A lag-phase in the reduction of flavin dependent thymidylate synthase (FDTS) revealed a mechanistic missing link†

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An unexpected substrate-dependent lag-phase, found in the single turnover reduction of FDTS bound flavin, sheds light on the molecular mechanism of this alternative thymidylate synthase.

In this communication we report a 2′-deoxythymidine-5′-monophosphate (dUMP) dependent lag-phase for the single turnover reduction of *tm*FDTS bound FAD by NADPH (Fig. 1). The lag-phase dependence on the dUMP concentration was sigmoidal with an apparent functional constant (K_f) for dUMP binding and enhancing FDTS bound FAD reduction by NADPH. These constants were determined at 37 °C and 80 °C (the physiological temperature for *Thermotoga maritima*). This functional binding constant for dUMP as the activator of FAD reduction is very different and has a very different temperature dependence than the Michaelis constant (K_M) of dUMP as a substrate. This comparison is valid because K_M is also an apparent functional constant and not a binding constant.[1] The differences between K_f and K_M can be understood as different binding modes and sites. All together, the very existence of the lag-phase suggests that previous kinetic experiments and their concluded mechanisms should be re-evaluated and revisited as discussed below. Furthermore, similarly unexplained delayed activation phenomena in biology[2–4] might benefit from studies of such well controlled lag-phase.

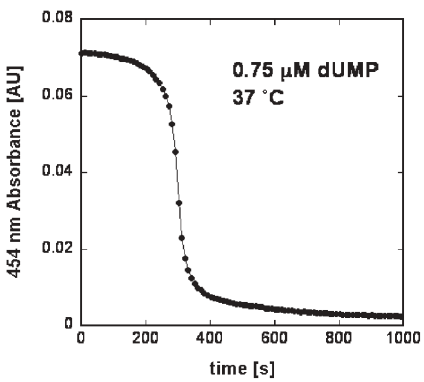

Fig. 1 Lag phase of FAD reduction with dependency on dUMP concentration. Lag phase shown for 0.75 μM dUMP at 37 °C.

dTMP is one of the building blocks of DNA and the last step in its *de novo* biosynthesis is the reductive methylation of 2′-deoxyuridine-5′-monophosphate (dUMP) catalyzed by thymidylate synthase (TS). Consequently, TS is a common target for antibiotic and chemotherapeutic drugs (*e.g.*, 5-fluorouracil). In most known organisms, including humans, TS (encoded by *ThyA*) is a highly conserved enzyme with a well-understood reaction mechanism.[5,6] TS uses methylene-5,6,7,8-tetrahydrofolate (CH_2H_4folate) as both a single carbon (methylene) donor and as a reductant (hydride) donor resulting in the formation of dihydrofolate (H_2folate). Tetrahydrofolate (H_4folate) is required for various biological functions and is therefore rapidly regenerated by the dihydrofolate reductase (DHFR) catalyzed reduction of H_2folate by reduced 2′-phosphate nicotinamide adenine dinucleotide (NADPH). Many organisms also have bi-functional enzymes that contain both TS and DHFR domains and activities.[5] However, recent genomic studies reported that several organisms lack the genes for TS and DHFR, and an alternative enzyme for the conversion of dUMP to dTMP has been identified.[7,8] This alternative protein is denoted flavin-dependent thymidylate synthase (FDTS) and is encoded by *ThyX*. These reports attract significant attention because many of these FDTS dependent organisms are pathogenic, bio-warfare agents, or parasites.[7–10] TS and FDTS share no sequence homology, making FDTS a promising target for antibiotic drug design. Such design requires a fundamental understanding of the FDTS mechanism and the differences between that mechanism and that of the classical (*e.g.*, human) TS.

In contrast to classical TS, little is known about the mechanism of FDTS and no drugs or even specific inhibitors that target this enzyme are known. FDTSs catalyze the conversion of dUMP to dTMP, but in contrast to classical TS, its activity depends on NADPH reduction, and CH_2H_4folate is converted to H_4folate, not H_2folate. The enzyme also requires flavin adenine dinucleotide (FAD) that is often tightly bound to the enzyme.[7–11] The crystal structures of FDTS from *Thermotoga maritima* (TM0449 - tmFDTS)[8] and from *Mycobacterium tuberculosis* (Rv2754c - MtbFDTS)[12] are similar to each other but contain no structural similarities to TS. These FDTS are homotetramers with four bound FADs and four identical active sites. tmFDTS is the model FDTS used in the studies presented here.

Three principal mechanisms were suggested to explain the molecular mechanism of FDTS. The first one invoked a bi-functional enzyme that first reacts like classical TS, but the product H_2folate rather than being released is reduced to H_4folate by the

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flavin that has been pre-reduced by NADPH (consecutive TS and DHFR activities). We have carefully tested this option by using *R*-[6-^{3}H]CH_2H_4folate that when used with TS leads to radioactive [7-^{3}H]dTMP and H_4folate.[11] We found that all the radioactivity remains on the H_4folate, which is not consistent with such a bi-functional mechanism. The second mechanism suggested that while both TS and FDTS depend on CH_2H_4folate as a methylene donor, they differ with respect to the reduction of that methylene to methyl. FDTS uses NADPH reduced $FADH_2$ as a reductant for the conversion of 5-methylene-dUMP intermediate to dTMP instead of H_4folate.[7–11,13] The third possible mechanism suggested that H_4folate binds to the reduced enzyme and transfers its methylene to an enzymatic residue (*e.g.*, Arg) followed by release of H_4folate. Then, dUMP binds, activated by a Ser at its C6 carbon and the methylene is transferred from the enzymatic Arg to C5 of dUMP, followed by reduction to dTMP (all Ping Pong mechanism).[9] The experimental evidence for this last mechanism was not compelling and could not be reproduced by us or Liebl's group (Kohen and Liebl unpublished data). Liebl and co-workers preferred the second mechanism and noted that the most efficient NADPH oxidation can be achieved when FDTS, NADPH, and dUMP are present under aerobic atmosphere in the absence of CH_2H_4folate.[10] Making the assumption that the mechanism of FAD reduction is not affected by the electron acceptors (dUMP or O_2), this led to the postulate that NADPH is oxidized by a pre-formed dUMP–FDTS complex. Such a mechanism would be consistent with an ordered binding that starts with dUMP binding to the free enzyme, followed by NADPH binding and FAD reduction. The first product, $NADP^+$, is released, CH_2H_4folate binds, transfers its methylene to dUMP, H_4folate is released and the exocyclic methylene is reduced by $FADH_2$ to form the final product (dTMP). These different observations and suggested mechanisms seem contradictory at first, but the findings presented here may shed light on this enigma.

The experiments are described in detail in the electronic supplementary material†. In short, the reduction of enzyme bound FAD to $FADH_2$ was measured by monitoring the 454 nm absorbance of the enzyme after initiating the reaction by adding a saturating concentration of NADPH (1 mM) in the absence or presence of different concentrations of dUMP. The experiments were conducted under anaerobic atmosphere at 37 and 80 °C. It was important to maintain an anaerobic atmosphere as the reduced $FADH_2$ can readily react with molecular oxygen to form hydrogen peroxide (Ref. 10 and our own unpublished results). An unexpected lag-phase was observed in the absence of dUMP (about 5 min. at 37 °C and 7 min. at 80 °C) that precedes a fast reduction of the tmFDTS bound FAD to $FADH_2$ (Fig. 1). The duration of the lag-phase was found to depend on the initial concentration of dUMP. As the concentration of dUMP was increased, the lag-phase shortened until it vanished at 2.8 and 9 μM dUMP at 37 and 80 °C, respectively (Fig. 2). When the lag-phase is plotted against the concentration of dUMP and fitted to a sigmoidal equation†, the apparent functional constant (K_f) of dUMP can be determined. The K_f was 1.4 ± 0.1 and 2.1 ± 0.1 μM at 37 and 80 °C, respectively. The binding enthalpy of this apparent K_f is very small ($\Delta H^\circ < 0.5$ kcal mol^{-1}). Since its ΔG° at 80 °C is quite large (about 10 kcal mol^{-1})[14] it is suggested that most of the effect is enthropic ($T\Delta S_{80\ ^\circ C} = 9.3 \pm 0.3$ kcal mol^{-1}). The entropic effect can be rationalized as the enzyme going through a conformational rearrangement prior to the formation of conformation that enables FAD reduction. It is not clear at this point whether that rearrangement occurs before or after the NADPH, but it is clear that binding of dUMP enhances that process.

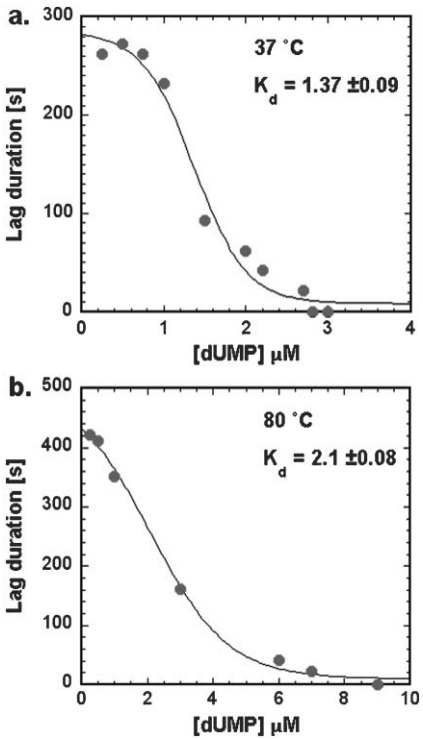

Fig. 2 Lag phase *vs.* dUMP concentration. Midpoint was calculated by fitting to sigmoidal equation.[16] Shown at 37 °C (panel **a**) and 80 °C (panel **b**).

The Michaelis constant K_M for dUMP in the half reaction in which dUMP is converted to dTMP and $FADH_2$ to FAD is 12.2 ± 0.7 μM at 80 °C.[15] This value is an order of magnitude higher than the apparent functional constant ($K_f = 2.1 \pm 0.1$ μM). The temperature dependence of this Michaelis constant (K_M) at 80 °C allowed estimation of $\Delta G^\circ_{80} = 8.0 \pm 0.04$,[17] $\Delta H^\circ = 6.7 \pm 1.6$, and $T\Delta S^\circ_{80} = 1.3 \pm 2.0$ kcal mol^{-1}.[15] Apparently, these two functional constants, K_M and K_f, represent different functions, and seem to have different temperature dependency. The differences in the energetics (entropy and enthalpy) suggest that the binding of dUMP to FDTS that activates NADPH oxidation is of a different nature than its capture as a substrate.

These findings are in accordance with a dUMP induced allosteric activation of the initial FAD reduction by NADPH. Two mechanisms are possible for this step: (i) dUMP binds prior to NADPH and synergistically enhances NADPH binding, (ii) dUMP binds to the enzyme–FAD–NADPH complex and induces the hydride transfer between the cofactors. Kinetic measurements cannot distinguish between these two options, and in the future calorimetric and fluorescence titrations with different ligands may further illumine this question (*e.g.*, dUMP titration of oxidized enzyme *vs.* reduced enzyme; NADPH titration of free enzyme *vs.* dUMP bound enzyme, *etc.*).

It is interesting to note that in previous cases where long (>1 min) delay in enzyme reactivity was observed (*e.g.*,

α-lytic protease[4]) no kinetic model was developed to explain the findings. A short (<1 second) and product dependent lag-phase has been identified with the enzyme soybean lipoxygenase-1 (SML-1).[2,3,18,19] A kinetic model was developed for this system that included a reactive (Fe^{III}) and non-reactive (Fe^{II}) enzyme and could fit data with lag-phase of up to 1 second. We have attempted to develop such a model for the findings reported here but models that could lead to reasonable fit to the time course of the FAD reduction (Fig. 1) could not explain the dUMP concentration dependency (Fig. 2). Such a kinetic model is described in the Electronic Supplementary Information† and attempts to develop a more comprehensive models are under way. We hope that the data presented here will lead to more theoretical work in the field of delayed reactivity.

As for the dUMP binding site, it might be bound at its reactive site and react with CH_2H_4folate after this second substrate binds the reduced enzyme, or it might be bound at a different site (an allosteric site or another dUMP binding site close to a different flavin in this tetrameric enzyme). Following the reduction of FAD and the release of $NADP^+$, the dUMP may react with CH_2H_4folate (in the first scenario), or a second dUMP may need to bind the reduced active site between the flavin and the nucleophilic Ser^8 (in the second case). Future trapping experiments with labeled dUMP using quench-flow methodology may resolve this question.

In summary, Liebl and co-workers[10] suggested a mechanism for the conversion of dUMP to dTMP catalyzed by FDTS. Their mechanism implied that dUMP binds to the FDTS with FAD in the oxidized state followed by NADPH binding and FAD reduction. Their main supportive evidence was the enhanced oxidation of NADPH by molecular oxygen in the presence of dUMP (followed by 340 nm absorbance reduction as the NADPH converted to $NADP^+$). McClarty and co-workers[9] on the other hand, used tritium-release kinetics (single point analyzed after a short reaction period using [5-^{3}H]dUMP) suggested a Ping Pong mechanism in which NADPH is oxidized and $NADP^+$ leaves prior to CH_2H_4folate binding, H_4folate release, dUMP binding and dTMP release. These experiments and findings appeared contradictory at first. Our finding of a dUMP dependent lag-phase can explain some of these different observations. The dUMP enhances the NADPH oxidation by deleting the lag-phase and thus appeared to bind first in Liebl's experiments. Its reactive binding as acceptor of the methylene (either from the CH_2H_4folate, or enzymatic methylene intermediate) may be of a different nature as suggested by the binding constant and energetics of dUMP as activator *vs.* its binding as a substrate. Such a mechanism could rationalize the different binding and release pattern suggested by McClarty and co-workers.[9] We believe that the new finding of a dUMP dependent lag-phase will redirect the thinking on FDTS mechanism, and will indicate how FDTS enzymes might be inhibited in a way that will not affect human or other classical TSs.

As a general note, the substrate dependent lag-phase reported here might be more general than realized hereto. In most cases it is not easy or even possible to follow the preliminary effect of one substrate in a multi-substrate reaction. Such phenomenon might be hidden in other systems either due to mixing effects or because the substrate concentration was above K_f. Consequently, it might be interesting to search for it in cases where different experimental settings suggest different orders of binding under different conditions.

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