CHEMBIOCHEM

Supporting Information

DOI: 10.1002/cbic.200900451

Supporting Information

for

Effect of Electrostatic Shielding on H-Tunneling in R67 Dihydrofolate Reductase

Atsushi Yahashiri, Guy Nimrod, Nir Ben-Tal, Elizabeth E. Howell, and Amnon Kohen*

Data Tables

Table S1.* Steady-state kinetic values for R67 DHFR in TE buffer in the presence of NaCl.[a,b]

[NaCl]	$K_{m(\mathrm{NADPH})}$	$K_{m(\mathrm{DHF})}$	k_H
0.13 M (μ = 0.15)	8.7 ± 0.4	13 ± 0.7	1.5 ± 0.1
0.20 M (μ = 0.22)	13 ± 0.8	29 ± 1.8	2.0 ± 0.1
0.30 M (μ = 0.32)	26 ± 1.3	54 ± 2.5	2.8 ± 0.1
0.40 M (μ = 0.42)	41 ± 2.3	81 ± 4.3	3.7 ± 0.2

[a] TE buffer contains 10 mM Tris and 1 mM EDTA. [b] Kinetic parameters from ref. [1]

* All the reported errors represent 1σ standard deviation from at least 5 replicates.

Table S2.* KIEs determined at various temperatures. D/T KIEs were only measured at 25 ˚C and extreme temperatures to establish that the KIEs are within Swain-Schaad relationship, so the chemistry is rate limiting throughout the temperature range.**

Ionic strength	μ = 0.42		μ = 0.15		
T [℃]	H/T KIE[a]	H/D KIE[a]	H/T KIE[b]	D/T KIT[b]	H/D KIE[c]
5	6.24 ± 0.08	3.55 ± 0.04	6.53 ± 0.09	1.76 ± 0.03	
15	6.02 ± 0.08		6.36 ± 0.07		
25	5.73 ± 0.10	3.40 ± 0.03	6.04 ± 0.11	1.73 ± 0.01	3.52 ± 0.04
35	5.41 ± 0.09		5.56 ± 0.16		
45	5.21 ± 0.06	3.14 ± 0.04	5.47 ± 0.06	1.68 ± 0.02	

[a] This work, [b] ref. [2], and [c] ref. [3]

* All the reported errors represent 1s standard deviation from at least 5 replicates.

** The observed KIE in this case (competitive experiment) is on the second order rate constant, denoted as ${}^{D}(V/K)$ and ${}^{T}(V/K)$ for H/D and H/T, respectively. Its relation to the intrinsic KIE follows:

$$ {}^{X}(V/K) = \frac{{}^{X}k + C}{1 + C} $$

where ${}^{X}k$ is the intrinsic KIE, X is D or T, and C the commitment for catalysis, representing the kinetic complexity (this is a simplified term but the full term will not change the trend in question). For all normal KIEs, the intrinsic H/D KIE is smaller than the intrinsic H/T KIE (${}^{D}k < {}^{T}k$). Since C is the ratio between the isotopically sensitive rate of H-transfer in one direction to the isotopically nonsensitive steps in the opposite direction, it is the same for the observed H/D and H/T. Consequently, the observed H/D will be deflated by the commitment (relative to its intrinsic value) more than H/T, and the observed Swain-Schaad relations (also denoted the Swain-Schaad exponent) will be inflated:

$$ \frac{\ln^{T}(V/K)}{\ln^{D}(V/K)} > \frac{\ln^{T}k}{\ln^{D}k} = 1.44\,. $$

As discussed in many books and reviews from the 1970s up through ref 2, this is a very sensitive probe as it can range from an intrinsic value of 1.44 up to ∞ due to commitment (in contrast to H/T vs. D/T that only varies between an intrinsic value of 3.3 to unity due to commitment).

Table S3.* Comparison of the isotope effect on the Arrhenius pre-exponential factors in accordance with the differences in k_{cat} relative to the wild-type chromosomal DHFR.

	R67 DHFR (Ionic strength)	
	μ = 0.42 M	μ = 0.15 M[a]
A_H/A_T	1.49 ± 0.08	1.36 ± 0.07
$\Delta\Delta E_{aA\text{-}B}$ [kcal/mol]	0.80 ± 0.03	0.87 ± 0.03
fold in k_H[b]	2.5	1

[a] ref.[2]. [b] Fold in k_H is relative to k_H at μ = 0.15 M.

* All the reported errors represent 1σ standard deviation from at least 5 replicates.

Experimental Section

Materials: Reagent-grade chemicals were used as purchased unless specified otherwise. β-Nicotinamide [Ad-^{14}C]adenine dinucleotide (267 mCi/mmol) ([Ad-^{14}C] NAD$^+$), and β-nicotinamide [2,5',8-^{3}H]adenine dinucleotide (25.0 Ci/mmol) ([Ad-^{3}H] NAD$^+$) were purchased from Amersham Pharmacia. Glucose dehydrogenase from *Bacillus megaterium* (*Bm*GDH) was purchased from USB Corporation. Phosphate and Tris base were purchased from Fisher. Congo Red was obtained from Aldrich. Ultima Gold liquid scintillation cocktail was purchased from PerkinElmer. 7,8-Dihydrofolate (DHF) was prepared by dithionite reduction of folic acid as described by Blakley.[4] All other materials were purchased from Sigma.

Methods

Calculation of the difference in electrostatic potentials on R67 DHFR. In order to produce a reliable image of the electrostatic potential on the surface of the protein scaffold, missing atoms were constructed using UCSF Chimera.[5] Tyr 69 in chain D was built by structural alignment with chain B that, among the other chains in the structure, has the least root mean square deviation (RMSD) from the Cα atoms of chain D.

The *p*-aminobenzoyl moiety of the DHF molecule (for which no electron density is defined in the crystal structure) was reconstructed as follows. Using Ligand Expo,[6] PDB entries with DHF molecules were searched, and each of the molecules in these entries was superimposed on the atoms available for the DHF molecule in the

original structure. Out of these alignments, we selected the one which formed the minimum number of atom clashes with R67 DHFR. Finally, we rotated the torsion angle defined by C6-C9-N10-C14, in order to avoid any atom clashes. Out of the resulting conformations, the one with the highest LigScore[7] value was selected using the Accelrys Discovery Studio package (v2.1).

Both the $NADP^+$ and the DHF molecules are mostly buried within the R67 DHFR pore and participate in hydrogen bonds with protein residues. Therefore, we assumed that the titratable groups of the ligands changed their protonation state and are neutral (but polar). Assuming a replacement of $NADP^+$ with NADPH does not induce a conformational change in R67 DHFR, we examined the effect of this replacement on the electrostatics of the complex. To this end, we repeated the calculations when the nicotinamide in $NADP^+$ is represented as in NADPH. However, the differences in the electrostatics of the complexes were negligible (data not shown).

The electrostatic potential on the protein surface was calculate by assigning partial charges and radii to each atom in the structure using the PDB2PQR server[8] and the AMBER force field[9]. The electrostatic potential was calculated with the Adaptive Poisson-Boltzmann Solver[10] using the Python molecular viewer[11]. The calculations were conducted with the nonlinear Poisson Boltzmann equation at low and high salt concentrations (μ = 0.15 and 0.42 M, respectively).

Enzyme Preparation. R67 DHFR was expressed, purified and lyophilized as described elsewhere.[12]

Synthesis of Labeled Cofactors for KIEs. [Ad-^{14}C]NADPH, (*R*)-[4-^{3}H]-NADPH, and (*R*)-[4-^{2}H;Ad-^{3}H]-NADPH were synthesized as described elsewhere[4, 14, 15]. Briefly, [Ad-^{14}C]NAD^+ was phosphorylated with NAD^+ kinase in creatine recycling system, and stereospecifically reduced at 4-pro*S* position on nicotinamide with *Bm*GDH in the presence of glucose. Similarly, (*R*)-[4-^{2}H;Ad-^{3}H]-NADPH was prepared with phosphorylation followed by 4*R* stereospecific reduction of $NADP^+$ with [D_8]propan-2-ol (>99.7% D at C2 as determined by ^{1}H NMR) using alcohol dehydrogenase from *Thermoanaerobium brockii*. All synthesized cofactors were purified by reversed-phase HPLC on a Supelco Discovery C18 column as described previously and lyophilized for long-term storage at -80 ℃ [13, 14].

Co-purification of isotopically labeled NADPH for Competitive KIE Experiments. [Ad-^{14}C]NADPH and (*R*)-[4-^{3}H]- NADPH (H/T experiments) were combined at a radio-

activity in DPM ratio close to 1:6 ($^{14}C/^{3}H$), or [Ad-^{14}C]NADPH and (*R*)-[4-^{2}H;Ad-^{3}H]-NADPH (H/D experiments) were combined at a ratio close to 1:4 to compensate for the lower efficiency of tritium scintillation counting. Each of the mixtures was copurified by reverse-phase HPLC on a Supelco Discovery C18 column (25 cm x 4.6 mm, 5 µL), divided into aliquots containing 360 000 DPM of ^{14}C, and flash frozen for short-term storage (up to 4 weeks) at -80 ℃.

Competitive H/D and H/T KIEs. All experiments at high ionic strength were conducted in TE buffer (10 mM Tris, 1 mM EDTA, and 400 mM NaCl) at pH 8.0. Since the reported KIEs at physiological ionic strength were determined in MTEN buffer (μ = 0.15 M), [15, 16] as control experiments, the effects of buffer components were also examined, but they did not affect intrinsic KIE values.

In each competitive KIE experiment, one aliquot of the copurified labeled NADPH was thawed immediately before use. DHF was added to a final concentration of 200 µM, approximately 50-fold excess over 4 µM of NADPH. The reaction was initiated by the addition of R67 DHFR enzyme solution, and incubated for up to 40 min. At various time points, 80 µL aliquots, which contained varied fractional conversions (*f*: equation 1) ranging from 15 to 85% as determined from the distribution of ^{14}C between NADPH and $NADP^+$, were withdrawn and quenched with 20 µL of Congo Red (final concentration of 5 mM). To ascertain complete fractional conversion, R67 DHFR was added to the residue of the mixture and two aliquots for t_∞ samples were quenched after 20 min of additional incubation after the completion of the NADPH consumption. All quenched samples were immediately frozen and stored at -80 °C.

Prior to the HPLC analysis, the sample was thawed, and oxygen was gently bubbled into the reaction mixture for 15 min to convert THF into its oxidized form.[14, 17] Aliquots were then injected onto an HPLC equipped with a Supelco reversed-phase C18 column, and the eluent was analyzed by Packard flo scintillation analyzer, or Liquid scintillation counter (LSC).[14] The detailed HPLC analysis followed refs. [13, 14]. In short, prior to HPLC-LSC analysis, the samples were thawed and oxidized through bubbling of oxygen for 15 min at room temperature. The samples were then injected into the reverse-phase HPLC system. Fractions (0.8 mL) were collected, mixed with 10 mL of Ultima Gold liquid scintillation cocktail (PerkinElmer), and stored in the dark for 24 h before radioactivity determination by LSC analysis (Packard Tri-

carb Tr2900 LSC). The fractional conversion (*f*) of NADPH was determined from the ratio of ^{14}C in the product to the total amount of ^{14}C.

$$f = \frac{[\text{Ad-}^{14}\text{C}]\text{NADP}^+}{[\text{Ad-}^{14}\text{C}]\text{NADP}^+ + [\text{Ad-}^{14}\text{C}]\text{NADPH}} \quad (1)$$

The observed KIEs were calculated according to the following equation.[18]

$$KIE = \frac{\ln(1-f)}{\ln\left[1-f\left(\frac{R_t}{R_\infty}\right)\right]} \quad (2)$$

where R_t is the ratio of ^3H to ^{14}C in products at various fractional conversions, and R_∞ is ratio at complete conversion from t_∞ samples. Experiments at one temperature point were performed at least in duplicate.

Those intrinsic KIEs were fitted to the Arrhenius equation for KIEs, and the isotope effects on the activation parameters were calculated using KaleidaGraph.

$$\frac{k_l}{k_h} = \frac{A_l}{A_h} e^{\frac{\Delta E_{a_{h-l}}}{RT}} \quad (3)$$

where *l* and *h* are the light and heavy isotopes. A_l/A_h and dEa_{h-l} are the isotope effect on the preexponential Arrhenius factors and the difference in activation energy between *l* and *h*, respectively.

References

[1] S. N. Hicks, Dissertation thesis, The University of Tennessee Knoxville (Knoxville, TN), **2003**.

[2] A. Yahashiri, E. E. Howell, A. Kohen, *ChemPhysChem* **2008**, *9*, 980-982.

[3] A. Yahashiri, Sen A. and Kohen A., *J. Label. Compd. Radiopharm.* **2009**, *Available on-line as Early View, July 29, 2009), DOI: 10.1002/jlcr.1660*.

[4] R. L. Blakley, *Nature* **1960**, *188*, 231-232.

[5] E. C. Meng, E. F. Pettersen, G. S. Couch, C. C. Huang, T. E. Ferrin, *BMC Bioinformatics* **2006**, *7*, 339.

[6] Z. Feng, L. Chen, H. Maddula, O. Akcan, R. Oughtred, H. M. Berman, J. Westbrook, *Bioinformatics* **2004**, *20*, 2153-5. Epub 2004 Apr 1.

[7] A. Krammer, P. D. Kirchhoff, X. Jiang, C. M. Venkatachalam, M. Waldman, *J Mol Graph Model* **2005**, *23*, 395-407. Epub 2004 Dec 25.

[8] T. J. Dolinsky, J. E. Nielsen, J. A. McCammon, N. A. Baker, *Nucleic Acids Res.* **2004**, *32*, W665-7.

[9] P. C. Junmei Wang, Peter A. Kollman,, *Journal of Computational Chemistry* **2000**, *21*, 1049-1074.

[10] N. A. Baker, D. Sept, S. Joseph, M. J. Holst, J. A. McCammon, *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 10037-41.

[11] M. F. Sanner, *J. Mol. Graph. Model.* **1999**, *17*, 57-61.

[12] H. Park, P. Zhuang, R. Nichols, E. E. Howell, *J. Biol. Chem.* **1997**, *272*, 2252-2258.

[13] K. A. Markham, A. Kohen, *Curr. Anal. Chem.* **2006**, *2*, 379-388.

[14] K. A. Markham, R. S. Sikorski, A. Kohen, *Anal. Biochem.* **2003**, *322*, 26-32.

[15] K. J. Ellis, J. F. Morrison, L. P. Daniel, *Methods Enzymol.* **1982**, *87*, 405-426.

[16] S. R. Stone, J. F. Morrison, *Biochemistry* **2002**, *23*, 2753-2758.

[17] L. Wang, S. Tharp, T. Selzer, S. J. Benkovic, A. Kohen, *Biochemistry* **2006**, *45*, 1383-1392.

[18] L. Melander, W. H. Saunders, *Reaction Rates of Isotopic Molecules*, 4th ed., Krieger, R.E., Malabar, FL, **1987**.