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# Flavoprotein photochemistry: fundamental processes and photocatalytic perspectives

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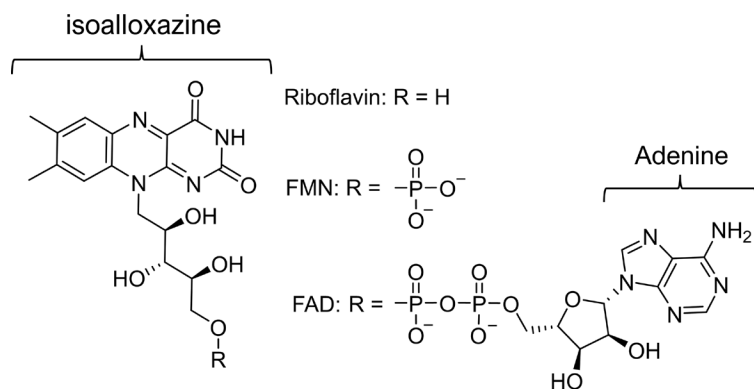
## ABSTRACT

Flavins are highly versatile redox-active and colored cofactors in a large variety of proteins. These do include photoenzymes and photoreceptors, although the vast majority performs non-light-driven physiological functions. Nevertheless electron transfer between flavins and specific nearby amino acid residues (in particular tyrosine, tryptophan, and presumably histidine and arginine) takes place upon excitation of flavin in many flavoproteins. For oxidized flavoproteins these reactions potentially have a photoprotective role. In this Perspective, we outline work on the characterization of early reaction intermediates not only in the relatively well-studied resting oxidized forms, but also in the fully reduced and the intrinsically unstable semi-reduced forms, where ultrafast photooxidation of flavin was recently demonstrated. Along different lines, flavoprotein-based novel photocatalysts for biotechnological applications are presently emerging, employing both substrate photooxidation and photoreduction strategies. Deep insight into the fundamental flavin photochemical reactions may help in guiding and optimizing their development and in the exploration of novel photocatalytic approaches.

## Introduction

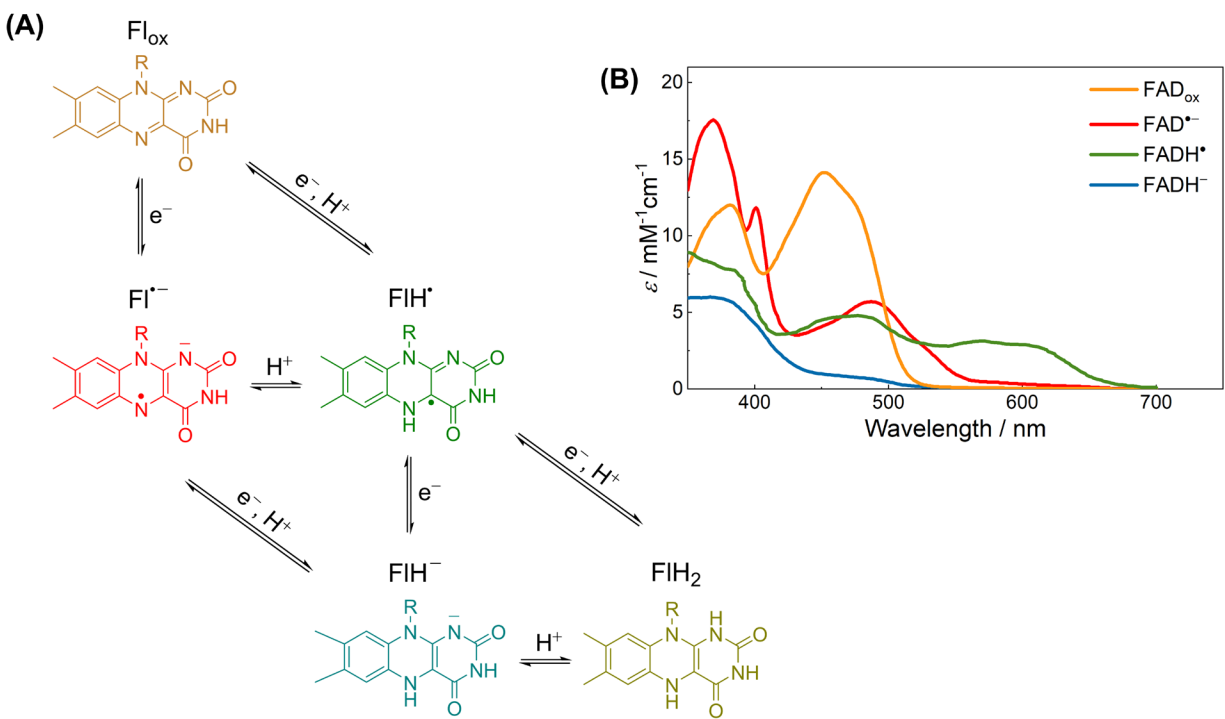
Colored biomolecules are ubiquitous in natural systems. Some of them, such as chlorophyll and retinal, are exclusively employed in photobiological processes. Others, such as heme-proteins and many metal center-containing proteins, do perform photochemistry, although not for known photobiological functions, and their spectroscopic properties can be used to follow biochemical reactions. Flavins form a highly versatile group of chromophores found in a large variety of enzymes and photoreceptors. In most of these systems they do not perform photobiological functions, but in some systems they do. The versatility of these systems makes them interesting starting points for the bioengineering of novel photocatalysts. In this Perspective, we will outline recent progress in the understanding of fundamental photochemical reactions in flavoproteins, and discuss how this insight may allow the exploration of novel photocatalytic approaches.

### Scheme 1. Molecular structure of flavins



Flavins (Fl) are derivatives of vitamin B<sub>2</sub> (riboflavin, Rfl) and are accommodated in proteins essentially in the form of flavin adenine dinucleotide (FAD) or flavin mononucleotide (FMN); see Scheme 1. The isoalloxazine ring system is at the heart of the reactivity of flavins and

of their spectroscopic properties. Flavins can adopt three different redox forms with various protonation states, leading to at least five physiologically relevant states: the oxidized form  $\text{Fl}_{\text{ox}}$ , the semi-reduced forms  $\text{Fl}^{\bullet-}$  and  $\text{FlH}^{\bullet}$  and the fully reduced forms  $\text{FlH}^-$  and  $\text{FlH}_2$  (Fig. 1A). Interacting with a manifold of substrates, as well as with other proteins, these properties allow flavoproteins to partake in a large variety of biochemical electron (one- and two-electron) and proton transfer reactions, including those that couple one- and two-electron reactions and bifurcation reactions<sup>1-2</sup>. The flavin color depends on the redox state (for the neutral states  $\text{Fl}_{\text{ox}}$ ,  $\text{FlH}^{\bullet}$  and  $\text{FlH}_2$ , respectively yellow, blue and virtually transparent in the visible). Hence the flavin absorption spectrum (with moderate extinction coefficients of up to  $\sim 13,000 \text{ M}^{-1}\text{cm}^{-1}$  in the visible range, see Fig. 1B) is widely used to monitor its redox states during biochemical reactions, for instance in rapid mixing experiments with external reactants.



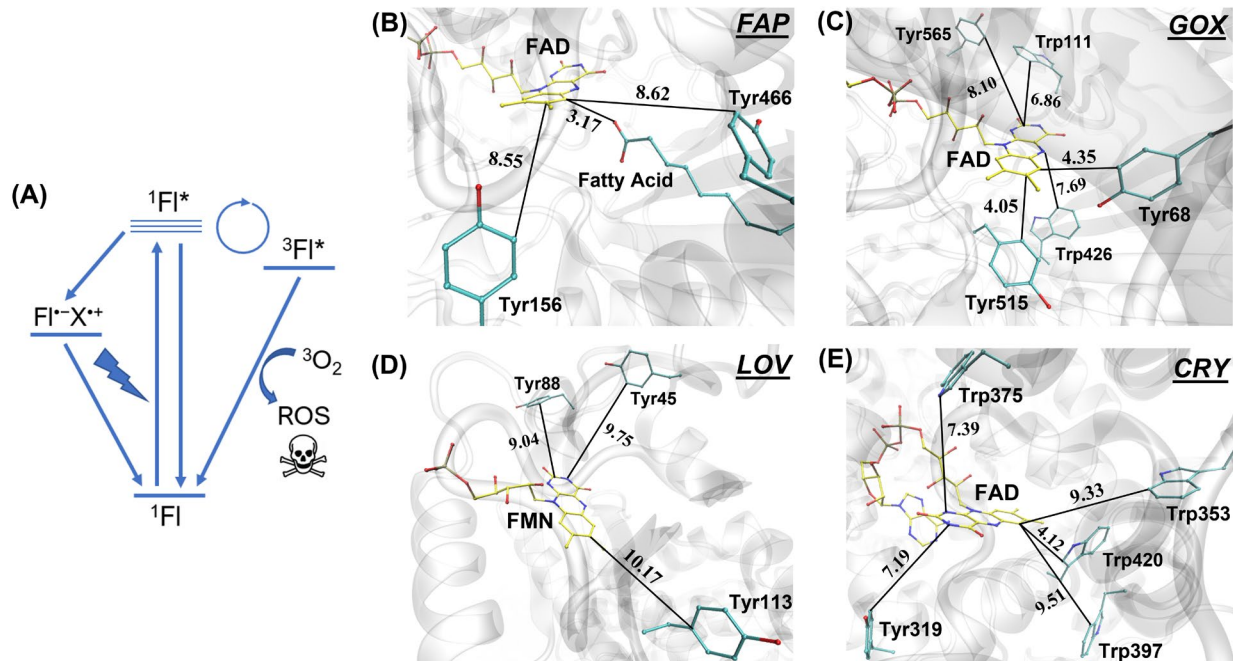
**Fig. 1 A.** Five redox and protonation states of flavins involved in the functional processes of flavoproteins. Flavin is represented by the isoalloxazine moiety. **B.** Steady-state absorption spectra of flavins in different redox and protonation states in glucose oxidase from *Aspergillus niger*. The spectrum of FADH<sup>•</sup> is reproduced from ref. <sup>3</sup>, and normalized with respect to the spectrum of FAD<sub>ox</sub>. Adapted with permission from Ref. <sup>4</sup>. Copyright 2022 the Authors.

### *Photochemistry of oxidized flavoproteins and photoprotection*

Flavin excited state formation by photon absorption can lead to fluorescence, and indeed fluorescence has been reported for most redox states, with the possible exception of the Fl<sup>-</sup> state<sup>5</sup>. The best-studied state is the Fl<sub>ox</sub> state, which is the resting state in the vast majority of flavoproteins. In solution, Rfl<sub>ox</sub> and FMN<sub>ox</sub> are highly fluorescent with lifetimes of the singlet excited state <sup>1</sup>Fl\* in the range of a few nanoseconds; for FAD<sub>ox</sub> there is a fraction ('open configuration') that is also highly fluorescent and a fraction in which the isoalloxazine ring is stacked with the adenine moiety ('closed configuration') leading to a lifetime of a few picoseconds<sup>6-8</sup> (see also Fig. 4). In proteins <sup>1</sup>Fl fluorescence is very often strongly quenched (although flavin fluorescence can still be observed spectroscopically and in microscopy of living materials in a useful<sup>9</sup> or perturbing<sup>10</sup> way). In many cases this quenching is due to a flavin photoreduction process involving close-lying aromatic amino acids: the radical states Fl<sup>-</sup>X<sup>•+</sup>, in which X is either tryptophan (TrpH) or tyrosine (TyrOH), lie energetically above the neutral ground state, but below the singlet excited state <sup>1</sup>Fl\*. The driving force for tryptophan and tyrosine photo-oxidation is estimated at ~1.2 eV<sup>11</sup> and 0.7-1.0 eV<sup>12-13</sup>, respectively. The electron transfer (ET) reaction towards the flavin thus competes with the intrinsic <sup>1</sup>Fl\* decay. The rate of such a reaction depends on the configuration of the donor-acceptor pair, which determines the electronic

coupling and the energetics involved<sup>14</sup>. Using a rough estimate based on distance and average protein density only<sup>15</sup>, direct electron donors located up to at most ~1 nm can efficiently compete, in the case of barrierless ET, with the nanosecond rates of intrinsic excited-state decay to the singlet or triplet ground state. In many cases tyrosines and/or tryptophanes are located much closer, up to near-van der Waals interaction with the flavin, and ET reactions with time constants down to the order of hundreds of femtoseconds regime have been documented<sup>16-24</sup>. The rates of such ET reactions are a sensitive probe of the interaction between the flavin cofactor and the amino acid electron donor. This feature can be exploited to study configurational heterogeneity and exchange rates between configurations in the active site of flexible flavoenzymes<sup>13, 25-26</sup>.

The presence of these intrinsic amino acid reactants incorporated in the protein thus prevents long-lived singlet flavin excited states (Fig. 1A). As long-lived (intrinsically microseconds) triplet states  $^3\text{Fl}$  can be formed from  $^1\text{Fl}^*$  by intersystem crossing on the timescale of ~10 ns<sup>27-28</sup>, and hence with quite sizeable quantum yields<sup>29</sup>, in this way the build-up of this state is also impeded. Ground state molecular oxygen ( $^3\text{O}_2$ ) can quench such states yielding  $^1\text{O}_2$ <sup>30</sup> and other reactive oxygen species (ROS)<sup>31</sup>. Therefore it has been put forward that the photoprotective effect of the presence of ET-quenchers of the  $^1\text{Fl}^*$  state yields a strong selection pressure in the evolution of flavoenzymes<sup>32</sup>.



**Fig. 2** **A.** Simplified scheme of photochemical reactions involving oxidized flavins. Excitation of the  $^1\text{Fl}$  ground state leads to population of the  $^1\text{Fl}^*$  state that can decay back to the ground state through fluorescence, to the triplet state  $^3\text{Fl}^*$  by intersystem crossing, or to a charge separated radical pair state  $\text{Fl}^-\text{X}^{++}$ , in which X is an amino acid electron donor (tryptophan or tyrosine). The triplet state can decay back to the singlet ground state via interaction with molecular oxygen ( $^3\text{O}_2$ ) yielding highly reactive singlet oxygen ( $^1\text{O}_2$ ) and other ROS. **B, C, D and E.** Closest tryptophan or tyrosine residues in the flavin environment in four characteristic flavoproteins: the photoenzyme Fatty Acid Photodecarboxylase from *Chlorella variabilis*, PDB entry: 6ZH7 (B) and the non-photoenzyme glucose oxidase from *Aspergillus niger*, PDB entry: 1CF3 (C), photoreceptors LOV domain protein from *Rhodobacter sphaeroides*, PDB entry: 4HJ3 (D) and cryptochrome from *Drosophila melanogaster*, PDB entry: 4JZY (E). In the latter, Trp 420 and Trp 397 are the first two constituents of the Trp triad ET chain<sup>33</sup> (see text). Distances are indicated in Å.

In most flavoproteins, photoinduced radical states rapidly decay by charge recombination, typically on the picosecond timescale, thus eventually thermally dissipating the photon energy. These closed reaction cycles thus do not lead to long-lived photochemical products, but rather help avoiding them. On the other hand, it is interesting to note that there is also a limited class of flavoproteins that support functional photobiochemical reactions. In such systems, competitive



quenching processes could be detrimental. An interesting system is the recently discovered photoenzyme Fatty Acid Photodecarboxylase (FAP)<sup>34</sup>. In FAP, a cascade of reactions is initiated by ET, in  $\sim 300$  ps, from the fatty acid substrate to the excited flavin cofactor<sup>35</sup>. ET from closeby tyrosine or tryptophan residues to the flavin would compete with this functional reaction. Inspection of the crystal structure (Fig. 1B) reveals that indeed the nearest aromatic residues are located over 8 Å (minimal ring-to-ring distance), much further than the carboxyl group of the substrate electron donor, and much further than the closest aromatic residues found typically in non-photoactive proteins (cf. glucose oxidase (GOX) structure, from the same GMC oxidase family of flavoenzymes as FAP, in Fig. 1C). Indeed, in FAP, replacement of the closest tyrosine residue, Tyr466, by ET-inert phenylalanine, does not enhance the activity<sup>35</sup>. Interestingly, in the absence of substrate, the enzyme is quite unstable and sensitive to light. In this case  $^3\text{FAD}^*$  is generated to a considerable extent by light absorption, which is likely, through ROS production, at the origin of the instability. We note that the instability has also been proposed to arise from harmful photooxidation of nearby residues<sup>36</sup>, but this proposal is inconsistent with the sheer lack of close-by oxidizable residues described above, and the corresponding long-lived fluorescence in the absence of substrates.

In this context, another interesting flavoprotein performing a completely different functional photochemical process is the light-oxygen-voltage (LOV) domain from plant phototropin photosensors, which contains an FMN cofactor. Here, upon formation of the triplet state  $^3\text{FMN}^*$  with high yield by intersystem crossing from the photo-generated singlet excited state  $^1\text{FMN}^*$  in  $\sim 2$  ns<sup>37</sup>, a flavin-cysteine adduct is formed on the microsecond timescale<sup>38</sup> that is a precursor of more remote structural changes<sup>39</sup>. Therefore, in this system the triplet state is part of the signaling pathway, and quenching of the singlet excited state by ET reactions competing with

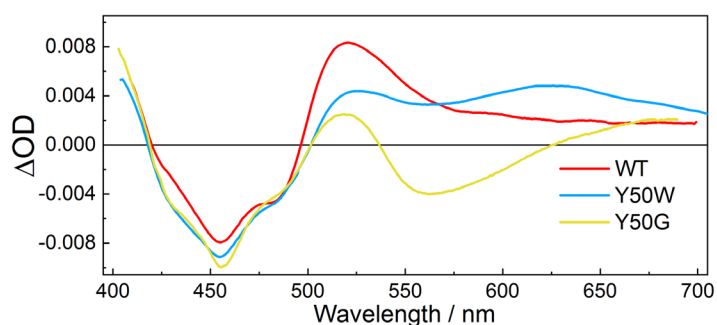
decay to the triplet state would be unfavorable. Indeed, tyrosine and tryptophan residues are located at  $> 9 \text{ \AA}$  from the isoalloxazine ring<sup>40</sup> (Fig. 1D) precluding quenching of the singlet state. It should be noted that in these systems under certain circumstances long-distance ET from tryptophan to the  $^3\text{FMN}^*$  state can compete with intrinsic  $^3\text{FMN}^*$  decay occurring on the microsecond timescale<sup>40</sup>. Based on the reactivity of their photogenerated triplets, variants of LOV domains are employed as optogenetic tools<sup>41</sup>. When ET in the triplet states can in addition be sufficiently reduced, they can also be tailored for use as light-induced generators of singlet oxygen (singlet oxygen generator, SOG) and other ROS<sup>31, 42</sup> using the mechanism described above.

As mentioned above, tryptophan and tyrosine are the only documented amino acid residues known to act as direct electron donor to photoexcited flavins. Interestingly, the involvement of tryptophan as electron donor was first explored not in a system with oxidized flavin, but in a photoenzyme that stabilizes the neutral radical  $\text{FADH}^\bullet$  state, DNA photolyase. In this protein, a chain of three tryptophan residues is located between the flavin cofactor and the solvent. Excitation of  $\text{FADH}^\bullet$  leads to a long-lived, and catalytically active,  $\text{FADH}^-$ . Here the initial ET step occurs in 30 ps and the primary  $\text{FADH}^- \text{TrpH}^{+\bullet}$  photoproduct is stabilized by subsequent ultrafast hopping along the chain and deprotonation of the solvent-exposed tryptophan yielding  $\text{FADH}^- \text{Trp}^{\bullet}$ <sup>43-45</sup>. To disentangle these steps, fully spectrally and polarization resolved transient absorption experiments were exploited along with the fact that the tryptophan radical species (weakly) absorb in the visible range, and solution model spectra for them are available from pulse radiolysis experiments. Similar approaches have been used for the homologous class of cryptochrome blue light sensors (Fig. 1E), in which the resting state of the flavin is the  $\text{FAD}_{\text{ox}}$  state that has a higher-lying lowest excited state, allowing  $\sim 2$  orders of magnitude faster ( $\sim 400 \text{ fs}$ ) initial charge separation towards the  $\text{FAD}^\bullet \text{TrpH}^{+\bullet}$  state<sup>16, 46</sup>, and subsequent stabilization of the radical through the triple tryptophan

chain or variations thereof involving additional tryptophans or tyrosines. These stabilized radical pairs are thought to serve as intermediates in the signal-transmission chain for light adaptation in a wide variety of ways, the precise nature of the more down-stream events still remaining uncertain in many cryptochromes. There are also increasingly strong indications that cryptochromes in the retinal of migratory birds are involved in their orientation sensing through an (earth) magnetic field effect on the spin conversion yields based on light-induced radical pair formation<sup>47</sup>. The latter possibility has inspired the development of *de novo* designed protein-riboflavin complexes in which the yield of neutral radicals involving flavin and tryptophan is magneto-sensitive, albeit at levels above the earth magnetic field<sup>48</sup>. Recent ultrafast spectroscopic approaches indicate that in these systems, other than in cryptochromes, the charge separation generating the initial radical pairs occurs on a much slower timescale, and strongly involves the riboflavin triplet excited state<sup>11</sup>.

The studies on native proteins discussed in the preceding paragraph made use of the distinct  $\text{TrpH}^{++}$  and  $\text{Trp}^{\bullet}$  spectral properties in the visible region (the latter is blue-shifted by  $\sim 60$  nm) that have been well characterized in solution using pulse radiolysis and photolysis approaches. The  $\text{p}K_a$  of  $\text{TrpH}^{++}$  is  $\sim 4$ , hence at physiological pH this species will eventually deprotonate, but it can be long-lived enough (hundreds of nanoseconds in DNA photolyase<sup>43</sup>) to be characterized as separate intermediate. For tyrosine radicals the situation is different. Whereas the  $\text{TyrO}^{\bullet}$  spectrum has been well characterized in solution, the  $\text{TyrOH}^{++}$  species had not been described, in general agreement with its estimated very low  $\text{p}K_a$  ( $\sim -2$ ). The question whether this latter species can be generated as a separate intermediate was solved recently using the bacterial flavoproteins, methyl transferase TrmFO and ferredoxin-NAD(P)<sup>+</sup> oxidoreductase (FNR, see Fig. 3), harboring a tyrosine close to the active site, where the primary photoproduct could be assigned to  $\text{FAD}^{\bullet-}\text{TyrOH}^{++}$  based on its spectral properties, substantially shifted with respect to those of  $\text{TyrO}^{\bullet}$  both in the visible<sup>19, 21-22</sup>

and in the infrared<sup>49</sup> spectral region. In these systems, the  $\text{FAD}^{\cdot-}\text{TyrOH}^{\cdot+}$  state is formed on the timescale of  $\sim 100$  fs – 1 ps and recombines within a few picoseconds. In GOX, where both tryptophan and tyrosine had previously been considered as primary electron donors<sup>12, 20, 50-51</sup>, using the newly identified  $\text{TyrOH}^{\cdot+}$  spectrum,  $\text{FAD}^{\cdot-}\text{TyrOH}^{\cdot+}$  was also found to form in  $\sim 1$  ps<sup>52</sup>. In competition with charge recombination and re-reduction of tyrosine by tryptophan, some deprotonation of the tyrosine cation radical by proton transfer to the flavin then takes place in a few picoseconds, yielding  $\text{FADH}^{\cdot}\text{TyrO}^{\cdot}$ . These studies thus imply that  $\text{TyrO}^{\cdot}$ , an important intermediate in a number of biochemical reactions<sup>53</sup>, does not necessarily have to be formed by strictly concerted electron and proton transfer. Thus, whereas the photochemistry in these flavoproteins themselves has no catalytic role, dissection of the mechanism does allow shining light on potential mechanisms in systems where short-lived intermediates cannot be accumulated.

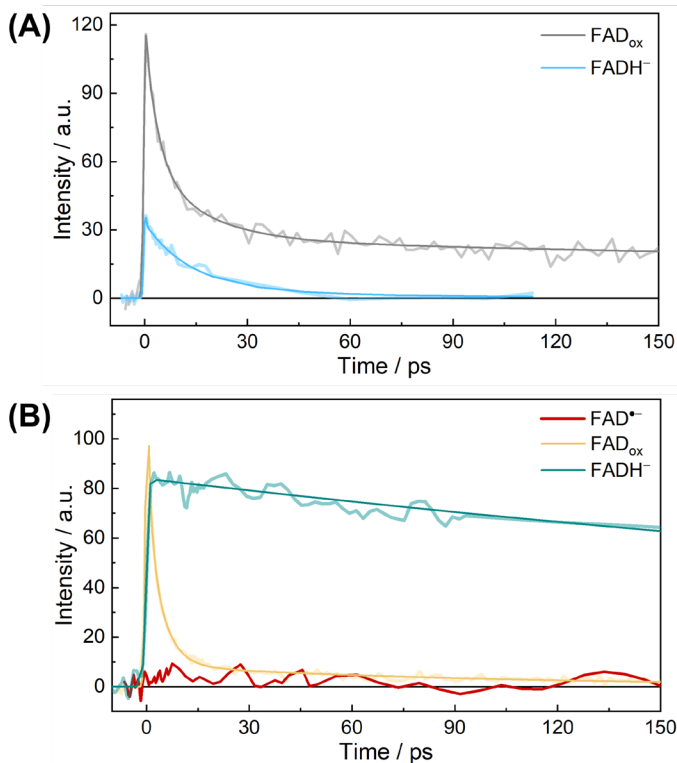


**Fig. 3** Transient absorption spectra, measured 3 ps after excitation of  $\text{FAD}_{\text{ox}}$ , of wild-type (WT) and the Y50W and Y50G variants of FNR from *Bacillus subtilis*, corresponding to the difference spectra of  $\text{FAD}^{\cdot-}\text{TyrOH}^{\cdot+}$ ,  $\text{FAD}^{\cdot-}\text{TrpH}^{\cdot+}$ , and  $^1\text{FAD}^*$ , respectively, with  $\text{FAD}_{\text{ox}}$ . The negative signals around 450 nm correspond to bleaching of the  $\text{FAD}_{\text{ox}}$  ground state. The positive signals above 500 nm are dominated by  $\text{TyrOH}^{\cdot+}$ ,  $\text{TrpH}^{\cdot+}$  and  $^1\text{FAD}^*$ , respectively, and the negative band around 560 nm in Y50G corresponds to stimulated emission from  $^1\text{FAD}^*$ . Reprinted with permission from Ref. <sup>22</sup>. Copyright 2022 American Chemical Society.

As a side remark, these approaches are facilitated by the fact that in the visible range the extinction of the oxidized and semi-reduced flavin species (maxima  $\sim 5,000$ – $13,000$   $\text{M}^{-1}\text{cm}^{-1}$ , Fig.

1) is of the same order of magnitude as the one of the tryptophan and tyrosine radicals ( $\sim 2,000$ – $15,000 \text{ M}^{-1}\text{cm}^{-1}$ ), as illustrated in Fig. 3. Studying such amino acid radicals generated by much stronger absorbing chromophore cofactors, such as chlorophylls or hemes (maxima up to  $\sim 100,000$  and  $200,000 \text{ M}^{-1}\text{cm}^{-1}$ , respectively), would lead to small signals that risk being buried in much larger chromophore spectral changes. Thus flavoproteins, due to their moderate extinction in the visible range, are systems of choice to study such radicals by transient optical spectroscopy.

In the context of photoinduced ET to  $\text{Fl}_{\text{ox}}^*$ , it is interesting to note that in the flavoenzyme choline oxidase, a low-yield highly fluorescent species with an unusual fluorescence excitation spectrum has been assigned to a flavin-histidyl adduct<sup>54</sup>, suggested to be formed in a light-induced way via photooxidation of the initially neutral, singly protonated close-lying histidine residue. Investigation of this hypothesis by time-resolved spectroscopic methods may shed light on such a mechanism, which should compete with cyclic photoinduced photooxidation of close-lying tyrosine and tryptophan residues occurring in  $\sim 300 \text{ fs}$ <sup>4</sup>. Interestingly, highly fluorescent flavin species cross-linked to the protein have also been reported in TrmFO<sup>55-56</sup>, and photo-transformation of such an adduct has been observed<sup>56</sup>. Investigation of the photochemical processes occurring in complexes of flavins and protein residues is yet a virtually unexplored domain that may unearth new photocatalytic roles of flavins.



**Fig. 4** Fluorescence decay on the picosecond timescale of free  $\text{FAD}_{\text{ox}}$  and  $\text{FADH}^-$  in solution (A) and of  $\text{FAD}^{\bullet-}$ ,  $\text{FAD}_{\text{ox}}$  and  $\text{FADH}^-$  in GOX. For  $\text{FAD}_{\text{ox}}$  the decay on the timescale of a few picosecond is assigned ET to the isoalloxazine ring system, in solution from adenine to which it is stacked in a fraction of the flavins, in GOX from closely tyrosine. For  $\text{FADH}^-$  in solution the decay is assigned to a conical intersection with the ground state reached by conformational flexibility of the isoalloxazine ring system. This conical intersection is thought to be more difficult to reach in a constrained protein environment, as in GOX. Panel B is reprinted with permission from Ref. <sup>4</sup>. Copyright 2022 the Authors.

### *Photochemistry of fully reduced flavoproteins*

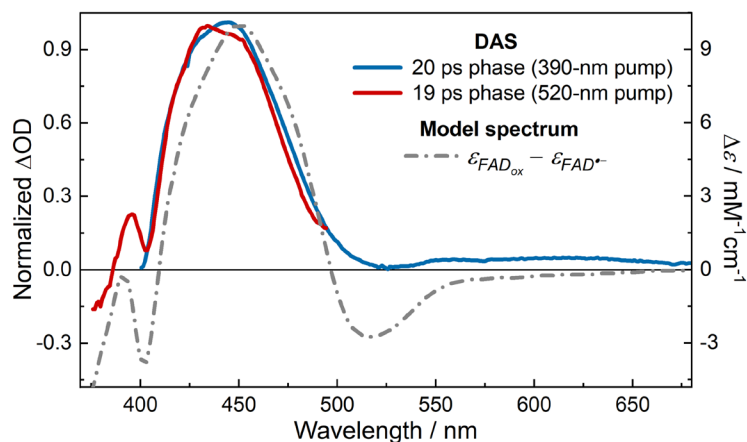
Photochemistry of flavin forms other than the oxidized resting state is interesting from a fundamental point of view, as well as for developing potential applications in using flavoprotein variants as photocatalysts (see below). Yet, studies of these forms remain scarce. As alluded to above, the excited-state and photoreduction properties of neutral semi-reduced radicals have been

studied mostly in the larger framework of the photoactivation of DNA photolyase<sup>43-45, 57-58</sup>. Fully reduced (neutral or anionic) flavins have very long been known to be virtually non-fluorescent in solution, but can have long-lived emissive excited states when their conformational dynamics are restricted by the protein environment or low temperatures<sup>59-61</sup>. This feature has been interpreted as a result of deactivation of the excited state via a conical intersection with the ground state that can be reached through butterfly bending motions of the isoalloxazine ring system. In confined environments such motions are hindered, leading to long-lived (up to nanoseconds) excited states. Such excited states are known to have a functional role in the photoenzyme DNA photolyase, where  $\text{FADH}^-*$  is the precursor of ET to the substrate, photodamaged DNA. The latter process occurs in  $\sim 200$  ps, an order of magnitude faster than the intrinsic  $\text{FADH}^-*$  decay in the absence of substrate.<sup>44</sup> Photochemical processes of fully reduced flavins are also thought to play a role in bio-engineered photocatalysts (see below); therefore a basic understanding of the competing excited-state deactivation processes is warranted. For instance, using transient absorption spectroscopy, the decay of  $\text{FADH}^-$  in the methyl transferase flavoenzyme ThyX has been interpreted in terms of the rigidity of the active site<sup>62</sup>. As fluorescence represents the most direct measure of the excited state, we are currently engaged in studying excited-state decays of fully reduced flavins in well-characterized model protein systems, as well as in aqueous solution, using higher time resolution and more sensitive fluorescence detection systems than available in the classical studies. Fig. 4 gives an example comparing fluorescence decay of  $\text{FADH}^-$  in GOX and in solution. A promising perspective of a system that is worth further exploring in this regard is FNR. It has been demonstrated that in some FNR, the lowest electronic transition band of the fully reduced flavins is significantly red-shifted by  $\sim 40$  nm compared with the commonly known absorption spectra of  $\text{FADH}^-$  or  $\text{FADH}_2$ .<sup>63</sup> It also appears that in FNR, the presence of this red-shifted band correlates

with the reactivity of the fully reduced enzyme toward  $\text{NADP}^+$ . Investigating the origin of this lower-lying electronic transition can provide useful insight into the electronic structures and surrounding protein environments of the fully reduced flavins in FNR, which may provide a better understanding of the function of this important class of flavoenzymes.

### *Photochemistry of semi-reduced flavoproteins*

Semi-reduced flavin species play an important role as reaction intermediates in many biochemical reactions. Yet they are intrinsically quite unstable and can be accumulated for steady-state investigation only for a limited number of cases. The most emblematic example concerns the neutral semi-reduced  $\text{FADH}^\bullet$  reaction intermediate form, which is the resting state of the isolated DNA-repair photoenzyme DNA photolyase, although its catalytically competent form is  $\text{FADH}^-$ . As alluded to above, the excited-state and photoreduction properties of neutral semi-reduced radicals have been studied mostly in the larger framework of the photoactivation mechanism of this enzyme<sup>43-45, 57-58</sup>, a second, substrate-independent, photoreaction that in DNA photolyase converts  $\text{FADH}^\bullet$  to  $\text{FADH}^-$ .

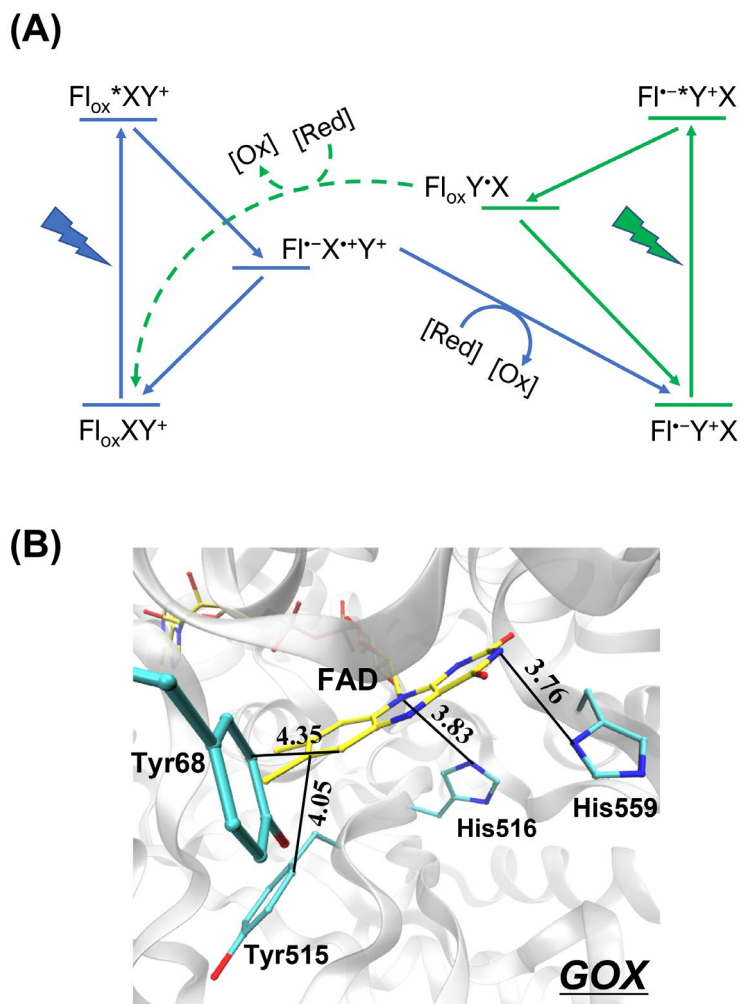




**Fig. 5** Decay associated spectra (DAS) obtained from transient absorption measurements of  $\text{FAD}^{\cdot-}$  in GOX upon excitation at 390 nm and 520 nm. The steady-state  $\text{FAD}_{\text{ox}}$  minus  $\text{FAD}^{\cdot-}$  spectrum difference spectrum is shown (dashed line) for comparison (cf. Fig. 1B). Reprinted with permission from Ref. <sup>4</sup>. Copyright 2022 the Authors.

The photophysical properties of the semi-reduced anionic radical species,  $\text{Fl}^{\cdot-}$ , have been even more scarcely studied<sup>46, 64</sup>, and photochemical products have been reported only this year<sup>4</sup>. By photochemical or chemical reduction of  $\text{Fl}_{\text{ox}}$  under anaerobic conditions,  $\text{Fl}^{\cdot-}$  can be stabilized to a substantial extent in some systems that harbor positively charged residues close to the flavin cofactors, in particular in flavoprotein oxidases where such residues may serve to maintain  $\text{O}_2^{\cdot-}$  radical intermediates in the active site. Our recent study on a number of such oxidases shows that the  $\text{Fl}^{\cdot-*}$  excited state is extremely short-lived (<100 fs, Fig. 4)<sup>4</sup> to a point that time-resolved fluorescence could not be observed; previous reports on  $\text{Fl}^{\cdot-*}$  fluorescence<sup>46, 64</sup> may have been complicated by admixture of minor quantities of other fluorescent forms of the flavin. Remarkably, in all flavin oxidases (including the GOX model system) transient absorption spectroscopy witnessed that  $\text{Fl}_{\text{ox}}$  was formed quasi-instantaneously with high yield, demonstrating photooxidation of  $\text{Fl}^{\cdot-}$  (Fig. 5).<sup>4</sup> Thus, the inverse of the ubiquitous photoreduction reaction of  $\text{Fl}_{\text{ox}}$  also occurs (Fig. 6). The electron acceptor in these oxidases is likely to be a positively charged protonated residue, probably histidine or arginine, located in near-van der Waals distance from the flavin. The driving force for  $\text{Fl}^{\cdot-*} \text{HisH}_2^+ \rightarrow \text{Fl}_{\text{ox}}\text{HisH}_2^{\cdot}$  ET was estimated at  $\sim 0.2\text{eV}$  from theoretical considerations<sup>4</sup>. The mechanism of this ultrafast, yet presumably low-driving force, electron transfer reaction can likely not be described in terms of classical Marcus theory with the high activation energies expected with reorganization energies in the often assumed 0.7–1.0 eV range<sup>14-15</sup>. Future theoretical and experimental studies will be required to investigate whether for instance two-state strong coupling, near adiabatic transitions and/or direct charge transfer optical

transitions may play a role. Also, identification of systems that stabilize  $\text{Fl}^-$  in the absence of a nearby positively charged acceptor (at present these are not available) may allow spectral characterization of the  $\text{Fl}^{\cdot-}$  precursor state.



**Fig. 6** **A.** General reaction pathways for photoinduced ET in flavoproteins that include the proposed cycle of the photooxidation of  $\text{Fl}^-$ . X stands for the electron-donating residue (tryptophan or tyrosine) and  $\text{Y}^+$  represents the electron-accepting residue (presumably histidine or arginine) in the protein active site. [Red] and [Ox] represent external reductants and oxidants. An exchange mechanism between reduced free flavins and protein-bound  $\text{Fl}_{\text{ox}}$  has also been proposed to explain the accumulation of protein-bound  $\text{Fl}^-$  under light illumination in the presence of hole scavenger<sup>65</sup>, which is not included in the present scheme. **B.** Active site in the crystal structure of GOX from *Aspergillus niger* (PDB entry: 1CF3). The presence of electron-donating tyrosines and tryptophans, as well as electron-

accepting histidines in close proximity of the flavin ring allows both photoreduction of  $\text{FAD}_{\text{ox}}$  and photooxidation of  $\text{FAD}^{\bullet-}$  to occur. Distances are indicated in Å.

In the five investigated systems in the aforementioned study<sup>4</sup>, the resultant photoproduct state (presumably  $\text{Fl}_{\text{ox}}\text{HisH}_2^{\bullet}$  in GOX) fully reverts (or ‘re-separates’) to the initial dipolar state ( $\text{Fl}^{\bullet-}\text{HisH}_2^+$  in GOX) where the radical is on the flavin moiety in 10–20 ps depending on the system. An interesting perspective from a more applied viewpoint would be the use of genetic engineering approaches for creating pathways in these systems that will allow hopping of the radical away from the flavin, thus stabilizing the photoproduct, and ultimately photoreduction of external reactants.

### *Photobiocatalysis perspectives*

Flavoproteins play an important role in the strongly developing field of photobiocatalysis, where the light sensitivity of -often engineered- proteins is employed for catalyzing chemical reactions in a sustainable way.<sup>66-67</sup> Indeed, in two of the only three known families of naturally occurring photoenzymes, the catalytic reaction is initiated by absorption of a photon by a flavin cofactor. These are DNA photolyase, where catalysis is initiated by light-induced electron transfer from fully reduced flavin to the pre-bound substrate<sup>44</sup>, and FAP, where catalysis is initiated by light-induced electron transfer, in ~300 ps, from the pre-bound substrate to oxidized flavin<sup>34</sup>. In particular for FAP, in the few years since its discovery in 2017, a strong world-wide activity has developed to explore variants with altered substrate-binding site residues produced by directed or semi-random mutagenesis strategies. In particular efforts have been made to adapt this enzyme to

catalyze decarboxylation of a variety of substrates, including molecules much smaller than the medium chain-length lipids of the native enzyme; furthermore decarboxylation at multiple positions of the same substrate was investigated<sup>68</sup>. Herewith efforts have mainly been directed towards optimizing the substrate positioning in the active site. Yet another important issue is the low photostability of the enzyme in the absence of substrate. As outlined above, this feature is likely due to the lack of photoprotective photoreduction excited-state decay processes that many non-photoactive flavoenzymes display. To optimize practical applicability it may therefore be useful to also (co)-engineer intrinsic flavin photoreduction pathways, introducing tyrosine or tryptophan residues allowing ET to occur in the hundreds of picosecond to nanosecond range. In this way these new pathways would moderately compete with the catalytic pathway in the presence of substrate, allowing substrate-dependent balances between photocatalytic efficiencies and photostability.

Light-activated flavoenzymes that are not naturally light-driven are also being used as a starting point for developing photocatalysts for a variety of chemical reactions. In particular, ene-reductases such as Old Yellow Enzyme that harbor a versatile substrate-binding site have recently been explored to this aim by the Hyster group<sup>69-72</sup>. In these systems, photoreduction of alternative substrates by one-electron transfer from excited fully reduced flavin was shown to initiate a variety of reactions, including stereoselective cyclization and reductive breaking of double bonds. In native and near-native enzyme systems the photochemical quantum yield of these reactions is often very low, possibly due to wasteful (from a catalytic point of view) early back reactions. Extensive screening of genetically modified variants has allowed the enhancement of yields up to the several-percent range<sup>71</sup> (still well below the >50% quantum yields observed for native photoenzymes<sup>34</sup>,

<sup>44</sup>), at least in part by modifying the substrate-flavin interaction so as to optimize the competition between forward reactive reactions and back reactions.

In ene-reductases, photocatalytic reduction of acrylamides has been shown to occur from a state where the flavin is in a well-defined, fluorescent, fully-reduced FMNH<sup>-</sup> form<sup>72</sup> and the reaction is initiated analogously to substrate photoreduction in DNA photolyase. On the other hand, addition of substrates for photo-cyclization to the fully reduced enzyme leads to a strong absorption increase in the blue spectral range that is assigned to a charge-transfer transition allowing direct photoreduction of the substrate<sup>69-70</sup>. Intriguingly, the spectral properties of this band are very similar to the ones of the semi-reduced flavin anion FI<sup>-</sup> (Fig. 1B) and the spectral properties of the initial photoproduct also show resemblance to those assigned to fully oxidized flavin in our recent photochemical study of flavoproteins containing semi-reduced flavin anion radicals<sup>4</sup>. Further theoretical and experimental investigations into such systems may help to better characterize the electronic properties of the starting and intermediate states and ultimately devise strategies to further improve their photocatalytic properties.

Photooxidation of substrates starting from oxidized flavoenzymes may also occur in non-native photoenzymes. In the dark, the bacterial monooxygenase PqsL does sustain activity with reduced 1-methylnicotinamide as a hydride donor, but not with NAD(P)H<sup>32</sup>. However, under light illumination the product can be formed with NAD(P)H. Based on time-resolved experiments under continuous illumination a scheme for this finding has been proposed that involves ET from this nicotinamide to FAD<sub>ox</sub>\* in two subsequent light-dependent steps. Each step yields FAD\*<sup>-</sup> that is subsequently rapidly transformed to FADH\*, ultimately leading to full reduction of the flavin. Consistently, as the nearest tyrosine or tryptophan residue is located 7 Å from the flavin ring<sup>73</sup>, ET from intra-protein donors is expected to occur on the timescale of tens of picoseconds at fastest<sup>15</sup>,

which leaves room for efficient competition of photoreduction by NAD(P)H. Further understanding and verification of the photoactivity of Pqsl, which may be extendible to other photocatalytic processes, can benefit from further characterization using high time resolution spectroscopic approaches.

### *Concluding remarks*

Altogether, recent fundamental spectroscopic and modeling work has highlighted the extreme diversity of photochemical redox processes in flavoproteins. At the same time, a variety of applied approaches are emerging in which flavoenzymes are sought to perform ‘green chemistry’ photocatalytic processes that differ from their native physiological function, sometimes using pseudo-random optimization strategies. It is foreseen that insight in the fundamental reactions that underlie the photocatalytic reactions will guide their development along directed pathways, as well as allow novel photocatalytic approaches to be explored.

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## **Biographies**

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TOC graphics

