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Determining the Role of Water and Proton Uptake/Release Upon Binding of Substrate DHF and Cofactor NADPH to the Enzyme Dihydrofolate Reductase's Active Site

Sharghi Rahmanian

University of Tennessee - Knoxville, srahmani@utk.edu

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**Determining the Role of Water and Proton Uptake/Release Upon Binding of the Substrate
DHF and Cofactor NADPH to the Enzyme Dihydrofolate Reductase's Active Site**

Sharghi Rahmanian

BCMB 457

Dr. Howell

Introduction

The enzyme dihydrofolate reductase (DHFR) catalyzes the conversion of 7,8-dihydrofolate (DHF) into 5, 6, 7,8-tetrahydrofolate (THF) using hydride transfer from its cofactor nicotinamide adenine dinucleotide phosphate (NADPH) and uptake of one proton for reduction (1). The product THF is necessary for the synthesis of methionine, purine nucleotides, thymidylate, and other compounds. Thus, the inhibition of DHFR leads to interruption of DNA synthesis and consequently leads to cell death, making this enzyme a crucial target in the treatment of cancer and other diseases. Trimethoprim (TMP) is an important bacterial inhibitor of DHFR that competes with the substrate DHF when binding to the enzyme. In the case of bacteria, either a chromosome (type I) or a R-plasmid (type II) can encode the enzyme DHFR. Certain strains of *E. coli* possess the plasmid-encoded R-67 DHFR that is resistant to TMP. Therefore, the drug TMP can be used to select for the *E. coli* that possess R-67 DHFR.

Regarding binding order of the substrate and the cofactor, random addition occurs for both DHFR enzymes. However, in R-67 DHFR, NADPH prefers to bind first to the enzyme, followed by the binding of DHF to form a DHFR·NADPH·DHF ternary complex. In the chromosomal DHFR, the substrate DHF can bind to the enzyme•NADP⁺ complex or NADP⁺ to the enzyme•DHF complex for the formation of ternary complex. In this complex, the nicotinamide ring of NADPH and the pteridine ring of the folate are situated close together to facilitate the transfer of hydride from C4 on the nicotinamide ring to the C6 of the pteridine ring (3). This hydride transfer occurs concomitantly with protonation of the N5 atom of DHF from the surrounding environment.

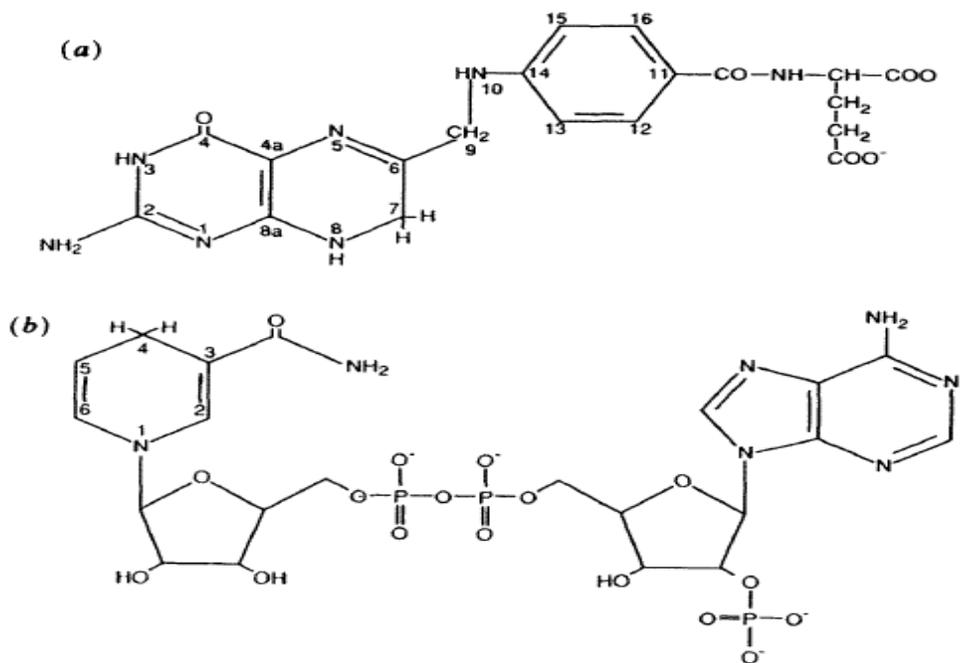


Figure 1: Chemical structures of (a) dihydrofolate and (b) NADPH (4)

The chromosomal DHFR of *E. coli* (EcDHFR) is a monomer of 18000 Daltons with eight strands of beta sheet and four helical connecting strands. The protein has two rigid sub-domains separated by a hinge region. The active site residues that have been conserved throughout evolution in different bacterial DHFRs are M20, P21, W22, D27, R44, R57, G95, G96, and T113 (2). In addition, the enzyme has a loop (β FG) that plays an essential role in the hydride transfer. It has been suggested that the β FG loop is involved in an interaction that spans throughout the protein to promote hydride transfer. Another loop that is important in this transfer is the Met20 loop that is in contact with the bound cofactor and the β FG loop at the same time. The mutagenesis of residues in these loops has been shown to decrease catalysis of the enzyme.

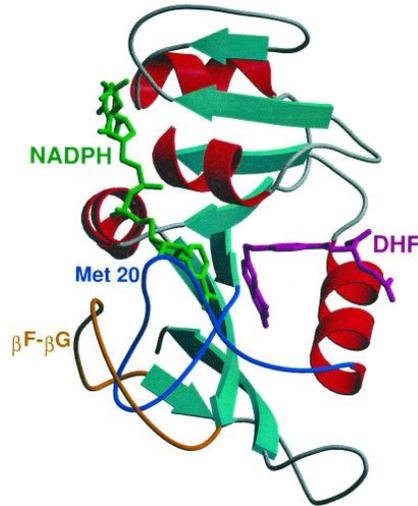


Figure 2: Structure of *E. coli* chromosomal DHFR (EcDHFR) (10)

In the earlier researches, it was noted that DHF binding to the binary complex R-67 DHFR•NADPH in the presence of osmolytes (small, typically uncharged molecules dissolved in buffer) is weaker than binding in buffer as reflected by a decrease in K_a (association constant) (6). This observation is likely due either to stabilization of the free substrate or to the destabilization of the transition state complex. To test the stabilization of the free substrate hypothesis, the same experiment was done in osmolytes with the enzyme chromosomal DHFR (EcDHFR) in order to be able to see if the same effect occurs with another enzyme scaffold. If the same observation is made with EcDHFR as with R-67 DHFR, then the lower K_a is coherent with the hypothesis that osmolytes stabilize free DHF.

Thus to understand the role of water in binding of the cofactor NADPH and substrate DHF to EcDHFR, the effect of osmolytes is noted. As the concentration of osmolytes increases, the effects of binding that result from the lower concentration of water molecules can be seen. Since different osmolytes are used in this experiment, the effect of the individual osmolytes on binding of the substrate and the cofactor are observed (8). If the osmolytes show the same effect

upon the binding of DHF and NADPH, then osmolytes are likely excluded from the active site and do not affect binding. If the osmolyte effect on binding, however, is different with each osmolyte, then the osmolytes likely interact with the binding site. Alternatively, the result could mean that osmolytes can interact with the protein surface, resulting in conformational change in the protein that is sensitive to osmolyte identity.

Also, the effect and the number of protons binding to the active site as the cofactor and the substrate bind can be monitored. In order to analyze the characteristics of proton transfer during binding, buffers that have different heats of ionization are used. In each of these buffers, as a proton is transferred from the solution to the active site or vice versa, the observed ΔH can change due to the different heats of ionization. These differences allow for the understanding of the release/uptake and the number of protons transferred during substrate or cofactor binding.

To carry out these experiments, Isothermal Titration Calorimetry (ITC) is used. In ITC, the thermodynamics of the interaction between two molecules (usually a ligand and a protein) are measured. Such measurements yield the K_a (the binding affinity of the molecules) and the enthalpy (ΔH). The entropy (ΔS) and Gibbs free energy (ΔG) can be calculated based on the equations:

Equation 1: $\Delta G = -RT \ln K = \Delta H - T\Delta S$ where T = temperature in Kelvin R = gas constant

Also, the stoichiometry of interaction between the ligands can be determined using data analysis software such as Origin and SEDPHAT. By employing ITC, it is hoped that the different measurements, such as the enthalpy, Gibbs free energy, and K_a show details on how osmolytes affect the binding of NADPH and DHF to the enzyme EcDHFR.

Methods

Chromosomal dihydrofolate reductase (EcDHFR) was grown in *E. coli* cells in TB media containing 200 µg/mL ampicillin and 20 µg/mL trimethoprim. The organism was grown in these media for 24 hours at 37°C. The cells were suspended in the loading buffer (50 mM Na₂HPO₄, 300 mM NaCl, 10 mM imidazole, 6 mM BME, pH= 8) for the 24 cm long Ni-NTA column (2 cm in diameter) and sonicated (Sonic Dismembrator 550). The content of the tube was next centrifuged (Sorvall RC5C Plus) for 30 minutes at 8000 rpm to collect the EcDHFR in the supernatant. Since DNA absorbs light mostly at 260 nm and protein absorbs mostly at 280 nm, the ratio of the absorbencies at 280 nm to 260 nm gives an estimation of how much DNA is present compared to protein (EcDHFR). The optimal ratio of A₂₈₀:A₂₆₀ is 1.5.

A Ni-NTA column was used as a first step in protein purification. The nitrilotriacetic acid (NTA) in the column is charged by Ni²⁺ that binds to the NTA resin. The nickel in turn binds to the six histidine residues that are attached to the modified protein (EcDHFR). After loading the supernatant into the Ni-NTA column, the protein was eluted off the column by a gradient. The gradient was setup using a solution of 50 mM Na₂HPO₄, 300 mM NaCl, 6 mM BME, and 10 mM imidazole at pH 8 and a solution of 50 mM Na₂HPO₄, 300 mM NaCl, 250 mM imidazole, and 6 mM BME at pH 8. The imidazole replaced the modified protein, and a fraction collector collected the protein. After elution, the activities of the protein in each fraction were assessed at saturating levels of DHF (0.72 mM) and NADPH (1 mM). To do this, the absorbance of the cuvette containing DHF and NADPH was measured upon the addition of a sample from the fraction at 340 nm, and the linear part of the photometric measurement correlated to the activity of the fraction. The fractions with the most activity (high changes in absorbance) were chosen for the gel electrophoresis to verify the fractions' purity.

The protein was further purified over a 13 cm long methotrexate column (3.5 cm diameter) and was dialyzed in phosphate buffer. Then, it was loaded and washed with a solution containing 0.2 M KH_2PO_4 , 1 M NaCl, 1 mM BME at pH 6.0 and a second solution of 50 mM sodium borate, 300 mM NaCl, and 6 mM BME at pH 8. For elution, a folate solution (50 mM sodium borate, 300 mM NaCl, 6 mM BME, and 1 mM folate) at pH 9.0 was used to compete for the DHFR binding site that was occupied with methotrexate, thus releasing the protein from the column. The process of enzymatic assay, gel electrophoresis, and dialysis was repeated as with the fractions from other columns. The protein was then dialyzed in 4 L of 1X TE with 6 mM BME at pH =8. The protein is usually dialyzed in a solution that is used for loading the protein onto the new column. The 1x TE (10 mM Tris and 1 mM EDTA) was the loading buffer for 44 cm long DEAE column (2.5 cm diameter), which separates eluents based on anionic charge. Gradient elution of TE plus BME at pH 8 (1x TE vs. 1x TE plus 0.4 M NaCl) was used to elute the protein off the column. After the protein was collected in the fraction collector, the same process of enzymatic assay, gel electrophoresis, and dialysis was done to determine the purity of the protein collected.

In these experiments, ternary binding of DHF and NADP^+ were examined using ITC. For the ternary complexes, DHF or NADP^+ was the ligand that was titrated into the protein saturated with either NADP^+ or DHF in the buffer alone. Additional experiments were performed in osmolytes to determine the effect of the water on binding. Also, the heat of ionization for each buffer was measured in ITC using water or HCl as the ligand and titrating these ligands into the buffer.

To perform these ternary-binding experiments, Isothermal Titration Calorimetry (ITC) was utilized applying the method noted before (5). Also, the use of the instrument has been

previously described (6). The parameters for the three buffers of SID (polybuffer of 33 mM succinic acid, 44 mM imidazole, and 44 mM diethanolamine), 100 mM MTH, and 100 mM Hepes + NaCl (each with EDTA and BME) used for the effect of ionization were pH 7 and 25 degrees Celsius. For the ternary complex when using DHF as the titrant, the concentration of DHF was about 0.5 mM while the concentration of protein and NADP⁺ was 0.012 mM and 0.2 mM, respectively. In the NADP⁺ ternary, NADP⁺ had a concentration of about 0.6 mM while the protein and DHF had a concentration of 0.012 mM and 20-100 μ M, respectively. The following extinction coefficients were used for the calculation of concentration of DHF, NADP⁺, and protein: DHF at 282 nm, 28,000 M⁻¹ cm⁻¹, NADPH⁺ at 259 nm, 18,000 M⁻¹ cm⁻¹, EcDHFR at 280 nm, 2.34 M⁻¹ cm⁻¹ (as determined by BCA). The buffers used to monitor the role of water were also made at pH 7 and at 25 degrees Celsius using different concentration of osmolytes in the MTH buffer (10% and 20% betaine, 7.5% and 15% sucrose, 15% and 30% PEG, 10% and 20% glycerol, 10% and 20% ethyl glycerol, 10% and 20% DMSO, and 7.5% and 15% TMAO). The titrations were done in duplicate. The ITC data were fit using Origin 7. Also, the duplicated data were fitted globally using SEDPHAT and the binary model. (11)

To monitor the role of water, the effect of osmolytes in the buffer was measured by using different concentrations of osmolytes. The osmolality of these solutions was measured using an osmometer (Vapro 5520). The osmometer was first standardized using solutions of 290 ± 3 mmol/kg, $1000 \pm$ mmol/kg, 100 ± 2 mmol/kg, according to the User's manual. Then, the osmolality of various buffers were measured based on their vapor pressure. The water activity is derived from the osmolality of the solution based on the equation:

Equation 2: $\ln(a_{\text{water}}) = - \text{osmolality} (\text{molal volume of water } (18/1000))$

The water activity of each buffer can be graphed as the independent variable in association with $\ln K_a$ of binding as the dependent variable to determine the molecules of water uptaken/released, as noted earlier (6).

For the protonation effect, the heat that was released during binding versus the heat of ionization for the buffer was graphed. The data was interpreted based on the equation (7):

$$\text{Equation 2: } \Delta H(\text{observed}) = \Delta H(\text{binding}) + n \Delta H(\text{ionization})$$

where n = protons released/uptaken

Results

The change in enthalpy upon the binding of the ligand to the enzyme•cofactor or enzyme•substrate complex (as in the case of ternary complexes) was monitored using ITC. As more ligand is titrated into the cuvette to form a complex, the heat release, as measured by microcal/sec in Figure 3, decreases due to saturation of the binding sites on the enzyme.

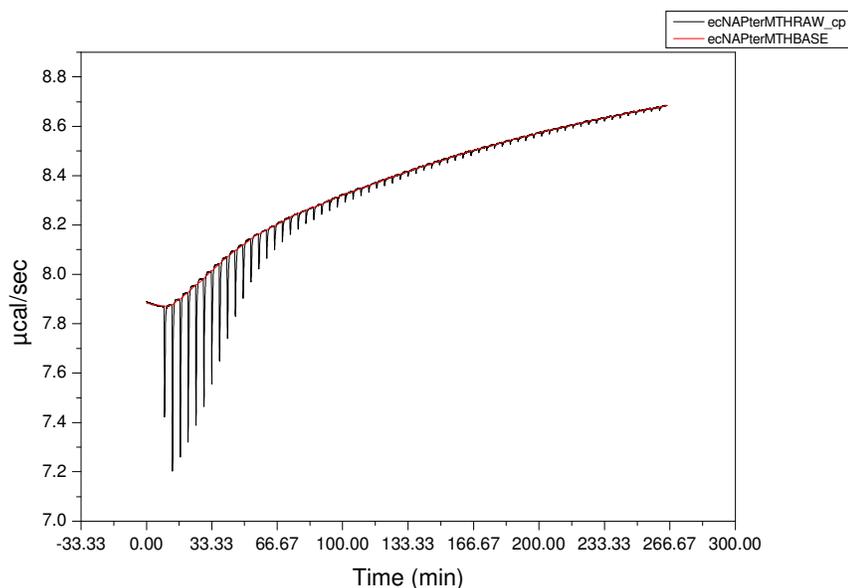


Figure 3: Raw ITC data for NADP⁺ ternary in 20% glycerol MTH buffer, showing the decrease in change of enthalpy upon the binding of ligand to the EcDHFR•DHF complex as the reaction proceeds.

Each raw ITC data was fitted using Origin 7 (Figure 4). This program integrates the area under the peaks of the raw ITC data; the result then could be fit to a single site model. This program determines the best-fit line for the data and calculates the stoichiometry between the ligand and the enzyme complex, the K_a (association constant) for the interaction, and the change in enthalpy upon binding. Since the titrations for each condition were repeated twice, the ITC data were also fit globally by SEDPHAT (Figure 5).

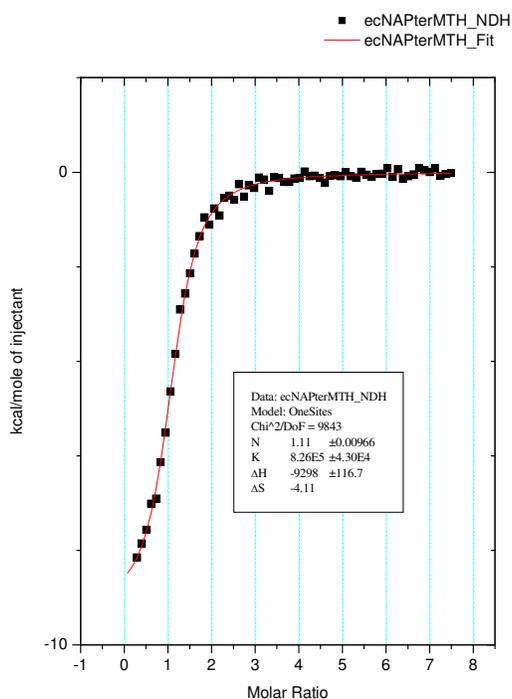


Figure 4: Fitting of NADP+ ternary in 20% Glycerol MTH, using Origin 7. The N-value corresponds to the stoichiometry of binding, the K-value corresponds to the K_a (association constant), allowing K_d (dissociation constant) to be calculated using the equation $K_a = 1/ K_d$. Lastly, Origin provides a ΔH value corresponding to the change in enthalpy, allowing for the calculation of ΔS , change in entropy (using equation 1 above). The plateau shows the saturation of the enzyme despite the addition of more ligands.

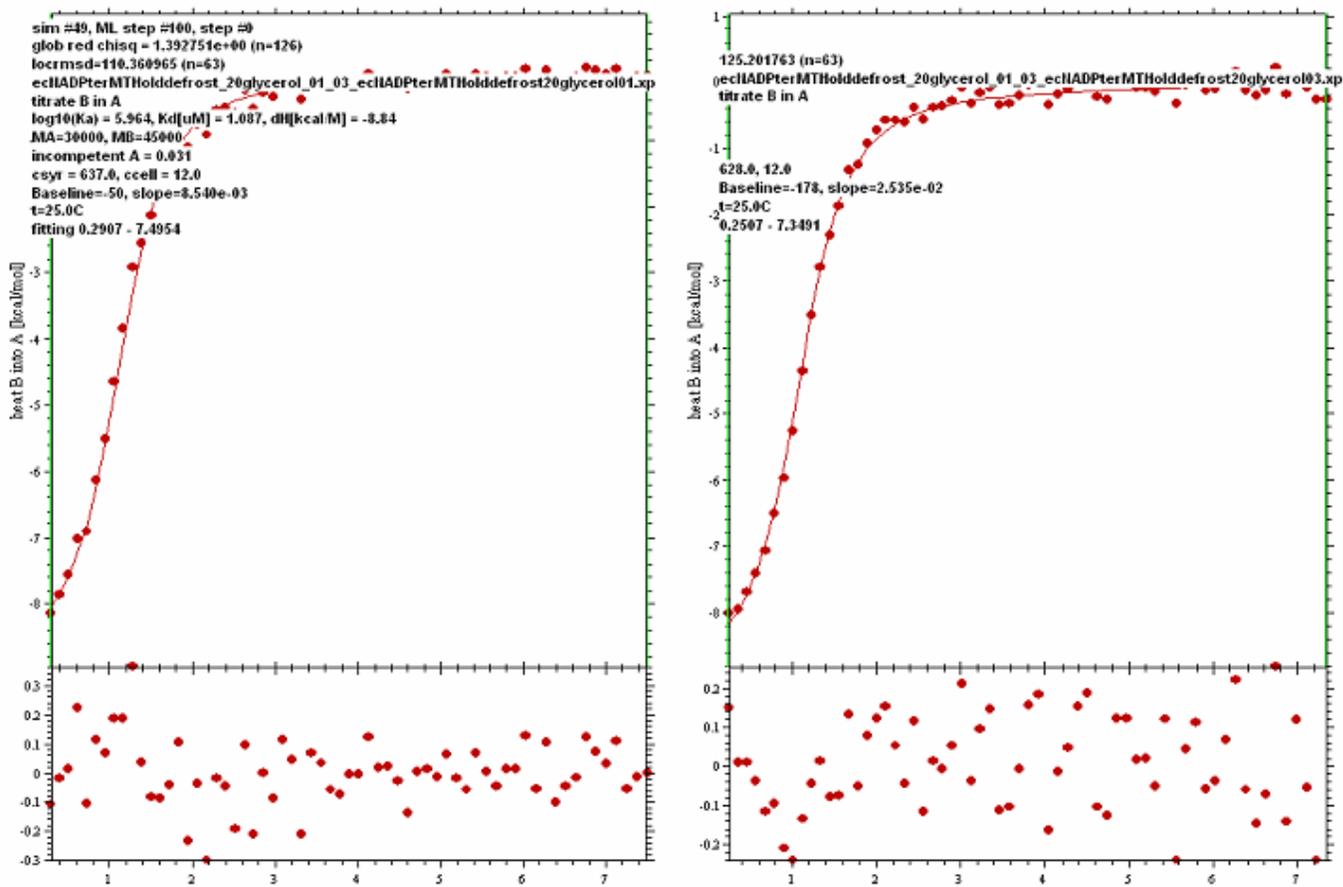


Figure 5: Global fitting of NADP+ ternary in 20% Glycerol MTH, using SEDPHAT, along with the calculation of K_a (association constant), K_d (dissociation constant), and ΔH (change in enthalpy upon binding). The values incompetent A and B represent the incompetency of the complex in the cuvette (Enzyme•DHF, for the above results) and of the ligand (NADP+, in this case), respectively. When the value incompetency A is calculated, this number can be subtracted from one to give the stoichiometry of binding while for incompetent B, the number is added to one to obtain the stoichiometry.

To analyze the role of water in the binding of NADPH and DHF to the active site of the EcDHFR, the uptake and release of water molecules in the presence of different osmolytes upon

the binding of the ligand to the enzyme complex was noted. The effect of osmolytes on the formation of the DHF and NADP+ ternary complexes are shown in Figure 6 and 7, respectively.

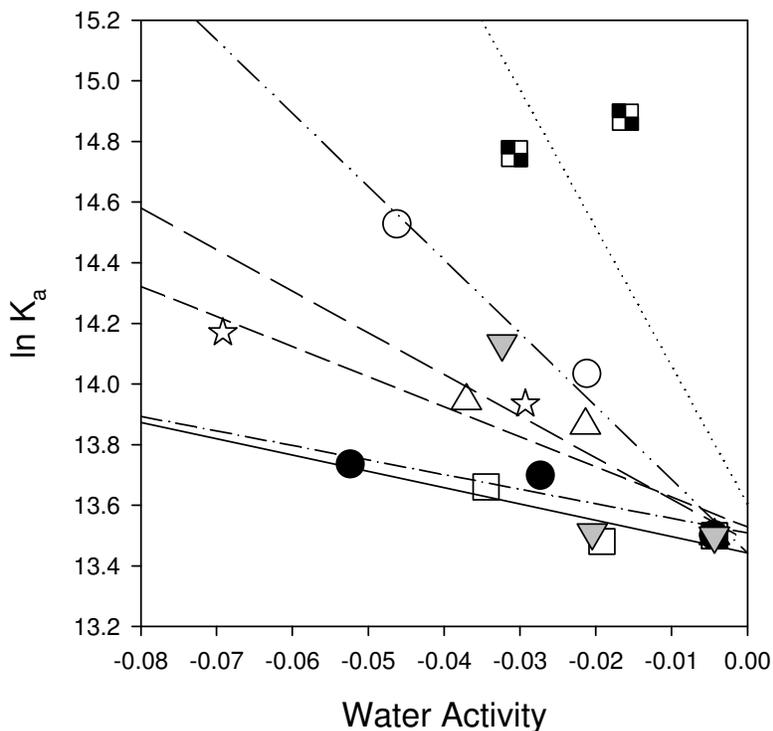


Figure 6: The above graph shows the osmolality effects on the ternary complex for NADP+. Data for glycerol (dark circles), ethylene glycol (star), TMAO (upside-down triangle), sucrose (rectangle), DMSO (open circle), glycine betaine (triangle) and PEG400 (checkered rectangle) are shown. The slope of $\ln K_a$ (association constant) vs. the water activity of the solutions corresponds to the net release/uptake of water molecules upon the binding of the ligand to the enzyme complex. The value of the slopes correlates to the number of water molecules released/uptaken. The panel shows the effect of osmolytes on the NADP+ ternary complex. Each osmolyte showed a net release of water, and the different slopes for the osmolytes used show that each osmolyte has unique interactions with the enzyme's binding site, on the protein surface, and/or on the ligand.

In the case of NADP+ ternary, the negative slope shows the net release of water molecules while the value of the slope shows the number of water molecules released in the presence of osmolytes. For DHF ternary, the positive slope shows the net uptake of water while the value of the slope shows the number of molecules uptaken. In both the NADP+ and DHF ternary results, each osmolyte has a different effect on the net release/uptake of water molecules due to the calculation of different slopes upon the binding of NADP+/DHF in the presence of osmolytes.

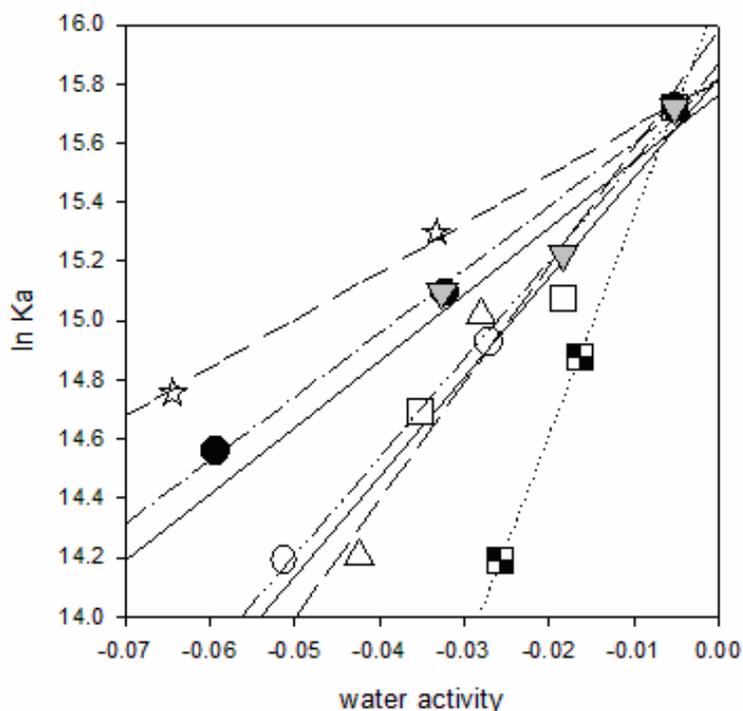


Figure 7: Water uptake is shown for DHF ternary. Data for glycerol (dark circles), ethylene glycol (star), TMAO (upside-down triangle), sucrose (rectangle), DMSO (open circle), glycine betaine (triangle) and PEG400 (checked rectangle) are shown. The different osmolytes interacted uniquely with the enzyme active site. However, the slope is positive, showing a net uptake of water.

The following table shows the number of molecules of water release/uptake upon the binding of NADP+ and DHF binding with each osmolyte:

Table 1: Water molecules released or uptaken upon binding of NADP+ or DHF, respectively, with each osmolyte.

Osmolyte	Slope for NADP+ plot	Slope for DHF plot
Glycerol	-5	21
Ethylene Glycol	-10	16
TMAO	ND	22
Sucrose	-5	34
DMSO	-24	33
Glycine Betaine	-14	40
PEG 400	-46	74

The protonation effect, which measures pKa perturbation of either protein residues and/or ligand, can be seen by graphing the heat observed against the different heat of ionization of buffers. As seen in the left panel of Figure 8, a buffer with lower heat of ionization shows a lower (more negative) exothermic enthalpy as proton is released into the buffer upon the binding of NADP+ to the enzyme•DHF complex. The buffer with higher heat of ionization, however, shows a higher exothermic (more positive) signal upon the release of a proton. Therefore, proton release occurs upon the binding of NADP+. With DHF binding, the opposite effect is shown, indicating that proton is uptaken from the buffer, with the buffer possessing higher heat of ionization showing a lower (more negative) exothermic signal upon the removal of proton to the buffer.

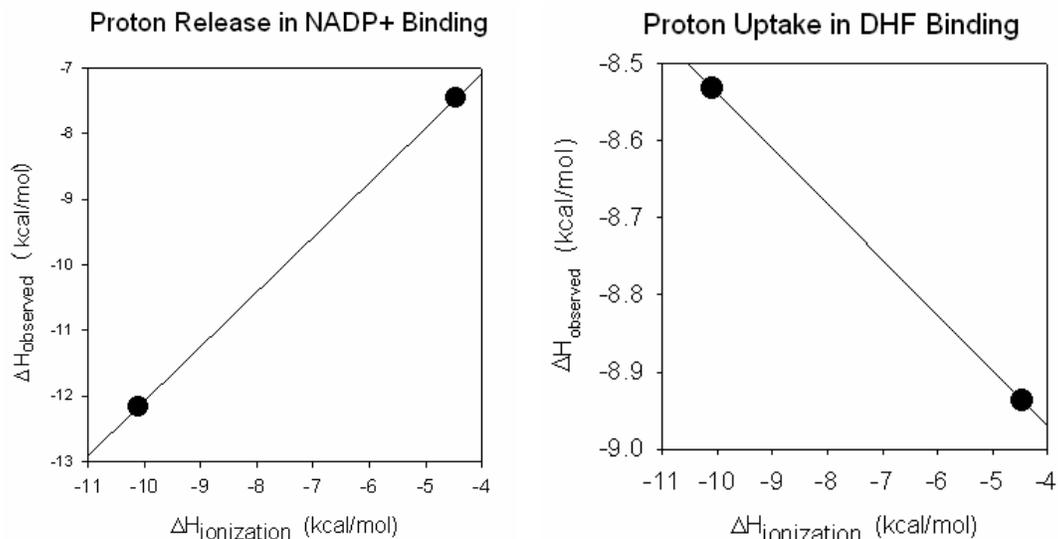


Figure 8: A plot of the enthalpy observed versus the enthalpy of buffer ionization shows proton release upon NADP⁺ binding to EcDHFR-DHF (Left Panel) and proton uptake for DHF binding to EcDHFR-NADP⁺ (Right Panel). The slope of each line corresponds to the net number of protons taken up or released. The DHF graph yields a slope of -0.1 (proton uptake) while the slope for NADP⁺ binding is 0.9 (proton release).

According to equation 2, the slope of the graphs in Figure 8 represents the n-value that is the net number of protons released or uptaken. With NADP⁺ binding, a net proton is released into the buffer while with DHF binding, the slope is -.1, with little net proton uptake.

Discussion

The chromosomal DHFR catalyzes the conversion of 7,8-dihydrofolate (DHF) into 5, 6, 7,8-tetrahydrofolate (THF) using a hydride from its cofactor NADPH and by employing the Met20 loop to aid this transfer. In this experiment, the role of water in the active site upon binding of the cofactor and DHF to the enzyme-DHF complex or enzyme-NADP⁺ complex, respectively, was monitored using different osmolytes in buffer. Upon NADP⁺ binding, water

was released while for the DHF binding, the graph shows water uptake. In both cases, the binding of NADP⁺ or DHF resulted in different number of molecules of water being released/uptaken, indicating that osmolytes interact with the active site of the enzyme and/or that the osmolytes exerted considerable pressure on the surface of the protein, thereby causing conformational change of the protein. Thus, the osmolytes are not excluded from the active site, supporting the idea of preferential interaction, as shown in the left panel of figure 8 below.

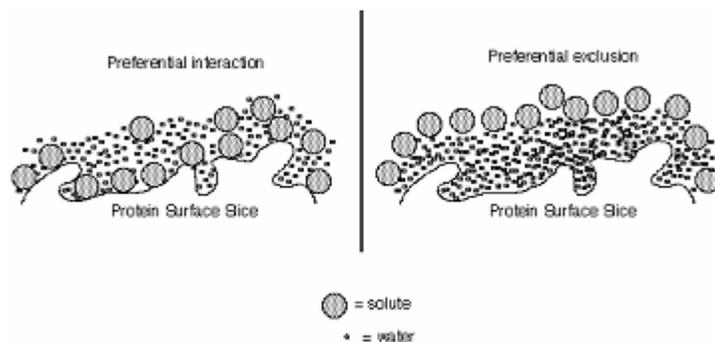


Figure 8: The left panel shows osmolytes interacting with the binding site. Since each osmolyte differentially interacts with the site, this concept is called preferential interaction. The right panel shows osmolytes are excluded from the binding site and form a shell around this site, thereby supporting the idea of preferential exclusion. (8)

Previously, it had been noted that upon the binding of NADP⁺ to R-67 DHFR-DHF, water was released and that there was water uptake for the binding of DHF to the enzyme-NADP⁺ (6). Preferential interaction, however, only occurred for the binding of DHF and not NADP⁺. The binding of NADP⁺ resulted in a single slope for the different osmolytes, supporting the idea of exclusion of osmolytes from the binding site (preferential exclusion, right panel in Figure 8). Since the R-67 enzyme has symmetry related sites for NADPH and DHF, with NADPH and DHF being able to bind to either one of four sites, this indicates that NADPH

serves as an internal control for DHF. The fact that NADPH showed exclusion while DHF showed preferential interaction shows that the structure of the free enzyme is not affecting the result seen with the binding of ligands. In addition, in comparing our results to the last experiment with R-67, since different results were found for the binding of the NADPH in R-67 DHFR that has a different active site structure than that of EcDHFR, it can be concluded that enzymes' active site structures do affect how osmolytes behave when the enzyme is bound to NADP+. Since osmolytes are displacing water, water also interacts differently with distinct enzyme active sites.

In the graph of NADP+ binding (Figure 6), the association constant, thereby the strength of the binding, decreases as the water activity increases, indicating that water is released for the binding of NADP+ to the enzyme• DHF complex. In DHF binding, however, the Ka increases as more water is present. This result supports the idea that water is uptaken upon the binding of DHF to the complex EcDHFR•DHF. The decrease in the strength of binding in the presence of increasing concentration of osmolytes shows that either the enzyme• DHF complex is destabilized or EcDHFR and DHF are individually stabilized, according to Figure 9 below:



Figure 9: In this figure, the different stages of the substrate binding are shown. The E refers to the ecDHFR plus NADP+ complex and the S corresponds to the substrate (free DHF).

Since the decrease in K_a in DHF binding was observed for both the R-67 and the chromosomal enzyme experiment in the presence of increasing concentration of osmolytes and these two enzymes vary widely in their binding structures, then it is thought that the free enzyme is most likely not the species affected. Thus, it is speculated that the free DHF is stabilized by the osmolytes to decrease K_a and increase the energy barrier for binding (and ultimately, catalysis). In previous studies, it was noted that DHF forms a dimer with another molecule of itself (9). This dimer could be stabilized by the presence of osmolytes, resulting in lower K_a . Doing NMR on folate/DHF in the presence of osmolytes can test this hypothesis.

Another effect that is noted is that upon the binding of two molecules, water exclusion/release is usually seen (Figure 10, upper panel), as is shown by the binding of NADP^+ . For DHF binding to the enzyme• NADP^+ complex, monomers would need to be formed. Since water is taken up upon the binding of DHF, it may be that the DHF-DHF dimer breaks apart and water molecules are attached to the sides of DHF that another DHF molecule previously occupied. Further, it is thought that water is displaced upon the binding of DHF to the protein-NADPH. The net uptake of water upon DHF binding can show the net effect of both water uptake upon monomerization of DHF and its binding to the protein-NADPH.

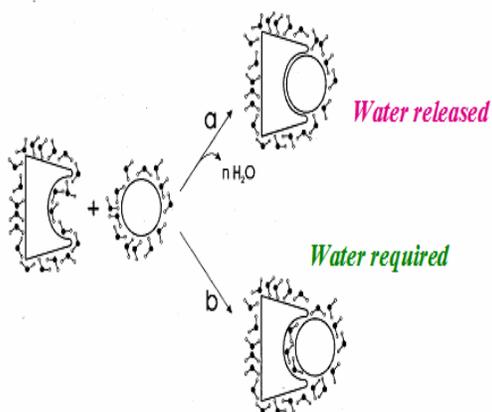


Figure 10: Upon the joining of two molecules, either water is released (upper panel) or water is required (lower panel). In the instance where water is released, water is displaced, allowing for the molecules to bind tightly. In the lower panel, a layer of water molecules remains between the molecules.

For the protonation effect, a net proton is released upon the binding of NADP⁺ to enzyme•DHF. The slope is 0.9, which correspond to a net release of one proton. This indicates that NADP⁺ binds as a protonated species with a perturbed pK_a, picking up one proton during binding. There is also the possibility that the pK_a of one or several residues of the protein change, resulting in a net effect of one proton uptake. Upon DHF binding, however, the slope of the graph was -.1, indicating that the net release of proton was not significant. This value could indicate that one or more proton is uptaken while the same amount of protons is released into the buffer.

References

1. Jackson, M., Chopra, S., Smiley, R. D., Maynard, P. O., Rosowsky, A., London, R. E., Levy, L., Kalman, T. I., Howell, E. E. (2005) *Biochemistry*. 44, 12420-12433.
2. Howell, E. E. (2005) *ChemBioChem*. 6, 590-600.
3. Loveridge, E. J., Evans, R. M., Allemann, R. K. (2008) *Chem. Eur.* 14, 10782-10788.
4. Brown, K. A., Kraut. J. (1993) *Faraday Discussion*. 31, 9813-9822.
5. Chopra, S., Dooling. R. M., Horner, C.G., Howell, E. E. (2008) *The Journal of Biological Chemistry*. 283 (8), 4690-4698.
6. Wiseman, T., Williston, S., Brandts, J.F., and Lin, L. N. (1989) *Anal. Biochem*. 179, 131-137.
7. Ortiz-Salmerón, E., Yassin, Z., Clemente-Jimenez, M.J., Heras-Vazquez, F.J.L., Rodriguez-Vico, F., Barón, C., García-Fuentes, L. (2001) *Eur. J. Biochem*. 268, 4307-4314.
8. USA. National Institute of Health. *Osmotic Stress*. Web. 15 Apr. 2010. <http://lpsb.nichd.nih.gov/photo_gallery/photo_gallery/index.htm>.
9. Poe, M. (1973) *The Journal of Biological Chemistry*. 248 (20), 7025-7032.
10. Proceedings National Academy Science. USA. (2002) 99(5) 2794–2799.
11. Houtman, J. C. D., Brown, P. H., Bowden, B., Yamaguchi, H., Appella, E., Samelson, L. E., Schuck, P. (2007) *Protein Science*. 16, 30-42.