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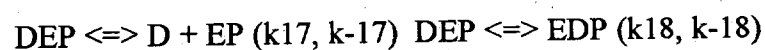
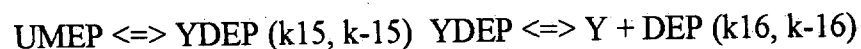
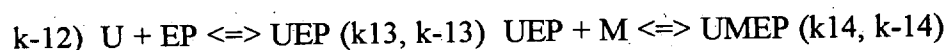
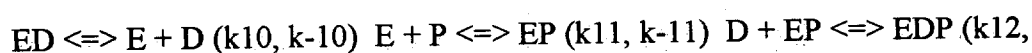
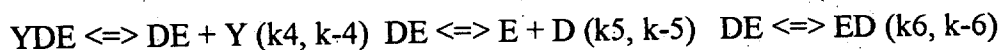
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Supporting information for kinetic simulation

The model is shown below for simulation of CH₂H₄F decomposition and H₂F formation in TS reaction, and H₂F consumption and H₄F production in DHFR reaction, and CH₂H₄F decomposition, H₄F formation and H₂F time courses under single turnover condition.



U = dUMP, E = Enzyme, M = CH₂H₄folate, Y = dTMP, D = H₂folate, P = NADPH, T = H₄folate and N = NADP⁺

In this model, DE \rightleftharpoons ED step represents the channeling step to transport the H₂F formed in the TS site to DHFR site. The k₃ and k₈ are the chemical step rate constants for TS and DHFR reactions, respectively, which are essentially irreversible. The focus of the simulation is to see whether H₂F can be accumulated in the single turnover of the TS-DHFR reaction with or without substrate channeling step and activation of DHFR activity. For this purpose, we have measured the association and dissociation and K_d constants of DHFR reaction in the bifunctional enzyme. The values are mostly consistent with that obtained from monofunctional DHFR. The stopped-flow experiments provide less kinetic information regarding TS reaction due to the instrumental limitation in measuring these rate constants. The rate constants obtained from recently reported monofunctional E. Coli TS was used as a reference in simulation. It was found that most uncertain rate constants actually do not change our simulation results. For example, in our single turnover experiments in TS site, high concentration of dUMP (500 μ M) was used to assure enzyme to be saturated. Thus, in the simulation, the dUMP

association and dissociation rate constants do not change the results. In the case of CH₂H₄F, the K_d is important for simulation. With smaller K_d (tighter binding), the predicted rate for TS reaction is faster than actual data and with higher K_d it is slower. This K_d effect can be compensated by small change of the TS chemical rate constant to fit the data. Our measured K_d for CH₂H₄F and chemical rate of TS reaction were therefore used to set constrain in the simulation. The release rate constant of dTMP from TS impacts on simulation according to our model where we have YDE ⇌ DE + Y prior to channeling step. Since H₂F is formed before the release of dTMP in the TS reaction and under the single turnover condition, H₂F is formed in TS active site or is ready to be channeled to DHFR site and used as substrate there, we simply put fast association and dissociation step for dTMP in our simulation. For H₂F release from TS site (k₅), a fast dissociation rate and relatively slow association rate were used to maximize the non-channeling possibility. All other chemical rate constants, association and dissociation rate constants are measurable. Under these reasonable assumption, several sets of rate constants were used to perform the simulation. The typical rate constants used in the model is shown below.

$$k_1 = 15 \text{ uM}^{-1} \text{ s}^{-1} \quad k_{-1} = 3 \text{ s}^{-1} \quad k_2 = 30 \text{ uM}^{-1} \text{ s}^{-1} \quad k_{-2} = 200 \text{ s}^{-1} \quad k_3 = 2.6 \text{ s}^{-1}$$

$$k_{-3} = 0 \text{ s}^{-1} \quad k_4 = 100 \text{ s}^{-1} \quad k_{-4} = 10 \text{ uM}^{-1} \text{ s}^{-1} \quad k_5 = 200 \text{ s}^{-1} \quad k_{-5} = 3 \text{ uM}^{-1} \text{ s}^{-1}$$

$$k_6 = 1000 \text{ s}^{-1} \quad k_{-6} = 0 \text{ s}^{-1} \quad k_7 = 21 \text{ uM}^{-1} \text{ s}^{-1} \quad k_{-7} = 1.8 \text{ s}^{-1}$$

$$k_8 = 120 \text{ s}^{-1} \quad k_{-8} = 0.001 \text{ s}^{-1} \quad k_9 = 127 \text{ s}^{-1} \quad k_{-9} = 47 \text{ uM}^{-1} \text{ s}^{-1}$$

$$k_{10} = 6.8 \text{ s}^{-1} \quad k_{-10} = 34 \text{ uM}^{-1} \text{ s}^{-1} \quad k_{11} = 21 \text{ uM}^{-1} \text{ s}^{-1} \quad k_{-11} = 1.8 \text{ s}^{-1}$$

$$k_{12} = 34 \text{ uM}^{-1} \text{ s}^{-1} \quad k_{-12} = 6.8 \text{ s}^{-1} \quad k_{13} = 15 \text{ uM}^{-1} \text{ s}^{-1} \quad k_{-13} = 3 \text{ s}^{-1}$$

$$k_{14} = 30 \text{ uM}^{-1} \text{ s}^{-1} \quad k_{-14} = 200 \text{ s}^{-1} \quad k_{15} = 2.6 \text{ s}^{-1} \quad k_{-15} = 0 \text{ s}^{-1}$$

$$k_{16} = 100 \text{ s}^{-1} \quad k_{-16} = 10 \text{ uM}^{-1} \text{ s}^{-1} \quad k_{17} = 6.8 \text{ s}^{-1} \quad k_{-17} = 34 \text{ uM}^{-1} \text{ s}^{-1}$$

$$k_{18} = 1000 \text{ s}^{-1} \quad k_{-18} = 0 \text{ s}^{-1}$$

Recall that our simulation is to see whether H₂F can be accumulated in the TS-DHFR single turnover reaction with or without channeling step and activation of DHFR activity. In the simulation process, channeling step rate constant (k₆ and k₁₈ in the model)

was changed to 0 s⁻¹ or high (1000 or 10000 s⁻¹) and the DHFR rate (k_8) is 14 s⁻¹ or 120 s⁻¹ as measured under different condition and all the remaining rate constants were kept constant. Under several conditions including (1) $k_6, k_{18} = 10000$ s⁻¹ and $k_8 = 120$ s⁻¹, (2) $k_6, k_{18} = 1000$ s⁻¹ and $k_8 = 120$ s⁻¹, (3) $k_6, k_{18} = 0$ s⁻¹ and $k_8 = 120$ s⁻¹, (4) $k_6, k_{18} = 10000$ s⁻¹ and $k_8 = 6.8$ s⁻¹, (5) $k_6, k_{18} = 0$ s⁻¹ and $k_8 = 6.8$ s⁻¹, the H₂folate concentration in the single turnover TS-DHFR reaction was simulated and compared to the our experimental chemical quench flow data. The rates constants were also used to simulate TS and DHFR single turnover reaction, respectively. For the simulation of DHFR single turnover reaction, $k_8 = 14$ s⁻¹ was used. It should be mentioned here that in the DHFR kinetic simulation using our simplified model, the dUMP concentration need to be set to zero otherwise the dUMP (U) will compete out E and slow the simulated reaction rate. In the actual experiments, we did not see difference in the DHFR reaction rate in the presence or absence of dUMP. The results for simulated curves with actual experimental data are shown in figures 1-3 in the paper.

In Figure 3A-D, we carried out simulation in the presence or absence of channeling step and DHFR activation. We found that only with both of channeling step (with a rate >1000s⁻¹) and DHFR activation (120 s⁻¹), the H₂F is not accumulated in the TS-DHFR single turnover reaction which is consistent with our observation in the chemical quench flow experiments. These rate constants also fit the actual data for TS and DHFR single turnover reaction shown in Figure 1 and 2. With only channeling but not activation or with only activation but not channeling, the H₂F in TS-DHFR reaction should be accumulated to a level detectable in our experiments. Taken together, these results suggest that in the single turnover of TS-DHFR reaction, the H₂F formed in the TS site must be transported to DHFR site at a very fast channeling step and consumed in the DHFR site with a chemical step rate of 120 s⁻¹.