Overlapping Electronic States With Nearly Parallel Transition Dipole Moments in Reduced Anionic Flavin Can Distort Photobiological Dynamics

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SUPPLEMENTARY INFORMATION

Experimental Methods

Stark spectrometer and cuvette. The sample cuvette was constructed from indium tin oxide (ITO) coated boro-aluminosilicate glass slides (Delta Technologies), separated by 50 μm thick kapton spacers. The two slides were held together by spring clips which also secure it to the cold finger. A 30–50 μL aliquot of the reduced sample was removed from the glovebox in an airtight syringe and loaded by capillary action into the cuvette.

Light from a 300 W Xe arc lamp (Oriel) was filtered through a 1/8 m monochromator (CVI) with a 2 nm bandpass. The polarization of the collimated beam was selected with a Glan-Taylor polarizer. The beam was focused onto the frozen sample held inside a dual chamber cryostat (JANIS) and the transmitted light collected and focused onto a Si photodiode. This signal was amplified with a current preamplifier (Stanford Research Systems) before being fed into the digital lock-in amplifier (Stanford Research Systems), which was used to generate a 3.5 kHz sinusoidal AC waveform. This waveform was amplified by a high voltage amplifier (TREK) to greater than $3x10^5$ V/cm. The AC field was applied to the sample via electrical leads connected to the conductive sides of the cuvette with alligator clips. Phase-sensitive detection was performed at the second harmonic of the field frequency with a 3 second time constant while the zero-field light intensity was obtained directly from the preamplifier output using an analog-to-digital converter at 16-bit resolution.

HPLC purification of flavin and analysis of photodegradation products. The purity of flavin samples was accessed by reversed phase high performance liquid chromatography on an Agilent 1100 HPLC equipped with a C18 J-sphere ODS-H80 column (150×4.6mm i.d., 5-4 μm particle size, 80Å pore size) and an isocratic mobile phase of 60% 100 mM ammonium formate (pH 3.8) and 40% methanol.[20] Re-purification of the flavin was performed as follows. Approximately 1 mg of flavin was dissolved in 300 μL methanol and then 750 μL of the ammonium formate solution was added to match the conditions of the mobile phase. This solution was injected onto the column in 100 μL aliquots, and the flow-through was collected between 36 and 42 minutes after injection. Solvent was removed by centrifugation under vacuum overnight.

For analysis of photodegradation products, a 30 μL aliquot of the flavin sample in organic solvent was added to 970 μL of the mobile phase, and a 100 μL of this solution was injected onto the column.

Photodegradation upon photoreduction - Lumichrome: Impurities in the flavin were observed by HPLC analysis in both "as received" and photoreduced samples. The major impurity has a retention time of ~17 min. (Figure S2), while the flavin elutes at

37–42 min.. The impurity increased by only about 1% after sample irradiation as determined by comparing the ratio of the areas of the two peaks in each chromatogram.

Two known photodegradation products of flavins are lumichrome and lumazine[34]. It was found that the retention time of lumichrome matched the impurity seen in the flavin sample (Figure S2). This was confirmed by UV/vis absorption spectroscopy of the major elution peaks (Fig. S3).

The Stark spectrum of lumichrome overlaps with most of the spectrum of FlH^- (manuscript in preparation), however these features are not apparent in the flavin spectrum. More importantly, lumichrome has no signal in the spectral region of the $S_0 \rightarrow S_1$ Stark spectrum of FlH^- (manuscript in preparation).

Photoreduction in the presence of $NaBH_4$: There are examples of photoreduction of chromophores in the presence of triethylamine in the literature[35], however, a stronger reductant would ensure formation of the desired redox/protonation state of the flavin without requiring long irradiation times that lead to the formation of lumichrome. Under the experimental constraints of Stark spectroscopy, it was found that the best conditions were the reduction of HPLC-purified flavin in ethanol in the presence of TEA and $NaBH_4$ with blue light irradiation. The room temperature absorption spectrum is indicative of formation of reduced anionic flavin, with an absorption band centered at 360 nm and a broad shoulder at longer wavelengths (data not shown).

A 30 µL aliquot of 100 mM $NaBH_4$ was added to 300 µL of deoxygenated 1 mM flavin in ethanol containing 0.02% triethylamine (v/v). A 30 µL portion of this mixture was removed from the glovebox in an air-tight syringe and loaded into a preassembled Stark cuvette. The sample was illuminated *in situ* with the handheld LED for 15 seconds and immediately plunged into liquid nitrogen.

The Stark spectrum of borohydride-photoreduced FlH^- in EtOH closely matched that of FlH^- in MeTHF/TEA, with a large feature of negative amplitude centered at the $S_0 \rightarrow S_2$ transition, and a much smaller feature in the spectral region of the $S_0 \rightarrow S_1$ transition. Differences in band shape and amplitude between the two reduction cases can be attributed to differing solvent polarities and hydrogen bonding ability. Additionally, and possibly more importantly, the dichroism exhibited by these features match those of the Stark spectra of FlH^- in MeTHF and of $FADH^-$ in DNA photolyase

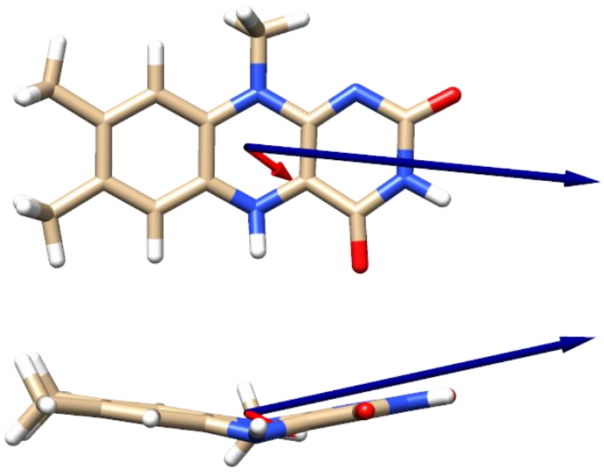

Figure S1: Calculated difference dipole moments of the $S_0 \rightarrow S_1$ (red) and $S_0 \rightarrow S_2$ (black) transitions of LfH^-

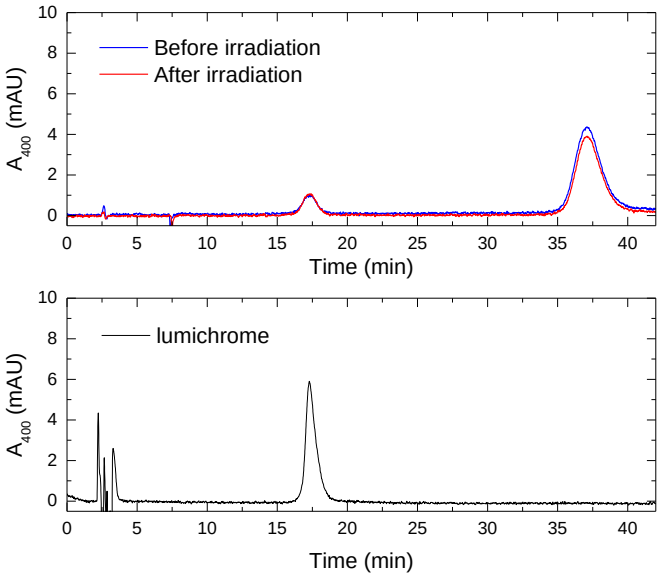

Figure S2: (top) HPLC analysis of an Fl sample before and after irradiation (bottom) HPLC analysis of lumichrome

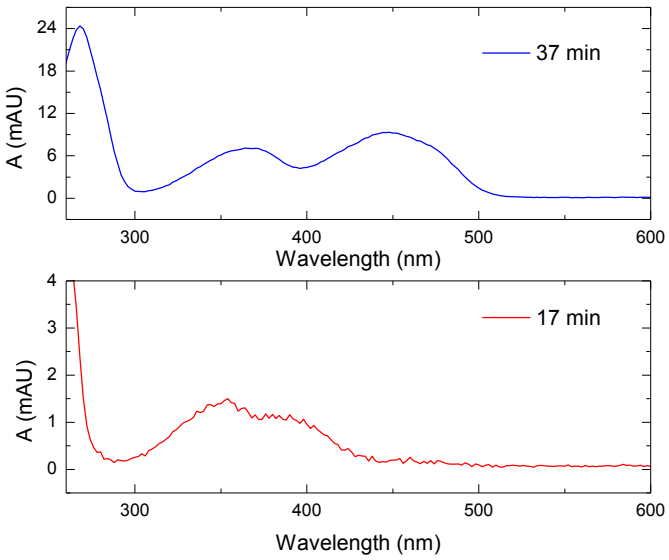

Figure S3: Absorption spectra of the peaks shown in Figure S2 (top) at 37 minutes (Fl) and 17 minutes (Lc).

Table S1: Vector components of the permanent dipole moments of LfH^- calculated by TD-DFT for a PCM with ε=4.9.

State	$\vec{\mu}_x$ (D)	$\vec{\mu}_y$ (D)	$\vec{\mu}_z$(D)	$\lvert\vec{\mu}\rvert$(D)
0	15.13	-0.60	-0.31	15.15
1	13.61	1.24	-1.27	13.73
2	5.02	0.59	1.13	5.18

Table S2: Vector components of the transition dipole moments of LfH^- calculated by TD-DFT as in Table S1.

	$\vec{m}_x$	$\vec{m}_y$	$\vec{m}_z$	$\lvert\vec{m}\rvert$
$S_0 \rightarrow S_1$	–0.3059	0.3008	–0.0162	0.4293
$S_0 \rightarrow S_2$	–1.1889	0.0081	–0.1393	1.1971