

Supporting Information

**Chemical Ligation and Isotope Labeling to Locate Dynamic Effects during Catalysis by Dihydrofolate Reductase\*\***

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## Experimental methods

**Chemicals.**  $^{15}\text{N}$ -ammonium chloride, [ $^{13}\text{C}_6, ^2\text{H}_7$ ]-glucose, 99.9%  $^2\text{H}_2\text{O}$ , folate, dithiothreitol, methoxyamine hydrochloride, 4-mercaptophenylacetic acid (MPAA) and sodium 2-mercaptoethanesulfonate (MESNA) were purchased from Sigma-Aldrich. Urea, NADPH,  $\text{NADP}^+$  and isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) were purchased from Melford (Ipswich, UK).  $\text{H}_2\text{F}$  was prepared by dithionite reduction of folate.<sup>[1]</sup> The concentrations of NADPH and  $\text{H}_2\text{F}$  were determined spectrophotometrically using extinction coefficients of  $6200 \text{ M}^{-1} \text{ cm}^{-1}$  at 339 nm and  $28000 \text{ M}^{-1} \text{ cm}^{-1}$  at 282 nm, respectively.<sup>[2]</sup>

**Preparation of EcDHFR-A29C.** The QuikChange site-directed mutagenesis kit and the following primers were used to prepare EcDHFR-A29C: 5' CCTGCCTGCCGATCTCTGCTG-GTTTAAACGCAACAC 3' and 5'-GGTGGTTGCTCTTCCGCAGAGATCGGCAGGCAGGT-TCCACG-3'.

**NMR experiments.** Wild type EcDHFR and EcDHFR-A29C were produced in M9 minimal medium containing  $^{15}\text{N}$ -ammonium chloride and purified as reported before.<sup>9</sup>  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra were recorded on a Bruker AVANCE III 600 MHz NMR spectrometer with QCI-P cryoprobe at 25 °C in 50 mM Tris-HCl buffer (pH 7.0) containing 1 mM NaCl and 10 mM  $\beta$ -mercaptoethanol. A 10-fold excess of  $\text{NADP}^+$  and folate was added to each enzyme. An equimolar solution of both ligands was prepared and adjusted to pH 7.0 before addition to the DHFR; the pH was then checked before measurement of the spectrum. 5%  $\text{D}_2\text{O}$  was added to all the NMR samples before acquisition. Spectra were processed using TopSpin 3.2.6.

**Preparation of N-terminal EcDHFR(1-28)- $\alpha$ -thioester.** To prepare a heavy N-terminal EcDHFR thioester that is completely isotopically labeled using synthetic techniques is almost economically unfeasible, thus a recombinant method was employed. The N-terminal EcDHFR gene fragment was amplified by PCR from a pJGetit-based vector containing the gene encoding EcDHFR. Forward primer (5'-GGTGGTCATATGATGATCAGTCTGATTGCGGCGTTAGC-3') encoding an *NdeI* restriction site and reverse primer (5'-GGTGGTTGCTCTTCCGCAGAG-ATCGGCAGGCAGGTTCCACG-3') encoding a *SapI* restriction site were used. The PCR product was cloned into the pTWIN1 (New England Biolabs) vector using the *NdeI* and *SapI* restriction sites. The resulting plasmid pNTecDHFR, which encodes EcDHFR(1-28) fused with intein *gyrA*, was shown to be free of mutations based on DNA sequencing results.

For protein expression, *E. coli* BL21(DE3) RP cells transformed with pNTecDHFR were used. An overnight culture of the relevant cells in LB medium containing 100  $\mu\text{g}/\text{mL}$  ampicillin and 35  $\mu\text{g}/\text{mL}$  chloramphenicol were washed once with M9 medium and then grown at 37 °C in 1 L M9 medium<sup>[3]</sup> with the same antibiotic selection until the  $\text{OD}_{600}$  reached 0.6. IPTG (0.5 mM) was added and the culture grown to an  $\text{OD}_{600}$  of 2.0. Cells were harvested by centrifugation at 16,000 rpm, resuspended in lysis buffer (25 mM HEPES, pH 7.5, 150 mM NaCl), and lysed by sonication. The pellet containing the desired fusion peptide was washed with lysis buffer containing 1.0% Triton X-100 and centrifuged at 16,000 rpm. The washed pellet was resolubilized in 8 M urea and then diluted to  $\sim 2$  M urea using thioester-exchange buffer (25 mM HEPES, pH 8.5, 500 mM NaCl, 1 mM EDTA, 200 mM MESNA). After overnight incubation at 4 °C, the crude solution containing the thioester peptide was re-acidified to pH 3.5 at 0 °C and centrifuged at 20,000 rpm. The pellet containing the desired product was further purified by reverse-phase HPLC (Phenomenex C-18, 250 x 10 mm, 10  $\mu\text{m}$ , 110 Å) using a linear gradient of 30-70% of buffer B in buffer A over 120 min (buffer A = 0.1% TFA in  $\text{H}_2\text{O}$ ; buffer B = 0.08%

TFA in acetonitrile). Isotopically substituted (heavy) thioester peptide (MESNA was not labeled) was produced as described for the unlabeled peptide, except that [ $^2\text{H}_2$ , 99.9%]  $\text{H}_2\text{O}$ , [ $^{15}\text{N}$ , 98%]  $\text{NH}_4\text{Cl}$  and [ $^{13}\text{C}_6$ , 99%; 1,2,3,4,5,6- $^2\text{H}_7$ , 97%] glucose were used to prepare the M9 medium, similar to a previously described protocol.<sup>[3]</sup> Typically 3-7 mg of peptide were obtained per liter of cell culture.

The purity and molecular masses of the N-terminal EcDHFR(1-28)- $\alpha$ -COSCH<sub>2</sub>CH<sub>2</sub>SO<sub>3</sub>H peptides were evaluated by Ultimate 3000 UHPLC/Bruker amaZonSL MS ion trap. Reverse phase chromatography was performed on ACE<sup>®</sup> C-18 (100 x 2.1 mm, 10  $\mu\text{m}$ , 300  $\text{\AA}$ ), using a linear gradient of 5-95% of buffer B in buffer A over 30 min (buffer A = 0.1% formic acid in  $\text{H}_2\text{O}$ ; buffer B = 0.1% formic acid in acetonitrile). The unlabeled and labeled peptides showed molecular masses of  $3280.6 \pm 0.1$  and  $3638.3 \pm 0.5$ , respectively (calculated: 3281.01 and 3641.8, respectively) (Figure S2). One extra methionine encoded by the plasmid is located at the N-terminal end of the peptide, but it does not cause any effect on catalysis (see text).

**Preparation of C-terminal EcDHFR(29-159, A29C).** Preparation of the C-terminal EcDHFR (29-159, A29C) was developed based on previous reported procedure.<sup>[4]</sup> The gene fragment was amplified by PCR from a pJGetit-based vector encoding the gene of EcDHFR. Forward primer (5'-GATCTCCATATGTGCTGGTTTAAACGCAACAC-3') encoding an *Nde*I restriction site and reverse primer (5'-GAGATTCTGGAGCGGCGGCTCGTACCTCGTGGATCTCTCG-AGCACCAC-3') encoding an *Xho*I restriction site were used. The PCR product was cloned into the pET31b vector (Novagen) using the corresponding restriction sites. The resulting plasmid pCTecDHFR, which encodes DHFR (29-159) fused with a thrombin recognition site (Leu-Val-Pro-Arg-Gly-Ser) and a polyhistidine tag (His<sub>6</sub>), was shown to be free of mutations as indicated by DNA sequencing.

For protein expression, *E. coli* BL21(DE3) Star cells transformed with pCTecDHFR was used. An overnight culture of the relevant cells in LB medium containing 100  $\mu\text{g}/\text{mL}$  ampicillin were washed once with M9 medium and then grown in 1 L M9 medium at 37  $^\circ\text{C}$  with the same antibiotic selection until the OD<sub>600</sub> reached 0.6. IPTG (1 mM) was added and the culture grown at 30  $^\circ\text{C}$  to an OD<sub>600</sub> of 2.0. Cells were harvested by centrifugation at 16,000 rpm, resuspended in lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl) and lysed by sonication. The pellet containing the desired fusion peptide was washed with lysis buffer containing 1.0% Triton X-100 and centrifuged 16,000 rpm. The pellet was resolubilized in loading buffer (8 M urea, 50 mM Tris, pH 8.0, 150 mM NaCl) and purified by Ni<sup>2+</sup> fast flow chromatography. The desired cysteine peptide was eluted with loading buffer containing 500 mM imidazole. The crude peptide was stirred overnight with MeO-NH<sub>2</sub> (250 mM) and TCEP (50 mM) at pH 8.0 and 37  $^\circ\text{C}$  to remove the thiazolidine adduct, similar to the previously described procedure.<sup>[4]</sup> The peptide was further purified using HPLC conditions as described above. Isotopically substituted (heavy) cysteine peptide was produced as described for the unlabeled peptide, except that [ $^2\text{H}_2$ , 99.9%]  $\text{H}_2\text{O}$ , [ $^{15}\text{N}$ , 98%]  $\text{NH}_4\text{Cl}$  and [ $^{13}\text{C}_6$ , 99%; 1,2,3,4,5,6- $^2\text{H}_7$ , 97%] glucose were used to prepare the M9 medium. The purity and molecular masses of the C-terminal EcDHFR peptide were assessed using the LC-MS system described above. The unlabeled and labeled peptides showed molecular masses of  $16699.7 \pm 2.0$  and  $18482.1 \pm 2.1$ , respectively (calculated: 16698.6 and 18505.4, respectively) (Figure S3). Typically 12-18 mg of peptide were obtained per liter of cell culture.

**Chemical ligation to form full-length EcDHFR.** Unlabeled, "light" EcDHFR was generated by expressed protein ligation of the two unprotected peptide segments and purification of the product 174-residue polypeptide, followed by folding and purification. N-terminal EcDHFR- $\alpha$ -

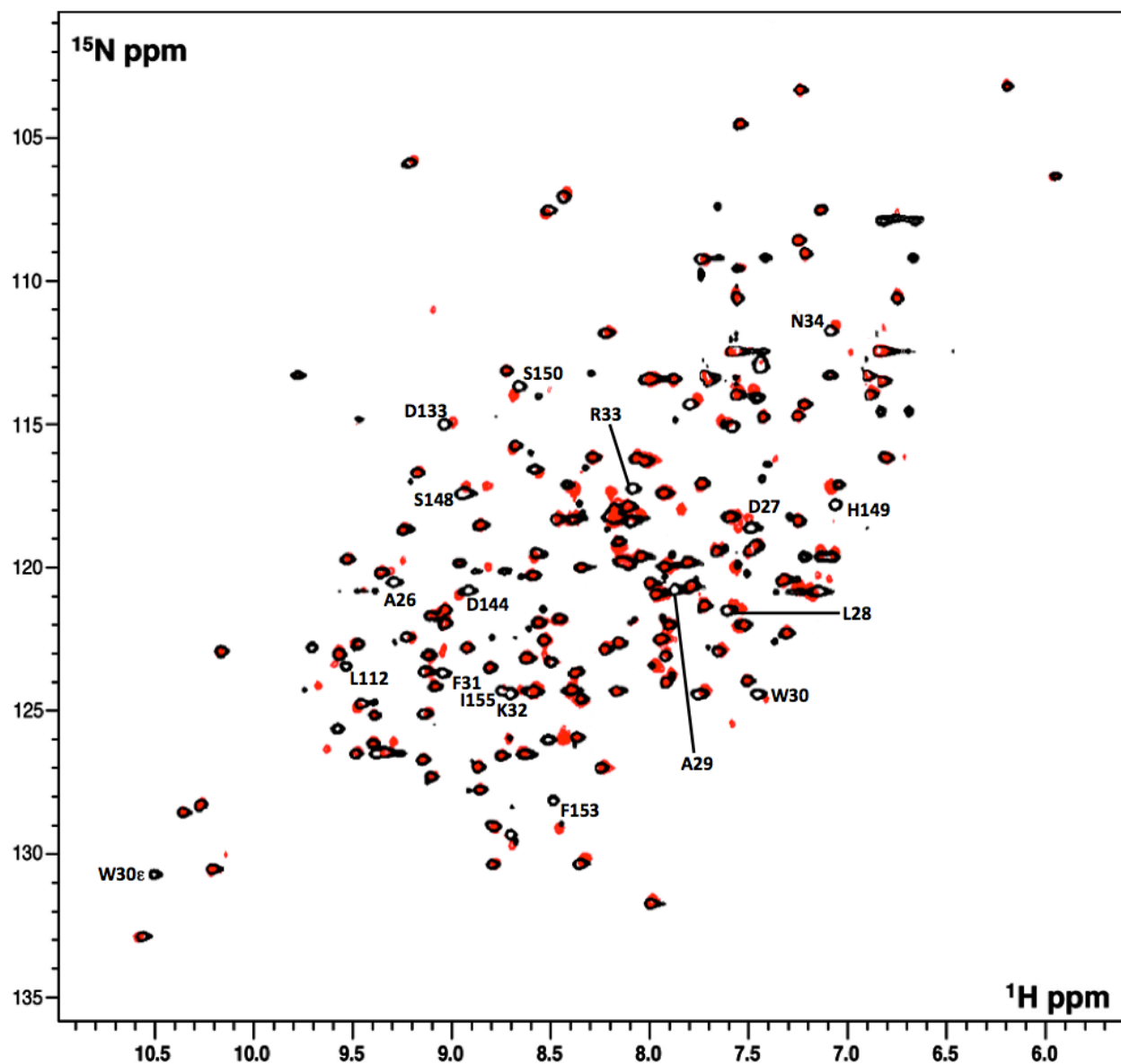
COSCH<sub>2</sub>CH<sub>2</sub>SO<sub>3</sub>H (4.3 mg, 1.31 μmol) and C-terminal EcDHFR cysteine peptide (A29C) (26.7 mg, 1.60 μmol) were dissolved in 2 mL of ligation buffer (6 M guanidine HCl, 0.2 M Na<sub>2</sub>HPO<sub>4</sub>, 10 mM TCEP hydrochloride, 10 mM MPAA). The pH was adjusted to 7.0 and the reaction kept at room temperature for 24 h. The linear polypeptide was dialyzed against folding buffer (50 mM potassium phosphate buffer, 100 mM NaCl, 5 mM DTT) purged under nitrogen. The unreacted cysteine peptide precipitated during the dialysis and was removed by centrifugation. Folded, chemically ligated EcDHFR was obtained after subjecting the crude product to size-exclusion chromatography using a Superdex75 column (GE Healthcare) in 50 mM potassium phosphate (pH 7.0) containing 5 mM DTT (yield = 4.0 mg, 0.2 μmol, 15% based on limiting peptide). The purity and molecular masses of the enzyme were assessed by LC-MS using conditions as described above (observed: 19836.7 ± 0.9, calculated: 19837.4).

The same procedure was used to generate the isotopic hybrids. For preparation of the "heavy" N-terminal EcDHFR (NT-EcDHFR), isotopically labeled N-terminal EcDHFR- $\alpha$ -COSCH<sub>2</sub>CH<sub>2</sub>SO<sub>3</sub>H (2.4 mg, 0.66 μmol) and natural abundance C-terminal EcDHFR cysteine peptide (7.7 mg, 0.46 μmol) were employed (yield = 2.1 mg, 0.10 μmol, 21% based on limiting peptide). For preparation of the "heavy" C-terminal EcDHFR (CT-EcDHFR), natural abundance N-terminal EcDHFR- $\alpha$ -COSCH<sub>2</sub>CH<sub>2</sub>SO<sub>3</sub>H (2.3 mg, 0.7 μmol) and isotopically labeled C-terminal EcDHFR cysteine peptide (12.8 mg, 0.69 μmol) were employed (yield = 3.3 mg, 0.15 μmol, 21% based on limiting peptide). The "heavy" N-terminal and C-terminal EcDHFR revealed molecular masses of 20196.6 ± 1.3 and 21620.6 ± 1.9, respectively (Figure S4).

**Circular dichroism spectroscopy.** Circular dichroism experiments were performed on an Applied PhotoPhysics Chirascan spectrometer using 16 μM protein in deoxygenated 10 mM potassium phosphate buffer (pH 7.0) at 20 °C. Spectra were measured between 190 nm and 400 nm in 10 mm quartz cuvettes under N<sub>2</sub> with a 50 nm/min scan speed, 0.5 nm data pitch, 1 nm bandwidth and 0.5 s response time (Figure S5).

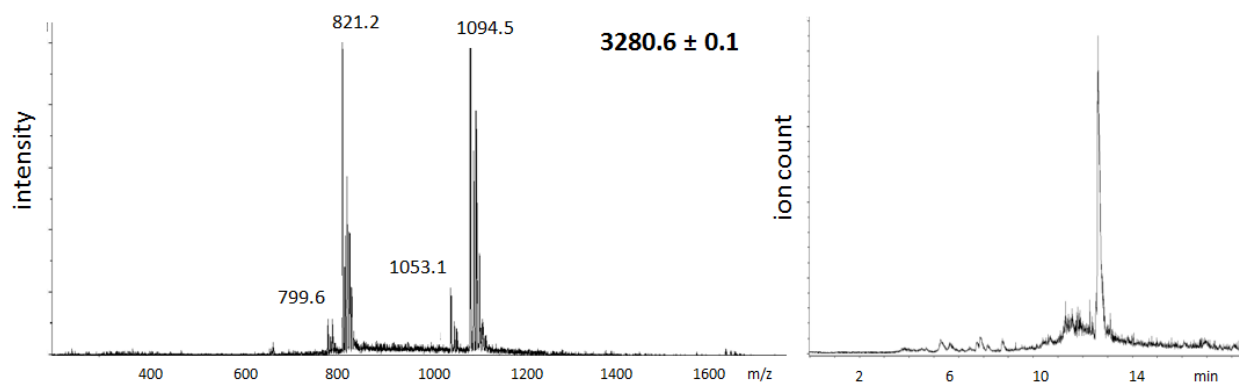
**Steady-state kinetic measurements.** Steady-state kinetic measurements were performed on a Shimadzu UV-2401PC spectrophotometer as described,<sup>[5]</sup> monitoring the decrease in absorbance at 340 nm during the reaction ( $\epsilon_{340}(\text{NADPH} + \text{H}_2\text{F}) = 11800 \text{ M}^{-1} \text{ cm}^{-1}$ ).<sup>[6]</sup> Because the chemically ligated enzymes are prone to oxidation, all the reaction buffers were always thoroughly degassed and 5 mM DTT added before use. The steady-state turnover rates were determined at pH 7.0 in 50 mM potassium phosphate buffer (containing 100 mM NaCl and 5 mM DTT) using 0.3-60 nM enzyme. The enzymes were preincubated with NADPH (250 μM) at the desired temperature for 2 min prior to addition of H<sub>2</sub>F (100 μM). Each data point (Figure S6) is the result of at least nine independent measurements. In order to determine Michaelis constants at pH 7.0, concentrations of NADPH were varied between 3 and 100 μM, and concentrations of H<sub>2</sub>F were varied between 0.5 and 100 μM, whilst keeping the concentration of the other reactant fixed at saturating concentration.

**Pre-steady-state kinetic measurements.** Hydride transfer rate constants were measured under single-turnover conditions on a Hi-Tech Scientific stopped-flow spectrophotometer as previously described.<sup>[7]</sup> Before mixing, the enzyme (7.5 μM) was preincubated with NADPH (1.5 μM) for at least 5 min in 100 mM potassium phosphate containing 100 mM NaCl and 5 mM DTT at pH 7.0, and the reaction started by rapidly mixing with H<sub>2</sub>F (200 μM) in the same buffer. Reduction of fluorescence of NADPH at 340 nm during the reaction was measured. All measurements were repeated at least twelve times. Rate constants (Figure S6) were extracted by fitting the kinetic data to the equation for a double-exponential decay.

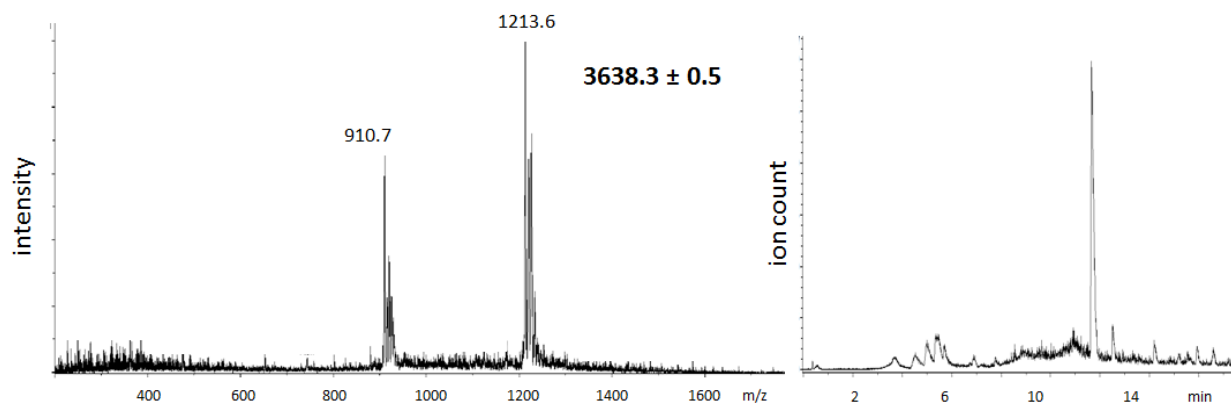


**Fig. S1.**  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra of the DHFR:NADP $^+$ :folate complexes of EcDHFR (black) and EcDHFR-A29C (red) at 25 °C. Contours are set so that EcDHFR resonances appear open, to show areas of resonance overlap. Residues showing the largest chemical shift perturbations are indicated. These are all localized around the mutation site or in neighboring regions of the enzyme.

### Light N-terminal EcDHFR- $\alpha$ -thioester

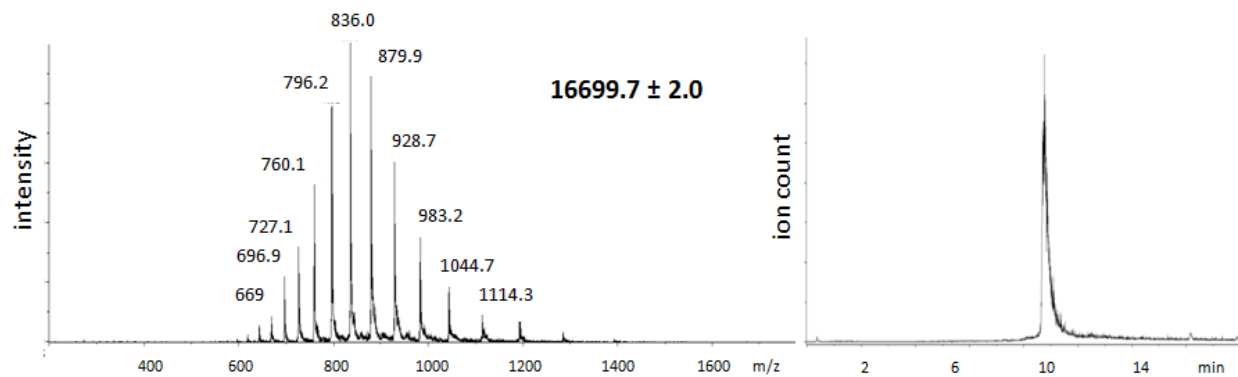


### Heavy N-terminal EcDHFR- $\alpha$ -thioester

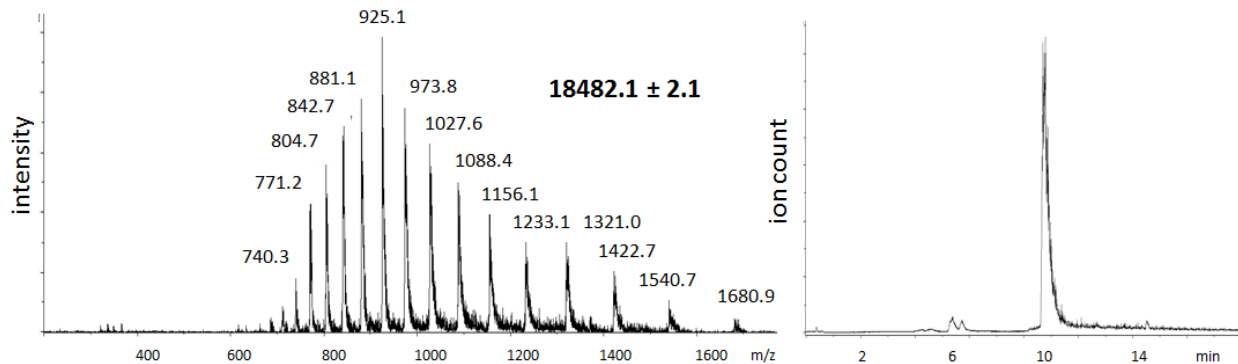


**Fig. S2.** Mass spectra (left) and total ion count chromatograms (right) of light and heavy N-terminal EcDHFR- $\alpha$ -thioester peptides.

**Light C-terminal EcDHFR (Ala<sup>29</sup>-Cys) peptide**

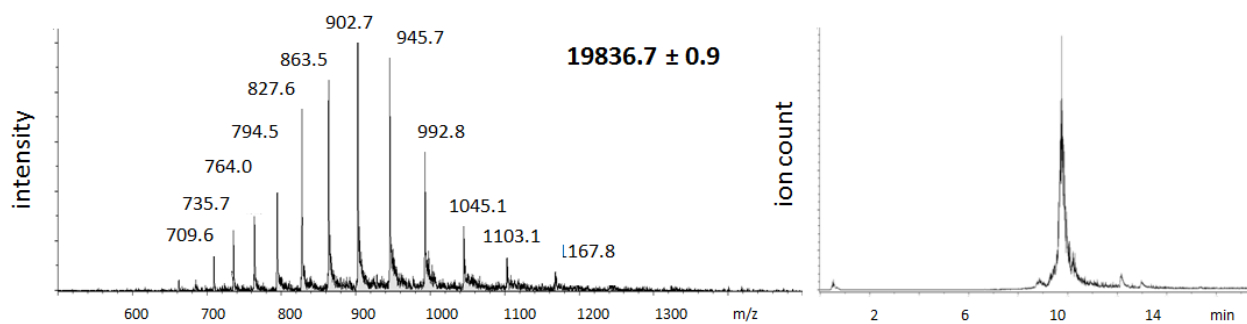


**Heavy C-terminal EcDHFR (Ala<sup>29</sup>-Cys) peptide**

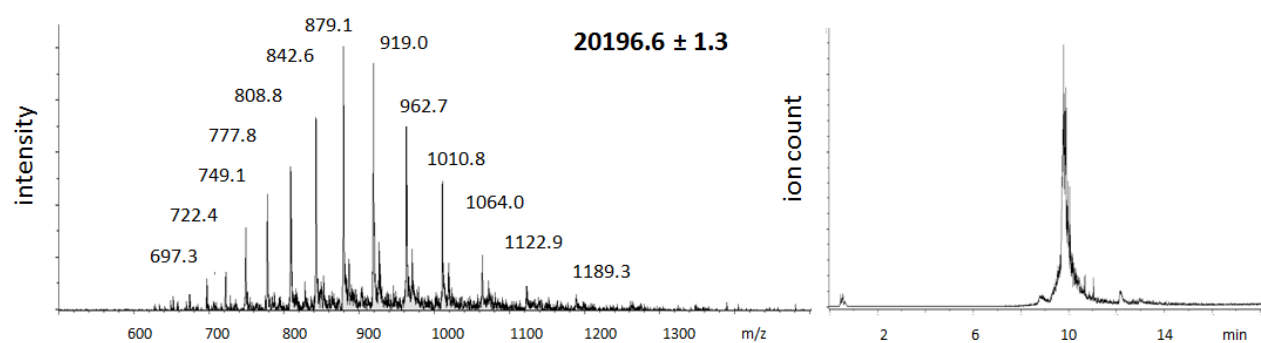


**Fig. S3.** Mass spectra (left) and total ion count chromatograms (right) of light and heavy C-terminal EcDHFR peptides.

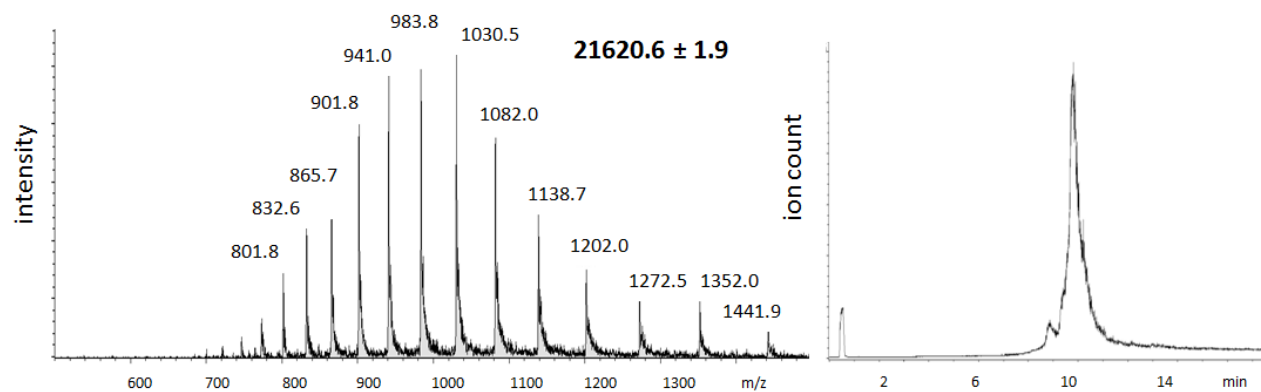
### Light, chemically ligated EcDHFR



### Heavy N-terminal EcDHFR

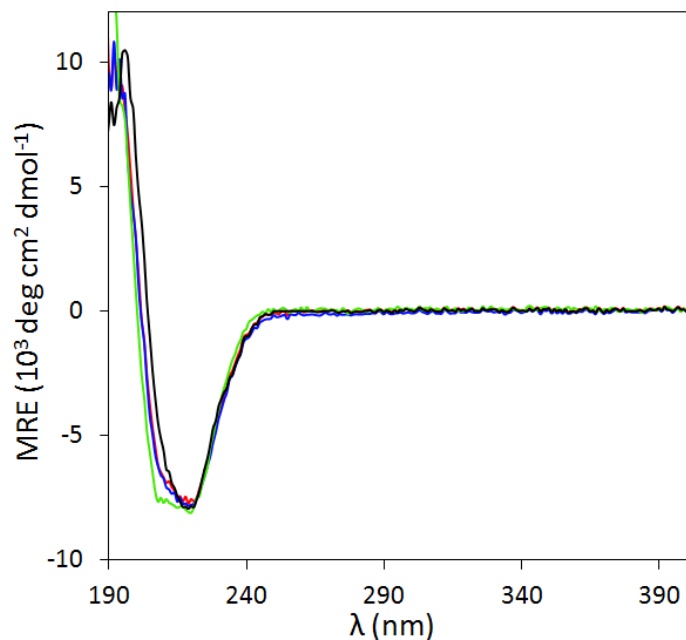


### Heavy C-terminal EcDHFR

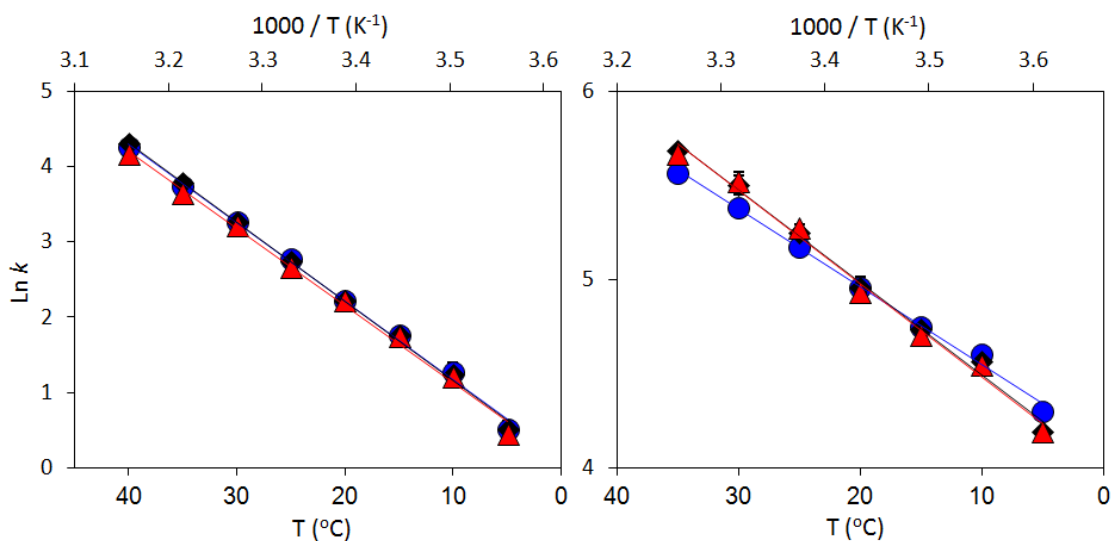


**Fig. S4.** Mass spectra (left) and total ion count chromatograms (right) of light chemically ligated EcDHFR and the corresponding isotopic hybrids.





**Fig. S5.** Circular dichroism spectra of light, chemically ligated EcdHFR (blue), NT-EcdHFR (red), CT-EcdHFR (green) and the light, wild type counterpart (black), measured in 10 mM potassium phosphate at pH 7.0, using 16  $\mu$ M protein.



**Fig. S6.** Arrhenius plots for  $k_{cat}$  at pH 7 (left) and  $k_H$  at pH 7 (right), for unlabeled, light EcdHFR (black diamonds), NT-EcdHFR (red triangles) and CT-EcdHFR (blue circles).

Table S1. Temperature dependence of the pre-steady-state rate constants ( $k_H$ ) for wild type EcDHFR and its A29C variant at pH 7.0.

Temp (°C)	$k_H / s^{-1}$	
	Wild type <sup>[a]</sup>	A29C
5	83.0 ± 3.1	85.5 ± 1.8
10	111.7 ± 1.3	108.1 ± 3.1
15	141.2 ± 1.8	134.6 ± 0.9
20	178.5 ± 2.9	181.0 ± 11.3
25	222.5 ± 6.6	207.2 ± 7.6
30	288.3 ± 33.0	255.8 ± 14.4
35	367.3 ± 23.1	316.5 ± 12.2

<sup>a</sup> Data from ref<sup>[8]</sup>

Table S2. Michaelis constants for light, ligated EcDHFR, NT-EcDHFR, CT-EcDHFR and EcDHFR-A29C (without His-tag) at pH 7.0

	Light	Heavy N-terminal	Heavy C-terminal
20 °C			
$K_M$ NADPH ( $\mu$ M)	7.6 ± 2.4	2.5 ± 0.5	5.80 ± 1.7
$K_M$ DHF ( $\mu$ M)	0.38 ± 0.10	0.17 ± 0.10	0.24 ± 0.04
35 °C			
$K_M$ NADPH ( $\mu$ M)	6.17 ± 1.42	1.78 ± 0.66	8.35 ± 2.10
$K_M$ DHF ( $\mu$ M)	0.69 ± 0.25	0.84 ± 0.10	1.03 ± 0.20

Table S3. Temperature dependence of the steady-state rate constants ( $k_{\text{cat}}$ ) and protein/segment isotope effects for light, ligated EcDHFR, NT-EcDHFR and CT-EcDHFR at pH 7.0.

Temp (°C)	$k_{\text{cat}} / \text{s}^{-1}$			Protein/segment isotope effect		
	Light	NT-EcDHFR	CT-EcDHFR	Whole enzyme <sup>a</sup>	Heavy N-terminal	Heavy C-terminal
5	1.66 ± 0.13	1.55 ± 0.07	1.66 ± 0.19		1.07 ± 0.10	1.00 ± 0.05
10	3.46 ± 0.54	3.33 ± 0.19	3.53 ± 0.03	0.98 ± 0.09	1.04 ± 0.09	0.98 ± 0.04
15	5.76 ± 0.16	5.75 ± 0.40	5.82 ± 0.13	1.01 ± 0.04	1.00 ± 0.04	0.99 ± 0.04
20	9.12 ± 0.57	9.13 ± 0.27	9.12 ± 0.07	1.04 ± 0.03	1.00 ± 0.03	1.00 ± 0.03
25	15.57 ± 0.36	14.33 ± 0.49	16.05 ± 0.33	1.06 ± 0.01	1.09 ± 0.02	0.97 ± 0.02
30	25.98 ± 1.21	25.07 ± 0.45	25.98 ± 1.74	1.11 ± 0.03	1.04 ± 0.03	1.00 ± 0.03
35	43.73 ± 1.82	38.43 ± 0.99	42.25 ± 3.45	1.16 ± 0.01	1.14 ± 0.03	1.04 ± 0.04
40	74.08 ± 2.58	63.99 ± 4.68	71.23 ± 2.12	1.16 ± 0.02	1.16 ± 0.05	1.04 ± 0.02
Ea (kcal mol <sup>-1</sup> )	18.3 ± 0.2	17.7 ± 0.1	18.0 ± 0.1			
ΔEa (kcal mol <sup>-1</sup> )				1.1 ± 0.2	0.6 ± 0.1	0.3 ± 0.1

<sup>a</sup> Data from ref<sup>[9]</sup>

Table S4. Temperature dependence of the pre-steady-state rate constants ( $k_{\text{H}}$ ) and protein/segment isotope effects for light, ligated EcDHFR, NT-EcDHFR and CT-EcDHFR at pH 7.0.

Temp (°C)	$k_{\text{H}} / \text{s}^{-1}$			Protein/segment isotope effect		
	Light	NT-EcDHFR	CT-EcDHFR	Whole enzyme <sup>a</sup>	Heavy N-terminal	Heavy C-terminal
5	66.2 ± 0.7	66.2 ± 1.6	73.4 ± 1.6	0.92 ± 0.02	1.00 ± 0.03	0.90 ± 0.02
10	95.8 ± 1.5	94.2 ± 2.5	99.7 ± 2.6	0.93 ± 0.03	1.02 ± 0.03	0.96 ± 0.03
15	113.7 ± 0.5	110.6 ± 1.9	115.3 ± 2.4	0.91 ± 0.02	1.03 ± 0.02	0.99 ± 0.02
20	141.8 ± 8.2	138.2 ± 0.9	142.3 ± 1.1	0.92 ± 0.06	1.03 ± 0.06	1.00 ± 0.06
25	189.6 ± 4.3	194.6 ± 6.9	175.9 ± 2.0	1.10 ± 0.03	0.97 ± 0.04	1.08 ± 0.03
30	245.0 ± 12.1	249.8 ± 0.8	217.1 ± 6.4	1.10 ± 0.06	0.98 ± 0.05	1.13 ± 0.06
35	293.3 ± 5.3	289.4 ± 6.8	260.5 ± 4.3	1.07 ± 0.07	1.01 ± 0.02	1.13 ± 0.08
40		Not determined		1.18 ± 0.09	Not determined	

<sup>a</sup> Data from ref<sup>[9]</sup>

Table S5. Temperature dependence of the deuteride transfer rate constants and kinetic isotope effects for the reaction catalyzed by wild type EcDHFR, its A29C variant and light, ligated EcDHFR at pH 7.0.

Temp (°C)	$k_D / s^{-1}$ (NADPD)		
	Wild type <sup>a</sup>	A29C	Light, ligated enzyme
5	25.9 ± 0.7	26.9 ± 1.2	19.5 ± 0.5
10	37.2 ± 8.1	35.7 ± 1.5	28.3 ± 1.5
15	48.1 ± 5.7	47.2 ± 0.12	36.2 ± 2.6
20	66.1 ± 2.8	64.0 ± 5.1	46.6 ± 1.5
25	83.3 ± 4.6	79.62 ± 4.0	64.5 ± 8.3
30	117.3 ± 3.1	111.2 ± 2.2	84.8 ± 1.3
35	172.2 ± 19.0	152.9 ± 9.2	111.6 ± 1.7
Hydride KIE ( $k_H/k_D$ )			
5	3.20 ± 0.04	3.17 ± 0.14	3.40 ± 0.10
10	3.00 ± 0.22	3.03 ± 0.11	3.39 ± 0.10
15	2.93 ± 0.12	2.85 ± 0.10	3.14 ± 0.11
20	2.70 ± 0.05	2.83 ± 0.25	3.04 ± 0.09
25	2.67 ± 0.06	2.60 ± 0.11	2.94 ± 0.19
30	2.45 ± 0.12	2.30 ± 0.12	2.89 ± 0.10
35	2.13 ± 0.13	2.07 ± 0.15	2.63 ± 0.03

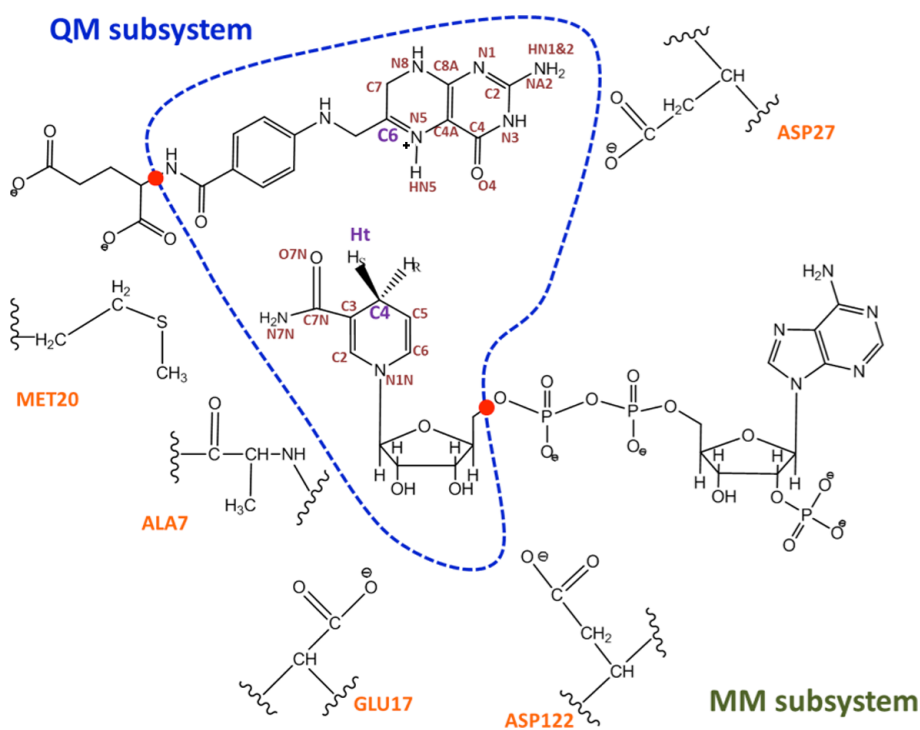
<sup>a</sup> Data from ref<sup>[8]</sup>

## Computational Details

**The simulation model.** Details of the computational model are given elsewhere.<sup>[10]</sup> Briefly, we employed a hybrid quantum mechanics/molecular mechanics (QM/MM) description for our system. The starting structure for dynamics simulations was obtained from the Protein Data Bank entry 3QL3.<sup>[11]</sup> The PROPKA3 program<sup>[12]</sup> was used to estimate the  $pK_a$  values of the titratable protein residues to verify their protonation states at pH 7 being histidines 45, 114, and 141 doubly protonated whereas all other histidine residues were protonated only on N $\delta$  or N $\epsilon$ . To neutralize the system, 13 sodium counterions were placed in optimal electrostatic positions around the enzyme. Finally, the system was solvated using a cubic box of water molecules with side length of 65.0 Å where water molecules with an oxygen atom found within 2.8 Å of any protein heavy atom was removed. The full system contained 27219 atoms, including the protein (159 residues, 2544 atoms), the substrate and cofactor (52 and 74 atoms, respectively), 13 sodium ions and 8196 water molecules (24132 atoms). Heavy EcDHFR was prepared *in silico* by modifying the masses of all <sup>14</sup>N, <sup>12</sup>C and non-exchangeable <sup>1</sup>H atoms to those of <sup>15</sup>N, <sup>13</sup>C, <sup>2</sup>H. Isotopically labeled NT-EcDHFR enzyme was prepared *in silico* by replacing the non-exchangeable <sup>14</sup>N, <sup>12</sup>C and <sup>1</sup>H atoms in residues 1 to 28 with <sup>15</sup>N, <sup>13</sup>C, <sup>2</sup>H isotopes respectively, whereas CT-DHFR enzyme was prepared by changing the mass of the corresponding non-exchangeable atoms from residues 29 to 159. The ratio of the mass of the simulated heavy enzyme to that of the light counterpart was 1.10987, which corresponds to the 100% isotope incorporation and is close to the experimentally observed molecular weight increase.<sup>[9]</sup> For NT-

EcDHFR and CT-EcDHFR, the corresponding ratios were 1.01944 and 1.09043 respectively, which are also close to the experimentally observed molecular weight increase.

In all the versions of the enzyme, the whole system was divided into a QM part and a MM part to perform combined QM/MM calculations (Figure S7). The quantum subsystem contained 76 atoms, including parts of the cofactor (nicotinamide ring and the ribose) and the substrate (pteridine ring and the N-methylene-substituted *p*-aminobenzoyl, pABA). Two hydrogen ‘link’ atoms were used to saturate the valence at the QM-MM boundary. The quantum atoms were treated by the AM1 Hamiltonian,<sup>[13]</sup> modified using specific reaction parameters (denoted as AM1-SRP) developed by Major and coworkers for DHFR.<sup>[14]</sup> The protein atoms and the ions were described by OPLS-AA<sup>[15]</sup> force field while the water molecules were described by the TIP3P potential.<sup>[16]</sup> 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 within the minimum image convention in all the simulations.



**Fig S7.** Schematic representation of the active site and definition of the QM/MM subsystem

*Calculation of the transmission coefficient.* Grote-Hynes (GH) theory can be applied to describe the evolution of the system along the reaction coordinate at the TS. In particular, the transmission coefficient can be obtained as the ratio between the reactive frequency and the equilibrium barrier frequency<sup>[17]</sup>:

$$\gamma_{GH} = \frac{\omega_r}{\omega_{eq}} \quad (S1)$$

with the equilibrium frequency derived from a parabolic fit of the PMF around the maximum and the reactive frequency  $\omega_r$  is obtained via the GH equation.<sup>[18]</sup>

$$\omega_r^2 - \omega_{eq}^2 + \omega_r \int_0^\infty \zeta_{TS}(t) e^{-\omega_r t} dt = 0 \quad (\text{S2})$$

$\zeta_{TS}(t)$  is the friction kernel obtained at the TS, assuming that recrossings take place in the proximity of this dynamic bottleneck:<sup>[18b, 19]</sup>

$$\zeta(t) = \frac{\langle F_{RC}(0)F_{RC}(t) \rangle}{\mu_{RC}k_B T} \quad (\text{S3})$$

$F_{RC}(t)$  is the force projected on the reaction coordinate (the antisymmetric combination of H<sub>r</sub>-C4 and H<sub>r</sub>-C6 distances) and  $\mu_{RC}$  the associated reduced mass. Initially we performed 100 ps of QM/MM molecular dynamics at 278, 283, 288, 293, 298, 303, 308, 313 and 318 K, constraining the reaction coordinate at the top of the PMF calculated in our previous work.<sup>[10]</sup> At each temperature, we also performed one dimensional PMF calculations in the vicinity of the transition state region. Finally, for the evaluation of the TS friction kernel, we ran 25 ps of constrained MD simulations at the top of the PMF. The simulations were carried out at each temperature using a Wilson's matrix-based RATTLE-like Velocity-Verlet algorithm.<sup>[20]</sup> A small time step of 0.05 fs was used to ensure the convergence of the algorithm and forces acting on the reaction coordinate were saved at each simulation step. We previously tested that the GH approach gives transmission coefficients in very good agreement with those obtained from activated trajectories initiated at the TS ensemble.<sup>[21]</sup> In the case of EcDHFR estimations obtained using the GH equation provide transmission coefficients close to those previously reported based on the analysis of free reactive trajectories.<sup>[10]</sup> Thus, the recrossing transmission coefficients,  $\gamma$ , were calculated using eq. S1 for the light and heavy versions of the EcDHFR enzyme prepared as described above. Values obtained at different temperatures are presented in Table S4, and the calculated isotope effects are presented in Table S5.

The measured recrossing coefficients could be used to explain the differences in the activation parameters. Both CT-EcDHFR and completely labeled 'heavy' EcDHFR have more negative activation entropies ( $\Delta S^\ddagger$ ) than the 'light' enzymes (Table 1).  $\Delta S^\ddagger$  is expected to be negative, because during the chemical transformation the enzyme needs to sacrifice its flexibility to provide a static, charge-complementary configuration.<sup>[22]</sup> However, the activation entropy is also affected by the intensity of dynamic coupling, as it is dependent on the magnitude and the temperature dependence of the recrossing coefficient, the contribution from which is described in equation (5):<sup>[22-23]</sup>

$$\Delta S^\ddagger(\gamma) = R \cdot \ln(\gamma) + \frac{RT}{\gamma} \cdot \frac{\partial \gamma}{\partial T} \quad (5)$$

where  $\Delta S^\ddagger(\gamma)$  is the fraction of activation entropy that depends on dynamic recrossing and  $\frac{\partial \gamma}{\partial T}$  is the change of the recrossing coefficient with temperature. As illustrated in the computational analysis, the recrossing coefficient deviates further from unity with increasing temperature (*i.e.*  $\frac{\partial \gamma}{\partial T} < 0$ ) due to thermal activation of protein motions that disturb the stability of the transition state. This effect is particularly pronounced in 'heavy' EcDHFR and in CT-EcDHFR, and the

magnitude of  $\frac{\partial\gamma}{\partial T}$  is approximately three-fold higher than for the ‘light’ enzyme (Table S4). Consequently, the activation entropies of these isotopically labeled enzymes are greater in magnitude and the hydride transfer rate constant becomes noticeably lower than for the ‘light’ enzyme at high temperature. Nevertheless, the activation free energies ( $\Delta G^\ddagger$ ) are identical for these enzymes as the activation enthalpies ( $\Delta H^\ddagger$ ) of both ‘heavy’ EcDHFR and CT-EcDHFR are lower than their ‘light’ counterparts. On the other hand, the activation free energy also remains unchanged in NT-EcDHFR since none of the mentioned parameters were affected by isotope labeling. These observations strongly suggest that the protein environmental motions along the EcDHFR reaction coordinate originate from the C-terminal segment.

Table S6. Recrossing coefficients ( $\gamma$ ) of light and heavy EcDHFR evaluated using GH theory.

Temp (K)	Light EcDHFR	NT-EcDHFR	CT-EcDHFR	Heavy EcDHFR
278	0.68 ± 0.01	0.67 ± 0.01	0.66 ± 0.01	0.65 ± 0.02
283	0.67 ± 0.01	0.63 ± 0.02	0.62 ± 0.02	0.59 ± 0.01
288	0.63 ± 0.01	0.62 ± 0.02	0.57 ± 0.01	0.57 ± 0.01
293	0.61 ± 0.01	0.60 ± 0.02	0.55 ± 0.01	0.55 ± 0.03
298	0.63 ± 0.02	0.58 ± 0.01	0.57 ± 0.02	0.55 ± 0.01
303	0.63 ± 0.02	0.58 ± 0.01	0.56 ± 0.01	0.55 ± 0.02
308	0.62 ± 0.01	0.57 ± 0.02	0.55 ± 0.01	0.51 ± 0.01
313	0.64 ± 0.01	0.64 ± 0.01	0.54 ± 0.01	0.54 ± 0.01
318	0.60 ± 0.01	0.59 ± 0.01	0.50 ± 0.01	0.47 ± 0.01
$\frac{\partial\gamma}{\partial T}(\text{K}^{-1})$	0.0013	0.0014	0.0031	0.0033

Table S7. Theoretical segment and enzyme KIE ( $\gamma^{\text{LE}}/\gamma^{\text{HE}}$ ).

Temp (K)	NT-EcDHFR	CT-EcDHFR	Heavy EcDHFR
278	1.01 ± 0.02	1.03 ± 0.02	1.05 ± 0.04
283	1.06 ± 0.04	1.07 ± 0.04	1.13 ± 0.03
288	1.02 ± 0.04	1.11 ± 0.03	1.11 ± 0.03
293	1.02 ± 0.04	1.10 ± 0.03	1.11 ± 0.06
298	1.08 ± 0.04	1.11 ± 0.05	1.14 ± 0.04
303	1.07 ± 0.04	1.11 ± 0.04	1.13 ± 0.05
308	1.08 ± 0.04	1.12 ± 0.03	1.20 ± 0.03
313	1.01 ± 0.02	1.18 ± 0.03	1.20 ± 0.03
318	1.02 ± 0.02	1.21 ± 0.03	1.29 ± 0.03

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