Supporting Material for:

Triple-isotopic labeling and kinetic isotope effects: Exposing H-transfer steps in enzymatic systems

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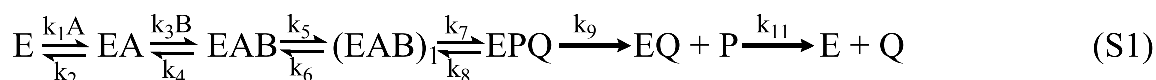
S1. Impact of kinetic complexity on KIE_{obs} for an enzymatic reaction

When the isotopically sensitive step is not rate limiting, the measured KIE will not be truly representative of the H-transfer reaction of interest, a phenomenon called kinetic complexity. Enzymatic reaction mechanisms provide many examples of such kinetically complex mechanisms, which generally involve several steps. A simple example is presented in ref (*1*): Consider the following enzymatic reaction where the isotopic label is on substrate B:



$$E \underset{k_2}{\overset{k_1 A}{\rightleftharpoons}} EA \underset{k_4}{\overset{k_3 B}{\rightleftharpoons}} EAB \underset{k_6}{\overset{k_5}{\rightleftharpoons}} (EAB)_1 \underset{k_8}{\overset{k_7}{\rightleftharpoons}} EPQ \xrightarrow{k_9} EQ + P \xrightarrow{k_{11}} E + Q \qquad (S1)$$

Here, the isotopically-sensitive step forward is represented by the rate constant k_7 (chemical conversion step). The intrinsic KIE is $k_{7(L)}/k_{7(H)}$, and as apparent from Eq. 2 in the text, the observed KIE (KIE_{obs}) is 'masked'. For the reaction shown in equation S1, C_f and C_r are the commitments to catalysis, and are given by

$$C_f = \frac{k_7}{k_6} + \frac{k_5 k_7}{k_4 k_6}$$

$$C_r = \frac{k_8}{k_9}$$

For the mechanism given in eq. S1, the *EIE* is simply $K_{eq(L)}/K_{eq(H)}$ where $K_{eq} = k_7/k_8$. For C-H-C transfer from sp^3 carbon in the reactant to sp^3 carbon product this value is not likely to be very different than unity. For C_r to be close enough to zero to satisfy the Northrop assumption, the reverse chemistry (k_8) needs to be at least 100 folds slower than the dissociation of the first product (k_9).

S2. Data analysis:

All data analysis was conducted following statistical techniques previously described in ref. 1; hence only an abridged description of the full data analysis is provided below.

a. Calculation of intrinsic KIEs

Observed H/D, H/T and D/T KIEs were measured independently; the results are shown in Table S1. The numerical evaluation of the intrinsic H/T KIE *via* the Northrop equations 3 and 5 (in the main text) were carried out using the Java-based program we post online at http://cricket.chem.uiowa.edu/~kohen/tools.html under Intrinsic KIEs. This program allows calculation of intrinsic KIEs when H and T are used as references, and the calculated values are in perfect agreement with the intrinsic values calculated using simple Mathematica scripts for equations 3 and 5. Calculation of the intrinsic H/T KIE using equation 4 (i.e. using D as reference isotope) was performed using Mathematica. Note that equations 3-5 use Swain-Schaad exponents of 1.427 and 3.34, calculated based on reduced-mass considerations as per ref (*2*). For all calculations, the average of the observed KIEs were used to calculate the intrinsic KIE value.

b. Calculation of errors on intrinsic KIEs

Since the Northrop equations contain transcendental functions, they must be solved numerically rather than analytically; the error propagation associated with such solutions is non-trivial. The procedure that follows describes the propagation of error on an intrinsic H/T KIE using observed H/D and H/T KIEs, but the same general method is used for all combinations of observed values. As described in more details in Ref (*3*), the individual observed values from several measurements where used to numerically calculate intrinsic values using Northrop equation (equations 3-5 in the main text). The intrinsic H/T KIEs so obtained were used to calculate the error on the average intrinsic H/T KIE calculated from the average observed values as described in the previous item. The procedure described in ref (*3*) assures that the number of different combinations of observed H/T and H/D does not surpass the number of experimental data points,.

Table S1. Observed KIEs using H, D and T as reference isotopes.

Enzyme /substrate	$^{T}(V/K)_{H}$	$^{D}(V/K)_{H}$	$^{T}(V/K)_{D}$
cDHFR/H_2F	4.90	2.89	1.65
	4.87	2.89	1.68
	4.87	2.96	1.66
	4.86	2.95	1.69
	4.84	2.92	1.65
	4.82	2.93	1.65
	4.81	2.89	1.69
	4.78	2.95	1.64

Table S1 Cont.

Enzyme /substrate	$^{T}(V/K)_H$	$^{D}(V/K)_H$	$^{T}(V/K)_D$
cDHFR/H_2B	7.20	3.54	1.98
	7.19	3.55	1.99
	7.10	3.56	1.99
	7.10	3.56	2.03
	6.97	3.54	2.02
R67-DHFR/H_2F	6.16	3.48	1.74
	6.07	3.54	1.76
	6.07	3.56	1.72
	6.05	3.50	1.74
	6.05	3.53	1.75
	6.01	3.53	1.72
	6.00	3.52	1.72
	5.99	3.29	1.75
	5.92	3.48	1.73

References

1. Cook, P. F., and Cleland, W. W. (2007) *Enzyme kinetics and mechanism*, Garland Science, New York.

2. Streitwieser, A., Jagow, R. H., Fahey, R. C., and Suzuki, S. (1958) Kinetic isotope effects in the acetolyses of deuterated cyclopentyl tosylates, *J. Am. Chem. Soc. 80*, 2326-2332.

3. Wang, L., Tharp, S., Selzer, T., Benkovic, S. J., and Kohen, A. (2006) Effects of a distal mutation on active site chemistry, *Biochemistry 45*, 1383-1392.