Comparing ultrafast excited state quenching of flavin 1,*N*⁶-ethenoadenine dinucleotide and flavin adenine dinucleotide by optical spectroscopy and DFT calculations

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Abstract

Flavins are photoenzymatic cofactors often exploiting the absorption of light to energize photoinduced redox chemistry in a variety of contexts. Both flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) are used for this function. The study of these photoenzymes has been facilitated using flavin analogs. Most of these analogs involve modification of the flavin ring, and there is recent evidence that adenine (Ade)-modified FAD can affect enzyme turnover, but so far this has only been shown for enzymes where the adenine and flavin rings are close to each other in a stacked conformation. FAD is also stacked in aqueous solution, and its photodynamics are quite different from unstacked FAD or FMN. Oxidized photoexcited FAD decays rapidly, presumably through PET with Ade as donor and Fl* as acceptor. Definitive identification of the spectral signatures of Ade^{•+} and Fl^{•-} radicals is elusive. Here we use the FAD analog Flavin 1,*N*⁶-Ethenoadenine Dinucleotide (ϵ FAD) to study how different photochemical outcomes depend on the identity of the Ade moiety in stacked FAD and its analog ϵ FAD. We have used UV–Vis transient absorption spectroscopy complemented by TD-DFT calculations to investigate the excited state evolution of the flavins. In FAD*, no radicals were observed, suggesting that FAD* does not undergo PET. ϵ FAD* kinetics showed a broad absorption band that suggests a charge transfer state exists upon photoexcitation with evidence for radical pair formation. Surprisingly, significant triplet flavin was produced from ϵ FAD*. We hypothesize that the dipolar (ϵ)Ade moieties differentially modulate the singlet–triplet energy gap, resulting in different intersystem crossing rates. The additional electron density on the etheno group of ϵ FAD supplies better orbital overlap with the flavin S₁ state, accelerating charge transfer in that molecule.

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Graphical abstract

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1 Introduction

Flavins are vital biological redox molecules that can undergo either one- or two-electron transfer, making them much more versatile than the $2e^-$ redox cofactor nicotinamide [1]. Riboflavin consists of an isoalloxazine (iso or flavin) moiety with a ribose group appended at N10 (see Scheme 1 for numbering and physiologically relevant oxidation states). Phosphorylated riboflavin, known as flavin mononucleotide (FMN), can be adenylated to form flavin adenine dinucleotide (FAD, see Fig. 1: top). These more complex derivatives (but not riboflavin) are the essential flavin cofactors participating in electron transfer reactions that are fundamental to many biochemical processes [2] such as photosynthesis gene expression [3, 4], metabolic pathway signaling [5, 6], and DNA repair [7, 8].

Of these photoenzymes, DNA photolyase and its homologue cryptochrome exhibit photoinduced electron transfer (PET) using photoexcited flavin as the electron donor. The orientation of the FAD in these proteins is such that the adenine and flavin moieties are stacked in a coplanar conformation at a distance of $\sim 5 \text{ \AA}$ (see color inset, Fig. 1) [9]. Although the kinetics of DNA repair have been elucidated by our group [10] and others [11–15], the role played by this unusual FAD conformation in the electron transfer pathway is still unclear [16–19]. We have addressed the role of the FAD adenine in DNA repair by replacing it with 1,*N*⁶-ethenoadenine (ϵ Ade) through the chemical modification of FAD (ϵ FAD, see Fig. 1, bottom). ϵ FAD was first synthesized by Kochetkov, et al. [20] and characterized by Harvey and Damle [21] and Barrio and Leonard [22–24]. We hypothesized that ϵ FAD would have a higher thermodynamic

Scheme 1 Flavin structure and oxidation states

driving force for PET between the FI* and ϵ Ade because the LUMO energy of ϵ Ade is lower relative to Ade [25, 26], making reduction of ϵ Ade more facile than Ade, and thus increasing the rate of PET for FIH[•]* ϵ Ade $\rightarrow$ FIH[•] ϵ Ade^{•-}. This was tested by reconstituting *E. coli* PL with ϵ FAD. *E. coli* PL accepted the reduced ϵ FAD (termed “ ϵ PL”) and CPD repair was found to increase about 15% relative to FAD-reconstituted PL [27].

The stacked FAD conformation is rarely found in other FAD-dependent proteins, but a very similar conformation of FAD occurs in aqueous solution [28]. Oxidized flavins (e.g., FMN, FAD, ϵ FAD) have a high emission quantum yield in solution, but FAD* emission is quenched by a factor of 7 or more, depending on solution conditions. It is commonly accepted that the quenching mechanism is due to ultrafast photoreduction of the oxidized flavoquinone (FI) $\rightarrow$ flavo-semiquinone anion (FI^{•-}) with the adenine moiety acting as the reductant (see Eq. 1a), followed by fast charge recombination. However, only two studies have reported direct spectroscopic evidence that this ultrafast deactivation pathway is the quenching mechanism [29, 30] while others studies provide indirect evidence [31]. Thus, the photodynamics of solution-state stacked FAD can be considered as a primitive model of the excited state catalytic FAD in PL.

To gain deeper insight into how this conformation affects the photophysics of FAD we start here by determining whether photoexcited oxidized ϵ FAD analog shows a similar photophysical evolution, since it is known that ϵ Ade in ϵ FAD is stacked on the flavin ring [24]. Although an imperfect comparison, we expect that the higher HOMO energy of ϵ Ade compared to Ade (see below) will result in faster electron donation to the acceptor flavin LUMO by increasing the thermodynamic driving force, ΔG° , all other things being equal (i.e. reorganization energy λ , and donor–acceptor distance r_{DA} , and orbital overlap V_0).

The photophysics of flavins have been widely studied. For example, *FMN has a high quantum yield for the formation of ³FMN, greater than that for radiative decay or other non-radiative decay routes [32]. In contrast, the rapid excited state dynamics of FAD* vs. FMN* might be due to PET quenching, leading to the radical pair given in Eq. 1a.

The degree of stacking is estimated at ~80 to 90% [24], but this value depends on solvent and the method used, which include steady-state [24, 28] and time-resolved fluorescence quenching [33, 34], time-resolved absorption, [35, 36], and more recently, protein nanopore analysis [37]

Fig. 1 Top—Flat structure of Flavin adenine dinucleotide (FAD). Bottom—Flavin 1,N6 ethenoadenine dinucleotide (eFAD). Color inset—The stacked FAD structure from *A. nidulans* photolyase (adapted from PDB 1TEZ [9])

(note—this is, regrettably, an incomplete listing of the large number of studies that have addressed this question since the mid-twentieth century). Many time-resolved studies [15,

29, 30, 36, 38, 39] have been performed on FAD* but the identification of the radical Ade spectra has been wanting, with two possible exceptions. Li and Glusac [29] reported

transient UV/IR kinetics of FAD in solution by observing changes in the Ade infrared band at 1623 cm^{-1} . They observed a 1.1 ps decay assigned to forward electron transfer followed by charge recombination with a 9 ps lifetime (Eq. 1a). Brazard et al. [30] obtained decay-associated spectra of FAD*. The data were fitted using riboflavin radical anion and Ade radical cation spectra to simulate the electron transfer reaction, giving a ~ 5 ps lifetime for forward electron transfer followed by charge recombination on a faster time scale. For comparison, Kao et al. reported [31] forward electron transfer in 4–5 ps with recombination in 30–40 ps. However, these data were obtained from fluorescence measurements and do not contain explicit Ade radical signals.

In this work, we present the analogous photodynamics of ϵ FAD in aqueous solution to ascertain whether PET is occurring between the stacked (ϵ)Ade and Fl given by Eq. 1b:

where excitation is indicated by $h\nu$, and the rate constants for electron transfer and radical recombination (back electron transfer) are k_{FET} and k_{BET} respectively. Semiclassical Marcus theory [40, 41] provides for the computation of these rate constants if the maximum electronic coupling (V_0), donor–acceptor distance (r_{DA}), driving force (ΔG^0), and reorganization energy (λ) between reactant and product states are known.

Subpicosecond UV–vis transient absorption spectra presented here show that ϵ FAD* demonstrates ϵAde^{++} formation within picoseconds of excitation followed by recombination at a slower rate. The predicted peak of the radical spectra is reasonably well identified by the TD-DFT calculations. In addition, ϵ FAD* produces significant triplet ϵ FAD compared to FAD*. In contrast, FAD* decay shows no hard evidence of radical intermediates.

2 Materials and methods

2.1 Sample preparation and photoreduction

FAD and FMN were purchased from TCI, Japan (> 95% purity). The sample purity was determined by reversed-phase C18 high pressure liquid chromatography (Agilent 1100 HPLC) and used as received. Ade and ϵ Ade or their nucleosides were purchased from Sigma and used as received. ϵ FAD was synthesized following the procedure of Barrio et al. [24]. Approximately 50 mg of FAD was stirred gently in 20 mL of 1.5 M chloroacetaldehyde (Sigma-Aldrich, 50 wt% in water) for 72 h. The reaction was carried

out at room temperature in a light-protected round-bottom flask.

Purification of ϵ FAD was accomplished in two steps adopting the procedure of Light et al. [42]. The reaction mixture was first purified on a weak anion exchange resin, DEAE Sephadex A-25 (Sigma Aldrich), using 100 mM potassium phosphate buffer, pH 6.8, for loading and elution. The eluate was dried under vacuum and then further purified using reversed-phase HPLC using a YMC-ODS-AQ 250 mm $\times$ 10 mm (C18) column. An isocratic method was applied using 25% methanol in 5 mM ammonium acetate, pH 6.0. ϵ FAD purity was assessed by steady-state fluorescence and UV–vis absorption spectroscopies, as well as by HPLC. The yield of the purified product was typically $\sim 40\%$. The purified sample was vacuum-dried and stored at -20°C until used. To further confirm the product identification we have shown, as was demonstrated by Barrio et al. [24] a dramatic increase in ϵAde^* emission (data not shown) upon enzymatic hydrolysis of the phosphodiester bond [24] due to the loss of energy transfer between ϵAde^* and flavin in the severed and destacked dinucleotide.

2.2 Steady-state spectroscopy

All absorption measurements were carried out on Hewlett Packard 8452A diode array spectrophotometer having 2 nm resolution. Steady-state fluorescence studies were carried out on a PTI Quantamaster 3 single photon-counting fluorimeter (Birmingham, NJ). Excitation spectra were obtained with an excitation slit band pass of 2 nm and emission slit band pass of 3 nm. The emission wavelength was set to 530 nm. For emission scans, excitation was set to 450 nm with 3 nm bandpass and a 2 nm bandpass emission slit width. A four-window quartz cuvette with inner dimensions of 4×10 mm was used for all measurements.

2.3 Transient absorption spectroscopy

A mode-locked Ti:sapphire laser (KLM, ~ 80 MHz, 780 nm, ~ 600 mW) was amplified using a regenerative amplifier (Positive Light Spitfire Lite) to produce ~ 300 μJ , 120 fs pulses at ~ 250 Hz. The pulse was split into pump and probe pulses. A white-light continuum probe beam (WLC, 325–620 nm) was generated by focusing a few microjoules ($\text{NA} \sim 0.04$) into a slowly translating CaF_2 crystal. Care was taken to attenuate the near-IR fundamental remaining in the WLC by using a Schott BG39 filter. Most of the 780 nm fundamental was used to generate the pump beam at the second-harmonic, 390 nm, using a 2 mm type-I BBO crystal.

The pump beam was focused using a 40 cm quartz lens, while the WLC was focused using an off-axis 90° aluminum paraboloid with a focal length of 10 cm. The angle between the focused pump and the probe beams at the sample

was $\sim 2^\circ$. Their relative polarization was set to magic angle (54.7°) using a half-wave plate in the pump beam. A CMOS camera with $25\ \mu\text{m}$ square pixels was placed at the focus to measure the diameters of the focused beams. This is a crucial operation as the pump beam must be no smaller than the probe beam to ensure homogeneous pumping of the sample volume. In addition, the optical alignment of the delay stage (pump beam) was checked to ensure overlap over the full 3 ns range. Typical pump and probe beam diameters were 264 and $126\ \mu\text{m}$ respectively. The pump power was measured before and after the completed scans using a calibrated silicon joulemeter (Molelectron J3-S10), which was digitized and averaged by a 400 MHz digital oscilloscope. For these studies, the pump fluence was set between 0.05 and $0.50\ \mu\text{J}/\text{cm}^2$.

The pump-probe delay was set using a motorized delay rail that adjusted the path-length of the pump beam to provide a delay range of up to 3.2 ns. The delay stage was stepped in a logarithmic fashion, typically 6–7 steps per decade, from *ca.* -30 to 3200 ps, except over the interval of -2 to 2 ps which was sampled at 0.2 ps per step to obtain the cross correlation between the pump and WLC. To build the difference absorption spectra, the sample was pumped at 125 Hz and the WLC intensity was dispersed on a 240 mm spectrograph (Spectral Products DK240i) equipped with a 300 groove/mm grating and a $150\ \mu\text{m}$ slit. The dispersed light was detected with a cooled CCD detector (Andor, operating at 950×32 pixels) at 250 Hz. The individual transmittance spectra were transferred to a computer for construction of the ΔA spectra using LabView software (National Instruments, Austin, TX). Typically, 2000 spectra were obtained per time point over five scans.

For the chirp correction and fitting of the instrument response function (IRF), the cuvette sample solution was replaced with water or buffer. The high intensity pump beam induces an optical Kerr effect in the solution that refracts a fraction of the probe beam away from the detector. The instrument response function (IRF) was found to be *ca.* 200 fs (data not shown).

For transient absorption, the flavins were dissolved in 100 mM potassium phosphate buffer or 50 mM Tris buffer at pH 7.0 over concentrations of 175–350 μM . The sample was held in a 2 mm path length fused silica cuvette that was fitted with an air-tight septum. This cuvette was maintained at 20°C using a thermostatted cuvette holder (Quantum Northwest FLASH 300). The thermostatted sample was continuously stirred with an 8 mm diameter by 0.6 mm thick magnetic stir disc. The beams overlapped about 2–4 mm above the stir disc.

Pre-processing of the data using MATLAB (2020b, The MathWorks, Natick MA). Baseline correction was performed by averaging at least 5 pre-zero spectra and subtracting this from the data set. In the raw spectra, scattered pump

light at *ca.* 390–400 nm is mostly removed by the baseline subtraction procedure.

Global fitting analysis was performed with Glotaran 1.5.1 [43]. The data sets were smoothed along the wavelength axis by averaging 8 pixels per point, giving a bandpass of 3 nm. The experimentally obtained Kerr instrument response function (IRF) was fitted using Glotaran. This IRF was very nearly identical to that obtained when Glotaran uses only the sample ΔA vs. time data to estimate the IRF. In subsequent analyses, Glotaran was used in this fashion to model the IRF. The pre-processed and averaged data from at least two replicates were simultaneously fitted to models that reflect possible photodynamics schema.

Time-dependent spectral shifts, $\Delta\lambda_{\text{max}}(t) = \lambda_{\text{max}}(t) - \lambda_{\text{max}}(t = 1.4\text{ps})$ were computed by performing a constrained weighted fit of the transient absorption spectra at each delay time to five gaussian functions, using the ‘gauss5’ model and Trust-Region nonlinear regression algorithm from MATLAB 2021b (Natick, MA). The spectra were first normalized to the maximum in the excited state absorption around 350 nm for consistency. Five Gaussians were necessary to account for the appearance of triplet flavin, which grows in as a positive signal in the 600–640 nm region.

2.4 Computational methods

The ground-state geometries and molecular orbital energies of 9-CH₃- ϵ -Ade and 9-CH₃-Ade radicals were optimized as before [44, 45] using the Density Functional Theory (DFT) package in Gaussian 16 [46], with UB3LYP/6-311 + G(d,p) functional and basis set respectively, as well as lumiflavin anionic semiquinone as a control. B3LYP/6-311 + G(d,p) calculations for neutral lumiflavin, 9-CH₃- ϵ -Ade, and 9-CH₃-Ade were performed as additional checks on the radical calculations. The solvent was modeled using the polarizable continuum model (PCM) developed by Mennucci et al. [47] Minimum energy structures were confirmed by vibrational frequency calculations at the same level of theory.

Simulated absorption spectra were calculated using TD-DFT calculations at the same level of theory as the ground-state optimized geometry. Radical transition energies from the TD-DFT calculations using 20 excited states were used to generate absorption spectra. Transition energies for the neutral molecules were performed using 10 excited states. The vertical absorption spectra were generated in Chemcraft ver. 1.8 [48].

Fig. 2 **a** Steady-state absorption spectra of oxidized FMN, FAD, εFAD, Ado, and εAdo. The inset shows the 305 nm absorption region. **b** The excitation (—) and emission (···) spectra of 10 μM FMN, FAD, and εFAD in phosphate buffer at pH 7, 20 °C. Excitation and emission were measured with a bandpass of 2 and 3 nm respectively. For excitation spectra, $\lambda_{\text{ex}} = 300\text{--}510$ nm with $\lambda_{\text{em}} = 530$ nm. For emission spectra, $\lambda_{\text{ex}} = 450$ nm with $\lambda_{\text{em}} = 470\text{--}700$ nm. For all scans, a 2 nm step size was used

3 Results

3.1 Steady-state spectroscopy of FMN, FAD, and εFAD

UV–vis absorption spectra of adenosine (Ado) and ε-adenosine (εAdo) in phosphate buffer at pH 6.8 are shown as red and blue dots, respectively, in Fig. 2a. The spectra of the nucleobases show why εFAD is such an attractive analog. Ado has its characteristic UV absorption at ~260 nm. εAdo is more highly conjugated and extended than Ado, leading to a red-shifted UVB absorption band centered around 305 nm. This band, serendipitously, lies in a spectral “valley” between the intense $S_0 \rightarrow S_2$ (~370 nm) and $S_0 \rightarrow S_3$ (~265 nm) transitions of flavin, with an extinction maximum of about $\epsilon \sim 3,200 \text{ M}^{-1} \text{ cm}^{-1}$, compared to about $\epsilon \sim 1200 \text{ M}^{-1} \text{ cm}^{-1}$ for Fl at this wavelength.

The room temperature ground state absorption spectra of oxidized FMN, FAD, and εFAD are plotted in Fig. 2a

(black, red, and blue lines respectively), with the flavin singlet $S_0 \rightarrow S_n$ transitions ($n = 1\text{--}3$, S_{0n}) indicated. The absorption spectra of εFAD and other ε-dinucleotides have been characterized by Barrio et al. [22–24] and others [21, 49]. The $S_0 \rightarrow S_1$ transition for εFAD spectrum is about 4 nm to the red of the FAD spectrum. Above 300 nm, εFAD and FAD deviate in extinction most significantly at ~310 nm (see Fig. 2a, inset), which is due solely to the εAde moiety.

The excitation and emission spectra of 10 μM FMN, FAD, and εFAD in phosphate buffer at pH 7, 20 °C are shown in Fig. 2b. The fluorescence intensity is governed by the emission quantum yield, $\Phi_f = k_R / (k_R + k_{\text{ISC}} + k_{\text{NR}})$, where k_R , k_{ISC} , and k_{NR} are the radiative, intersystem crossing, and non-radiative rate constants, respectively. k_{NR} can be further broken down into k_{IC} and k_{ET} , which are the rate constants for internal conversion and electron transfer respectively. For excitation spectra, $\lambda_{\text{ex}} = 300\text{--}510$ nm with $\lambda_{\text{em}} = 530$ nm. For emission spectra, $\lambda_{\text{ex}} = 450$ nm with $\lambda_{\text{em}} = 470\text{--}700$ nm. For all scans, a 2 nm step size was used. The data have been normalized to the maximum intensity of the FMN excitation spectrum.

The steady state fluorescent properties of εFAD were identical with previous measurements [23]. Under these conditions, the ratio of the integrated emission intensities for FAD and εFAD relative to FMN are 0.20 and 0.14, respectively. Thus, εFAD is quenched about 7× more than FMN, compared to the quenching observed for FAD relative to FMN (5× lower).

3.2 Calculated spectra of Ade and εAde radical ions

To our knowledge, calculations of the energies and spectra of εAde radicals have not been reported, although high level calculations of εAde are available [25, 26, 50]. The TD-DFT-derived spectra are shown in Fig. 3a for 9-CH₃-N1,N6-ethenoadenine (εAde, black line), 9-methyladenine (Ade, red line). Additionally, the computed lumiflavin anionic semiquinone is shown in Fig. 3a (blue line). The transition energies, oscillator strengths (f_{osc}), and their orbital characteristics are tabulated in SI Table 1. Transitions that have an oscillator strength greater than 0.05 (dashed line) and within the wavelength range of the probe pulse have been labeled with their peak wavelength. The inset in Fig. 3a shows the ground state dipole moments of (ε)Ade, which become relevant in the discussion of stacking orientation below.

The TD-DFT derived spectra of the 9-methyl (ε)Ade neutral species at the same level of theory using a water PCM are plotted in Fig. 3a, as a validation of the radical calculations. For 9-CH₃-Ade, $f_{\text{OSC}} = 0.267$ and $\lambda_{\text{max}} = 254$ nm, as compared to the experimental value, $f_{\text{OSC}} = 0.2$ [51–53]. Our result is in reasonable agreement with the experiment. The permanent dipole moment of 9-CH₃-Ade in a water PCM is $|\vec{\mu}_{\text{Ade}}| = 3.7D$ (red arrow) and points parallel to the

Fig. 3 UV-vis spectra generated by TDDFT/PCM calculations of **a)** the neutral bases along with lumiflavin radical anion and **b)** 9-methyladenine and 9-methyl-ethenoadenine radical cations. Water was used as the polarizable continuum. Transitions with oscillator strengths above 0.05 and $\lambda_{\max} > 300$ nm are labeled by wavelength. In panel a) the permanent ground state dipole moments for ϵ Ade (black arrow) and Ade (red arrow) are shown on their molecular framework

AC8-AN1 axis. This can be compared with the result of Hanus et al. for 9H-adenine using the RI-MP2 level using the TZVPP basis set and a C-PCM solvation model [54] obtaining $|\vec{\mu}_{\text{Ade}}| = 2.8D$. The experimental value for 9-methyladenine in CCl_4 is $3.0D$ [55]. The transition is dominated by a $\pi\pi^*$ HOMO $\rightarrow$ LUMO (MO 39A $\rightarrow$ 40A), and the Ade HOMO (39A—donor orbital) is plotted below, along with the Lf LUMO acceptor MO (68A, see SI Table 1 and SI Fig. 1).

We find the lowest energy bright transition for 9-CH₃- ϵ Ade at 4.08 eV (MOs 45A $\rightarrow$ 46A, $\pi \rightarrow \pi^*$, see SI Table 1). The transition lies at $\lambda_{\max} = 304$ nm with $f_{\text{osc}} = 0.073$ (black line, Fig. 3a). This compares favorably with our experimental results ϵ Ado in 0.1 M phosphate buffer at pH 6.8, shown in the top panel of Fig. 2, where $\epsilon \sim 3400 \text{ M}^{-1} \text{ cm}^{-1}$ ($f_{\text{osc}} \sim 0.06$, as estimated from Fig. 2a using $f_{\text{osc}} \sim 4.32 \times 10^{-9} \int \epsilon(\tilde{\nu}) d\tilde{\nu}$), and $\lambda_{\max} = 296$ nm respectively. By comparison, Major and Fischer [26] published theoretical results for neutral ϵ Ade using B3LYP functional with the aug-cc-pVDZ basis

set in aqueous solution. They reported that the lowest energy transition is at 4.08 eV in good agreement with our method. Given the different solvent conditions, a small difference in $\lambda_{\max}$ is understandable. We report the first calculation of the permanent ground state dipole moment $|\vec{\mu}_{\epsilon\text{Ade}}| = 7.3D$, which points roughly along the $\epsilon\text{AC4-C5}$ axis (black arrow). To the best of our knowledge, the calculated oscillator strength of ϵ Ade in water is not available. However, Holmen et al. obtained the gas phase value of $f_{\text{osc}} = 0.057$ from a Pariser–Parr–Pole calculation [25], and the larger f_{osc} for solvated ϵ Ade obtained here is reasonable. This $\pi\pi^*$ transition is essentially HOMO $\rightarrow$ LUMO (MO 45A $\rightarrow$ 46A), and the ϵ Ade HOMO (45A—donor orbital) is plotted below, along with the Lf LUMO (68A—acceptor orbital, see SI Table 1). The computed (ϵ)Ade MOs (natural bond order representation) used in this study are shown in SI Fig. 1.

As a test of computed radical spectra, the calculated lumiflavin radical anion spectrum is shown in blue. Characteristic bands at 439, 385, and 362 nm are observed in experimental [6, 56] and computational [57–59] studies. Experimental comparisons are difficult because Lf^{•-} is not stable in water, but can be estimated from the spectroelectrochemical work [60] of Niemz et al. on *N*(3)-methyl-*N*(10)-isobutylflavin in CH_2Cl_2 by comparing the absorbance of the oxidized flavin at 450 nm with the anionic semiquinone at 360 nm. Based on this, Fl^{•-} in solution has an extinction at 360 nm of $\epsilon \sim 8,000 \text{ M}^{-1} \text{ cm}^{-1}$ and $\sim 5,000 \text{ M}^{-1} \text{ cm}^{-1}$ from 450–500 nm, an 1.6:1 ratio. This can be compared to the computed ratio of $f_{\text{osc}} = 0.33/0.08$ ($\sim 4:1$).

(ϵ)Ade radicals have rich UV-Vis spectra with transitions that would have sufficient extinction to be seen in TA experiments. The computed spectra of radical cations $\epsilon\text{Ade}^{\bullet+}$ and $\text{Ade}^{\bullet+}$ in a water PCM are shown in Fig. 3b. Of particular importance here, the spectrum of 9-CH₃- $\epsilon\text{Ade}^{\bullet+}$ radical cation (black line) has only one transition at 460 nm that has significant oscillator strength in the probe wavelength range. The computed $f_{\text{osc}} \sim 0.089$ suggests that the extinction of the 9-CH₃- $\epsilon\text{Ade}^{\bullet+}$ radical cation is approximately $0.089 \times 3400 \text{ M}^{-1} \text{ cm}^{-1}/0.073 = 4145 \text{ M}^{-1} \text{ cm}^{-1}$, stronger than the 9-CH₃- ϵAde absorption at 304 nm (Fig. 3a). It also has a larger oscillator strength than 9-CH₃- $\text{Ade}^{\bullet+}$, $f_{\text{osc}} = 0.056$ at $\lambda_{\max} = 547.5$ nm (red line). This is significant because $\text{Ado}^{\bullet+}$ has been observed in transient absorption experiments, with an estimated extinction $\epsilon \sim 1500 \text{ M}^{-1} \text{ cm}^{-1}$ at about 590 nm [61, 62]. Thus, the calculated cation radical spectrum and extinction coefficient of 9-CH₃- $\epsilon\text{Ade}^{\bullet+}$ appear reasonable based on the agreement between the computed 9-CH₃- $\text{Ade}^{\bullet+}$ and the cited experimental adenosine radical spectra.

If PET takes place in ϵFAD^* , then the Fl_{OX} ground state bleach at ~ 460 nm ($\epsilon_{\text{Fl}} \sim 10,700 \text{ M}^{-1} \text{ cm}^{-1}$, see Fig. 2a) should be followed by the appearance of the Fl^{•-}: $\epsilon\text{Ade}^{\bullet+}$ radical pair (RP) spectrum. Adding their extinctions gives $\epsilon_{\text{RP}} \sim (4145 + 5000) \text{ M}^{-1} \text{ cm}^{-1} = 9145 \text{ M}^{-1} \text{ cm}^{-1}$. This is

within 10% of the extinction lost in the flavin ground state bleach and we would expect this spectral region to collapse as the $\Delta\epsilon_{\text{RP}} \sim -\Delta\epsilon_{\text{Fl}}$. This is discussed below in the analysis of the transient absorption data.

3.3 Molecular orbital energies and the thermodynamics of PET

DFT calculations (see Sec. 2.4 and SI Tables 2–7 for further details) of the molecular orbital (MO) energies for the neutral and radical species described above are plotted in Fig. 4 in atomic units. All calculations were performed using 20 excited states and a water PCM. The energies of the highest occupied MOs (HOMO) are rendered in black, singly-occupied molecular orbitals (SOMO) are rendered in blue, and the lowest unoccupied MOs (LUMO) are rendered in red. The left hand side of the figure shows energies of MOs involved in forward PET, $\text{Fl}^+ \cdots (\epsilon)\text{Ade} \xrightarrow{k_{\text{FET}}} \text{Fl}^- \cdots (\epsilon)\text{Ade}^+$ and the right hand side shows the energetics of the recombination reaction, $\text{Fl}^- \cdots (\epsilon)\text{Ade}^+ \xrightarrow{k_{\text{BET}}} \text{Fl} \cdots (\epsilon)\text{Ade}$.

The calculations suggest that forward electron transfer for ϵFAD^* is slightly faster than FAD^* due to its higher driving force ($\Delta\Delta E = \Delta E(\epsilon\text{FAD}) - \Delta E(\text{FAD}) = -0.009$ a.u. = -0.24 eV), assuming the reorganization energies are the same. The driving force for back electron transfer is more than $8 \times$ larger for each radical pair, although the difference in ΔE between the two systems is smaller than for FET ($\Delta\Delta E = 0.003$ a.u. = -0.06 eV). If the reorganization energy is about the same as for FET, then BET may be in the Marcus inverted region, leading to slower recombination. Again, there is no accounting for differences in electronic coupling (orbital overlap, donor–acceptor distance), which may be the most important differential factor in the rate of charge separation.

3.4 Ultrafast transient absorption spectroscopy of oxidized flavins—general observations

It has been widely accepted that the fluorescence of oxidized FAD^* is quenched due to stacking with the adenine moiety [24, 30, 33, 38, 63]. To investigate the role of adenine in this non-radiative decay pathway of photoexcited FAD^* , and to provide a reference point for comparison to ϵFAD^* , FMN* transient measurements were made to model the unstacked excited state dynamics of the flavin core.

Time-resolved difference spectra for FMN from 320 to 620 nm and 1 ps to 3.2 ns are shown in Fig. 5a at selected time points. The data are shown as points, and the spectra returned from the kinetic modeling (see below) are shown as lines, with the residuals between the kinetic model fit and data are given as fine lines dispersed around $\Delta A = 0$. The time-dependent spectral shifts, $\Delta\lambda_{\text{max}}(t) = \lambda_{\text{max}}(t) - \lambda_{\text{max}}(t = 1.4\text{ps})$, as determined by a five

Fig. 4 DFT results for the molecular orbital energies of Ade, ϵAde , and their radical cations, as well as the lumiflavin LUMO and its radical anion SOMO. Calculations on neutral and radical molecules were performed at the B3LYP/6-311+G(d,p) (10 states) and U3LYP/6-311+g(d,p) (20 states) level of theory respectively. The MOs used are identified by number below each level, and further information about the functional/basis set used can be found in SI Tables 2–7. The solid arrows indicate forward electron transfer (FET) for each pair of reactants. The dashed arrows indicate back electron transfer (BET, or recombination)

gaussian fit to the data, are plotted for the major spectral bands (see below) and shown in Fig. 5a, inset. The amplitudes of the fitted Gaussians as a function of delay time are shown in SI Fig. 2a for FMN.

The excited state absorption band centered at 350 nm, ESA_{350} , shows little change over time in either magnitude or λ_{max} (inset Fig. 5a, black line). Similarly, the ground state bleach (GSB₄₄₄, in red) is relatively constant in amplitude and λ_{max} but undergoes a small red shift starting around 1 ns. A second excited state absorption at ~ 507 nm (ESA_{507} , in green) shows little decay or shift. The fitted Gaussian amplitudes, normalized to the amplitude of the ESA_{350} at each time point, are shown in SI Fig. 2a for comparison.

The behavior of the signal beyond 520 nm become complex. The amplitude of the SE_{578} band (blue) is relatively constant but decays rapidly after 1 ns. The large red shift is due to vibrational cooling in the excited state manifold. After 1 ns the band appears to blue shift by ~ 10 nm. Evidence for triplet flavin formation ($^3\text{Fl}_{600}$, cyan in the inset) is seen in the rising TA signal around 600 nm, which is shown more definitively in the modeling below. The spectral shift for this region (cyan in the inset) shows a large red shift almost immediately after excitation, but with an accompanying small amplitude (SI Fig. 2a, cyan), putting into doubt the accuracy of the gaussian fitting in this

Fig. 5 Representative data sets for FMN, FAD, and ϵ FAD transient absorption spectra in phosphate buffer, pH 7, with excitation at 397 nm. The legend on the right side shows the time (in ps) at which the spectra were taken. Residuals between the data (•) and fitted models (—) are given as fine lines around $\Delta A = 0$. Arrows indicate isosbestic points. The insets in each panel capture the spectral shifts of these major bands vs. time, as determined using a five gaussian model

region. This is to be expected when the peak of the triplet [64] (> 650 nm) outside of wavelength range used here.

To facilitate a qualitative comparison of FAD* and ϵ FAD* with FMN*, it will be useful to reference the maximal ΔA at 1.4 ps for the spectral bands to the ESA_{350} . For FMN*, these ratios are $GSB_{444}/ESA_{350} = 0.50$, $EAS_{507}/ESA_{350} = 0.25$, and $SE_{578}/ESA_{350} = 0.20$ (tabulated in Table 1 and also apparent in SI Fig. 2a).

As observed in prior studies [30, 63], FAD* shows rapid excited state decay, losing about half of its extinction within ~ 5 ps (Fig. 5b) due to the quenching effect of adenine stacked on the isoalloxazine ring ($\sim 80\%$ of the sample). Isosbestic points are observed at 407 and 536 nm. The 536 nm isosbestic point, missing in the FMN* decay, suggests that triplet formation is quenched by the adenine. The

loss of the isosbestic point at 478 nm may be reporting on the creation of the anionic flavosemiquinone, $FI^{\bullet-}$, which has significant extinction in this spectral region [6, 56, 65], but no concomitant change is observed in the 320 and 550 nm regions where Ade $^{++}$ is expected to absorb.

The $\Delta\lambda_{max}(t)$ for FAD* are similar to FMN* but do not show any evidence of triplet formation. There is a small blue

Table 1 the ratios of ΔA_{max} referenced to $\Delta A_{max}(ESA_{350})$

	GSB_{444}/ESA_{350}	EAS_{507}/ESA_{350}	SE_{578}/ESA_{350}
FMN	0.50	0.25	0.20
FAD	0.50	0.25	0.17
ϵ FAD	1.0	0.67	0.07

shift of the FAD* ESA₃₅₀ (see inset Fig. 5b, black) where FI^{•−} has significant extinction. The GSB₄₄₄ band also blue shifts slightly within 10 ps after excitation (inset Fig. 5b, red). The time dependence of the ESA₅₀₇ band (green) is more complex, beginning to blue shift after ~20 ps but then red shifting after 1000 ps. The SE₅₇₈ band red shifts, again probably due to vibrational cooling in the S₁ manifold. Unlike FMN*, there is no positive ΔA in the 600 nm region. We ascribe this behavior to the quenching of the stacked FAD*, leaving behind the unstacked FMN-like species that has stimulated emission to longer wavelength. The decay-associated difference spectra from the kinetic model (see below) shows evidence for a small amount of ³FAD at long times, consistent with the unstacked FAD population (~20%).

FAD* transient absorption spectra are nearly identical in shape and proportion to FMN* around ~1 ps. Maximal ΔA ratios for FAD* are GSB₄₄₄/ESA₃₅₀ = 0.50, ESA₅₀₇/ESA₃₅₀ = 0.25, and SE₅₇₈/ESA₃₅₀ = 0.17. These are virtually identical to those for FMN* (also see SI Fig. 2b). The largest contrast is the ESA₃₅₀/SE₅₇₈, which is about 15% lower for FAD*, consistent with the steady-state fluorescence quenching observed (c.f. Fig. 2b). As mentioned above, there is no obvious spectral signature for the adenine radical cation. This suggests that quenching in stacked FAD* may proceed by a different mechanism than PET.

For εFAD, as with FAD, a distribution of stacked to unstacked conformations are also expected at the moment of excitation. εFAD* photodynamics evolve more rapidly than FAD* initially, with only a single isosbestic point at 408 nm (Fig. 5c). The faster decay kinetics are consistent with the lower emission quantum yield (c.f. Fig. 2b) indicative of a higher quenching rate compared to FAD.

The εFAD* spectra show several differences compared to FAD*. Δλ_{max}(t) (inset Fig. 5c) shows that the ESA₃₅₀ and ESA₅₀₇ bands blue shift significantly within 10 ps of excitation, with the largest shifts in the ESA₅₀₇ region. Of special significance is the behavior of the ESA₃₅₀ band, which initially has a λ_{max} = 360 nm, consistent with the ultrafast formation of the FI^{•−} radical. The band then blue shifts to 350 nm in about 10 ps, looking like FMN*. The GSB₄₄₄ band decays rapidly with little change in λ_{max}. This can be rationalized formation of FI^{•−} whose extinction could negate and shift the FI_{ox} ground state bleach. This is also the spectral region where the TD-DFT calculated spectrum shows that εAde^{•+} has oscillator strength. The amplitude of the 507 nm band (SI Fig. 2c, green) at early time also supports this possibility. Within 5 ps of excitation the SE₅₇₈ band exhibits a large red-shift (~10 nm) but blue shifts after 1 ns. The early time behavior of this band suggests the formation of a new species with significantly greater extinction than for stimulated emission pumping. This behavior was not seen for FAD*. Finally, and unexpectedly, ³FI [66, 67] appears

immediately after excitation, as evidenced by the positive ΔA above 600 nm. This assignment is also supported by the increase in amplitude of the ³FI band (SI Fig. 2c, cyan) in the first few picoseconds.

The ratios of ΔA(t = 1.4 ps) relative to the ESA₃₅₀ are: GSB₄₄₄/ESA₃₅₀ = 1.0, ESA₅₀₇/ESA₃₅₀ = 0.67, and SE₅₇₈/ESA₃₅₀ = 0.07, as tabulated in Table 1. The ESA₅₀₇/ESA₃₅₀ ratio for εFAD* is more than twice that for FAD*. This is consistent with the formation of εAde^{•+} and FI^{•−}, which have sufficient extinction to raise the ΔA compared to FAD* over the same wavelength range (see the discussion above, Sect. 3.2), supporting ultrafast charge separation leading to the diradical FI^{•−}:εAde^{•+} intermediate, a supposition also consistent with the 0.07 ratio for SE₅₇₈/ESA₃₅₀. For the unstacked εFAD* fraction, Δλ(t) should shift to FMN*-like values and decay radiatively or through intersystem crossing to the triplet.

3.5 Band integral time courses

The temporal evolution of the four major spectral bands were retrieved using Lustres' band integral (BI) method [68], according to

$$\text{BI}(\lambda_1, \lambda_2; t) = \int_{\lambda_1}^{\lambda_2} \frac{\Delta A(\lambda, t)}{\lambda} d\lambda.$$

The band integral method removes artifacts in the time courses due to band shifts. The ESA₃₅₀, GSB₄₄₄, ESA₅₀₇, SE₅₇₈, and ³FI₆₀₀ nm ranges highlight the evolution of radical ions FI^{•−}, [60] εAde^{•+}, Ade^{•+} [61, 62, 69], and triplet flavin, respectively. The time courses were normalized by $\text{BI}(\lambda_1, \lambda_2; t)_{\text{norm.}} = \text{BI}(\lambda_1, \lambda_2; t > 1 \text{ ps}) / \max(|\text{BI}(\lambda_1, \lambda_2; t > 1 \text{ ps})|)$ and are plotted from −3 to 3200 ps, with the first +25 ps plotted on a linear time scale, and the remaining decay on a logarithmic time scale (Fig. 6a–e). Integrated data are given as symbols. The fits from the kinetic models (see below) are plotted as solid lines.

In Fig. 6a, the FMN* ESA₃₅₀ BI(t) (data—black dots, fit—black line) rises to a quasi-constant value within 10 ps after excitation. It is relatively constant out to 300 ps after which it drops about 40%, consistent with intersystem crossing to ³FMN. The EAS₃₅₀ BI(t) for FAD* (data—red dots, fit—red line) decreases to ~40% of its initial amplitude in less than 20 ps, presumably from stacked conformations undergoing immediate quenching. Further evolution is clearly not mono-exponential, probably due to dynamic stacking over its excited state lifetime [70].

In contrast, the significantly more rapid decay of the EAS₃₅₀ for εFAD* (blue symbols and lines) falls to ~30% of its t = 0 value by 15 ps and is FMN*-like after this time. This suggests that dynamic stacking in εFAD* may occur

Fig. 6 Band integral analysis (•) and fits (—) for FMN, FAD, and ϵ FAD. In contrast to FMN and FAD, at 578 nm ϵ FAD shows a rapid increase before turning negative. The FAD transient rises more rapidly than ϵ FAD, but its decay appears slightly slower. See Tables 2, 3 for lifetimes for the fits based on the models in Scheme 2

to a lesser degree than for FAD*, that the stacked conformation of ϵ FAD is better stabilized than for FAD. Roughly the same behavior is seen in the decay of the GSB₄₄₄ spectral band (Fig. 6b). Taken together, they suggest that about 70% of ϵ FAD* decays within 15 ps with no changes in stacking thereafter.

The ESA₅₀₇ BI(*t*) for FMN (Fig. 6c) rises to a constant value after 5 ps. After about 300 ps, it increases slightly and then decays after 1 ns. Interestingly, FAD* and ϵ FAD* share a faster rise time, but the BI(*t*) for ϵ FAD* decays more quickly and again exhibits the plateau behavior as if it were FMN-like after about 20 ps.

The SE₅₇₈ band integrals (Fig. 6d) show a deviation from the previous trends. Here, FMN* has a negative BI(*t*). The sharp drop in the FMN* BI(*t*) is followed by a 1–5 ps evolution assigned to the S₂ state undergoing solvation and

internal conversion to S₁. This is mirrored in the growth of the ESA₅₀₇ BI(*t*). FAD* BI(*t*) shows similar behavior to FMN*, with a concomitant rise in the ESA₅₀₇. It is unclear whether the results support radical pair formation or a fast deactivation via a conical intersection or some other internal conversion process.

The ϵ FAD* BI(*t*) for this band is strikingly different from FMN* and FAD*. Instead of a negative ΔA , the BI(*t*) rises significantly above $\Delta A = 0$ and starts decaying after about 2 ps (the sharp spike at *t* ~ 0 ps is due to cross phase modulation that is not removed by the Glotaran [43] instrument response algorithm). After 5 ps, $\Delta A < 0$ and ϵ FAD*, presumably the unstacked form, once again tracks the evolution of the FMN* BI(*t*). We assign this positive ΔA as evidence that PET and back electron transfer are occurring for some, if not all, of the stacked ϵ FAD*. Triplet formation is suggested by $\Delta A > 0$ at long times, and this is confirmed below. Thus, ϵ FAD*, unlike FAD*, has a non-negligible triplet quantum yield.

The ³FI₆₀₀ band integrals (Fig. 6e) are similar to the SE₅₇₈ decays, with the important difference that the negative FAD* signal decays more slowly than in other spectral regions, suggesting that some fraction of unstacked FAD* is picked up by this band integral.

3.6 Modeling the transient absorption kinetics

For a parallel, decay scheme, each of *n* species, *l* = 1 → *n*, starts at the same initial concentration, and decays with its characteristic rate constants *k_l*, as modelled by $\sum c_l = \exp(-k_l t)$ (Sect. 2.4.1 of the Stokkum reference [71]). The spectra from this analysis are termed Decay Associated Difference Spectra, or DADS. Clearly this model does not apply here since the triplet is not an independent species, decaying in parallel with FI^{•−}, but rather building up through intersystem crossing from FI*.

Instead of a DADS model, it is often useful to employ a sequential model, termed Evolution-Associated Difference Spectra (EADS). The EADS can be quite different from the DADS for the same data (e.g., see Fig. 1 in the Stokkum reference [71]). As a control, we have fitted the FMN data using a 4-state EADS model (see SI Figs. 3 and 4). It matches the analysis of FMN photodynamics by Andrikopoulos et al. [72] nearly perfectly. However, that model is incomplete because it implies that all of the FMN* population evolves to ³FMN.

In contrast, a compartmental or target analysis (Species-Associated Difference Spectra or SADS, Stokkum Sect. 2.4.3 [71]), consists of *nc* compartments, where the time rate of change of the species concentration *c*(*t*) = [*c*₁ *c*₂ ... *c_{nc}*]^T in each compartment is given by $\frac{dc(t)}{dt} = Kc(t) + j(t)$, where *j*(*t*) = *i*(*t*)[*x*₁ *x*₂ ... *x_{nc}*]^T are the populations of the different states, *i*(*t*) is the IRF, and *K* is the transfer matrix of rate

constants that control population kinetics between compartments. This approach is useful for modeling sequential or branched kinetic models for species that have zero population initially, i.e. that are not initially excited by the pump pulse. It is also possible to set the initial populations of the states to take into account stacked vs. unstacked fractions of (ϵ)FAD. This is the most appropriate model for (ϵ)FAD* studied here.

3.7 FMN transient kinetics modeling

The photodynamics of FMN* must be consistent with the observed competition between fluorescence (radiative decay) and evolution to ^3FMN (intersystem crossing). Ultrafast internal conversion and solvent dynamics at early time should be represented as well. Therefore, the photodynamics of FMN* were modelled using 4 states with one branch. The kinetic SADS scheme is given in Scheme 2a, with the states labeled by boxed numbers. For the FMN SADS model, excitation at 390 nm populates both S_1 and S_2 states. The S_2 state decays by internal conversion/solvent relaxation to S_1 with rate constant k_1 . The FMN kinetics were fitted assuming simultaneous population of S_1 and S_2 states with fixed populations $j_1=0.29$ and $j_2=0.71$. This partitioning comes from a spectral deconvolution of the S_{10} and S_{20} electronic transitions from a study of the 77 K absorption spectroscopy of oxidized flavin [73].

The S_1 state evolves with a subpopulation emitting fluorescence (radiative decay) and a larger population crossing over to the triplet state (intersystem crossing) [74]. This involves a branching step represented by rate constants b_1k_2 and b_2k_2 , where b_1 and b_2 are coefficients that weight k_2 depending on the branching of population. The branching coefficients, $b_1=0.33$ and $b_2=0.67$ represent the radiative and triplet quantum yields respectively [74]. The triplet decays with a lifetime of 10 μs , which is fixed at this value, as others have done, because the limited experimental time range cannot afford a good fit of this parameter [72].

Four spectra are retrieved from this SADS model (see Fig. 7a). Qualitatively, the spectra are similar to those reported previously (e.g., Stanley and MacFarlane [35], Li et al. [29], Plaza's group [30], Fuertes' group [72], and, regrettably, others too numerous to cite here). However, these previous analyses are based on parallel or sequential models, and many of them do not account for the bifurcation of population that leads to radiative decay or triplet decay. The four FMN SADS have identical spectral profiles and time constants as those obtained from the 4 state sequential model of Andrikopoulos et al. [72] (see Tables 2, 3 for fitted lifetimes, and the 4 state EADS in SI Fig. 3 for comparison). The relatively weak features of the 1.1 ps SADS1 (black line) is attributed to a combination of internal conversion

[35] from $S_2 \rightarrow S_1$ and solvent reorganization, which, given our IRF width of ~ 200 fs, is difficult to capture with high fidelity.

SADS2 represents the branch between fluorescence and triplet formation. It should be emphasized that the branching of State 2 has an apparent lifetime, $(k_2)^{-1}=4.7$ ns. Applying the branching coefficients gives two rate constants. Here the branch that corresponds to radiative decay, $b_1=0.33$, yields 14.2 ns, which is very close to the fluorescence lifetime of 17.1 ns observed by Tyagi et al. for riboflavin in water [75]. The lifetime for the evolution of $^1\text{FMN} \rightarrow ^3\text{FMN}$ is about 4.0 ns ($=1/(b_2k_2)$). Both lifetimes are probably solvent- and conformation-dependent, although we have not tested these assumptions here. However, these lifetimes are in reasonable agreement with the TR-IR work of Li et al. [29] and Plaza et al. [30] when solvent conditions are taken into account.

The FMN SADS2 and SADS3, red and green lines respectively, are nearly identical in shape (see normalized SADS in SI Fig. 4). In SADS2 and 3, the signature oxidized flavin excited-state absorptions at about 350 and 500 nm, a ground state bleach around 439 nm, and stimulated emission at around 570 nm are consistent with those studies cited above. The 4th SADS (blue) reproduces ^3FMN quite well, with a minimum ΔA at 445 nm [29, 72, 76]. As mentioned above, our 4 state SADS fit returns rate constants that are virtually identical to the 4 state EADS Fuertes study [72] (c.f. SI Fig. 3), whose TA measurements utilize as long a time window as was employed here.

3.8 FAD transient kinetics modeling

FAD and ϵFAD transient data require a more complex SADS model that reflects both forward and back electron transfer (FET and BET respectively). However, attempts to add these two extra states to the FMN SADS model failed, most likely due to imperfect signal to noise and cross-phase modulation (XPM) in the data sets. Under the assumption that $S_2 \rightarrow S_1$ internal conversion and solvent relaxation signals for stacked FAD* are subsumed in the ET kinetic signatures, we constructed the FAD SADS model shown in Scheme 2b. Here, the pump pulse excites the stacked FAD species ($\sim 80\%$) and the open FMN-like population (20%) directly. FAD* evolution has been shown to undergo dynamic stacking/destacking that occurs over the excited state lifetime of the molecule [70], illustrated using a cartoon of open and stacked FAD conformations at the top of the Scheme. This will produce a distribution of rate constants, but we have not included this in our model.

To begin the fit, the populations of the open (State 1) and stacked (State 4) populations were fixed to $j_1=0.2$ and $j_4=0.8$ (see, for example, van der Berg et al. [70] and Radoszkowicz et al. [77]). Because of dispersion in the UV and XPM, it was necessary to fix $k_1=10$ ps $^{-1}$. The branch

assumption that open and stacked molecules are excited in a 0.2:0.8 ratio. Scheme **a** was incorporated to model the open conformation but simplified by excluding $S_2 \rightarrow S_1$ internal conversion (labeled as S_2/S_1). Here, a branched step represented by b_1k_1/b_2k_1 where $b_1:b_2$ is 0.33:0.67 as in **a**. Stacked molecules are assumed to be excited into S_2/S_1 but decay in picoseconds to a radical ion pair, followed by recombination, or by ultrafast ISC to the T_1 state. The wide double-headed arrow is to remind the reader that dynamic interconversion of stacked and unstacked conformations of FAD will occur during the lifetime of the excited state

Fig. 7 Species-associated Decay Spectra (SADS) for FMN, FAD, and ϵ FAD. Significantly, in **c**, SADS4 and 5 suggest ultrafast charge separation in ϵ FAD*, while FAD* (**b**) shows little evidence for radical formation. The time constants for the models are given in each panel and are tabulated in Tables 2, 3. These and the models used are discussed in more detail in the text

Table 2 Lifetimes, in ps unless otherwise indicated, from the SADS fitting of FMN, FAD, and ϵ FAD (see Scheme 2, * indicates fixed parameter)

	τ_1	τ_2	τ_3	τ_4	τ_5
FMN	1.07 ± 0.02	4690 ± 350	138 ± 6	$10 \mu\text{s}^*$	NA
FAD	0.1^*	1562 ± 47	$10 \mu\text{s}^*$	3.15 ± 0.08	8.6 ± 0.1
ϵ FAD	0.1^*	3706 ± 253	$10 \mu\text{s}^*$	0.67 ± 0.01	2.44 ± 0.02

coefficients $b_1 = 0.33$ (radiative decay) and $b_2 = 0.67$ were fixed using the steady-state values for the quantum yields for these processes [74]. The rate constant for the decay of the FMN-like triplet was also fixed at $k_3 = 10 \mu\text{s}^{-1}$ [72, 76].

SADS from the target analysis for FAD* are shown in Fig. 7b. As mentioned above, the SADS1 captures spectral chirp and cross phase modulation and is omitted from Fig. 7b (the SADS1 for (ϵ)FAD* are shown in SI Fig. 5, along with SADS2 for comparison). SADS2 (red line) is very similar to the FMN* SADS2. With $b_1 = 0.33$ radiative decay takes $\tau_{\text{rad}} = 4.7$ ns. For $b_2 = 0.67$, the average time for the evolution to the conformation that yields ^3FMN is 2.3 ns. Again, the lifetime of the triplet (state 3) was fixed at $\tau_3 = 10 \mu\text{s}$. The corresponding SADS3 (blue line) is supposed to capture the triplet state, but little evidence for its characteristic spectrum is seen (c.f. SADS4 for FMN). However, the normalized SADS (see SI Fig. 6a) provide for the calculation of difference SADS3-SADS2 (SI Fig. 6b, plotted in black), which demonstrates a small amount of triplet for this state.

Evidence for electron transfer should be detected in SADS 4 and 5 (Fig. 7b, green and magenta lines respectively), representing the stacked conformation, but there is no evidence for the formation of the $\text{Fl}^{\bullet-} \cdots \text{Ade}^{\bullet+}$ radical pair. The two SADS are almost identical (see also SI Fig. 6a where the SADS are shown normalized). SADS4 has a lifetime of $\tau_4 = 3.2$ ps, and the putative recombination has a lifetime of $\tau_5 = 8.6$ ps for the associated SADS5 spectrum. If PET occurred, SADS5 should show an increased absorption around 550 nm [78] but the ΔA_{550} is lower than SADS4. If the anionic flavosemiquinone were formed then a large positive change in ΔA should be observed around 360 nm, but the SADS5 wavelength maximum remains at 350 nm and has lower extinction than SADS4 (see SI Fig. 6c for the difference spectrum).

3.9 ϵ FAD transient kinetics modeling

The photodynamics of excited state ϵ FAD* were the most interesting. Unfortunately, direct evidence for ϵ Ade radical cation formation by monitoring the GSB of ϵ Ade at ~ 305 nm was outside our scan range. The observation of significant triplet yield required a modification of the kinetic model used for FAD*. Comparing the evolution of FMN* (Fig. 5a) with Fig. 5c, triplet production seems to be associated with a fraction of stacked ϵ FAD. This conclusion also follows from the lower emission quantum yield of ϵ FAD* compared to FAD*, which is inconsistent if ϵ FAD* prefers an open, triplet-prone, conformation to the stacked (quenched) one, as compared to FAD*. Thus, a branching of State 4 (Scheme 2b) was added, affording bifurcation of the excited stacked ϵ FAD* (State 4) into a charge transfer population

Table 3 Lifetimes in ps, unless otherwise indicated, from the SADS fitting of FMN, FAD, and ϵ FAD, accounting for model-dependent branching ratios (see Scheme 2)

Lifetime	Open ($b_1=0.33$, $b_2=0.67$)					Stacked ($b_3=0.3$, $b_4=0.7$)		
	$(k_{ic}^{S_2 \rightarrow S_1})^{-1}$	$(k_{rad}^{S_1 \rightarrow S_0})^{-1} = (b_1 k_2)^{-1}$	$(k_{isc}^{S_1 \rightarrow T_N})^{-1} = (b_2 k_2)^{-1}$	$(k_{ic}^{T_N \rightarrow T_1})^{-1}$	$(k_{isc}^{T_1 \rightarrow S_0})^{-1}$	$(k_{FET})^{-1} = (b_3 k_4)^{-1}$	$(k_{isc}^{S_1 \rightarrow T_1})^{-1} = (b_4 k_4)^{-1}$	$(k_{BET})^{-1}$
FMN	1.07 ± 0.02	14.2 ± 0.3 ns	7.0 ± 0.8 ns	138 ± 6	$10 \mu\text{s}^b$	N/A	N/A	N/A
FAD	0.9^b	4.7 ± 0.4 ns	2.3 ± 0.1 ns ^a		$10 \mu\text{s}^b$	3.2 ± 0.1^c	N/A ^c	8.6 ± 0.1
ϵ FAD	0.9^b	11.2 ± 0.2 ns	5.5 ± 0.6 ns ^a		$10 \mu\text{s}^b$	2.2 ± 0.1	1.0 ± 0.2	2.44 ± 0.02

^aIndicates that both ($S_1 \rightarrow T_N$) intersystem crossing and ($T_N \rightarrow T_1$) internal conversion are occurring due to conformational evolution of (ϵ)FAD*

^bFixed parameter

^cThere is only one branch used to fit FAD* dynamics, thus $b_3=1$

(State 5) and one that undergoes ISC as if it were FMN-like (State 3).

SADS from the modified target analysis for ϵ FAD* are shown in Fig. 7c. As before, the SADS1 is omitted from Fig. 7c (but see SI Fig. 5). The unstacked FMN-like ϵ FAD* spectra are captured in SADS2 and 3 (normalized difference spectra are seen in SI Fig. 7d, e). SADS2 for ϵ FAD* is very similar to the FAD* SADS2, with a maximum at 350 nm. With $b_1=0.33$, radiative decay for ϵ FAD* takes $\tau_{rad} \sim 11$ ns, more than twice as long as that for FAD. Interestingly, this would suggest that the steady-state fluorescence from ϵ FAD* will be higher than for FAD*, but this was not observed (see Fig. 2b). For $b_2=0.67$, the average time for the evolution to $^3\epsilon$ FAD is 5.5 ns on this branch. The corresponding SADS3 (blue line) clearly shows triplet character (c.f. Fig. 7a, SADS4), suggesting that some ϵ FAD conformations enhance triplet formation in ϵ FAD* relative to FAD*. We discuss this more thoroughly below.

SADS4 and 5 have shorter lifetimes than the corresponding FAD* SADS. The overall shape of SADS4 suggests a loss of extinction throughout the spectrum as compared with FAD, and a positive ΔA beyond 550 nm is suggestive of a broad charge transfer band (see SI Fig. 6f) [79]. The ESA₅₀₇ and SE₅₇₈ regions are depleted in SADS4, but become positive in SADS5 after about 2.4 ps. In addition, the GSB₄₄₄ has blue-shifted, supporting the hypothesis of radical pair formation. Significantly, SADS5 show a shift in the 360 nm peak to 350 nm, consistent with the formation of the anionic flavosemiquinone. As shown in Table 3, the FET rate in ϵ FAD is about 30% faster than for FAD, but there is a strong competing ultrafast pathway to the triplet (State 3 in Scheme 2b). Apparently, those ϵ FAD* that undergo charge separation in 2.2 ps recombine to form the ground state in about the same time.

It must be considered that the chosen branch point ($3 \leftarrow 4 \rightarrow 5$) is not the only possibility. The branch point could be State 5, the CT state formed directly from the stacked ϵ FAD*, instead of State 4 (see SI Fig. 7). This

possibility was tested and the results are shown in SI Fig. 8c (SADS for FMN, 8a, and FAD, 8b, are retained for comparison). The two branching coefficients for this second alternative branch were set to $b_3=b_4=0.5$. That is, we have no a priori way of knowing the efficiency of branching from the CT state (5), as this must involve a singlet to triplet radical pair evolution ($^1\text{CT} \leftrightarrow ^3\text{CT}$). The SADS spectra are nearly identical, as are the lifetimes, but the amplitudes of the component spectra differ proportionally based on changing the branch point from State 4 $\rightarrow$ 5. We cannot choose between these two models, but they lead to essentially the same behavior and conclusions. The assumption about the branching ratio rules out an accurate estimate of the relative triplet quantum yields for the flavins.

4 Discussion

To examine the role of the Ade in the PL FADH[−] cofactor, we hypothesized that by changing the free energy of the donor–acceptor (*FIH[−]/Ade) pair in the electron transport pathway, the rate of electron transfer would be altered. To test this, apo-PL was reconstituted with ϵ FAD and successfully photoreduced [27]. Reduced ϵ PL was shown to repair CPD DNA with a 15% increase in efficiency. Since this outcome may be due to how stacking of ϵ Ade differs from Ade, we have tried to approach this by determining the subpicosecond excited-state dynamics of oxidized ϵ FAD. The motivation for doing so was to document the excited state photoredox behavior of the modified cofactor in the absence of protein to test whether PET occurs between the FI* and ϵ Ade, and to compare these results with the dynamics of FAD*. Of course, further experiments on the reduced cofactors will be necessary for direct applicability to the ϵ PL experiments.

To summarize the data and fits, no evidence for radical intermediates were seen for FAD* (Figs. 5b, 6b, 7b), suggesting that stacked FAD* decays by a different mechanism

than widely supposed, such as deactivation by a conical intersection involving a charge transfer state between adenine and flavin [80]. On the other hand, ϵFAD^* shows clear evidence of the formation of a short-lived (~ 5 ps) intermediate whose spectral intensity is largest around ~ 505 nm (see Figs. 5c, 7c). Because of its strikingly different behavior compared to FAD^* , we state that PET takes place in stacked conformations of ϵFAD^* to produce a radical pair, and that the ϵAde acts as a real electron donor as opposed to a virtual intermediate through superexchange mechanism. However, there appears to be a new channel that affords a higher triplet yield compared to FAD .

4.1 Calculations

While the MO energies computed here support faster PET by ϵFAD^* , the expected ϵAde^{++} band at 460 nm was not obvious, although an increase in ΔA at ~ 505 nm could be this evidence. This is in contrast to the good agreement between our calculation of the spectrum of Ade^{++} and experiment. Unfortunately, the ethenoadenine radical cation spectrum has not been measured. The TD-DFT calculation in water as a continuum solvent somewhat overestimates the energy of this transition. It is likely that the inclusion of hydrogen bonding made by water molecules will red-shift the transition in silico. Future calculations modeling the stacked conformation are warranted based on the observed transient behavior.

4.2 Ultrafast kinetics of FAD^* and the lack of radical pair formation

Estimates for the lifetimes of the excited state decay of FAD^* have been reported by many groups [24, 33, 35, 38, 63], but here we focus on comparable pump-probe spectroscopic studies in similar solvents. At the earliest time (0–5 ps), intramolecular vibrational relaxation in the excited state manifold is undoubtedly taking place since the pump wavelength (~ 390 nm) excites both S_2 and S_1 states. Solvent reorganization also operates on this time scale [81, 82].

This same time window overlaps with possible radical pair formation or competing deactivation pathways. If the anionic flavosemiquinone were formed by PET from $\text{Ade} \rightarrow \text{Fl}^{\bullet-}$, then the loss of intensity around 350 nm relative to the GSB₄₄₄ would be expected as given in the second step in Eq. 1. The 3.1 and 8.6 ps lifetimes are also consistent with the Li study [29], in which they attribute a 9 ps lifetime with charge recombination in $\text{Fl}^{\bullet-} \cdots \text{Ade}^{++}$ in the stacked conformation. In this study, there is no evidence for radical formation. This could be due to PET and recombination kinetics that are too fast to be observed with our 0.2 ps IRF, or that quenching in FAD^* proceeds by a different mechanism. The

two FAD^* studies cited earlier supporting PET in FAD^* suffer from possible artifacts of dimerization due to high flavin concentration [29], or a very low radical pair yield, in spite of the purported fast k_{ET} constant [30]. As we have observed a putative charge transfer band in oxidized PL [79], this CT intermediate could rapidly decay through a conical intersection, as has been suggested by theory [83–85]. A computational study of a phenothiazine-phenyl-isoalloxazine dyad also concludes that excitation of the flavin results in an ultrafast evolution to a dark CT state [83].

If Eq. 1a is operative, then Ade^{++} and $\text{Fl}^{\bullet-}$ are both formed. Ade^{++} has a low extinction at 500 nm, $\epsilon \sim 1000 \text{ M}^{-1} \text{ cm}^{-1}$, making it difficult to detect [61]. In this study we could not obtain reasonable signal to noise below 330 nm. However, Candeias and Steenken report that dAMP radical cation has a band maximum extinction at 330 nm of $\sim 5000 \text{ M}^{-1} \text{ cm}^{-1}$. Recent pulse radiolysis work by Majima's group [69] shows that d Ade^{++} (from dAMP) at pH 7 has a broad absorption band at 595 nm with presumably low extinction ($< 2000 \text{ M}^{-1} \text{ cm}^{-1}$), in agreement with earlier studies [61, 69, 86], but increasing sharply below 350 nm, the lower limit of their scan range. Nonetheless, there is no evidence of this band showing up in the blue edge of our transient spectra (Fig. 5b). The semiquinone anion $\text{Fl}^{\bullet-}$ has an extinction of $\sim 5000 \text{ M}^{-1} \text{ cm}^{-1}$ from 450 to 500 nm, and a larger extinction at 360 nm of $\epsilon \sim 8000 \text{ M}^{-1} \text{ cm}^{-1}$ in organic solvent, estimated from the spectroelectrochemical work of Niemz et al. [60] on *N*(3)-methyl-*N*(10)-isobutylflavin in CH_2Cl_2 by comparing the absorbance of the oxidized flavin at 450 nm with the anionic semiquinone at 360 nm. At early time there is, in fact, a maximum at 353 nm that slowly shifts to 350 nm after about 500 ps, in like fashion with FMN* dynamics.

4.3 Triplet flavin yields in FAD and ϵFAD

The EADS analysis of FMN* yielded the expected triplet spectrum for the 10 μs component. The long emission lifetimes and large emission quantum yields of oxidized flavin that have been reported by Song et al. [87] Moore et al. [67] Grodowski et al. [66] and others [88–93] have shown that the FMN quantum yield due to intersystem crossing is $\Phi_{\text{isc}} \sim 0.4$, on the order of the internal conversion yield [32]. Other reports suggest a higher value of 0.67 [74].

In contrast the triplet yield of FAD^* and ϵFAD^* differ dramatically, as shown in the persistence of the large $\Delta\lambda(t)$ values after the fraction of stacked ϵFAD^* undergoes recombination. To rationalize this difference, we look to theoretical treatments of the ISC process in FMN* as a starting point. Climent and Serrano-Andres used gas-phase CASPT2 theory [94] to try to explain the photochemistry of LOV photoreceptor proteins [64, 95–97]. These FMN-dependent proteins absorb blue light that leads to an ultrafast FMN-Cys photo-adduct (signaling state), and that a possible conical

intersection (CI) for intersystem crossing is responsible [94]. Climent et al. argue that blue light excitation to the flavin S_1 state leads to ultrafast ISC to the flavin T_N ($N > 1$) state. There is a second CI (T_N/T_1) yielding the T_1 state, which is then trapped behind a large energy barrier against relaxation to the S_0 state. They concluded that the T_N state would be very sensitive to solvent polarity, such that increasing polarity leads to ISC that is faster than current experimental methods can resolve. The computed spin–orbit coupling constant (SOC) for $T_N \rightarrow S_1$ is about 5 cm^{-1} for flavin in vacuum, but ISC in flavoproteins could be much faster than this SOC value would provide, particularly when an electron-rich residue is near the flavin N_5 atom [64, 95, 98].

Marian's computational group analyzed ISC in the YtvA bacterial LOV domain [99, 100], including large-scale water solvation and amino acid residues close to the FMN for QM/MM calculations. They found that the T_N manifold of states suggested above is specifically the T_2 state. At their relaxed minima, the T_1 and T_2 states are about 0.9 and 0.2 eV below the S_1 minimum.

Both groups support the picture that ISC rates in flavin are extremely sensitive to the S_1 – $T_{1,2}$ energy gaps. It is worth noting that Climent and Serrano-Andres computed that the (gas phase) T_1 dipole moment, $|\mu_{T_1}| = 6.0D$, which was comparable to $|\mu_{S_1}| = 9.4D$, [94] while Salzmann et al. found $|\mu_{T_1}| = 8.2D$, which was comparable to $|\mu_{S_1}| = 9.8D$ [99]. Additionally, a computational analysis from the Schütz group suggests that a conical intersection between S_1 and S_0 states is responsible for the lack of triplet formation in BLUF photoreceptor protein [101] and in the YtvA LOV domain as well [85]. We believe that the orientation of the (ϵ)Ade S_0 dipole moment may be a lever by which the triplet quantum yields are modulated (see below).

4.4 Stacking and dinucleotide orientation of ϵ FAD vs. FAD

Our kinetic data suggest that in ϵ FAD* about 70% of stacked conformations undergo electron transfer, while 30% are in non-ET conformations but branch via ultrafast ISC to form triplets. The relative conformation of the flavin molecule to the (ϵ)Ade moiety is likely to play a large role in the different triplet yields of the two dinucleotide molecules. Molecular dynamics simulations of stacking in FAD by van der Berg et al. [70] found that Ade stacks so that the long axes of the molecules are roughly parallel, $\angle \vec{\mu}_{\text{Ade}}, \vec{\mu}_{\text{Iso}} \sim 18^\circ$ on average, with a 2:1 preference for the *Si* face of the flavin. The Ade was found mostly over the pyrazine ring, although a smaller fraction of molecules were found with overlapping pyrimidine and xylene rings. Thus, the Ade, positioned over N_5 , could alter the intersystem crossing rate, which may affect the triplet quantum yield. Further details of the relative orientations of the rings were not given.

A 2014 study of gas phase FAD conformation by Molano-Arevalo et al. using ion mobility spectrometry measurements [102] showed that the most stable FAD conformation has the Ade about 4.1 Å from the Re face of the isoalloxazine. Using the Ade C8-N1 and isoalloxazine C8-N3 coordinates from that study we computed the dihedral angle between the planes of the molecules. The Ade N7-C8-N9 five-membered ring is situated over the flavin xylene ring at a distance of 4.32 Å with the dihedral angle $\angle \vec{\mu}_{\text{Ade}}, \vec{\mu}_{\text{Iso}} = 35.4^\circ$, with the Ade N1 atom situated over the isoalloxazine N5 atom. Interestingly, these pseudo-axes coincide with the directions of the DFT-derived ground state dipole moments of Ade and lumiflavin obtained here.

Another ion mobility study including solvent by Bull et al. [103] gave a similar stacking result for FAD with both phosphates deprotonated. No coordinate data is given, but in this case the ground state dipole moment dihedral angle appears to be smaller than 35° , with stacking of the six-membered Ade ring over the *Si* face of the isoalloxazine xylene ring, and with permanent dipole moments $\angle \vec{\mu}_{\text{Ade}}, \vec{\mu}_{\text{Iso}} \sim 0^\circ$.

Perhaps the most interesting and relevant study on FAD conformation is based on the FAD storage protein dodecin. The crystal structure of dodecin by Bieger et al. [104] shows stacked FAD resolved to 1.85 Å. The authors suggest that the protein has little effect on the solution conformation. Here, Ade is stacked parallel to the Re face of the isoalloxazine ring, which are separated by 3.7 Å (Iso- N_5 to Ade- N_1) in two conformations. The dihedral angle between Ade and isoalloxazine is $\angle \vec{\mu}_0^{\text{Ade}}, \vec{\mu}_0^{\text{Iso}} = 12^\circ$, in which case their permanent dipole moments are again roughly parallel. This structure has been extracted from the PDB file and is shown in Fig. 8a depicted as a green arrow.

To summarize, these studies support the picture that both Re and Si stacking are stable in FAD, and that the six-membered ring of Ade stacks over the pyrazine moiety of the isoalloxazine at a distance of ~ 4 Å. In the lowest energy conformation, the permanent dipole moments of the two species are parallel. In fact, this alignment should destabilize stacking, which is consistent with the band integral analysis for FAD given above. It must be the case that the conformation is dominated by reducing strain in the ribityl-phosphate moiety and by hydrophobic interactions between molecules, but not dipole–dipole forces.

4.5 ϵ Ade and Ade S_0 dipoles have a differential effect on the ISC energy gap and triplet yield

Based on the very low triplet yield in FAD* as compared to ϵ FAD*, and the somewhat contradictory evidence that stacked ϵ FAD* decays faster than FAD*, we hypothesize that the orientation and magnitude of the ground state dipole moment of Ade significantly depresses k_{ISC} in FAD

Fig. 8 **a** The stacked geometry of Ade and isoalloxazine rings in FAD from the dodecin crystal structure (PDB: 2CJC) of Gringer et al. [105]. The orientation and magnitude of the DFT-computed ground state dipole moment ($\vec{\mu}_0^{Ade}$) for Ade is shown as a green vector relative to the approximate isoalloxazine center of mass. The experimentally determined isoalloxazine S_1 dipole moment vector [107], $\vec{\mu}_1^{Iso}$, is shown to scale in red and is nearly parallel to $\vec{\mu}_0^{Ade}$. **b** The ground state

ϵ Ade and S_1 isoalloxazine dipoles ($\vec{\mu}_0^{\epsilon Ade}$ in black) are superimposed on a model of ϵ FAD assuming the ϵ Ade moiety binds in the same orientation as Ade in FAD. Note that the dipole moments are nearly perpendicular. **c** The flavin LUMO (MO 68) is superimposed over the Ade HOMO (MO 39). **d** The flavin LUMO (MO 68) is superimposed over the ϵ Ade HOMO (MO 45). The molecular orbitals shown here were obtained using the Natural Bond Orbital (NBO) analysis available in Gaussian [46]

by changing the energy gap between the FAD T_2 and S_1 states, while the ground state dipole moment of ϵ Ade has little effect in this regard, thus appearing FMN-like.

We construct a simple model based on the stacking geometries cited above, and on calculations of the energies and permanent dipole moments of the isoalloxazine S_1 and T_2 states, and the ground state dipole moments for (ϵ)Ade found here. The structure of stacked ϵ FAD is not known, but based on the arguments presented above, we place the ϵ Ade moiety in the same orientation as the Ade moiety in FAD from the dodecin structure (PDB 2CJC) [105]. A cartoon of this hypothetical orientation is shown in Fig. 8b, with computed magnitude and direction of $\vec{\mu}_{\epsilon Ade}^{S0}$ (black arrow) as shown in Fig. 3a. The isoalloxazine S_1 dipole is plotted for reference, based on calculations by Alexandre et al. [106] indicating that the permanent dipole moment of the lumiflavin S_1 state is inclined about 15° to the long axis of the flavin with

$\left| \vec{\mu}_{Iso}^{S1} \right| \sim 10.3D$. Stark spectroscopic studies of oxidized flavin at 77 K show that $\vec{\mu}_{Iso}^{S1}$ (red arrow) and $\vec{\mu}_{Iso}^{S0}$ are approximately parallel to N3-C8 axis of the molecule [73, 107], thus both excited state dipole moments would be roughly parallel to $\vec{\mu}_{Ade}^{S0}$.

Alexandre et al. computed the direction and magnitude of the flavin T_1 dipole, which is parallel to $\vec{\mu}_{Iso}^{S1}$, with $\left| \vec{\mu}_{Iso}^{T1} \right| \sim 7.9D$. [106] Calculations by Salzmann et al. suggest that the T_2 dipole, $\left| \vec{\mu}_{Iso}^{T2} \right| \sim 7.0D$ for the gas phase, undergoes charge redistribution along the long axis of the molecule [99], suggesting that $\vec{\mu}_{Iso}^{T2} \parallel \vec{\mu}_{Iso}^{T1}$, or that the T_2 dipole moment is roughly parallel with T_1 . Thus, the $\vec{\mu}_{Iso}^{T2}$ and $\vec{\mu}_{Iso}^{S1}$ dipoles are likely parallel with the flavin C8-N3 axis.

To continue, estimates of the ground state dipole moments of (ϵ)Ade are taken from this study. The adenine ground state dipole moment $|\vec{\mu}_{\text{Ade}}^{\text{S0}}| \sim 3.7D$ in a water PCM. The dipole runs parallel to the A₈C8-AN1 axis, and as such is roughly parallel to $\vec{\mu}_{\text{Iso}}^{\text{S1}}$ and $\vec{\mu}_{\text{Iso}}^{\text{T2}}$. The interaction energy of two dipoles depends on their orientation through the dot product. For two parallel dipoles, the dipole–dipole interaction will change the energy gap between T_2 and S_1 , given by Eq. 2:

$$\begin{aligned} \Delta E_{S1-T2}(\text{Ade}) &= E_{S1}(\vec{\mu}_{\text{Ade}}^{\text{S0}}) - E_{T2}(\vec{\mu}_{\text{Ade}}^{\text{S0}}) \\ &= \frac{|\vec{\mu}_{\text{Ade}}^{\text{S0}}|^2}{4\pi\epsilon_0 r^3} \left(\left| \vec{\mu}_{\text{Iso}}^{\text{S1}} \right| - \left| \vec{\mu}_{\text{Iso}}^{\text{T2}} \right| \right) = 0.13 \text{ eV} \end{aligned} \quad (2)$$

The computed ΔE_{S1-T2} in the absence of $\vec{\mu}_{\text{Ade}}^{\text{S0}}$ is about 0.14 eV [94, 99], thus $\Delta E_{S1-T2}(\text{Ade})$ causes the energy gap to double, which should reduce k_{ISC} , even without a change in the spin–orbit (or spin–vibronic) coupling constant. This interaction could partly explain why FAD* produces so little triplet relative to ϵFAD^* .

Interestingly, in our hypothetical stacked ϵFAD structure, $\vec{\mu}_{\epsilon\text{Ade}}^{\text{S0}} (= 7.3D)$ is roughly orthogonal to that for Ade. Thus, $\vec{\mu}_{\epsilon\text{Ade}}^{\text{S0}} \cdot \vec{\mu}_{\text{Iso}}^{\text{S1}}$ (or $\vec{\mu}_{\text{Iso}}^{\text{T2}}$) = 0 and there will be no dipole–dipole interactions of ϵAde with the flavin S_1 - T_2 system. Based on this model, ϵFAD should be FMN-like in terms of triplet production.

4.6 Electron transfer in ϵFAD^*

We have obtained evidence that ϵFAD^* undergoes radical pair production in less than 5 ps. Electron transfer rates are a function of the driving force, ΔG° , the donor acceptor distance, r_{DA} , and the orbital overlap between donor and acceptor orbitals involved in the ET reaction. We have addressed driving force issues through calculations of relevant donor and acceptor orbital energies (see above). If we assume that a donor–acceptor distance of $\sim 4 \text{ \AA}$ is applicable to both FAD and ϵFAD , then this leaves changes in orbital overlap (electronic coupling) afforded by the ϵFAD etheno group which are absent in Ade.

A qualitative comparison of orbital overlaps are shown in Fig. 8c, d for FAD and ϵFAD respectively. In Fig. 8c, the orbital isosurface (isovalue = 0.03) of the lumiflavin LUMO (MO 68) is shown with blue lines. The Ade HOMO isosurface (MO 39, red lines, C atoms in cyan) is superimposed on flavin in the dodecin-based orientation. The same was done for ϵFAD in Fig. 8d (ϵAde HOMO,

MO 45, red lines, C atoms in lime green). For both, (ϵ)Ade imidazole ring density overlaps about equally with the Iso xylene ring. The most significant difference is better overlap between ϵAde density located on the etheno group with the Iso C4–C4a–N5 region, suggesting that there is greater electronic coupling between ϵAde and isoalloxazine (S_1) and therefore a predicted increase in k_{ET} relative to FAD. New calculations are necessary to test this conjecture, but the ultrafast radical pair formation taking place in stacked ϵFAD^* suggests it may be so.

5 Conclusions

In this paper, we presented subpicosecond optical broadband transient absorption measurements of Flavin 1, N^6 -Ethenoadenine Dinucleotide and made a direct comparison to FMN* and FAD*. Complementing the results with TD-DFT calculations, our observations suggest that in free solution, ϵFAD^* and FAD* display decidedly different charge transfer, triplet evolution, and deactivation dynamics. FAD* does not appear to form radical pairs indicative of electron transfer between Ade and Fl*, in spite of a preconception that this must be the major deactivation pathway. The extended electron density afforded by ϵAde in ϵFAD^* points to increased electronic coupling, promoting fast intramolecular electron transfer in this dyad. The orientation of permanent dipoles of the stacked (ϵ)FAD moiety appears to be important in adjusting the intersystem crossing rate constant and triplet yield, in contrast to the negligible triplet yield in FAD*.

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Author contributions RJS designed the research; KJM, DTB, RAM, and VRS performed the transient absorption experiments; MN performed the TDDFT and Chimera docking calculations; MRB made the fluorescence measurements; KJM, MN, and RJS wrote the paper.

Declarations

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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