Supporting information:

Two parallel pathways in the kinetic sequence of the Dihydrofolate Reductase from *Mycobacterium tuberculosis*

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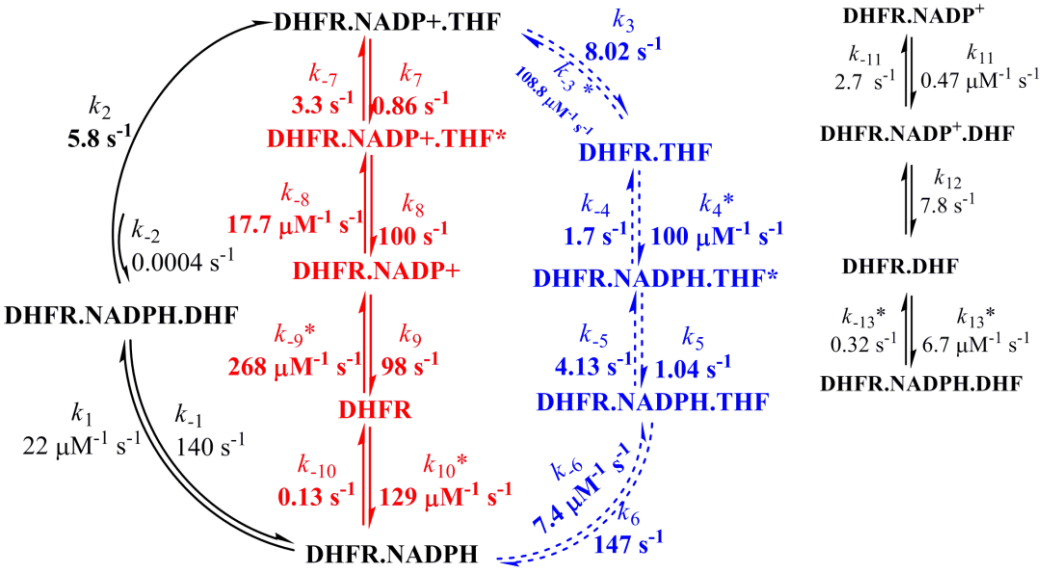
The first portion of the supporting information (pages 2-9) summarizes the experiments conducted in order to obtain each one of the rate constants presented. The rate constants for each step are named according to the scheme below (Schemes 2 and 4 in the paper). For more information concerning the equations used for data fitting, see (*1*) and (*2*).



★Determination of k_1, k_{-1}

DHF binding to *Mt*DHFR-NADPH – Single turnover experiments monitoring protein fluorescence (Excitation 280 nm, emission above 400 nm). Two transients with opposite amplitudes were observed, and the rate constant of the first transient showed a linear dependence on the DHF concentration indicating that this transient is associated with DHF binding to *Mt*DHFR-NADPH and that this binding process occurs via a single step mechanism.

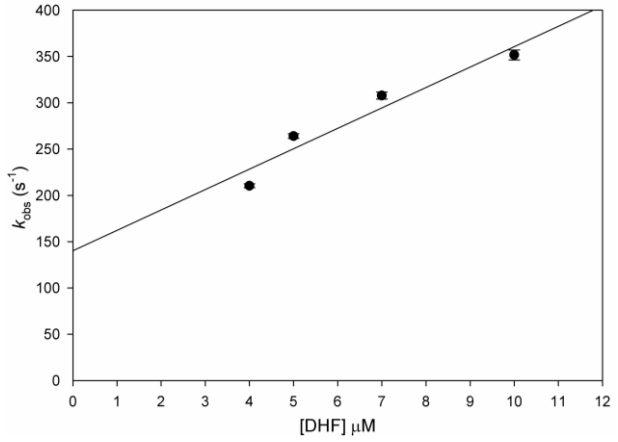


Figure S1: Replot of observed rate constants obtained at 4, 5, 7, 10 μM concentrations of *Mt*DHFR.
$k_{obs} = k_1[DHF] + k_{-1}$
$k_{obs1} = 22.03[DHF] + 140.20$

$K_{ov} = k_{-1}/k_1 = 6.4 \pm 1.8$

★Determination of k_2

Single turnover experiments monitoring protein fluorescence (Excitation 280 nm, emission above 400 nm) or decrease in absorbance at 340 nm. Two transients with opposite amplitudes were observed, and the rate constant of the second transient showed a hyperbolic DHF concentration dependence indicating that this transient is associated with a step after DHF binding to *Mt*DHFR-NADPH, including chemistry. Hence the observed rate constant at saturating DHF concentrations permits to estimate the maximal rate constant for chemistry k_2

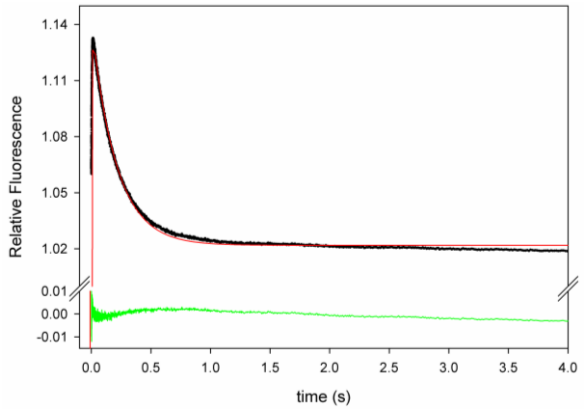


Figure S2: Representative trace of single turnover experiment monitoring protein fluorescence with 4 µM *Mt*DHFR, 100 µM NADPH and 1 µM DHF. The red line is a fit to a double exponential equation, where the first phase corresponds to DHF binding, followed by a second phase associated with the chemical step. The rate for the second phase was $k_2 = 4.83 \pm 0.03$ s^{-1}.
The green line is the residual of fit.

★Determination of k_{-2}

Single turnover experiments of the reverse reaction monitoring increase in absorbance at 340 nm.

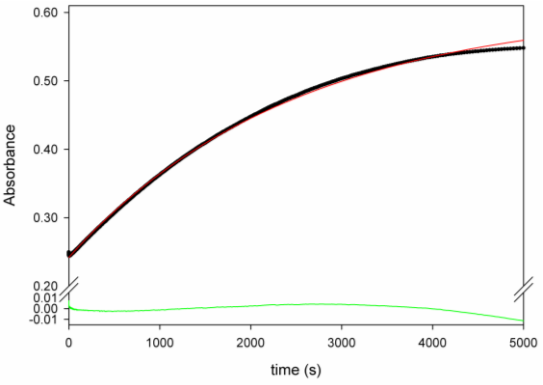


Figure S3: Single turnover experiment mixing 2 µM THF with 8 µM *Mt*DHFR-$NADP^+$. Red line is a fit to a single exponential equation, yielding a $k_{-2} = 0.0004$ s^{-1}. The green line is the residual of the fitting.

★Determination of k_3

NADPH competition with *Mt*DHFR-NADP$^+$-THF monitoring protein fluorescence (Excitation at 280 nm, emission above 305 nm). *Mt*DHFR-NADP$^+$ was preincubated with 20 μM THF, and then mixed with increasing concentrations of NADPH (2, 4, 6, 8, 10 μM) to displace NADP$^+$, causing increase in fluorescence. The observed dissociation rate constant was independent of competitor (NADPH) concentration, and assumed to be equal to k_3.The events that occur in this experiment are shown below.

$$\textit{Mt}\text{DHFRNADP}^+\text{-THF} \underset{}{\overset{k_3}{\rightleftharpoons}} \text{NADP}^+ + \text{NADPH} + \textit{Mt}\text{DHFR-THF} \rightleftharpoons \textit{Mt}\text{DHFR-NADPH-THF} + \text{NADP}^+$$

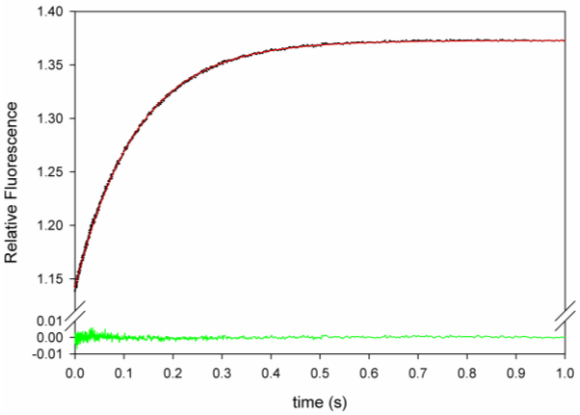


Figure S4: Representative trace of a competition experiment mixing 10 μM NADPH to 0.5 μM *Mt*DHFR-NADP$^+$-THF. Red line is a fit to a single exponential equation yielding k_3 = 8.0 s^{-1}, and the green line is the residual of the.

★Determination of k_{-4}

NADP$^+$ competition with DHFR-NADPH-THF monitoring protein fluorescence (Excitation at 280 nm, emission above 305 nm). *Mt*DHFR-NADPH was preincubated with 20 M THF, and then mixed with increasing concentrations of NADP$^+$ (1, 2, 5, 10 mM) to displace NADPH, causing decrease in fluorescence. The events that occur in this experiment are shown in the scheme below:

$$\textit{Mt}\text{DHFR-NADPH-THF} \underset{}{\overset{k_{-4}}{\rightleftharpoons}} \text{NADP}^+ + \text{NADPH} + \textit{Mt}\text{DHFR} \rightleftharpoons \textit{Mt}\text{DHFR- NADP}^+\text{-THF} + \text{NADPH} + \text{NADP}^+$$

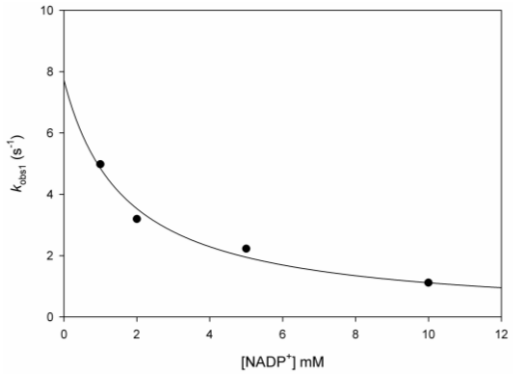


Figure S5: Relationship between the observed rate constants of NADPH displacement obtained with different concentrations of competitor (1, 2, 5, 10 mM). Line is a fit to a hyperbolic equation that extrapolates to k_{-4} = 1.69 ± 0.6 s^{-1}

★Determination of k_6, k_{-6}, k_5, k_{-5}

1.THF binding to DHFR-NADPH – Equilibrium binding

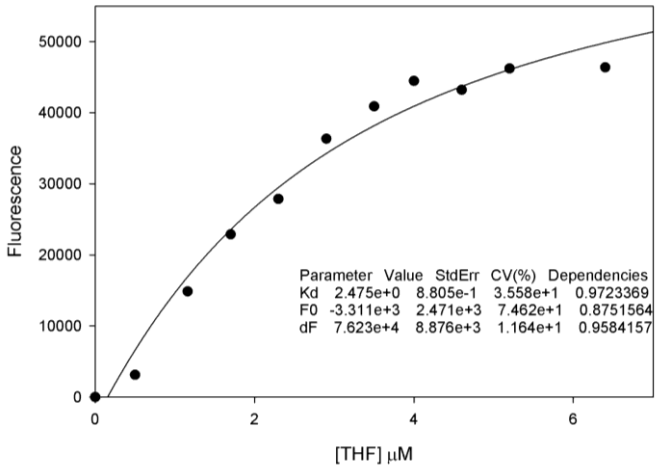


Figure S6: Equilibrium binding with 1 μM *Mt*DHFR-NADPH and increasing concentrations of THF. Excitation: 280 nm, Emission 440 nm.

Data were fit to the equation below:

$$F = \frac{(E_0 + S_T + K_d) - \sqrt{(E_0 + S_T + K_d)^2 - 4.E_0.S_T}}{2.E_0}$$

(see text for more detail)

$K_d = 2.47 \pm 0.88$ μM

2.THF binding to DHFR-NADPH – pre-steady state FRET; excitation at 280 nm, emission above 400 nm. Two transients of opposite amplitudes were observed, and fitted to a double exponential equation. The observed rate constant of the first transient (kobs1) showed a linear THF concentration dependence, while the observed rate constant of the second transient (kobs2) showed a hyperbolic concentration behavior. Hence THF binds via a two-step binding mechanism. Fitting of the replots of the observed rate constants yield rate constants for the first (k_6, k_{-6}) and second phases (k_5, k_{-5}).

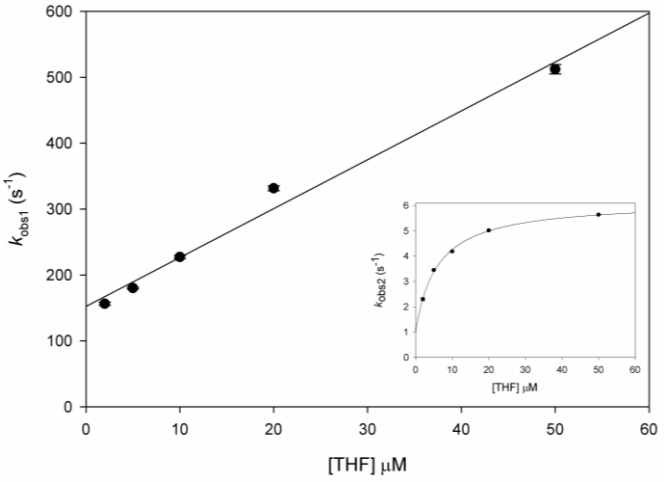


Figure S7: THF concentration dependency of the observed rate constants for the first and second phases.

In the main plot, the line is a fit to a linear equation where

$k_{obs} = k_{-6}[THF] + k_6 + k_{-5} + k_5$

$k_{obs1} = 7.41[THF] + 152.40$, so that

$k_{-6} = 7.41$ μM^{-1}s^{-1}.

Knowing from the hyperbolic fit (inset) that $y_{max} = k_{-5} + k_5 = 5.17$, it's possible to obtain

$k_6 = 147.2$ s^{-1}.

The inset represent the observed rate constant for the second phase, fitted to

$$k_{obs2} = \frac{k_{-6}[S](k_5 + k_{-5}) + k_6 k_5}{k_{-6}[S] + k_6 + k_5 + k_{-5}}$$

to give

$k_{-5} = 4.1$ s^{-1}

$k_5 = 1.04$ s^{-1}

3. A competition experiment using increasing concentrations (10, 20, 50, 100 μM) of Methotrexate (MTX) was conducted to better estimate the slow dissociation rate constant (k_{-5}). MTX displaces THF from the *Mt*DHFR-NADPH-THF complex, as represented in the scheme below:

$$\textit{Mt}\text{DHFR-NADPH-THF} \underset{}{\overset{k_{-5}}{\rightleftharpoons}} \text{THF} + \text{MTX} + \textit{Mt}\text{DHFR} \rightleftharpoons \text{THF} + \text{MTX-}\textit{Mt}\text{DHFR}$$

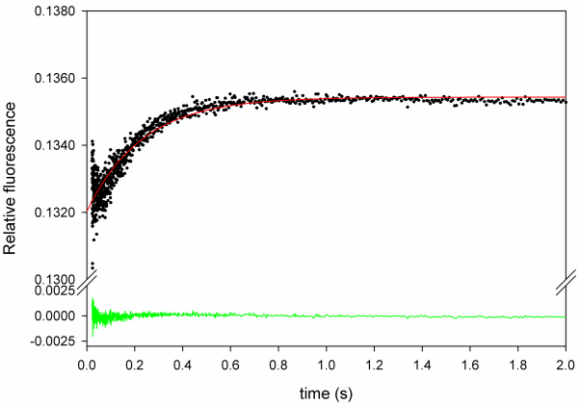


Figure S8: Representative trace of 10 μM MTX, mixed with 0.5 μM *Mt*DHFR-NADPH pre-incubated with 10 μM THF. Increasing concentrations of the competitor were used to guarantee that the dissociation rate constant obtained was equivalent to k_{-5} only. The red line is a fit to a single exponential equation, giving $k_{-5} = 4.8 \pm 0.1\ s^{-1}$, and the green line is the residual of the fit.

★Determination of k_7, k_{-7}, k_8, k_{-8}

1.THF binding to DHFR-$NADP^+$ – Equilibrium binding

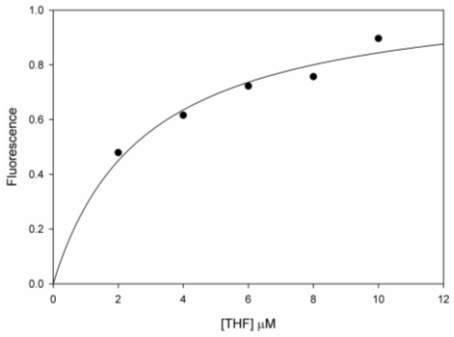


Figure S9: Equilibrium binding with 0.5 μM *Mt*DHFR-$NADP^+$ and 2, 4, 6, 8, 10 μM of THF. Excitation: 280 nm, Emission above 305 nm.

Data were fit to a hyperbola to give a K_d = 2.8 μM

2.THF binding to DHFR-$NADP^+$ – Protein Fluorescence excitation at 280 nm, emission above 305 nm. Two transients of opposite amplitudes were observed, and fitted to a double exponential equation. The observed rate constant of the first transient (kobs1) showed a linear THF concentration dependence, while the observed rate constant of the second transient (kobs2) showed a hyperbolic concentration behavior. Hence THF binds via a two-step binding mechanism. Fitting of the replots of the observed rate constants yield rate constants for the first (k_8, k_{-8}) and second phases (k_7, k_{-7}).

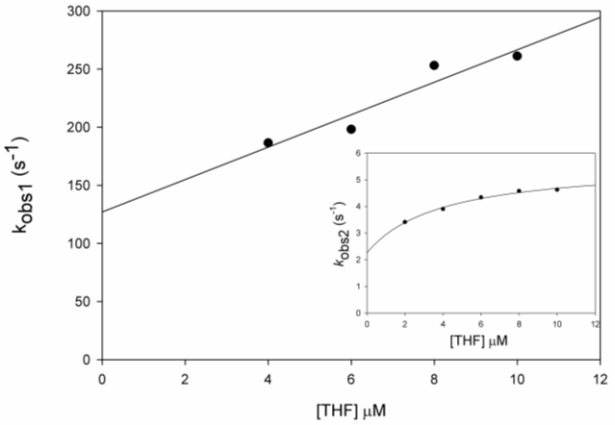


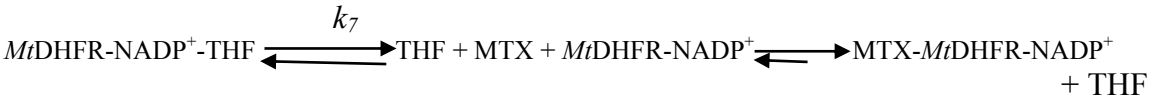
Figure S10: THF concentration dependency of the observed rate constants for the first and second phases.
In the main plot, the line is a fit to a linear equation where
$k_{obs} = k_{-8}[THF] + k_8 + k_{-7} + k_7$
$k_{obs1} = 17.67[THF] + 104.15$
$k_{-8} = 7.41\ \mu M^{-1}s^{-1}$.
Knowing from the hyperbolic fit (inset) that $y_{max} = k_{-7} + k_7 = 4.13$, it's possible to obtain $k_8 = 100\ s^{-1}$.

The inset represent the observed rate constant for the second phase, fitted to

$$k_{obs2} = \frac{k_{-8}[S](k_7 + k_{-7}) + k_8 k_7}{k_{-8}[S] + k_8 + k_7 + k_{-7}}$$

to give
$k_{-7} = 3.26\ s^{-1}$
$k_7 = 0.86\ s^{-1}$

3.A competition experiment using increasing concentrations (10, 20, 50, 100 μM) of Methotrexate (MTX) was conducted to better estimate the slow dissociation rate constant (k_7). MTX displaces THF from the *Mt*DHFR-$NADP^+$-THF complex, as represented in the scheme below:



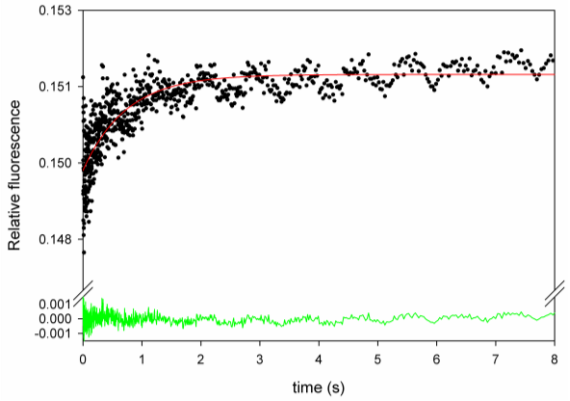


Figure S11: Representative trace of 10 μM MTX, mixed with 0.5 μM *Mt*DHFR-$NADP^+$ pre-incubated with 10 μM THF. Increasing concentrations of the competitor were used to guarantee that the dissociation rate constant obtained was equivalent to k_7 only. The red line is a fit to a single exponential equation to give $k_7 = 1.89 \pm 0.15\ s^{-1}$, and the green line is the residual of the fit.

★Determination of k_9

A competition experiment using increasing concentrations of NADPH was conducted to better estimate the dissociation rate constant of NADP+ from the *Mt*DHFR.$NADP^+$ (k_9). NADPH displaces $NADP^+$ from the *Mt*DHFR-$NADP^+$ complex, as represented in the scheme below:

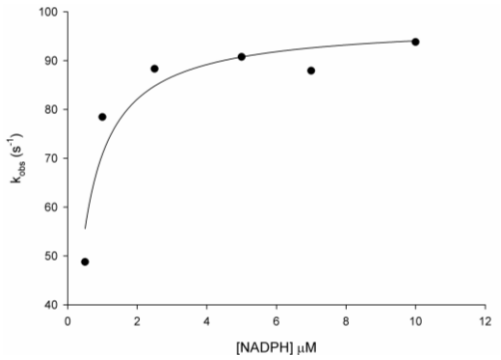


Figure S12: Relationship between the observed rate constants of $NADP^+$ displacement obtained with different concentrations of competitor (0.5, 1, 2.5, 5, 7, and 10 μM). Line is a fit to a hyperbolic equation that extrapolate to $k_9 = 97.6 \pm 4.0\ s^{-1}$.

★Determination of k_{-10}

A competition experiment using increasing concentrations of NADP+ was conducted to better estimate the dissociation rate constant of NADPH from the *Mt*DHFR.NADPH complex (k_{-10}). NADP+ displaces NADPH from the *Mt*DHFR-NADPH complex, as represented in the scheme below:

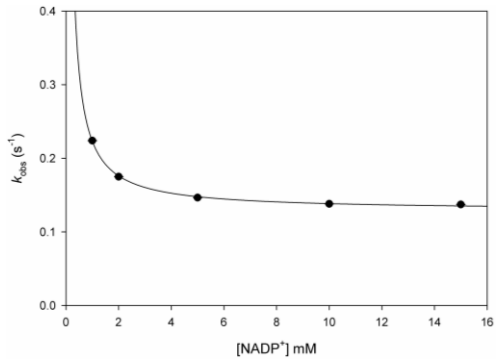


Figure S13: Relationship between the observed rate constants of NADPH displacement obtained with different concentrations of competitor (1, 2, 5, 10, and 15 mM). Line is a fit to a hyperbolic equation that extrapolate to $k_{-10} = 0.13 \pm 0.01\ s^{-1}$.

★Determination of k_{11} and k_{-11}

1.DHF binding to DHFR-$NADP^+$ – Protein Fluorescence excitation at 280 nm, emission above 305 nm. One single transient was observed, and fitted to a single exponential equation yielding rate constants for k_{11} and k_{-11}.Calculation of an apparent Kd was conducting by analyzing the DHF concentration dependency of the amplitudes of each binding curve.

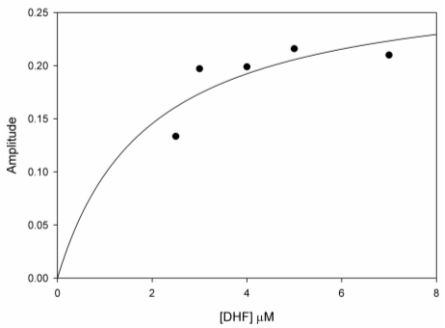


Figure S14: Equilibrium dissociation constant for DHF was obtained from

$$A_1 = \frac{A_{max}[S]}{K_d^{app} + [S]}$$

yielding an apparent $K_d = 1.9 \pm 1.3$.

2. DHF binding to *Mt*DHFR-NADP$^+$ – Protein Fluorescence excitation at 280 nm, emission above 305 nm. A single transient was observed, indicating that DHF binds via a single step binding mechanism to MtDHFR-NADP+. Fitting of the replot of the observed rate constant yield rate constants for the association and dissociation rate constants (k_{-11} and k_{11} respectively)

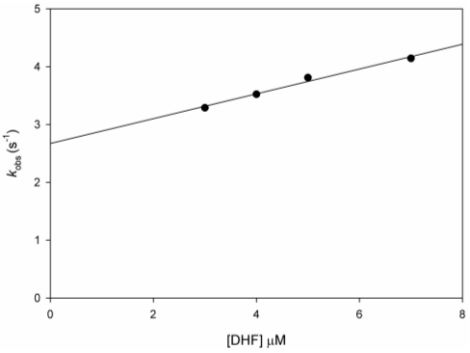


Figure S15: Concentration dependency of the observed rate constants and DHF concentration.
The line is a fit to a linear equation where
$k_{obs} = k_{-11}[DHF] + k_{11}$
$k_{obs} = 0.21[DHF] + 2.67$

3. MTX competition with DHFR-NADP$^+$-DHF A competition experiment using increasing concentrations (2, 4, 20, 40 μM) of Methotrexate (MTX) was conducted to better estimate the slow dissociation rate constant (k_{-11}). MTX displaces DHF from the *Mt*DHFR-NADP$^+$-DHF complex, as represented in the scheme below:

$$\textit{Mt}\text{DHFR-NADP}^+\text{-DHF} \underset{}{\overset{k_{-11}}{\rightleftharpoons}} \text{DHF} + \text{MTX} + \textit{Mt}\text{DHFR-NADP}^+ \rightleftharpoons \text{MTX-}\textit{Mt}\text{DHFR-NADP}^+ + \text{DHF}$$

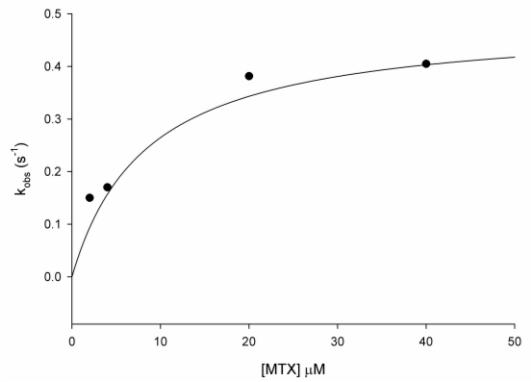
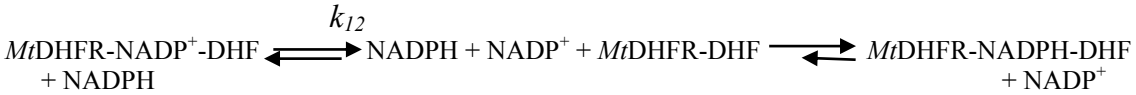


Figure S16: Plot of observed rates constants in function of competitor (2, 4, 20, 40 μm MTX). The line is a fit to a hyperbola whose y_{max} gives $k_{-11} = 0.47 \pm 0.04$ s^{-1}.

★Determination of k_{12}

A competition experiment using increasing concentrations of NADPH was conducted to estimate k_{12}, monitoring protein fluorescence with excitation at 280 nm, emission above 305 nm. NADPH displaces $NADP^+$ from the *Mt*DHFR-$NADP^+$-DHF complex, as represented in the scheme below:



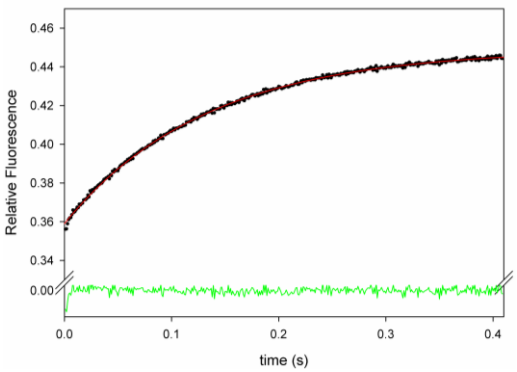


Figure S17: Representative trace of 50 μM NADPH, mixed with 0.5 μM *Mt*DHFR-$NADP^+$ pre-incubated with 10 μM DHF. Increasing concentrations of the competitor were used to guarantee that the dissociation rate constant obtained was equivalent to k_{12} only. The red line is a fit to a single exponential equation to give k_{12} = 7.99 ± 0.01 s^{-1}, and the green plot line is the residual of the fit.

Temperature dependence of k_{cat} and k_H were additionally fitted to EqS2, and the results are combined in Table S1 and Figure S18.

$$\ln k = (E_a/R)(1/T) + \ln A \quad \text{(S1)}$$

$$\ln(k/T) = [\ln\kappa + \ln(\kappa_B/h) + \Delta S^{\neq}/R] - \Delta H^{\neq}/RT \quad \text{(S2)}$$

$$\Delta G^{\neq} = -RT\left(\ln(k_i) - \ln(\kappa_B T/h)\right) \quad \text{(S3)}$$

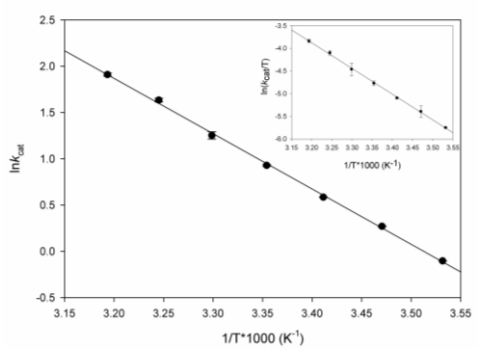
For eq. S2, S3, and S4, k represents the rate being measured (k_H or k_{cat}), k_i is the rate constant of the reaction step, E_a is the experimental activation energy, T is the temperature in Kelvin, R is the gas constant (1.98 cal mol^{-1}), A represents a pre-exponential factor that correlates collision frequency, and the proper orientation of colliding molecules with the rate of the reaction, κ_B and h are the Boltzmann and the Planck constants, respectively, $\Delta S^{\neq}$ and $\Delta H^{\neq}$ are the entropy and enthalpy of activation, respectively, and $\Delta G^{\neq}$ is the free energy of activation. It's important to note that the activation energy and the enthalpy of activation are related by $E_a = \Delta H^{\neq} + RT$ (*3*).

Supplementary Table 1[a]

	Parameter	Value	Obtained by
Steady-state conditions: k_{cat}	E_a	11.9 ± 0.2	Fitting to eq. S1.
	$\Delta G^{\neq}$	11.6 ± 0.2	$\Delta G^{\neq} = \Delta H^{\ddagger}_{(kcat)} - T\Delta S^{\ddagger}_{(kcat)}$
	$\Delta H^{\neq}$	6.0 ± 0.1	$E_a = RT + \Delta H^{\neq}$[b]
	$T\Delta S^{\neq}$	-5.6 ± 0.1	Fitting to eq. S2.
Pre-steady state conditions: k_H	E_a	13.2 ± 0.3	Fitting to eq. S1.
	$\Delta G^{\neq}$	15.1	Calculation using eq. S3.
	$\Delta G^{\neq}$	10.6	$\Delta G^{\neq} = \Delta H^{\ddagger}_{(kH)} - T\Delta S^{\ddagger}_{(kH)}$
	$\Delta H^{\neq}$	7.3 ± 0.3	$E_a = RT + \Delta H^{\neq}$[b]
	$T\Delta S^{\neq}$	-3.3 ± 0.1	Fitting to eq. S2.
Equilibrium	ΔG	-9.5	$\Delta G = RT\ln K_{eq}$, where $K_{eq} = 5.06 \times 10^7$[c]
	ΔG	-8.5 (Pathway B) -8.7 (pathway A)	Calculation from rate constants (eq. S3).

[a] all values are at 25°, pH 7.5, and are in kcal mol^{-1}.
[b] see Winzor *et al. (3)*
[c] from Czekster *et al. (4)*

A



B

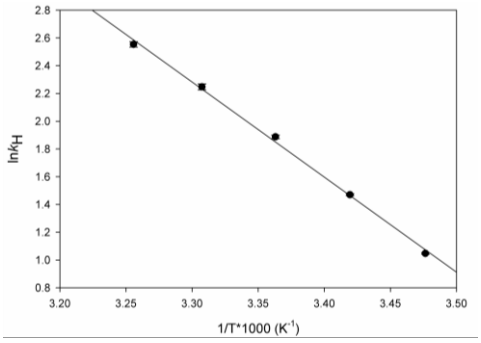


Figure S18: Temperature dependence of k_{cat} (A) and k_H (B). Rates were measured at different temperatures, with saturating concentrations (100 mM) of each substrate. Each point is the average of at least three measurements, errors on k_{cat} are standard errors, while errors on k_H represent the error for each exponential fitting. Lines are fits to eq. 1 (A, and B), or eq.2 (A, inset), yielding 11.7 ± 0.2 kcal mol^{-1}, and 13.2 ± 0.3 kcal mol^{-1}, for the activation energies of k_{cat} and k_H, respectively. Table S1 summarizes the thermodynamic parameters obtained. k_{cat} and k_H are expressed in s^{-1}.

1. Johnson, K. A. (1992) Transient State kinetic analysis of enzyme reaction pathways, *The Enzymes 20*, 1-61.
2. Hiromi, K. (1979) *Kinetics of Fast Enzyme Reactions: Theory and Practice*, Halsted Press, New York, USA.
3. Winzor, D. J., and Jackson, C. M. (2006) Interpretation of the temperature dependence of equilibrium and rate constants, *Journal of Molecular Recognition 19*, 389-407.
4. Czekster, C. M., Vandemeulebroucke, A., and Blanchard, J. S. (2011) Kinetic and Chemical Mechanism of the Dihydrofolate Reductase from Mycobacterium tuberculosis, *Biochemistry 50*, 367-375.