Further Studies on the Role of Water in R67 Dihydrofolate Reductase

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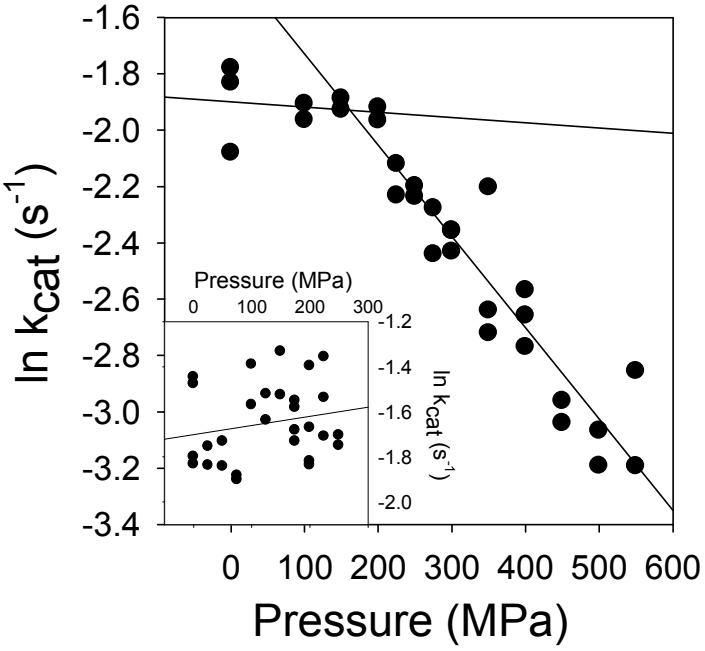
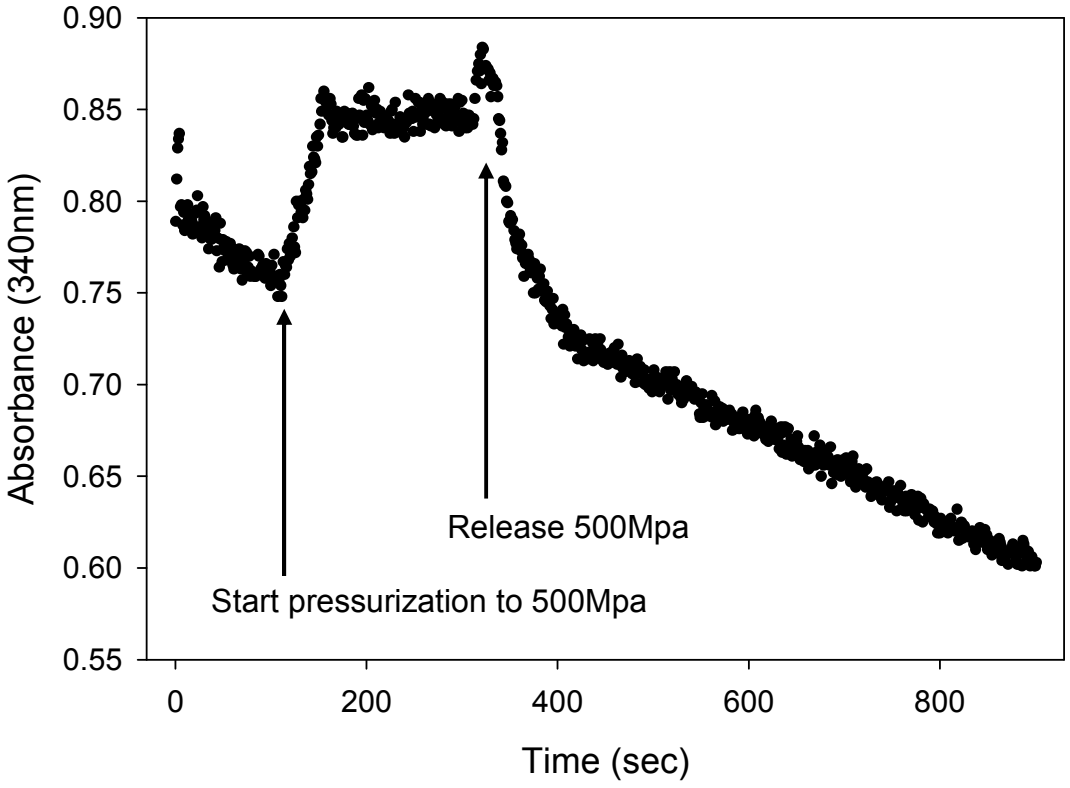
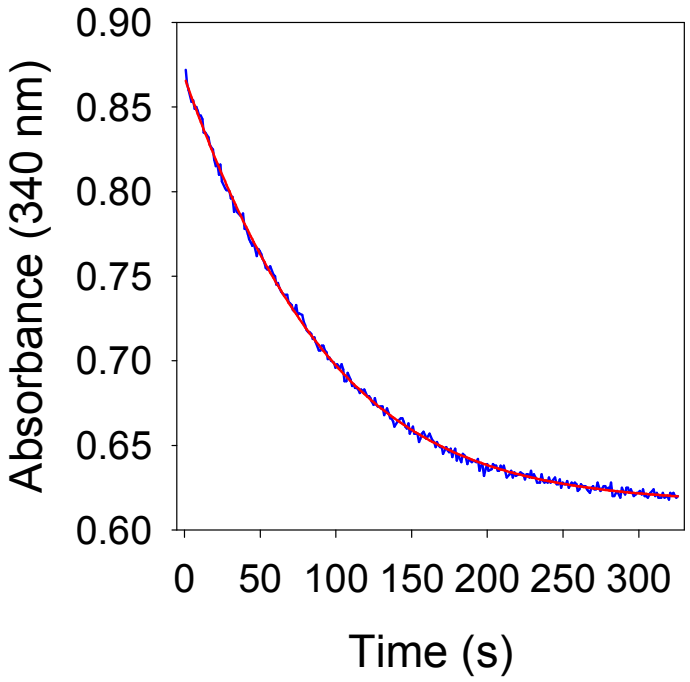


Figure S1. Plot of ln k_{cat} vs. pressure. Substrate (70-120 μM) and cofactor (70-100 μM) were added to R67 DHFR and the rate monitored as a function of pressure. These concentrations are >10x the K_m values at ambient pressure. Additional data in the 0.1-250 MPa range were collected a year later with a different protein preparation. These data are shown as an inset. Note that the scale change in the inset compared to the full figure.



Supplemental Figure S2. Saturating concentrations of DHF and NADPH were added to R67 DHFR and the steady state rate monitored at ambient pressure. Then the pressure was raised to 500Mpa (first arrow). Upon pressure release (second arrow), the rate returned to roughly 70% of the original rate.



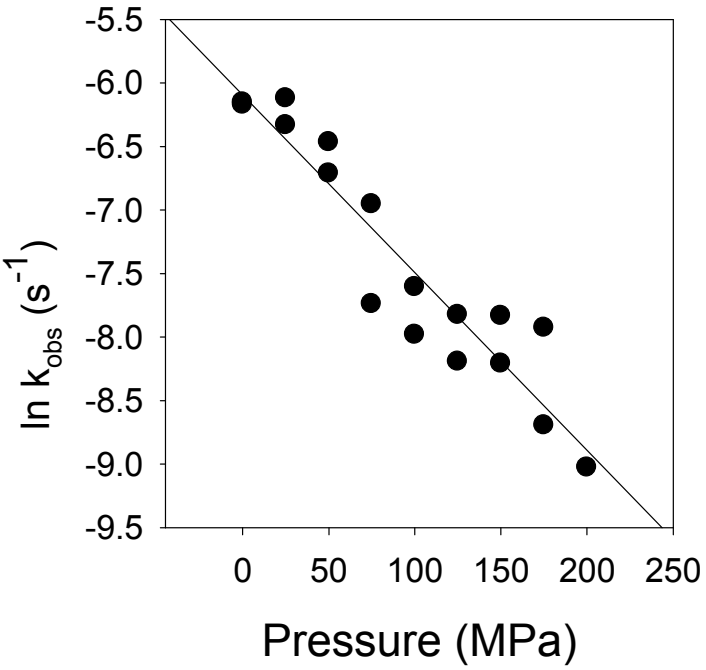
Supplemental Figure S3. A progress curve trace for the reduction of DHF (65 μM) in the presence of excess NADPH (165 μM) cofactor by R67 DHFR (400 nM) at 175 Mpa. The blue line shows the data and the red line gives the fit.

HHP kinetics of Y69L mutant of R67 DHFR It is difficult to use a steady state kinetics approach to monitor the effect of HHP on DHF binding in wt R67 DHFR. This constraint arises as initial rates are not measured since it typically takes up to 2 minutes to mix the solutions and achieve high pressure. Thus we turned to a Y69L mutant R67 DHFR and used k_{cat}/K_m conditions to measure rates.

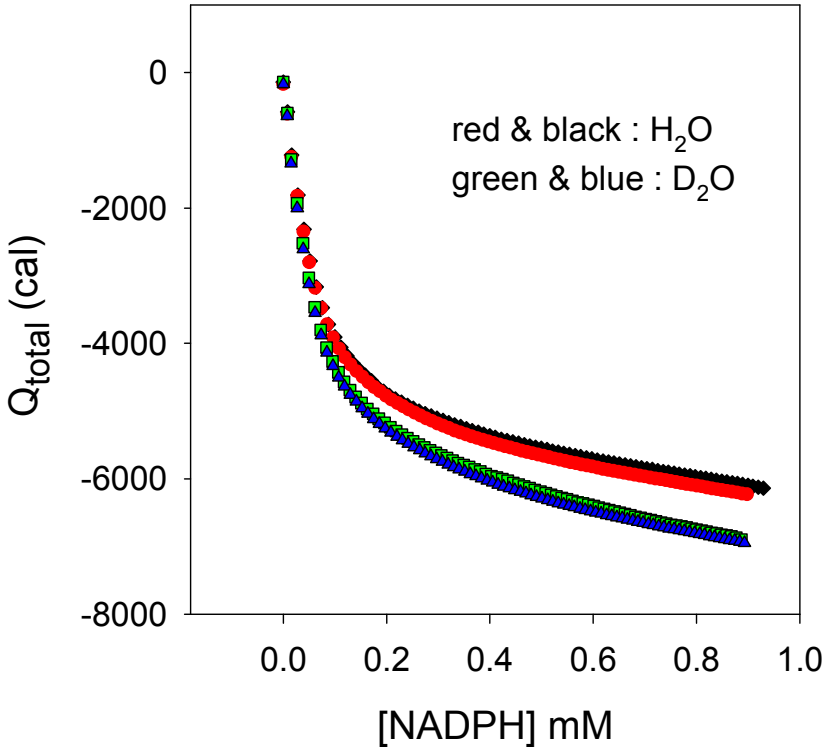
The k_{cat} for a Y69L R67 DHFR mutant is ~10 fold lower than for wt R67 DHFR and its K_m values are 68 μM for NADPH and 180 μM for DHF.[1] To monitor rates under $k_{cat}/K_{m\ (DHF)}$ conditions for this mutant, absorbance readings in the linear range of the spectrometer needed to be balanced against the level of NADPH saturation. At an absorbance above 1.8, the instrument response is not linear but at too low a concentration, the enzyme is not saturated with NADPH. We settled on using 300 μM NADPH (4.4x K_m) and 50 μM DHF (0.28x K_m). Rates were constant for up to 8 min. While other mutants were available, they either displayed substrate inhibition or their K_m values were too low to allow monitoring of rates under k_{cat}/K_m conditions.

Based on the above reasoning, the rate of the Y69L mutant R67 DHFR was measured. While pressure should weaken binding of NADPH (as seen for the wt R67 DHFR in Figure 4 of the main text), the NADPH concentration should remain higher than its K_m value. As the DHF concentration will remain below its K_m value, the predominant HHP effects are expected to report on substrate capture and subsequent transition state formation. Figure S4 below shows the observed rate for Y69L R67 DHFR steadily decreases with increasing HHP. The slope of the ln k_{cat}/K_M plot vs. HHP yields an activation volume of 35.2 ± 3.2 $cm^3 \cdot mol^{-1}$.

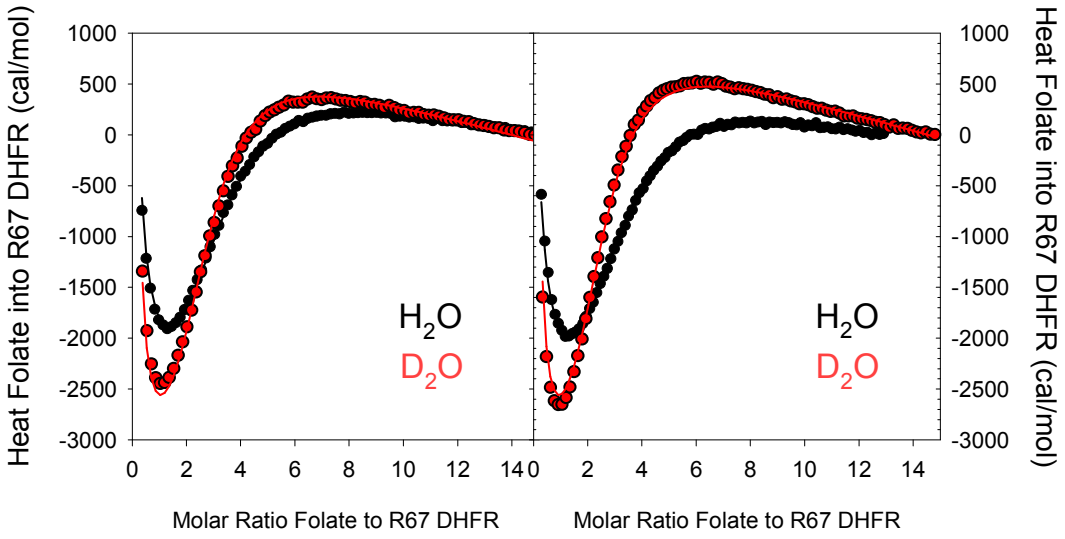
We note that the activation energy for the Y69L mutant is substantially higher than for wt R67 DHFR (3.3 ± 0.5 $cm^3 \cdot mol^{-1}$, see progress curve analysis in main text). This difference may arise due to the presence of the Y69L mutations and perhaps some contribution from non-saturating NADPH concentrations. However the activation energies associated with $k_{cat}/K_{m\ (DHF)}$ for both wt and Y69L R67 DHFRs are positive, consistent with weaker substrate capture.



Supplemental Figure S4. Plot of ln k_{obs} for the Y69L mutant R67 DHFR vs. HHP. Rates were collected for the Y69L mutant under $k_{cat}/K_{m\,(DHF)}$ conditions where the DHF concentration was 0.28 times its K_m at ambient pressure and the NADPH concentration was 4.4 times its K_m. The activation volume is $35.2 \pm 3.2\ cm^3 \cdot mol^{-1}$.



Supplemental Figure S5. Total heat plots for NADPH binding to R67 DHFR. The enzyme concentration varied from 102-111 μM while the syringe concentration varied from 5.6-5.9 mM NADPH. The red and black data points correspond to ITC titrations performed in H_2O while the green and blue points correspond to titrations performed in D_2O.



Supplemental Figure S6. Comparison of the folate titrations into apo R67 DHFR in water and D_2O based buffers. The D_2O points are shown in red while the H_2O points are depicted in black. The enzyme concentrations for the D_2O experiments were 104 and109 μM (left and right panels respectively), while the syringe concentration was 8 mM folate. The enzyme concentration for the H_2O experiments was 109 μM for both panels, while the syringe concentration was 6.6 mM folate. To reach equilibrium, four minutes were allowed between each injection. Each dataset was first opened using Origin v7 software and the first two points removed. Next, two different titrations were imported into SEDPHAT v8.2 [2] and globally fit to an A+B+B ↔ AB + B ↔ ABB model with 2 symmetric sites and macroscopic K_d values. The fits from the SEDPHAT analysis are listed in Table 1 in the main text.

Table S1. Binding enthalpies determined by ITC for assorted R67 DHFR constructs.

Protein	Complex	K_d	ΔH
Wt R67 DHFR[a]	Folate into enzyme•NADPH	10.6 ± 0.4	-8500 ± 500
Wt R67 DHFR[a]	DHF into enzyme•$NADP^+$	4.8 ± 1.0	-11700 ± 300
Wt R67 DHFR[b]	DHP into enzyme•NADPH	25 ± 0.4	-6900 ± 50
Wt R67 DHFR[b]	DHB into enzyme•NADPH	K_i = 160 ± 18	no signal
Q67H R67 DHFR[c]	Folate into enzyme•NADPH	1.5 ± 0.3	–4800 ± 300
Quad3[d]	DHF into enzyme•$NADP^+$	4.9 ± 0.1	-11500 ± 800
Q67H:1+2+3 in Quad3[d]	DHF into enzyme•$NADP^+$	6.6 ± 0.2	-8500 ± 590
Q67H:1+2+3+4 plus K32M:1+3 in Quad3[e]	DHF into enzyme•$NADP^+$	38 ± 2	-13700 ± 600

[a] from [3]
[b] from [4]
[c] from [5]
[d] from [6]
[e] from [7]

Discussion of enthalpy effects in Table S1 A comparison of ternary folate versus ternary DHF binding shows a more negative enthalpy, however to prevent catalysis, $NADP^+$ was used for the DHF titration.[3] As DHF is truncated to dihydropteroate, the enthalpy term becomes less negative, reaching zero when dihydrobiopterin is used.[4] These results indicate the pABA-glu tail is quite important in binding. Another contributing factor to this series of titrations may be ligand connectivity. Previous studies on connectivity have described how binding is more than the sum of the component parts.[8-10]

Addition of the Q67H mutation to wt R67 DHFR resulted in tighter binding of folate with a reasonable enthalpic signal.[5] To be able to construct asymmetric mutations, a tandem array of four fused R67 DHFR genes was constructed.[6] This variant, named Quad3, was almost fully functional and ternary DHF binding still provided a full enthalpic signal. Addition of three Q67H mutations to Quad3 also provided a good ΔH value.[6] These various studies indicate the introduction of Q67H mutations does not abrogate a reasonable enthalpy associated with ternary DHF/folate binding.

Finally, to evaluate the role of K32 in DHF binding, two K32M mutations were added asymmetrically to Quad3, such that both mutations occurred in one half of the active site pore (see Figure 1 in [11]). While very weak DHF binding was observed, this protein tended to aggregate, not allowing ITC analysis. To try and tighten binding, the Q67H mutation was additionally added to each gene copy and the resulting protein (Q67H:1+2+3+4 plus K32M:1+3 in Quad3) showed tighter binding of DHF to enzyme•$NADP^+$ and was sufficiently stable to allow ITC analysis.[7] DHF ternary complex formation displayed a large and clear ΔH signal. We conclude that loss of an ion pair between K32 and the glu tail of DHF does not diminish the observed enthalpy. This observation, in combination with the variation in ΔH observed with the various Q67H mutants, suggests other origins for the enthalpic signal should be considered.

ASA calculations Using the protocol described in Grubbs et al.,[12] the change in accessible surface area (ΔASA) for ligands binding to R67 DHFR was calculated. The ΔASA for $NADP^+$ binding to R67 DHFR using the enzyme•$NADP^+$ structure is -996 $Å^2$.[13] Since the pABA-glu tail of DHF is disordered in the R67 DHFR ternary complex structure, we used a model of protonated DHF bound to R67•NADPH.[14] For ternary complex formation, a ΔASA value of -835 $Å^2$ was calculated. If we assume an area of 9 $Å^2$ for a water molecule,[15] we estimate release of 93-111 water molecules for binding of either ligand to R67 DHFR. These calculations agree with the trend monitored in our present HHP studies, where increasing HHP shifts the equilibrium for both ligands towards the unbound state.

Pressure perturbation calorimetry (PPC) PPC measures the effect of small pulses of pressure on the volume of solutes in solution,[16, 17] and has been used to study the role of water in binding of small molecules to proteins.[18-20] PPC experiments were performed using a VP-Differential Scanning Calorimeter (DSC) with a PPC attachment from MicroCal (Northampton, MA) and a nitrogen tank to supply the pressure. Samples were prepared using stoichiometric amounts of ligand and protein at concentrations such that 90% of the molecules in solution are in the bound state. These conditions enable measurement of the volumetric properties of the bound complex, without large effects arising from free protein or ligand. Typically, concentrations of 1 mM protein-ligand complex were required. Sample was loaded into the sample cell of the DSC and matching MTA buffer (100 mM Tris, 50 mM MES, 50 mM Acetic Acid, pH 7.0) was loaded into the reference cell. For the EcDHFR samples, the MTA buffer also contained 1 mM EDTA and 5 mM β-mercaptoethanol. The samples were equilibrated for 10 minutes at 25 °C prior to starting the experiment. A total of 25 cycles of decompression and compression (0.32-0.34 MPa) were performed with five minutes of equilibration between each cycle. Experiments were repeated at least five times on freshly prepared samples. The heat released or adsorbed, ΔQ, was measured by integrating the change in power with time. The thermal expansion coefficient, α_s^o, was calculated from ΔQ for each sample using equation S1:[16]

$$\alpha_s^o = \alpha_0 - \frac{\Delta Q}{\Delta P T m \overline{V}_s} \quad \text{(S1)}$$

where α_o is the thermal expansion coefficient of the buffer, ΔP is the difference in pressure between the compression and decompression pulses, T is the temperature, m is the mass of solute in the sample, and $\overline{V}_s$ is the partial specific volume of the solute. A buffer-buffer control was performed and the heat evolved was subtracted from the ΔQ of the sample. In order to determine the thermal expansion coefficient of the buffer, α_o, PPC was performed on buffer versus water.[16] Equation S1 was solved for the thermal expansion coefficient of buffer using a value of 2.5705×10^{-4} K^{-1} for the thermal expansion coefficient of water.[21] The molar expansivity, E°, for the solutes was then calculated as:

$$E^o = MW * \overline{V}_s * \alpha_s^o \quad \text{(S2)}$$

where MW is the molecular weight of the solute. Error in E° was calculated from propagation of the experimental error for $\overline{V}_s$ and ΔQ. The difference in molar expansivities between the complex and free protein and ligand species, $\Delta E°$,[18, 22] can be used to estimate the number of waters released upon complex formation, Δn_w. The $\Delta E°$ was calculated by subtracting mean E° values for the free and complex species. The number of waters released is calculated using the below equation:

$$\Delta E^o \approx \Delta n_w (E_h - E_0) \quad \text{(S3)}$$

where $(E_h - E_0)$ is the difference in molar expansivities between water in the hydration layer and bulk solution, respectively. The value of $(E_h - E_0)$ was estimated from the analysis of the hydration of small molecule to be 0.006[23] or 0.01[24] cm^3 mol^{-1} K^{-1}. For the ternary DHFR•$NADP^+$•DHF complexes of EcDHFR and R67 DHFR, $\Delta E°$ was calculated for DHF binding to the DHFR•$NADP^+$ binary complexes.

Bootstrap Analysis A SAS (Cary, NC) program was written to test for mean statistical differences between binary and ternary complexes, using experimental replicates for the error term. This program is given at the end of the Supporting Information document.

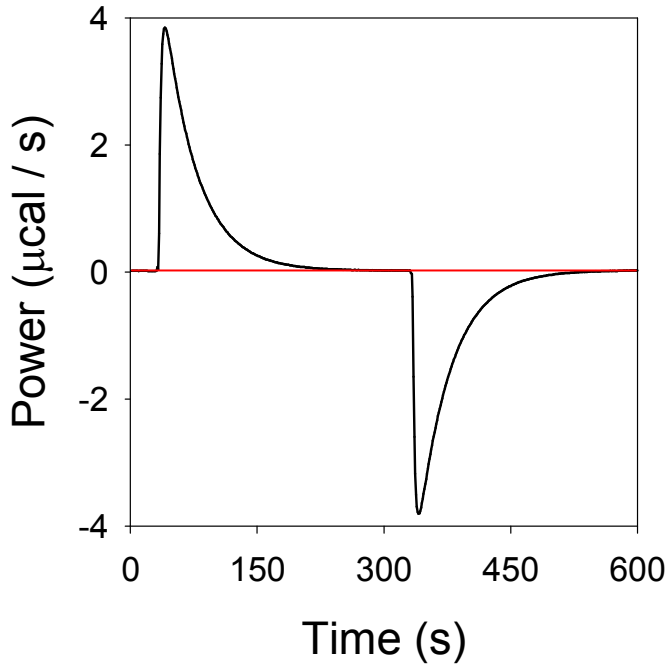
Partial Specific Volume The partial specific volumes for EcDHFR and its ligand complexes were taken from the literature.[25] The partial specific volumes of R67 DHFR and its ligand complexes were obtained from density measurements. Densities were measured using an Anton Paar DMA 35 vibrating tube density meter. The partial specific volume was then determined from equation S4:

$$\overline{V}_s = \left(\frac{1}{C}\right)\left[1 - \frac{\rho - C}{\rho_0}\right] \quad \text{(S4)}$$

where $\overline{V}_s$ is the partial specific volume of the solute, C is the concentration of the solute in mg·mL^{-1}, ρ is the density of the sample solution and ρ_0 is the density of the buffer without solute.[26]

PPC Results Pressure perturbation calorimetry (PPC) was used as a complementary technique to examine the change in the expansivity of the R67 DHFR●ligand complexes. An example of a typical PPC thermogram is given in Figure S7. Volumetric and thermodynamic parameters determined from densitometry and PPC experiments are given in Table S2. However the molar expansivities for R67 DHFR and the two ligand complexes are within error of each other. PPC studies examining the binding of an inhibitor binding to lysozyme and cytidine-2'-phosphate binding to RNAse also noted that the E°'s for the apo and ligand bound forms of the protein were within error.[18] For each protein ligand complex examined, the difference in molar expansivity between the complex and its individual components was calculated. The difference between the ternary complex with the free DHF plus the protein-NADP$^+$ binary complex was used to analyze ternary complex formation. For all the DHFR ligand complexes, ΔE° was negative indicating that the volume of the complexes was smaller than the sum of their component parts. However, as can be noted in Table S2, the errors in the E° values are larger than the ΔE° values themselves. When the error is propagated from the values of E° to the ΔE° values, then the large errors do not allow statistically robust analysis of the data. Therefore, for our conditions, PPC cannot be used to determine the change in hydration between the apo and holo forms of the protein from equation S3. A statistical power analysis found that 500 replicates would be needed to declare ΔE° statistically different from zero, given the replicate variation.

PPC Discussion The PPC data possess an ~1% instrumental error for a single sample. Performing replicate experiments with fresh samples resulted in an independent sampling error of 2-6% (Table S2), depending upon the complex species. While these errors might otherwise be considered adequate, the difference between the molar expansivities of the apo and holo forms of the protein yields values that are indistinguishable due to the error. Bootstrap analysis of the data as per Cameron et al.[18] was used to try and reduce the error. Our bootstrap analysis of the R67 DHFR•NADP$^+$ and R67 DHFR•NADP$^+$•DHF PPC replicates indicates that the molar expansivity values are not sufficiently different to allow us to reach a conclusion about water uptake or release upon ligand binding. Additionally, student t-tests performed on the apo and holo protein data indicate that the two data sets were not sufficiently different to make a conclusion about water uptake or release. Another potential way to improve the error is to analyze the 25 compression/decompression cycles typically performed by PPC on each sample. The data from each cycle could be analyzed individually to increase the total number of data points and improve the error; however, these data points are a measure of the sample (instrumental) error and not an accurate measure of the experimental (replicate) error.



Supplemental Figure S7. Raw PPC thermogram for 1 mM R67 DHFR in MTA buffer, pH 7.0 (solid black line). PPC experiments were performed at 25 °C using ±48 PSI pressure for decompression (left side of the plot) and compression (right side of the plot) pulses. Heat effects from pressure events were measured as the area underneath the curve using a baseline at 0 µcal / s (red line).

Supplemental Table S2. Volumetric and thermodynamic parameters determined from PPC for R67 DHFR and its stoichiometric ligand complexes in MTA buffer, pH 7.0 at 25 °C.

Species	$\overline{V}_s$ ($cm^3 \cdot mol^{-1}$)	ΔQ (μcal)	α_s^o (K^{-1}, x10^3)	E° ($cm^3 \cdot mol^{-1} \cdot K^{-1}$)	ΔE° ($cm^3 \cdot mol^{-1} \cdot K^{-1}$)	Bootstrap analysis[d]
DHF	228±8[a]	-4.3±0.3	1.61±0.08	0.367±0.019	N/A[b]	N/A
$NADP^+$	428±11	-5.3±0.3	1.33±0.06	0.566±0.027	N/A	N/A
R67 DHFR	24200±100	-147±4	0.759±0.007	18.7±0.2	N/A	N/A
R67 DHFR•$NADP^+$	24900±100	-147±4	0.764±0.013	19.0±0.5	-0.24±0.57[c]	-0.02±0.04
R67 DHFR•DHF•$NADP^+$	24800±100	-154±4	0.779±0.012	19.2±0.4	-0.24±0.65[c]	-0.07±0.06

[a] Partial specific volume for folate was used for DHF. [b] Not applicable. [c] Error calculated from the propagation of the experimental error. [d] This value determined from bootstrap analysis (100,000 samples) of the E° values for the apo and holo protein-ligand complexes. This value indicates whether the difference between the two E° values is statistically different. The E° values for the respective ligands were not taken into account in this analysis.

SAS file for bootstrap analysis (many data pts used, only 2 given below to save space)

```
Data one;
input Treat$ value rep;
datalines;
R 18.72 18.71
R+ 19.17 19.08
;

proc mixed data=one;
 class treat rep;
 model value = treat;
 random rep(treat);
 lsmeans treat/diffs;
run;

****** bootstrap one value per rep datasets for permutation test;
%macro bstrap(iter,seed);
ods noresults;
options nonotes;
data results; run;  ***empty dataset;
data pop; set one;
proc sort data=pop; by treat rep;
run;
%do ii=1 %to &iter;
 data sample; set pop end=aa;
 retain seed &seed;
 call ranuni(seed,xx);
 if aa then call symput('seed',seed);
 run;
 proc sort data=sample; by treat rep xx;
 data sample; set sample; by treat rep ;
   if first.rep;
   iter=&ii;
 run;
 proc means data=sample noprint; by iter treat;
  var value;
  output out=mmm mean= mv;
 run;
 data results; set results mmm;
  if iter=. then delete;
 run;
 %end;
 ods results;
 options notes;
%mend;

%bstrap(100,3478);
proc sgplot data=results;
 vbar mv / group=treat;
run;

data diffs; set results; by iter;
retain mean1;
 if first.iter then mean1=mv;
 if last.iter then do; diff=mean1-mv;
  if -0.07145<diff<0.07145  then pcntextreme=0; else pcntextreme=100;
```

```
  output; end;
run;
proc means data=diffs n mean std min max p95 p5;
 var diff pcntextreme;
run;
```

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