Excited-State Properties of Flavin Radicals in Flavoproteins: Femtosecond Spectroscopy of DNA Photolyase, Glucose Oxidase, and Flavodoxin

Jie Pan,[†] Martin Byrdin,[‡] Corinne Aubert,[§] André P. M. Eker,[||] Klaus Brettel,[‡] and Marten H. Vos*[,†]

Laboratory for Optical Biosciences, INSERM U451, CNRS UMR 7645, Ecole Polytechnique-ENSTA, 91128 Palaiseau Cedex, France, Service de Bioénergétique, CEA, and URA 2096 CNRS, CEA Saclay, 91191 Gif-sur-Yvette Cedex, France, Laboratoire de Bioénergétique et Ingénierie des Protéines—CNRS, 31 chemin J. Aiguier, 13402 Marseille Cedex 20, France, and Department of Cell Biology and Genetics, Medical Genetics Centre, Erasmus University Medical Centre, PO Box 1738, 3000 DR Rotterdam, The Netherlands

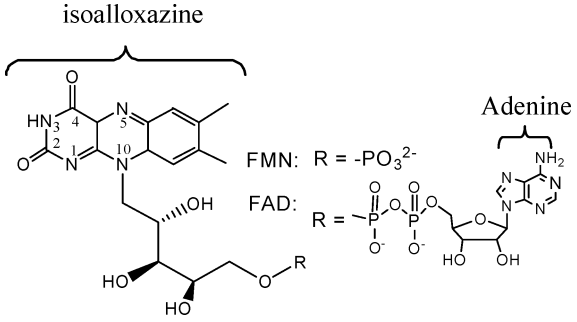
Received: December 12, 2003; In Final Form: February 20, 2004

In isolated, catalytically inactive, DNA photolyase from *Escherichia coli* (E. coli), the flavin adenine dinucleotide cofactor is in its neutral radical state $FADH^{\bullet}$. It can be activated by a unique light-induced reduction of the flavin, a process initiated by the formation of the excited-state $FADH^{\bullet}$*. As the photophysical properties of this state are essentially unknown, we performed a comparative characterization by femtosecond transient absorption spectroscopy of $FADH^{\bullet}$* in DNA photolyase from *E. coli* and glucose oxidase from *Aspergillus niger*, and of the excited neutral radical flavin mononucleotide ($FMNH^{\bullet}$*) in flavodoxin from *Desulfovibrio gigas.* In contrast to photolyase, in glucose oxidase and flavodoxin no electron-transfer products are observed after selective excitation of the flavin radical. In glucose oxidase, $FADH^{\bullet}$* decays to the ground state in 59 ± 5 ps, close to the 80-ps intrinsic lifetime of the excited state in photolyase, and we discuss that the intrinsic lifetime of the excited state of flavin radical in protein environment is in the 50−80 ps range. $FMNH^{\bullet}$* in flavodoxin decays much faster (2.3 ± 0.3 ps), possibly because of quenching by formation of a very short-lived (< 0.7 ps) electron-transfer intermediate. Spectroscopically, the excited state of $FADH^{\bullet}$ in photolyase displays a pronounced spectral feature that is absent in the other systems studied. Further characterization by polarized photoselection experiments identifies the feature as an additional induced absorption band at $\sim$ 550 nm superimposed on the ground-state bleaching signal. In view of the unique U-shape configuration of FAD in photolyase, we suggest it to reflect a flavin-adenine charge-transfer interaction.

Introduction

Flavoproteins are ubiquitous proteins in which the flavin cofactor plays the role of electron-transfer intermediate in various biochemical reactions.[1,2] Flavins (Scheme 1) display a rich redox chemistry as they can adopt three different redox states: oxidized, semi-reduced (radical), and fully reduced. In addition, the redox changes can be accompanied by protonation changes. The different forms of the flavin chromophore have characteristic absorption spectra in the visible and near UV, but the physiological functions of most flavoproteins are light-independent. Among the exceptions[3] is a class of proteins that comprises DNA photolyases and cryptochrome blue light receptors. DNA photolyase photochemically catalyzes the repair of some UV-induced lesions in DNA.[4] Here, a catalytic repair cycle is initiated by photoinduced electron transfer (ET) from the fully-reduced flavin adenine dinucleotide cofactor ($FADH^{-}$) to the substrate.[4,5] In isolated CPD (cyclobutane pyrimidine dimer) photolyase from many organisms, the flavin is in the neutral radical form ($FADH^{\bullet}$),[6] and the active fully-reduced form can be generated by another photochemical process (photoreduction).[7−9] The key initial step for this photoreduction process has been demonstrated to be the intraprotein ET from a tryptophan residue located near the photoexcited $FADH^{\bullet}$ in $\sim$ 30 ps[9] with a quantum efficiency of $\sim$ 65%.[10] The formed $FADH^{-}$ is subsequently stabilized, presumably by charge displacement along a highly-conserved triple tryptophan chain[9] (Figure 1a).

SCHEME 1: Molecular Structure of FAD and FMN



The initial charge separation in this process constitutes a unique example of a redox reaction initiated by formation of the excited state of a flavin in the semi-reduced form (semiquinone form). An intriguing question is whether the properties of the $FADH^{\bullet}$ excited state in this protein are adapted so as to allow effective ET. However, no characterization of this state

* Corresponding author. Phone: 33-1-69334777. Fax: 33-1-69333017. E-mail: Marten.Vos@polytechnique.fr.
[†] Laboratory for Optical Biosciences.
[‡] Service de Bioénergétique.
[§] Laboratoire de Bioénergétique et Ingénierie des Protéines—CNRS.
[||] Erasmus University Medical Centre.

10.1021/jp037837b CCC: $27.50
Published on Web 06/08/2004

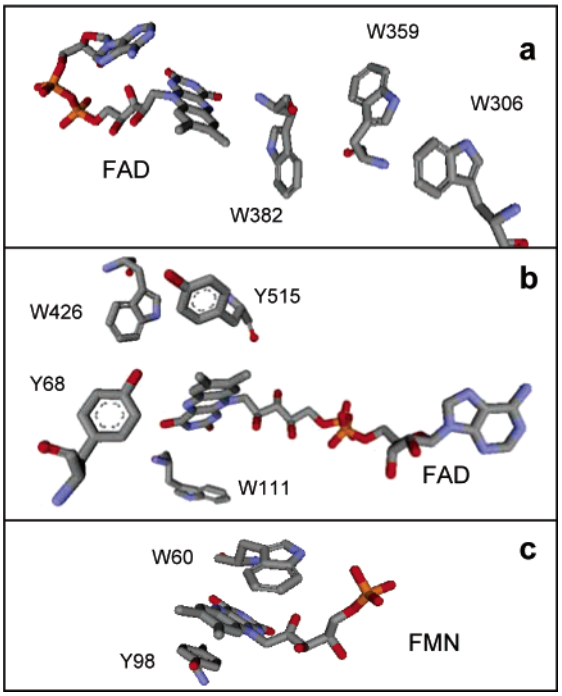


Figure 1. Conformation of the flavin cofactor and selected aromatic residues in DNA photolyase from *E. coli* (PDB 1DNP[19]) (a), in glucose oxidase from *A. niger* (PDB 1CF3[20]) (b), and in flavodoxin from *D. vulgaris* (PDB 3FX2[17]) (c). Three tryptophan residues playing a role in radical transfer during photoreduction of *E. coli* photolyase are shown. Aromatic residues near FAD in glucose oxidase, and FMN in flavodoxin, respectively, are also shown, which may participate in photoinduced electron transfer to oxidized FAD and FMN. See text for details. The figure was produced using the program ViewerLite 5.0 (Accelrys Inc.).

in other environments has been reported; $FADH^{\bullet}/FMNH^{\bullet}$ cannot be stably produced in sizable amounts in solution and to our knowledge no other proteins are isolated with a flavin in the neutral radical state. In this study we compare photolyase with two other flavoproteins, glucose oxidase and flavodoxin, where the flavin cofactor can be brought to the radical state by chemical or photochemical reduction and the spectral and kinetic properties of the photoexcited flavin radicals can be studied.

Previous studies on the excited-state properties of flavin in proteins focused on the stable oxidized[11−14] or fully reduced forms,[15] and aimed to probe the flavin environment in proteins that are *not* naturally photoactive. In addition, such studies have been performed on DNA photolyase to probe the interaction of the substrate with the fully-oxidized flavin cofactor[16] (this form is nonphotoactive). The excited state of oxidized flavin is a good electron acceptor, which has been demonstrated to be readily reduced by nearby aromatic residues. For example, photoinduced ET in 1.8 ps has been reported from tyrosine and/or tryptophan to FAD in glucose oxidase[14] (Figure 1b). Even shorter time constants of less than 200 fs were reported for photoexcited oxidized flavin mononucleotide (FMN) in flavodoxin and riboflavin in riboflavin binding protein (RBP).[12−14] In both latter cases, the isoalloxazine ring is sandwiched between a tyrosine and a tryptophan[17,18] (Figure 1c), presumably providing a favorable configuration for ET. In all these cases, the back reaction to the ground state occurs in tens of picoseconds, and no net ET products remain on a longer time scale.[14]

Besides the unique photochemical feature, the configuration of the FAD cofactor in photolyase is rather unusual among the flavin binding proteins. The FAD is in a hydrophobic protein environment, with a U-bent shape approaching the flavin and

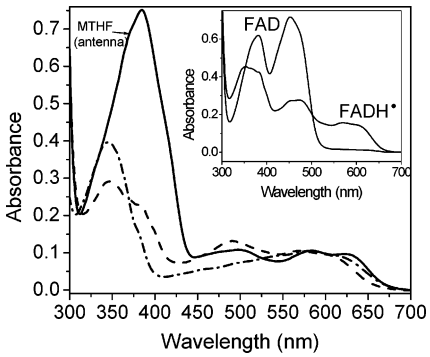


Figure 2. Steady-state absorption spectra of isolated photolyase from *E. coli* (—), semi-reduced glucose oxidase from *A. niger* (− − −, taken from Massey et al.[23]), and flavodoxin from *G. gigas* (−•−•−). The strong near-UV band in photolyase is due to the methenyltetrahydrofolate (MTHF) antenna chromophore. The $FADH^{\bullet}$ in photolyase has a similar UV band[4] as that in glucose oxidase. The spectra of semi-reduced glucose oxidase and flavodoxin are normalized to the maximum of the α band (580 nm) of the $FADH^{\bullet}$ in photolyase. Inset: anaerobic EDTA-photoreduction of glucose oxidase at pH 6.0, before (FAD) and after ($FADH^{\bullet}$) blue light illumination. See text for details.

adenine (Ade) moieties of the cofactor[19] (Figure 1a). In other FAD containing proteins of known structure, an extended configuration exists, as for example in glucose oxidase[20] (Figure 1b). In photolyase, the adenine has been suggested to play a role in ET to the DNA substrate.[16,21]

Comparative studies were carried out on the excited-state spectral properties and dynamics of the neutral flavin radical in photolyase, and in *nonphotoactive* glucose oxidase and flavodoxin by using femtosecond transient absorption spectroscopy. Remarkable differences among these three systems were found. In particular, specific features for excited-state $FADH^{\bullet}$ in photolyase are discussed in the light of interaction between the flavin and adenine rings, which might contribute to formation of the stable charge separation in this protein.

Materials and Methods

Sample Preparation. Recombinant *Escherichia coli* photolyase was obtained as described before.[9] The sample was stored at −80° in 10 mM phosphate buffer (pH 7.0) containing 0.25 M NaCl, 18% (w/w) glycerol and 5 mM 2-mercaptoethanol (ME). Buffer exchange with polyacrylamide chromatography microcolumns was performed immediately before the experiment. Enzyme in 20 mM Tris·HCl (pH 7.4), 200 mM NaCl buffer was used with a concentration of 200 μM with respect to the flavin radical as determined from the absorbance at 580 nm by using $\epsilon = 5000\ M^{-1}\cdot cm^{-1}$.[7]

Glucose oxidase from *Aspergillus niger* was purchased from Sigma, and prepared in 60 mM phosphate buffer, pH 6.0, at a flavin concentration of ∼ 500 μM. The semi-reduced form was obtained by anaerobic photoreduction following Massey's method[22] with some modifications. Briefly, in the presence of 10 mM EDTA and 25 μM riboflavin (Merck), the sample was extensively degassed in a 1-mm path length cell. Subsequently, at 0 °C it was illuminated with a 150 W tungsten halogen lamp through an optical fiber (diameter 18.3 mm) and a BG1 blue light filter. To follow the reduction of the flavin, absorption spectra were measured with a Shimadzu UV−Vis 1601 spectrophotometer. Oxidized FAD in glucose oxidase was reduced to the neutral radical form ($FADH^{\bullet}$) by EDTA under blue light illumination in a few minutes (inset of Figure 2), until ∼ 60% was converted (using the extinction coefficient of the oxidized FAD ($\epsilon_{452} \sim 14000\ M^{-1}\cdot cm^{-1}$), and of $FADH^{\bullet}$ ($\epsilon_{570} \sim 4400$

$M^{-1}\cdot cm^{-1}$) in glucose oxidase[23]). Further treatment with blue light leads to the formation of fully-reduced $FADH^-$, as reported by Massey et al.[22] Under our experimental conditions of excitation with red light, probing with a very weak continuum, small excitation volume, and continuously moving the sample (see below), this did not occur during the femtosecond experiments.

Flavodoxin from *Desulfovibrio gigas*[24] (*D. gigas*) was prepared in 50 mM MOPS buffer, pH 7.0, and brought completely to the semi-reduced form,[25] with a final flavin concentration of 200 μM. Either biochemical reduction (oxidized flavodoxin was added to a cocktail of 250 mM $NADP^+$, 1mM glucose-6-phosphate, 1 $U\cdot mL^{-1}$ glucose-6-phosphate dehydrogenase, and 0.05 $U\cdot mL^{-1}$ spinach ferredoxin−$NADP^+$−oxidoreductase) or the same photochemical method as described above for glucose oxidase, was used.

Transient Absorption Measurements. Femtosecond transient absorption measurements were performed using a pump−probe setup with a 30 Hz repetition rate as described previously.[26] Briefly, the amplified fundamental centered at 620 nm was employed as a pump pulse (stretched to 200 fs fwhm to avoid continuum generation in the sample cell), and a compressed white light continuum as probe pulse. About 10% of the flavins were excited at each pulse. The polarization of the pump pulse was set parallel, perpendicular, or at the magic angle (54.7°) with respect to that of the probe pulse using a Polarcor polarizer and a half-wave plate. The sample was kept in a 1-mm path length cell moving continuously perpendicular to the beams (diameter of focal spot $\sim$ 50 μm). Measurements on photolyase and glucose oxidase were performed at 10 °C, under anaerobic conditions for the latter. Flavodoxin was measured at room temperature. The redox-state of photolyase and flavodoxin samples did not change over the duration of the experiments. For glucose oxidase, a small fraction of the sample (< 20%) returned to the oxidized form during the measurements. This fraction can be reconverted to the neutral radical form under blue light illumination.

For the flavodoxin experiments, the data in the 390−600 nm region were obtained in two different data sets, where the group velocity dispersion in the probe pulse was minimized at $\sim$ 420 nm and $\sim$ 520 nm, respectively, using a pair of BK7 prisms. The remaining chirp (for the most dispersive blue region less than 300 fs below 460 nm and $\sim$ 700 fs between 460 and 500 nm) was determined from the cross-phase modulation signal of the sample, and corrected for to $\pm$200 fs in the shown data. For photolyase and glucose oxidase chirp correction was unnecessary as the decay was much slower. Meanwhile, in independent experiments, it was confirmed that in these two systems no significant spectral evolution occurred on the subpicosecond time scale.

Global exponential fitting of the data in terms of decay-associated spectra was performed with a procedure involving singular value decomposition as described in detail previously.[27]

Results

UV−Vis Absorption. Figure 2 shows the UV−Vis absorption spectra of isolated photolyase, and semireduced glucose oxidase and flavodoxin, respectively. In the visible region, the two weak broad absorption bands have been assigned to the two transition dipole moments of the isoalloxazine ring (α band at $\sim$ 580 nm and β band at $\sim$ 475 nm).[28] The neutral radical $FADH^\bullet$ in photolyase is stable.[6] $FMNH^\bullet$ in flavodoxin is also relatively stable due to hydrogen-bonding interaction between the proton on *N* (5) of the isoalloxazine ring and nearby amino acid residues, which decreases greatly the flavosemiquinone/flavohydroquinone midpoint potential of FMN with respect to its solution value.[29−31] The spectrum of $FMNH^\bullet$ in *D. gigas* flavodoxin obtained in our experiment is similar to those reported for flavodoxins from other species.[29,30] In contrast to flavodoxin and photolyase, the redox potentials for the flavoquinone/flavosemiquinone and flavosemiquinone/flavohydroquinone couples of FAD in glucose oxidase are close, and therefore it is more difficult to maintain in the neutral radical form (see materials and methods for details).[32] For clarity, the absorption spectrum of $FADH^\bullet$ in glucose oxidase was taken from Massey et al.[23] to compare with that in photolyase and $FMNH^\bullet$ in flavodoxin (note that the semi-reduced glucose oxidase produced as by Massey et al.[23] was not stable enough for our experiments). The absorption spectrum of $FADH^\bullet$ in glucose oxidase is similar in shape to that in photolyase, but is blue shifted by $\sim$ 10 nm.

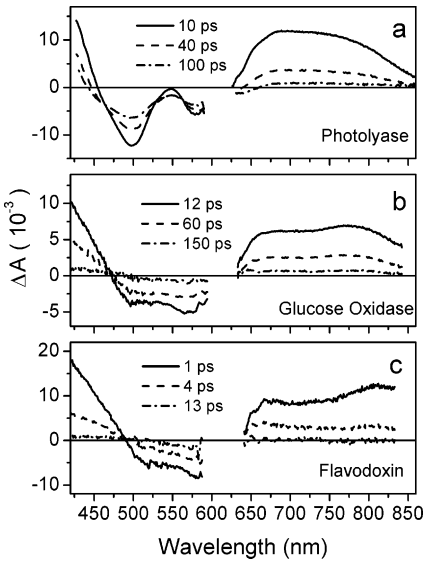


Figure 3. Visible and near-IR transient absorption spectra of $FADH^\bullet$ in photolyase (a) and in glucose oxidase (b), as well as $FMNH^\bullet$ in flavodoxin (c) at different delay times after excitation at 620 nm. The polarization of the pump and probe beams was set to the magic angle (54.7°). For flavodoxin, spectra are shown after correction for the group velocity dispersion in the probe light (see Materials and Methods for details).

Transient Absorption Measurements. Figure 3 shows transient absorption spectra in the visible/near-infrared range for the neutral flavin radicals $FADH^\bullet$ in photolyase and glucose oxidase, and $FMNH^\bullet$ in flavodoxin, respectively, at different delay times after 620 nm excitation. The overall spectral shape in these three samples has some common features. A negative absorption change in the 450−600 nm region is observed, which we attribute to the bleaching of the neutral flavin radical ground state. Two positive bands appear in the blue (400−450 nm) and red (650−860 nm) regions. These two bands are formed instantaneously (< 200 fs, see Figure 4) and we can ascribe these two bands to the excited-state absorption (ESA) of the flavin radicals, in line with Okamura et al.[33] For photolyase, the near-infrared signal decays completely with a time constant of $\sim$ 30 ps. In the visible region a signal remains after completion of this phase, which has been shown to reflect the $FADH^- - Trp^{\bullet+}$ state.[9] By contrast, both in the case of glucose oxidase (Figure 3b) and flavodoxin (Figure 3c), the decay of the ESA of the flavin radicals correlates well with the ground-state recovery, indicating that the excited neutral flavin radicals return to the original ground state.

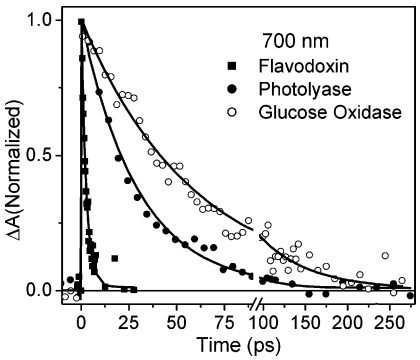


Figure 4. Transient absorption kinetics at 700 nm for FMNH• in flavodoxin (■), as well as FADH• in photolyase (●) and in glucose oxidase (○), after laser excitation at 620 nm. Symbols are experimental data, and the solid line is a single-exponential fit with the following time constants: 2.3 ± 0.3 ps, 30 ± 3 ps and 59 ± 5 ps, respectively, for these three samples. See text for details.

We also note that in none of the three systems is a clear feature that can be ascribed to stimulated emission (which, if observable, would be expected as a transmission increase at the red of the ground state absorption) observed. This is generally consistent with the notion that neutral radical flavin is nonfluorescent.

Besides the persisting ground-state bleaching signal after the disappearance of the ESA signals, interestingly, the transient spectra for photolyase at early times are also remarkably different from those for glucose oxidase and flavodoxin. Compared to the flat bleaching signal for both glucose oxidase and flavodoxin at the 475−600 nm region (Figure 3b, 3c), for photolyase there is a pronounced maximum in the transient absorption signal centered at ∼ 550 nm (Figure 3a). This feature may be due to an additional absorption, or to a band shift, superimposed on the bleaching signal of the FADH•* in photolyase (see below).

The kinetic traces at 700 nm are shown in Figure 4 to compare the decay dynamics of FADH•* in photolyase and glucose oxidase, and FMNH•* in flavodoxin. All kinetics can be fit with single exponentials, but with different time constants of 2.3 ± 0.3 ps for flavodoxin, 30 ± 3 ps for photolyase, and 59 ± 5 ps for glucose oxidase. The observed 30-ps time constant for the decay of the FADH•* in photolyase reflects the sum of the rates for FADH•* intrinsic decay and the ET process. A longer lifetime of 80 ps for FADH•* was observed in the *E. coli* photolyase W382F mutant, where virtually no quenching by ET occurred.[10]

Anisotropy Measurement. To investigate the origin of the pronounced spectral feature in the initial transient absorption of photolyase (Figure 3a), we performed comparative polarization anisotropy measurements for photolyase and the other two semi-reduced flavoproteins. Figure 5 shows the polarized spectra at $t = 0$ upon laser excitation (parallel or perpendicular), extrapolated from the data by a global analysis.[27]

For single linear transitions, the polarization ratio $\Delta A_{\parallel}/\Delta A_{\perp}$ should be constant over the spectral region and related to the angle θ between the pumped and probed transition as

$$\frac{\Delta A_{\parallel}}{\Delta A_{\perp}} = \frac{1 + 2\cos^2\theta}{2 - \cos^2\theta} \qquad (1)$$

that gives values between 1/2 and 3. In spectral regions where more than one transition contributes, the polarization ratio can vary with wavelength, because each transition has a different polarization ratio. Moreover, if transitions with opposite ΔA signs contribute, the polarization ratio values can go beyond the range expected for single transitions due to compensation effects.

For flavodoxin (Figure 5c), the polarization ratio is essentially constant at < 470 nm, and 500 nm < λ < 580 nm (note that $\Delta A_{\parallel}/\Delta A_{\perp}$ is not well defined near the zero crossing points), indicating that mainly one transition is dominant in each of these two regions. These two spectral regions correspond to the induced absorption of FMNH•* and the bleaching of the ground state, respectively (see above). The $\Delta A_{\parallel}/\Delta A_{\perp}$ value of ∼ 3 for the bleaching region fits well with the theoretical value for pumping and probing the same linear transition ($\theta = 0$). A similar polarization spectrum was observed for FADH•* in glucose oxidase, apart from a more gradual decrease of $\Delta A_{\parallel}/\Delta A_{\perp}$ values from ∼ 3 to 1.5 in the ground-state bleaching region (Figure 5b). At the region of maximum bleaching (550−590 nm), the $\Delta A_{\parallel}/\Delta A_{\perp}$ value of ∼ 3 again indicates pumping and probing the same linear transition. The gradual polarization decrease in the 460−550 nm region can be explained by increasing contributions from the second flavin transition moment (β band, the transition moment of this band presumably makes an angle of ∼ 20° with that of the α band,[28] corresponding to a polarization ratio of ∼ 2.5) and from ESA (see below). The corresponding polarization change in flavodoxin is less gradual, probably related to the fact that the β band of the isoalloxazine ring for FMNH• in flavodoxin is far less pronounced, as shown in Figure 2.

For photolyase, however, the polarization varies in a more complicated pattern (Figure 5a). Besides the flat polarization

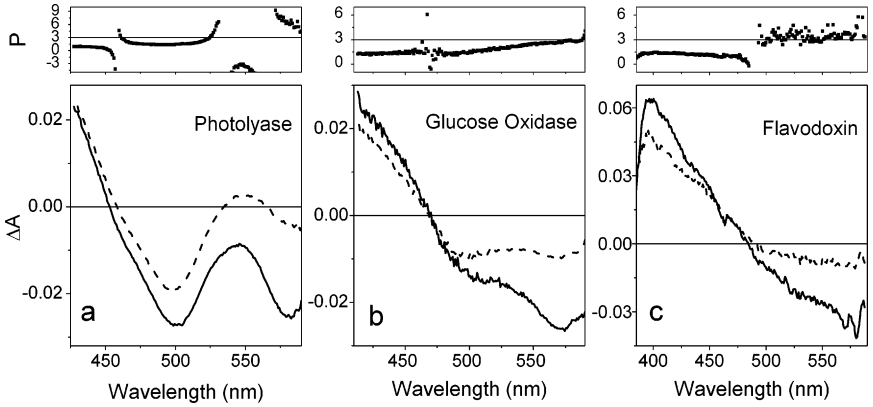


Figure 5. Polarized transient absorption spectra at $t = 0$ extrapolated from a global analysis for FADH• in photolyase (a) and in glucose oxidase (b), as well as FMNH• in flavodoxin (c). The pump and probe pulses were polarized in parallel (—) or perpendicular (− − −). The upper panels depict the polarization ratio $P = \Delta A_{\parallel}/\Delta A_{\perp}$ as a function of wavelength.

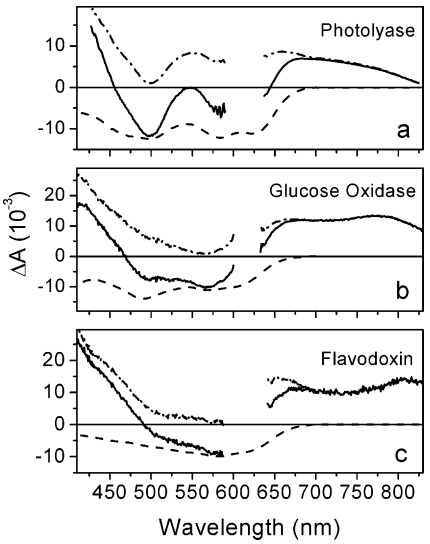


Figure 6. Transient absorption spectra at $t = 0$ (—) extrapolated after global exponential fitting to the magic angle kinetics for the FADH• in photolyase (a) and in glucose oxidase (b), as well as FMNH• in flavodoxin (c). The ground-state absorption spectra of these flavin radicals are shown as negative signals (− − −). For glucose oxidase and flavodoxin, the relative amplitude of the negative signals was normalized to the maximum of the bleaching signal, because contribution of ESA to this region is small as inferred from anisotropy measurements (see Figure 5b, 5c). For photolyase, the spectra were normalized using the spectrum remaining after the 30-ps phase (Figure 3a). This spectrum can be well-decomposed in contributions from FADH• bleaching and $FADH^-$ and $Trp^{\bullet+}$ induced absorption.[9] The contribution of the bleaching of FADH• in the $t = 0$ spectrum was determined using the estimated quantum yield of 65% for electron transfer from FADH•*.[10] "Pure" ESA (−•−•−) of the flavin radical in these three flavoproteins were constructed by subtracting the negative signals from the transient spectra at $t = 0$.

spectrum in the regions below 450 nm, and between 465 and 515 nm, as in the cases of flavodoxin and glucose oxidase, a dramatic change in polarization is observed at 520−590 nm. Moreover, the polarization ratio takes values outside the range of 1/2−3 expected for a single transition. These findings clearly imply that there is more than one transition in this spectral region, which corresponds exactly to the higher ΔA region in photolyase. The fact that this polarization variation is limited to this spectral region indicates that this spectral feature for photolyase should be interpreted as an induced absorption superimposed on bleaching, rather than a band shift. Taking the decomposition of the photolyase FADH•* spectrum of Figure 6a (see Discussion) and assuming that predominantly the α band contributes to the ground-state absorption at $\lambda > 575$ nm (cf. Eaton et al.[28]), we can estimate the polarization ratio associated with the induced absorption at ∼ 0.90,[34] corresponding to an angle of 58° between the transition moment of the induced absorption and that of the ground-state α band.

In the 700−850 nm region (Figure 3), we observed a constant polarization ratio of ∼ 0.8 for both photolyase and flavodoxin (data not shown). This value corresponds to an angle of ∼ 62° between the transition moments of the ground-state α band and the ESA band. The lack of variation of this value indicates that only one transition contributes, and confirms that stimulated emission (for which a near-parallel transition moment would be expected) does not significantly contribute to the spectra.

Discussion

(1) Excited-State Properties of the Flavin Radical. We have performed the first comparative study on the excited-state properties of flavin radicals in proteins. One general result is that the excited-state lifetimes we measured are all less than 100 ps, much shorter than the intrinsic lifetimes of a few nanoseconds for the oxidized state or the fully-reduced state.[15,35,36] These much shorter lifetimes can be due both to intrinsically faster decay and to quenching of the excited state in all systems studied. Whereas the difficulty in accumulating flavin radicals in solution precludes the determination of their excited-state properties, in the following we argue that the excited-state decay of flavin radicals at least in proteins is probably intrinsically fast.

Previous studies have demonstrated that in *E. coli* photolyase, the excited FADH• is able to extract an electron from the nearest tryptophan W382 in 30 ps.[9] In the W382F mutant, the FADH•* decay slows down to 80 ps and the yield of the ET product decreases by a factor of ∼ 200 (the remaining product formed is probably due to the ET from a second tryptophan residue W359).[10] Therefore, the 80 ps lifetime has been interpreted as the intrinsic lifetime of FADH•* in photolyase.[10]

A much shorter lifetime (2.3 ps) was observed for FMNH•* in flavodoxin (Figure 4). The absence of an adenine moiety in the FMN molecule is unlikely to be at the origin of this difference, since the same 1.5 ns lifetime was observed for the fully-reduced FAD ($FADH^-$) in glucose oxidase and the fully-reduced FMN ($FMNH^-$) in the flavodehydrogenase component of flavocytochrome b_2;[15] similar lifetimes have been reported for oxidized or fully-reduced FMN and FAD (provided it is in the extended state) in solution.[35,36] We suggest that the 2.3 ps lifetime for FMNH• in flavodoxin implies significant quenching. As mentioned in the Introduction, ET from proximate amino acids was demonstrated to contribute to the excited-state quenching of the oxidized FMN in flavodoxin and riboflavin in RBP in a few hundred femtoseconds.[12−14] In these proteins, ultrafast ET is thought to occur mainly because of a favorable spatial stacking, where the flavin isoalloxazine ring is sandwiched between conserved aromatic residues (a tyrosine and a tryptophane residue), with a very short interplane distance.[17,18] The structure of the FMNH• in flavodoxin is nearly identical to that in the oxidized state.[17] The midpoint potential for the flavosemiquinone/flavohydroquinone of FMN in flavodoxin from *D. gigas* has been measured as −0.44 V vs NHE,[37] similar to flavodoxin from other species,[29,30,38] and for $Trp^{\bullet+}$/Trp buried in an artificial protein as ∼ 1.1 V vs NHE.[39] Thus, the photoexcited FMNH• should be able to provide enough driving force for the ET reaction (estimated $\Delta G \sim -0.4$ eV, by using $E_{0-0} \sim 2.0$ eV). Accordingly, we suggest that the short lifetime of FMNH•* in flavodoxin can result from the quenching by an ET reaction with proximate aromatic residues. Assuming electron transfer between the same partners is at the origin of quenching, the shorter lifetime of FMN* (∼ 160 fs[13]) than that for FMNH•* (2.3 ps; this work) in flavodoxin may be related to the higher driving force (estimated $\Delta G \sim -1.1$ eV, by using $E_{0-0} \sim 2.35$ eV, and a midpoint potential of −0.15 V for the flavoquinone/flavosemiquinone couple of FMN in flavodoxin[29]). Our transient absorption measurements did not directly detect any ET products, as the ground state of FMNH• is regenerated concomitant with the disappearance of the excited state in 2.3 ps (Figure 3c). Therefore we suggest that if ET takes place, the product is extremely unstable and decays faster than it is formed. Absorption of the potential ET products $FMNH^-$ and $Trp^{\bullet+}$, is expected at < 500 nm[29,30] and 560 nm,[40] respectively. From the published relative extinctions of FMNH• and $FMNH^-$ at 490 nm (the isosbestic point for the FMNH•* minus FMNH• spectrum), as well as of FMNH• at 580 nm and our noise level

at 490 nm, we estimate an upper limit for the charge recombination time constant of ~ 0.7 ps.[41]

If this mechanism is correct, the origin of the much higher speed of charge recombination following photoexcitation of $FMNH^{\bullet}$, compared to FMN, remains to be determined. One possible explanation may be that electron transfer to excited FMN is followed by fast (energetically favorable) protonation of the flavin, so that the flavin product state would be $FMNH^{\bullet}$ rather than $FMN^{\bullet-}$. Back electron transfer may then be preceded by energetically-unfavorable deprotonation of $FMNH^{\bullet}$, and the overall back reaction may be relatively slow for this reason. The validity of this proposal for flavodoxin remains to be investigated, but we note that, upon excitation of the oxidized flavin cofactor in riboflavin-binding protein and glucose oxidase, substantial induced absorption at 630 nm has been observed in the product state that lives for 10 ps or longer.[14] Absorption at this wavelength is expected for the protonated flavin radical, rather than for the flavin radical anion or oxidized amino acids, as suggested by the authors.[14]

We note that charge recombination between reduced flavin and a nearby oxidized aromatic residue (Trp 382 in *E. coli* photolyase), as discussed here for flavodoxin, may in principle also play a role in the photoreduction reaction in photolyase. If so, the competing further charge stabilization by charge transfer along the chain of tryptophans must be faster than recombination to explain the high yield of the photoreduction reaction.

In the case of oxidized glucose oxidase, ET from nearby aromatic residues was observed to quench FAD*. ET rates of $(1.8\ \text{ps})^{-1}$ and $(10\ \text{ps})^{-1}$, an order of magnitude slower than in oxidized flavodoxin, were explained by the larger spatial separation of these residues to the FAD cofactor.[14] In photolyase apoprotein reconstituted with oxidized FAD, a moderate quenching phase of 30 ps was observed, and a substantial fraction of the excited state appears unquenched and decays in nanoseconds.[16] Altogether, apart from quenching due to long-lived charge separation in semi-reduced wild type photolyase, the neutral radical forms of the flavin have the same order of observed excited-state lifetimes (flavodoxin < glucose oxidase < photolyase) as the oxidized flavins. Taking together the findings that (a) quenching in the neutral radical states, if present, appears substantially slower than in the oxidized forms (possibly due to the driving-force difference discussed above for flavodoxin) and (b) little and relatively slow quenching is observed in oxidized photolyase, we suggest that the lifetime of $FADH^{\bullet}$* in photolyase observed in the absence of flavin photoreduction ($\tau \sim 80$ ps), corresponds to the intrinsic excited-state lifetime of $FADH^{\bullet}$*. $FADH^{\bullet}$* in glucose oxidase has a lifetime of 59 ps, which is only slightly less than that of the intrinsic $FADH^{\bullet}$* lifetime in photolyase. The small difference between these two rates may arise from minor quenching (in which case also electron-transfer products would recombine so fast that they are not detected) and/or the difference in protein environment.

We emphasize that our suggestion of an intrinsic excited-state lifetime of 50−80 ps originates from an indirect line of reasoning, and no direct experimental evidence for or against this proposal is available. We therefore do not exclude the possibility that quenching by electron transfer determines the rate of $FADH^{\bullet}$* decay in glucose oxidase and photolyase as well. If this was the case, ultrafast charge recombination between $FADH^{-}$ and its oxidized redox partner would appear to be a general feature in flavoproteins. This hypothesis is hard to test as $FADH^{-}$ absorbs only weakly in the visible (below 500 nm) and as its putative redox partner is unknown.[42] A possible exception is photolyase from *E. coli*, where we know that it is W382 that is oxidized in ~ 30 ps,[10] and charge separation is stabilized by further electron transfer along the triple-tryptophan chain, with unknown time constants. If a mutant could be made that disrupts this chain at the level of the next tryptophan (W359), the kinetics of charge recombination in the pair $FADH^{-}-Trp_{382}^{\bullet+}$ might be resolved by following the absorption of $Trp^{\bullet+}$ around 580 nm. In summary, we favor the possibility that the intrinsic lifetime of the instantaneously (< 200 fs) populated, nonfluorescent, excited state of neutral flavin radicals ($FADH^{\bullet}$ or $FMNH^{\bullet}$) in a protein environment is on the order of 50−80 ps, without excluding the possibility that quenching by electron-transfer plays a role on this time scale.

(2) Spectral Features of the $FADH^{\bullet}$ Excited State in Photolyase and Implications. In all systems studied, a bleaching in the ground-state absorption region was observed upon 620 nm excitation in the lowest-lying band of the flavin radical. This finding confirms that it is this spectrum that corresponds to the initially-formed excited state of $FADH^{\bullet}/FMNH^{\bullet}$. It contrasts with previous assignments[4,33] to an excited state of $FADH^{\bullet}$ of a spectrum characterized by featureless induced absorption in the entire visible/near-infrared region. However, the latter was obtained upon excitation of DNA photolyase in the near-UV region, where all flavin redox forms absorb.

A striking feature was observed in the excited-state transient spectra of $FADH^{\bullet}$ in photolyase that is absent in both other flavoproteins studied (Figure 3). Our anisotropy measurements (Figure 5) demonstrate that this feature is due to an additional induced absorption band at ~ 550 nm, superimposed on the bleaching signal. To further elucidate this spectral feature, we attempt to separate the ground-state bleaching and ESA contributions from the transient spectra associated with the initially-formed excited state. To this aim, the transient spectra at $t = 0$ were obtained from the global exponential fits of the isotropic data (magic angle), as shown as solid lines in Figure 6. The ground-state neutral flavin radical spectra are shown as negative signals (dashed lines), representing the expected bleaching due to ground-state depletion. "Pure" ESA spectra (upper-most spectra (dash−dot lines) in Figure 6) of these flavin radicals were constructed by subtracting the ground-state bleach signals from the $t = 0$ transient spectra. For flavodoxin and glucose oxidase only two broad positive absorption bands in the blue (< 480 nm) and red (650−850 nm) were obtained (Figure 6b, 6c), corresponding to regions with constant anisotropy. For photolyase, where $FADH^{\bullet}$ has a similar ground-state absorption spectrum as in glucose oxidase, an additional peak at ~ 550 nm is clearly present, besides the similar two bands in the blue and red regions (Figure 6a).

In principle, the specific modulation in the absorption spectrum of $FADH^{\bullet}$* in photolyase may be due to (a) an interaction between the flavin and the protein environment, and (b) the configuration of the $FADH^{\bullet}$ itself. Regarding possibility (a), for example, excited-state charge transfer could occur between the $FADH^{\bullet}$* and a tryptophan residue, as $Trp^{\bullet+}$ is known to absorb around 560 nm.[40] However, this spectral feature is present also in a mutant where the most close-lying Trp residue (and primary electron donor) is replaced by phenylalanine.[10] Therefore this possibility is highly unlikely.

Regarding possibility (b), we note that the 550-nm spectral feature is absent in glucose oxidase that employs the same FAD as cofactor, but with a markedly different configuration.[20] The FAD cofactor in photolyase is in a unique U-shape with the isoalloxazine ring and the adenine moiety close to each other (Figure 1a), instead of the extended conformation in glucose

oxidase (shown in Figure 1b). We suggest that the spectral feature for photolyase in Figure 6a may result from interaction between the close-lying isoalloxazine and adenine rings. In particular, it is possibly due to a change in charge distribution between the photoexcited flavin radical and the adenine ring.

A precise assessment of the influence of interaction between the isoalloxazine ring and adenine moieties on the $FADH^{\bullet}$* properties in photolyase must await further experiments and theoretical investigations. Nevertheless, here we note that in principle the population of excited states with $Ade^{\bullet+}FADH^{-}$ and $Ade^{\bullet-}FADH^{+}$ charge transfer character appears feasible; in addition one might even invoke the possibility of an optical transition to such states from a singlet excited state. The following observations appear generally consistent with the proposal of charge-transfer involving adenine being at the origin of the 550-nm band: (1) the oxidized[43] as well as the reduced adenine radical,[44,45] absorb around 550 nm in solution and (2) inspection of the photolyase structure indicates that the orientation of the transition corresponding to the 550-nm band (58° from the $FADH^{\bullet}$ α band, cf. Eaton et al.[28]) is within the possible range for transitions in the plane of the adenine.[46]

The characteristic conformation of FAD in photolyase has previously led to suggestions of interaction between the isoalloxazine and adenine moieties in studies involving all three redox states of the FAD cofactor.[16,21,47] Transient absorption measurement on the apoprotein reconstituted with oxidized FAD reported a broad induced absorption band above 490 nm at 5 ps after excitation on FAD, which was suggested to reflect a charge-transfer complex between isoalloxazine and the adenine ring.[16] In a quantum chemistry calculation on the fully-reduced $FADH^{-}$ in photolyase, a functional role of this special U-shape has been proposed in that the adenine moiety might constitute an ET bridge between the $FADH^{-}$ and the CPD dimer in the catalytic repair cycle.[21] Theoretical calculations on the electronic structure of $FADH^{\bullet}$ in photolyase,[47] taking into account hydrogen-bonding interaction with the protein environment,[19,48] suggested that its specific protein environment may cause significant redistribution of both the spin and electron densities in the isoalloxazine ring of $FADH^{\bullet}$, thus enhancing the $\pi-\pi$ interaction with the adjacent adenine ring. However, altogether no unambiguous experimental evidence has been reported on the possible interaction between these two moieties in photolyase. The results of our comparative study generally support the notion of close isoalloxazine-adenine interaction.

As discussed above, only in photolyase is the excited flavin radical able to attract and stabilize an electron from a nearby aromatic residue, and ultrafast charge stabilization along the triple tryptophan chain may be required to avoid wasteful charge recombination. In addition, one may speculate on a functional implication for the special spectral feature of the $FADH^{\bullet}$* in photolyase. The putative excited-state charge delocalization between the isoalloxazine and adenine rings appears not to considerably stabilize the excited-state itself (a comparable excited-state lifetime is found in glucose oxidase). Yet, redistribution of electron density from the isoalloxazine ring to the adenine may be favorable for extracting an electron from tryptophan W382 (see Figure 1a). The same charge redistribution might help to prevent back reactions between oxidized tryptophan and reduced FAD.

Conclusion

We carried out comparative studies on the excited-state properties of flavin radicals in photolyase, glucose oxidase, and flavodoxin. We suggest that the intrinsic excited-state decay of flavin radicals in a protein environment is on the order of 50−80 ps. $FMNH^{\bullet}$* in flavodoxin decays much faster with a time constant of 2.3 ps, pointing to an additional quenching channel, possibly involving an unstable charge-separated state. Photolyase differs from the other flavoproteins by undertaking effective photoinduced ET to the flavin radical. The excited state of $FADH^{\bullet}$ in photolyase displays a pronounced and unique spectral feature, which was characterized by anisotropy measurement as an additional induced absorption band at $\sim$ 550 nm superimposed on the ground-state bleaching signal, which we suggest to reflect a flavin-adenine charge transfer interaction, possibly favoring the $FADH^{\bullet}$ photoreduction reaction.

Acknowledgment. We thank Dr. Ulrich Mühlenhoff for a gift of flavodoxin from *Synechococcus* sp. PCC 7002 and advice on its chemical reduction to the neutral radical state.

References and Notes

(1) Massey, V. *Biochem. Soc. Trans*. **2000**, *28* (part 4), 283−296.
(2) Walsh, C. *Acc. Chem. Res*. **1980**, *13*, 148−155.
(3) Van der Horst, M. A.; Hellingwerf, K. J. **2004**, *Acc. Chem. Res. 37*, 13−20.
(4) Sancar, A. *Chem. Rev*. **2003**, *103*, 2203−2238.
(5) MacFarlane, A. W.; Stanley, R. J. *Biochemistry* **2003**, *42*, 8558−8568.
(6) Jorns, M. S.; Sancar, G. B.; Sancar, A. *Biochemistry* **1984**, *23*, 2673−2679.
(7) Heelis, P. F.; Okamura, T.; Sancar, A. *Biochemistry* **1990**, *29*, 5694−5698.
(8) Li, Y. F.; Heelis, P. F.; Sancar, A. *Biochemistry* **1991**, *30*, 6322−6329.
(9) Aubert, C.; Vos, M. H.; Mathis, P.; Eker, A. P. M.; Brettel, K. *Nature* **2000**, *405*, 586−590.
(10) Byrdin, M.; Eker, A. P. M.; Vos M. H.; Brettel, K. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 8676−8681.
(11) Yagi, K.; Tanaka, F.; Nakashima, N.; Yoshihara, K. *J. Biol. Chem.* **1983**, *258*, 3799−3802.
(12) Mataga, N.; Chosrowjan, H.; Shibata, Y.; Tanaka, F.; Nishina, Y.; Shiga, K. *J. Phys. Chem. B* **2000**, *104*, 10667−10677.
(13) Mataga, N.; Chosrowjan, H.; Taniguchi, S.; Tanaka, F.; Kido, N.; Kitamura, M. *J. Phys. Chem. B* **2002**, *106*, 8917−8920.
(14) Zhong, D.; Zewail, A. H. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 11867−11872.
(15) Enescu, M.; Lindqvist, L.; Soep, B. *Photochem. Photobiol.* **1998**, *68*, 150−156.
(16) MacFarlane IV, A. W.; Stanley, R. J. *Biochemistry* **2001**, *40*, 15203−15214.
(17) Watt, W.; Tulinsky, A.; Swenson, R. P.; Watenpaugh, K. D. *J. Mol. Biol*. **1991**, *218*, 195−208.
(18) Monaco, H. L. *EMBO J*. **1997**, *16*, 1475−1483.
(19) Park, H. W.; Kim, S.-T.; Sancar, A.; Deisenhofer, J. *Science* **1995**, *268*, 1866−1872.
(20) Wohlfahrt, G.; Witt, S.; Hendle, J.; Schomburg, D.; Kalisz, H. M.; Hecht, H. J. *Acta Crystallogr*. **1999**, *D55*, 969−977.
(21) Antony, J.; Medvedev, D. M.; Stuchebrukhov, A. A. *J. Am. Chem. Soc*. **2000**, *122*, 1057−1065.
(22) Massey, V.; Stankovich, M.; Hemmerich, P. *Biochemistry* **1978**, *17*, 1−8.
(23) Massey, V.; Matthews, R. G.; Foust, G. P.; Howell, L. G.; Williams, C. H.; Zanetti, G.; Ronchi, S. In *Pyridine Nucleotide-Dependent Dehydrogenases*; Sund, H., Ed.; Springer-Verlag: Berlin, 1969; p 393−411.
(24) Moura, I.; Moura, J. J. G.; Bruschi, M.; Le Gall, J. *Biochim. Biophys. Acta* **1980**, *591*, 1−8.
(25) The yield and stability of the $FMNH^{\bullet}$ were tested for flavodoxin from *Synechococcus* sp. PCC 7002 and *D. gigas*. The latter was found to be more favorable.
(26) Martin, J.-L.; Vos, M. H. *Methods Enzymol*. **1994**, *232*, 416−430.
(27) Liebl, U.; Lambry, J.-C.; Leibl, W.; Breton, J.; Martin, J.-L.; Vos, M. H. *Biochemistry* **1996**, *35*, 9925−9934.
(28) Eaton, W. A.; Hofrichter, J.; Makinen, M. W.; Andersen, R. D.; Ludwig, M. L. *Biochemistry* **1975**, *14*, 2146−2151.
(29) Swenson, R. P.; Krey, G. D. *Biochemistry* **1994**, *33*, 8505−8514.
(30) Lostao, A.; Gómez-Moreno, C.; Mayhew, S. G.; Sancho, J. *Biochemistry* **1997**, *36*, 14334−14344.
(31) Chang, F.-C.; Swenson, R. P. *Biochemistry* **1999**, *38*, 7168−7176.
(32) Stankovich, M. T.; Schopfer, L. M.; Massey, V. *J. Biol. Chem*. **1978**, *253*, 4971−4979.

(33) Okamura, T.; Sancar, A.; Heelis, P. F.; Hirata, Y.; Mataga, N. *J. Am. Chem. Soc*. **1989**, *111*, 5967−5969.

(34) Around 575 nm, the signals for glucose oxidase and flavodoxin are dominated by ground-state bleaching, as indicated by the polarization ratio of 3, as expected for pumping and probing the same transition. For photolyase, we assume the signal in this region to be a superposition of ground-state bleaching of the flavin (ΔB) and induced absorption (ΔA). For the ground-state bleaching $\Delta B_{||}/\Delta B_{\perp} = 3$. From the decomposition of Figure 6a, we can estimate the ratio α of the contributions of isotropic ΔA and ΔB, $\alpha = (\Delta A_{||} + 2\Delta A_{\perp})/(\Delta B_{||} + 2\Delta B_{\perp})$. From the polarization data of Figure 5a we can estimate the polarization ratio β of the total signal: $\beta = (\Delta A_{||} + \Delta B_{||})/(\Delta A_{\perp} + \Delta B_{\perp})$. Solving this set of equations yields the polarization ratio $\Delta A_{||}/\Delta A_{\perp} = (\beta + 2\chi)/(1 - \chi)$, with $\chi = (\beta - 3)/5\alpha$. For $\alpha \sim -0.4$ and $\beta \sim 8$, we find a value of ~ 0.9.

(35) Stanley, R. J. *Antioxidants & Redox Signaling* **2001**, *3*, 847−866.

(36) van den Berg, P. A. W.; Feenstra, K. A.; Mark, A. E.; Berendsen, H. J. C.; Visser, A. J. W. G. *J. Phys. Chem. B* **2002**, *106*, 8858−8869.

(37) Moura, J. J. G. Personal communication.

(38) Curley, G. P.; Carr, M. C.; Mayhew, S. G.; Voordouw, G. *Eur. J. Biochem*. **1991**, *202*, 1091−1100.

(39) Tommos, C.; Skalicky, J. J.; Pilloud, D. L.; Wand, A. J.; Dutton, P. L. *Biochemistry* **1999**, *38*, 9495−9507.

(40) Solar, S.; Getoff, G.; Surdhar, P. S.; Armstrong, D. A.; Singh, A. *J. Phys. Chem*. **1991**, *95*, 3639−3643.

(41) Briefly, we analyze the data around the 490-nm isosbestic point of the initially observed $FMNH^{\bullet}$*-$FMNH^{\bullet}$ spectrum, because any shift from this isosbestic point would be most directly observable. We estimate that the absorption change during the decay at this wavelength is less than 10% of the signal at 580 nm, which can be assigned solely to bleaching of $FMNH^{\bullet}$ (Figure 6c). Ignoring for simplicity potential contributions from amino acid electron donors, formation of $FMNH^-$ is expected to give rise to a negative ΔA ($FMNH^- - FMNH^{\bullet}$). This is about 0.6 times that of the initial bleaching of $FMNH^{\bullet}$ at 580 nm, as can be inferred from published extinction coefficients[29]. Together this indicates that the maximal intermediate population of $FMNH^-$ is $\sim$ 17%. Assuming a linear reaction scheme

$$FMNH^{\bullet}* \xrightarrow{k_1} FMNH^- \xrightarrow{k_2} FMNH^{\bullet}$$

and solving the corresponding coupled linear differential equations with all population in $FMNH^{\bullet}$* at $t = 0$, we find for the time course of the $FMNH^-$ population $[k_1/(k_2 - k_1)](e^{-k_1 t} - e^{-k_2 t})$. The maximum of this function depends on the ratio k_2/k_1 and amounts to ~ 0.17 for $k_2/k_1 = 3.5$. Taking $k_1 = 1/(2.3\ \text{ps})$, we find $k_2 > 1/(0.7\ \text{ps})$.

(42) Electron donors could be aromatic residues. For photolyase it could also be the adenine moiety of the cofactor.

(43) Jovanovic, S. V.; Simic, M. G. *J. Phys. Chem*. **1986**, *90*, 974−978.

(44) Visscher, K. J.; de Haas, M. P.; Loman, H.; Vojnovic, B.; Warman, J. M. *Int. J. Radiat. Biol*. **1987**, *52*, 745−753.

(45) Candeias, L. P.; Steenken, S. *J. Phys. Chem*. **1992**, *96*, 937−944.

(46) Transition moment dipole directions for adenine radicals have not been reported, but supposedly lie in the adenine plane.

(47) Weber, S.; Möbius, K.; Richter, G.; Kay, C. W. M. *J. Am. Chem. Soc*. **2001**, *123*, 3790−3798.

(48) Schelvis, J. P. M.; Ramsey, M.; Sokolova, O.; Tavares, C.; Cecala, C.; Connell, K.; Wagner, S.; Gindt, Y. M. *J. Phys. Chem. B* **2003**, *107*, 12352−12365.