catalysis on a more general basis.

Binding of Ligands to R67 DHFR

Although 4 symmetry related binding sites must exist in R67 DHFR due to its 222 symmetry, both ligands are bound to the enzyme in a different manner. Fluorescence anisotropy and Isothermal Titration Calorimetry (ITC) studies were performed and data supported the binding and catalytic scheme shown in Figure 4. The enzyme can accommodate 2 NADPH molecules or 2 DHF/folate, or one of each ligand with the latter being the reactive complex. The studies indicated negative cooperativity between the 2 NADPH molecules, meaning that one NADPH molecule would bind tightly to the apo-enzyme and a second NADPH molecule would require a much higher concentration to bind. This pattern may arise due to symmetry-breaking upon binding of the first NADPH molecule. The opposite was true for the DHF/folate complexes. Positive cooperativity was indicated in that a large concentration of DHF/folate was required for initial binding to the apo-enzyme and another molecule would bind tightly to the second site due to stacking of the pteridine rings. These data point to NADPH occupying the first site allowing DHF/folate to occupy the second site with positive cooperativity (12). Once this complex is formed, the catalytic reaction proceeds.

Salt Effects on R67 DHFR

As ionic interactions could play a role in the binding of DHF/folate, perturbation of the ionic strength of the solution by increasing salt concentration should have large effects on the DHF/folate binding constants. Different salt species were used to increase the ionic strength of solutions and similar results were obtained (13). As the ionic strength of the solution is increased, k_{cat} is also increased; however, K_m values for both NADPH and DHF are increased which is consistent with ionic strength weakening the binding of each ligand. Figure 5 plots the steady state kinetic data vs. ionic strength. It can be seen from the log k_{cat}/K_m plots for both NADPH and DHF that the slope is negative, meaning that increasing ionic strength is slowing k_{cat}/K_m. This is opposite to the effect of ionic strength on k_{cat} alone as k_{cat} increases with increased ionic strength. The inserts of the k_{cat}/K_m plots are the plots of the K_m alone. The slope is positive as the K_m is increased or weakened in the presence of increasing ionic strength (14).

The k_{cat} of the reaction increases consistent with the disruption of a salt bridge involved in transition state formation. Higher K_m values for both DHF and NADPH were also observed with increasing salt concentrations. The slope of log-log plots of K_m or k_{cat} vs. ionic strength can quantitate the number of salt bridges being broken within the reaction (15). As the salt concentration within the system is increased, the formation of ionic interactions will be decreased as the presence of more salt will competitively inhibit the interactions between the proteins and the ligands. The disruption of ionic bonds will increase as the ionic interactions will prefer to pair with ions in solution. Slopes of close to -2 for the K_m of each ligand were found, however a slope of 1 was found for k_{cat}/K_m conditions. These data indicate that formation of 2 salt bridges is important for initial ligand binding and 1 of these interactions is broken upon moving from the ground state to the transition state (14).

In addition to the above steady state kinetic data, ITC experiments demonstrated that increasing the ionic strength resulted in the loss of enthalpic interactions for both NADPH and folate. For NADPH, the K_d becomes weaker and the observed enthalpy becomes less negative. For folate binding to enzyme-NADPH, a titration in enthalpy is observed while the K_d remains constant (14).

Thermodynamics of Ligand Binding to R67 DHFR

Previous ITC and kinetic inhibition studies within the lab used structurally different ligands to determine the important interactions between the ligand and protein during binding (16). The truncation of each ligand resulted in weaker binding and a smaller exothermic signal. Ligand connectivity is important for binding to occur. The nicotinamide ring of NADPH contributes substantially to the enthalpic signal associated with binding. However, no enthalpic contribution was observed in the binding of an analogue with just the nicotinamide ring and a phosphate to R67 DHFR, indicating that multiple interactions are important, not just the nicotinamide ring (13). In the case of folate/DHF analogues, similar observations were seen as the use of Dihydrobiopterin (DHB) provided no enthalpic signal. Figure 6 shows the structures of DHF and DHB. The main difference is the lack of the PABA-glu tail in the DHB molecule when compared to DHF. When the analogues were tested with inhibition kinetics, K_i's were observed, indicating binding, albeit with much weaker interaction strength. It was hypothesized that the loss in ITC signal was due to a loss in enthalpy derived from a disruption of an ionic interaction between the carboxylate residues in the DHF tail and the two symmetry related K32 residues in the half pore of the protein (16). This view is consistent with the previous findings for salt effects. These results also are consistent with analysis of the crystal structure. All of these findings are important to understand the difference in DHF binding to R67 DHFR and NADPH binding to R67 DHFR with respect to water studies.

Osmolyte Studies on R67 DHFR

As addition of sucrose showed an effect on the k_{cat}/K_m for DHF value in R67 but did not particularly impact k_{cat} (13), a study on the involvement of water in the reaction was undertaken. Water activity was altered by the presence of increasing osmolytes of different natures. While k_{cat} was not significantly affected, the DHF K_d and NADPH K_d values were effected in opposite directions. As osmolytes of different natures were increased in concentration, the NADPH K_d tightened. Similar effects among different osmolytes are consistent with the interpretation that NADPH binds with a net release of approximately 38 water molecules (17) (18) (19). This effect is normal in ligand binding as both ligand and protein are solvated and water typically needs to be removed for binding. In the case of osmolytes and DHF binding to R67 DHFR, the K_d was weakened but to different extents depending on osmolyte type. This result potentially indicates DHF binding with a net uptake of water with different values for each osmolyte. Water uptake is unusual for protein/ligand interactions (20). A plot of the ln K_a for both DHF and NADPH binding to enzyme vs. osmolality can be seen in Figure 7 below. The osmolality of a solution can be measured using an osmometer and from that the activity of water (α_{H2O}) can be found by the relationship:

$$\alpha_{H2O} = e^{-0.018*\text{osmolality}} \qquad \text{(Eq. 1)}$$

The plot of ln K_a vs. osmolality is based on the equation:

$$\delta \ln K_a / \delta \ln \alpha_{H2O} = v_{H2O} + v_s [\, \delta \ln \alpha_s / \delta \ln \alpha_{H2O}] \quad \text{(Eq. 2)}$$

where v_{H2O} and v_s are the stoichiometries of the water and osmolytes respectively. Thus the slope of a plot of ln K_a versus osmolality or water activity will yield the number of water molecules involved in the

experimental reaction if osmolality is plotted, then the slopes can be converted to Δn_w using the relationship given in equation 3:

$$d \ln K_a / d [\text{Osmolal}] = - \Delta n_w / 55.6 \quad \text{(Eq 3)}$$

Water reorganization was found by Chervenak and Toone (21) to be correlated with change in heat capacity values. The change in heat capacity values can be obtained by measuring the enthalpy at different temperatures. The effects seen on water in the R67 DHFR reaction were complex and showed that water seemed to be playing an important part in the binding of both ligands. The measured heat capacity for the first NADPH site was – 178 ± 15 cal/K mol while the heat capacity measured for DHF binding to R67 DHFR:$NADP^+$ was – 199 ± 16 cal/K mol. Each of these values is small and within error of one another. Both NADPH and DHF binding to R67 DHFR show enthalpy-entropy compensation (20), considered to be a hallmark of water involvement.

Heavy Water Studies on other protein complexes

As wild type R67 DHFR has been hypothesized to utilize water as a co-substrate based on the studies with osmolytes, an investigation into the reorganization of solvent from the protein and ligand would provide valuable information on the role of water. ITC studies on protein-ligand interactions in H_2O and D_2O can lead to an understanding of solvent reorganization upon ligand binding. The Born-Haber cycle diagrammed in Figure 8 shows the interactions involved with ligand binding and the observed enthalpy of the reaction. As water solvates all molecules in the reaction, water molecules will hydrogen bond to the solvent exposed surfaces of these molecules. As the molecular interaction occurs, the molecules will be desolvated. The observed enthalpy during a binding interaction is a net effect and all interactions being formed or broken will contribute to the overall enthalpy of the interaction. As water molecules will need to be removed, or in some cases added, to the molecules for the interaction

to occur, an enthalpy of solvent interaction (ΔH_{si}) can potentially be observed in the enthalpy as well as the interaction of the two molecules. Equation 4 describes the contributors to the observed enthalpy.

$$\Delta H_{obs} = \Delta H_i + \Delta H_s \quad \text{(Eq. 4)}$$

Chervenak and Toone (21) propose that the enthalpy of solvent reorganization can be determined by the difference in the observed enthalpy of an interaction in D_2O and H_2O. As deuterium bonds are stronger (approximately 10% greater) than regular protium bonds as well as more localized, an increase in the negative enthalpy of the interaction in D_2O versus H_2O might be predicted to be observed. Ten times this $\Delta\Delta H$ difference might then account for differences in solvation. This hypothesis is made with the assumption that receptor and ligand structures remain the same in D_2O as they do in H_2O. The interactions studied in Chervenak and Toone studies showed that solvent interaction contributed to the observed enthalpy of the reactions approximately 25-100% of the total enthalpy. All of the measured enthalpies were less negative in D_2O than H_2O.

The above results are not always the case and not always this simple. For example, Ozen et. al. performed similar studies on a promiscuous enzyme with different results (22). Aminoglycoside phosphotransferase(3')-IIIa (APH) is an antibiotic resistance enzyme that binds several structurally different ligands that are chemically synthesized antibiotics. The two ligand families studied via ITC were neomycins and kanamycins. For kanamycins, a more negative enthalpy was observed upon binding to APH in D_2O with respect to H_2O, while the neomycins showed a less negative enthalpy upon binding to APH in D_2O. Chervenak and Toone only observed less negative enthalpies in D_2O (21). Ozen et. al. hypothesized the existence of an alteration of water structure around areas other than the active site (22).

High Hydrostatic Pressure (HHP) on Protein systems

Le Chatelier's principle suggests that when systems at equilibrium are subjected to a stress, the equilibrium will be shifted to relieve that stress. In the case of hydrostatic pressure, a chemical system will be put under a stress. tending to decrease volume as pressure and volume are inversely related. Therefore, according Le Chatelier's principle, the system will shift the equilibrium towards the smaller volume state. Northrop states that volume changes reported from shifts in the equilibrium under pressure do not have to represent real space, as pressure can change other elements of the system (23).

High Hydrostatic Pressure (HHP) can be applied to protein systems to better understand the mechanism involved. HHP has been commonly recognized as a denaturant of protein structure, but different studies with different protein complexes have shown HHP to have little effect on structure as a denaturant (24). The major effect of pressure is on the volume change of the system noted as ΔV, which can be determined from equation 5:

$$(\delta \ln K/\delta P)_T = -(\Delta V/RT) \quad \text{(Eq 5)}$$

Where K is the equillibrium constant

P is the change in pressure

T is Temperature in K

ΔV is the change in volume

R is the gas constant in terms of pressure

The effect of the change in pressure on the equilibrium constant of the pressure can yield the ΔV. The major factors that contribute to the change in volume and determine the pressure effects are

electrostriction of polar and charged groups, elimination of packing defects, and the solvation of the molecules (25).

The change in volume of covalent bonds upon pressurization is relatively minimal and usually the primary structure of the protein remains intact. The literature is contradictory on the effects of hydrostatic pressure on hydrophobic interactions. Boonyaratanakornkit et al. state that water molecules will form clathrate structures around the hydrophobic residues as pressure is increased (25). Figure 9 is a schematic showing the direct relationship between pressure and the hydration level of molecules of different bond interactions in proteins. As pressure is increased, so is the level of hydration of the molecules. Cavities within proteins are penetrated with water and eventually cavities within the protein will lead to molten globule formation. Ion pairs within the system are not favored by pressure as they become solvated. The solvation of an ion pair results in electrostriction, which is a decrease in the volume as an alignment of solvent molecules in the electric field occurs. The dissociation of ion pairs with increasing pressure results in breakage of ionic interactions and increasing HHP can increase the dielectric constant of the system (25) (26). Studies using hydrostatic pressure have been developed to study complex systems. Many different enzymes have been used and the effects on catalytic rate as well as binding of ligands have been explored (25) (27).

*Ec*DHFR has been an enzyme of interest under hydrostatic pressure. Ohmae et. al. explored the effects on hydrostatic pressure on the catalytic rate constant and binding constants of all the ligands for *Ec*DHFR (27). *Ec*DHFR has many conformations during the catalytic process and is probably very susceptible to stress by hydrostatic pressure. In the 0-50 Mpa pressure range, plots of ΔG vs pressure suggest the presence of different conformations. The data sets taken from 50 Mpa to 200 Mpa showed linear relationships, however the first point from 0-50 Mpa did not fit the line. These data indicate that

the preferred conformation of the protein was likely different at higher pressures. The catalytic rate constant decreased with increasing pressure and yielded an activation volume of 7.8 ± 0.6 ml/mol. These data were collected at pH 7.0 and 30°C, where release of product, THF, is the rate-determining step.

Purpose of this study

This current study approaches water involvement in R67 DHFR function two ways, using heavy water and hydrostatic pressure. Perturbing the water structure is difficult to do without changing other elements of the solution and can therefore be difficult to determine the specific role of water in a reaction. The previous osmolyte studies on R67 DHFR showed that water has a role in the binding of both ligands to R67 DHFR, but no role in the catalytic rate constant. DHF bound with a net uptake of water and NADPH bound with a net release of water. As desolvation of each binding partner to provide specific interactions between the protein and ligand is common, a net release of water upon binding is a typical result. The net uptake of water upon DHF binding was intriguing and posed the question of water being a co-substrate in the R67 DHFR binding process. As hydrostatic pressure is proposed to hydrate molecules and increase the water concentration which are the opposite of osmotic pressure effects, our initial hypothesis was that increasing hydrostatic pressure on R67 DHFR will weaken the binding of NADPH, tighten the binding of DHF, and have little to no effect on the catalytic rate constant.

Introducing heavy water into the system and measuring the enthalpy can give information on the solvent reorganization of the system. As deuterium bonds are stronger and require more energy to break, the removal or uptake of water molecules will contribute to the observed enthalpy of the system. Assuming that the slightly different properties of D_2O versus H_2O have no effect on the protein or ligand structure, the enthalpy of solvent interaction can be determined by the difference in observed enthalpy

monitored in H_2O and D_2O. As the osmolyte studies indicate different trends for water upon binding of DHF and NADPH, it would be hypothesized that the same would be true for the enthalpy differences in H_2O and D_2O.

As stated earlier, elucidating the role of water is a difficult task as currently there is no way to only effect the water structure and not perturb anything else in solution. As it stands, to accurately determine the role of water, many different studies must be done with different properties that all effect the water concentration and possibly structure. In solution, molecules are surrounded by solvent molecules, generally water. The water molecules are often facilitated as a means to aid and accomplish the interaction of molecules and must also be dealt with as they surround both molecules involved in the interaction. This study will help determine the role of water in R67 DHFR and possibly help future investigators in their water endeavors as well.

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Appendix

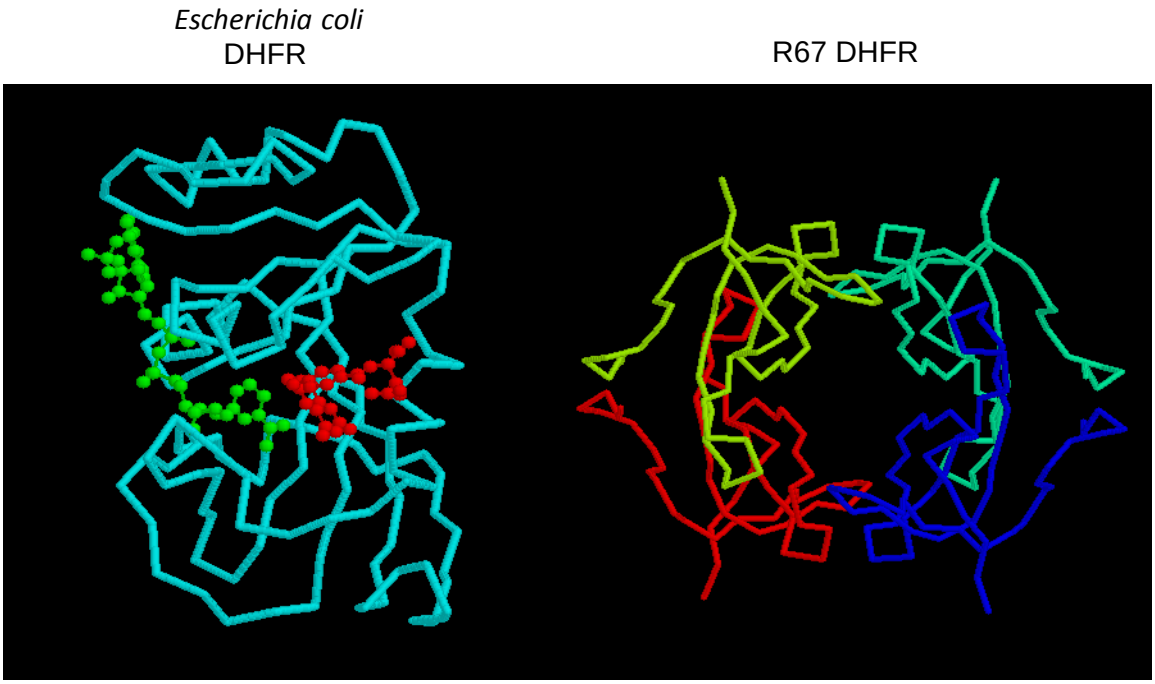


Figure 1: Crystal structures of *E. coli* DHFR (PDB: 1RX2) and R67 DHFR (PDB: 1VIE)

***Ec*DHFR is a monomer with a flexible loop that opens and closes the active site. The red and green molecules are Folate and $NADP^+$ respectively. R67 DHFR is a homotetramer with an active site pore open to the environment. The four different colors represent different subunits in the tetramer.**

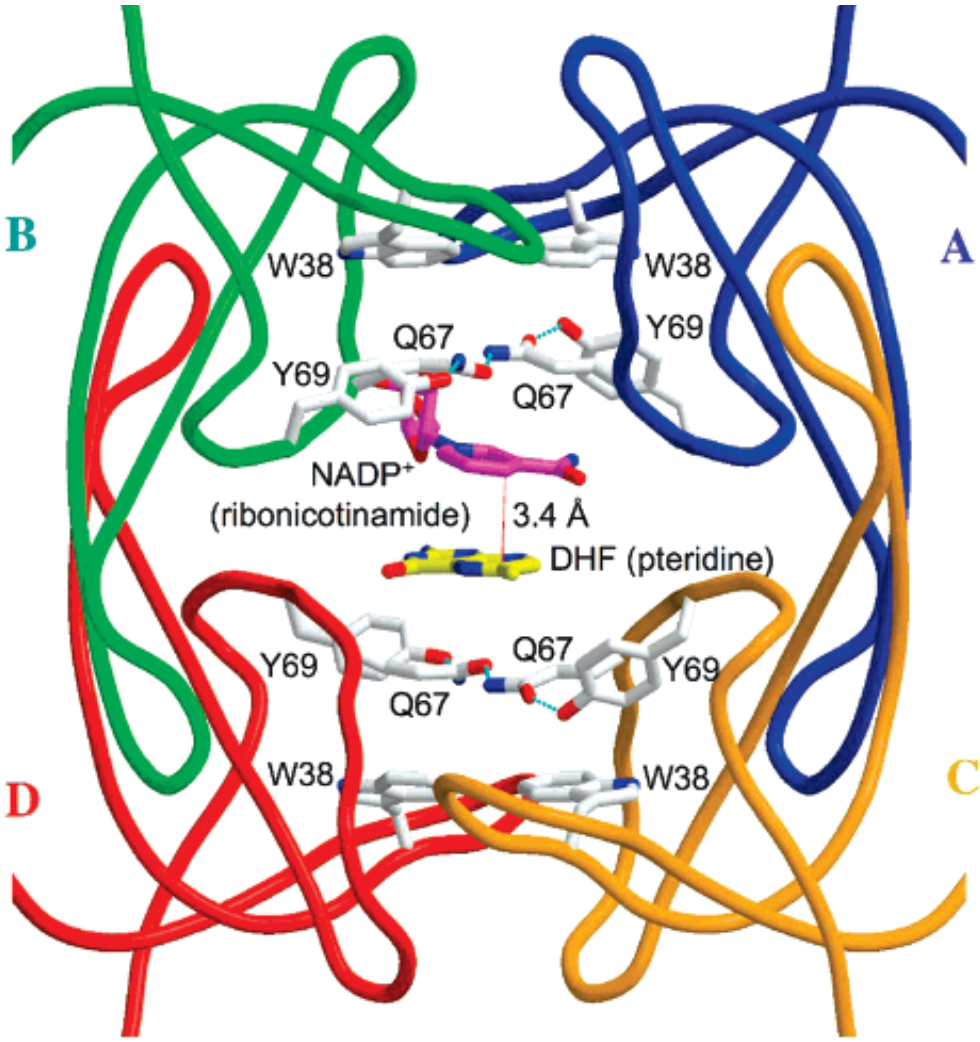


Figure 2: "Club Sandwich Motif" (8)

The endo conformation of the ring in substrate and cofactor allows for proper hydride transfer to occur. It can be seen that the W38, Q67, Y69 residues play critical roles in stacking of the rings for transfer of the proton. $NADP^+$ is in magenta and DHF is in yellow. The $NADP^+$ molecule has been truncated for simplicity, only showing the ribonucletide. The important residues mentioned above side chains are shown in white with oxygens in blue and nitrogens in red.

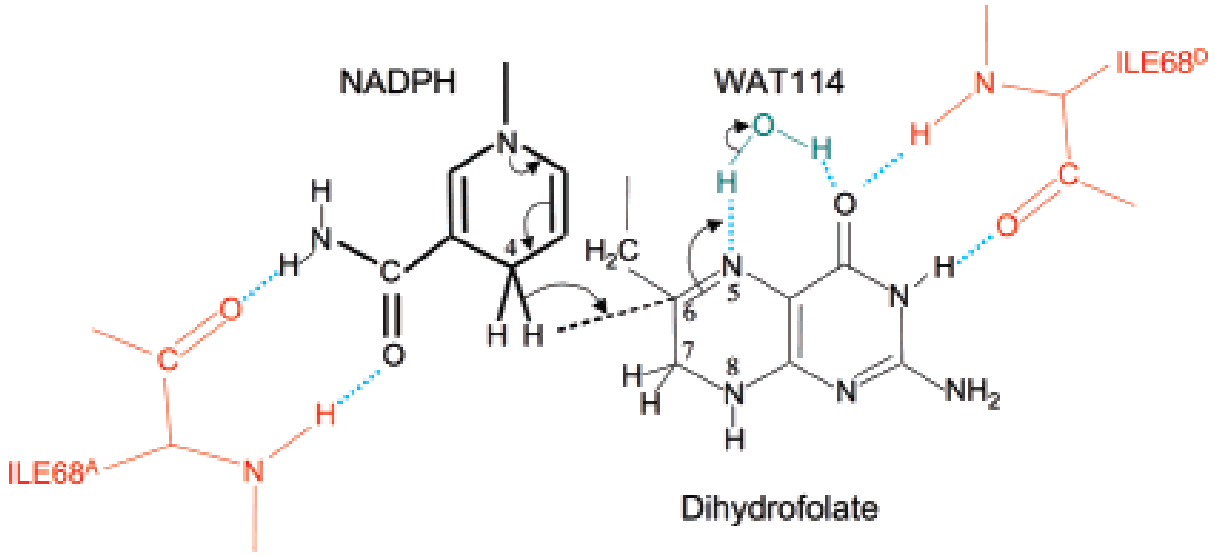


Figure 3: Hydride Transfer and Protonation Step (8)

The backbone carbonyls and nitrogens of symmetry related ILE68 residues use hydrogen bonds to position correctly the pteridine and nicotinamide rings. Several water molecules can be seen within the active site and it is hypothesized that a water molecule donates a proton to the N5 of DHF.

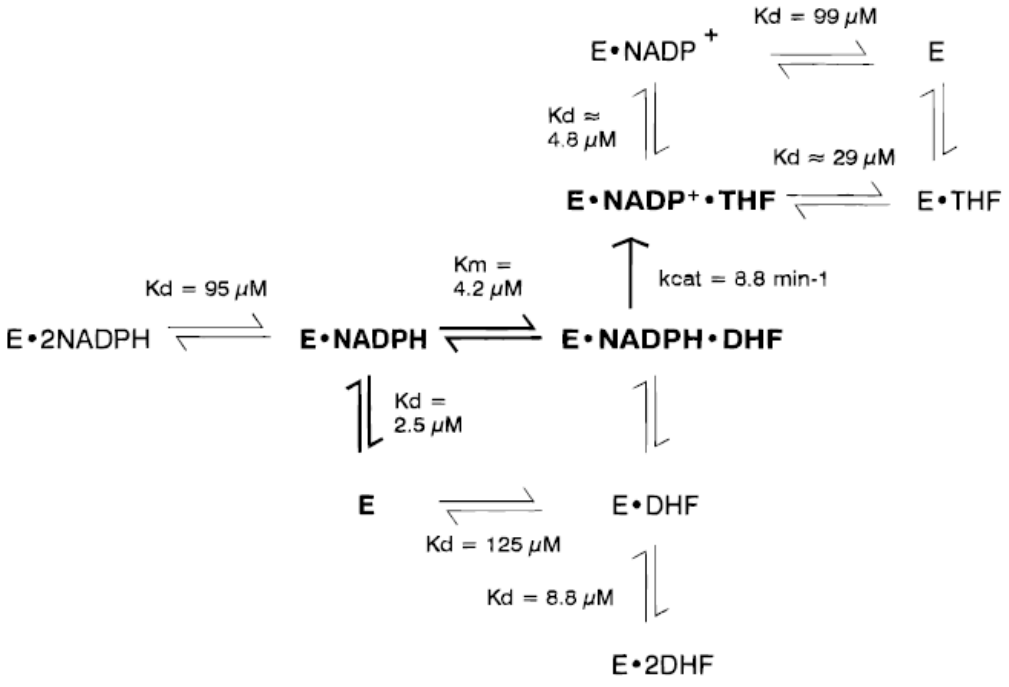


Figure 4: Proposed Scheme of Binding and Catalysis of R67 DHFR (12)

The binding constants of the ligands and catalytic rates involved in R67 DHFR indicate that cooperativity between bound NADPH and DHF leads to preferential formation of the active complex which facilitates the reaction.

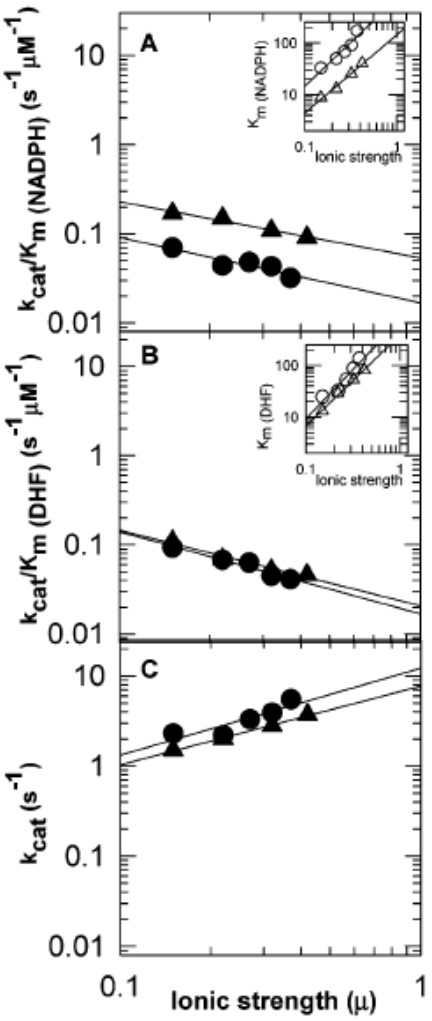
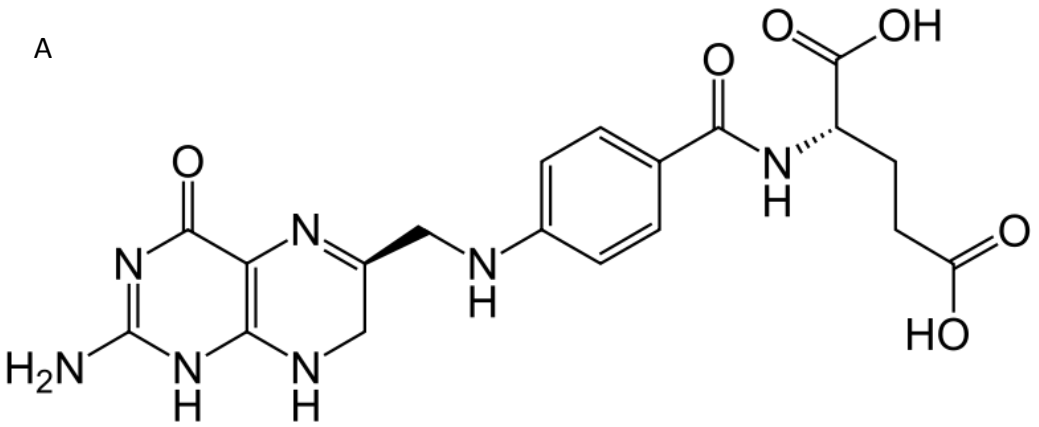


Figure 5: Log Steady State Kinetic Plots vs. Ionic strength (14)

The ionic strength of solution was increased with increasing concentrations of NaCl and plotted against the change in the constants for steady state kinetic data collection. Panel A represents the log k_{cat}/K_m for NADPH with an insert showing the plot of K_m NADPH vs. ionic strength. Panel B represents the log k_{cat}/K_m for DHF with an insert showing the plot of K_m DHF vs. ionic strength. Panel C represents the log of k_{cat} vs. ionic strength.

A



B

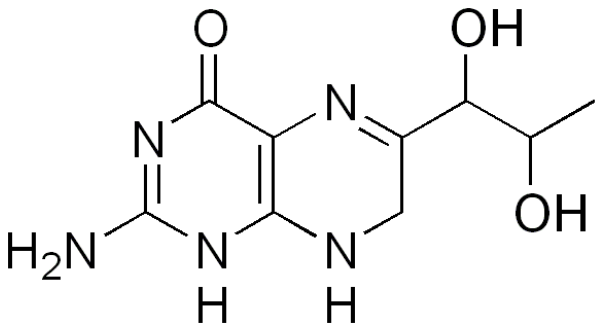


Figure 6: Dihydrofolate(DHF) and Dihydrobiopterin(DHB) Structures

Panel A is the structure of DHF, in this structure the pteridine ring is seen attached to PABA-glu tail. Panel B is the structure of DHB, the pteridine ring is the same as DHF but the tail is truncated in the case of DHB.

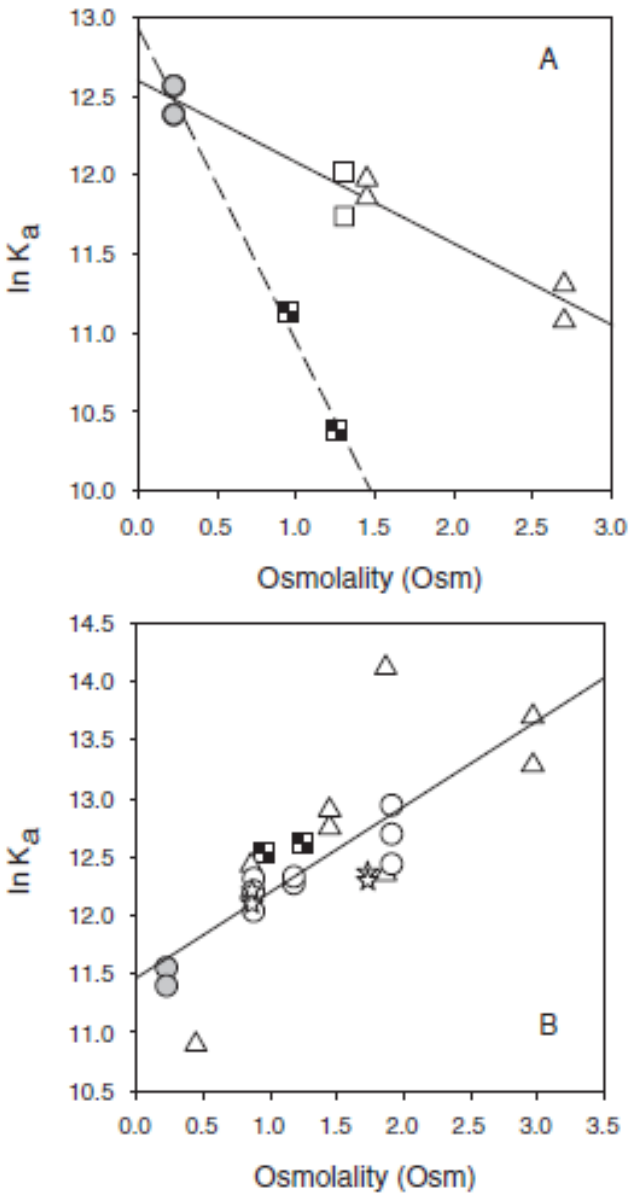


Figure 7: Osmolyte effects on ligand binding to R67 DHFR (20)

Panel A is the ln K_a for DHF titrated into R67:$NADP^+$, collected by ITC in the presence of osmolytes. The osmolality of solution was measured and the lnK_a plotted versus the osmolality to get the Δn_w. Panel B is the ln K_a of NADPH binding to apo-enzyme in the presence of osmolytes. The different symbols represent the different osmolytes, buffer (grey circles), sucrose (open square), glycine betaine (open triangle), DMSO (open circle), and PEG400 (checkerboard square).

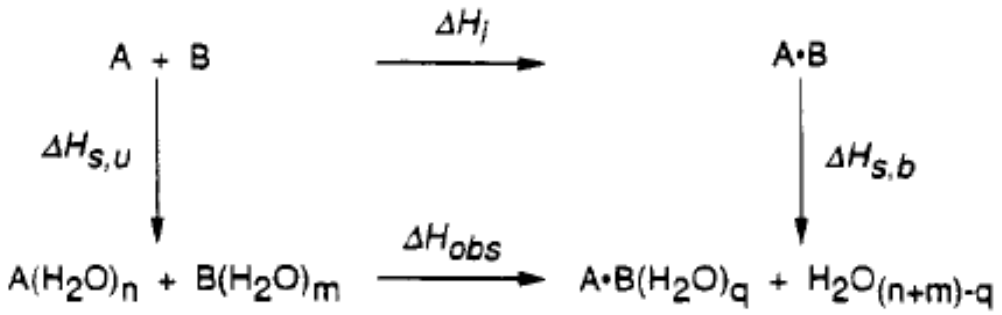


Figure 8: Born-Haber Cycle (21)

The ΔH_{obs} can be measured by ITC, but is broken up into 2 parts. The ΔH_i is the enthalpy that comes from the interactions of receptor and ligand interaction. The ΔH_s is termed the enthalpy of solvent reorganization and comes from $\Delta H_{s,u} - \Delta H_{s,b}$.

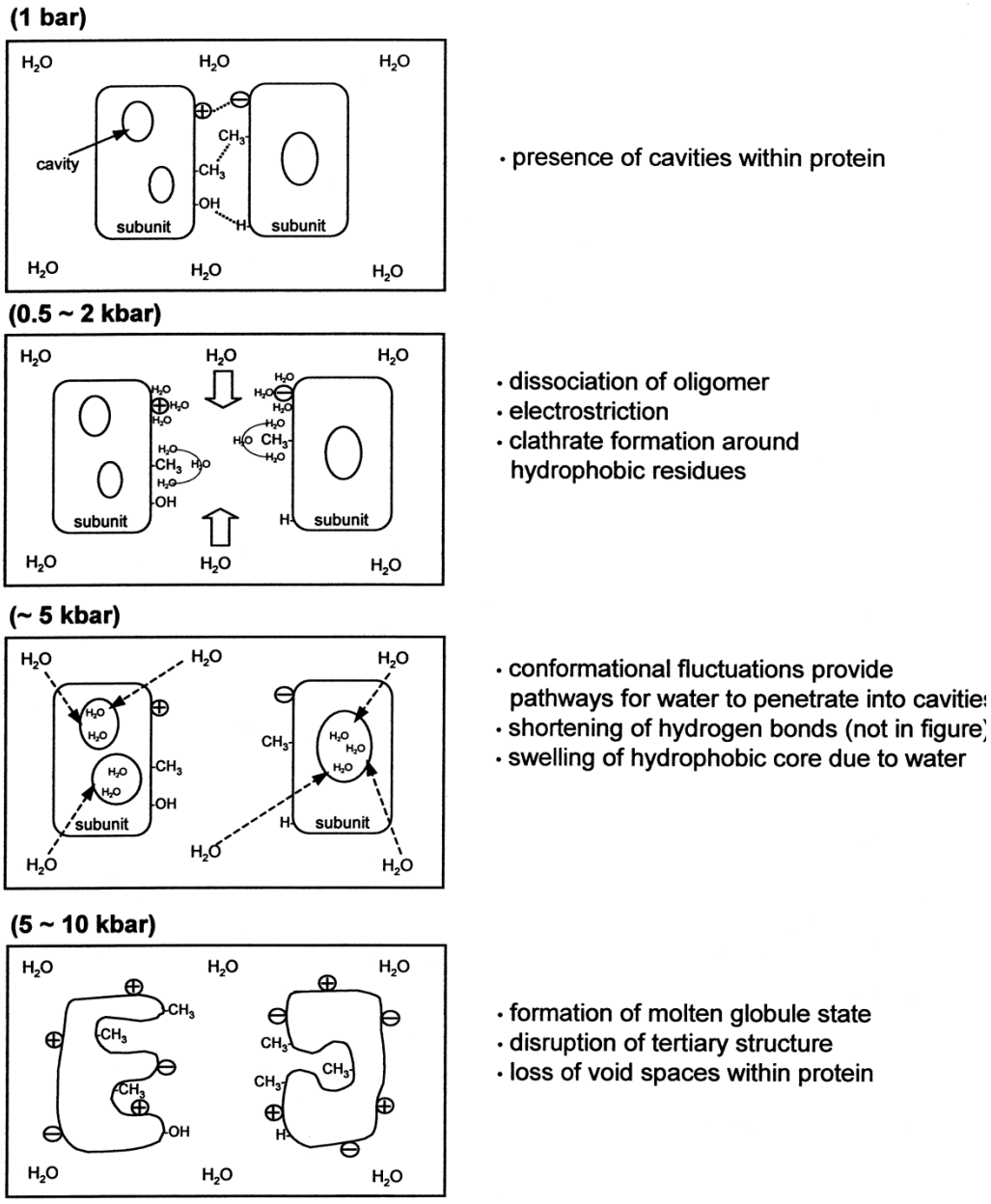


Figure 9: Schematic Representation of Proteins Under Pressure (25)

As pressure is increased the molecules become compact. With the application of 0.5 ~ 2 kbar pressure, the solvent molecules can disrupt the molecular interactions that are holding the oligomer together. As pressure is increased, solvent molecules begin to penetrate the cavities within the protein and begin to dissociate other interactions.

Chapter II

Materials and Methods

The materials in these studies were provided by the University of Tennessee and funded by the National Science Foundation. Dr. Elizabeth Howell has been working with this system for over 20 years and many of the procedures described below have been previously used in her lab. A collaborator, Jose Reyes-DeCorcuera at the University of Florida, provided the instrument for the hydrostatic pressure studies.

Protein Expression and Purification

The R67 DHFR gene was inserted into a pUC94 vector and transformed into competent SK383 *Escherichia coli* cells as previously described (1). An isolated *E. coli* colony shown to express resistance to ampicillin (AMP) and trimethoprim (TMP) was grown in terrific broth (TB) with 20 μg/ml TMP and 200 μg/ml AMP. The presence of two antibiotics allowed for selection of only the cells expressing the plasmids, detected by the presence of AMP, and those expressing viable R67 DHFR, detected by the presence of TMP. Previous growth curves showed best expression at approximately 65 hours, thus cultures were grown at 37°C for that time. No IPTG induction was needed as the promoter is the constitutive, up promoter for *E. coli* chromosomal DHFR (1).

Cells grown for 65 hours were centrifuged and resuspended in 50 mM phosphate and 10 mM EDTA (1xPE) buffer at pH 8.0. Following complete resuspension, the cell solution was titrated slowly to pH 12.5 with 1M NaOH and centrifuged. The supernatant was slowly titrated to pH 2.0 with 1 M HCl and this was also centrifuged. The solution was titrated to pH 7.5 with

1M NaOH and then subjected to ammonium sulfate precipitation. Once the ammonium sulfate was added to 50% saturation, the solution was centrifuged. The resulting ammonium sulfate pellet was dialyzed into 1x PE buffer at pH 8.0 for further purification using anion exchange (DEAE and Mono-Q) and size exclusion (G75-Sephadex) columns. Separation of wild type R67 DHFR from other cellular proteins was accomplished using a DEAE anion exchange column in 1x PE buffer at pH 8.0. The phosphates within this buffer are more negatively charged than R67 DHFR and therefore competitively bind to the DEAE anion exchange column. Other proteins within the solution do bind to the column and provide a separation step. The collected fractions with high levels of activity were run on SDS-PAGE to determine purity and pooled. This solution was dialyzed in 10 mM Tris and 1 mM EDTA (1x TE) buffer at pH 8.0 and loaded onto a DEAE column in 1x TE. Tris does not contain a negative charge at pH 8.0 and will therefore not bind to the DEAE; however R67 DHFR will bind and can be eluted with an increasing salt gradient from 0 M – 0.4 M KCl. After R67 was eluted from the DEAE column, the solution was run on a G75 Sephadex size exclusion column at pH 8.0. If R67 DHFR was not completely separated from the other cellular proteins after this column, another G75 Sephadex column was run at pH 5.0 where R67 DHFR is an inactive dimer. The change in pH alters the molecular weight and allows the R67 DHFR to be separated from different proteins. In a few cases where the protein was not pure after these steps, the solution was loaded onto a Mono-Q anion exchange column and eluted with a 0-1 M KCl salt gradient. The purity of the protein during the purification process was monitored by SDS-PAGE analysis and protein concentration was measured by a BCA assay (Pierce) and spectrophotometric analysis (29).

Isothermal Titration Calorimetry

For the purpose of these studies, isothermal titration calorimetry (ITC) was carried out to elucidate the effects of water reorganization in the binding of either substrate or cofactor to R67 DHFR. A VP-ITC microcalorimeter from Microcal interfaced to a personal computer was used for data collection.

Titrations in H_2O

R67 DHFR was present in the ITC cell at a concentration of approximately 100 μM (99-115 μM range) in 1x TE buffer at pH 7.5 and 102 mM NaCl was added to maintain the ionic strength of 0.11 M at 25˚C. All of the complexes were analyzed at pH 7.5. Each titration was run at least in duplicate. The "c-values" (P_{tot}/K_d) ranged from 1-40 within the acceptable value ranges of 1-1000.

The titrations of ligand were collected until saturation of the binding site or sites had been successfully reached and were analyzed using both Origin7 and SEDPHAT (3) fitting systems. For the NADPH binary complex, additional datasets were collected where low concentrations of cofactor (3μl injections of 0.37-0.63 mM) were injected into 103-106 μM R67 DHFR. For these experiments, all the cofactor binds, resulting in heats of injection which are reasonably constant. This allows calculation of molar enthalpy values (after subtraction of a heat of dilution term) without use of a model (4).

Export of Origin fits into SEDPHAT allows global fitting of replicate data sets. In some cases a slope associated with saturation was observed and SEDPHAT was able to calculate values for the binding constants, enthalpy, and slope. As Origin requires a slope of zero for fitting, the slope derived from the SEDPHAT fit can be used in Origin to allow construction of the total heat plots.

Titrations in D_2O

Titrations in D_2O were carried out under the same conditions as in H_2O. D_2O, DCl, NaOD and deuterated Tris were obtained from Cambridge Isotopes. In addition, lyophilized protein, folate, NADPH, $NADP^+$ and EDTA went through a cycle of reconstitution in D_2O, equilibration (1 hr for small ligands and 3 days for protein), followed by lyophilization. This cycle was repeated. For the ITC measurements, the deuterated protein and ligand were reconstituted in deuterated TE buffer plus salt. All solutions were titrated to pH 7.5. Uncorrected pH-meter readings were used as typically the 0.4 pH meter offset is similar to any shifts in pK_a value (5). Since the stability of DHF is limited once synthesized, it is frozen and stored as a suspension in 5mM HCl. In these experiments, we washed the DHF pellet twice with 5mM DCl prior to use. Each titration was collected at least twice and fit to the same programs as the H_2O titrations.

Hydrostatic Pressure Analysis

A high hydrostatic pressure (HHP) system was constructed by connecting a micropump MP5 (Unipress) to a DH-2000 UV-VIS-NIR light source (Mikropack) with deuterium and halogen lamps. The latter provided light through the high-pressure optical vessel U103 to the HR4000CG-UV-NR high resolution spectrometer (Ocean Optics, Inc). This system was provided by Dr. Jose Reyes-De-Corcuera at the University of Florida Citrus Research and Education Center (Figure 10 panel A). The system was controlled by the program LABVIEW (National Instruments) and accommodated cylindrical quartz cuvettes with a pathlength of 0.7 cm. The cylindrical nature of the cuvette allows it to withstand high pressure. Cuvettes with joints and glue do not survive high pressure well; this prevents an excessive amount of breakage while the system is under pressure.

Pressure Effects on Protein Structure of R67 DHFR

The peak of R67 DHFR protein fluorescence at 340 nm shifts during denaturation to around 360 nm and when the protein transitions from a homotetramer to an inactive dimer at lower pH (6) (1). The protein fluorescence under 0.1-500 Mpa hydrostatic pressures was monitored by exciting the protein with an open spectrum and collecting the emission spectra from 300 nm to 400 nm. (The deuterium and halogen lamps provide a broad excitation band. While filters could be used to provide more specificity for excitation of tryptophan residues, they substantially decreased the signal and were not used.) R67 DHFR (10 μM) was dissolved in 89 mM Tris and 11 mM KH_2PO_4 (Tris/phosphate) buffer at pH 7, as this buffer was previously found to be resistant to pH changes upon application of pressure (34). Protein free buffer was used as a control and its spectrum was subtracted from the corresponding protein spectrum.

Kinetics under Hydrostatic Pressure

Steady state kinetics requires the assessment of initial rates at varying substrate concentrations. The time required to load the sample and bring the system up to pressure made it difficult to obtain initial rates of the reaction at various subsaturating substrate concentrations. Thus saturating substrate concentrations were typically used.

Approximately 100 nM R67 DHFR was mixed with saturating levels of both ligands and the reaction was allowed to proceed for 10 minutes under pressures ranging from 0.1-500 Mpa. To obtain comparable rates for each pressure range, mixing time and time to reach pressure before the recording of the decrease in absorbance at 340 nm began was monitored and found to be in the range of 69-110 seconds. The data were then fit to the most linear portion of the reaction, typically from 5-7 minutes. The reaction was mostly linear for 10 minutes, but occasionally showed a rate decrease (perhaps due to product inhibition). An activation volume,

defined as the change in molar volume associated with the ternary E-NADPH-DHF complex going to the transition state, can be determined from a plot of the natural log of k_{cat} vs pressure (29). The data were fit to equation 6, with the slope equal to - $\Delta V^{\ddagger}p/R_pT$.

$$\ln k = \ln k_o - \Delta V^{\ddagger}p/R_pT \text{ (Eq. 6)}$$

where:

k- rate constant
k_o - rate constant at pressure 0.1 Mpa
$\Delta V^{\ddagger}p$- activation volume in terms of pressure
R_p- gas constant in terms of pressure
T- temperature in ^oK

To test for reversibility of the pressure effects, the reaction was run at saturating levels of both ligands at 500 Mpa for 3 minutes and then the pressure was released, the reaction was allowed to proceed, and the rates before and after pressurization were compared.

Fluorescence Quenching

The intrinsic fluorescence of 10 μM R67 DHFR was determined using a modified version of the Zhuang et al. protocol (3). Aliquots of different NADPH concentrations, from 0 μM to 100 μM, and 10 μM R67 DHFR in Tris/Phosphate Buffer (4) resistant to changes in pressure, were made as needed. Each individual protein and NADPH aliquot was brought up to a range of different pressures, from 0.1 Mpa (ambient room pressure) to 250 Mpa and the emission spectrum monitored. Data were imported into Excel and analyzed. The data were fit to the following equation 7:

$$Fl = F_o - 0.5F_o[P_{tot} + K_d + L_{tot} - [(P_{tot} + K_d + L_{tot})^2 - 4P_{tot}L_{tot})^{1/2}] \text{ (Eq. 7)}$$

where:

Fl – observed fluorescence
L_{tot} – total ligand
P_{tot} – Micromolar protein concentration
K_d – dissociation constant
F_o – fluorescence yield per unit concentration of enzyme

To allow ready comparison, data were normalized using equation 8:

$$F_{app} = (Fl_{apo} - Fl_{obs}) / (Fl_{apo\ -} Fl_{bound}) \quad \text{(Eq. 8)}$$

where F_{app} is a fractional value between 0 and 1 and Fl_{obs}, Fl_{apo}, and Fl_{bound} are the fluorescence values associated with the observed, apo and ligand bound forms, respectively.

Rates of the Y69L Mutant Under k_{cat}/K_m Conditions at High Hydrostatic Pressure

To determine the effect of pressure on the K_m of DHF, a previously constructed Y69L mutant of R67 DHFR (8) was used under various levels of HHP. The concentration of enzyme ranged from 0.8-3.2 µM and 300µM NADPH (approximately 5x the K_m) and 50 µM DHF (approximately 1/3 the K_m) were added. The rate of the reaction was monitored for 10 minutes under pressures ranging from 0.1 Mpa – 200 Mpa.

These data collected under k_{cat}/K_m conditions were normalized based on the concentration of protein. The observed rate under these conditions should correspond to substrate capture. was determined a plot of the natural log k_{cat}/K_m versus the pressure was determined. A plot of the natural log of k_{obs} versus the pressure was constructed. The slope of this plot was used to collect an activation volume for substrate capture using equation 6.

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Materials and Methods Figure

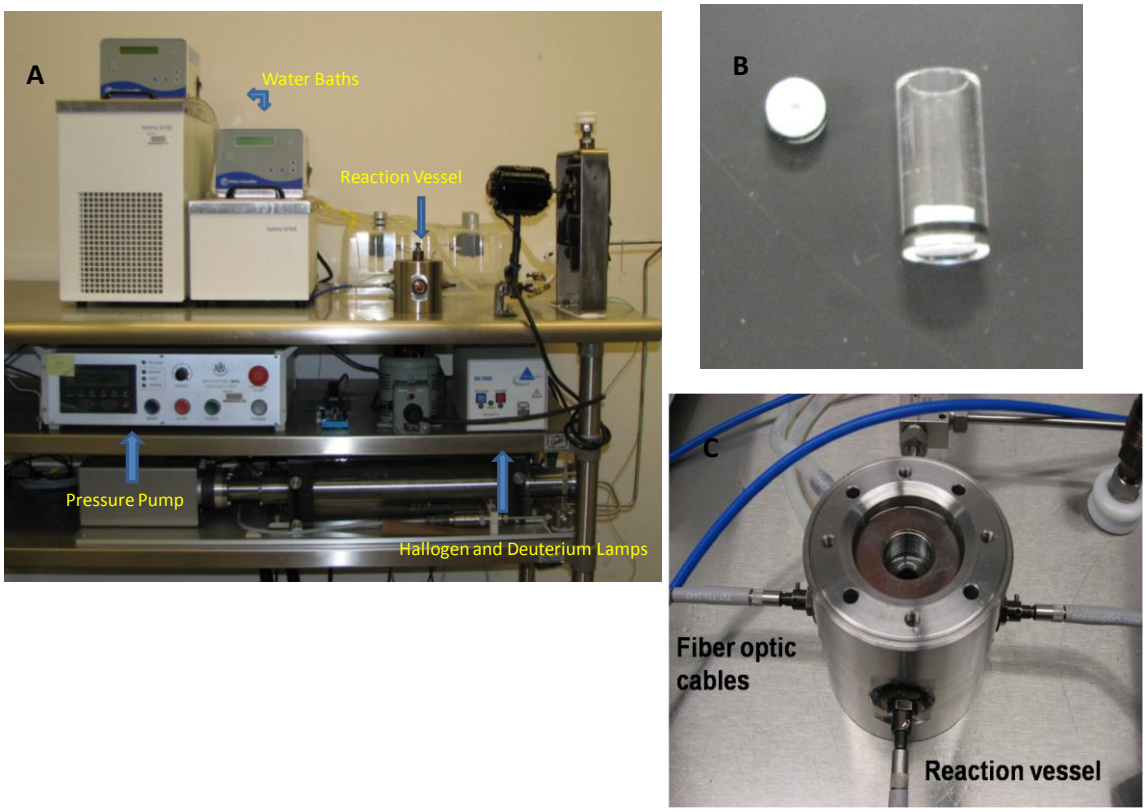

Figure 10: HHP System
Panel A is the pressure system connected to the water baths and the reaction vessel. Panel B is a close up of the cylindrical cuvette into which the sample was loaded. Panel C is a close up of the reaction vessel and the fiber optic cables.

Chapter III

Results

The application of hydrostatic pressure and the use of D_2O based solutions provide different techniques with respect to perturbing water involvement in the R67 DHFR mechanism. Though other factors are involved in changing the isotopic structure of the solvent and introducing pressure into the system, perturbation of water is common between the two.

Solvent Reorganization Studies

ITC studies were carried out to determine the role of solvent reorganization in the R67 DHFR reaction mechanism. As previously shown by osmotic pressure, "water uptake" is required for DHF binding and water release is required for NADPH binding (1), thus solvent reorganization may play a large part in ligand binding. In solution, solvent would be expected to occupy open crevices and binding sites within the protein as well as the ligand surface. Before the ligand binds to the protein, a reorganization of the solvent molecules from the binding site and around the ligand may be expected. In the case of a conformational change of protein or ligand during binding, solvent molecule reorganization may also be expected. As ITC measures the enthalpy for the association of molecules including, protonation, conformational change, and water effects, perturbation of any bond formation in the interaction by the presence of D_2O should also contribute to the observed change in enthalpy.

Data were collected in H_2O and D_2O with 1x TE buffer at pH 7.5 with 0.102 M NaCl added. A binary complex is a ligand titrated into the apo-enzyme, so that only the compound and macromolecule are interacting with one another. A ternary complex is when a second ligand is titrated into an already bound first ligand:protein complex, so that 3 species are involved in the interaction. The use of binary and ternary complexes can yield different information on how the ligands bind to different complexes.

NADPH binding to R67 DHFR

Figure 11 shows the ITC titrations describing NADPH binary complex formation in H_2O and D_2O with the residuals below the fit. Figure 12 is a total heat plot of the NADPH binary complex formation in H_2O and D_2O. The data are imported from Origin into Sedphat and both data sets are globally fit to a single-site model.

Previous studies have shown that two NADPH molecules bind to R67 DHFR with negative cooperativity. In our studies, the data fit reasonably well to a one-site model and showed no significant change in enthalpy when fit to a two-site model as well; however the second site was more variable. Thus both data sets are compared using the 1-site model. As shown in Table 1, the K_d values are comparable in H_2O and D_2O, indicating that binding to the single site is not significantly affected. The observed enthalpy of binding in H_2O and D_2O ($\Delta\Delta H = \Delta H_{H2O} - \Delta H_{D2O}$) are the same within error. It can be seen in Figure 11 that the titrations describing NADPH binary complex formation in H_2O and D_2O are not much different, however Figure 12 shows the total heat plot and the titrations in D_2O give a slightly more negative total heat than the titrations in H_2O. A separate set of experiments, using a low concentration of NADPH titrated into R67 DHFR, were conducted to obtain information on the first site binding. The data were not fit to a model and the molar enthalpy was determined to be - 6.06 ± 0.3 kcal/mol in D_2O buffer and - 6.08 ± 0.4 kcal/mol. As the concentration of NADPH is low, the first site is the main site that will be contributing to the enthalpy of binding. Combining these data, it seems reasonable to suggest that the change in enthalpy for NADPH binding to R67 DHFR is not significantly different between H_2O and D_2O.

Chervenak and Toone make the assumption that D_2O yields 10% of the enthalpy of reorganization because of the difference in bond strength between a proton and a deuteron. This leads

to the assumption that 10x the difference is the amount of enthalpy related to solvent reorganization, if D_2O does not have an effect on protein, ligand, or protein:ligand complex structure (2). A ΔΔH value of zero indicates that solvent reorganization does not contribute to the observed binding enthalpy for NADPH binding to apo-enzyme.

Folate binding to R67 DHFR

Binding of 2 folates has previously been shown to display positive cooperativity (3) and isotherms have been fit to a 2 site model. A more negative enthalpy in the presence of D_2O was observed for the first site and a less negative enthalpy was observed for the second site. The ΔΔH for the first site is 1.7 kcal/mol consistent with a 17 kcal/mol enthalpy of solvent reorganization which is much higher than the observed enthalpy in H_2O (2). The difference in the observed enthalpy in H_2O and D_2O (ΔΔH) of the second site is - 0.4 kcal/mol indicating solvent reorganization contributes - 4 kcal/mol to the overall enthalpy. The value for ΔΔH of the first site is much higher than the value of the second site. The second site enthalpy is also in an opposite direction for the ΔΔH. While binding of NADPH and DHF have not shown any perturbed pKas, binding of folate has previously shown proton uptake (4). Since the N3-O4 enol tautomer pKa for DHF is 10.8 (5), while this pKa in folate is 8.4 (6), the Howell Lab previously proposed that folate prefers to bind in its N3 protonated form (4).

Figure 13 shows the titrations of folate into apo-R67 DHFR in both H_2O and D_2O. It can be seen from these plots that the positive cooperativity of the folate binary complex is still present in the titrations in D_2O buffer. A more negative heat can be observed in D_2O as the hook that occurs is at a much more negative heat.

DHF binding to R67 DHFR:$NADP^+$ complex

A DHF ternary complex was formed in the sample cell at 25 °C at pH 7.5. The sample cell was loaded with a 1:5 ratio of R67 DHFR:$NADP^+$ and DHF was titrated into the cell. $NADP^+$ binds weakly to only one site of R67 DHFR, therefore, the complex that is formed should be R67 DHFR:$NADP^+$:DHF. The observed enthalpy trend change is less negative in D_2O versus H_2O, the same as for the second binding site of the folate binary complex. The ΔΔH for the DHF ternary complex is - 1.1 kcal/mol and this translates into an - 11 kcal/mol enthalpy related to solvent reorganization. Figure 14 shows the titrations of the DHF ternary complex in both H_2O and D_2O. It can be seen from this figure that D_2O provides a less negative heat than H_2O at a similar molar ratio of DHF to R67 DHFR.

Table 1: Table of ITC data

Complex	H_2O K_d µM	H_2O ΔH kcal/mol	H_2O TΔS kcal/mol	D_2O K_d µM	D_2O ΔH kcal/mol	D_2O TΔS kcal/mol K	ΔΔH
NADPH Binary	11.9 ± 0.3	-6.8 ± 0.06	-0.09	10.8 ± 0.5	-6.8 ± 0.09	-0.05	0
Folate Binary 1^{st} site	152.2 ± 14.7	-2.7 ± 0.2	2.4	141.6 ± 11.3	-4.4 ± 0.2	0.9	+ 1.7
2^{nd} site	43.6 ± 3.3	-9.3 ± 0.3		43.3 ± 8.1	-8.6 ± 0.4		- 0.7
DHF Ternary	2.6 ± 0.07	-10.4 ± 0.05	-2.7	2.5 ± 0.04	-9.3 ± 0.04	-1.6	- 1.1

Hydrostatic Pressure Studies on R67 DHFR Binding and Catalysis

High hydrostatic pressure (HHP) studies can yield interesting results in concert with osmolyte studies. For example, Robinson et. al (7) found osmotic pressure (OP) results were opposite to those of HHP. Thus we used HHP to ascertain whether it weakens the binding of NADPH to R67 DHFR and/or tightens the binding of DHF to R67 DHFR. Osmolytes are used to dehydrate the molecules involved in the interactions and can help determine the involvement of water in the interaction. However, these studies involve the introduction of osmolytes into the system which can cause interactions between the osmolytes and protein/ligand. The use of hydrostatic pressure can be a good complement for determining the role of water in the reaction. As hydrostatic pressure can have unfolding/denaturing effects on proteins, studies were carried out to determine under what pressures R67 DHFR remained intact.

Hydrostatic Pressure Effects on R67 Structure

Figure 15 A shows the fluorescence spectra of apo enzyme from 250 nm – 500 nm under various HHP. A slight decrease in the fluorescence intensity at 340nm was observed, but no red shift in the spectra occurred until high pressure. When the fluorescence intensity at 360nm is plotted, a break point at approximately 300 Mpa is observed. This can be seen in Figure 15 B as a plot of pressure versus the fluorescence intensity at 360 nm.

Figure 16 shows the effect of HHP on the k_{cat} of R67 DHFR. Minimal effects were observed until an application of pressure greater than 200 Mpa. An activation volume, defined as the change in molar volume associated with the ternary E-NADPH-DHF complex going to the transition state, can be determined by a plot of the natural log of k_{cat} vs. pressure. The slope of this line is equal to $-\Delta V/R_pT$ (8). The small slope observed until 200 Mpa (calculated from the inset data of Figure 16) gave an activation

volume of -1.03 ± 0.97 cm^3/mol. A second slope, equal to 8.16 ± 0.69 cm^3/mol, describes the activation volume associated with increasing pressure from 200 Mpa to 500 Mpa. Combined with the effects of HHP on apo protein fluorescence, these two data sets define a range of pressure from 0.1-200 MPa which appear to have minimal effects on protein structure and activity. This is also the pressure range most often used to study enzyme function (9) (10) (11) (7) (13). Therefore this pressure range was used in our experiments.

It is clear from these studies that R67 DHFR is losing activity at pressures above 300 Mpa. To determine if these pressure effects were reversible, the enzyme activity was monitored before and after application of pressure. An initial rate (before the application of pressure) was followed by pressurization to 500 Mpa. This was followed by the release of pressure. While the protein was under pressure, the rate was greatly decreased. Figure 17 shows the reversibility plot, which demonstrates that at the beginning of the reaction, the rate was 16 turnovers per minute. As pressure increased, the rate decreased. And once pressure was released, the rate returned to 11 turnovers per minute. This is slightly slower than the original rate indicating that the effect of 500 Mpa of pressure on the catalytic rate constant Mpa is either not entirely reversible in this time frame and/or that substrate depletion or product inhibition may be occurring.

Hydrostatic Pressure Effects on NADPH binding to R67 DHFR

Cofactor binding was monitored by quenching protein fluorescence. A sample series of emission curves at 100Mpa are shown in Figure 18A as the concentration of NADPH increases. Figure 18B shows a sample set of 3 fluorescence quenching curves at 0.1, 100, and 200 Mpa. The curves shift to the right at higher pressure, indicating weaker binding. K_d values were calculated at each pressure and Figure 19 plots ln K_a vs. pressure. The slope of this plot yields the volume change for association of

NADPH to R67 DHFR, which is 11.4 ± 0.5 cm^3/mol. Ohmae et. al. determined a volume of dissociation for NADPH to EcDHFR which is - 33.5 cm^3/mol (11).

Hydrostatic Pressure Effects for the Y69L mutant using k_{cat}/K_m conditions

To probe the effects of pressure on DHF binding, several experiments were attempted, including fluorescence quenching and progress curves. The first site for DHF binding to R67 DHFR is weak and could not be determined from fluorescence quenching. Titrating DHF into apo-enzyme and measuring the aromatic amino acid quenching under hydrostatic pressure did not yield consistent results. Addition of folate to R67 DHFR:NADPH under increasing hydrostatic pressure was also attempted by fluorescence quenching. However, since the protein fluorescence was already greatly quenched, it was difficult to monitor an additional signal loss. No clear trend could be determined from these studies. Progress curves were also monitored and fit to an equation to determine the effects of HHP on the DHF Km. However, these data did not yield consistent results as well, most likely as the cuvette was cylindrical and different placement in the HPP cell alters the pathlength and thus the absorbance, which introduces variability in the calculated starting concentration of DHF.

Finally, we decided to monitor $k_{cat}/K_{m\ (DHF)}$ in a previously constructed Y69L mutant. Here, catalysis was measured under very low substrate concentrations, or k_{cat}/K_m conditions of DHF. The mutant allowed for a collection of a steady rate at kcat/Km conditions for DHF in the time allotted for the system to be loaded and reach pressure. This mutant has a measured rate of 0.16 s^{-1}, a K_m NADPH of 68 µM, and a K_m DHF of 180 µM (14). Our present studies were performed at concentrations that were 5 times the K_m of NADPH and 0.3 times the K_m of DHF. The k_{cat}/K_m rate decreased as pressure increased, likely indicating that binding of DHF is weakened under HHP. If we assume that there is very little effect on k_{cat} from figure 16 and that high concentrations of NADPH mostly keep the enzyme in the R67:NADPH

complex, then the calculated activation volume of 33.5 ± 0.4 cm^3/mol should mostly describe DHF binding. This plot can be seen in figure 20.

As pressure was shown to weaken the binding of NADPH to the apo-enzyme, some of the effects that are seen on the k_{cat}/K_m conditions of Y69L could be the result of losing saturation of NADPH. However, because DHF was at a concentration below its K_m and the NADPH concentration was 5x higher than its K_m, the majority of the effects should be on substrate capture.

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Appendix

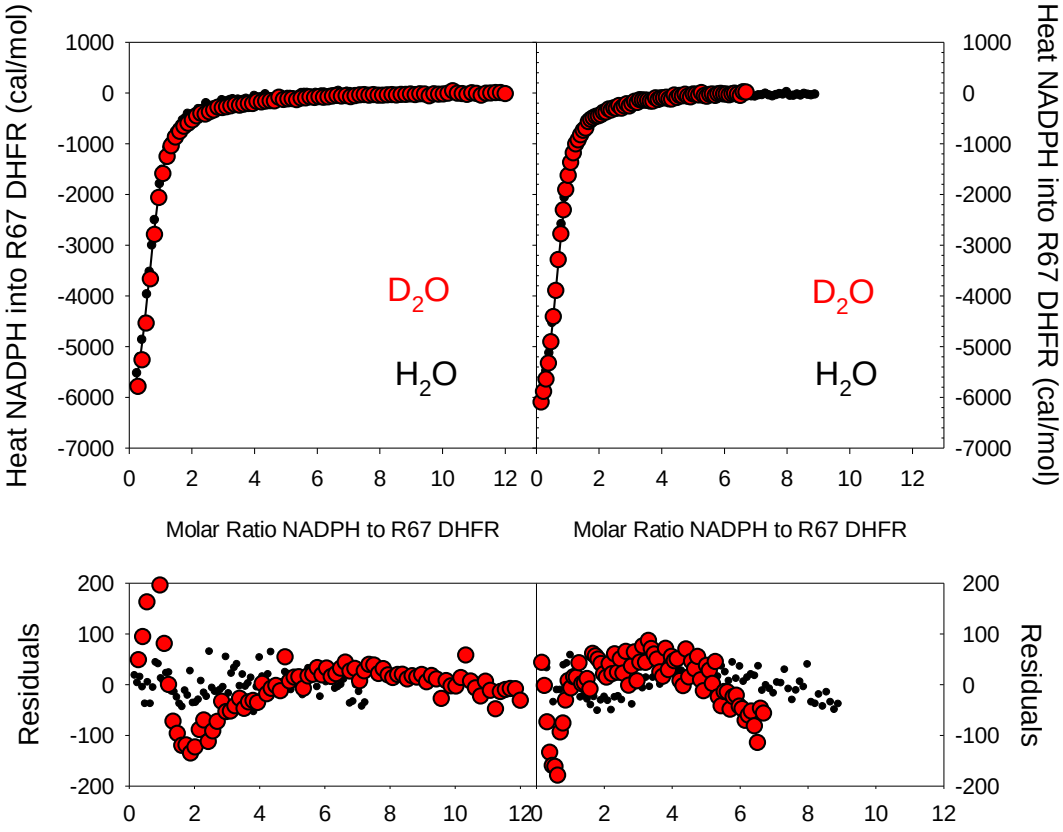

Figure 11: NADPH Binary ITC

The observed heat is on the y-axis as the concentration of NADPH increases. Once binding has reached saturation, the heat goes to zero. The line is the calculated fit from SEDPHAT and the bottom graphs show the residuals.

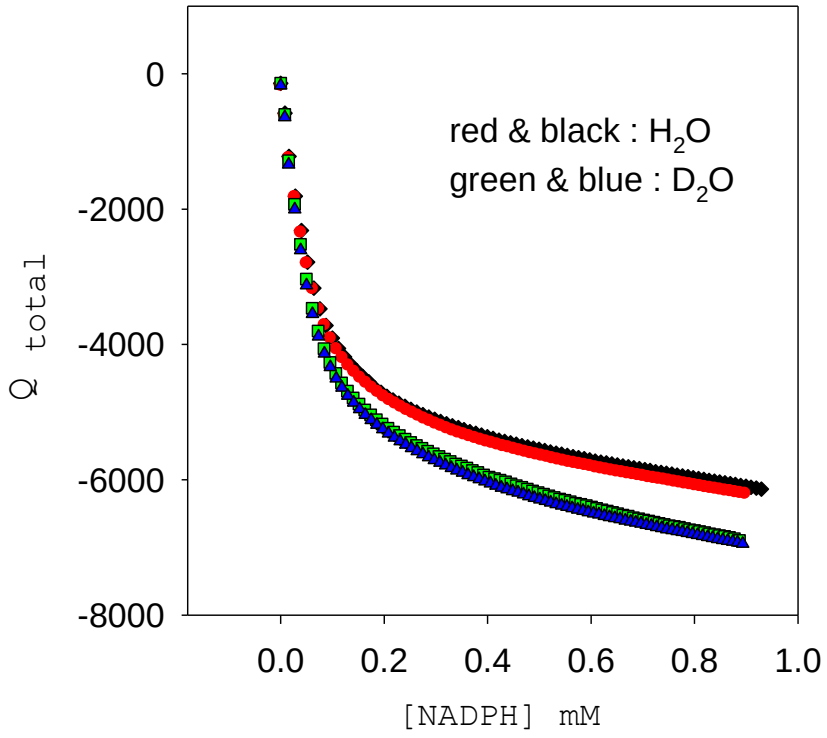

Figure 12: Total Heat Plot of NADPH binding to R67 DHFR

Above is a graph of the total heat for NADPH binding to R67 DHFR measured by ITC. The red and black dots correspond to H_2O and the green and blue dots correspond to D_2O.

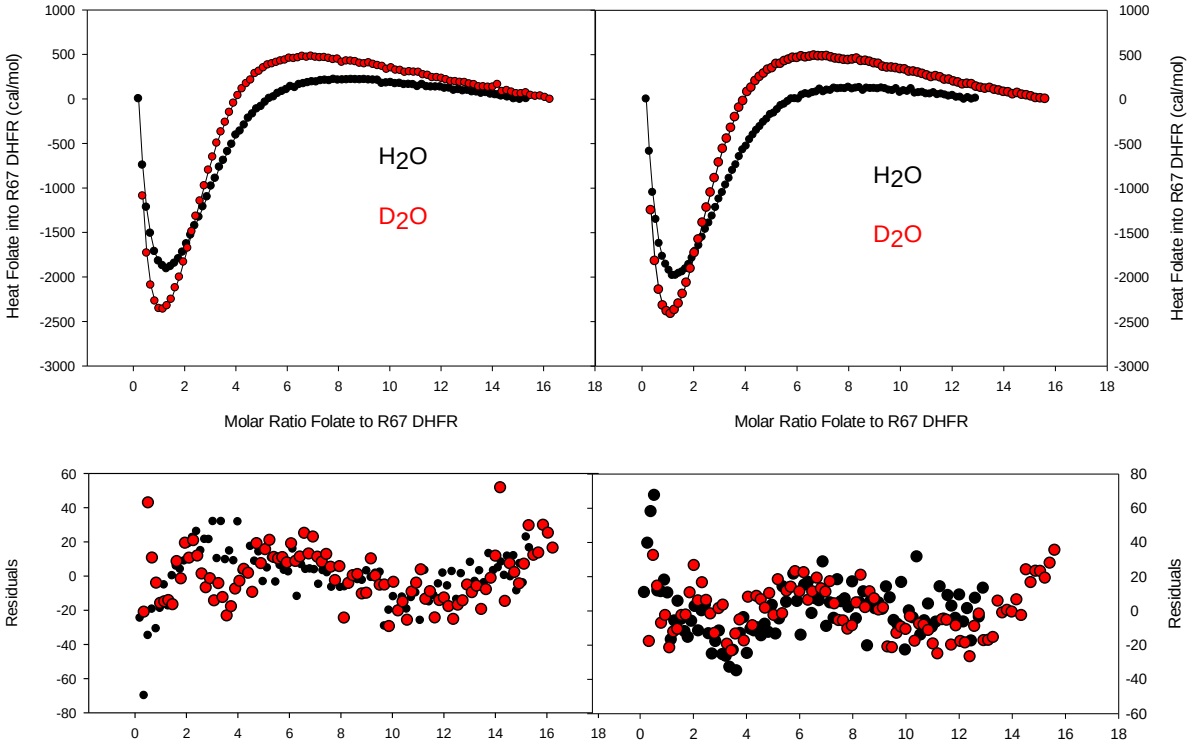

Figure 13: Folate Binary ITC

The titrations of folate into apo-R67 DHFR in both H_2O and D_2O can be seen overlaying one another. The red dots correspond to the titrations performed in D_2O and the black dots correspond to the titrations done in H_2O. Below the titrations are the residuals of the Sedphat fits.

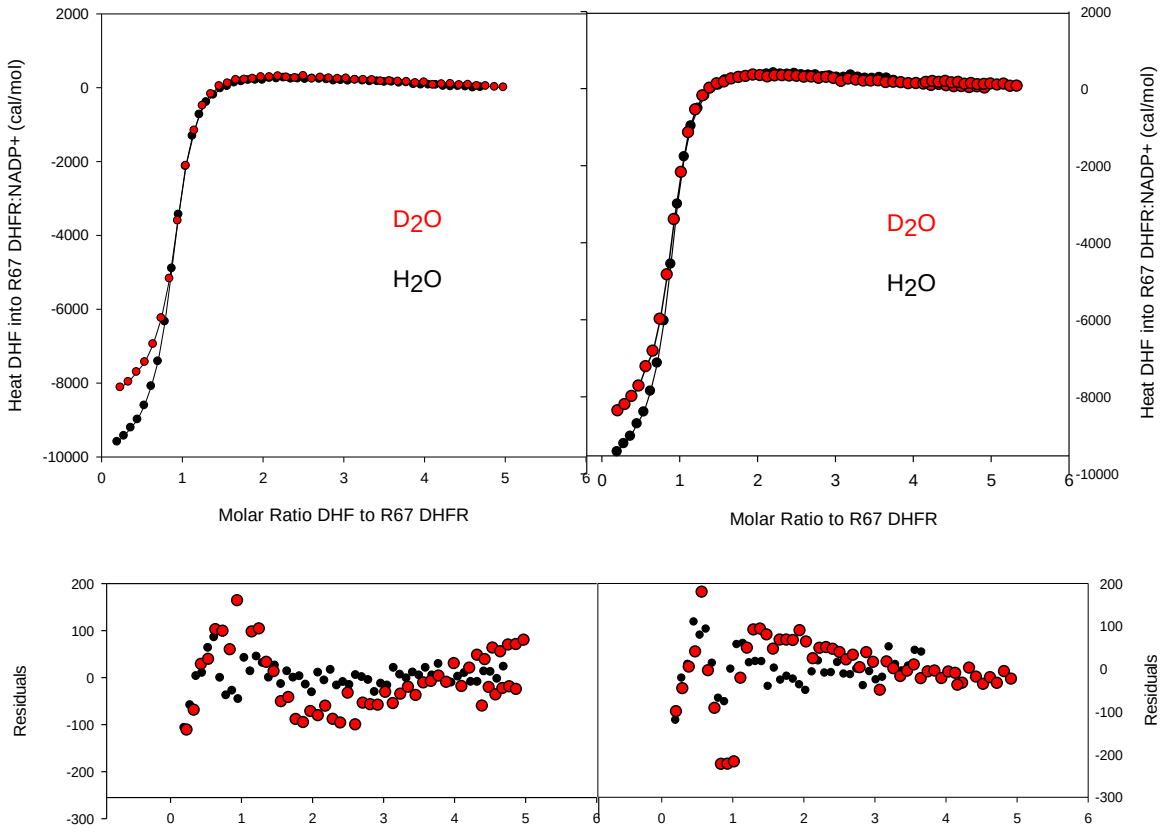

Figure 14: DHF Ternary ITC

Above is a plot of the molar ratio for each DHF to R67 DHFR versus the heat in cal/mol for DHF ternary complex formation in H_2O and D_2O. The red dots correspond to the titrations performed in D_2O and the black dots correspond to the titrations done in H_2O.

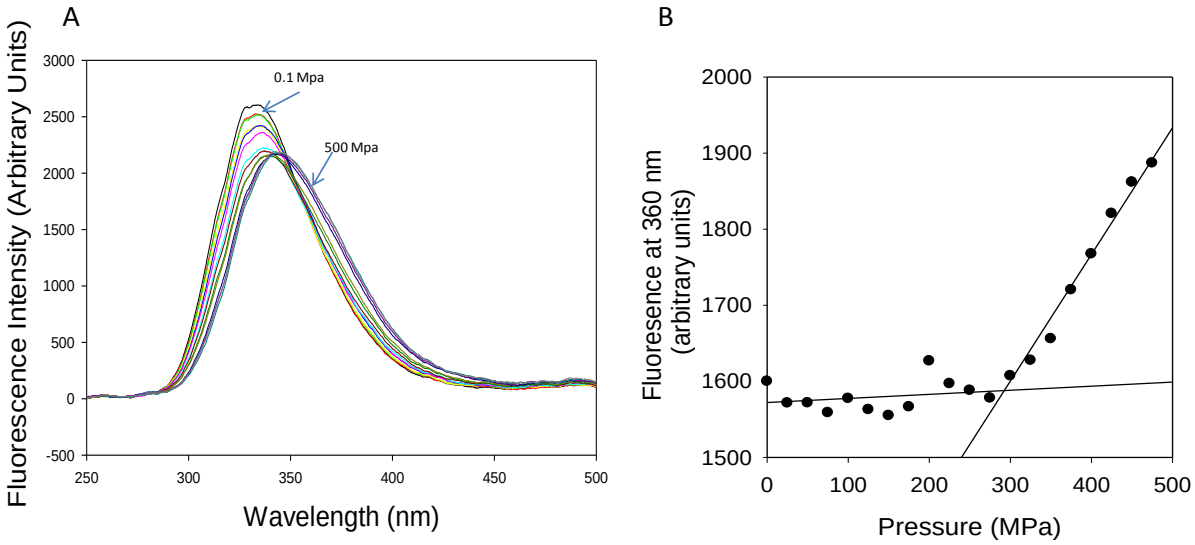

Figure 15: HHP effects on the Structure of apo R67 DHFR

Panel A plots the fluorescence intensity of wild type R67 DHFR over a wavelength from 250 nm – 500 nm. The peak of the fluorescence intensity can be seen at around 340 nm. As pressure is increased, the peak begins to decrease and by approximately 250 Mpa the peak shifts to approximately 360 nm. Panel B shows a graph of the fluorescence intensity at 360 nm as pressure is increased. The intensity begins to increase at around 250 Mpa of pressure.

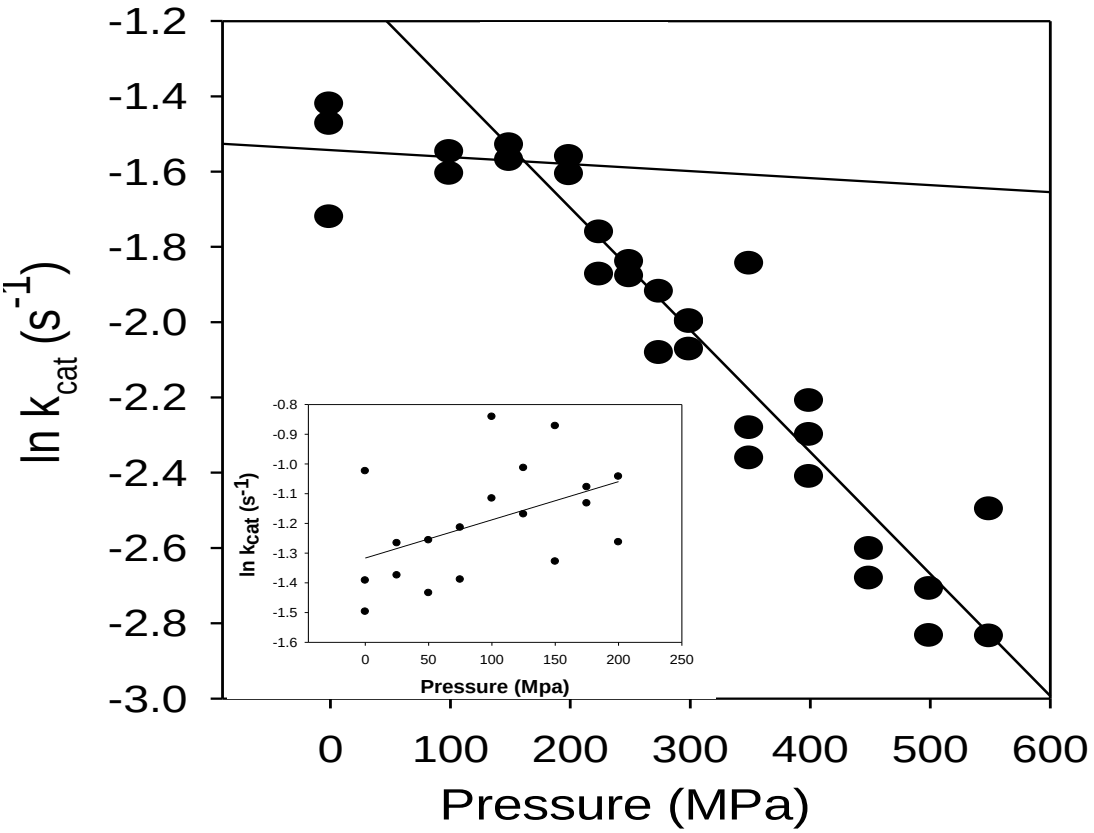

Figure 16: HHP effects on k_{cat} of R67 DHFR

The catalytic rate constant was determined under various pressures and the ln k_{cat} was plotted versus pressure. Using equation 4 an activation volume can be determined from the slope of the plot. The change of k_{cat} from pressures 0-250 Mpa appears to be small and a large decrease begins to occur around 275 Mpa. The inset describes data from a different protein preparation of R67 DHFR performed on a different day.

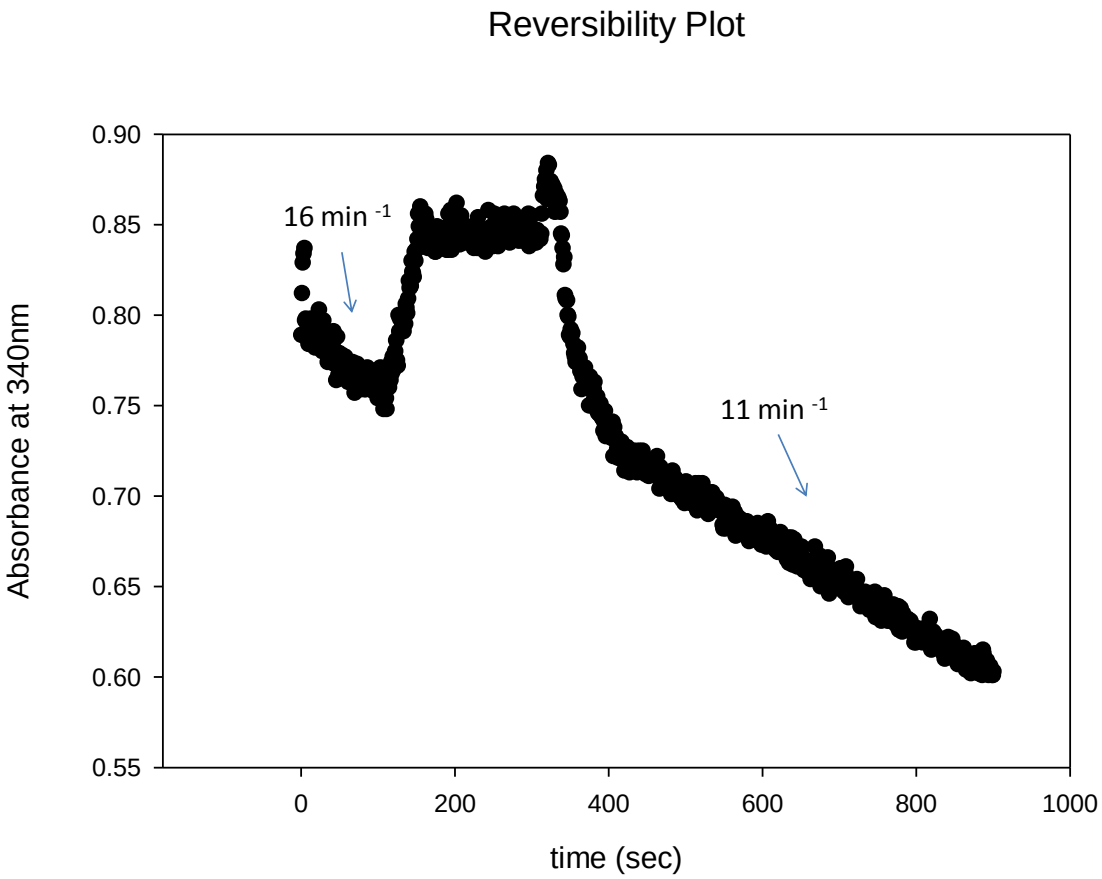

Figure 17: Reversibility Plot

An R67 DHFR assay with saturating levels of NADPH and DHF was performed and the activity was measured prior to the application of pressure. Then the system was pressured to 500Mpa followed by release of pressure to determine the reversibility of the HHP effects. The y-axis shows the absorbance measured at 340 nm and the x-axis is the time in seconds. As pressure is increased, the absorbance increases due to the smaller volume (and thus higher concentration) and once the pressure is released the absorbance returns to ambient pressure values.

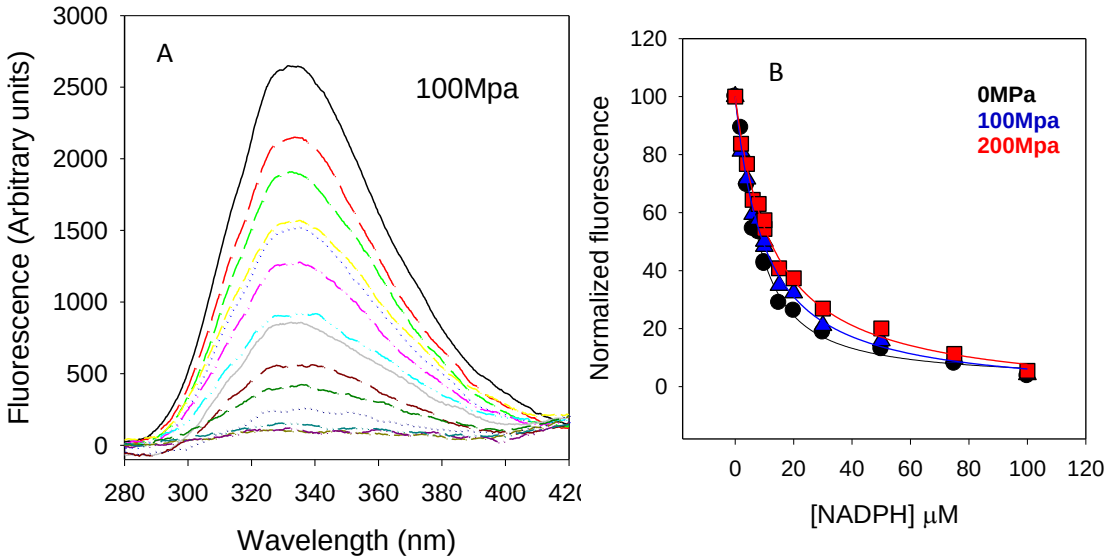

Figure 18: NADPH binding to R67 DHFR under HHP

Panel A shows the quenching of tryptophan fluorescence in R67 DHFR upon the addition of increasing concentrations of NADPH from 0 µM – 100 µM. Panel B plots the fluorescence intensity at 340 nm as a function of [NADPH]. To facilitate comparison, data were normalized according to equation 2. Three titrations are presented, corresponding to 0.1, 100 and 200Mpa pressures. An increase in K_d as pressure increases can be seen as a higher concentration of ligand is required for saturation.

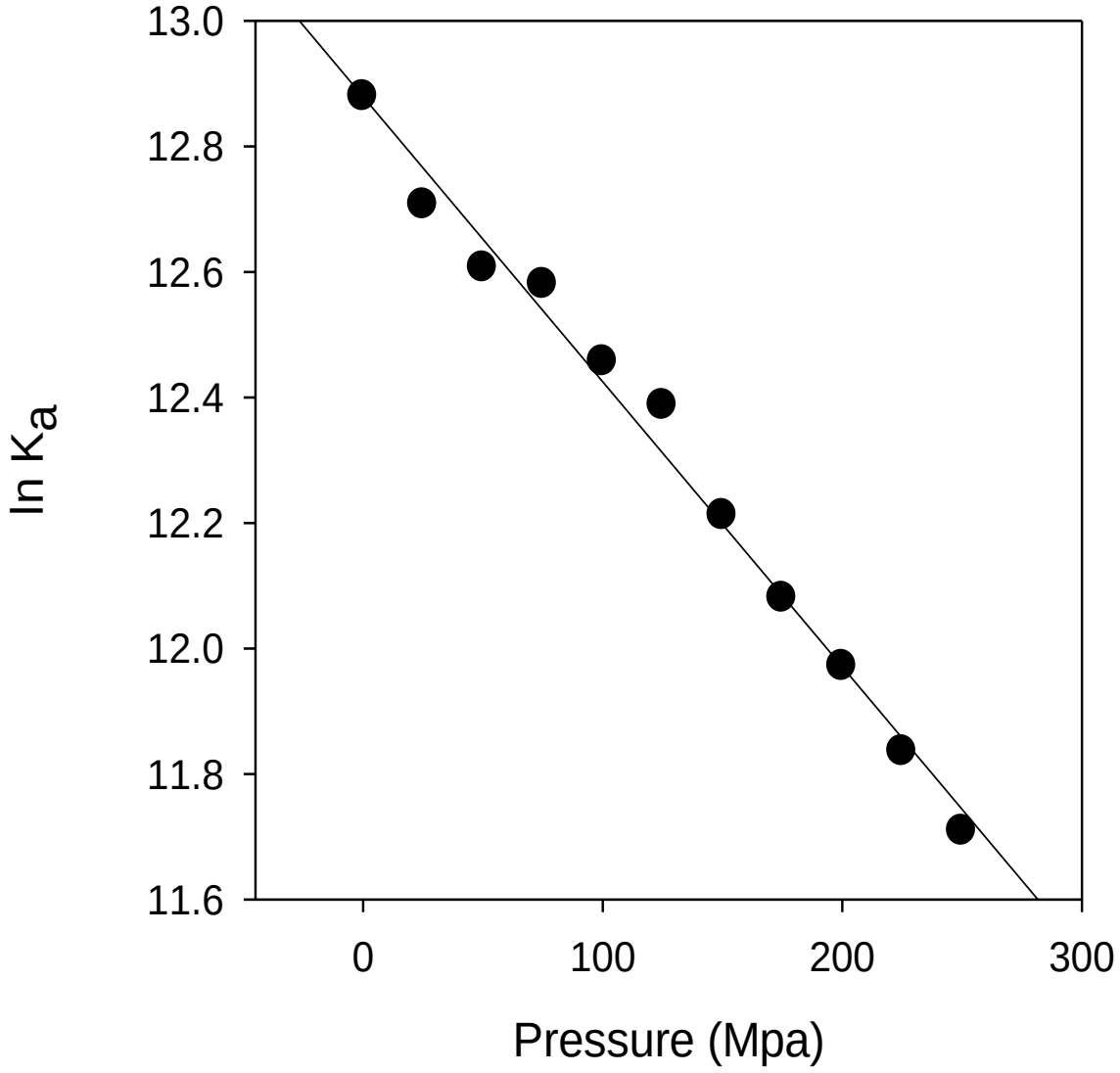

Figure 19: Plot of association constants monitoring NADPH binding to R67 DHFR versus HHP

Different pressures yielded higher K_d values. A plot of the natural log of the K_a determined at each pressure shows a positive volume of association of 11.4 ± 0.5 cm^3/mol.

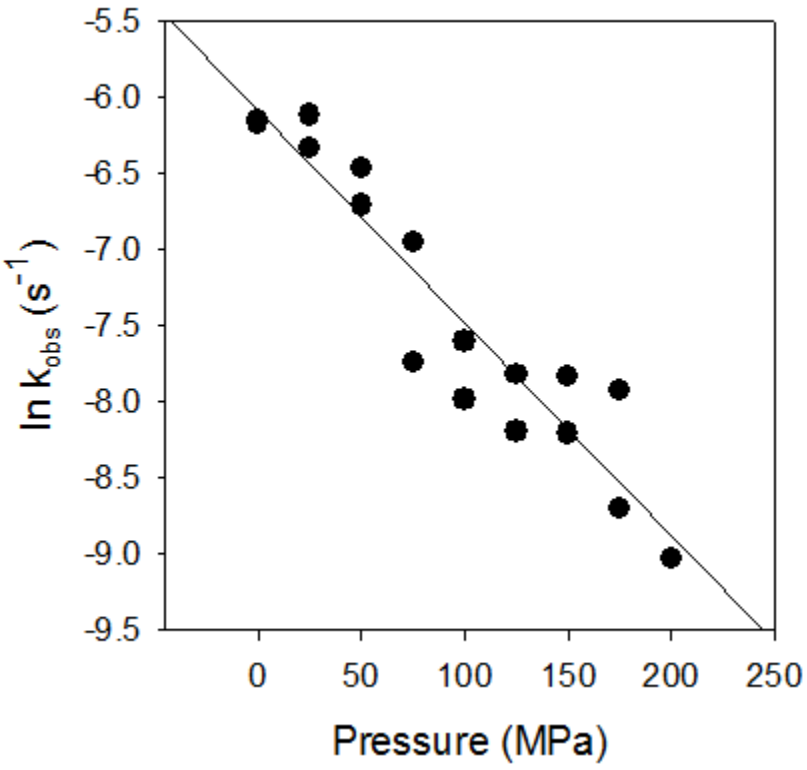

Figure 20: A plot of ln k_{cat}/K_m for DHF in the Y69L mutant vs. Pressure

Various rates under HHP at k_{cat}/K_m conditions with the mutant Y69L were collected and plotted. The trend was a downward slope, consistent with the interpretation that the DHF substrate capture becomes more difficult as pressure is increased. An activation volume of 33.5 ± 0.4 cm^3/mol is observed.

Chapter IV

Discussion

The role of water in a reaction is difficult to determine for many reasons. As the solvent, water molecules are present at a high concentration throughout the reaction. Unlike other experimental parameters, it is difficult to manipulate water without altering some other aspect that may contribute to the reaction mechanism. Despite these difficulties, it is possible to conduct several different experiments that will affect water within the reaction. Combining the information obtained from these experiments can contribute to the understanding of the role of water within a system.

Solvent Reorganization Associated with Binding of Ligands to R67 DHFR

Few studies have been conducted with ITC using D_2O and H_2O. Chervenak and Toone studied carbohydrate binding to proteins (1) and reported that replacing H_2O with D_2O results in a 10% increase in hydrogen bond strength between the solvent molecules in D_2O. The replacement of hydrogen with deuterium allows for H/D exchange in the ligand and protein as well as the replacement of water by D_2O in solvent shells and in water mediated interactions. These changes are attributed to the enthalpy of solvent interaction because of the added strength in the deuterium bonds. It may exert a change in the measured enthalpy upon exchange of the D_2O molecules when compared to the exchange of the H_2O molecules. Therefore, the difference in the observed enthalpy of H_2O and D_2O would yield the enthalpy of solvent reorganization when multiplied by 10 as the signal change is approximately 10%. Chervenak and Toone (1) found a less negative enthalpy observed in the presence of D_2O. In each of these cases, they found the percent of enthalpy that could be attributed to solvent reorganization was between 25-100% depending on the complex. When the same hypothesis is applied to the studies of solvent reorganization in R67 DHFR, the percent of enthalpy change can be seen in Table 2 for NADPH binary at

0% , for folate binary the first site at approximately -630% and the second site at 75%, and for DHF ternary complex formation at approximately 110%.

Chervenak and Toone's (1) hypothesis is based on the assumption that the presence of D_2O does not alter the structures of the protein or the ligands and does not take into account potential pK_a shifts due to the presence of D_2O. Their explanation for the enthalpy change upon introduction of D_2O may perhaps be too simple. For example, Ozen et al. (2)used a similar approach to study Aminoglycoside Phosphotransferase(3')-IIIa (APH), which is a promiscuous enzyme with a flexible binding site. Two groups of antibiotics, Kanamycins and Neomycins bind to APH in similar thermodynamic patterns in that they are enthalpically favored and entropically disfavored. Ozen et al. reported that neomycins bound to APH with a more negative enthalpy in D_2O than H_2O and kanamycins bound to APH with a less negative enthalpy in D_2O than H_2O. Because this was the first observation of this pattern, they suggested that shifts in pK_a and solvent structure around the protein may also have effects.

In the present study, opposite enthalpy changes were also observed for different ligands binding to the protein. R67 DHFR binds the cofactor NADPH tightly to the first site and then DHF prefers to bind to the protein with the first site occupied (3). Therefore, NADPH was studied in a binary complex with only the first site and DHF was studied in a ternary complex with $NADP^+$ at a saturating level. DHF is not as stable as folate, therefore the binary complex used folate because it requires a high concentration to saturate the first site. In the present study, binding of NADPH to apo-enzyme showed no significant enthalpy change upon the addition of D_2O as a solvent. This result suggests water reorganization does not contribute to the overall enthalpic signal during cofactor binding to R67 DHFR. The present study found enthalpy changes for all complexes of folate/DHF. These changes were not consistent as the enthalpy of the first binding site for folate was more negative in D_2O and the second binding site was

less negative in D_2O. The DHF ternary complex was similar to the second binding site of folate and less negative in D_2O. Considering the data obtained from the previous study, it appears that solvent reorganization contributes to the enthalpic term of DHF/folate binding to R67 DHFR, but in the case of NADPH binding to R67 DHFR the enthalpic term appears to mostly result from interactions between the enzyme and cofactor.

Chervenak and Toone (1) worked primarily with sugars binding to lectins and sugars have a high hydrophobic character. A hydrophobic molecule may require more solvent reorganization to bind than a non-hydrophobic molecule as water would be required to be removed from the surfaces of both the binding site and the molecule. Calculation of log P from the LigX option in MOE v2009.10 (Chemical Computing Group) predicts NADPH to be less hydrophobic than DHF/folate with logP values of -7.774 and -3.875 respectively. These values indicate folate is much more hydrophobic than cofactor, and as such may show more effects on water behavior. This may explain the lack of change in enthalpy for NADPH binding to R67 DHFR.

What is the origin of the ΔΔH upon DHF binding to R67 DHFR?

The dominant contribution of solvent reorganization to the enthalpic signal in the DHF ternary complex is interesting because the enthalpic term for DHF binding has been previously attributed to the ion pair or solvent separated ion pair formation between the pABA-glu tail and the K32 residues in R67 DHFR. The pABA-glu tail has been shown to be disordered in the crystal structure (4), NMR (5), and molecular dynamics (6). Previous studies within the Howell lab using mutagenesis of R67 DHFR have shown a large enthalpic contribution associated with preorganization of the ternary complex (7). A plot of the natural log of k_{cat}/K_m for both DHF and NADPH versus the enthalpies for ternary complex formation in the 6 mutants demonstrated a linear relationship. This plot shows that high catalytic

efficiencies are correlated with more negative enthalpies of binding. The original hypothesis was consistent with closer contacts leading to larger enthalpic contributions for folate binding to NADPH (or DHF binding to enzyme:$NADP^+$) consistent with the proposal by Williams et al. (8; 9).

This hypothesis was further tested by the construction of a Q67H:1+2+3+4 plus K32M:1+3 multimutant (10). The introduction of K32M in half of the pore prevents DHF from forming ionic interactions between the α- and γ- carboxylates of the pABA-glu tail and the K32 residues. The DHF can still bind due to the compensatory nature of the Q67H mutations that tighten binding, presumably by stacking with the pterin and nicotinamide rings (11). The Q67H:1+2+3+4 plus K32M:1+3 multimutant showed a more negative enthalpic signal than the quadruplicated R67 DHFR gene product upon DHF binding to enzyme:$NADP^+$. Two possibilities consistent with this result are that ion pair formation between the pABA-glu tail and K32 residues does not contribute to the ΔH of binding and/or that introduction of interactions due to the Q67H mutations compensated for the loss of ΔH signal. The addition of the Q67H mutation to the original wild type R67 DHFR resulted in an enthalpy signal of -4.8 kcal/mol for the formation of the folate ternary complex (12). The wild type R67 DHFR resulted in an enthalpy signal of -13.1 kcal/mol for the formation of the folate ternary complex in the same buffer (13), which is more negative than the Q67H mutation. Based on this result, it seems unlikely that the addition of the Q67H mutations would compensate for the loss of ΔH signal. If ion pair formation does not contribute to the ΔH signal, then pteridine ring contacts with NADPH (4) or solvent reorganization are left to contribute to the ΔH signal for DHF binding.

The change in enthalpy attributed to solvent reorganization for DHF binding to R67 DHFR:$NADP^+$ may not be specific to water in the active site. As NADPH binding to apo-enzyme showed no major change in D_2O, it is possible that the change for DHF correlates with the movement of the pABA-glu tail

in DHF and water reorganization during this process. The water within the active site could be solvating the ion pairs and the introduction of D_2O could increase the enthalpic term. The results of this study show that the ΔΔH for DHF ternary complex to be -1.1 kcal/mol. The multiplication of this by 10 as discussed earlier suggests the solvent reorganization enthalpy to be -11 kcal/mol which is approximately 110% of the observed enthalpy for the DHF ternary complex formation. This suggests that the enthalpy term for DHF ternary complex formation is entirely composed of solvent reorganization.

To consider the results for binary folate binding to R67 DHFR, several observations may need to be considered. Previous proton studies using ITC have shown folate binding to R67 DHFR is associated with proton uptake upon binding in the ternary complex (14). For binding of the other ligands, DHF and NADPH, no proton uptake or release occurs (14). Previous measurement of the folate ternary complex at pHs 8.0 and 9.0 found proton uptake associated with binding. This was proposed to occur as folate needs to be protonated to interact with backbone I68 atoms (4). Deprotonation of the N3-O4 tautomer, (pK_a of 8.4) leads to the loss of a hydrogen bond (see Figure 3 in the introduction) (15) (14). Since all of the complexes in this study were analyzed at pH 7.5 and the folate ternary complex was the only one to show protonation effects, it may be that some level of protonation and/or perturbation of the N3-O4 pK_a by D_2O could potentially account for the more negative enthalpy change in D_2O compared to H_2O. Alternatively there may be alterations in either the apo-enzyme or R67 DHFR:2 folates structure.

Table 2: Percent of Enthalpy Contributing to Solvent Reorganization

Complex	ΔH_{H2O}	$\Delta\Delta H * 10$	% ΔH for Solvent Reorganization
NADPH Binary	-6.8 kcal/mol	0 kcal/mol	0%
Folate Binary (1^{st} site)	-2.7 kcal/mol	+17 kcal/mol	-630%
Folate Binary (2^{nd} site)	-9.3 kcal/mol	-7 kcal/mol	75%
DHF Ternary	-10.1 kcal/mol	-11 kcal/mol	110%

HHP effects on R67 DHFR

Multiple studies have shown opposite results upon the applications of OP and HHP to the system. For example, enolase dissociates into two subunits in a simple step. The application of HHP favors dissociation as the subunits become hydrated, therefore a separation of the interactions that keep them together occurs. The application of OP favors association (16) (17). In the case of enolase the relationship between the effects of HHP and OP supports a hypothesis of water involvement in the binding reaction. In order to test the previous hypothesis of water uptake during DHF binding and water release during NADPH binding determined from R67 DHFR using OP, HHP would be predicted to have weakening effects on the K_m of NADPH and tightening effects on the K_m of DHF, while k_{cat} would remain unchanged. This pattern would support NADPH binding to a "dry interface" and DHF binding to a "wet interface."

Hydrostatic pressure has many different effects on a system as were discussed in detail within the introduction. The purpose of the present study was to determine if HHP affected the system in a manner opposite of our previous osmotic pressure studies. In several cases of proteins studied both with HHP and osmotic pressure (OP), the opposite trend was observed for each type of pressure. In the case of OP the introduction of osmolytes shifts the equilibrium of the system to a dehydrated state. With HHP the opposite is expected as bulk water has a larger volume than bound water and therefore the equilibrium would be shifted towards the hydrated state (18).

Introducing osmolytes into the system may result in interactions between the osmolytes and ligand and/or protein. The use of several osmolytes with different properties can help determine the effect of water. Introducing HHP could also have the opposite effect of water on the system, without the introduction of another molecule. However, increasing HHP can also have effects on other

interactions such as ionic, hydrophobic, van der Waals, and ring stacking. The use of osmolytes can also have effects on these interactions and not always in an opposite manner (18). Therefore, a combination of OP and HHP effects on a system can help better understand the role of water in an enzymatic mechanism.

It appears from our fluorescence studies on the apo structure and k_{cat} that R67 DHFR stayed intact from 0.1-200 Mpa. However, as pressure is increased from 250-500 Mpa, the catalytic rate decreased and the fluorescence showed a red shift in the emission spectra as seen in figure 12 of the results section. In a reversibility test, the rate was decreased as the reaction was assessed at 500 Mpa. After the pressure was released the rate increased but only to 70% of the original rate. This result is consistent with the physical event at 500 Mpa of pressure being reversible. The application of such a high pressure could be unfolding the protein or converting the active homotetramer to inactive dimer. The event caused by an increase in HHP seems to be a shift in equilibrium that is corrected quickly upon return to ambient pressure. Alternatively, the rate of conversion from substrate to product begins to decrease over time as the amount of substrate decreases and product inhibition may begin to occur. Therefore, some decrease in rate may be due to these factors as well.

Increasing the concentration of osmolytes tightened NADPH binding to the first R67 DHFR site, consistent with water being released from the binding site in order to bind NADPH. As hydrostatic pressure is thought to hydrate the molecules, NADPH binding under hydrostatic pressure weakened the binding. This is consistent with the hypothesis that NADPH binds to a dry interface of R67 DHFR. The results of these 2 studies are consistent with water release upon NADPH binding.

As described in the Results section, it was more difficult to monitor DHF binding due to the positive cooperativity associated with DHF binding to apo R67 DHFR and the time it took to reach HHP,

making it difficult to monitor initial rates. Therefore DHF effects were monitored under k_{cat}/K_m conditions with the Y69L mutant. The k_{cat}/K_m rate decreased as pressure increased. Assuming that pressure is not having an effect on k_{cat} or the structure of the Y69L mutant, this is consistent with hydration of R67 DHFR weakening DHF binding. This result is also similar to the observation by Ohmae et al. (19) where HHP weakens DHF binding to EcDHFR. OP studies on EcDHFR also showed similar results with respect to DHF binding to R67 DHFR as can be seen in Table 3. Similar results between the two different enzymes with different structures are consistent with increased hydration weakening DHF binding.

Impact of Both HHP and D_2O Effects on R67 DHFR

The initial hypothesis from the osmolyte studies of R67 DHFR was that water played a role in both substrate and cofactor binding (20). The results obtained for NADPH binding to R67 DHFR with several different osmolytes were clear, demonstrating that NADPH bound to R67 DHFR with a net release of water. This observation is common as two species form contacts, the solvent molecules around each species would need to be released in order for the bonds to be formed. The results for DHF binding to R67 DHFR with several different osmolytes were less clear. Though each osmolyte consistently showed a net uptake of water upon DHF binding to R67 DHFR, the amount of change was different for each osmolyte. This is consistent with the osmolytes interacting preferentially with either the free DHF, the free enzyme, or the enzyme:DHF complex. The osmolyte studies gave approximately the same slope for different osmolytes during NADPH binding to R67 DHFR. As the same site is occupied by NADPH and DHF, it seems unlikely that the osmolytes are interacting with the free enzyme alone since all osmolytes used had identical effects for NADPH.

To investigate this hypothesis further, osmolyte studies were performed in the Howell lab on chromosomal *E. coli* DHFR (21). These studies showed the same results for DHF binding to chromosomal DHFR as R67 DHFR, i.e. weaker binding in the presence of osmolytes consistent with "water uptake". The NADPH results were similar (tighter binding in the presence of osmolytes), but showed different slopes indicating that the osmolytes were likely interacting with either the apo-enzyme, NADPH, or the EcDHFR:NADPH complex. From these studies, it seems likely that the osmolytes are affecting the free DHF in the case of R67 DHFR and chromosomal DHFR, as this molecule is the same between the two systems while the protein structures are entirely different. This conclusion is also consistent with calculations of accessible surface area (ASA) changes upon ligand binding, i.e. ASA changes of 700-900 $Å^2$ were calculated for either cofactor or substrate binding,. These changes predict water release upon binding for both cofactor and substrate.

Ohmae et al. conducted a study on HHP for EcDHFR and found the same trend for DHF K_m and K_d values as found in this study (19). They found a disassociation volume of – 33.5 ± 2.0 ml/mol for NADPH binding to apo-enzyme and an disassociation volume of – 7.3 ± 0.4 ml/mol for folate binding to enzyme:NADPH. For the purpose of this study, the disassociation volumes were converted to association volumes to compare to R67 DHFR HHP results in Table 3. As $K_a = 1/K_d$, a plot of the natural log of K_a and K_d would yield a slope in the same quantity with a different direction of slope, i.e. disassociation would be negative and association would be positive. They were able to carry out progress curve analysis and found an increase in K_m for both NADPH and DHF as pressure was increased from 0.1 Mpa to 200 Mpa.

Table 3 compares the activation and association volumes and the Δn_w's for the complexes in both EcDHFR and R67 DHFR under pressure. The activation and association volume and the Δn_w are

Table 3: Comparison of OP and HHP results for EcDHFR and R67 DHFR

	R67 DHFR[a]	EcDHFR[b]
DHF Ethylene Glycol Δn_w	25 ± 8 [20]	13 ± 1 [21]
DHF Glycerol Δn_w	16 ± 3 [20]	18 ± 1 [21]
DHF TMAO Δn_w	22 ± 1 [20]	17 ± 4 [21]
DHF DMSO Δn_w	41 ± 7 [20]	29 ± 1 [21]
DHF Sucrose Δn_w	40 ± 4 [20]	30 ± 2 [21]
DHF Glycine Betaine Δn_w	60 ± 13 [20]	34 ± 9 [21]
DHF PEG400 Δn_w	78 ± 11 [20]	64 ± 5 [21]
DHF/folate association volume	35.2 ± 0.3 ml/mol	7.3 ± 0.4 ml/mol [19]
NADPH association volume	11.4 ± 0.5 ml/mol	33.5 ± 2.0 ml/mol [19]
k_{cat} activation volume	-1.03 ± 0.9 ml/mol < 200 Mpa 8.06 ± 0.8 ml/mol > 200 Mpa	7.8 ± 0.6 ml/mol [19]

[a] Obtained from Steady State Kinetics

[b] Obtained from ITC Experiments

both derived from the slope of the graph when comparing the pressure applied to the system, either HHP or OP. In the case of DHF binding to both enzymes in the presence of OP, the Δn_w are fairly close in magnitude. This is consistent with the osmolyte having the same effect on both enzyme systems. DHF binds very differently in R67 DHFR and EcDHFR, as they have structurally different binding sites. This is interesting as the binding site for DHF in EcDHFR allows for a closed loop and the "uptake of water" upon DHF binding, which does not make as much sense as DHF binding to R67 DHFR with the open active site. The similarity in OP per osmolytes may be consistent with the osmolytes having a direct effect on DHF and potentially, may be due to an interaction between osmolyte and DHF. The activation volumes in EcDHFR and R67 DHFR have similar trends, but different magnitudes, likely due to the different active site architectures and/or different levels of osmolyte interactions with the different protein scaffolds.

Both OP and HHP studies are consistent with water release upon NADPH binding to R67 DHFR; however, the studies with D_2O show no effect on the enthalpy. Switching the solvent from H_2O to D_2O should give an indication of solvent reorganization as deuterium bonds are stronger than hydrogen bonds. The surface of all the molecules in a reaction is covered with water molecules. These water molecules must be displaced in order for a ligand to bind to the protein. The water molecules are pretty easily exchangeable with different exchange rates (22). Ringe et al (23). proposes that water surrounding the biomolecules can be categorized in three ways. The first and second categories of waters are found in the binding site and make contacts with the protein surface. They are usually ordered and require entropy to displace them from the active site (1). The third category of waters are disordered and easily displaced from the binding site. Ringe proposes that the displacement of category three water molecules have an absence of a solvent entropy term (23). The lack of change in enthalpy

for NADPH binding to R67 DHFR for the first binding site may be due to the displacement of category three water molecules.

The pressure studies in this project are consistent with release of water upon DHF binding to R67 DHFR. In contrast, the OP results from R67 DHFR and EcDHFR suggest "water uptake" upon DHF binding. Because of this difference, as well as the observation of similar OP effects in two different DHFRs, we have begun to consider another model where the osmolytes bind to free DHF and need to be released before DHF can bind to the enzyme. Here the osmolytes act as inhibitors for DHF binding to the enzyme active site. Another possible model is that the osmolytes can shift free DHF into the dimer state, which would also serve to weaken the binding affinity and would require water uptake as the dimer interface becomes solvated upon monomer formation. Figure 21 illustrates these models side by side with panel A depicting osmolytes interacting with free DHF and panel B showing a shift in the DHF dimer equilibrium via the application of HHP or OP. While we originally considered the DHF dimer model recent NMR studies in the Howell lab do not support the ability of osmolytes to weaken folate dimerization. In contrast, calculation of preferential interaction coefficients (24) with free folate as well as difference absorbance and NMR experiments support the ability of folate to weakly interact with osmolytes.

Summary and Future Directions

The goals of this project were to determine the effects of D_2O and HHP on the R67 DHFR mechanism of both binding and catalysis. In the case of NADPH binding to apo-enzyme, the ΔΔH was approximately 0 kcal/mol, making the observed enthalpy not significantly different in D_2O versus H_2O. This is interesting as the DHF/folate complexes all yield a large solvent reorganization enthalpy. The folate binding to apo-enzyme gave a difference of enthalpy in H_2O versus D_2O (ΔΔH) of + 1.7 kcal/mol for

the first binding site and -0.7 kcal/mol for the 2nd binding site. DHF binding to R67 DHFR:$NADP^+$ in D_2O also gave a less negative enthalpy with a ΔΔH value of - 1.1 kcal/mol. The ambiguity of the folate binary complex could be due to the altered pK_a effects in D_2O. A way to test this in future studies would be to assess the pK_a of folate in D_2O to see if it is shifted by more than 0.4 (the value corrected by the pH meter reading (25)) . As it was suggested earlier, solvent reorganization may be more important for hydrophobic molecules and DHF is calculated to be more hydrophobic than NADPH. This could potentially account for the lack of significant change in enthalpy upon NADPH binding to R67 DHFR. If this were the case, other enzymes that use both DHF and NADPH should show similar results upon NADPH binding to the enzyme. Another investigation using D_2O with EcDHFR and other enzymes that use NADPH as a cofactor could lead to a better understanding of solvent reorganization for NADPH binding to R67 DHFR.

Another future direction would be to collect data for all of the complexes in D_2O with deuterated osmolytes and compare the enthalpies with regular water and osmolytes. This could yield information on how the osmolytes might be affecting the water structure within the R67 DHFR mechanism. It may also help in determining the water interaction involved in the R67 DHFR mechanism.

As the two enzymes, R67 DHFR and EcDHFR with different structures have shared similar results upon DHF binding in the presence of osmolytes, it appears that osmolytes may be interacting with the DHF molecule free in solution. In order to support this hypothesis, other enzymes that also bind DHF and have different structures could be tested under osmotic pressure. As DHF is the common denominator, it would be expected that the presence of osmolytes for most of these enzymes would show similar results as EcDHFR and R67 DHFR.

The effect of HHP on the R67 DHFR reaction mechanism was similar to osmolytes in that k_{cat} and oligomerization of the protein did not seem to be affected. The K_d of NADPH upon binding to the first site of R67 DHFR showed weaker binding with a volume of association value of 11.4 ± 0.5 ml/mol. This correlates well with the hypothesis from the osmolyte studies indicating that NADPH binds with a net release of water.

When a mutant Y69L of R67 DHFR was measured under k_{cat}/K_m(DHF) conditions, a weakening in the k_{cat}/K_m(DHF) rate was observed yielding an activation volume of 35.2 ml/mol. This does not correlate well with the osmolyte studies as DHF was proposed to bind with a net uptake of water and the K_m/K_d of DHF would have been predicted to get tighter as pressure was increased. Pressure is not a perfect system and tends to disrupt ionic bonds causing electrostriction. Electrostriction is the contraction of solvents because of an alignment of dipolar solvent molecules in the electric field of an exposed charge (26) which can be caused by pressure. The tail of DHF has been proposed to be involved in electrostatic interactions within the protein (4; 13). Both studies done with salt and a crystal structure of the ternary complex have supported this hypothesis (13) (4). Increasing temperature under HHP should cancel the volume reduction caused by electrostriction as the temperature increase should disrupt the highly ordered structure of electrostricted waters (27). Previous data from Parsons (28) and Nickerson (29) have shown that electrostriction becomes less significant as temperatures are increased and may be entirely eliminated at the temperature is increased to 90 - 115^{o} C. Therefore temperature studies of R67 DHFR under HHP could yield more information about whether the effects observed are from HHP effects on ionic interactions (30). As temperature may denature R67 DHFR or DHF, some control studies may need to be performed with increasing temperature under pressure to determine the effect on stability. The change in the activation volume or volume of association would change in correspondence to the amount of contribution from electrostriction.

Another possibility for why DHF may not be correlating with the osmolytes studies could be due to the use of a Y69L mutant. The k_{cat} of Y69L is measured approximately 8 fold lower than wild type R67 DHFR (31). The measure of the change in rate for k_{cat}/K_m conditions under hydrostatic pressure can yield information of the pressure effect on the K_m of DHF if the mutant remains stable and the k_{cat} remains unchanged. This mutant may have a different rate determining step because the original k_{cat} is lower than wild type and/or may not be as stable under pressure, so a control experiment could be done using the Y69L mutant. The fluorescence spectrum could be generated as above and the amount of unfolding could be determined as pressure was increased. Another future direction would be to determine if the k_{cat} of the mutant was affected upon the increase of pressure within this system. The amount of substrate required to saturate the mutant Y69L may be beyond the limits of the current pressure system and could therefore become a problem in determining the effect of pressure on k_{cat} of Y69L. The use of NADPD with the Y69L mutant could determine if the rate determining step had changed and whether or not pressure changes the rate determining step for this enzyme. Because the rate determining step is the chemical process (32), it seems unlikely that this has been changed.

The rate determining step for R67 DHFR has been previously shown to be hydride transfer (32). Though it seems likely that the rate determining step is not changed under pressure, as the k_{cat} remains unchanged, an experiment with NADPD should be executed. A comparison of kinetics with NADPD under pressure should not be different, if the rate determining step has not changed.

Other future directions for determining the role of water in the R67 DHFR reaction mechanism by the use of hydrostatic pressure could use different instruments. A Pressure Perturbation Calorimeter could aid in the understanding of binding DHF to R67 DHFR and yield information on the volume change associated with binding (33). Another instrument that could also lead to better understanding would be

a stopped flow spectrophotometer connected to a hydrostatic pressure pump (34). A stopped flow mechanism would allow for mixing to occur while under pressure and eliminate the difficulties with collecting an initial rate.

All of the future directions could help provide additional information on the effect of water on R67 DHFR mechanism more accurately once completed. With the information available at the moment, it seems that water may not be acting as a co-substrate in the active site as originally thought and the solvent interaction of water molecules may be more involved with the pABA-glu tail of DHF. Osmolytes may be binding to DHF, inhibiting the molecule from binding to the protein, and be required to be displaced from DHF in order for DHF to bind to R67 DHFR. As similar magnitudes of slope for the same osmolyte appear in both EcDHFR and R67 DHFR and DHF is the common denominator, it seems likely that this may be the case. The results of these data support a role for water in the R67 DHFR mechanism, but do not support water as a co-substrate to the reaction.

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Appendix

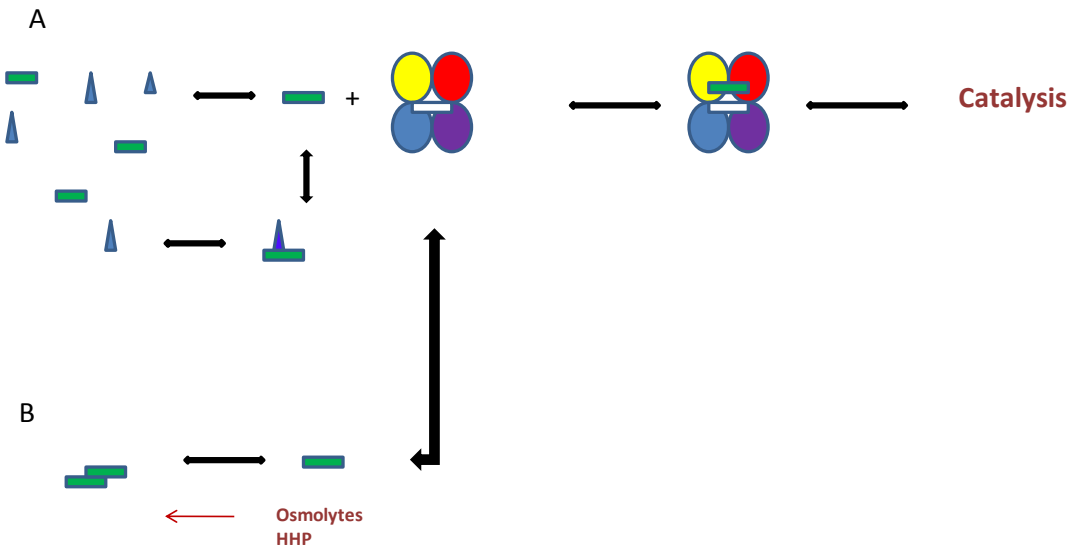

Figure 21: Model of Potential Mechanisms of DHF binding in the Presence of Osmolytes

R67 DHFR is portrayed by the yellow, red, blue, and purple circles which represent the active tetramer. The white rectangle across the active site represents bound NADPH. The binding of DHF is represented by the green rectangle. The blue triangles represent the osmolytes within the system. Scenario A is the interaction of the osmolytes with the free DHF. As the osmolytes interact with free DHF, they would need to be removed before the DHF could bind to R67 DHFR. Scenario B is the presence of osmolytes or HHP shifting the equilibrium of the free DHF into a dimer formation.

Vita

Mary Jane Timson graduated from Missouri State University in May of 2007 with a bachelor's degree in Cell and Molecular biology with a minor in Chemistry. She went to the University of Tennessee for her Master's Degree in Biochemistry, Cellular and Molecular Biology. Here she became interested in an antibiotic resistance enzyme called R67 Dihydrofolate Reductase.

She currently lives in Denver, CO and works for an asbestos testing lab as a Transmission electron microscope analyst.