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Excited-State Properties of Fully Reduced Flavins in Ferredoxin-NADP⁺ Oxidoreductase

Bo Zhuang,[†] Alexey Aleksandrov,^{*†} Daisuke Seo,^{*‡} and Marten H. Vos^{*†}

[†]LOB, CNRS, INSERM, École Polytechnique, Institut Polytechnique de Paris, 91120 Palaiseau, France

[‡]Division of Material Science, Graduate School of Natural Science and Technology, Kanazawa University, 920-1192 Kanazawa, Ishikawa, Japan

AUTHOR INFORMATION

Corresponding Author

*alexey.aleksandrov@polytechnique.edu (A.A.)

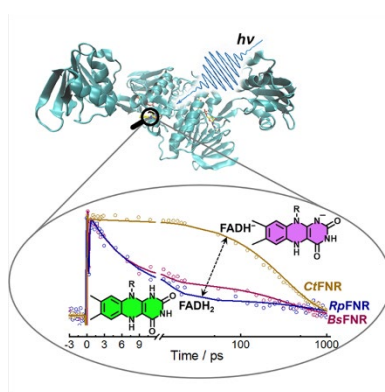
*dseo@se.kanazawa-u.ac.jp (D.S.)

*marten.vos@polytechnique.edu (M.H.V.)

ABSTRACT: The fully reduced flavin cofactor (FAD_{red}) in ferredoxin-NADP⁺ oxidoreductase (FNR) is a functional intermediate that displays different catalytic and steady-state spectral properties for enzymes from *Bacillus subtilis* (BsFNR), *Chlorobaculum tepidum* (CtFNR) and

Rhodopseudomonas palustris (*RpFNR*). Using ultrafast spectroscopy, we reveal that at physiological pH, photoexcited FAD_{red} in *BsFNR* and *RpFNR* exhibits unprecedentedly fast decays (dominantly in 6 and 8 ps, respectively), whereas in *CtFNR* the decay is much slower (~ 400 ps), as in other flavoproteins. Correlating these observations with the protonation states of FAD_{red} and the dynamic properties of the protein environment, we conclude that the excited state of neutral FAD_{red} can be intrinsically short-lived even in proteins, contrasting with the well-documented behavior of the anionic form that systematically displays markedly increased excited-state lifetime upon binding to proteins. This work provides new insight into the photochemistry of fully reduced flavins, which are emerging as functional initial states in bioengineered photocatalysts.

TOC GRAPHICS



Flavins are redox-active cofactors found in a large variety of enzymes and photoreceptors. They are accommodated in proteins essentially as flavin adenine dinucleotide (FAD) or flavin mononucleotide (FMN), and can exist in at least five physiologically relevant redox and protonation states.¹ In the majority of flavoenzymes, the oxidized quinone form is the resting state. For enzymes participating in two-electron redox processes, the (two-electron) fully reduced hydroquinone form can also be viewed as a quasi-stable active state that reacts with various

substrates (usually external electron acceptors) to complete the catalytic cycle.²⁻⁴ In recent years, inspired by the catalytic cycle of the photoenzyme, DNA photolyase,⁴ employing fully reduced flavins as the functional state has become a promising strategy in the development of bioengineered flavin-dependent photocatalysts.⁵⁻⁷ Therefore, fundamental knowledge of the properties of protein-bound fully reduced flavins is required both for understanding the catalytic mechanism of many natural flavoenzymes, and for enabling the rational design of flavoprotein variants as photocatalysts. However, time-resolved spectroscopic studies of fully reduced flavins in proteins⁸⁻¹⁴ are scarce compared to those in the oxidized state.¹⁵

Fully reduced flavins naturally occur either in the anionic (FADH^- or FMNH^-) or protonated, neutral (FADH_2 or FMNH_2) form.¹ It is commonly assumed that fully reduced (both neutral and anionic) flavins are virtually nonfluorescent in aqueous solution, but they can have long-lived, and therefore highly fluorescent, excited states in a protein environment or at low temperatures.^{8,9,11} This feature has been interpreted as a result of excited-state deactivation via a conical intersection (CI) with the ground state that can be reached through butterfly bending motions of the isoalloxazine ring system.^{10,12} In confined environments or at low temperatures such motions are hindered, leading to long-lived (up to nanoseconds) excited states. Yet, time-resolved spectral characterization of protein-bound fully reduced flavins has thus far been limited to the anionic forms (or to conditions with the anionic forms as the major fraction). Such studies included those concerning lactate oxidase, D- and L-amino acid oxidase, flavodoxin,⁹ DNA photolyase,¹⁰ glucose oxidase (GOX), the flavodehydrogenase component from flavocytochrome b_2 ¹¹ and thymidylate synthase ThyX.¹² To the best of our knowledge, the excited-state properties of the protonated neutral form remain unexplored in a protein environment.

In this work, we apply time-resolved fluorescence and absorption spectroscopy to investigate the excited-state dynamics and spectral properties of the fully reduced FAD cofactor (FAD_{red}) in three homologous ferredoxin-NADP⁺ oxidoreductases (FNRs): FNR from *Bacillus subtilis* (*BsFNR*), *Chlorobaculum tepidum* (*CtFNR*), and *Rhodospseudomonas palustris* (*RpFNR*). FNR is flavoenzyme that catalyzes the two-electron transfer reaction between the iron-sulfur protein ferredoxin and NAD(P)⁺/H.^{16,17} *BsFNR*, *CtFNR* and *RpFNR* (for sequence information see Figure S1) belong to a subgroup of NADPH-thioredoxin reductase (TrxR)-type FNRs found in green sulfur bacteria, firmicutes and several alpha-proteobacteria.¹⁸ This group is distinct from other types of FNR because it forms a homodimer in solution as a functional catalytic unit, and conserves structural topology with bacterial TrxR.^{19–22} Crystal structures of these enzymes are available, but only for the oxidized form FAD_{ox} (Figure 1A–C) and they reveal a very similar flavin environment. Histidine (His324), phenylalanine (Phe337), and tyrosine (Tyr328) stack on the *re*-face of the isoalloxazine ring moiety of the flavins in *BsFNR*, *CtFNR*, and *RpFNR*, respectively.²² Threonine residues are located close to the N5 atom of the flavins in the active site of *BsFNR* and *RpFNR* (Thr326 and Thr330, respectively), while a serine residue is located at a similar position in *CtFNR* (Ser339). These residues form hydrogen bonds with the N5 atom of the oxidized flavins, which may help maintain the position of the C-terminal extension region in contact with the FAD-binding domain.²² Previous studies have demonstrated that, whereas in the oxidized state *BsFNR*, *CtFNR* and *RpFNR* exhibit similar kinetic behavior in the redox reactions with NADPH, the reactions of the fully reduced enzymes with NADP⁺ are diverse in terms of charge-transfer complex formation and hydride transfer rates between FAD_{red} and NADP⁺.^{17,18,22} Altogether, despite the structural similarities of the three oxidized FNRs, the cofactors presumably differ in their electronic properties in the reduced state.

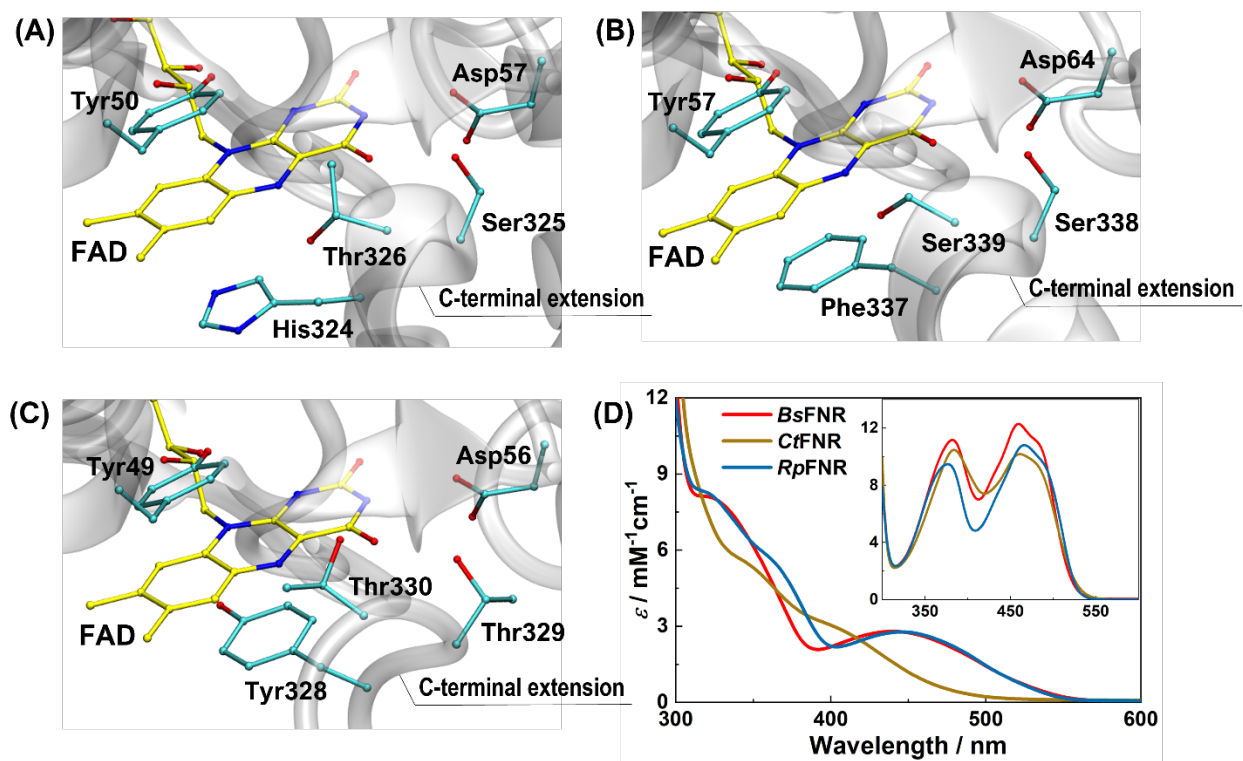


Figure 1. Active sites of *BsFNR* (A, PDB entry: 3LZW), *CtFNR* (B, PDB entry: 3AB1) and *RpFNR* (C, PDB entry: 5YGQ) in the oxidized state. (D) Steady-state absorption spectra of FAD_{red} and FAD_{ox} (inset) in *BsFNR*, *CtFNR* and *RpFNR* at pH 7. The reduced samples were prepared by chemical reduction using dithionite.

As shown in Figure 1D, at pH 7, the steady-state absorption spectra of FAD_{red} in *BsFNR* and *RpFNR* are both characterized by an absorption band in the visible peaking near 450 nm, whereas that of *CtFNR* lacks such a peak and displays a shoulder around 400 nm. Comparison with the spectra of fully reduced flavins obtained in aqueous solution and other flavoproteins systems (Figure S2) may suggest that FAD_{red} in *CtFNR* is predominantly in the anionic form while in *BsFNR* and *RpFNR* it is predominantly in the neutral form. However, the strong variation in the reference spectra precludes a definitive assignment based on these spectra alone (Section SI2). Therefore, we investigated the effects of pH on the absorption spectra of FAD_{red} in these FNRs

(Figure S3). In the pH range where the proteins remain stable (pH 6–10 for *Bs*FNR and *Ct*FNR, and 6–9 for *Rp*FNR), the spectra were found to change with pH, indicating conversion between different protonation states. The pK_a associated with the spectral changes of FAD_{red} can be roughly estimated (Section SI3) as close to 6 in *Ct*FNR, between 7 and 8 in *Bs*FNR, and higher than 8 in *Rp*FNR. Therefore, it can be inferred that at pH 7, FAD_{red} in *Ct*FNR exists predominately as $FADH^-$ and that in *Rp*FNR as $FADH_2$, while in *Bs*FNR it is a mixture of $FADH_2$ and $FADH^-$, with the former being preponderant. Compared with free FAD_{red} in aqueous solution ($pK_a = \sim 6.7$),²³ the pK_a of FAD_{red} in *Bs*FNR and *Rp*FNR are clearly upshifted. From the active-site structures of the three FNRs (Figure 1A–C), it can be inferred that the C-terminal residues contribute to the pK_a shift; in particular, whether it is a hydrophobic phenylalanine, or a hydrophilic histidine/tyrosine stacking on the *re*-face of the flavins may have a direct effect on the hydrogen-bonding networks in the protein active sites.

In many flavoproteins, determination of the protonation states of fully reduced flavins can be challenging as they are usually not titratable in the pH ranges where the proteins are stable.⁸ Therefore, with the protonation states unambiguously identified, here the three FNRs can be considered as suitable systems to investigate the excited-state properties of protein-bound FAD_{red} , in particular, those of the essentially unexplored neutral form $FADH_2$.

We performed time-resolved fluorescence measurements on FAD_{red} in the three FNRs at pH 7 (Figures S9 and Figure 2, 3). For comparison, experiments were performed under the same experimental conditions on FAD_{red} in aqueous solution and in GOX, which have also been previously studied by others.^{10–12,24}

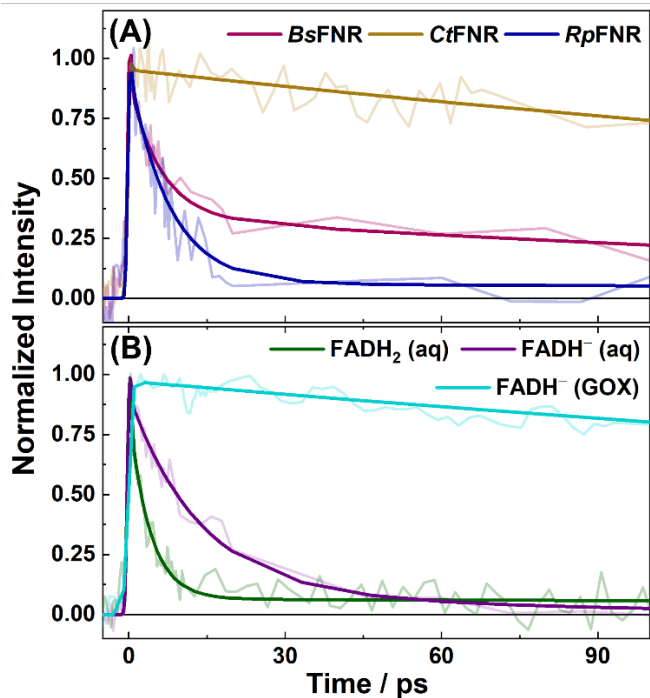


Figure 2. Fluorescence decays of FAD_{red} in *BsFNR*, *CtFNR* and *RpFNR* at pH 7 monitored at 587, 532 and 589 nm (A), as well as FADH⁻ and FADH₂ in aqueous solution (at pH 10 and 4), and FADH⁻ in GOX (at pH 7), monitored at 569, 593 and 530 nm, respectively (B). The intensities are normalized, and exponential fits are shown as smooth curves.

In aqueous solution, based on global analysis (Figure 3F), we determined that the fluorescence of free FADH⁻ mainly decays in 20 ps, with a 2-ps fast phase, which has been assigned to an excited-state relaxation process (Figure 2B).^{10,12} The fluorescence of free FADH₂ predominantly decays in 4 ps, and no additional relaxation phase is required to fit the data (Figures 2B, 3E). These kinetics are consistent with previous observations.^{10,12,25} The obtained decay associated spectra (DAS), which are reported here for the first time, demonstrate that in solution, excited-state FADH⁻ emits with a maximum at ~575 nm (Figure 3F), in contrast to FADH₂ where an emission maximum positioned at a much longer wavelength (~620 nm) was observed (Figure 3E).

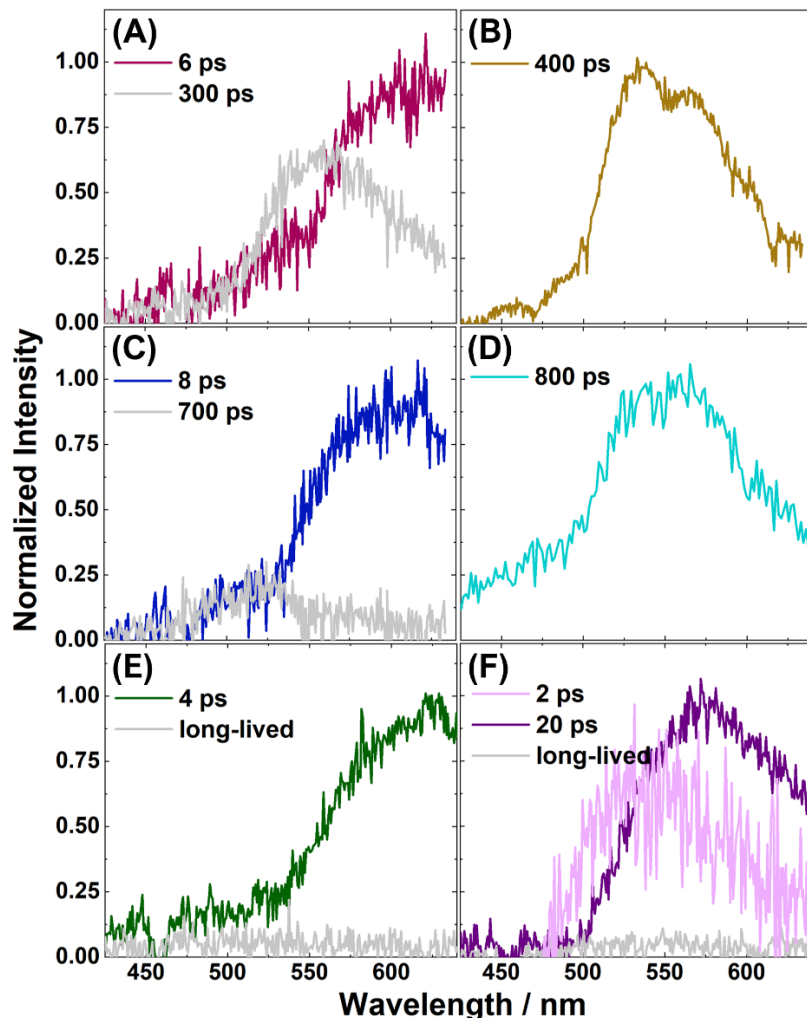


Figure 3. DAS from the global analysis of the time-resolved fluorescence spectra of FAD_{red} (see Figure S9) in *BsFNR* (A), *CtFNR* (B) and *RpFNR* (C) at pH 7, and FADH^- in GOX (D),²⁶ as well as FADH_2 (E) and FADH^- (F) in aqueous solution at pH 4 and pH 10, respectively. Intensities are normalized on the maxima of the dominant phases.

The fluorescence of FAD_{red} in *CtFNR* can be reasonably fitted with a monophasic decay with a time constant of 400 ps (Figure 2A), and the corresponding DAS has an emission maximum at ~ 550 nm, which is very similar to the DAS of the FADH^- fluorescence in GOX that decays in

~800 ps (Figure 3B, D).²⁶ These findings are in full agreement with the above assessment that at pH 7, the FAD_{red} cofactor in *CtFNR* exists as single species, i.e., FADH⁻.

On the other hand, the fluorescence of FAD_{red} in *BsFNR* and *RpFNR* is dominated by much faster decay phases (Figure 2A). Both can be reasonably fitted with two components. For *BsFNR*, the time constants are determined as 6 and 300 ps, and those for *RpFNR* are 8 and 700 ps. The DAS of the 6-ps phase of *BsFNR* and the 8-ps phase of *RpFNR* display emission maxima at ~620 nm (Figure 3A, C) as for free FADH₂ (Figure 3E). The 700-ps phase for *RpFNR* is very small, and the maximum of the corresponding DAS at ~525 nm is close to what would be expected for oxidized flavins.²⁷ Therefore, we attribute this long-lived phase to a trace amount of FAD_{ox} (either in non-bound or protein-bound form) and the 8-ps phase to *RpFNR*-bound FADH₂, which is the dominant form of FAD_{red} in *RpFNR* at pH 7, as revealed by the steady-state spectral titrations (*vide supra*).

The 400-ps phase of *BsFNR*, however, has a more substantial relative amplitude, and its DAS exhibits an emission maximum at ~550 nm, similar to those of FADH⁻ in *CtFNR* and GOX (Figures 3A, B, D). The lifetime of 300 ps of this phase is also close to that of 400 ps in *CtFNR*. This result corroborates the assessment that at pH 7, FAD_{red} in *BsFNR* comprises two protonation states; the FADH₂ majority contributes to the 6-ps phase in the fluorescence decay while the FADH⁻ fraction accounts for the remaining 300-ps phase.

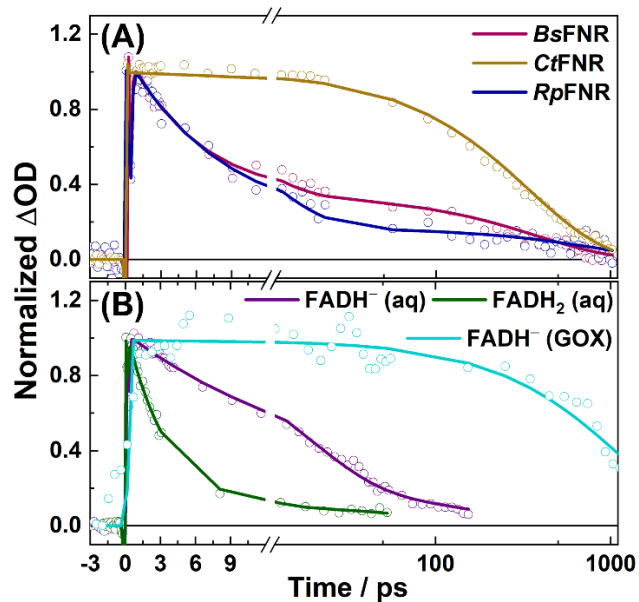


Figure 4. Kinetic traces at 520 nm from the transient absorption measurements of FAD_{red} in *BsFNR*, *CtFNR* and *RpFNR* at pH 7 (A), as well as FADH_2 and FADH^- in aqueous solution at pH 4 and pH 10, respectively, and FADH^- in GOX (B). Intensities are normalized on the maxima. The time axes are in linear-logarithmic scale for clarity.

As illustrated by *CtFNR* and GOX, the excited state of protein-bound FADH^- generally decays in hundreds of picoseconds to nanoseconds, more than an order of magnitude slower than that of free FADH^- in solution (~ 20 ps).^{9–12} However, as we here measured by time-resolved fluorescence, the excited state of FADH_2 in *BsFNR* and *RpFNR* display much shorter lifetimes (6 and 8 ps, respectively), which is close to the 4-ps lifetime of excited free FADH_2 (Figures 2, 3). To investigate whether the excited states of FADH_2 in *BsFNR* and *RpFNR* decay directly back to the ground state as in other systems, and not via other radiationless pathways such as electron transfer, we performed transient absorption measurements. The resulting kinetic traces at 520 nm from the measurements are shown in Figure 4, transient spectra at selected decay times in Figure S10, and the DAS from the global analysis of the data are shown in Figure 5.

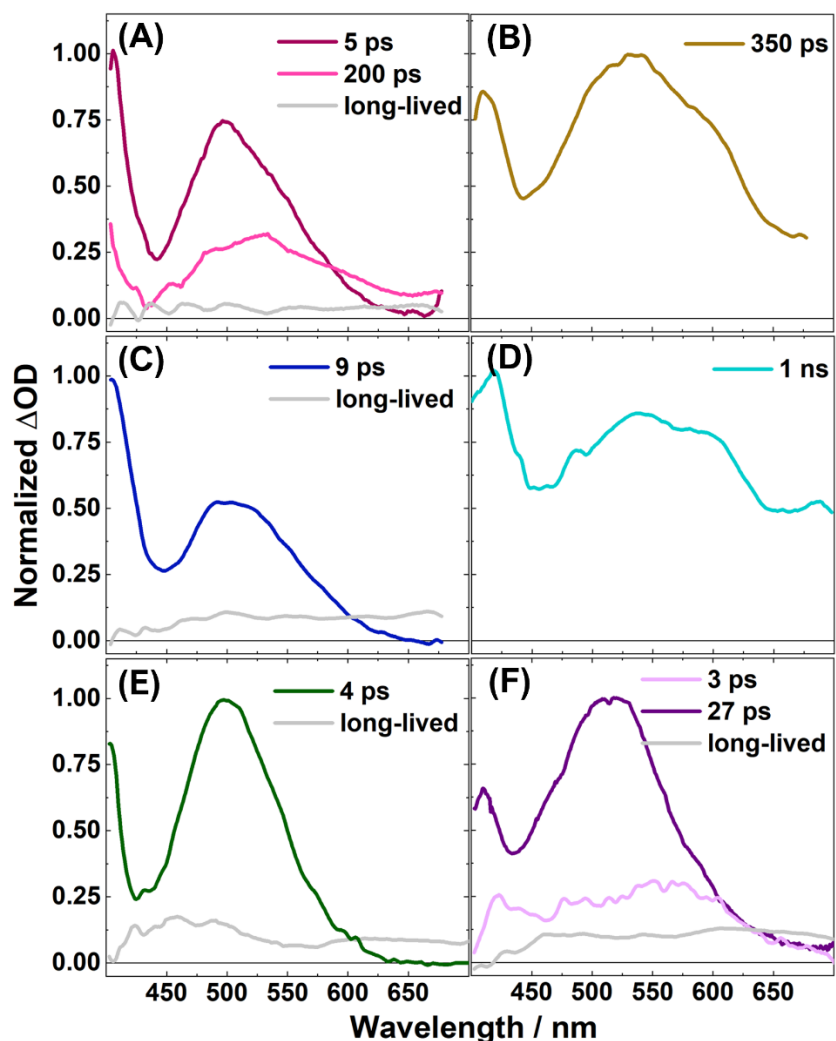


Figure 5. DAS from the global analysis of the transient absorption spectra of FAD_{red} (see Figure S10) in *BsFNR* (A), *CtFNR* (B) and *RpFNR* (C) at pH 7, and $FADH^-$ in GOX (D),²⁶ as well as $FADH_2$ (E) and $FADH^-$ (F) in aqueous solution at pH 4 and pH 10, respectively. Intensities are normalized on the maxima of the dominant phases.

For all the systems, including those of *BsFNR* and *RpFNR*, the time constants of the dominant components correspond reasonably well with those found in time-resolved fluorescence (Figure 3 and Section SI6). This indicates that the excited states deactivate back to the ground states without population of longer-lived intermediate states. Altogether, the observed transient

spectra comprise contributions from the ground-state bleaching (GSB, ~400–550 nm) superimposed on broad excited-state absorption (ESA). Stimulated emission (SE, with maxima at ~550 nm for FADH⁻ and at ~620 nm for FADH₂, cf. Figure 3) presumably also contributes to the spectra as negative transient absorption on the red side of the spectra. (Here the negative features of GSB and SE are not obvious as they overlap with the strong ESA; cf. Refs. 11, 12.)

Our transient absorption spectra (Figure 5E, F) of FADH₂ and FADH⁻ in aqueous solution agree with previous reports.^{11,24,25} The broad and featureless spectra of the minor long-lived phases have been previously assigned to FADH[•]/hydrated electron and FADH₂^{•+}/hydrated electron pairs as a result of biphotonic ionization of FAD_{red}.^{24,25} The DAS of FADH⁻ in *Ct*FNR displays two major bands centered at ~530 and ~415 nm, similar to those resulting from excited FADH⁻ in GOX. Both are broader compared with that of FADH⁻ in aqueous solution and exhibit a shoulder at ~600 nm. The two major bands in the DAS of the 5-ps phase of *Bs*FNR and the 9-ps phase of *Rp*FNR, which result from the FADH₂ species, are centered at ~500 and 410 nm. These maxima, as well as the overall spectral features, are almost identical to those of the DAS of the 4-ps phase of FADH₂ in solution. The DAS of the 200-ps phase of *Bs*FNR resembles the FADH⁻ transient spectrum as in *Ct*FNR, further confirming the coexistence of two protonation states of FAD_{red} in *Bs*FNR at pH 7.

These results, along with transient absorption measurements at different pH conditions (Section SI4), unambiguously demonstrate that, in these FNRs, FADH₂ intrinsically has a much shorter lifetime than FADH⁻. However, whereas excited free FADH₂ in solution decays ~7 times faster than free FADH⁻, in FNRs FADH₂ decays ~50 times faster than FADH⁻. This comparison suggests that other factors may also contribute to this extremely fast excited-state decay of FADH₂ in the proteins.

In 50% (v/v) glycerol/water mixture, the fluorescence of free FADH₂ and FADH⁻ decays in 6 and 31 ps, ~1.5 times slower than the decays in the absence of glycerol (4 and 20 ps, respectively, Section SI5). This indicates that an increase in solvent viscosity has a similar effect on the excited-state dynamics of FADH⁻ and FADH₂, imposing a denser environment which hinders the radiationless excited-state decay via a CI, as discussed previously for other flavin species in solution.^{8,10,28} Yet, when bound to *Ct*FNR, the excited-state decay of FADH⁻ is slower by a factor of ~20 compared with that of free FADH⁻ (400 ps *versus* 20 ps), whereas the lifetimes of excited FADH₂ in *Bs*FNR and *Rp*FNR were found to be close to that of free FADH₂ (6 and 8 ps *versus* 4 ps). In the framework of the assignment of FAD_{red} excited-state deactivation through a CI attained via butterfly bending motions, this comparison suggests that the active site of fully reduced *Ct*FNR is more rigid than those of *Bs*FNR and *Rp*FNR at pH 7, as a result of changes in intrinsic protein conformation and flexibility accompanying the FAD_{red} protonation/deprotonation.

Active-site structural dynamics probed by molecular dynamics (MD) simulations (Section SI7) strongly support this suggestion. As illustrated in Figure 6C, in the MD simulations of *Ct*FNR, the C-terminal extension of *Ct*FNR maintains close interactions with the FADH⁻ cofactor. On the other hand, in *Rp*FNR, the C-terminal extension changes its conformation and moves away from the FADH₂ cofactors, opening up the active site and exposing the cofactors more to the solvent (Figure 6B). The simulations of *Bs*FNR show two main active-site configurations (Figure 6A; note that the C-terminal extension in *Bs*FNR is much shorter than those in *Ct*FNR and *Rp*FNR); one in which the C-terminal extension stays close to the cofactor, similar to that in *Ct*FNR; and another in which the C-terminal extension changes its orientation and opens up the active site, similar to the conformation observed in *Rp*FNR. These observations are likely attributable to the difference in charge distributions of the flavin ring in the anionic and neutral forms and long-range

electrostatic effects, as the additional proton in FADH₂ (at the C1 atom of the flavin ring) does not directly interact with the C-terminal residues.

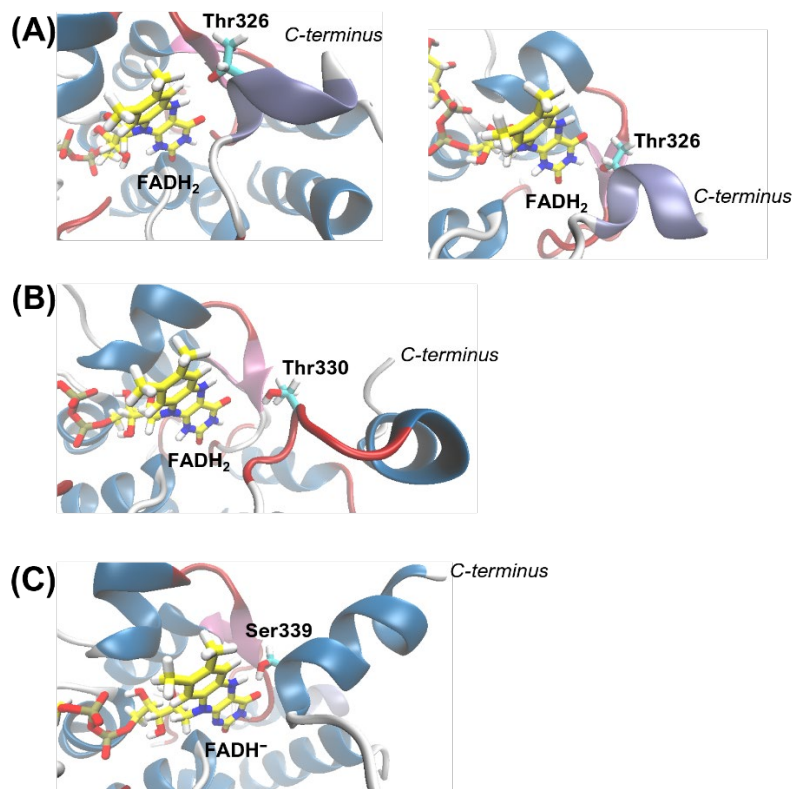


Figure 6. Selected snapshots from the MD simulations of fully reduced *BsFNR* (A), *RpFNR* (B) and *CtFNR* (C).

In addition to the intrinsically short-lived nature of excited FADH₂, we conclude that the extremely fast excited-state decays of FADH₂ in the FNRs (with lifetimes comparable to that of free FADH₂ in solution) may also in part be due to a lack of constraints from the C-terminal extension and a largely solvent-exposed active site. For FADH⁻, as illustrated in the simulations of *CtFNR*, the closer interactions between the C-terminal extension and FADH⁻ provide a rigid environment that hinders the radiationless decay pathway of the excited state. In this regard, we note that the transient absorption results indicate that biphotonic ionization reactions occur for

FADH₂ in *Bs*FNR and *Rp*FNR, but not for FADH⁻ in *Ct*FNR (*vide supra*), leading to the formation of hydrated electrons. Thus, in the fully reduced state, the active sites of *Rp*FNR and *Bs*FNR should contain enough water molecules to allow the detached electron to be solvated, which is not the case for *Ct*FNR.

It has been reported that in spinach FNR, FADH₂ is bound much less tightly to the protein than FAD_{ox}, with a dissociation constant four order of magnitude larger than that of FAD_{ox} (50 μM and 3.4 nM, respectively).²⁹ One may speculate that in general, an open, solvent-exposed (therefore, less constrained) active site is required for the formation of FADH₂ in a flavoprotein; as a result, the excited state of protein-bound FADH₂ is likely to be intrinsically short-lived, similar to that of free FADH₂ in solution. To verify this hypothesis, further studies on other systems that can stabilize FADH₂ (e.g. spinach FNR²⁹ and TrxR^{30,31}) are warranted. We note that, consistent with our suggestion, for FADH₂ in TrxR, a lack of (steady-state) fluorescence has been previously reported.⁸

In summary, we elucidate the excited-state properties of protein-bound FAD_{red} in three well-defined flavoprotein systems. In particular, we present for the first time a full dynamics and spectral investigation of the photophysical properties of protein-bound FADH₂. We demonstrate that excited FADH₂ in *Bs*FNR and *Rp*FNR exhibit very fast decays (6–8 ps), which contrast to the general notion that when bound to proteins, fully reduced flavins have long-lived excited states as is the case in *Ct*FNR (400 ps). We rationalize these observations with the intrinsically short-lived nature of excited FADH₂, possibly combined with the more solvent-exposed, less constrained nature of the active sites harboring an FADH₂ cofactor. Our findings significantly deepen our understanding of the photochemistry of fully reduced flavins, and exemplify how their properties can differ in structurally homologous flavoproteins that yet display significantly different

conformational properties in the fully reduced states under physiological conditions. Our results also imply that protonation state and flexibility should be taken into account in the design of bioinspired photocatalysts based on fully reduced flavoprotein complexes.

ASSOCIATED CONTENT

The Supporting Information is available free of charge on the ACS Publications website.

Experimental and computational methods; steady-state spectral properties of fully reduced flavins; pH dependence of steady-state spectra of fully reduced FNRs; transient absorption measurements at different pH conditions; effects of viscosity on the excited-state decays of FAD_{red}; summary of time-resolved spectroscopic measurements; structural dynamics probed by MD simulations (PDF)

AUTHOR INFORMATION

Notes

The authors declare no competing financial interests.

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REFERENCES

- (1) Miura, R. Versatility and Specificity in Flavoenzymes: Control Mechanisms of Flavin Reactivity. *Chem. Rec.* **2001**, *1*, 183–194.
- (2) Carrillo, N.; Ceccarelli, E. A. Open Questions in Ferredoxin-NADP⁺ Reductase Catalytic Mechanism. *Eur. J. Biochem.* **2003**, *270*, 1900–1915.

- (3) Dijkman, W. P.; De Gonzalo, G.; Mattevi, A.; Fraaije, M. W. Flavoprotein Oxidases: Classification and Applications. *Applied Microbiology and Biotechnology*. Springer June 3, 2013, pp 5177–5188.
- (4) Brettel, K.; Byrdin, M. Reaction Mechanisms of DNA Photolyase. *Curr. Opin. Struct. Biol.* **2010**, *20*, 693–701.
- (5) Biegasiewicz, K. F.; Cooper, S. J.; Gao, X.; Oblinsky, D. G.; Kim, J. H.; Garfinkle, S. E.; Joyce, L. A.; Sandoval, B. A.; Scholes, G. D.; Hyster, T. K. Photoexcitation of Flavoenzymes Enables a Stereoselective Radical Cyclization. *Science* **2019**, *364*, 1166–1169.
- (6) Huang, X.; Wang, B.; Wang, Y.; Jiang, G.; Feng, J.; Zhao, H. Photoenzymatic Enantioselective Intermolecular Radical Hydroalkylation. *Nature* **2020**, *584*, 69–74.
- (7) Sandoval, B. A.; Clayman, P. D.; Oblinsky, D. G.; Oh, S.; Nakano, Y.; Bird, M.; Scholes, G. D.; Hyster, T. K. Photoenzymatic Reductions Enabled by Direct Excitation of Flavin-Dependent “Ene”-Reductases. *J. Am. Chem. Soc.* **2021**, *143*, 1735–1739.
- (8) Ghisla, S.; Massey, V.; Lhoste, J.-M.; Mayhew, S. G. Fluorescence and Optical Characteristics of Reduced Flavines and Flavoproteins. *Biochemistry* **1974**, *13*, 589–597.
- (9) Visser, A. J. W. G.; Ghisla, S.; Massey, V.; Müller, F.; Veeger, C. Fluorescence Properties of Reduced Flavins and Flavoproteins. *Eur. J. Biochem.* **1979**, *101*, 13–21.
- (10) Kao, Y. T.; Saxena, C.; He, T. F.; Guo, L.; Wang, L.; Sancar, A.; Zhong, D. Ultrafast Dynamics of Flavins in Five Redox States. *J. Am. Chem. Soc.* **2008**, *130*, 13132–13139.
- (11) Enescu, M.; Lindqvist, L.; Soep, B. Excited-State Dynamics of Fully Reduced Flavins and Flavoenzymes Studied at Subpicosecond Time Resolution. *Photochem. Photobiol.* **1998**,

- 68, 150–156.
- (12) Dozova, N.; Lacomat, F.; Lombard, M.; Hamdane, D.; Plaza, P. Ultrafast Dynamics of Fully Reduced Flavin in Catalytic Structures of Thymidylate Synthase ThyX. *Phys. Chem. Chem. Phys.* **2021**, *23*, 22692–22702.
- (13) Zhao, R. K.; Lukacs, A.; Haigney, A.; Brust, R.; Greetham, G. M.; Towrie, M.; Tonge, P. J.; Meech, S. R. Ultrafast Transient Mid IR to Visible Spectroscopy of Fully Reduced Flavins. *Phys. Chem. Chem. Phys.* **2011**, *13*, 17642–17648.
- (14) Pauszek, R. F.; Kodali, G.; Siddiqui, M. S. U.; Stanley, R. J. Overlapping Electronic States with Nearly Parallel Transition Dipole Moments in Reduced Anionic Flavin Can Distort Photobiological Dynamics. *J. Am. Chem. Soc.* **2016**, *138*, 14880–14889.
- (15) Zhuang, B.; Liebl, U.; Vos, M. H. Flavoprotein Photochemistry: Fundamental Processes and Photocatalytic Perspectives. *J. Phys. Chem. B* **2022**, *126*, 3199–3207.
- (16) Medina, M.; Gómez-Moreno, C. Interaction of Ferredoxin-NADP⁺ Reductase with Its Substrates: Optimal Interaction for Efficient Electron Transfer. *Photosynthesis Research*. Springer 2004, pp 113–131.
- (17) Seo, D.; Soeta, T.; Sakurai, H.; Sétif, P.; Sakurai, T. Pre-Steady-State Kinetic Studies of Redox Reactions Catalysed by *Bacillus Subtilis* Ferredoxin-NADP⁺ Oxidoreductase with NADP⁺/NADPH and Ferredoxin. *Biochim. Biophys. Acta - Bioenerg.* **2016**, *1857*, 678–687.
- (18) Seo, D.; Kitashima, M.; Sakurai, T.; Inoue, K. Kinetics of NADP⁺/NADPH Reduction–Oxidation Catalyzed by the Ferredoxin-NAD(P)⁺ Reductase from the Green Sulfur Bacterium *Chlorobaculum Tepidum*. *Photosynth. Res.* **2016**, *130*, 479–489.

- (19) Waksman, G.; Krishna, T. S. R.; Williams, C. H.; Kuriyan, J. Crystal Structure of Escherichia Coli Thioredoxin Reductase Refined at 2 Å Resolution: Implication for a Large Conformational Change during Catalysis. *J. Mol. Biol.* **1994**, *236*, 800–816.
- (20) Muraki, N.; Seo, D.; Shiba, T.; Sakurai, T.; Kurisu, G. Asymmetric Dimeric Structure of Ferredoxin-NAD(P)⁺ Oxidoreductase from the Green Sulfur Bacterium Chlorobaculum Tepidum: Implications for Binding Ferredoxin and NADP⁺. *J. Mol. Biol.* **2010**, *401*, 403–414.
- (21) Komori, H.; Seo, D.; Sakurai, T.; Higuchi, Y. Crystal Structure Analysis of Bacillus Subtilis Ferredoxin-NADP⁺ Oxidoreductase and the Structural Basis for Its Substrate Selectivity. *Protein Sci.* **2010**, *19*, 2279–2290.
- (22) Seo, D.; Muraki, N.; Kurisu, G. Kinetic and Structural Insight into a Role of the Re-Face Tyr328 Residue of the Homodimer Type Ferredoxin-NADP⁺ Oxidoreductase from Rhodospseudomonas Palustris in the Reaction with NADP⁺/NADPH. *Biochim. Biophys. Acta - Bioenerg.* **2020**, *1861*, 148140.
- (23) Lowe, H. J.; Clark, W. M. Studies on Oxidation-Reduction. XXIV. Oxidation-Reduction Potentials of Flavin Adenine Dinucleotide. *J. Biol. Chem.* **1956**, *221*, 983–992.
- (24) Brazard, J.; Usman, A.; Lacomat, F.; Ley, C.; Martin, M. M.; Plaza, P. New Insights into the Ultrafast Photophysics of Oxidized and Reduced FAD in Solution. *J. Phys. Chem. A* **2011**, *115*, 3251–3262.
- (25) Brazard, J. Photoactivation Femtoseconde de Deux Nouvelles Protéines de La Famille Des Cryptochrome/Photolyase, Issues de l'algue Verte Ostreococcus Tauri. Photocycle Ultrarapide et État Signalant de La Photophobie Du Protozoaire Blepharisma Japonicum,

PhD Thesis, Université Pierre-et-Marie-Curie, Paris, France, 2009.

- (26) Zhuang, B.; Ramodiharilafy, R.; Liebl, U.; Aleksandrov, A.; Vos, M. H. Ultrafast Photooxidation of Protein-Bound Anionic Flavin Radicals. *Proc. Natl. Acad. Sci.* **2022**, *119*, e2118924119.
- (27) Zhuang, B.; Seo, D.; Aleksandrov, A.; Vos, M. Characterization of Light-Induced, Short-Lived Interacting Radicals in the Active Site of Flavoprotein Ferredoxin-NADP⁺ Oxidoreductase. *J. Am. Chem. Soc.* **2021**, *143*, 2757–2768.
- (28) Domcke, W.; Yarkony, D.; Köppel, H. *Conical Intersections: Electronic Structure, Dynamics and Spectroscopy*; World Scientific: Singapore, 2004.
- (29) Corrado, M. E.; Aliverti, A.; Zanetti, G.; Mayhew, S. G. Analysis of the Oxidation-Reduction Potentials of Recombinant Ferredoxin-NADP⁺ Reductase from Spinach Chloroplasts. *Eur. J. Biochem.* **1996**, *239*, 662–667.
- (30) Zanetti, G.; Williams, C. H. Characterization of the Active Center of Thioredoxin Reductase. *J. Biol. Chem.* **1967**, *242*, 5232–5236.
- (31) Prongay, A. J.; Williams, C. H. Oxidation-Reduction Properties of Escherichia Coli Thioredoxin Reductase Altered at Each Active Site Cysteine Residue. *J. Biol. Chem.* **1992**, *267*, 25181–25188.