

Supplementary Information

Experimental details:

Expression and purification of I14A DHFR: Plasmid construction, protein expression, purification and storage of I14A DHFR was performed as described elsewhere.¹

Competitive KIEs: All experiments were performed in MTEN buffer (50mM MES, 25mM Tris, 25mM EtOH-NH₂ and 100mM NaCl) at pH8.0, 4 μ M NADPH, and a 200-fold excess of H₂folate (final concentration of 0.85 mM). The pH was adjusted at each experimental temperature, after the electrode has been calibrated in appropriate buffers at that temperature. These conditions are identical to those used in previous kinetic studies of *wt*DHFR and its mutants.²⁻⁵ Reactions were quenched with an excess of methotrexate ($K_d=1$ nM). The irreversibility of the H-transfer was assured by performing the reaction under aerobic conditions, and bubbling oxygen through the reaction after quenching to insure the rapid oxidation of H₄folate. Samples were stored at -80C prior to HPLC analysis. The reactions were quenched at various time points and after completion, and then analyzed to assess the ratio of tritium to ¹⁴C in the product to obtain the observed V/K KIE.

Table 1S: Observed V/K KIEs and their standard errors.

Temp. °C	Observed D/T KIE	Observed H/T KIE
45	1.81 ±0.01	5.94 ±0.03
35	1.82 ±0.01	5.95 ±0.03
25	1.82 ±0.01	5.95 ±0.01
15	1.83 ±0.01	5.96 ±0.03
5	1.84 ±0.02	5.98 ±0.03

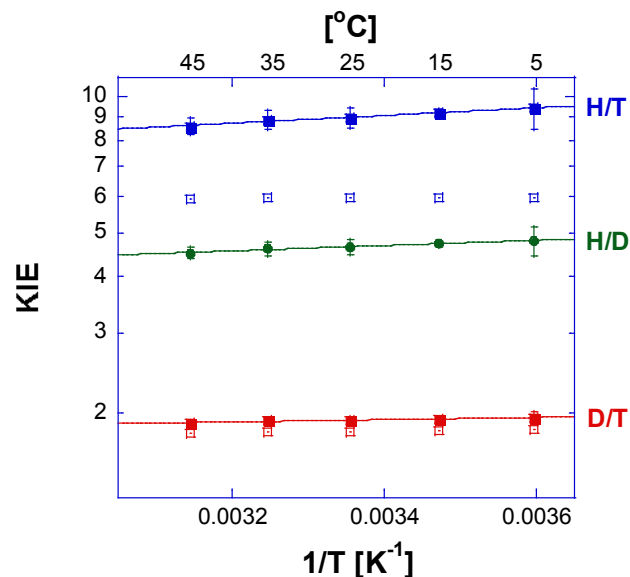
Data analysis

Calculation of Intrinsic KIEs and Curve fitting: The Northrop equation for intrinsic H/T, H/D and D/T KIEs was solved numerically using a program presented elsewhere,⁵ and the tool we posted on <http://cricket.chem.uiowa.edu/~wang11/temp/intrin.html>. Figure 1 and Figure 1S present the average values of the KIEs and the standard deviations of intrinsic values calculated from the observed values, while the lines and the fitted parameters are from nonlinear regression using all the experimental points as described in ref 5. In short, Figure 1 in the communication, and Figure 1S here, present KIEs on a logarithmic scale vs. the reciprocal of the absolute temperature. Curve fitting was carried out as a least root-mean-square fit exponential regression for proper error analysis, yielding the isotope effects on the preexponential factor and on the energy of activation of the Arrhenius equation.

Table 2S: Intrinsic KIEs and their standard errors.

Temp. °C	H/T KIE	H/D KIE	D/T KIE
45	8.59 ±0.15	4.51 ±0.09	1.90 ±0.01
35	8.87 ±0.17	4.61 ±0.06	1.92 ±0.01
25	8.97 ±0.14	4.65 ±0.05	1.93 ±0.01
15	9.18 ±0.09	4.73 ±0.03	1.94 ±0.01
5	9.44 ±0.34	4.81 ±0.13	1.96 ±0.02

Figure 1S: Arrhenius plot of observed (open structures) and intrinsic (closed structures) 1° KIEs for I14A DHFR



Molecular simulation

System Setup

The simulations were performed starting from the crystal structure of wild type DHFR, determined at 1.8 Å resolution (PDB entry 1RX2.pdb). The crystal structure contains a total of 159 amino acid residues, 153 crystallographic water molecules, and Folate and NADP⁺ ligands. Missing hydrogens were added to the PDB structure using the leap module of Amber package.^{6,7} Folate and NADP⁺ ligands were replaced by N5 protonated 7,8-dihydrofolate (H₂folate) and NADPH respectively. Protonation state for all ionizable residues were set corresponding to pH 7. Thus, histidines residues were modeled as neutral residues with the proton on Nε or Nδ as determined on the basis of possible hydrogen bond interactions deduced from X-ray crystallographic structure. Only His-45 was modeled charged based on both NMR⁸ and crystallographic⁹ studies which indicate salt bridge formation between His-45 and the nicotinamide 5'-phosphate of NADP⁺. Crystallographic studies have also establish the existence of a salt bridge between Arg-44 and Adenine 2'-phosphate.¹⁰ The estimated pK_a for His-45 determined by PropPKa¹¹ was 8.83 in accordance with experimental observation. The resulting system has a net charged of -15e including N5-protonated- H₂folate (-1) and NADPH(-4).¹² The final protein structure was solvated with a previously equilibrated 9 Å truncated octahedron box of TIP3P water molecules. Overlapping solvent molecules were removed leaving 5879 water molecules. Mutations were performed in silico by changing the corresponding amino acid in the original structure.

MD Simulation Parameters

All simulations were done at 300 K and 1 bar, regulated with the Berendsen barostat and thermostat,¹³ using periodic boundary conditions and Ewald sums for treating long range electrostatic interactions.^{14,15} The SHAKE algorithm¹⁶ was used to keep bonds involving hydrogen atoms at their equilibrium length. A 2 fs time step for the integration of Newton's equations was used. The nonbonded cutoff radius of 12 Å was used. The AMBER99 force field¹⁷ parameters were used for all residues. The NADP⁺ and NADPH parameters were taken from the Amber parameter data base.¹⁸ Gaff force field¹⁹ parameters together with RESP²⁰ charges calculation at the HF/6-31G* level were used to generate the parameter files for H₂folate.

MD runs

All simulations were run with the PMEMD module of the AMBER9 package.^{6,7} An equilibration protocol was applied that consists in performing an energy minimization by optimizing the initial structure, followed by a slow heating to the desired temperature using a linear temperature ramp from 100 to 300 K during 80 ps at constant volume and a subsequent pressure stabilization run at 300 K and 1 bar during 100 ps. Position frames, which were used for analyzing trajectories, were collected at 2 ps intervals. Production MD simulations consisted of 15 ns simulations: (i) wtDHFR- H₂folate -NADPH; (ii) I14ADHFR- H₂folate -NADPH.

Figure 2S: Time evolution of RMSD between the average simulated structure and trajectory snapshots (red) and between crystal structure and trajectory snapshots (black) for ternary complex: wtDHFR- H₂folate -NADPH (PDB:1rx2).

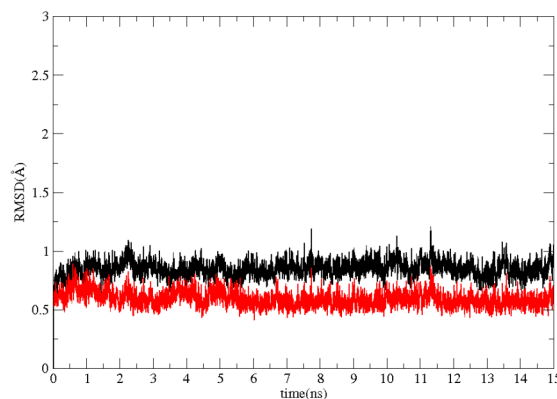
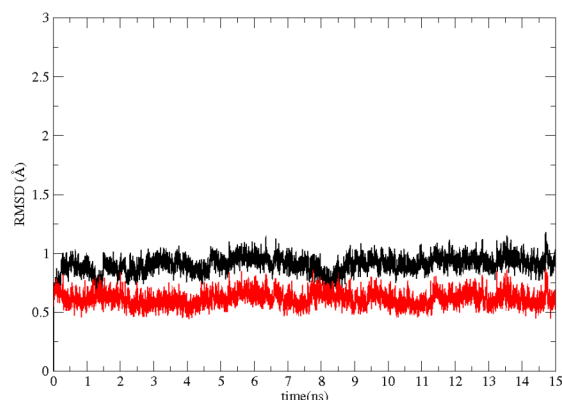


Figure 3S: Time evolution of RMSD between the average simulated structure and trajectory snapshots (red) and between crystal structure of *in silico* mutant I14A DHFR and trajectory snapshots (black) for ternary complex: I14ADHFR- H₂folate -NADPH.



The DAD, however, adopts two main populations (Figure S4), one that is similar to that of the wild type and one that is almost an angstrom longer. The later conformation involves a short distance between the nicotinamide ring (the H-donor) and the β -carbon of A14 (Figure S5). As mentioned in the main text, in the first conformation (wt-like) one or even two water molecules enter the active site filling the void between the nicotinamide ring and A14.

Figure 4S: Time evolution of DAD distance for wtDHFR (red) and I14A DHFR (blue) during the 15ns simulation.

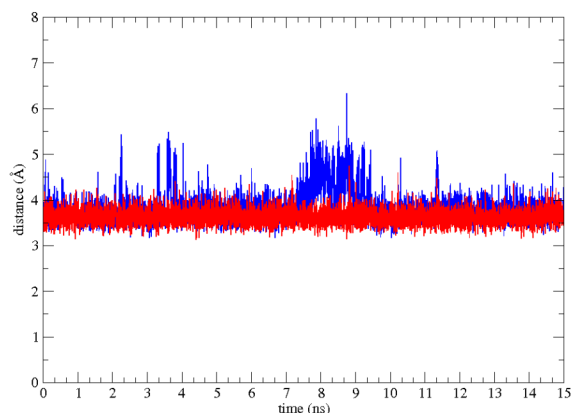
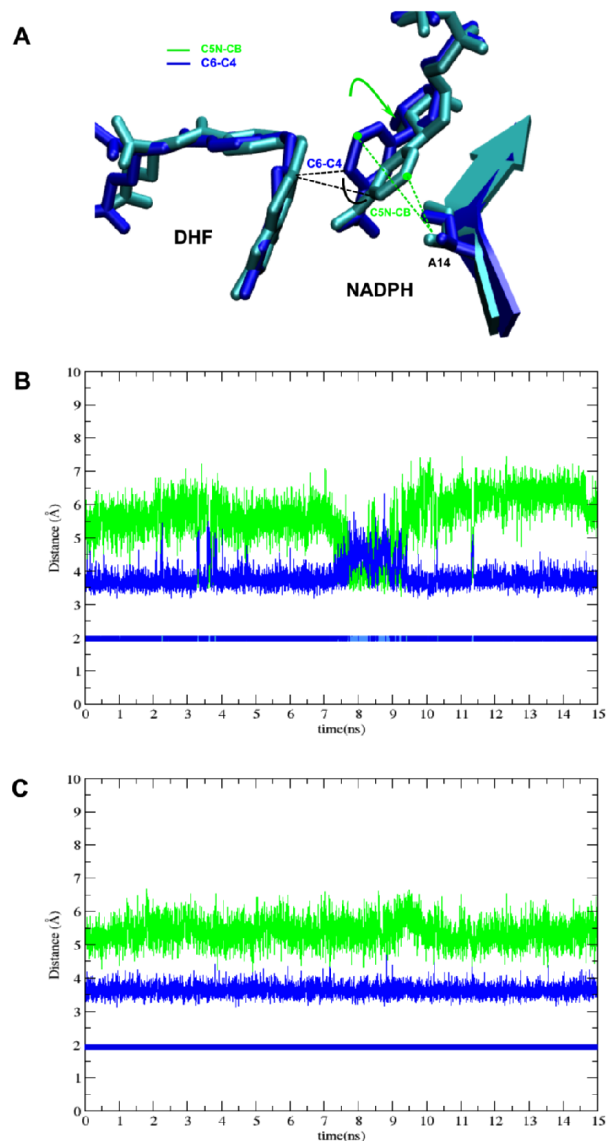


Figure 5S: A) View of the two active site conformations of I14A DHFR. The two overlapping structures indicate the wt-like conformation (dark blue) and the conformation with the largest DAD (light blue). Panels B and C present the time evolution of DAD distance (blue) and C5N-C β distance (green) for I14A DHFR (B) and wtDHFR (C), respectively. Straight line indicates the occurrences where C5N-C β distance becomes shorter than DAD (light blue regions). Unlike in the case of wtDHFR, where nicotinamide ring is held relatively stiffly in the close proximity to pterin ring, in I14A DHFR nicotinamide ring shows more fluctuations, falling towards the hydrophobic residue 14 number of times during the 15ns simulation.



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