Electronic Supplementary Information

Materials

dUMP, dTMP, NADPH, dithiothreitol (DTT), Tris(hydroxymethyl)aminomethane, NaCl, and o-dianisidine were purchased from Sigma Co. CH_2H_4folate was a generous gift from Eprova Inc, Switzerland. [2-^{14}C]-dUMP (specific radioactivity, 52 Ci/mol) was from Moravek Biochemicals. FDTS (TM0449) was cloned, expressed and purified as described before.[1]

Methods

To test the dependence of the lag phase on the concentration of dUMP, 12.5 μM enzyme purified with bound FAD in its oxidized state (50 μM active sites using ε_{454}=12.5 $mM^{-1}cm^{-1}$) was incubated in 20 mM Tris Buffer (pH 7.9, adjusted at the experimental temperature), 150 mM NaCl, and 0.25 mM DTT under Ar, with varying concentrations of dUMP (0 – 100 μM), and the 454 nm absorbance was followed by a UV/Visible spectrophotometer (Hewlett-Packard 8453 series diode-array UV/Vis spectrophotometer equipped with a water-jacketed cuvette holder under Ar atmosphere). Internal absorbance reference at 550-570 nm was subtracted from the 454 nm absorbance (independent measurements showed no absorbance change at the reference wavelength range during the course of the reaction). The reaction was initiated with the addition of a large excess of NADPH (1 mM final concentration) to the reaction mixture preincubated at the experimental temperature. A typical absorbance trace is presented in Figure 1. The lag phase was defined as the time before the reduction of FAD (454 nm absorbance - yellow) to $FADH_2$ (no 454 nm absorbance – colorless). To determine the duration of the lag phase a tangent was drawn to the initial absorbance (time independent during the lag phase) and from the linear reduction phase (see Figure1). The time of the intersection of these two lines was defined as the duration of the lag phase. Alternative definitions of the duration of the lag phase (e.g., the time from initiation to inflection point, maximal rate change, or first derivative of the OD, or value of zero of its second derivative) did not significantly affect the constant calculated. The reaction was carried out at 37°C and 80°C. The apparent binding constant of dUMP was determined by fitting the duration of the lag phase vs. the concentration of dUMP to a sigmoid equation by non-linear regression of the data to Equation 1 (represented by the solid lines in Figures 2):

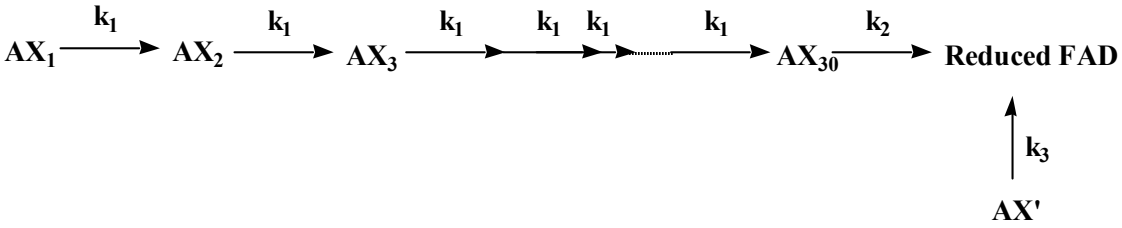
$$a+b/(1+\exp((mp-[dUMP])/c)) \qquad [1]$$

The midpoint (mp) of the sigmoid yields the apparent/effective binding constant (K_f) of dUMP to enhance FDTS-FAD reduction by NADPH.

Kinetic model

Hitherto, attempts to develop a more rigorous kinetic model that will explain and fit all the experimental data in a meaningful manner were not sufficiently successful. The challenge is to construct a kinetic model that will be fitted to the time course of the FAD reduction and its dependency on the dUMP concentration. Previous studies could only simulate and fit very short lag-phases (<1 second).[2,3] We used the package Scientist 2.0 to fit the experimental data to possible kinetic models. The program numerically solves complex differential equations as function of several variables (e.g., time, dUMP

concentration, etc.). Many kinetic models were attempted and the most relevant one we found that can fit the time dependence of the reaction involves 30 consecutive steps (of equal rate constants) that follow the formation of the ternary complex where only the last complex leads to FAD reduction (with different rate constant). Additionally, an independent one step starting from a different ternary complex leading to the reduction with a different rate constant was required (Scheme S1). An example of the fitting of this model to the experimental data is presented in Figure S1. This model is relevant as the two starting states could represent two different bound states and the multi non-reactive steps could represent enzymatic conformations that lead to the reactive one. Branching-models (similar to those developed for lipoxygenase[2,3]) or models with more starting states did not lead to a better fit. At this time, this model cannot address the dUMP concentration effect on one hand, and is not unique on the other hand.



Scheme S1: A schematic illustration of the model described in the text, where AX is the complex of the enzyme in different states. States X_1 to X_{29} are not reactive and only X_{30} and X' lead to FAD reduction.

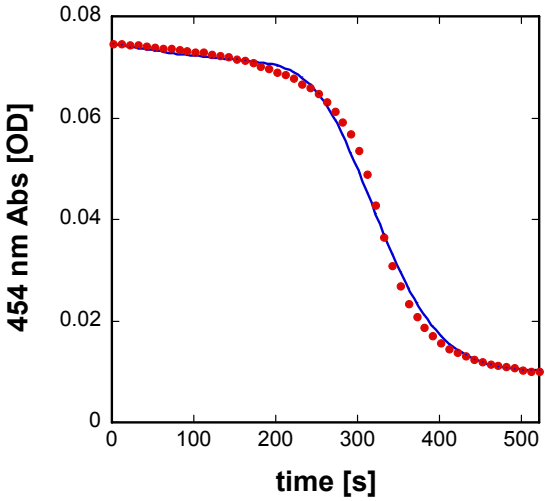


Figure S1: The reduction of *tm*FDTS bound FAD in the presence of 0.25 μM dUMP and excess of NADPH is presented with the experimental points (red) fitted to the kinetic model described above (blue line).

References

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