Supporting Information

Single Hydrogen Bond Donation from Flavin N_5 to Proximal Asparagine Ensures FAD Reduction in DNA Photolyase

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Expression of Enzymes. The unlabeled, uniform ^{13}C-labeled, and Asn reverse-labeled *E. coli* CPD photolyase enzyme was made from the E109A mutant which does not carry MTHF, used as WT enzyme, and prepared as N-terminal His_6-tagged protein using an expression system in *E. coli* strain BL21(DE3) as previously reported with slight modifications.[16,31] For the uniform ^{13}C labeled enzyme, *E. coli* with the CPD photolyase gene was grown in M9 minimum medium with ^{13}C glucose (Cambridge Isotope Laboratories; 99.0% purity). The ^{12}C asparagine in ^{13}C enzyme, termed Asn reverse-labeled enzyme, was prepared as follows. The ^{12}C (unlabeled) Asn (100 mg/L) was added 30 min prior to the addition of IPTG into *E. coli* CPD photolyase grown in ^{13}C M9 minimum medium. Cells were grown at 24 °C until the OD_{660} reached 0.4, IPTG was added to a final concentration of 1 mM, and the culture was incubated at 18 °C for 18 h, then harvested by centrifugation at 8,000 rpm for 5 min.

Purification of Enzymes. The harvested cells were resuspended in lysis buffer [50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, and 5% (*v/v*) glycerol (pH 8.0)]. Using a French press to break the cells, the insoluble fraction was removed by centrifugation (17,000 g) for 60 min. The supernatant was loaded onto a Co-NTA column (TALON Metal Affinity Resin, Clontech) which was then washed in a 20-bed volume wash buffer [50 mM NaH_2PO_4, 300 mM NaCl, and 20 mM imidazole (pH 8.0)]. The fusion protein was eluted with elution buffer [50 mM NaH_2PO_4, 300 mM NaCl, and 250 mM imidazole (pH 8.0)]. For the enzyme resting in a semiquinoid form in an unphotolyzed state, the purified protein in elution buffer was then exchanged into storage buffer with an electron donor [50 mM HEPES, 100 mM NaCl, 25 mM β-mercaptoethanol, and 20% (*v/v*) glycerol (pH 7.0)] by dilution and ultrafiltration (Amicon Ultra 30K device, Millipore) prior to being stored at −80 °C prior to use. Meanwhile, for oxidized enzyme in an unphotolyzed state, to induce

oxidation, the purified enzyme was exchanged with storage buffer without an electron donor [50 mM HEPES, 100 mM NaCl, and 20% (*v/v*) glycerol (pH 7.0)] stored at 4 °C and aerated for several days until the green enzyme visibly turned yellow. The presence of ^{12}C Asparagine in ^{13}C enzyme does not change or alter the stability of the enzyme, since both enzyme has the same stability in room temperature upon sample preparation for spectroscopic measurement. From 1 L of *E. coli* culture, approximately 6 mg of E109A (WT) and 3 mg of uniform ^{13}C-labeled and Asn reverse-labeled enzyme were obtained from each liter of LB and M9 media, respectively. Isotope-labeling is believed to have yielded >90% of enzyme (see Results section).

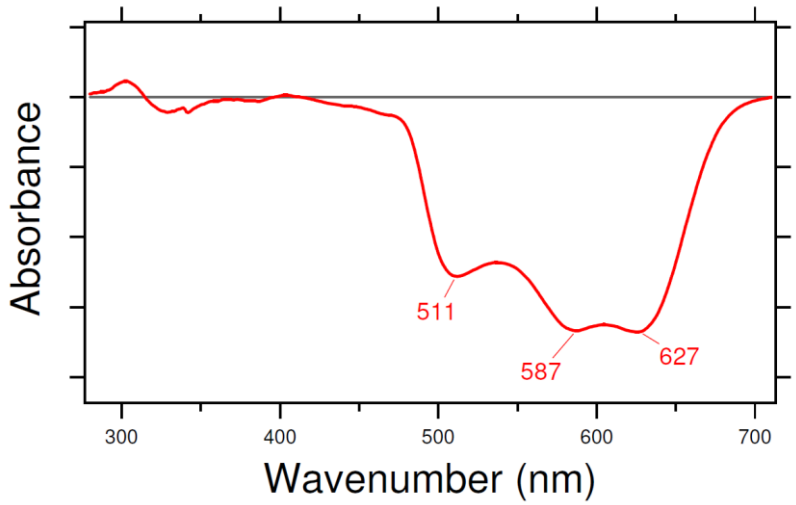

Figure S1. Light-induced difference UV-visible spectra of $FADH^{\bullet}$ into $FADH^{-}$ of unlabeled enzyme by illumination of >550 nm light for 2 min; one division is 10^{-2} absorbance units. Peak close to 300 nm is due to sample absorbance exceeded detector limitation.

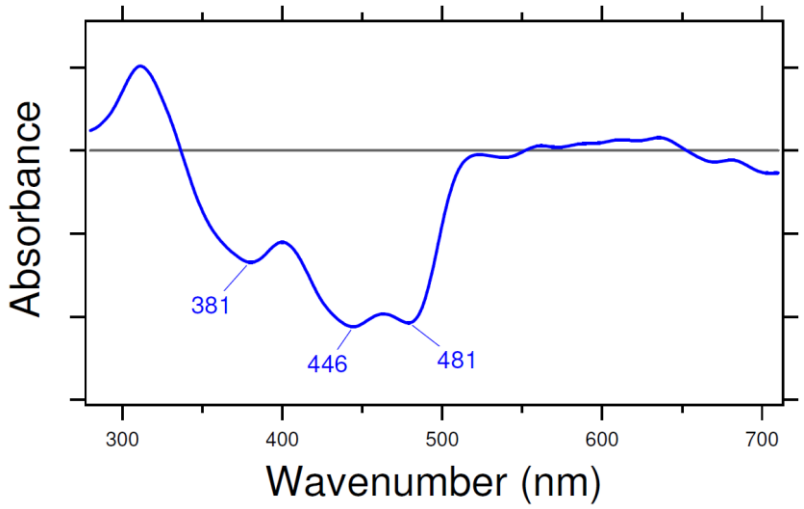

Figure S2. Light-induced difference UV-visible spectra from FAD^{ox} into $FADH^{-}$ of unlabeled enzyme by illumination of >390 nm light for 2 min followed by >550 nm for 30 s; one division is 10^{-3} absorbance units. Peak close to 300 nm is due to sample absorbance exceeded detector limitation.

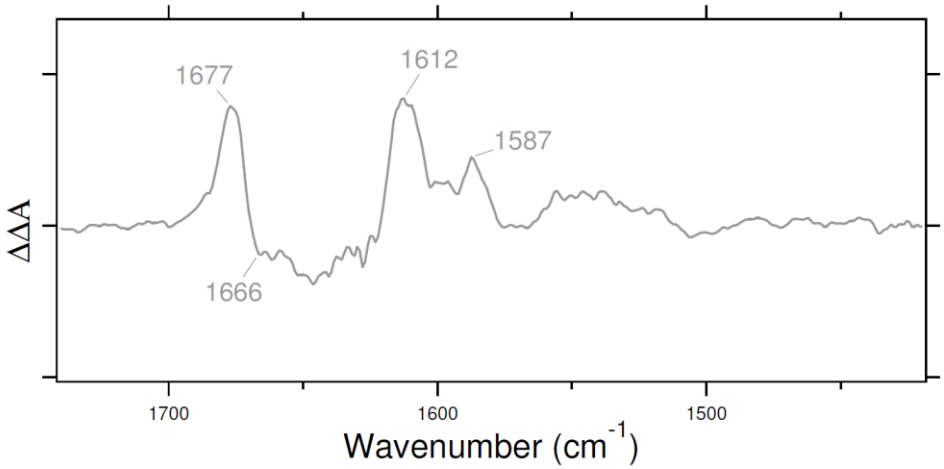

Figure S3. Double difference spectra of Asn reverse-labeled (blue) *minus* Uniformly ^{13}C-labeled (red) in Figure 1b, where one division equals 0.003 absorbance unit.

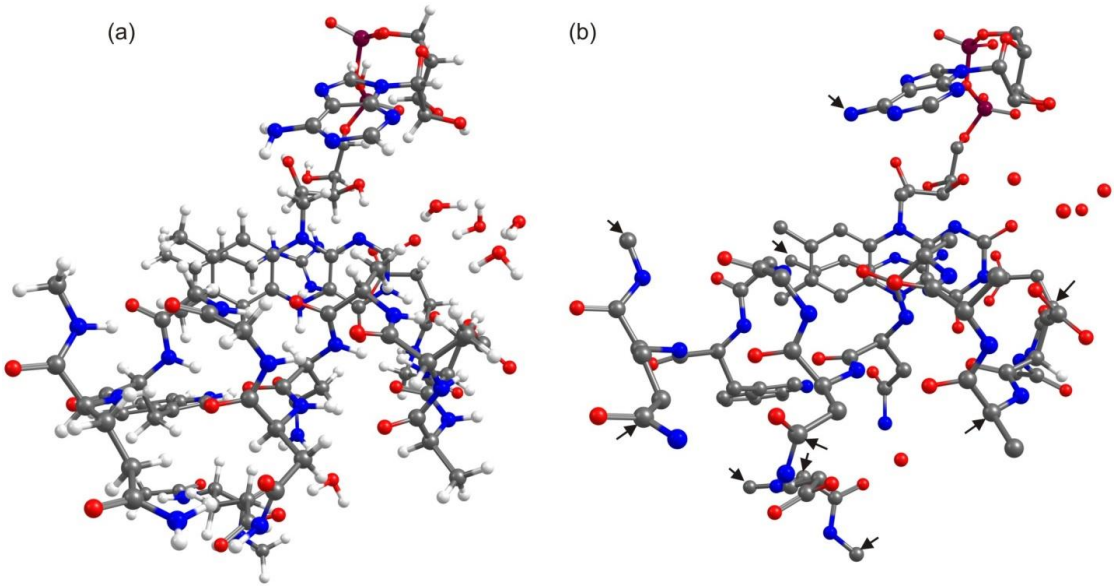

Figure S4. The computational model of the *E. coli* photolyase active site. (a) All atoms of the model in the $FADH^-$ state and (b) the heavy atoms of the model shown in the panel (a). The black arrows point to the atoms with the Cartesian coordinates fixed to the respective PDB values during geometry optimization.

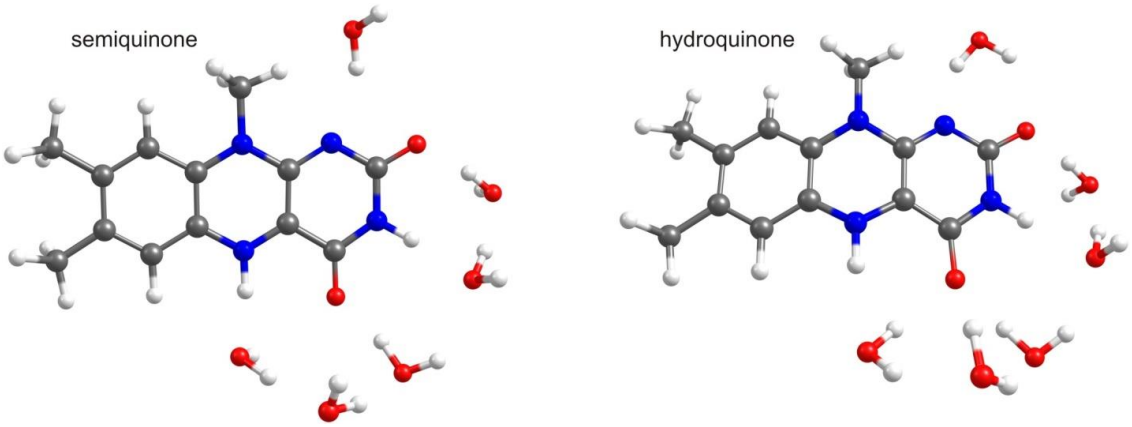

Figure S5. Structure of the lumiflavin-water complex $LfH{-}6H_2O$ in the neutral semiquinone and anionic hydroquinone states. Geometry optimization was carried out with the (U)B3LYP/6-31G* method.

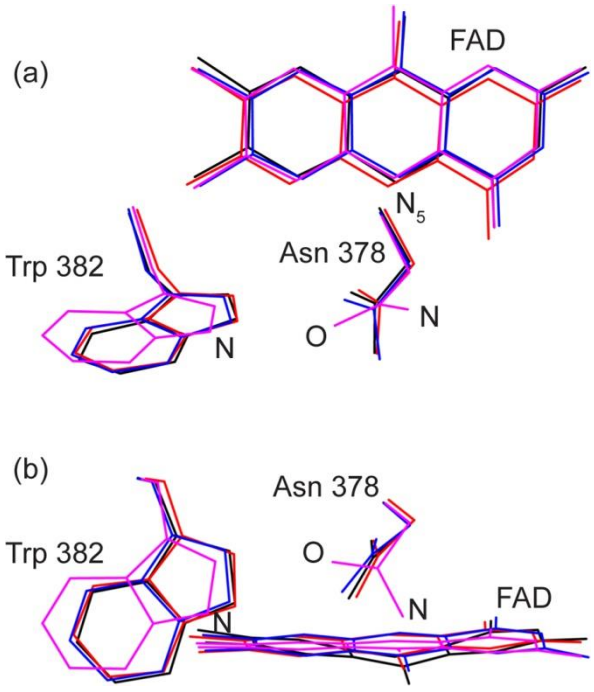

Figure S6. Geometry of the model in the FAD^{ox} state with rotated Asn378 (magenta) in comparison to the geometries of the models presented in Figure 4. The N and O atoms are indicated for rotated Asn378.

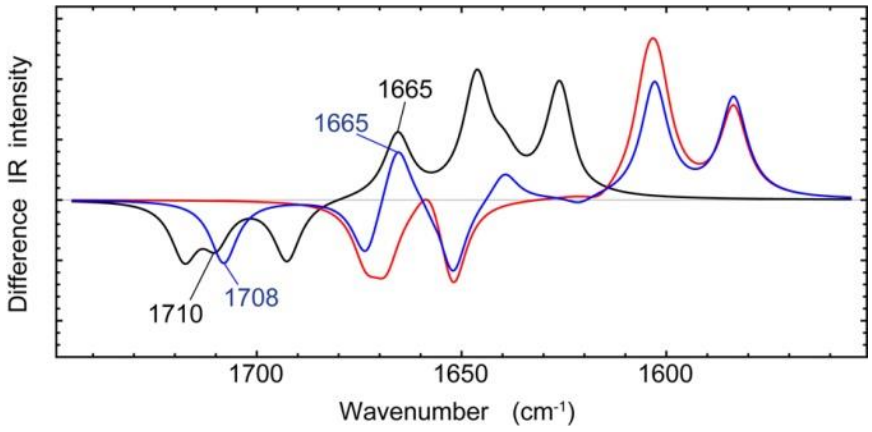

Figure S7. Computed infrared difference spectra FADH$^-$ *minus* FAD^{ox}. The model with FAD^{ox} contains rotated Asn378. Unlabeled (black), uniformly ^{13}C-labeled (red) and Asn reverse-labeled (blue) spectra. The numbers indicate the assignment of the Asn378 frequencies. Assignments of

other frequencies in the region of the double bond stretches are presented in Table S1. IR intensity is in Debye/AMU·Å^2.

Table S1. Assignments of the computed infrared spectra in the region of the double bond stretches.

^{12}C			^{13}C			Fl-^{13}C, Asn-^{12}C		
Frq. [a]	IR Int.[b]	Assign.[c]	Frq.	IR Int.	Assign.	Frq.	IR Int.	Assign.
				FAD^{ox}				
1730	9.79	Fl C=O_2	1686	10.21	Fl C=O_2	1686	9.98	Fl C=O_2
1702	11.95	Fl C=O_4	1665	3.8	Asn	1677	11.6	Asn
1677	10.23	Asn	1661	13.33	Fl C=O_4	1662	9.52	Fl C=O_4
1645	3.27	Asn	1613	6.29	Asn	1645	3.13	Asn
1613	1.03	Fl	1559	2.13	Fl	1559	2.11	Fl
1568	15.56	Fl	1523	15.28	Fl	1522	15.38	Fl
1530	16.68	Fl	1496	2.63	Fl	1495	2.64	Fl
1516	3.24	Fl	1489	0.43	Fl	1490	0.42	Fl
1488	0.65	Fl	1469	2.7	Fl	1469	2.71	Fl
				FAD^{ox}-(Asn^{rot})				
1718	9.1	Fl C=O_2	1673	8.31	Fl C=O_2	1709	10.42	Asn
1710	6.46	Asn	1668	9.22	Asn	1673	10.28	Fl C=O_2
1693	19.07	Fl C=O_4	1652	14.95	Fl C=O_4	1652	12.81	Fl C=O_4
1621	1.4	Asn	1616	1.83	Asn	1621	1.44	Asn
1614	0.18	Fl	1559	0.51	Fl	1559	0.53	Fl
1565	11.21	Fl	1520	11.93	Fl	1520	11.98	Fl
1527	14.31	Fl	1494	5.05	Fl	1494	5.04	Fl
1508	0.09	Fl	1481	1.78	Fl	1481	1.78	Fl
1491	0.41	Fl	1468	0.33	Fl	1572	0.56	Fl
				FADH$^{\cdot}$				
1688	16.79	Fl C=O_2	1657	5.27	Asn	1663	9.28	Asn
1664	4.07	Asn	1644	19.0	Fl C=O_2	1644	18.98	Fl C=O_2
1659	20.78	Fl C=O_4	1617	12.08	Fl C=O_4	1626	12.23	Fl C=O_4
1626	6.05	Asn	1587	8.20	Asn	1616	5.82	Asn
1603	0.17	Fl	1556	1.79	Fl	1557	1.43	Fl
1591	2.81	Fl	1546	0.94	Fl	1547	0.68	Fl
1530	16.26	Fl	1494	12.18	Fl	1494	12.64	Fl
1516	2.37	Fl	1491	1.65	Fl	1492	1.16	Fl
1502	0.78	Fl	1472	3.2	Fl	1472	3.19	Fl
				FADH^{-}				
1666	10.48	Asn	1658	6.02	Asn	1666	10.94	Asn
1646	19.30	Fl C=O_2	1603	14.66	Asn	1640	5.06	Asn
1640	5.02	Asn	1602	15.29	Fl C=O_2	1603	19.01	Fl C=O_2
1626	19.13	Fl C=O_4	1584	14.61	Fl C=O_4	1584	16.4	Fl C=O_4
1599	0.28	Fl	1544	0.38	Fl	1544	0.35	Fl
1584	3.39	Fl	1530	2.57	Fl	1530	2.63	Fl
1555	3.64	Fl	1505	2.81	Fl	1505	2.8	Fl
1505	4.75	Fl	1482	0.85	Fl	1482	0.83	Fl
1493	1.36	Fl	1474	0.42	Fl	1474	0.42	Fl

[a] harmonic normal mode frequency, scaled by 0.965 (in cm^{-1})

[b] infrared intensity (in Debye/AMU·Å^2)

[c] assignments of the normal models to the C=O and NH_2 vibrations of Asn378 (indicated as "Asn"), $C=O_2$ and $C=O_4$ of flavin ("Fl $C=O_2$" and "Fl $C=O_4$") and C=C and C=N of flavin ("Fl").