ELECTRONIC SUPPLEMENTARY INFORMATION

Ultrafast Dynamics of Fully Reduced Flavin in Catalytic Structures of Thymidylate Synthase ThyX

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Contents

S1. Transient absorption spectroscopy of FADH⁻ in solution

S1.1. Transient spectra

To provide a reliable reference to the transient absorption spectra of the *Tm*ThyX systems presented in the main text, we studied pure FADH⁻ in aqueous solution (pH 8; 1.4 mM) under the same experimental conditions (excitation at 360 nm; see Section 2.2).
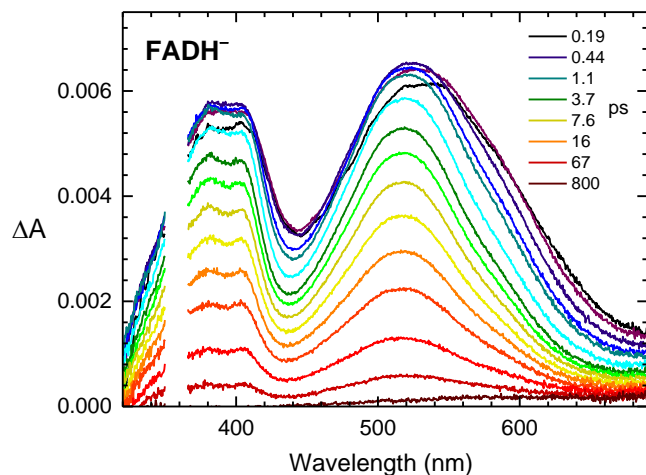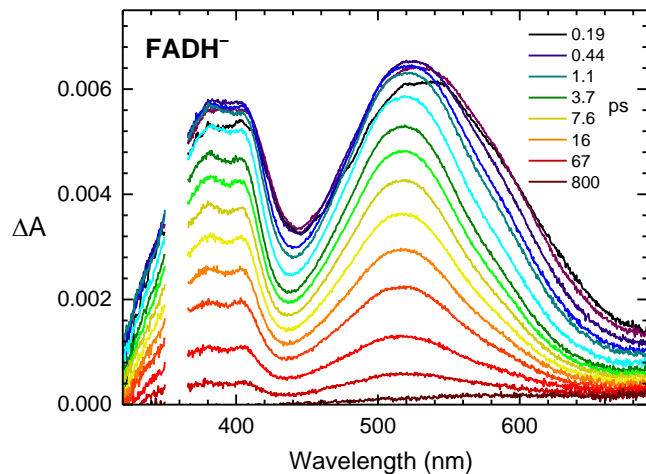

Fig. **S1** displays transient absorption spectra at selected pump-probe delays. They exhibit the characteristic bands of excited FADH⁻ (or FADH$_2$, FMNH⁻, FMNH$_2$), at ~390 and ~520 nm, previously described in the literature.[1-4] The excitation energy was low enough to reduce the amount of biphotonic ionization, known to be easily formed under ultrashort pulse excitation,[4] to a small long-lived background (see 800 ps delay). The latter shows the typical short wavelength tail of the hydrated electron absorption (peaking at 720 nm), above 600 nm.[4-7]

Fig. S1. Femtosecond transient absorption spectra of FADH⁻ in aqueous solution at selected pump-probe delays, with excitation at 360 nm.

S1.2.   Global analysis

   Global analysis of the above data yielded a sum of four exponentials followed by plateau. The time constants of the exponentials are: 0.27 ± 0.04 ps, 1.5 ± 0.1 ps, 8.4 ± 0.7 ps, 32 ± 1 ps. The plateau corresponds to the state (hydrated electron plus flavin semi-quinone radical) produced by biphotonic ionization. We provide in Fig. S2A the spectra of pre-exponential factors (decay-associated difference spectra; DADS). Putting DADS5 aside as it does not belong to the one-photon induced dynamics of FADH$^-$, the first four DADS were recast into evolution-associated difference spectra (EADS), corresponding to a virtual cascading model with unit quantum yield between successive states, shown in Fig. S2B. EADS1 is the transient spectrum extrapolated at t=0, deconvoluted from the instrument response function, which evolves in 0.27 ps towards EADS2, and so on. The final decay is that EADS4 in 32 ps.
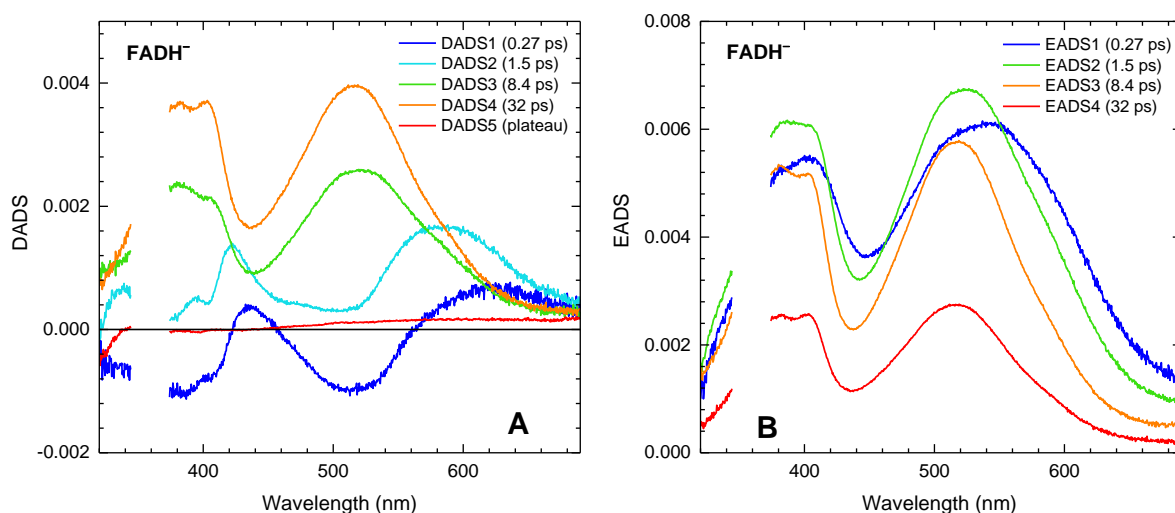
Fig. S2. Decay-associated difference spectra (DADS; A) and evolution-associated difference spectra (EADS; B) produced by the global multiexponential analysis of the absorption spectra of FADH$^-$. DADS5 (plateau) was excluded from the calculation of the EADS as it belongs to the separate dynamics induced by biphotonic ionization.

*S1.3.   Analysis of the integral decay trace*

   As already noted several times,[4,8] the transient absorption evolution of FADH$^-$ in solution may be considered as a continuous one —resulting from the progressive movement along the butterfly bending coordinate until reaching a conical intersection with the ground state. In that sense it is pointless to seek any precise meaning for each of the time constants produced by our (discrete multiexponential) global analysis.

In order to quantify more globally the excited-state decay, we used an alternative approach. The transient spectra were first integrated between 380 and 700 nm, using a $d\lambda/\lambda$ integral to yield for each time delay a number proportional to an oscillator strength (or Einstein B coefficient).[9] The obtained kinetic trace (restricted to $t \geq 0.25$ ps in order to get rid of experimental artifacts at short times) was then fitted to a sum of three exponentials plus a plateau, convoluted by instrument response function (see Fig. S3). The time constants (τ_i) and relative amplitudes (a_i, in parentheses) were found to be: 0.38 ps (0.02), 41 ps (0.41), 114 ps (0.55) and plateau (0.02). Finally, the average decay time ($\bar{\tau}$) of the distribution of time constants was calculated using Equation S1 (the plateau was excluded from this calculation). To give a sense of the width of the distribution, the standard deviation of decay times (σ) was calculated with Equation S2. We found $\bar{\tau}$ = 19.2 ps and σ = 13.8 ps.

$$\bar{\tau} = \sum_i a_i \tau_i / \sum_i a_i \tag{S1}$$

$$\sigma^2 = \sum_i a_i \tau_i^2 / \sum_i a_i - \bar{\tau}^2 \tag{S2}$$
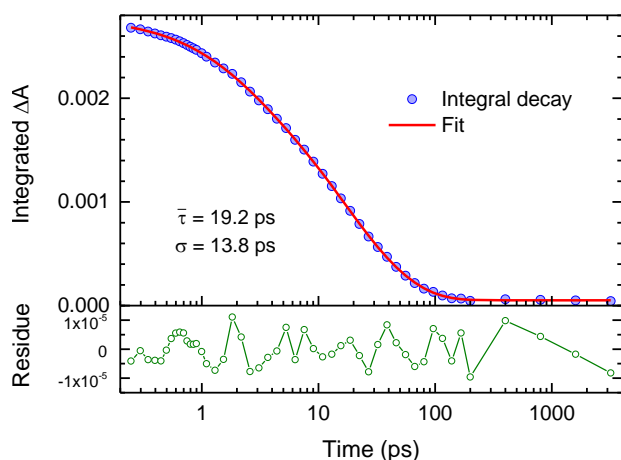
Fig. S3. Integral transient absorption decay of FADH$^-$ in solution (blue circles) and multiexponential fit (red line). The residues are shown in green below.

It may be noted that the integral analysis did not produce any sub-ps time component, contrary to the spectrally-resolved global analysis. This is because the global 0.27 ps component essentially represents a change of shape of the spectra without evolution of the integral. It can be understood as a mere relaxation of the excited state without modification of oscillator strength of the transitions contributing to the transient spectra (between 380 and 700 nm) and without loss of excited-state population.
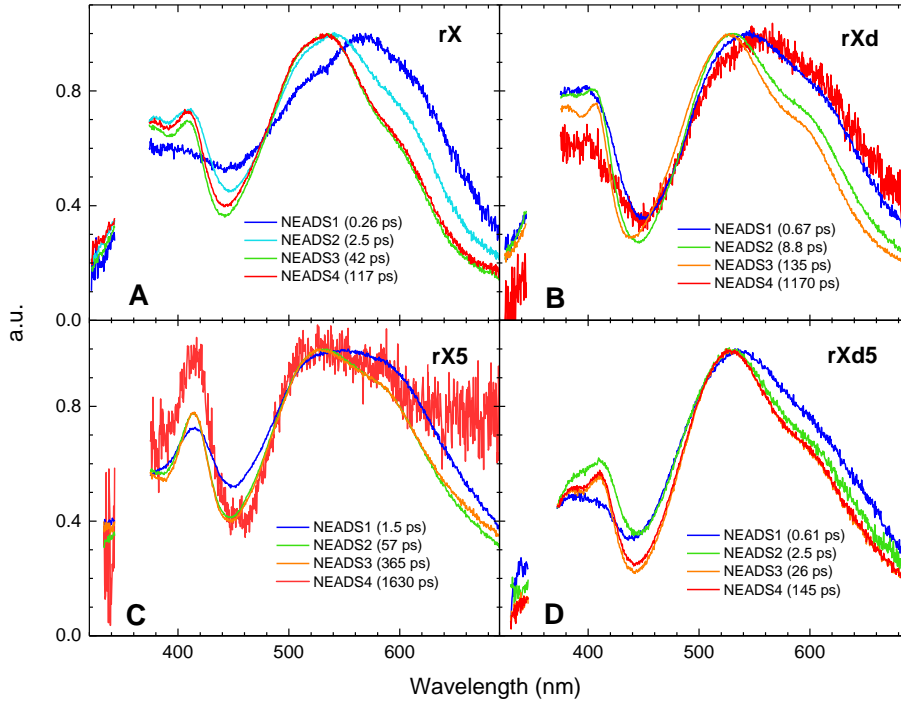
S2. Transient absorption spectroscopy of the *Tm*ThyX systems
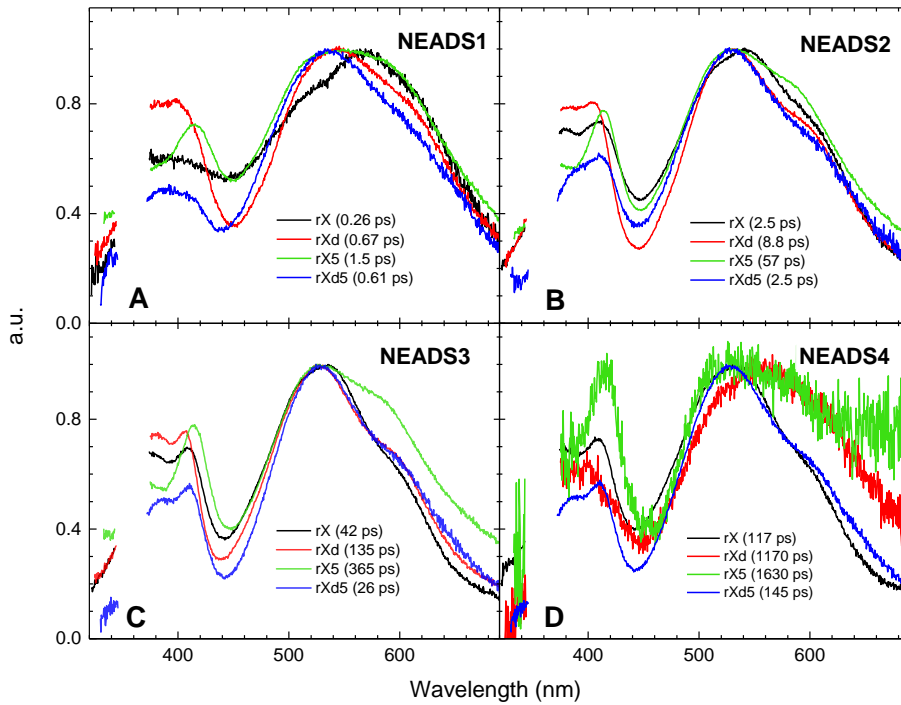
S2.1. DADS
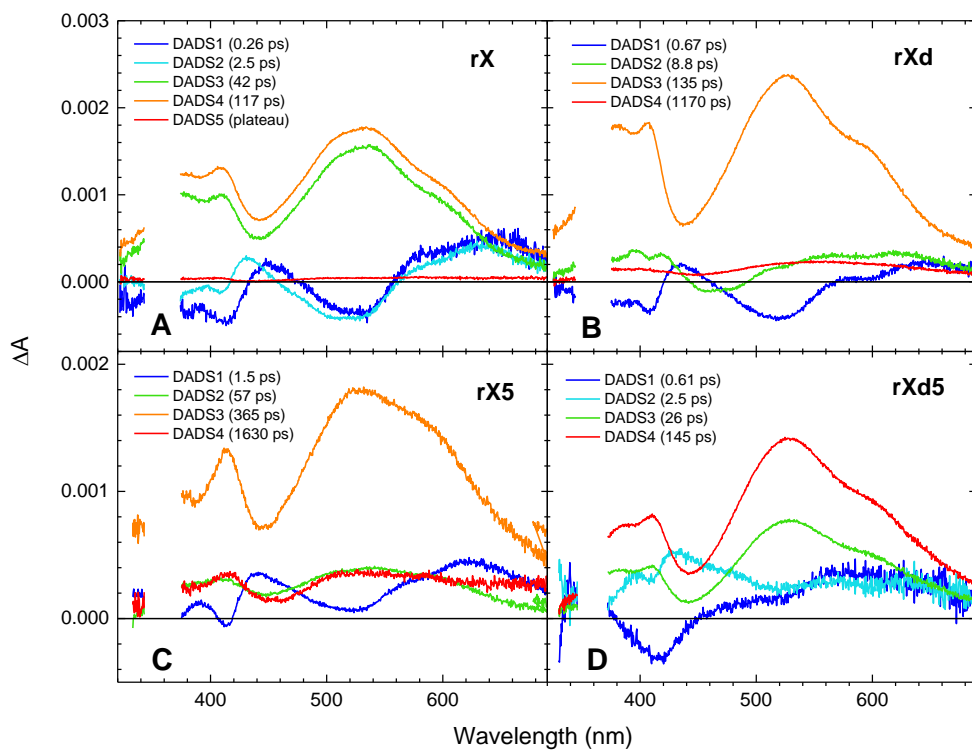
Fig. S4. DADS of the four *Tm*ThyX systems under study (see notations in the main text, Section 3.1).

Fig. S5. Normalized EADS of the four *Tm*ThyX systems under study. Normalization was arbitrarily made at the maximum of the TA_2 band. In the case of complex rX, the small ill-defined plateau (DADS5) has been removed from the calculation of the EADS.

Fig. S6. Comparison of the successive normalized EADS (Fig. S5) of the four *Tm*ThyX systems.

Supplementary references

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