Supplementary online data for

Different dynamical effects in mesophilic and hyperthermophilic dihydrofolate reductases

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Experimental Methods

Chemicals. ^{15}N-ammonium chloride, [$^{13}C_6$,$^{2}H_7$]-glucose, 99.9% $^{2}H_2O$ and folate were purchased from Sigma-Aldrich. NADPH, $NADP^+$ and isopropyl-β-D-thiogalactopyranoside (IPTG) were purchased from Melford (Ipswich, UK). H_2F was prepared by dithionite reduction of folate.[1] The concentrations of NADPH and H_2F 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. TmDHFR, and ^{15}N-, ^{13}C-, ^{2}H-labelled (heavy) TmDHFR were prepared in M9 media using a modification of the protocol described by Falzone *et. al.*[3] *E. coli* BL21(DE3)-RP harboring a cDNA for TmDHFR[4] from an overnight culture in LB medium containing 100 μg/mL ampicillin and 35 μg/mL chloramphenicol were washed three times with M9 medium and then grown in 1 L M9 medium until the OD_{600} reached 0.6. 0.5 mM IPTG was added and the culture grown to an OD_{600} of 2.0. The cells were harvested and the enzyme purified using HiPrep 16/10 SP XL cation-exchange column (GE Healthcare) as previously described.[4] Protein concentrations were determined spectrophotometrically assuming an extinction coefficient of 22 800 M^{-1} cm^{-1} at 280 nm.[5] Electrospray ionization mass spectrometry (ESI-MS) indicated masses of 19392.2 and 21443.6 Da for light and heavy enzymes, respectively (Figure S2). Quality of the mass spectra was somewhat compromised due to enzyme precipitation in mass spectrometry solvent (1:1 H_2O:acetonitrile + 0.1% trifluoroacetic acid).

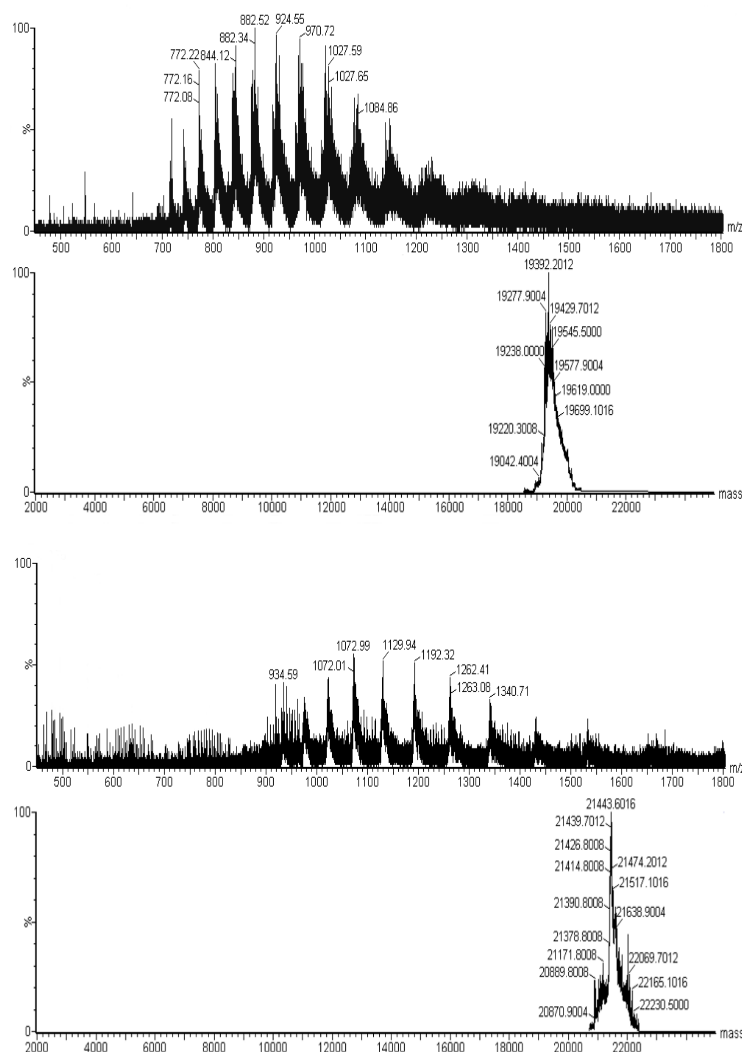

Figure S1. Electrospray ionization mass spectrometry of light (top two panels) and heavy (bottom two panels) TmDHFR. For each enzyme the raw (upper panel) and deconvoluted (lower panel) spectrum is shown.

Circular dichroism spectroscopy. Circular dichroism experiments were performed on an Applied PhotoPhysics Chirascan spectrometer using 10-12 μM protein in deoxygenated 10 mM potassium phosphate buffer (pH 7). Spectra were measured between 200 nm and 400 nm in 10 mm quartz cuvettes under N_2 with a 50 nm/min scan speed, 0.5 nm data pitch, 1 nm bandwidth and 0.5 s response time.

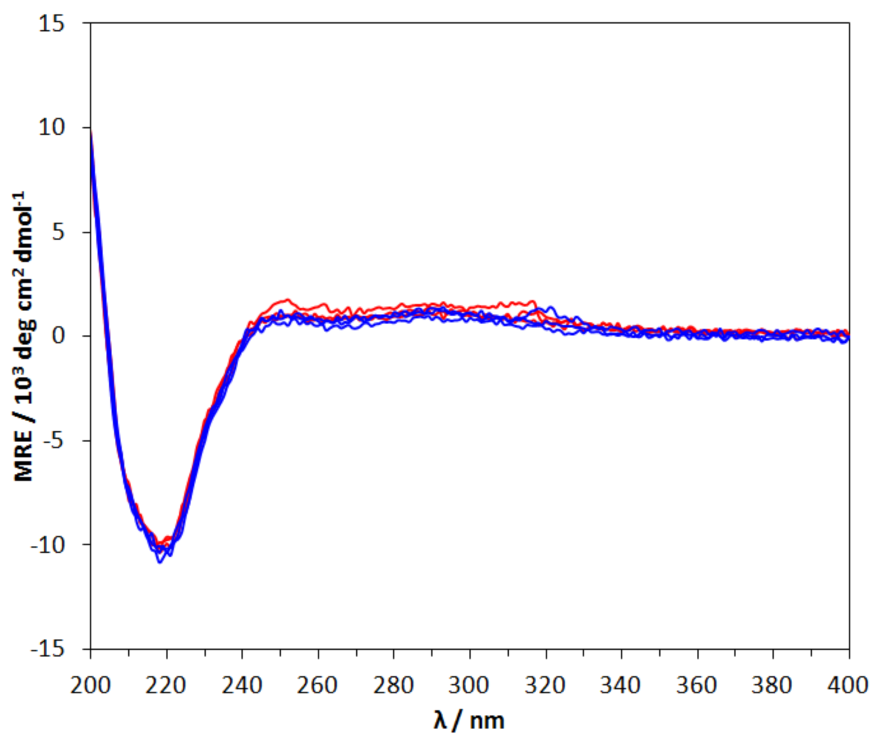

Figure S2. Circular dichroism spectroscopy of light (red) and heavy (blue) TmDHFR (three scans each), measured in 10 mM potassium phosphate buffer at pH 7 and 20 ^{o}C using 12 μM protein.

Steady-state kinetic measurements. Steady-state kinetic measurements were performed on a JASCO V-660 spectrophotometer as described,[6] monitoring the decrease in absorbance at 340 nm during the reaction (ε_{340} (NADPH + H_2F) = 11800 M^{-1} cm^{-1}).[7] The steady-state turnover rates of TmDHFR were determined at pH 7 in 50 mM potassium phosphate buffer (100 mM NaCl and 5 mM β-mercaptoethanol) or in MTEN buffer (50 mM morpholinoethanesulfonic acid, 25 mM Tris, 25 mM ethanolamine, 100 mM NaCl and 5 mM β-mercaptoethanol) using 0.3-60 nM enzyme. The enzymes were preincubated with NADPH (100 μM) at the desired temperature for 5 min prior to addition of H_2F (100 μM). Each data point is the result of three independent measurements. In order to determine Michaelis constants at pH 7, concentrations of NADPH or H_2F were varied between 0.1 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 by measuring the reduction of fluorescence resonance energy transfer (FRET) from the enzyme to NADPH on a Hi-Tech Scientific stopped-flow spectrophotometer as previously described.[8] Before mixing, the enzyme (20 μM) was preincubated with NADPH (8 μM) for at least 1 min in 100 mM potassium phosphate containing 100 mM NaCl and 5 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-mercaptoethanol) for pH-dependent measurements, and the reaction started by rapidly mixing with H_2F (200 μM) in the same buffer. Where MTEN buffer was used, the pH was carefully adjusted at each experimental temperature to account for the temperature dependence of the pK_a of organic amines. The sample was excited at 297 nm and measured 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.

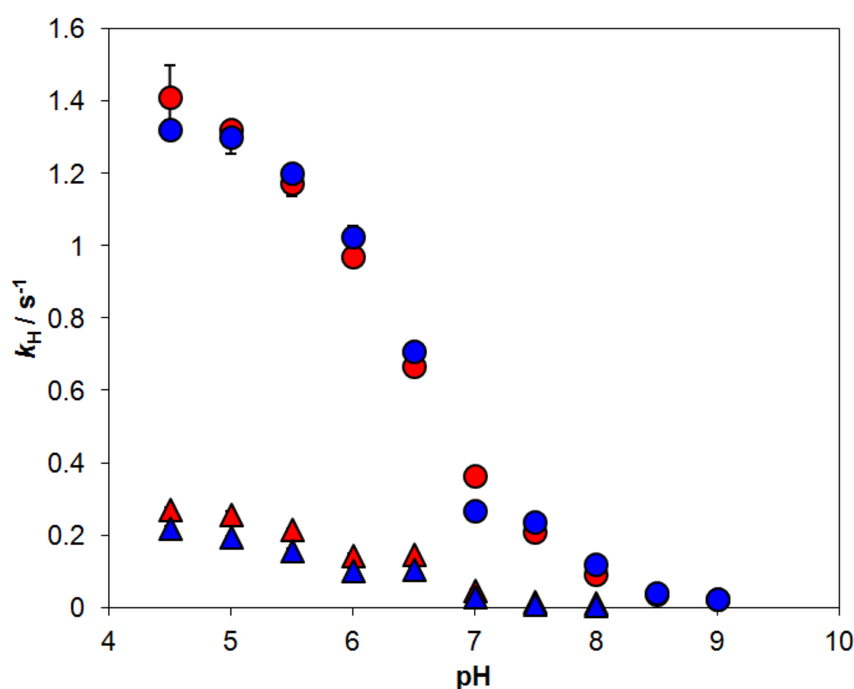

Figure S3. pH dependence of the pre-steady state turnover for the chemical step during catalysis by light (red) and heavy (blue) TmDHFR at 10 °C (triangles), and 40 °C (circles). All data points are the average of at least 6 measurements; the standard errors of the mean are given for one sigma.

Table S1: Temperature dependence of the steady-state rate constants (k_{cat}) and the pre-steady-state rate constants (k_H) at pH 7 during catalysis by light and heavy TmDHFR.

	Pre-steady state		Steady state	
T (ºC)	k_H^{LE} (s^{-1})	k_H^{HE} (s^{-1})	k_{cat}^{LE} (s^{-1})	k_{cat}^{HE} (s^{-1})
5	0.051 ± 0.001	0.051 ± 0.001		
7			0.041 ± 0.002	0.024 ± 0.002
10	0.060 ± 0.001	0.061 ± 0.001	0.046 ± 0.002	0.031 ± 0.002
15	0.086 ± 0.001	0.087 ± 0.002	0.067 ± 0.002	0.050 ± 0.001
20	0.128 ± 0.001	0.128 ± 0.001	0.090 ± 0.003	0.066 ± 0.003
25	0.189 ± 0.003	0.187 ± 0.003	0.139 ± 0.006	0.102 ± 0.003
30	0.269 ± 0.002	0.267 ± 0.003	0.205 ± 0.005	0.147 ± 0.001
35	0.380 ± 0.003	0.373 ± 0.004	0.279 ± 0.002	0.198 ± 0.001
40	0.547 ± 0.009	0.547 ± 0.005	0.362 ± 0.008	0.265 ± 0.009
45	0.743 ± 0.015	0.743 ± 0.011	0.488 ± 0.020	0.357 ± 0.015
50	0.997 ± 0.011	0.995 ± 0.012	0.633 ± 0.002	0.466 ± 0.018
55	1.313 ± 0.004	1.324 ± 0.025	0.792 ± 0.002	0.575 ± 0.050
60	1.731 ± 0.007	1.688 ± 0.044	1.026 ± 0.021	0.752 ± 0.044
70	2.197 ± 0.024	2.167 ± 0.045	1.211 ± 0.007	0.994 ± 0.005

Table S2: Temperature dependence of the heavy enzyme effect on the steady state constant (k_{cat}) and pre-steady state constant (k_H) at pH 7 during catalysis by TmDHFR.

T (ºC)	Pre-steady state k_H^{LE}/k_H^{HE}	Steady state $k_{cat}^{LE}/k_{cat}^{HE}$
5	0.992 ± 0.012	
7		1.73 ± 0.01
10	0.995 ± 0.012	1.49 ± 0.01
15	0.987 ± 0.009	1.33 ± 0.04
20	1.001 ± 0.008	1.37 ± 0.04
25	1.010 ± 0.011	1.36 ± 0.04
30	1.009 ± 0.007	1.39 ± 0.04
35	1.020 ± 0.007	1.41 ± 0.04
40	0.999 ± 0.010	1.37 ± 0.04
45	1.000 ± 0.013	1.37 ± 0.04
50	1.003 ± 0.008	1.36 ± 0.04
55	0.992 ± 0.009	1.38 ± 0.04
60	1.025 ± 0.014	1.38 ± 0.04
65	1.014 ± 0.012	1.22 ± 0.04

Table S3: Steady state kinetic parameters for light and heavy TmDHFR

	At 20 ºC	
	Light	Heavy
k_{cat} (s^{-1})	0.090 ± 0.003	0.066 ± 0.003
K_M (μM) NADPH	0.10 ± 0.02	0.13 ± 0.05
K_M (μM) DHF	0.32 ± 0.04	0.22 ± 0.07
	At 10 ºC	
	Light	Heavy
k_{cat} (s^{-1})	0.046 ± 0.002	0.031 ± 0.002
K_M (μM) NADPH	0.16 ± 0.04	0.10 ± 0.05
K_M (μM) DHF	0.24 ± 0.08	0.26 ± 0.05
	At 45 ºC	
	Light	Heavy
k_{cat} (s^{-1})	0.488 ± 0.020	0.357 ± 0.015
K_M (μM) NADPH	0.99 ± 0.19	1.04 ± 0.18
K_M (μM) DHF	0.90 ± 0.04	1.20 ± 0.20

Table S4: pH dependence of the pre-steady-state rate constants (k_H) during catalysis by light and heavy TmDHFR.

	10 ºC		40 ºC	
pH	k_H^{LE} (s^{-1})	k_H^{HE}(s^{-1})	k_H^{LE} (s^{-1})	k_H^{HE}(s^{-1})
4.5	0.270 ± 0.011	0.220 ± 0.010	1.410 ± 0.200	1.320 ± 0.065
5	0.255 ± 0.005	0.200 ± 0.003	1.322 ± 0.090	1.298 ± 0.028
5.5	0.215 ± 0.010	0.160 ± 0.001	1.173 ± 0.011	1.199 ± 0.045
6	0.143 ± 0.002	0.103 ± 0.003	0.970 ± 0.036	1.023 ± 0.020
6.5	0.144 ± 0.005	0.106 ± 0.002	0.667 ± 0.015	0.707 ± 0.032
7	0.060 ± 0.001	0.061 ± 0.001	0.547 ± 0.009	0.547 ± 0.005
7.5	0.015 ± 0.002	0.010 ± 0.001	0.208 ± 0.003	0.235 ± 0.010
8	0.011 ± 0.001	0.010 ± 0.001	0.092 ± 0.004	0.012 ± 0.005
8.5			0.034 ± 0.002	0.038 ± 0.002
9			0.023 ± 0.002	0.022 ± 0.006
pK_a	**6.26 ± 0.29**	**6.01 ± 0.23**	**6.38 ± 0.03**	**6.48 ± 0.07**

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