Electronic Supplementary Information
for

Increased dynamic effects in a catalytically compromised variant of *Escherichia coli* dihydrofolate reductase

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This Supplementary Information includes more detail regarding the:

(1) experimental methods and full experimental data
(2) computational methods
Experimental Methods

Chemicals. NADPH, NADP$^+$ and isopropyl-β-D-thiogalactopyranoside (IPTG) were purchased from Melford (Ipswich, UK). $^{15}$N-ammonium chloride, $[^{13}\text{C}_6,^{2}\text{H}_7]$-glucose, 99.9% $^2\text{H}_2\text{O}$ and folate were purchased from Sigma. $^2\text{H}_2\text{F}$ was prepared by dithionite reduction of folate.$^1$ The concentrations of NADPH and $^2\text{H}_2\text{F}$ were determined spectrophotometrically using extinction coefficients of 6200 M$^{-1}$ cm$^{-1}$ at 339 nm and 28000 M$^{-1}$ cm$^{-1}$ at 282 nm, respectively.$^2$

Enzyme preparation. EcDHFR-N23PP/S148A and $^{15}$N-, $^{13}$C-, $^2$H-labelled (‘heavy’) EcDHFR-N23PP/S148A were prepared as described for wild-type EcDHFR.$^3$ *E. coli* BL21(DE3) cells harboring a cDNA for EcDHFR-N23PP/S148A$^4$ from an overnight culture in LB medium containing 100 µg/mL ampicillin were washed three times with M9 medium and then grown in 1 L M9 medium until the OD$_{600}$ reached 0.6. IPTG (to 0.5 mM) was added and the culture grown to an OD$_{600}$ of 2.0. Cells were harvested and the enzyme purified as previously described.$^5$ Heavy EcDHFR-N23PP/S148A was produced as described for the unlabelled enzyme in M9 medium in $[^2\text{H}_2$, 99.9%] H$_2$O supplemented with 1 g/L [$^{15}$N, 98%] NH$_4$Cl and 2 g/L $[^{13}\text{C}_6$, 99%; 1,2,3,4,5,6,6-$^2$H$_7$, 97%] glucose. Typically ~10 mg of apparently homogenous (by SDS-PAGE) EcDHFR-N23PP/S148A was obtained from 1 L culture. Enzymes were stored at 4 °C for up to 3 weeks without detectable loss of activity. Electrospray ionization mass spectrometry indicated masses of 17996.5 and 19933.6 for light and heavy EcDHFR (Figure S1),$^3$ and 18062.9 and 19906.4 for light and heavy EcDHFR-N23PP/S148A, respectively (Figure S2).
Figure S1. Electrospray ionization mass spectrometry of light (top two panels) and heavy (bottom two panels) EcDHFR. In each case the raw (upper panel) and deconvoluted (lower panel) spectrum is shown.
Figure S2. Electrospray ionization mass spectrometry of light (top two panels) and heavy (bottom two panels) EcDHFR-N23PP/S148A. In each case the raw (upper panel) and deconvoluted (lower panel) spectrum is shown.
Circular dichroism spectroscopy. Circular dichroism experiments were performed on an Applied PhotoPhysics Chirascan spectrometer using 11 µM protein in deoxygenated 10 mM potassium phosphate buffer (pH 7). Spectra (Figure S3) were measured between 200 nm and 400 nm in 10 mm quartz cuvettes under N₂ with a 50 nm/min scan speed, 0.5 nm data pitch, 1 nm bandwidth and 0.5 s response time.

Figure S3. Circular dichroism spectroscopy of light (red) and heavy (blue) EcDHFR-N23PP/S148A (three scans each), measured in 10 mM potassium phosphate at pH 7 using 11 µM protein.

Steady-state kinetic measurements. Steady-state turnover at pH 7 and pH 9.5 was followed on a JASCO V-660 spectrophotometer as described, monitoring the decrease in absorbance at 340 nm during the reaction \( \varepsilon_{340} (\text{NADPH} + \text{H}_2\text{F}) = 11,800 \text{ M}^{-1} \text{ cm}^{-1} \). \(^7\) Experiments were performed in MTEN buffer (50 mM morpholinoethanesulfonic acid, 25 mM Tris, 25 mM ethanolamine, 100 mM NaCl and 10 mM β-mercaptoethanol) using 30 nM enzyme. The pH was carefully adjusted at each experimental temperature to account for the temperature dependence of the pKₐ of organic amines. H₂F and NADPH (100 µM each) were incubated at the desired temperature for 5 min prior to addition of the enzyme (50 nM to 300 nM). Each data point is the result of three independent measurements. To determine Michaelis constants at pH 7, concentrations of NADPH were varied between 3 and 100 µM (pH 7) or 1 and 100 µM (pH 9.5), and concentrations of H₂F were varied between 0.5 and 100 µM, whilst keeping the concentration of the other reactant fixed at 100 µM.
Pre-steady-state kinetic measurements. Hydride transfer rate constants were measured under single-turnover conditions on a Hi-Tech Scientific stopped-flow spectrophotometer essentially as described before. Before mixing, the enzyme (40 µM) was preincubated with NADPH (16 µM) for at least 1 min in 100 mM potassium phosphate containing 100 mM NaCl and 10 mM β-mercaptoethanol at pH 7, or in MTEN buffer (50 mM morpholinoethanesulfonic acid, 25 mM Tris, 25 mM ethanolamine, 100 mM NaCl, and 10 mM β-mercaptopethanol) for pH dependence measurements (the pH of MTEN buffer was adjusted at the experimental temperature), and the reaction started by rapidly mixing with H₂F (200 µM) in the same buffer. The reaction was monitored by following the reduction of fluorescence resonance energy transfer from the enzyme to NADPH, by exciting the sample at 297 nm and measuring emission using a 400 nm cut-off filter. All measurements were repeated at least six times. Rate constants were extracted by fitting the kinetic data to the equation for a double exponential decay.

Figure S4. pH dependence of the pre-steady state rate constants during catalysis by light (red) and heavy (blue) EcDHFR-N23PP/S148A at 20 °C (squares) and 35 °C (circles). All data points are the average of at least 6 measurements; the standard errors of the mean are given for one sigma.
Table S1: Temperature dependence of the steady-state rate constants at pH 9.5 and pH 7 ($k_{\text{cat}}$) and the pre-steady-state rate constants at pH 7 ($k_H$) during catalysis by light and heavy EcDHFR-N23PP/S148A.

| $T$ (°C) | $k_{\text{cat}}^\text{LE}$ (s$^{-1}$) | $k_{\text{cat}}^\text{HE}$ (s$^{-1}$) | $k_H^\text{LE}$ (s$^{-1}$) | $k_H^\text{HE}$ (s$^{-1}$) | $k_{\text{cat}}^\text{LE}$ (s$^{-1}$) | $k_{\text{cat}}^\text{HE}$ (s$^{-1}$) |
|---------|----------------|----------------|----------------|----------------|----------------|----------------|
| 5       | 0.038 ± 0.002  | 0.035 ± 0.001  | 16.19 ± 0.26  | 12.38 ± 0.54  | 0.434 ± 0.035  | 0.466 ± 0.057  |
| 10      | 0.057 ± 0.002  | 0.045 ± 0.002  | 20.19 ± 0.08  | 14.95 ± 0.55  | 0.522 ± 0.036  | 0.536 ± 0.035  |
| 15      | 0.085 ± 0.005  | 0.067 ± 0.003  | 26.90 ± 0.35  | 20.72 ± 0.44  | 0.867 ± 0.044  | 0.865 ± 0.028  |
| 20      | 0.145 ± 0.012  | 0.112 ± 0.009  | 34.63 ± 0.53  | 25.60 ± 0.90  | 1.643 ± 0.191  | 1.680 ± 0.213  |
| 25      | 0.237 ± 0.011  | 0.187 ± 0.010  | 40.32 ± 0.79  | 30.41 ± 0.80  | 2.367 ± 0.325  | 2.398 ± 0.335  |
| 30      | 0.351 ± 0.021  | 0.268 ± 0.012  | 47.23 ± 1.28  | 34.44 ± 1.18  | 3.304 ± 0.369  | 3.417 ± 0.476  |
| 35      | 0.476 ± 0.029  | 0.345 ± 0.017  | 51.78 ± 0.72  | 37.45 ± 2.16  | 5.374 ± 0.425  | 5.283 ± 0.559  |
| 40      | 0.695 ± 0.029  | 0.519 ± 0.031  | 59.62 ± 0.81  | 41.62 ± 2.54  | 6.589 ± 1.08  | 6.368 ± 0.956  |

Table S2: Temperature dependence of the enzyme KIE on catalysis by EcDHFR-N23PP/S148A at pH 9.5 and 7.

| $T$ (°C) | $k_{\text{cat}}^\text{LE} / k_{\text{cat}}^\text{HE}$ | $k_H^\text{LE} / k_H^\text{HE}$ | $k_{\text{cat}}^\text{LE} / k_{\text{cat}}^\text{HE}$ |
|---------|----------------|----------------|----------------|
| 5       | 1.14 ± 0.02    | 1.31 ± 0.03    | 0.93 ± 0.07    |
| 10      | 1.27 ± 0.02    | 1.35 ± 0.03    | 0.97 ± 0.05    |
| 15      | 1.26 ± 0.01    | 1.30 ± 0.02    | 1.00 ± 0.03    |
| 20      | 1.30 ± 0.01    | 1.35 ± 0.03    | 0.98 ± 0.08    |
| 25      | 1.27 ± 0.03    | 1.33 ± 0.02    | 0.99 ± 0.10    |
| 30      | 1.31 ± 0.03    | 1.37 ± 0.03    | 0.97 ± 0.09    |
| 35      | 1.38 ± 0.01    | 1.38 ± 0.04    | 1.02 ± 0.07    |
| 40      | 1.34 ± 0.03    | 1.43 ± 0.04    | 1.03 ± 0.12    |
### Table S3: Steady state kinetic parameters for light and heavy EcDHFR-N23PP/S148A.

|                  | At 20 ºC |                  | At 35 ºC |                  |
|------------------|----------|------------------|----------|------------------|
|                  | Light    | Heavy            | Light    | Heavy            |
| $k_{\text{cat}}$ (s$^{-1}$) | 0.145 ± 0.012 | 0.112 ± 0.009 | 1.643 ± 0.191 | 1.680 ± 0.213 |
| $K_M$ (µM) NADPH  | 0.83 ± 0.20 | 1.06 ± 0.41 | 1.29 ± 0.11   | 1.32 ± 0.26   |
| $K_M$ (µM) DHF    | 0.75 ± 0.23 | 0.71 ± 0.20 | 0.45 ± 0.10   | 0.40 ± 0.08   |
|                  | Light    | Heavy            | Light    | Heavy            |
| $k_{\text{cat}}$ (s$^{-1}$) | 0.476 ± 0.029 | 0.345 ± 0.017 | 5.374 ± 0.425 | 5.283 ± 0.559 |
| $K_M$ (µM) NADPH  | 0.68 ± 0.21 | 0.77 ± 0.41 | 3.00 ± 0.70   | 5.07 ± 0.78   |
| $K_M$ (µM) DHF    | 0.90 ± 0.20 | 1.67 ± 0.50 | 0.75 ± 0.20   | 0.64 ± 0.20   |

### Table S4: pH dependence of the pre-steady-state rate constant at pH 7 ($k_H$) during catalysis by light and heavy EcDHFR at 20 ºC and 35 ºC.

| pH   | 20 ºC                  | 35 ºC                  |
|------|------------------------|------------------------|
|      | Light                  | Heavy                  | Light                  | Heavy                  |
| 5    | 139.00 ± 13.22         | 128.56 ± 15.96         | 315.59 ± 3.30          | 310.16 ± 7.50          |
| 6    | 93.85 ± 0.67           | 88.30 ± 0.354          | 150.57 ± 1.60          | 160.68 ± 2.66          |
| 6.5  | 55.93 ± 0.31           | 43.83 ± 1.80           | 84.82 ± 0.55           | 77.68 ± 0.85           |
| 7    | 34.63 ± 0.11           | 25.60 ± 0.53           | 51.78 ± 0.72           | 37.45 ± 2.16           |
| 8    | 7.475 ± 0.160          | 4.72 ± 0.32            | 11.58 ± 0.04           | 8.29 ± 0.12            |
| 9    | 0.71 ± 0.01            | 0.49 ± 0.01            | 0.82 ± 0.02            | 0.68 ± 0.03            |
Computational methods

The model. The initial coordinates were taken from the X-ray crystal structures of EcDHFR together with the substrate and the cofactor, using PDB files 3QL3 for the wild type EcDHFR and 3QL0 for EcDHFR-N23PP/S148A. EcDHFR is a monomer with one active site and a total of 159 residues in the case of the wild type and 160 in the N23PP/S148A variant. The protonation state of each residue was determined at pH 7.0 for both EcDHFR and EcDHFR-N23PP/S148A, employing the PropKa 3.1 program. Once the protonation state of each titratable residue was determined, hydrogen atoms were added by means of the HBUILD tool included in the fDYNAMO package. 13 sodium cations were added in order to equilibrate the total charge of the system, with their initial positioning selected according to the protein’s electrostatic potential. The system was then placed inside a pre-equilibrated cubic box of water molecules (65.2 Å sides) centered on the substrate-cofactor pair. Water molecules within 2.8 Å of any non-hydrogen atom were removed.

The wild type enzyme system was formed by the monomer of the protein (159 residues), the substrate and cofactor (52 and 74 atoms, respectively), 13 sodium ions, 152 crystal water molecules (306 atoms) and 8044 solvation water molecules. On the other hand, the variant protein system was formed by the monomer of the protein (160 residues), the substrate and cofactor (52 and 74 atoms, respectively), 13 sodium ions, 198 crystal water molecules (594 atoms) and 8024 solvation water molecules. The protein atoms and the ions were described by means of the OPLS-AA force field while the water molecules were described by the TIP3P potential. The quantum mechanical (QM) subsystem was formed by the parts of the cofactor and the substrate that are directly involved in the chemical process (Figure S5), comprising 29 atoms from the NADPH coenzyme (dihydronicotinamide and ribose rings) and 38 atoms from the protonated substrate (pterin ring, N-methylene-substituted p-aminobenzoyl (pABA) moiety, and NH atoms of the glutamate moiety). To saturate the valence of the QM/MM frontier the link atom procedure was used, placing these link atoms between the carbon atoms of the ribose ring and the Cα for the cofactor and the nitrogen atom and Cα for the substrate. Therefore, the QM part was formed by 67 atoms plus 2 link atoms. The QM subsystem was treated by the semiempirical Hamiltonian AM1 as modified by Major et al. to provide a more accurate energetic description.
Figure S5. QM/MM partitioning scheme. Black dots represent the quantum hydrogen link atoms. Atoms in the nicotinamide ring and pteridine ring, most notably the transferred hydride, Ht, and its donor and acceptor carbon atoms, have been labelled.

Heavy EcDHFR and EcDHFR-N23PP/S148A were prepared by modifying the masses of all $^{14}$N, $^{12}$C and non-exchangeable $^1$H atoms to those of $^{15}$N, $^{13}$C, $^2$H. The ratio between the masses of the simulated heavy and light variant enzymes was 1.10987, similar to the experimentally observed molecular weight increase. After full minimization and heating dynamics, the systems were equilibrated at 300 K using the NVT ensemble with the Langevin-Verlet integrator\textsuperscript{22} and a time step of 0.5 fs over a total of 150 ps. Cutoffs for the nonbonding interactions were applied using a switching function within a radius range of 13.0 to 9.0 Å. Periodic boundary conditions were employed in all simulations.

The uncatalysed reaction in aqueous solution was also studied following the same protocol, except that the substrate and the cofactor were first optimized together in the gas phase using the MP2/6-31+G(d,p) \textit{ab initio} method within the Gaussian09 package.\textsuperscript{23} The optimized structure and 5 sodium ions (in order to compensate the total charge of the system) were then placed inside of a cubic box (60.2 Å sides) of TIP3P water molecules centered on the substrate-cofactor pair.

\textit{Potential of Mean Force (PMF).} One-dimensional PMFs, $W_{CM}$, were computed using the antisymmetric combination of distances describing the hydride transfer, $z=\text{d}_{C4Ht}−\text{d}_{HtC6}$, as the reaction coordinate. The umbrella sampling approach\textsuperscript{24} was used with constraints on the value of
the reaction coordinate by means of the addition of a harmonic potential with a force constant of 2500 kJ·mol$^{-1}$·Å$^{-2}$, which allows good overlap between windows. The reaction coordinate was then explored in a range from –2.07 to 1.57 Å, with a window width of 0.07 Å (the total number of windows was 53). The probability distributions obtained from molecular dynamics (MD) simulations within each individual window were combined by means of the weighted histogram analysis method (WHAM). 25 20 ps of relaxation and 40 ps of production MD, with a time step of 0.5 fs, in the canonical ensemble (NVT, with a reference temperature of 300 K) and the Langevin–Verlet integrator, 22 were used in the simulations.

Figure S6. Classical mechanical AM1-SRP/MM PMF obtained from five different structures of the TS selected and optimized from the constrained MD simulation performed at the initial TS structure of EcDHFR-N23PP/S148A. The plot shows that the PMFs are well converged.

Four additional PMFs were computed at the AM1-SRP/MM level to check the robustness of our method and to get averaged energy values. The starting structures were selected from snapshots of a long QM/MM MD simulation with the reaction coordinate restrained to the value obtained for the TS of the first PMF. The results (Figure S6) show very small deviations between the profiles, and between the averaged structures of the three states involved in the reaction. From these PMFs, the classical mechanical activation free energy barrier obtained for EcDHFR-N23PP/S148A, $W^\dagger$, is
$17.5 \pm 0.6 \text{ kcal-mol}^{-1}$. A slightly higher dispersion was observed than for the wild type ($15.8 \pm 0.4 \text{ kcal-mol}^{-1}$), but the result is also in agreement with experimental data.

Selected geometries of the reactant state (RS) and TS were used as starting points to run 2 ns AM1-SRP/MM MD simulations with the reaction coordinate restrained to the corresponding values, to investigate the structural properties of the RS and TS in more detail (Table S5). The effect of the dihedral angle $\text{C}_{\beta}-\text{C}_{\gamma}-\text{S}_{\delta}-\text{C}_{\varepsilon}$ in Met20 on the PMF was investigated in more detail (Figure S7).

Figure S7: Potential of Mean Force (PMF) obtained as a function of the dihedral angle $\text{C}_{\beta}-\text{C}_{\gamma}-\text{S}_{\delta}-\text{C}_{\varepsilon}$ of the Met20 residue obtained for the wild type EcDHFR (blue) and the variant EcDHFR-N23PP/S148A (red).
Table S5: Key averaged structural parameters of the reactant state, RS, and transition state, TS, from 2 ns MD simulations at the AM1-SRP/MM level of the RS and TS of EcDHFR and EcDHFR-N23PP/S148A, performed at 300 K. Distances are in Å and angles in degrees.

|                    | EcDHFR | EcDHFR-N23PP/S148A |
|--------------------|--------|--------------------|
|                    | RS     | TS     | RS     | TS     |
| C4\textsubscript{NADPH}–Ht–C6\textsubscript{H2F} | 141 ± 15 | 163 ± 7 | 144 ± 16 | 164 ± 7 |
| C4\textsubscript{NADPH}–Ht–C6\textsubscript{H2F} | -1.9 ± 0.4 | -0.18 ± 0.04 | -1.9 ± 0.4 | -0.24 ± 0.04 |
| C4\textsubscript{NADPH}–C6\textsubscript{H2F} | 3.9 ± 0.3 | 2.63 ± 0.06 | 3.9 ± 0.4 | 2.66 ± 0.06 |
| C4\textsubscript{NADPH}–Ht | 1.09 ± 0.03 | 1.24 ± 0.03 | 1.09 ± 0.03 | 1.22 ± 0.03 |
| C6\textsubscript{H2F}–Ht | 2.9 ± 0.4 | 1.42 ± 0.04 | 3.1 ± 0.4 | 1.46 ± 0.04 |
| HN7\textsubscript{NADPH}–O\textsubscript{ALA7} | 2.2 ± 0.5 | 2.1 ± 0.2 | 2.6 ± 0.9 | 2.1 ± 0.2 |
| HN7\textsubscript{NADPH}–N\textsubscript{ALA7} | 3.7 ± 0.5 | 3.5 ± 0.2 | 4.1 ± 0.8 | 3.6 ± 0.3 |
| HN7\textsubscript{NADPH}–S\textsubscript{MET20} | 4.6 ± 0.7 | 2.6 ± 0.3 | 4.3 ± 0.4 | 3.0 ± 0.4 |
| HO3\textsubscript{NADPH}–N\textsubscript{ASN18} | 3.8 ± 0.6 | 4.1 ± 0.6 | 5.3 ± 0.5 | 4.0 ± 0.5 |
| HO2\textsubscript{NADPH}–O\textsubscript{ALA19} | 3.2 ± 0.4 | 3.2 ± 0.5 | 3.4 ± 0.6 | 3.9 ± 0.6 |
| HO2\textsubscript{NADPH}–N\textsubscript{ALA19} | 3.1 ± 0.2 | 3.0 ± 0.2 | 3.2 ± 0.4 | 3.3 ± 0.4 |
| HN7\textsubscript{NADPH}–O\textsubscript{ILE14} | 3.0 ± 0.9 | 3.5 ± 0.4 | 3.0 ± 0.8 | 3.5 ± 0.5 |
| HN5\textsubscript{H2F}–S\textsubscript{MET20} | 3.1 ± 0.4 | 2.9 ± 0.4 | 3.7 ± 0.6 | 3.1 ± 0.5 |
| HN3\textsubscript{H2F}–O\textsubscript{ASP27} | 1.8 ± 0.1 | 1.9 ± 0.2 | 1.8 ± 0.1 | 1.9 ± 0.2 |
| HN2\textsubscript{H2F}–O\textsubscript{ASP27} | 1.8 ± 0.2 | 1.8 ± 0.1 | 1.8 ± 0.1 | 1.9 ± 0.2 |
| Ca–Cβ–Cγ–S\textsubscript{MET20} | 166 ± 20 | 177 ± 13 | 175 ± 14 | 179 ± 16 |
| Cβ–Cγ–S\textsubscript{MET20} | -83 ± 19 | -75 ± 15 | 98 ± 40 | -83 ± 28 |
| HN\textsubscript{GLU17}–O\textsubscript{ASP122} | 4.0 ± 0.6 | 4.0 ± 0.7 | 3.2 ± 0.4 | 2.4 ± 0.6 |

Free Energy Surfaces (FES). The FESs for the hydride transfer in aqueous solution and catalyzed by EcDHFR and EcDHFR-N23PP/S148A were obtained using umbrella sampling and WHAM.\textsuperscript{24,25} For EcDHFR and EcDHFR-N23PP/S148A the solvent coordinate was sampled from +30 kcal-mol\textsuperscript{-1}·e\textsuperscript{-1} to -10 kcal-mol\textsuperscript{-1}·e\textsuperscript{-1} with simulation windows in which the coordinate was incremented by 4.8 kcal-mol\textsuperscript{-1}·e\textsuperscript{-1} using a force constant of 0.024 kcal\textsuperscript{-1}·mol·e\textsuperscript{2}, giving a good compromise between the number of simulation windows needed and the simulation time per window.\textsuperscript{26} The chemical coordinate was sampled from -2.07 to 1.57 Å with increments of 0.07 Å and using a force constant of 2500 kJ·mol\textsuperscript{-1}·Å\textsuperscript{-2}. For the reaction in solution, the solvent coordinate was sampled from +43 kcal-mol\textsuperscript{-1}·e\textsuperscript{-1} to -33 kcal-mol\textsuperscript{-1}·e\textsuperscript{-1} with increments of 4.8 kcal-mol\textsuperscript{-1}·e\textsuperscript{-1} and a force constant of 0.024 kcal\textsuperscript{-1}·mol·e\textsuperscript{2}; while the chemical coordinate was sampled from -3.12 to 1.57 Å with an increments of 0.07 Å and a force constant of 2500 kJ·mol\textsuperscript{-1}·Å\textsuperscript{-2}. The FESs for the wild type and variant enzymes were obtained from a total of 583 windows, while the FES for the water solution reaction needed a total of 1190 windows. Each window consisted in 5 ps of relaxation and 20 ps of production. All simulations were performed at 300 K using the Langevin-Verlet integrator and periodic boundary conditions.
Ensemble Averaged Variational Transition State Theory. Deviations from classical Transition State Theory (TST) as a result of dynamic recrossings and quantum tunnelling effects can be estimated by means of Ensemble-Averaged Variational Transition State Theory (EA-VTST).\textsuperscript{27–29} Thus, our theoretical estimation of the rate constant can be written as:

\[
k(T) = \Gamma(T,z) \frac{k_B T}{h} \exp \left( -\frac{\Delta G^\text{QC}_\text{act}(T,z)}{RT} \right)
\]

(S1)

The transmission coefficient, \(\Gamma(T,z)\), is obtained as the product of recrossing (\(\gamma\)) and tunnelling (\(\kappa\)) contributions:

\[
\Gamma(T,z) = \gamma(T,z) \cdot \kappa(T)
\]

(S2)

\(\Delta G^\text{QC}_\text{act}\) is the quasi-classical activation free energy at the transition state, obtained from the classical mechanical (CM) PMF and including a correction for quantizing the vibrations orthogonal to the reaction coordinate and the vibrational free energy of the reactant mode that correlates with motion along the reaction coordinate, and is calculated as:

\[
\Delta G^\text{QC}_\text{act} = \left[ W^\text{CM}(T,z^*) + \Delta W_{\text{vib}}(T,z^*) \right] - \left[ W^\text{CM}(T,z_R) + \Delta W_{\text{vib,}R}(T) + G^\text{CM}_{R,T,F} \right]
\]

(S3)

where \(\Delta W_{\text{vib}}(T,z^*)\) corrects \(W^\text{CM}(T,z^*)\) to account for quantized vibrations orthogonal to the reaction coordinate along which the PMF is defined, \(z^*\); \(\Delta W_{\text{vib,}R}(T)\) corrects \(W^\text{CM}(T,z_R)\) for quantized vibrations at the reactant minimum of the PMF, \(z_R\); and \(G^\text{CM}_{R,T,F}\) is a correction for the vibrational free energy of the reactant mode that correlates with motion along the reaction coordinate.\textsuperscript{27}

To correct the classical mechanical PMF, \(W^\text{CM}\), normal mode analyses were performed for the quantum region atoms. We localized 13 TS structures starting from different configurations of the corresponding simulation windows in the heavy and light enzymes. After intrinsic reaction coordinate (IRC) calculations we optimized the corresponding reactant structures and obtained the Hessian matrix for all the stationary structures. The final quantum mechanical vibrational corrections (Table S6) were obtained as an average over these structures as indicated in equation
(S4). As far as the masses of the substrate and cofactor remain unchanged these contributions are statistically identical for the heavy and light enzymes.

\[
\Delta W_{\text{vib}} = \Delta W_{\text{vib}}(T,z^*) - \Delta W_{\text{vib},R}(T) = \\
\left[ ZPE_{\text{TS}} - \left\langle ZPE_{R} \right\rangle \right] + \left[ \sum_{i=1}^{3N-7} RT \ln \left( 1 - e^{\frac{-h \nu_i}{k_B T}} \right)_{TS} - \left\langle \sum_{i=1}^{3N-6} RT \ln \left( 1 - e^{\frac{-h \nu_i}{k_B T}} \right) \right\rangle_R \right] - \\
\left[ \sum_{i=1}^{3N-7} RT \ln \left( 1 - e^{\frac{-h \nu_i}{k_B T}} \right)_{TS} - \left\langle \sum_{i=1}^{3N-6} RT \ln \left( 1 - e^{\frac{-h \nu_i}{k_B T}} \right) \right\rangle_R \right]
\]  

(S4)

Table S6: Components of the quasi-classical activation free energy: \( W^\ddagger \) is the PMF difference between the TS and the reactants \( [W^\ddagger = W^{CM}(T,z^*) - W^{CM}(T,z_R)] \); \( G^{CM}_{R,T,F} \) is the vibrational free energy corresponding to the reaction coordinate at the reactants and \( \Delta W_{\text{vib}}(T) \) is the correction for quantized vibrations \( [\Delta W_{\text{vib}}(T) = \Delta W_{\text{vib}}(T,z^*) - \Delta W_{\text{vib},R}(T)] \). Data for light and heavy wild type (WT) EcDHFR are from ref 3. All values are in kcal·mol\(^{-1}\).

| Enzyme                  | \( W^\ddagger \) (kcal·mol\(^{-1}\)) | \( G^{CM}_{R,T,F} \) (kcal·mol\(^{-1}\)) | \( \Delta W_{\text{vib}} \) (kcal·mol\(^{-1}\)) |
|------------------------|---------------------------------------|------------------------------------------|-----------------------------------------------|
| C4\text{NADPH–H}–C6\text{H2F} | 141 ± 15                             | 163 ± 7                                  | 144 ± 16                                      |
| C4\text{NADPH–H}–C6\text{H2F–H} | -1.9 ± 0.4                            | -0.18 ± 0.04                             | -1.9 ± 0.4                                    |
| C4\text{NADPH–C6\text{H2F}} | 3.9 ± 0.3                             | 2.63 ± 0.06                              | 3.9 ± 0.4                                    |
|                         | 1.09 ± 0.03                           | 1.24 ± 0.03                              | 1.09 ± 0.03                                  |

Reactive trajectories (activated dynamics) simulations. We ran a 2 ns NVT QM/MM MD trajectory restrained to the transition state (TS) region with a time step of 0.5 fs for the reaction in both enzymes. The simulation temperature was 300 K and one configuration was saved every 10 ps, resulting in 200 configurations that were used to compute free (unrestrained) downhill trajectories. The velocity associated with the reaction coordinate is not properly thermalized in these initial configurations (because of the reaction coordinate restraint). Thus, following a procedure similar to that used by Gao and coworkers\(^{30}\) and used in our previous studies,\(^{31–34}\) we selectively removed the projection of the velocity on the reaction coordinate and added a random value taken from a Maxwell-Boltzmann distribution. For each of the saved TS configurations with modified velocities, we ran free MD simulations (\emph{i.e.} with no reaction coordinate restraint) within the micro-canonical ensemble (NVE). Separately, for each configuration we integrated the equations of motion forwards and backwards, just changing the sign of the velocity components. Downhill trajectories were
propagated from $-2$ ps to $+2$ ps using a time step of 0.5 fs. The trajectories obtained in the enzyme and in solution were then classified as reactive trajectories when reactants connect to products (RP trajectories) or nonreactive trajectories leading either from reactants to reactants (RR) or from products to products (PP). Both reactive and nonreactive trajectories may exhibit recrossings of the dividing surface. To compute the recrossing transmission coefficient, we used the ‘positive flux’ formulation, assuming that the trajectory is initiated at the barrier top with forward momentum along the reaction coordinate:

$$
\gamma(t) = \frac{\langle j_+ \theta[z(+t)] \rangle - \langle j_+ \theta[z(-t)] \rangle}{\langle j_+ \rangle}
$$

(S5)

where $z$ is the reaction coordinate, $j_+$ represents the initially positive flux at $t=0$, given by $z(t=0)$, and $\theta(z)$ is a step function equal to one in the product side of the reaction coordinate and zero on the reactant side. The average is calculated over all the trajectories to get the recrossing transmission coefficient $\gamma$ (Figure S8).

Figure S8. Time-dependent evolution of the recrossing transmission coefficient $\gamma(t)$ for the hydride transfer catalysed by the light (blue) and heavy (green) EcDHFR-N23PP/S148A.

Quantum Tunnelling Effects and Phenomenological Free Energy. The tunnelling transmission coefficient, $\kappa$, was calculated with the small-curvature tunnelling (SCT) approximation, which includes reaction-path curvature appropriate for enzymatic hydride transfers.\(^{36,37}\) Previously, the SCT approximation has been applied successfully to enzymatic reactions catalysed by DHFR and aromatic amine dehydrogenase, yielding KIEs and phenomenological activation energies that are in
good agreement with the experimental results.\textsuperscript{36,37} The final tunnelling contribution is obtained as the average over the reaction paths of 13 TS structures.

Once recrossing and tunnelling transmission coefficients are computed, Eq (S1) can be transformed into Eq (S6) by incorporating the transmission coefficient into the exponential term as a correction to the effective classical free energy barrier, giving a phenomenological free energy of activation, $\Delta G_{\text{eff}}$:

$$k_{\text{theo}}(T) = \frac{k_B T}{h} \exp\left(-\frac{\Delta G_{\text{eff}}}{RT}\right)$$

(S6)

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