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Understanding flavin electronic structure and spectra

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Abstract

Flavins have emerged as central to electron bifurcation, signaling, and countless enzymatic reactions. In bifurcation, two electrons acquired as a pair are separated in coupled transfers wherein the energy of both is concentrated on one of the two. This enables organisms to drive demanding reactions based on abundant low-grade chemical fuel. To enable incorporation of this and other flavin capabilities into designed materials and devices, it is essential to understand fundamental principles of flavin electronic structure that make flavins so reactive and tunable by interactions with protein. Emerging computational tools can now replicate spectra of flavins and are gaining capacity to explain reactivity at atomistic resolution, based on electronic structures. Such fundamental understanding can moreover be transferrable to other chemical systems. A variety of computational innovations have been critical in reproducing experimental properties of flavins including their electronic spectra, vibrational signatures, and nuclear magnetic resonance (NMR) chemical shifts. A computational toolbox for understanding flavin reactivity moreover must be able to treat all five oxidation and protonation states, in addition to excited states that participate in flavoprotein's light-driven reactions. Therefore, we compare emerging hybrid strategies and their successes in replicating effects of hydrogen bonding, the surrounding dielectric, and local electrostatics. These contribute to the protein's ability to modulate flavin reactivity, so we conclude with a survey of methods for incorporating the effects of the protein residues explicitly, as well as local dynamics. Computation is poised to elucidate the factors that affect a bound flavin's ability to mediate stunningly diverse reactions, and make life possible.

This article is categorized under:

Structure and Mechanism > Computational Biochemistry and Biophysics
Electronic Structure Theory > Combined QM/MM Methods
Theoretical and Physical Chemistry > Spectroscopy

KEYWORDS

computational methodology, electronic structure, flavin, flavoprotein, hybrid QM/MM

1 | INTRODUCTION

Flavins, in the common biochemical forms of flavin mononucleotide (FMN) or flavin adenine dinucleotide (FAD), are versatile electron carriers that couple electron transfer to proton transfer. They also enable enzymes to redistribute electrochemical energy among electrons, producing strongly reducing electrons from a more modest source and thereby enabling demanding reactions.[1] Thus, flavins are central to the activity of hundreds of enzymes, signaling, receptors, circadian rythm, magneto-navigation, DNA repair, electron transfer, energy transduction, and more.[2–7] Electron bifurcation is a more recent addition to the list of flavin talents and exploits the fact that flavins' extensive π system including heteroatoms poises their chemistry on the cusp between single electron transfers and pair-wise closed shell hydride transfers. In bifurcation, a flavin reduced by a pair of electrons dispatches them individually to spatially and energetically disparate acceptors, such that one electron ends up on a much more reducing electron carrier, in apparent violation of the laws of thermodynamics, but in fact "paid for" by highly exergonic transfer of the other electron to a more oxidizing carrier (Figure 1).[8] Tight coupling between the two electron transfers is critical, and rests on the energy of a semiquinone state that is too short-lived to characterize by most experiments.[9,10] This is one example of several critical knowledge gaps that can be addressed by computations, if they can be sufficiently accurate. To understand the fundamental bases for the diverse activities of flavins, we need to obtain accurate descriptions of the electronic structure in each of the flavin's states and in its biochemical contexts.

Formal structures of the flavin oxidation states encountered in proteins are shown in Figure 2. The conversions between the oxidized (OX), semiquinone (SQ), and hydroquinone (HQ) states occur by acquisition of electron(s). The SQ exists in two protonation states, namely anionic SQ radical (ASQ, $FAD^{\cdot -}$) or neutral SQ radical (NSQ, FADH·). Similarly, the HQ state exists as anionic (AHQ, $FADH^-$) or neutral forms (NHQ, $FADH_2$, for the example of FAD). The associated pK_a values and sites of protonation vary with the flavin oxidation state, so protonation of the flavin in turn modulates the E°s, and the sources of the protons are often among the questions to be resolved. Besides exploring the properties of states that participate in mechanism but are not amenable to experimental study, computation can also identify protein interactions able to *explain* observed phenomena at the fundamental level of flavin electronic structure. Such insights can be transferable to other comparable systems. Two of the flavin oxidation/protonation states have net charge and all have significant dipoles, so they are affected by local electrostatics.[12,13] Similarly, multiple positions capable of hydrogen bonding (H-bonding) participate in the extended π system,[14] providing additional points at which noncovalent interactions can significantly alter the flavin E°s and electron density distribution.[15] Besides their electronic plasticity, flavins report on their electronic structure via strong distinctive UV/visible spectra that make them attractive targets for experiments, and indeed were instrumental to their discovery.[16,17] However, the sensitivity of their electronic structure to their environment makes quantum chemical calculations a demanding challenge.

Crystallographic structures are available for several bifurcating flavin sites.[18–20] It is noteworthy that in the cases known, the bifurcating flavin is bound between protein domains, where movement of either one could modulate the flavin's activity (Figure 3).[19,21,22] Conversely, flavin oxidation or protonation state changes could alter the domain

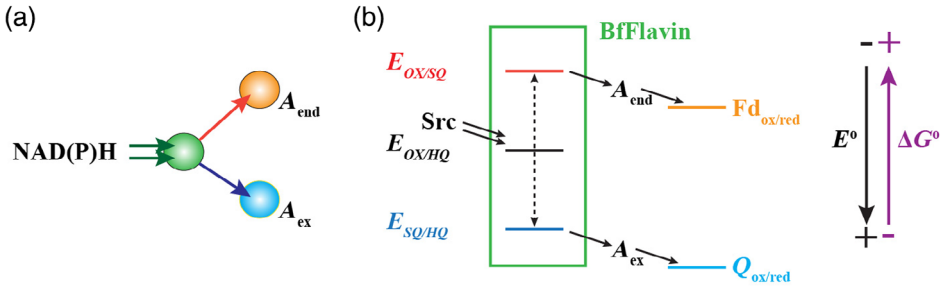


FIGURE 1 (a) Cartoon of the essence of bifurcation based on reduced nicotinamide adenine dinucleotide (NADH) or the phosphorylated version NADPH. (b) Energy landscape for steps and electron carriers involved in flavin-based electron bifurcation, wherein the three reactions of the bifurcating flavin (BfFlavin) are within the green box. The positions of the horizontal lines indicate the reduction potentials, E°s, of the reactions of each of the carriers ($E^\circ = -\Delta G^\circ/nF$ is the negative change in free energy per electron). The 2e E° of the BfFlavin is constrained to be the average of the two 1e E°s. A_{end} and A_{ex} indicate electron acceptors whose reduction is endergonic and exergonic, respectively, Q indicates a generic quinone as an ultimate electron acceptor as in the case of several bifurcating electron transfer flavoproteins[11] and Fd indicates ferredoxin, a common low-E° ultimate electron acceptor

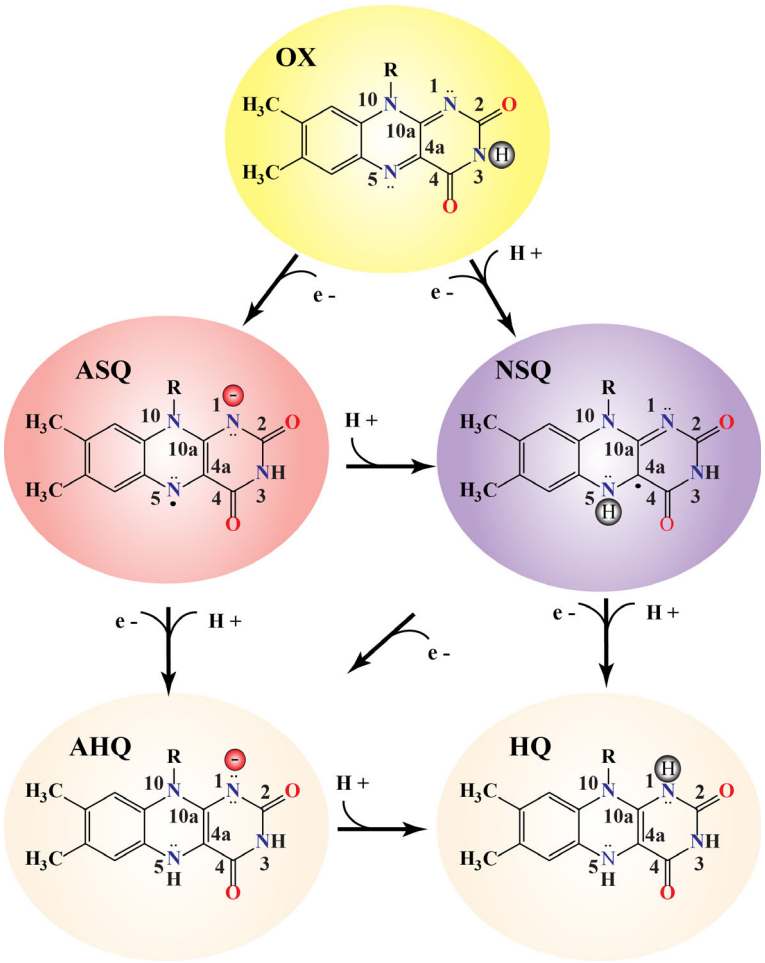
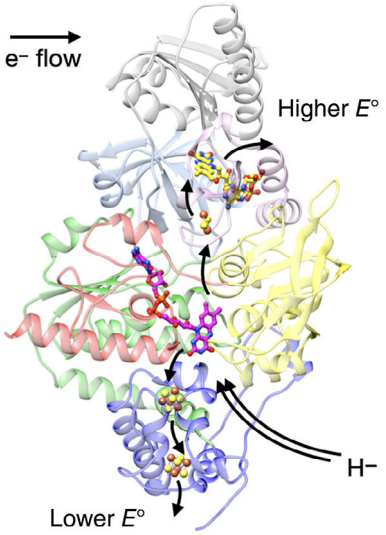


FIGURE 2 Oxidation and protonation states of the flavin, showing numbering of a few key positions and accepted resonance structures. The backgrounds depict the observed colors of flavin in its OX, ASQ, and NSQ states, illustrating the diagnostic response to fundamental chemical events such as acquisition of a single electron or proton. The visible electronic spectra of AHQ and HQ are also responsive, but the extinction coefficients are much weaker. R = ribityl phosphate for FMN, R = ribityl adenosine diphosphate for FAD, and R = CH_3 for lumiflavin

FIGURE 3 Bifurcating electron flow in NfnI diagramming exergonic flow (up) that pays for endergonic flow to ferredoxin (down). NfnI stands for NADH-dependent reduced ferredoxin: NADP oxidoreductase I. The BfFlavin in the large subunit of NfnI is bound at the interface of four domains, colored blue, green, yellow, and red (from N to C in the amino acid sequence). The flavin in the small subunit is also bound between domains (pale blue, gray, pink, in N to C order). Based on PDB accession code 5JCA, and also see 4YRY[19,21]



interface and thus trigger conformational change. Computational methods able to extract overall features of the sites such as charge distribution or polarity, may reveal common themes. In general, important goals are to understand how the protein context modulates the electronic structure and spectral signatures of bound flavins, to understand their reactivity.

Herein we review methods for calculating electronic transitions and the corresponding observed spectra of flavins, because they provide stringent tests of quantum chemical calculations. In particular, methods that have been vetted on flavin-based photoreceptors are highly relevant. Thus, we provide the following account of such efforts including their success in describing the photochemistry of flavin-based sensors, as a foundation for new studies of bifurcating flavins, and flavin-based systems in general.

2 | MODELS OF FLAVIN-BASED SYSTEMS

2.1 | Gas phase

The electronic absorption spectrum provides very stringent validation of the flavin electronic structure because it rests on descriptions of excited states as well as the ground state. Although the flavin exists as FMN and FAD in biochemistry, the substituent ("R"—in Figure 2) does not contribute to the visible absorption because it is not a part of the conjugated system. Therefore, truncation of "R" to CH_3, serves as a valid starting point. For gas phase calculations we begin with an isolated flavin to study properties intrinsic to it. The advantage of this simple model is its power to identify the spectroscopic details such as electronic transitions which are dark in nature. Furthermore, such information serves as a reference for calculations on embedded flavins and supports experimental assignments. Gas-phase calculations were used by several groups to benchmark quantum chemical methods for OX flavin[23–25] and to elucidate electronic differences characterizing other oxidation states of flavin.[26,27] However, gas phase approaches cannot describe the response of the correlated polarizable π system to solvation[28] and protein environments.[29]

2.2 | Implicit continuum model

The mismatch with experiment and inability to account for variations in flavin behavior from one protein to another demonstrate the need to account for the protein. An intermediate approach acknowledges that the flavin is not in vacuum by including a continuum dielectric medium to capture zero-order effects of flavin' surroundings. This approach uses a dielectric constant (ε) and thereby provides a polarizable reaction field.[30] This implicit medium can use $\varepsilon = 78.4$ to model aqueous solvation[28] or, for example, $\varepsilon = 9.2$[31,32] to model protein environments of flavins. Use of implicit water decreased by a factor of 2 the offset between the calculated and observed excitation energies for OX FMN.[33] Recently, more comprehensive analyses of dielectric effects on electronic structure of OX flavin dimers[34] and SQ states[35] as well as a charge-transfer state[36] were reported. This method is also advantageous as it helps in understanding the solvatochromism of flavins.[37]

2.3 | Explicit description of the protein environment

It is widely accepted that the protein environment plays a crucial role in tuning the electronic structure of chromophores such as flavins.[38,39] The correlated and highly delocalized electrons in the isoalloxazine triple ring system of flavins are highly responsive to the amino acids and water molecules present in protein active sites (Figure 2).[40] For instance, the C=O groups at C2 and C4 have H-bond accepting character and doing so modulates their spectroscopic signatures.[41] Significant discrepancies between experimental frequencies and results of simple calculations were attributed to the treatment of the solvent.[42] H-bond donation by N3H likewise affects flavin stretching vibrational frequencies.[43–45] Conversely, non-bonding lone pairs on N1 and N5 can each accept an H-bond, with strong effects on their nuclear magnetic resonance (NMR) chemical shifts.[46,47] Additionally, long range electrostatic and van der Waals interactions are critical in determining the flavin's overall properties. Protein-bound flavin thus experiences a highly constrained, interactive, and perturbing environment, which is able to bring about a wide range of reactivities, favor different redox and protonation states,[48] and produce different spin configurations.[49–51] Thus, explicit treatment of the

immediate environment (amino acid side-chains and solvent molecules) is essential for an accurate description of structural or electronic properties of flavin cofactors. Several strategies for doing so are implemented in the so-called quantum mechanical approaches, which will be described in detail in following sections.

3 | METHODS

3.1 | Classical force-field (molecular mechanics) methods

Although reactivity is fundamentally an electronic property, it must be developed in the context of the protein structure. A solved crystal structure is the ideal starting point, but the best approximation of the protein structure is the lowest energy one. This is obtained using molecular mechanics (MM) energy minimizations, as established 45 years ago and now accepted as part of the standard *modus operandi*, thanks to hardware improvements that make this relatively fast.[52] The fundamental objective of MM is to represent the energies associated with the protein's structure as functions of the individual atoms' coordinates. Dynamics trajectories are then helpful in obtaining populated (ensemble) states in the low-energy region of the potential energy surface at thermal equilibrium. As a simple example, the intramolecular conformational dynamics of FAD in presence of urea was studied using time-resolved fluorescence spectroscopy and correlated with the dominant conformations obtained from molecular dynamics simulations.[53] These are considered to occur on the potential energy surface describing motions corresponding to the ground state.[54] Some of the classical force-fields which are most widely used for investigation of structure, dynamics, enzyme reaction mechanism, denaturation, and protein folding are AMBER,[54,55] CHARMM,[56] GROMACS,[57] and OPLS-AA.[58] While available force-field parameters provide good descriptions of the standard amino acids, ions, and water models, an additional effort is required to parameterize crucial non-standard residues such as chromophores. Crude parameters however can be obtained using the general AMBER force field (GAFF)[59] and CHARMM General Force Field (CGenFF).[60] A simple but clever improvement is obtained by combining parameters obtained from generalized force-fields with charges derived from the restrained electrostatic potential (RESP) method[61] as shown in Ref. 62.

In order to study the biophysical properties of flavoproteins classically, several groups have derived MM parameters for the flavin, for example Refs. 63–65. Some are compatible with AMBER force field[66,67] whereas others work with the CHARMM force field.[64,65,68,69] As described by Sjulstok et al.,[40] the key challenge in parameterization of flavin is accurate treatment of the delocalized electron density in the isoalloxazine ring, which cannot be fragmented into smaller groups to fit charge, angle, and dihedral parameters. In addition, the different oxidation states incorporate different bonding, and in some cases an unpaired electron, so each state requires different parameterization. The validity of these model parameters can be assessed on the basis of their ability to reproduce the bond lengths and torsion angles of the isoalloxazine ring (e.g., butterfly bend[70]) as predicted by quantum mechanics (QM) in gas phase.[71]

3.2 | Quantum mechanical approaches

While local protein structure is necessary, it is rarely sufficient. MM methods also enable treatment of flavoprotein motions that may be coupled to cofactor activity or may gate it via access to solvent or substrate or partners in the reactions. Thus, classical simulations are highly informative regarding domain motions and amino acid reorientations that can affect flavin reactivity through electrostatic, H-bonding and Van der Waals (vdW) interactions.[15,44] This has been widely used in flavin-based systems.[40] In contrast, the electronic properties of cofactors and their reactivity also reflect faster electronic motions. Along with spectroscopic properties, they require description at the quantum level, which is rarely possible to extend to the entire protein. A useful compromise is achieved by integrating MM with the QM, which is discussed below.

3.2.1 | Hybrid quantum mechanics/molecular mechanics

Figure 4 compares a range of quantum chemical methods, the simplest being the polarizable continuum model. At the other extreme, Martin Karplus, Michael Levitt, and Arieh Warshel earned a Nobel prize for their introduction of multiscale QM/MM models for studying complex chemical systems.[72,73] These integrate QM at the site of interest with MM

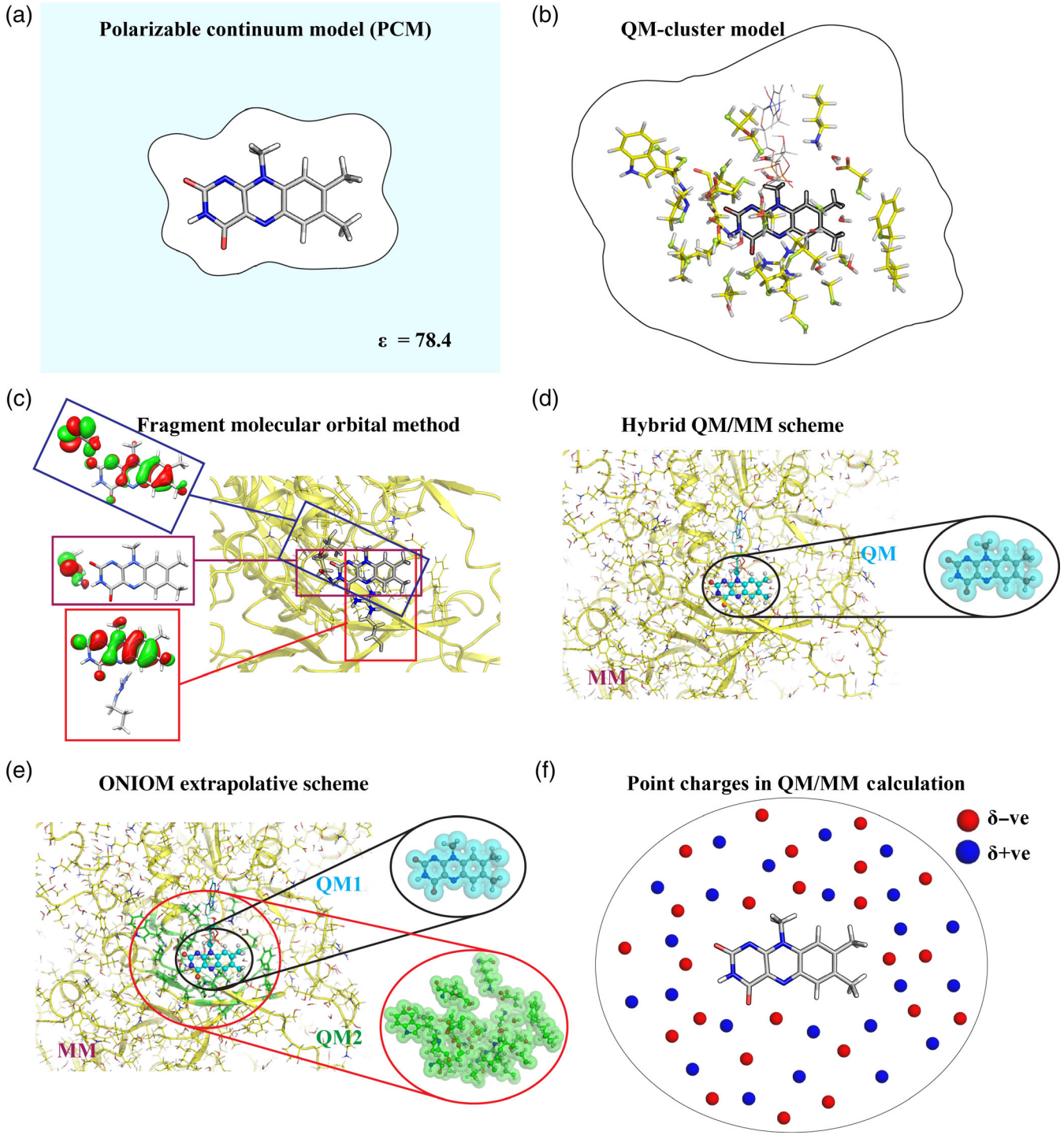


FIGURE 4 Illustration of quantum chemical methods that are used to study the photochemistry of flavoproteins. (a) Polarizable continuum model, where the environment (cyan) can be tuned via the value of the dielectric constant. (b) QM-cluster model showing lumiflavin and sidechains of amino acids in stick representation. (c) Fragment molecular orbital (FMO) method featuring MOs of fragments each containing lumiflavin and a different amino acid side chain. (d) Hybrid QM/MM method combining a central QM part containing lumiflavin, and the remaining system as the MM part. (e) Extrapolative scheme, known as ONIOM where multiple QM layers are treated with different levels of theory (QM1 and QM2) and the remainder of the system with MM. (f) Scheme of QM/MM calculations where the MM atoms are replaced with their partial (point) charges

for the rest of the system (hybrid QM/MM). For photoreceptors and other flavoproteins, this method does justice to both (a) the chemical system that undergoes bonding changes, which must be described using QM because they amount to redeployment of electrons, and (b) the effect of the environment and coordinated motions of atoms during

reaction, as described by MM. Relevant application of the method to photoreceptor proteins has been discussed in a recent review.[74] This strategy has become a ubiquitous method with which to study enzymatic reaction mechanisms, including photocycle intermediates of flavin-centered receptors.[68,75–77] Additional successful applications of this hybrid method in flavin-based system include the following:

1. Geometry optimization using hybrid QM/MM to impose restraints from the protein environment.[78,79]
2. Energetics from electronic transitions (ground-state $S_0 \rightarrow$ excited state S_n) that explains UV/Vis absorption (one-photon and two-photon absorption) and emission properties.[34,80]
3. Vibrational properties underlying infrared, Raman, and resonance Raman spectra.[81–84]
4. Electromagnetic properties such as NMR chemical shifts and EPR hyperfine structure.[47,85,86]
5. Time-dependent propagation of excited states which describes conical intersection and intersystem crossing.[76,87]
6. Protonation state changes coupled with electron transfer processes.[75,88]

3.2.2 | Comparison of the performance of different quantum chemical methodologies

Due to its success, QM/MM has been elaborated in different flavors to reduce computational cost and increase accuracy, with different pros and cons. These variations mainly address the QM component, as it tends to be the bottleneck. A graphical comparison of approaches that have been applied to flavins in proteins is shown in Figure 4.

The QM cluster model considers the flavin as well as surrounding amino acids as the QM region (Figure 4(b)). The residues are truncated at the Cα atom and hydrogen atoms are added to complete the valence shell of electrons. Since the remainder of the protein system is truncated, the Cα atoms are fixed at their initial positions, to prevent unrealistic atomic movements. A few examples of application of QM cluster at the density functional theory (DFT) level are illustrated by Huang and Gauld, who studied enzymatic catalysis mediated by flavins[89] and by Dourado et al., in their study of flavin-mediated electron transport.[90] Notably, the size of QM cluster needs to encompass all considered reaction mechanisms for proton transfer or bond-breaking. However, consideration of long-range interactions is not included in this method, which often makes it less attractive. An all-inclusive comparison of QM cluster treatment with the full QM/MM model has been carried out in calculations of vertical excitation energy in blue light photoreceptors (BLUF).[91] The study concluded that local effects of individual amino acids on flavin spectroscopic properties can be accurately estimated by the QM cluster approach including a sufficiently large cluster; however, the full QM/MM model is always beneficial to minimize theoretical errors.

QM calculations can also be carried out using a fragment molecular orbital (FMO) scheme (Figure 4(c)). This method is advantageous as it avoids truncation of any protein region, and allows linear scaling between the computational cost and the system size.[92] FMO can extend a QM calculation to 10 K atoms with retention of accuracy, whereas the same accuracy is only transferrable from 60 to 1000 atoms by DFT, and a full analytical quantum mechanical solution is rarely possible for molecules larger than a few atoms.[93] In principle, the FMO method divides the QM system into several fragments and performs quantum chemical calculations on each. However, decisions regarding the fragmentation require careful consideration, especially to not include any conjugated system in the fragment boundary. Successful applications of this method to a flavin embedded in protein were reported for cryptochromes (CRYs)[66,67] where the molecular fragments involved in electron transfer were defined in the QM region and fragment orbitals were calculated using the density functional based tight binding (DFTB) method. In another study on DNA photolyase, the fragment orbitals were obtained as DFT Kohn–Sham (KS) orbitals.[94]

Multiscale hybrid QM/MM modeling using a high-resolution quantum level description for the QM region, and classical parameters for the description of the remaining MM system is particularly appropriate for photoreceptor proteins (Figure 4(d)).[95] Cost-effectiveness in such simulations can be achieved by keeping the backbone atoms fixed to their initial positions while allowing flexibility to atoms in the QM region and all sidechains that contains at least one atom within 5 Å of the chromophore. This method has been successful for photoreceptors such as retinal,[96] and could also be informative regarding flavoproteins. Another approach is the ONIOM scheme.[97] The method was developed with the objective of enabling multiple QM layers of different levels of theory in a versatile way, for example, *ab initio* treatment of the QM1 region and application of semi-empirical methods to the QM2 region with MM treatment of the rest of the protein, to reduce the cost of computation while nonetheless obtaining reliable structure and energetics (Figure 4(e)).

In the electrostatic embedding version of QM/MM, the QM region alone is considered for simulation of the spectroscopic properties, using point-charge schemes to incorporate the effects of the protein (MM) environment.[98] This involves replacement of individual atoms in the MM region with their partial charges at their positions as optimized by MM using classical force-field parameters (Figure 4(f)).[99] Thus, the cutoff used in selecting surrounding point charges for inclusion must be sufficiently large to fully account for the electrostatic force. Indeed, the polarization produced by the environment, as replicated by a spatial distribution of charge, can prove critical for accurate modeling and elucidation of spectral features. An example has been reported for CRY with different cut-off distances.[100]

Electrostatic spectral tuning maps are a complementary approach; such maps can elucidate the effect of the anisotropic environment, and thus are useful in designing mutants that may be able to tune the flavin's optical spectrum.[29] Schwinn et al. used the electrostatic potential fitting (ESPF) method in calculating the flavin excitation energy, using B3LYP/TZVP and predicting the lowest lying state at 2.69 eV[79] versus the experimental value of 2.79 eV.[101] Likewise, the ESPF method was recently applied in conjunction with unrestricted wavefunctions, demonstrating potential utility for the semiquinone states.[27,79] Thus, there are several demonstrated approaches for considering the environment of flavins, both in solution and in proteins.[102]

3.2.3 | Conformational sampling methods

A single optimized geometry suffices for calculation of visible and vibrational spectra, yielding a single value for each vertical excitation energy along with the associated oscillator strength (f). Convolution with a Gaussian line-shape can be used to model the effects of slight geometric and environmental differences between sites in experimental samples.[103] Such methods are adequate for smaller systems in which the ground-state equilibrium geometry is well defined. However, in proteins, the enormous number of degrees of freedom and low energy modes complicate the notion of a unique global minimum. Hybrid QM/MM based sampling of populated conformations has been used to generate spectral predictions for the statistical ensemble, which can significantly improve agreement with experiment both regarding line shapes and absorption maxima.[102–104] This strategy has been clearly outlined in Ref. 105 to model the absorption spectrum of riboflavin in aqueous medium. QM/MM based conformational sampling can be initiated based on an energy-minimized geometry[64,91] or a long-time scale classical dynamics trajectory wherein snapshots are extracted and optimized using QM/MM.[106,107] Different levels of theory have been tested ranging from semi-emperical AM1,[104] ZINDO,[108] to Hartree–Fork,[109] and self-consistent charge (SCC-DFTB) methods.[66,67] Besides reproducing spectral properties, sampling methods have been valuable in studying the reaction mechanisms[110] and reduction potentials[104] in flavin-based systems.

4 | INSIGHTS INTO ELECTRONIC STRUCTURE CALCULATIONS

4.1 | Butterfly-like bending motion of the chromophore

The chemical virtuosity of the flavin chromophore is primarily attributed to the heterocyclic isoalloxazine ring (Figure 5). As noted above, it can exist in various oxidation states which participate in electron transfer reactions but are challenging to model computationally.[48] Redox reactivity is concentrated in the N1=C10a–C4a=N5 diazabutadiene system (OX state is shown in Figure 5(a)).[27] The isoalloxazine ring conformation is also involved because the system is essentially planar in the OX state, whereas it undergoes "butterfly-like" bending that is related to altered frontier orbital natures, energies, and overall charge distribution, in the radical and reduced states.[70,111,112] The dihedrals that describe these geometrical distortions are shown in Figure 5(b). *Ab initio* methods have found that the radical state adopts close to planar structure whereas significant bending tends to occur about the N5 and N10 axis in the 2e reduced HQ state.[104,113,114] Such a bending motion also distinguishes the geometrical parameters of the ground state (S_0) from those of the excited states (S_n) which in turn, modulates electron transfers to and from the isoalloxazine ring. For example in protein, structural deformation allows the reactive (N5–C4a) to assume sp^3 configuration instead of sp^2 and thereby favors formation of reduced states and photoproducts (Figure 5(c)).[115] Studies of the excited state dynamics at femtosecond resolution further reveal that such motion also affects the lifetimes of excited states before they switch between potential energy surfaces (conical intersection).[48]

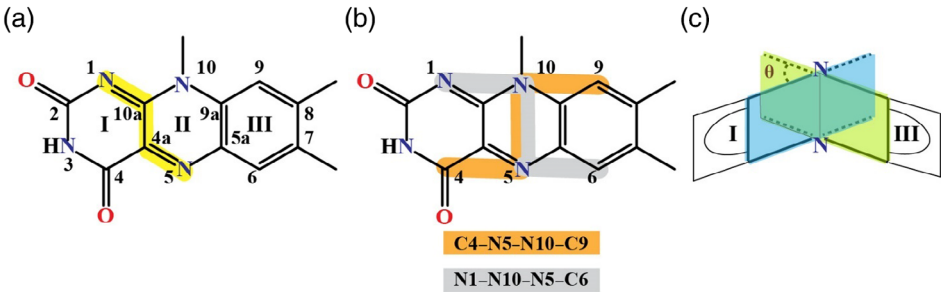


FIGURE 5 Geometric perspectives on the chemical structure of OX lumiflavin. (a) Bond order changes upon reduction are concentrated in the highlighted N1=C10a–C4a=N5 framework. (b) Important dihedrals participating in butterfly-like bending in flavin. (c) Distortions in different oxidation states can also be described by changes in the angle θ between the planes of rings I and III

On the other hand, if one compares the bending angles in free flavins to those of flavins in proteins, a wide distribution can be found, indicating that the environment has a strong effect, and also reflecting variations in flavin oxidation state in the observed systems.[79,116] This argues that the environment could modulate the E°s by imposing a bend on the chromophore.[117] Bhattacharyya et al. used the hybrid QM/MM simulation method to compute the E°, accounting for the puckering of the iso-alloxazine ring, as shown in Figure 5.[104]

Overall, the MM tools established for proteins must be considerably augmented to adequately address flavins at even the basic level of their geometries. The best solution at present is not to rely on MM for the flavin itself, but to incorporate quantum mechanical treatment of it.

4.2 | Quantum-based insights from bond length alternation in flavin

Optimization of the geometrical parameters (bond lengths and angles) is one of the most important steps toward obtaining the minimum energy configuration of a molecule. Based on quantum chemical methods, optimized geometries have been reported for flavin and its derivatives by several groups, using the TD-DFT methods for excited states.[24,25,28,113] Among geometrical parameters obtained, the bond-length alternation (BLA) change between the S_0 and S_1 geometries[118] or between different redox states of flavin[119,120] informs on the degree of electron delocalization, as smaller BLA indicates greater electron delocalization.[121] This has been shown by Bois and Körzdörfer by correlating the effect of BLA with the optical excitation energies in π-conjugated polymers.[122] The study also highlights the importance of correct description of ground-state BLA and use of the correct level of theory to reduce the theoretical error in calculated excitation energies. BLA can thus be linked to the localization of electron density during excited-state transition which affects the calculated vertical excitation energy. For example, the $S_0 \rightarrow S_1$ transition of lumiflavin is associated with a decrease in electron density at N1 and an increase over the N5–C4a atoms, because bonds tend to be longer in the S_1 state, especially those of the N5–C4a pair.[118]

5 | ELUCIDATION OF SPECTROSCOPIC PROPERTIES

5.1 | Calculation of electronic spectra of lumiflavin

To assess the success of calculations, researchers have been able to exploit the bounty of spectral data available for flavins. However, these are far from simple to replicate, due to the complexity of flavin electronic structure and the heterogeneity of protein environments. The simplest form of flavin, namely lumiflavin (Figure 2) has been extensively studied using quantum chemistry, both isolated in the gas phase as well as embedded in protein systems using hybrid QM/MM schemes. While more than one bright state must be considered to describe the flavin UV/Vis spectra, we will concentrate on the computed vertical excitation energy corresponding to the $S_0 \rightarrow S_1$ transition (Exp. 2.79 eV, obtained for riboflavin in aqueous solvent[101]), in this section. In the gas-phase, TD-DFT obtained lowest vertical excitation energies of 3.02 eV (B3LYP), 3.31 eV (CAM-B3LYP), and 3.05 eV (PBE0) using double-ζ type basis sets, where CAM-B3LYP is a range separated hybrid functional.[80,123] Neiss et al. have compared results obtained using hybrid DFT functionals and

explained the overestimated vertical excitation energies in terms of the proportion of Hartree–Fock exchange used.[23] For example, B3LYP which incorporates 20% HF exchange, overestimates the excitation energy by 0.26 eV, whereas the deviation is greater than 1 eV using the BHLYP functional which includes 50% HF exchange. Wavefunction-based methods such as coupled cluster at second order (CC2) and algebraic diagrammatic construction at second order ADC (2) yield excitation energies of 3.06 and 2.94 eV with triple-ζ type basis sets, which are in much better agreement with the experimental value.[25,34] Interestingly, application of multireference methods, for example, symmetry-adapted cluster-configuration interaction (SAC-CI) have led to underestimates of the experimental energy, at 2.46 eV.[124] However another high level of theory, extended multiconfiguration quasi-degenerate perturbation theory at second order (XMCQDPT2) was reported to overestimate the excitation energy at 3.09 eV, in-line with the trend produced by other levels of theory.[91] Note that this latter method uses wavefunction solutions based on complete active space self-consistent field (CASSCF) methods. Application of these multireference methods requires accurate chemical insight for selection of active space orbitals, which otherwise is a challenge. To obtain preliminary insight into this, black-box methods (TD-DFT or coupled cluster) can be used to construct the π-orbitals in the conjugated system employing Hückel theory. The frontier molecular orbitals of lumiflavin are shown in Figure 6. These are the starting point for configuration of the active space in multireference methods. Application of second-order perturbation theory (PT2) is then used to account the dynamic electron correlation, which otherwise is not available with the CASSCF method.[125]

5.2 | Absorption spectrum calculations for the flavin oxidized state

Electronic structure calculations in isolated systems are important for understanding the fundamental optical properties of flavins, as well as to benchmark the method of choice for a given chemical situation. Gas phase calculations serve as references against which to compare spectroscopic properties predicted for flavins in complex chemical environments, for example, embedded in proteins. Valuable examples of flavins in proteins include studies of BLUF, light, oxygen, and voltage sensitive domains (LOV) LOV1 and LOV2, and CRY. Studies of the photochemistry and

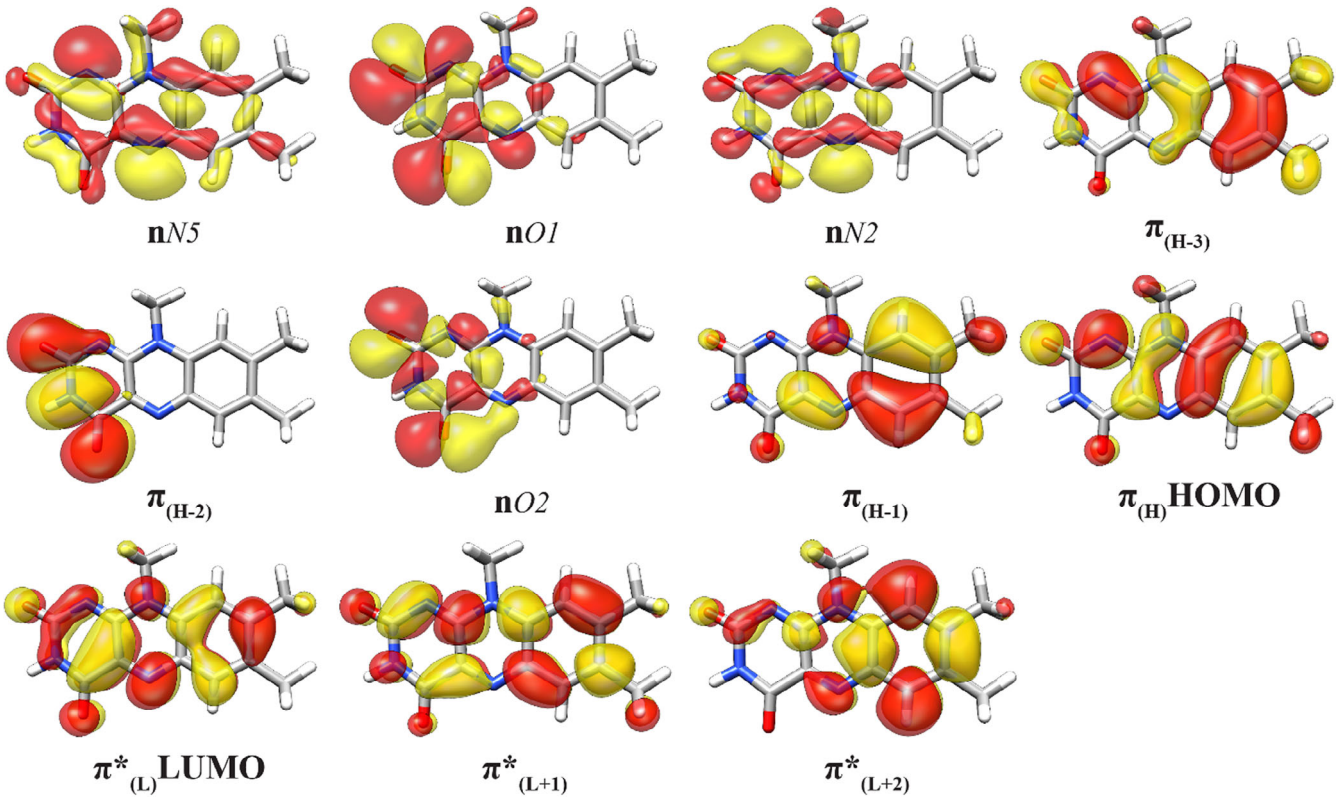


FIGURE 6 Frontier molecular orbitals of lumiflavin involved in lowest transitions. The MOs were obtained based on CC2/CC-pVTZ calculation in gas phase. The figure is adopted with permission from Ref. 25. Copyright 2018 John Wiley & Sons

photobiology of these flavins have mostly focused on the dark-adapted state "resting" state, containing the OX flavin. The influence of HF exchange in the DFT functionals used has been assessed in BLUF using the hybrid QM/MM method,[126] which was motivated by a reference study in the gas phase.[23] In the protein context, TD-DFT was again found to overestimate the excitation energy[127] by amounts of 0.06 eV (B3LYP) and 0.54 eV (BHLYP).[126] Application of the CAM-B3LYP functional, which can describe charge transfer (CT) states reasonably well[25,128] results in excitation energies that are high by 0.03 eV in AppA BLUF protein and 0.25 eV in Slr1694 BLUF protein, albeit with different basis set sizes.[129,130] Meanwhile the CC2 method produces an overestimate of 0.24 eV. In another study, the application of scaled opposite-spin configurational interactions with perturbative doubles (SOS-CIS(D)), yielded an overestimate of 0.17 eV.[131] CASSCF based wavefunction have been applied by Udvarhelyi et al. employing XMCQDPT2, CASPT2, and multistate CASPT2 methods.[91] The choice of active space orbitals has a large effect on the excitation energy, for example active space (4e, 3o) 4 electrons in 3 orbitals yields a value of 3.09 eV, (8e, 8o) gives 2.90 eV, (12e, 12o) gives 2.96, and even larger system (14e, 13o) gives 2.90 eV, versus the experimental value of 2.82 eV.[132] It is also important to note that in these higher levels of theory, larger active spaces may be prohibited by the high associated computational costs. In a more recent work, another multireference method namely N-electron valence state perturbation theory second order (NEVPT2) based on CASSCF wavefunction was shown to give a better description of the excitation energy.[133,134] However, the work by Sayfutyarova et al. considered lumiflavin as well as counter ion residues' side chains in the QM region, thereby using a large active space of (18e, 15o). Interestingly the multireference method was also used to calculate absorption spectra including the effects of vibrational structure. This has been achieved using CASSCF/CASPT2 with an active space of (10e, 8o). Even with this, the calculations overestimated the experimental excitation energy with their value of 3.25 eV.[87] The consistent tendency of theory to overestimate the excitation energy, raises the intriguing possibility that the protein environment may be soft on a very short timescale, allowing excitation into vibrational excited states lower than those accessed in theory.

5.3 | Calculations of electronic spectra of other oxidation states

Calculation of excitation energies for the semiquinone and hydroquinone remains a challenge, due to these states' anionic natures and/or because of their radical characters. Since the semiquinone states possess an unpaired electron, there is a concern regarding spin contamination, especially in studies using the DFT method with unrestricted HF wavefunctions.[109] Special schemes have been developed that are suitable to these situations and can be expected to provide accurate descriptions of absorption properties.[29,79,80] Using these, B3LYP/cc-pVTZ predicted the lowest excitation energy at 2.83 and 2.13 eV, for the anionic and neutral SQ states versus experimental values of 2.55 and 2.17 eV. Similarly, 3.59 and 3.01 eV were obtained for the anionic and neutral HQ states versus experimental values of 3.63 and 3.14 eV.[26] Application of the time-dependent path integral method with a larger basis set (def2-TZVPP) slightly overestimates the energies at 2.93, 2.31, 3.57, and 3.09 eV, respectively.[27]

With a protein-embedded system modeling the BLUF domain, Sadeghian et al. have shown that the unrestricted-B3LYP (UB3LYP) functional offered reasonable agreement with the experimental geometries and transition energies of the transient semiquinone states.[75] However, the level of theory used fails to provide correct evaluation of the energetics associated with the electron transfer steps. In particular, the biradical state wavefunction describing flavin and amino acid radicals requires multireference treatment, which otherwise can be obtained with the CASSCF method or higher theories based on CASSCF wavefunctions.[135] Spin contamination originating from the application of unrestricted HF wavefunctions has been found to be significant, on the order of 25% or more, in a study of the semiquinone states in LOV domain.[109] Therefore, an alternative recommendation is to consider restricted open-shell-B3LYP (ROB3LYP) treatment. Open-shell treatment must also be the choice, when triplet states are of interest, as for example can be learned from a study in LOV domain.[109]

The two SQ states of flavin differ only in regards to their protonation state, yet have sufficiently distinct visible spectra to have earned the names "neutral blue" versus "anionic red" SQ (Figure 2).[136] Studies addressing protonation states of OX flavin used B3LYP calculations in FMN (cationic, neutral, anionic models), mimicking acidic, neutral, and alkaline condition, and showed that the HOMO and LUMO remain largely unaffected by the changes in protonation state.[137] Instead, the lower lying orbitals change dramatically.

5.4 | Calculations of excited states involved in photochemistry

Besides different oxidation states, flavin also accesses excited states with different spin multiplicities, and therefore different capacities to engage in chemistry. Computations able to reproduce transition energies can be used as sources of insight into transition dipole moments and corresponding polarizabilities, which in turn are required to obtain mechanistic insights regarding electron transfer, solvation effects and natures of H-bonding. They also reveal the existence of dark states ($f \sim 0.000$), and the extent to which different orbitals contribute to transitions. Mechanistic insights based on changes in dipole moments between ground state and excited states in conjunction with the intramolecular charge redistribution have been discussed in several works from the Stanley group's studies using TD-DFT methods.[138–141]

Calculations of triplet states of flavin are relevant for understanding flavin photochemistry, which is required to follow intersystem (singlet–triplet) crossing.[142,143] This has been studied using B3LYP with double and triple-ζ type basis sets, obtaining that the lowest-lying triplet excited state of flavin is 1.82 eV (42 kcal/mol), higher in energy than the ground state.[144] Calculations are also useful in accounting for the spin orbit coupling between singlet and triplet states that must be invoked to explain the ISC observed for FMN in LOV domain.[145] A notable issue in these studies is to include Tamm–Dancoff approximation (TDA) in TD-DFT calculations,[146] which has produced dramatic improvement in the singlet–triplet energy gaps.[25,123] However, such singlet–triplet splitting in flavin can also be studied with more accurate methods as reported by Climent et al. with the CASPT2//CASSCF method.[147]

In the case of LOVs, accurate computation of the photochemistry has been challenging. In these systems, a full description requires consideration of ISC between singlet and triplet excited states of the flavin, before modeling of the formation of a neutral biradical state.[142] The experimental absorption maximum of this photoreceptor in its dark-adapted state occurs at a range of experimental values, 2.75–2.82 eV depending on which LOV domain is observed, as documented by Wingen et al.[148] Computationally, the B3LYP functional has provided good agreement with the excitation energy, with overestimates ranging from 0.11 to 0.27 eV, when simulated with different starting structures.[149]

The steps of nonradiative decay from the excited singlet state to the triplet state were explored by Nakagawa et al., using DFT/MRCI methods in LOV domain[150] and compared with the results of Salzmann et al.[28] in a gas phase model. In conjunction with the use of multireference methods, the effect of dynamic electron correlation was clearly noted in the work by Domratcheva et al. in the context of a protein environment.[151] This was demonstrated by comparing the calculated vertical excitation energies based on CASSCF and MCQDPT2 methods using active spaces of (2e, 2o) and (4e, 4o), in the context of a LOV domain. Improvements based on the CASPT2 method and its multistate variant (MS-CASPT2) were also demonstrated using the CASSCF-based wavefunctions. However, this instance included calculations on a larger active space of (10e, 8o) and (12e, 9o).[152] The work sheds light on the virtue of using larger active spaces and bigger basis sets to simulate complex photochemical reaction pathways. CRYs are another class of flavoprotein in which photochemical events lead to conformational changes. Although this photoreceptor is present in most living systems, systematic investigation of the photochemistry underlying function is still at early stages. Biradical states of this protein may even serve as magnetosensors that enable avian navigation,[153,154] demanding computations able to elucidate factors mediating ISC. The first computational characterization of oxidized and reduced FMN in a CRY protein was reported by Cannuccia et al. with the help of hybrid QM/MM simulations, employing TD-DFT methodology. The authors replicated the observed red-shift in their calculated absorption spectrum.[106] They also found that geometrical fluctuations induced in the chromophore by the protein environment affected the vertical excitation energy, especially for the AHQ state of the flavin, and to a lesser degree for the OX flavin. A more comprehensive analysis of FAD absorption spectra in six different CRYs was provided based on advanced QM/MM methods including the polarizable embedding (PE) technique.[155] This specialized scheme not only accounts for the electrostatic effect of the protein environment, but also the responsive potential (localized multipole and dipole–dipole polarizabilities). The reported work was based on the PE/CAM-B3LYP method.[156]

5.4.1 | Interplay between electronic and vibronic states

Addressing the vibronic structure and its contribution in computed spectra was not an object of reports listed above. Nevertheless, vibrational effects contribute to the absorption spectra of all flavin oxidation states. Accounting for vibrational energies can also improve the agreement with experiment, because calculated vertical transitions describe excitation into higher vibrational sub-states of the excited electronic state. Vibrational effects have been discussed with

application of Franck–Condon (FC) analysis using vertical and adiabatic approaches in combination with time-independent and time-dependent formalizations.[103]

The experimental spectra of flavins manifest vibrational structure as well as broadening,[157] which are challenging to describe informatively using standard methods.[27] Broadening of electronic transitions for flavins reflects contributions of higher vibronic states that can be populated by vertical transitions, and calculated with the FC principles.[103,158] They are also attributed to changes in chromophore conformation, that is, the butterfly like bending. The effect of isoalloxazine ring bending or puckering coupled to the $S_0 \rightarrow S_n$ transition has been explicitly discussed.[138,159,160] This is more relevant to the SQ and HQ states, as the effect of geometric changes on the spectral properties is significant.[24,138] For flavin embedded in protein, electrostatic interactions or vdW forces are also capable of inducing geometrical changes, which then give rise to complications in calculating the FC overlaps.[26] However, recent reports have shown that computation of a Hessian based on normal mode analysis of the QM/MM system is effective in reproducing the vibrationally resolved absorption spectra. This has been tested on all the oxidation states of flavin in plant CRY.[79,161] This method requires calculation of Huang–Rhys vibronic (electron-nuclear) coupling to construct the spectral densities and obtaining the vibrationally resolved spectrum. An overview of various methods used for calculation of UV/Vis spectra of flavin-based systems, using quantum chemical methods, is presented in Table 1.

5.5 | Calculations of vibrational spectra

Infrared (IR) and Raman vibrational spectroscopies have been very informative as to the environments of protein-bound flavins because many of the assigned bands are highly sensitive to electronic structure and interactions with protein groups and solvent molecules.[164] The intense diagnostic peaks of the C2=O and C4=O carbonyl groups are significantly affected by the H-bonding environment. Other important vibrational modes arise from the redox active N5=C4a–C10a=N1 unit (Figure 5(a)) which is active in both IR and Raman spectra.[165] Because the observed vibrational bands reflect principally the ground state, established QM methods are fully competent and a methodological consensus has emerged. In brief, DFT methods (using B3LYP) have been found to be the most suitable for predicting vibrational spectra of flavins, and we refer interested readers to some excellent examples show-casing flavin systems (Table 2). Such studies have compared various approaches including explicit water molecules,[163] accounting for excited states using TD-DFT, and evaluating band shifts due to hydrogen/deuterium (H/D) exchange.[163,166]

5.6 | Calculations of nuclear magnetic resonance chemical shifts

Quantum chemical methods are also useful in assignment and understanding of NMR chemical shifts of flavins, both in simple as well as complex environments.[174] The established method for predicting the magnetic properties is the Gauge-Independent Atomic Orbital (GIAO) approach.[175] Nevertheless, the multiscale simulations established to date for such application are based on DFT and MP2 levels of theory. In the context of DFT, the B3PW91 functional showed better agreement with experiment than B3LYP for chemical shielding values for both OX and HQ states of flavin.[46] Furthermore, the method was also reported to be effective in reproducing striking changes in the chemical shift of N5 in response to H-bonding interactions.[47,176] In a comparison of the accuracy of DFT (B3LYP) versus MP2 for flavin in BLUF domain, the former method gave good agreement.[177] It should be noted that the choice of basis set has a significant effect on the obtained values, as reported in.[25] In-line with recent advances, the use of a segmented contracted basis set, also known as a Jensen basis set,[178] could be a better alternative for calculation of NMR properties for flavin systems.[179] Last but not the least, GIAO methods were shown to replicate hyperfine coupling constants for flavin semiquinone states in isolated systems,[85] COSMO solvent models[180] or a protein-embedded system.[181]

6 | CONCLUDING REMARKS: EMBRACING RECEPTORS, SIGNALING, AND ENERGY TRANSDUCTION

The diagnostic electronic spectra of flavin in its various oxidation states has enabled researchers to use it as an internal probe of enzyme mechanism, protein biophysics and redox status. The very molecular properties enabling the

TABLE 1 Theoretical studies reporting calculated excitation energies of flavin-based systems

Flavin-based system studied	Level of theory for geometry optimization	Level of theory for excitation energy calculation	Oxidation state studied	References
Lumiflavin (gas phase)	B3LYP/6-31G*	CIS/6-31G*, B3LYP/(6-31G*, SV(P)]), BHLYP/SV(P) DFT/MRCI/SV(P)	OX	23
Lumiflavin (gas phase)	B3LYP/6-311G(d,p)	B3LYP/(cc-pVDZ, d-aug-cc-pVDZ)	OX	24
Lumiflavin (gas phase)	B3LYP/6-31G*	B3LYP, CAM-B3LYP, RI-CC2, RI-ADC (2)/cc-pVTZ	OX	25
Lumiflavin (gas phase)	B3LYP/TZVP	DFT/MRCI/TZVP	OX	28
Lumiflavin (gas phase)	B3LYP/6-31G*	B3LYP/6-31G*	OX	29
Lumiflavin (PCM)	B3LYP/cc-pVTZ	B3LYP/cc-pVTZ	OX, SQ, HQ	26
Lumiflavin (microsolvation)	CAM-B3LYP/cc-pVDZ	RI-ADC(2)/cc-pVDZ, aug-cc-pVDZ	OX	34
FMN (COSMO)	B3LYP/TZVP	B3LYP/TZVP	OX	137
FMN	B3LYP/(G-31G*, 6-31G**)	B3LYP/(G-31G*, 6-31G**)	HQ	141
FMN	B3LYP/6-311++G(2d,2p)//AMBER	B3LYP/6-311++G(2d,2p)//(AMBER, PCM)	OX, SQ, HQ	162
FMN (microsolvation)	B3LYP/Def2-TZVP//PCM	B3LYP/Def2-TZVP//PCM	OX	145
Riboflavin	B3LYP/6-31G(d)	B3LYP/aug-cc-pVDZ	OX	105
Riboflavin (microsolvation)	B3LYP/TZVP	B3LYP/TZVP, B3LYP/TZVP//PCM	OX	163
FMN LOV1 photoreceptor	B3LYP/6-31G(d)//PCM	B3LYP/6-31G(d)//PCM, MCQDPT2/6-31G (d)//PCM	OX	149
Flavin (FMN) in Nitronate monooxygenase	B3LYP/cc-pVTZ	B3LYP/cc-pVTZ/PCM	OX, SQ, HQ	35
FAD BLUF	B3LYP/TZVP//CHARMM	BP, B3LP, BHLYP, RI-CC2/def-TZVP//CHARMM	OX	126
Slr1694 BLUF photoreceptor	ωPBEh/6-31G*//CHARMM	LRC-ωPBEh/6-31++G**	OX, HQ	77
Slr1694 BLUF photoreceptor	ABPO/CHARMM	CAM-B3LYP/6-31G*	OX	130
FAD BLUF photoreceptor	B3LYP/6-31G(d)	MCQDPT2/CASSCF	OX	78
FAD cryptochrome	B3LYP/TZVP/TZVPFit//AMBER	B3LYP/TZVP/TZVPFit//AMBER	OX, SQ, HQ	79
FAD BLUF photoreceptor	B3LYP/DZV(P), CASSCF/DZV(P)	XMCQDPT2, RI-CC2, MRCI, SOS-CIS/DZV(P), TZVP, cc-pVDZ	OX	135
FMN phototropin 2	B3LYP/6-31G*	PE-CAM-B3LYP/cc-pVDZ, PERI-CC2//cc-pVDZ	OX	80
FMN BLUF photoreceptor	CASSCF/(cc-pVDZ, DZV(P))	XMCQDPT2/DZV(P)//AMBER	OX	91
FMN iLOV photoreceptor	BLYP/TZV2P(GPW)//AMBER	XMCQDPT2/cc-pVDZ//AMBER	OX	118
FMN iLOV photoreceptor	B3LYP/6-31G(d)//AMBER	B3LYP/6-31G(d)//AMBER	OX	158
FAD Bifurcating ETF	B3LYP/(6-31G(d), 6-31++G(d,p))	B3LYP/(6-31G(d), 6-31++G(d,p))//CHARMM	OX	33
FAD Bifurcating ETF	CAM-B3LYP/cc-pVDZ, ωB97X-D/6-31G(d,p)	CAM-B3LYP/cc-pVDZ//PCMωB97X-D/6-31G(d,p)//PCM	OX, HQ	36

TABLE 2 Theoretical studies reporting calculated vibrational frequencies of flavin-based system

Flavin-based system studied	Level of theory	Oxidation state studied	Reference
Lumiflavin (gas phase)	B3LYP/6-31++G, B3LYP/6-31G*	OX, SQ	165144
Lumiflavin (gas phase)	B3LYP/6-31G**	HQ	167, 168
FAD (microsolvation H_2O/D_2O)	B3LYP/TZVP	OX	163
FMN in LOV domain and AppA BLUF domain	B3LYP/6-31G**	OX	169
FMN BLUF domain	B3LYP/6-31G(d,p)//PCM, B3LYP/6-31+G(d,p)//AMBER, B3LYP/def-TZVP//CHARMM	OX	44,170,126
FAD AppA BLUF domain	B3LYP/6-31G**	OX	171
FAD flavodoxin	B3LYP/6–31++G(d,p)	SQ	172
FMN flavodoxin	B3LYP/6-311++G(2d,2p)//AMBER	OX, SQ, HQ	162
FMN-containing flavoenzyme NrdI	(U)B3LYP/6-31+G(d,p)//AMBER	OX, SQ	84
FMN Slr1694 BLUF photoreceptor	LRC-ωPBEh/6-31++G**//CHARMM	SQ	77
FAD DNA photolyase	(U)B3LYP/6-31G*	OX, SQ, HQ	173
FAD cryptochrome	B3LYP/6-31G*//AMBER	OX, SQ, HQ	81

strong and responsive electronic spectroscopy make computational elucidation of the spectra an unusually subtle and theoretically demanding task. Moreover, upon reproduction of electronic excitation, the job has only just begun, because this event precipitates a cascade of transitions involving ISC, redistribution of electron density and even macromolecular conformational changes that can occur on very long time scales. Thus, this field can look forward to replication of transient absorption studies elucidating the basis for CRY, LOV, BLUF signaling, and the light-driven chemistry of photolyase[182] and fatty acid photodecarboxylase, that can produce biofuel.[183] While the flavins participating in bifurcation may not be as showy, their ability to separate coupled electrons and redistribute energy among them represents chemical and energetic control at the most fundamental level. Thus, via its modulation of the electronic structure of protein-bound flavins, life achieves enviable control using only mild and benign materials. Our intent in this review is to provide a springboard to calculations in these systems and we note that it is precisely the fact that the photochemistry is less dramatic that makes them more demanding, computationally, because smaller-energy and more subtle distinctions must be replicated. A very recent study on a bifurcating electron transfer flavoprotein demonstrates successful application of TD-DFT to distinguish flavins present in separate domains, based on their spectroscopic properties.[33] We anticipate that future studies will be able to inform not only on bifurcation, but also on the design of novel photonic molecules and even materials. Like flavins, these could leverage highly correlated extended π-conjugated systems including heteroatoms and H-bonding interactions that are exploited with such virtuosity, in proteins.

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CONFLICT OF INTEREST

The authors have declared no conflicts of interest for this article.

AUTHOR CONTRIBUTIONS

Rajiv Kar: Investigation; methodology; writing-original draft. **Anne-Frances Miller:** Writing-original draft; writing-review & editing. **Maria-Andrea Mroginski:** Methodology; writing-review & editing.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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