Supporting Information

Hydride Transfer in DHFR by Transition Path Sampling, Kinetic Isotope Effects and Heavy Enzyme Studies

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Assumptions in Using Northrop’s Method to Extract Intrinsic KIEs for ecDHFR

Based on the kinetic mechanism of ecDHFR (Scheme 1), the V/K expression for NADPH (under conditions $[\text{NADPH}] \to 0$, with the approximation that $k_{-3} \approx 0$) is readily derived:

$$
\left( \frac{V}{K} \right)_{\text{NADPH}} = \frac{k_2 k_3 k_6 [\text{DHF}]}{k_3 (k_2 k_6 [\text{DHF}] + k_2 k_{-5} [\text{DHF}] + k_{-5} k_{-6} [\text{THF}]) + k_{-2} k_{-5} k_{-6} [\text{THF}]} \quad (S1)
$$

This equation can be written for both H and T isotopes (i.e., hydride transfer reactions of NADPH and NADPT, respectively), and the H/T V/K KIE is:

$$
\frac{V}{K} = \frac{k_{3H}}{k_{3T}} + \frac{k_{3H} X_1}{1 + k_{3H} X}, \quad T_k^3 \text{ is the intrinsic tritium KIE of the hydride transfer,}
$$

and the forward commitment factor ($C_f$) for NADPH is:

$$
C_{fH} = k_{3H} X = \frac{k_{3H}}{k_{3T}} \left(1 + \frac{k_2 [\text{DHF}]}{k_6 [\text{THF}]} \left(1 + \frac{k_8}{k_{-5}}\right)\right) \quad (S3)
$$

Similarly, the D/T V/K KIE can be derived ($C_f^D$ is the $C_f$ for NADPD):

$$
\frac{V}{K} = \frac{k_{3D}}{k_{3T}} + \frac{k_{3D} X_1}{1 + k_{3D} X}, \quad T_k^3 \text{ is the intrinsic tritium KIE of the hydride transfer,}
$$

and the forward commitment factor ($C_f$) for NADPH is:

$$
C_{fD} = k_{3D} X = \frac{k_{3D}}{k_{3T}} \left(1 + \frac{k_2 [\text{DHF}]}{k_6 [\text{THF}]} \left(1 + \frac{k_8}{k_{-5}}\right)\right) \quad (S5)
$$

If $X$ is invariant between different KIE experiments (the first assumption), then the magnitudes of $C_f$ for different isotopes are related by the intrinsic KIEs:

$$
X = \frac{C_{fH}}{k_{3H}} = \frac{C_{fD}}{k_{3D}} = \frac{C_{fT}}{k_{3T}} \quad (S6)
$$

$$
\Rightarrow \quad C_{fH} = C_{fT} \cdot T_k^3, \quad C_{fD} = C_{fT} \cdot T_k^3 \quad (S7)
$$
This allows expressions of H/T and D/T V/K KIEs (eqs S2 and S4) as functions of intrinsic KIEs and $C_f$:

\[
\Gamma \left( \frac{V}{K} \right)_H = \frac{1 + C_f}{1/k_3 + C_f} \tag{S8}
\]

\[
\Gamma \left( \frac{V}{K} \right)_D = \frac{1 + C_f}{\Gamma (k_3)_D + C_f} \tag{S9}
\]

Thus,

\[
\left[ \Gamma \left( \frac{V}{K} \right)_H \right]^{-1} - 1 = \left[ \Gamma (k_3)_H \right]^{-1} - 1
\]

\[
\left[ \Gamma \left( \frac{V}{K} \right)_D \right]^{-1} - 1 = \left[ \Gamma (k_3)_D \right]^{-1} - 1 \tag{S10}
\]

With SSE relationship$^{1,2}$ (the second assumption; $\mu$ is the reduced mass, where the isotope mass of $^{12}\text{C}$ is used for carbon):

\[
\text{SSE: } \frac{\ln \left( \Gamma (k_3)_H \right)}{\ln \left( \Gamma (k_3)_D \right)} = \frac{1}{\sqrt{\mu_{C.H}}} - \frac{1}{\sqrt{\mu_{C.T}}} \left/ \left( \frac{1}{\sqrt{\mu_{C.D}}} - \frac{1}{\sqrt{\mu_{C.T}}} \right) \right. = 3.34 \tag{S11}
\]

The intrinsic KIEs can be numerically solved from the observed V/K KIEs:

\[
\left[ \left[ \Gamma \left( \frac{V}{K} \right)_H \right]^{-1} - 1 \right]^{-1} = \left[ \left[ \Gamma (k_3)_H \right]^{-1} - 1 \right]^{-1}
\]

\[
\left[ \left[ \Gamma \left( \frac{V}{K} \right)_D \right]^{-1} - 1 \right]^{-1} = \left[ \left[ \Gamma (k_3)_D \right]^{-1} - 1 \right]^{-1} \tag{S12}
\]

Although Northrop’s method is very instrumental for extracting intrinsic KIEs from V/K KIEs measured in competitive experiments, care should be taken to consider the validity of the two assumptions noted above. First, $C_f$ cannot be eliminated (to obtain eq S10 from eqs S2 and S4) if $X$ is not constant, like the case of ecDHFR, where $X$ is dependent on the concentration of unstable THF. Secondly, it remains controversial whether SSE holds for enzymatic hydride transfer reactions that may involve QM.
tunneling.\textsuperscript{2,3} Previous studies have suggested deviation from the semi-classical SSE (eq S11) even in the absence of QM tunneling.\textsuperscript{3-5}
Supplementary Figures and Tables

Figure S1. Representative plots of the fluctuations in $d_{DA}$ (red), $d_{DH}$ (blue), and $d_{HA}$ (green) during the hydride transfer reactions of l- and h-DHFR at different temperatures. Plots on the left show the full trajectories in 500 fs simulation time, and plots on the right are zoomed into the region where committor probability (black curve) changed from 0 to 1. Committor analysis allows identification of TS (magenta) as the structure for which the committor probability was found to be 0.5. The TS structures marked here are shown in Figure 2B in the main text.
Figure S2. The l- and h-DHFRs show variations in dynamic fluctuations of the distances previously proposed to be part of the “network of coupled motions”. Examples shown here are: (A) distance between $C_\alpha$ of Gly121 and $C_\beta$ of Met42; (B) distance between $C_\zeta$ of Phe31 and C11 of DHF; (C) distance between $C_\delta$ of Ile14 and the side-chain oxygen.
of Tyr100. These distances are plotted against the antisymmetric combination of \(d_{DH}\) and \(d_{AH}\) distances (i.e., \(d_{DH} - d_{AH}\) represents the hydride transfer reaction progress). Different colors in each figure represent 12 different reactive trajectories (for which the committor analysis was performed) of each system, and the thick black line marks the approximate location of TS identified by committor analysis (Figure S1). The variations in these protein dynamics do not cause any change in the hydride transfer TS geometry, barrier-crossing time, or activation free energy (Figures S3, Table S1, and Figure 2 and Table 1 in the main text).

**Figure S3.** The l- and h-DHFRs show very similar dynamic fluctuations of (A) the donor-acceptor distance \((d_{DA})\) and (B) the donor-hydride-acceptor angle, plotted against the antisymmetric combination of \(d_{DH}\) and \(d_{AH}\) distances. Different colors in each figure represent 12 different reactive trajectories (for which the committor analysis was
performed) of each system, and the thick black line marks the approximate location of TS identified by committor analysis (Figure S1).

**Figure S4.** (A) At 5 °C, pH 9, the FRET signal shows biphasic exponential kinetics when mixing DHFR•NADPH with DHF on a stopped-flow instrument. (B) The observed rate constant of the fast exponential phase (FRET increase) increases linearly with DHF concentration (eq 3). (C) The observed rate constant of the second exponential phase (FRET decay) increases with DHF concentration as a hyperbolic function (eq 4).

**Table S1.** Key geometric parameters of TS are the same for l- and h-DHFRs (in addition to the parameters listed in Table 1 in the main text).

<table>
<thead>
<tr>
<th>DHFR</th>
<th>Temperature</th>
<th>$d_{DH}$ (Å)$^a$</th>
<th>$d_{AH}$ (Å)$^a$</th>
<th>$\theta_{DHA}$ (°)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>light</td>
<td>280 K</td>
<td>1.55 ± 0.03</td>
<td>1.34 ± 0.05</td>
<td>155 ± 9</td>
</tr>
<tr>
<td></td>
<td>300 K</td>
<td>1.50 ± 0.04</td>
<td>1.32 ± 0.04</td>
<td>150 ± 6</td>
</tr>
<tr>
<td>heavy</td>
<td>280 K</td>
<td>1.48 ± 0.03</td>
<td>1.37 ± 0.04</td>
<td>154 ± 7</td>
</tr>
<tr>
<td></td>
<td>300 K</td>
<td>1.55 ± 0.08</td>
<td>1.34 ± 0.05</td>
<td>154 ± 6</td>
</tr>
</tbody>
</table>

$^a$ The $d_{DH}$ and $d_{AH}$ are the distances between the donor (D), hydride (H), and acceptor (A) atoms. The $\theta_{DHA}$ is the donor-hydrogen-acceptor angle.
Table S2. The pre-steady state rate constants and KIEs measured for the hydride transfer reactions of l- and h-DHFRs.

<table>
<thead>
<tr>
<th>DHFR</th>
<th>Temperature</th>
<th>$k_{\text{burst}}^{\text{NADPH}}$ (s$^{-1}$)$^a$</th>
<th>$k_{\text{burst}}^{\text{NADPD}}$ (s$^{-1}$)$^a$</th>
<th>H/D KIE on $k_{\text{burst}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>light</td>
<td>5 °C</td>
<td>123 ± 3</td>
<td>39.9 ± 0.8</td>
<td>3.08 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>15 °C</td>
<td>173 ± 3</td>
<td>57.8 ± 0.8</td>
<td>3.00 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>25 °C</td>
<td>270 ± 7</td>
<td>84 ± 1</td>
<td>3.2 ± 0.1</td>
</tr>
<tr>
<td>heavy</td>
<td>5 °C</td>
<td>126 ± 2</td>
<td>39.0 ± 0.5</td>
<td>3.23 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>15 °C</td>
<td>176 ± 3</td>
<td>59 ± 1</td>
<td>2.98 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>25 °C</td>
<td>264 ± 6</td>
<td>83.8 ± 0.9</td>
<td>3.15 ± 0.08</td>
</tr>
</tbody>
</table>

$^a$ The $k_{\text{burst}}^{\text{NADPH}}$ and $k_{\text{burst}}^{\text{NADPD}}$ are the observed rate constants for the pre-steady state FRET decay measured by stopped-flow experiments at pH 7, when NADPH and NADPD were used as the cofactor, respectively. H/D KIE on $k_{\text{burst}}$ is $k_{\text{burst}}^{\text{NADPH}}/k_{\text{burst}}^{\text{NADPD}}$. The rates and H/D KIEs are plotted in Figure 3 in the main text.

Table S3. The kinetic and equilibrium constants of the hydride transfer and overall reaction$^a$ catalyzed by l- and h-DHFRs at 5 °C, pH 9.

<table>
<thead>
<tr>
<th>DHFR</th>
<th>$k_{\text{cat}}^\text{forward}$ (s$^{-1}$)$^b$</th>
<th>$k_{\text{cat}}^\text{reverse}$ (s$^{-1}$)$^b$</th>
<th>$k_{\text{hyd}}^\text{forward}$ (s$^{-1}$)$^c$</th>
<th>$K_{\text{eq}}^\text{overall}$ (M$^{-1}$)$^d$</th>
<th>$K_{\text{eq}}^\text{hyd}$ $^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>light</td>
<td>1.25 ± 0.02</td>
<td>0.062 ± 0.007</td>
<td>5.89 ± 0.05</td>
<td>(8 ± 3) x 10$^{11}$</td>
<td>95 ± 11</td>
</tr>
<tr>
<td>heavy</td>
<td>1.34 ± 0.05</td>
<td>0.065 ± 0.004</td>
<td>5.80 ± 0.07</td>
<td>(7.9 ± 0.6) x 10$^{11}$</td>
<td>89 ± 6</td>
</tr>
</tbody>
</table>

$^a$ The overall reaction: \[ \text{NADPH} + \text{DHF} + \text{H}^+ \xrightarrow{K_{eq}} \text{NADP}^+ + \text{THF} \]

and [H$^+$] = 10$^{-9}$ M at pH 9.

$^b$ The $k_{\text{cat}}^\text{forward}$ and $k_{\text{cat}}^\text{reverse}$ are the forward and reverse steady state rate constants of the overall reaction. Our stopped-flow experiments suggest the hydride transfer is rate limiting in the reverse reaction; thus, $k_{\text{cat}}^\text{reverse}$ is also the reverse hydride transfer rate $k_{\text{hyd}}^\text{reverse}$. 

$^c$ $K_{\text{eq}}^\text{overall}$ = $K_{eq}^\text{hyd}$.
The forward hydride transfer rate $k_{hyd}^{forward}$ was measured by stopped-flow experiments (Figure S4).

The equilibrium constant of the overall reaction $K_{eq}^{overall}$ was measured by the total change in 340 nm absorbance in the reverse reaction. The equilibrium constant of the hydride transfer step $K_{eq}^{hyd}$ was calculated from $k_{hyd}^{forward}$ and $k_{hyd}^{reverse}$.

References


