Supplementary Information

A Remote Mutation Affects the Hydride Transfer by Disrupting Concerted Protein Motions in Thymidylate Synthase

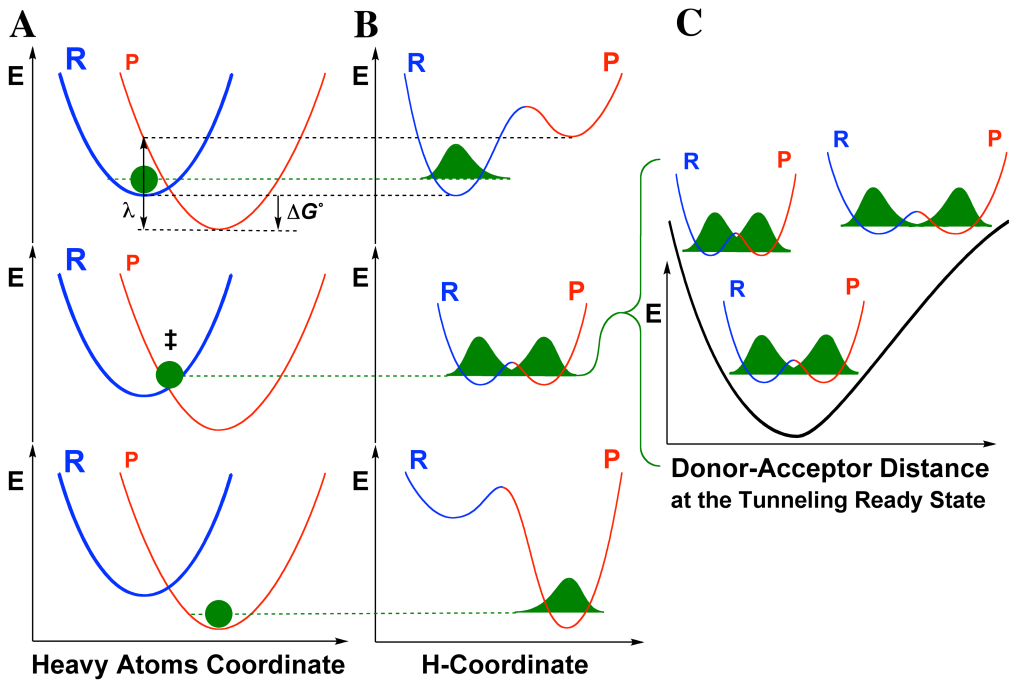
Zhen Wang,[1,§] Thelma Abeysinghe,[1,§] Janet S. Finer-Moore,[2] Robert M. Stroud,[2] and Amnon Kohen[1,*]

[1] Department of Chemistry, University of Iowa, Iowa City, IA 52242-1727, USA
[2] Department of Biochemistry & Biophysics, University of California, San Francisco, CA 94158-2517, USA

* Author to whom correspondences should be addressed. E-mail: amnon-kohen@uiowa.edu Phone #: 319-335-0234
[§] These authors equally contribute to this work.

Table of Content:

1. **Figure S1.** Marcus-like model for H-transfer reactions. As an extension of the Marcus theory for electron transfer,[1] this model makes a Born-Oppenheimer-like separation between the fast motions of the transferring hydrogen (on H-Coordinate, B) and the slower motions of the rest of the system (on Heavy Atoms Coordinate, A).[2,3] Enzyme motions could contribute to an H-transfer reaction by affecting pre- and re-organization of the protein environment, as well as the donor-acceptor distances (DADs) at the tunneling ready state (TRS). (A) The "preorganization" generates the diabatic states of the reactants (R, blue) and products (P, red) on the heavy atoms coordinate. The heavy atom "reorganization" brings the system (green) from the reactant state (top) to the TRS (middle, denoted by "‡"), and finally to the product state (bottom). The rate of reaching the TRS depends on the reorganization energy (λ) and the reaction's driving force (ΔG°). (B) The double-well potential for H-tunneling is modulated by the heavy atom motions throughout the reaction. At a given donor acceptor distance (DAD), the tunneling probability of H-nucleus is proportional to the overlap ($F(m, DAD)$) between the hydrogen wavefunctions (green) at the TRS. (C) The heavy atom motions also generate a range of possible DADs following the Boltzmann distribution at the TRS.[4] The thermal fluctuation of DADs is the source for the temperature dependence of KIEs on the H-transfer. Reproduced with permission from Springer.[5]



2. Analysis of products and by-products in the TSase reaction. RP-HPLC separation for the analysis of reactions catalyzed by wild type (WT) and Y209W *E. coli* TSase (ecTSase) in the presence of reactive thiols (Scheme 2 in the main text). We used an Agilent Technologies model 1100 HPLC system with a Supelco Discovery® C18 reverse phase column.

HPLC mobile phase solutions:

A = 20 mM KH_2PO_4/H_3PO_4 buffer (pH = 5.0)
B = 40% methanol, 60% A
C = methanol

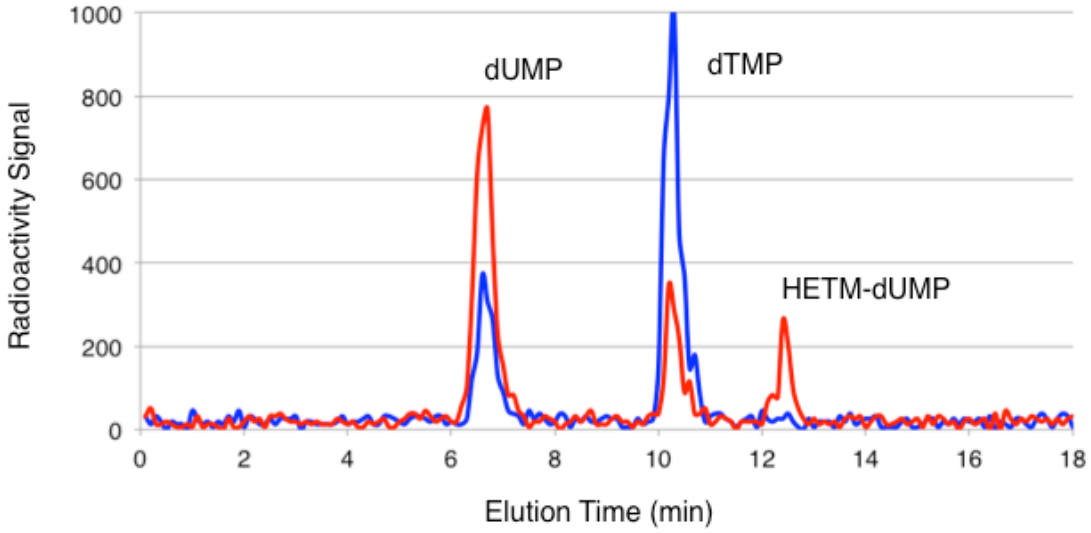
Table S1. HPLC mobile phase gradient (flow rate 0.8 ml/min):

t (min)	% A	% B	% C
0	100	0	0
8	70	30	0
14	0	0	100
17	0	0	100

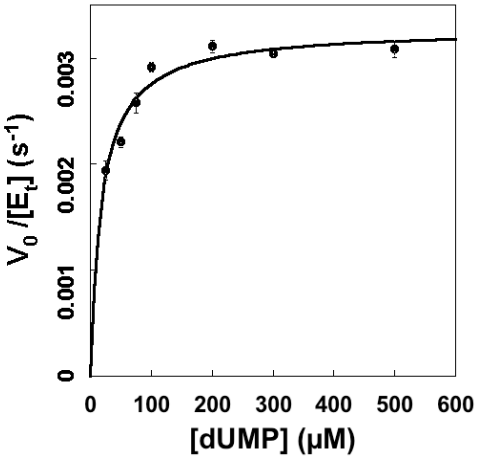
Table S2. Retention time (min) of the by-products found for the Y209W-catalyzed reaction in the presence of different thiols.

Thiol reagent	dUMP	dTMP	thiol-trapped Intermediate
β-mercaptoethanol	6.8	10.2	12.4
DTT			14.0
3-mercapto-1-propanol			14.2
1-pentanethiol			14.2
Cysteine			N/A

Figure S2. Representative radiograms of the reaction mixtures of WT (blue) and Y209W (red) ecTSase in the presence of β-mercaptoethanol. The third peak indicates formation of 5-(2-hydroxyethyl)thiomethyl-dUMP (HETM-dUMP, see Scheme 2 in main text) in the reaction mixture of the mutant..



3. **Figure S3**. Steady state initial velocities of Y209W measured with 50 μM CH_2H_4folate and varying concentrations of dUMP at 25 ˚C.



4. **Table S3**. Steady-state kinetic parameters (Eq. 1 in main text) of Y209W ecTSase at different temperatures.

	k_{cat} (s^{-1})	$K_m^{CH_2H_4folate}$ (mM)	$\frac{k_{cat}}{K_m^{CH_2H_4folate}}$ (s^{-1} mM^{-1})	$K_I^{CH_2H_4folate}$ (mM^2)
5 °C	0.008 ± 0.003	0.6 ± 0.3	0.013± 0.008	0.5± 0.2
15 °C	0.013 ± 0.003	0.3 ± 0.1	0.04 ± 0.02	1.2 ± 0.5
25 °C	0.020 ± 0.001	0.21 ± 0.03	0.10 ± 0.01	2.8 ± 0.4
35 °C	0.11 ± 0.02	0.8 ± 0.2	0.14 ± 0.04	2.9 ± 0.8

5. **Table S4**. KIE on the hydride transfer catalyzed by Y209W (Figure 3 in the main text).

Temperature °C	Observed KIE		$SSE_{obs.}$ [a]
	H/T KIE	D/T KIE	
5	8.5 ± 0.1	1.90 ± 0.02	3.32 ± 0.06
15[b]	7.68 ± 0.04		
25	7.77 ± 0.04	1.85 ± 0.01	3.33 ± 0.03
35	6.6 ± 0.1	1.81 ± 0.03	3.18 ± 0.09
35[c] ($KIE_{int.}$)	7.8 ± 0.6	1.85 ± 0.05	

[a] The Swain-Schaad exponent for the observed KIEs: $SSE_{obs.} = \ln\left(KIE_{obs.}^{H/T}\right) \Big/ \ln\left(KIE_{obs.}^{D/T}\right)$.
The errors of the SSE_{obs} ($\Delta SSE_{obs.}$) are calculated from the errors associated with the observed H/T and D/T KIEs by the error propagation rule:

$$\Delta SSE_{obs} = \sqrt{\left(\frac{\Delta\left(KIE_{obs.}^{H/T}\right)}{KIE_{obs.}^{H/T}} \Big/ \ln\left(KIE_{obs.}^{D/T}\right)\right)^2 + \left(\frac{\Delta\left(KIE_{obs.}^{D/T}\right)}{KIE_{obs.}^{D/T}} \cdot \frac{\ln\left(KIE_{obs.}^{H/T}\right)}{\left[\ln\left(KIE_{obs.}^{D/T}\right)\right]^2}\right)^2}$$

[b] Since the observed KIEs at 5 and 25 °C are the same as the intrinsic values (based on SSE_{obs} close to 3.3), it is not necessary to measure the D/T KIE at 15 °C.

[c] At 35 °C, $SSE_{obs} < 3.3$. Therefore, we used the Northrop method to calculate the intrinsic KIE at this temperature, with SSE_{int} of 3.34.[6] Error propagation in this case has been described elsewhere.[6]

6. References

(1) Marcus, R. A., Sutin, N. *Biochim. Biophys. Acta* **1985**, *811*, 265-322.

(2) Borgis, D., Hynes, J. T. *J Chem Phys* **1991**, *94*, 3619-3628.

(3) Kuznetsov, A. M., Ulstrup, J. *Can. J. Chem.-Rev. Can. Chim.* **1999**, *77*, 1085-1096.

(4) Here we assume that all the functional motions of the protein are in equilibrium in the Marcus-like model, which agree with most established theories. However, "protein promoting vibrations", which are not necessarily equilibrium dynamics, have also been proposed to contribute to chemical transformations.

(5) Cheatum, C. M., Kohen, A. In *Dynamics in Enzyme Catalysis*; Hammes-Schiffer, S., Klinman, J.P., Ed.; Springer DE: 2012, In Press.

(6) Sen, A., Yahashiri, A., Kohen, A. *Biochemistry* **2011**, *50*, 6462-6468.

(7) Schwartz, S. D., Schramm, V. L. *Nat. Chem. Biol.* **2009**, *5*, 551-558.