

Exploring the Light-Emitting Agents in *Renilla* Luciferases by an Effective QM/MM Approach

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Abstract

Bioluminescence is a remarkable natural process in which living organisms produce light via specific biochemical reactions. Among these organisms, *Renilla* luciferase, derived from the sea pansy *Renilla reniformis*, is notable for its blue light emission, making it an ideal candidate for bioluminescent tagging. Our focus lies at RLuc8, a variant with eight amino acid substitutions, recent research has shown that its emitter, coelenteramide, can exist in different protonation states, significantly influenced by proximal proton acceptor residues at the active site. Herein employing the Quantum Mechanical Consistent Force Field (QCFF/PI) method and the semi-macroscopic Protein Dipole-Langevin Dipole method with Linear Response Approximation (PDL/D/S-LRA), we show that the phenolate state of coelenteramide in RLuc8 is the predominant light-emitting species, corroborating experimental results. Our calculations also demonstrate that proton transfer from neutral coelenteramide to Asp162 is integral to the bioluminescence mechanism. Furthermore, our calculations accurately reproduce the observed emission maximum for the amide anion in RLuc8-D120A, which is responsible for chemiluminescence. In the case of RLuc8-D162A, we observed that the pyrazine anion, existing in the presence of a Na⁺ counterion, has an emission maximum consistent with experimental data, suggesting its primary role as potential emitter. This study not only showcases an effective way for investigating the bimolecular states of chromophores in light emission but also introduces an efficient approach that integrates the proton transfer process into the calculations of

the emission spectrum, proving vital for further research of proton transfer and light emission in photoproteins.

Introduction

Bioluminescence is a remarkable phenomenon in which living organisms emit light as a result of a specific biochemical reaction. In recent years, bioluminescent proteins have garnered considerable attention owing to their potential applications in a variety of fields, including biosensors, biothreat assessment, and notably, as a sought-after reporter gene to observe gene expression, promoter activity, post-translational regulation assays, as well as in the realm of protein engineering.^{1–6} Organisms with bioluminescence properties produce light through the oxidation of a substrate known as luciferin, a reaction that is catalyzed by a specific class of enzymes referred to as luciferases.^{2,7} Among the array of bioluminescent proteins, Renilla luciferase (RLuc) is particularly appropriate for its applications as bioluminescent tags.² Unlike other bioluminescent proteins that depend on ATP, these enzymes function without ATP reliance. Instead, they require only the substrate and molecular oxygen, setting them apart in the field of bioluminescence. This 36 kDa enzyme, derived from the sea pansy, *Renilla reniformis* catalyzes the oxidation of the substrate coelenterazine, an aromatic compound specifically characterized as 2-(p-hydroxybenzyl)-6-(p-hydroxyphenyl)-8-benzylimidazo(1,2-a)pyrazine-3-(7H)-one. During this catalytic process, the substrate undergoes an oxidative decarboxylation reaction in the electronic ground state (S_0). This leads to the opening of the imidazole ring, and eventually the release of carbon dioxide and coelenteramide, the light emitter in the first singlet excited state (S_1). The radioactive decay of this light emitter in the ground state give rise to the emission of blue light – a phenomenon known as bioluminescence^{8–11} (see Scheme 1).

Several bioluminescent proteins utilize coelenterazine as a substrate, and high-resolution crystal structures have been determined for calcium-stimulated photoproteins like aequorin and obelin.^{12–16} Within these proteins, coelenterazine, while not covalently attached, resides in a snug cavity. It interacts with multiple residues that contribute to the

efficiency of light emission and the modulation of its color. Typically, coelenteramide, the light emitter, is thought to be deprotonated at the 6-para hydroxyphenyl group (forming a phenolate ion), a process likely facilitated by a neighboring histidine residue (His22).^{14,16,17} This results in the emission of blue light in the range of 470-490 nm. However, if the emitter remains protonated, it emits a photon of purple light (405 nm).^{13,16,17} Additionally, earlier studies by McCapra *et al.*¹⁸ and Shimomura *et al.*¹⁹ have also explored the possibility of the amide anion formed as a result of chemiluminescence^{20,21} as the potential emitter. Consequently, coelenteramide is believed to exist in various bimolecular states, distinguished by their protonation states (either neutral or anionic) and the specific site of deprotonation (either phenolate or amide).

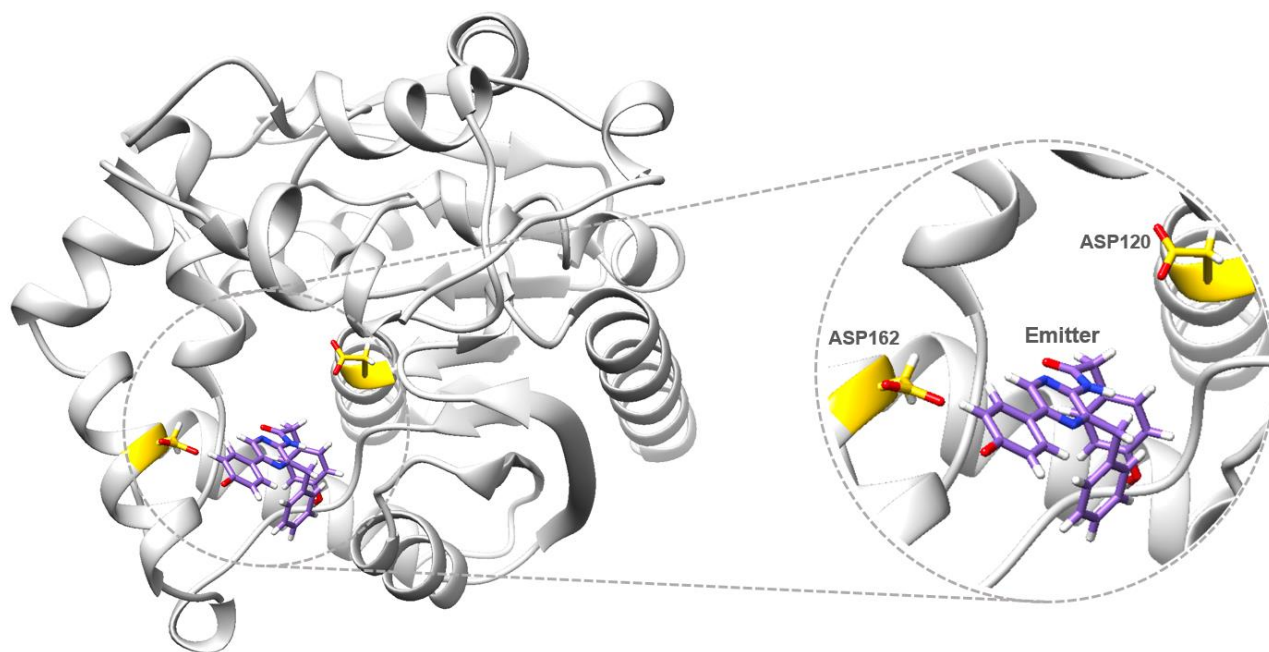
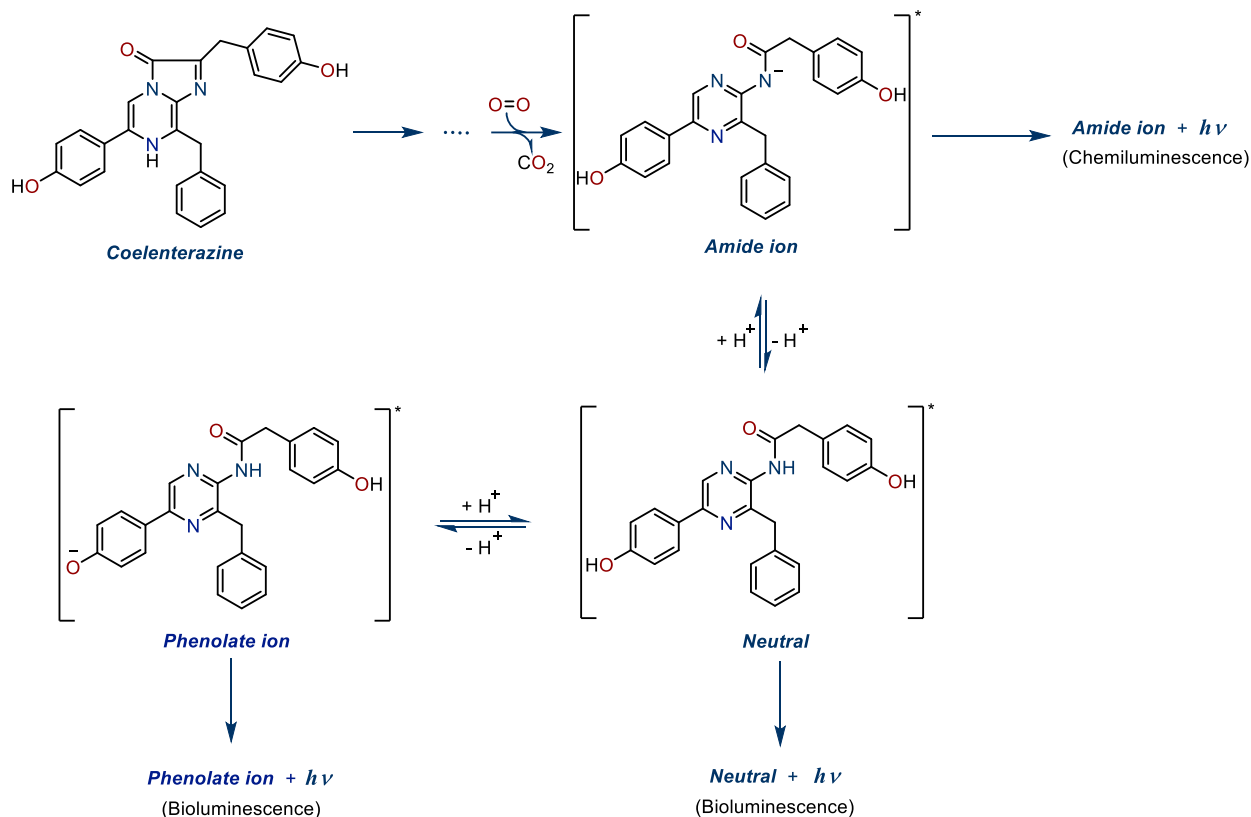


Figure 1. Schematic representation of RLuc8 (7OMR), with coelenteramide as emitter, and nearby Asp162, 120 residues in sticks.



Scheme 1. Bioluminescence and Chemiluminescence path showing coelenteramide light emitter in different protonation states. The asterisk indicates molecule in the excited state. The final product is relaxed to the ground state with the emission of light ($h\nu$).

Since RLuc utilizes the same substrate as the aequorin and obelin proteins, produces identical products, and emits light with similar spectral characteristics, the reaction mechanisms are likely analogous. However, understanding the detailed catalytic mechanisms and spectral properties of RLuc has proven to be a significant challenge, largely due to the scarce availability of structural data for substrate- and product-bound enzyme complexes. To address this issue, mutagenesis experiments were conducted to create mutants that are more conducive to crystallization. In one of the earlier investigations, Loening *et al.*²² determined the structure of a stabilized form of RLuc, which contains eight amino acid substitutions (RLuc8), both with and without the coelenteramide product bound. While the coelenteramide was found in one of these structures (PDB ID: 2PSJ), its location on the outer edge of the active site was interpreted as a nonproductive binding mode.²³ Recently, Damborksy *et al.*²⁴ co-crystalized

coelenteramide bound RLuc8 complex with a resolution of 1.9 Å (PDB ID: 2PSF) in the presence of excess of the substrate coelenterazine, providing biological relevant structural insights. In another recent study conducted by Marek *et al.*,¹¹ a crystal structure of the RLuc8-coelenteramide complex was obtained with a high-resolution of 1.5 Å (PDB ID: 7OMR). The authors of this work carried out a detailed mechanistic study for this complex, taking into account multiple co-crystal structures of the stabilized AncFT surrogate enzyme and RLuc8 luciferases, given their structural similarities. This study was backed by mutagenesis, experimental approaches, and molecular dynamics studies. Furthermore, they assigned the bioluminescence and chemiluminescence states of the coelenteramide emitter in RLuc8 and its mutants, which is the main focus of our work.

In a study by Marek *et al.*, the phenolate form of coelenteramide was identified as a potential emitter in RLuc8. This emission is hypothesized to be attributed to the deprotonation of the R2-6-(p-hydroxyphenyl) substituent, influenced by the nearby aspartic acid D162, which is only 2.9 Å away. On the other hand, the RLuc8-D162A and RLuc8-D120A mutants see the pyrazine and amide ion as light emitters, as shown in Scheme 1. This latter finding corroborates earlier research by Shimomura and Teranishi,²⁰ who investigated the luminescence of various biomolecular states of coelenteramide in different solvent polarities, and in the absence of the protein environment. While the X-ray crystallography method used in the above study has provided insights into the luminescence mechanism, it is crucial to understand the molecular details associated with the luminescent reaction. This is because X-ray crystallography captures only a single spatial structure at a specific moment in time, without considering the entire reaction coordinates. In particular the previous studies did not explore the relationship between the proton transfer coordinate to the emission spectra.

In this article, we have explored the biomolecular states of the chromophore and spectroscopic properties in RLuc8 and its mutants, also the proton transfer mechanism involve in the luminesce process. Utilizing the quantum mechanical consistent force field with perturbation interaction (QCFF/PI) along with the semi-macroscopic protein dipole-Langevin dipole (PDL/D/S) method, combined with the linear response approximation

(LRA), our findings affirm the significance of the phenolate and pyrazine states of coelenteramide in RLuc8 and its D162A mutant. These states, resulting from proton transfers of neutral emitter to D162 and the presence of counter ion respectively, are pivotal for consistent bioluminescence emission energy. Notably, our computed emission wavelength for the amide ion in D120A, due to chemiluminescence, matches closely with experimental results. Additionally, our approach offers a robust mechanism for integrating the energetics of the proton transfer in bioluminescence reactions. This is done by determining the free energy of the reaction landscape by assessing the pK_a differences of the donor-acceptor proton transfer (PT) process in both the excited and ground states using the PDL/D/S-LRA method (see section 2.2 and 3). This deprotonation energy is then added to the emission energy derived from the QCFF/PI (see section 2.1). This combined approach proves to be the most effective in studying the bioluminescence process that involve proton transfer.

2. Materials and Methods

2.1. The Quantum Mechanical Consistent Force Field (QCFF/PI) Method

Understanding the bioluminescence and chemiluminescence of the chromophore in protein environment requires a reliable QM/MM model to accurately map the excited state potential energy surface. While high-level ab initio calculations have been applied to study the spectroscopic properties of the coelenteramide emitter in the absence of protein environment, their application to studying RLuc8 with various protonation state of the emitter requires immense computational resources. A pragmatic approach is using a QM/MM variant of the semiempirical QCFF/PI method^{25–27} developed in our group, which we utilized in our prior studies. For our current study, we leveraged the QCFF/PI quantum mechanical potential surface^{26,28,29} combined with the protein force field of the ENZY MIX program interfaced in the MOLARIS-XG software suite.³⁰ Our previous studies have shown the success of this method, which integrates ENZY MIX³¹ and QCFF/PI, to investigate conjugated molecules in solutions or in the presence of protein environment. The program package and additional details are accessible on our website. An overview of our potential surface and simulation methodologies is provided below. For a deeper

understanding of the QCFF/PI method, the readers are referred to the specified references^{26–28,32}.

The combined QM/MM potential surface for the emitter's N^{th} electronic excited state within its protein active site is expressed as.

$$V^N(\mathbf{r}) = V_{\pi}^N(\mathbf{r}_S) + V_{\sigma}(\mathbf{r}_S) + V_{SS}(\mathbf{r}_S, \mathbf{r}_s) + V_{SS}(\mathbf{r}_s) \quad (1)$$

where S and s designate, respectively, the “solute” (i.e., the emitter) and the “solvent” which involves in our case the protein and its internal and surrounding water molecules. V_{π}^N is the π -electron contribution to the N^{th} excited state of the emitter, V_{σ} is the σ -electron contribution to the chromophore surface. V_{SS} is the potential that couples the protein (solvent) and the chromophore and V_{SS} is the protein force field of the protein and solvent system. The surfaces V_{π} and V_{σ} are obtained by the QCFF/PI method.^{26,28} This method uses ZDO (Zero Differential Overlap) approximation when all $\phi_a^*(1) \phi_b(1)$ are set to zero when $a \neq b$ in the frame of Pariser-Parr-Pople approximation assuming separation of σ and π electrons in V_{π}^N , while representing V_{σ} by an empirical force field, thus rendering the computation process more feasible, and enabling the depiction of conjugated heteroatomic molecules in their ground and excited π to π^* states by theoretically disentangling σ and π electrons. The QCFF/PI method is well documented and widely available.³² This method has been used extensively in studies of retinal and related systems^{28,33,34} and is more efficient than QM/MM approaches. Our study encompasses all coelenteramide atoms within the QM region. The QM treatment involves a configuration interaction treatment of all the single excitations for the three highest occupied and the three lowest unoccupied orbitals. The full parameters for our systems used are those from our earlier study.²⁶

The coupling potential between the solute and solvent, denoted as V_{SS} is derived using the method presented in detail in our prior studies.^{34,35} This integration involves including the electrostatic potential from the solvent (or protein) into the solute Hamiltonian, as explained in ref³⁴, using,

$$F_{\mu\mu} = F_{\mu\mu}^0 - \sum_k (q_k/r_{k\mu} + \mathbf{m}_k r_{k\mu}/r_{k\mu}^3) = F_{\mu\mu}^0 - U_{\mu}$$

$$F_{\mu\nu} = F_{\mu\nu}^0 \quad (2)$$

Here $F_{\mu\nu}^0$ is the corresponding element of the solute's SCF matrix and k spans over the solvent atoms. q_k and \mathbf{m}_k represent the residual charge and induced dipole on the k^{th} atom of the solvent. Overall, the second term in $F_{\mu\mu}$ captures the potential U_μ , on the μ^{th} atom from its environment. The induced dipoles are determined self-consistently and are an important part of our treatment. The solute charges are computed at each molecular dynamics (MD) step with the current \mathbf{U} vector components, mirroring the present solvent setup. Concurrently, the solvent molecules respond at each step to the solute charges in the electronic state whose surface drives the given MD simulation. The approach for handling solvent and protein-induced dipoles in excited-state calculations is modeled on the procedure from eq 17 in ref³⁴. Within this framework, we let the solvent's permanent and induced dipoles adapt to the charge distribution of the considered electronic state. It's important to highlight that no existing QM/MM method offers a completely consistent way of polarizing the solvent across every excited state, as discussed in ref³⁴. A perturbation approach, though suitable under certain conditions (refer to the Appendix in ref³⁴), doesn't yield results matching those from eq 17 in ref³⁴. In the latter, induced dipoles are polarized distinctively in each electronic state. Regardless, our approach to solvent-induced dipoles gives a more nuanced depiction of excited states within proteins and solutions.

The interaction term between the solute and solvent in Eqn. 2 encompasses the classical van der Waals interactions between solute and solvent atoms. The potential of the combined protein and solvent, V_{SS} , is characterized by the ENZYMIK force field. This includes the polarization constraints from the Surface Constrained All-Atom Solvent (SCAAS) model^{31,36} and the Local Reaction Field (LRF) approach for long-range interactions.³⁷ The reference system was immersed in a SCAAS sphere with a 20 Å radius. The surface-constrained water sphere was surrounded by a 10 Å surface of Langevin dipoles and then by a bulk.

Furthermore, to ensure accurate mapping of the excited-state potential energy surface in the emission energy calculations, we supplemented our approach with reference TD-DFT calculations for the chromophore in a water environment. We then adjusted the

QCFF/PI emission energy calculated within the protein environment by incorporating any differences observed between our QCFF/PI emission energy and the TD-DFT results in the water environment. The adjustment is quantified as follows:

$$\Delta E_{QCFF/PI}^{Total} = \Delta E_{QCFF/PI}^P + (\Delta E_{QCFF/PI}^W - \Delta E_{TD-DFT}^W) \quad (3)$$

In this equation, $\Delta E_{QCFF/PI}^{Total}$ represents the total emission energy in the protein environment. $\Delta E_{QCFF/PI}^P$ is the emission energy calculated within the protein environment using the QCFF/PI method. $\Delta E_{QCFF/PI}^W$ and ΔE_{TD-DFT}^W are the emission energies calculated in water using the QCFF/PI and TD-DFT methods, respectively.

The ionization states of the protein residues is determined using the PDL/D/S-LRA titration method (see section 2.2 for details) for the RLuc8 and its variants, remained consistent during the simulations. The simulation domain contained water molecules filling the available spaces and surrounding the protein. The system was first equilibrated by slowly heating from 5 K to 300 K for an overall relaxation of 200 ps, and then the QM/MM MD was executed for 10 ps in the first excited state with a time step of 0.5 fs at 300 K.

The ground and excited state charges of the chromophore were determined using the DFT method (detailed further below). Following this, we employed antechamber to obtain the RESP charges for the QM region (see Supporting Information for the full parameter).

2.2 The Semi-macroscopic protein dipole-Langevin dipole (PDL/D/S-LRA) method

The term ionization configuration (IC) is defined as the most favored combination of protonation states of ionizable residues in a protein at certain pH. It is crucial to have a knowledge of the protonation states of the residues around the emitter as it can significantly influence the spectroscopic properties of the emitter. The IC of the studied enzyme-emitter complexes were obtained by pK_a calculations using the Protein Dipole Langevin Dipole (PDL/D) method.³⁸ The pK_a of the i^{th} ionizable residue in protein can be calculated by Eqn. (4):

$$pK_{a_i}^p = pK_{a_{int,i}}^p + pK_{a_{int,i}}^{charges} \quad (4)$$

Where $pK_{a_{int,i}}^p$ refers to intrinsic pK_a of the i^{th} residue, while all the other residues are kept neutral; $pK_{a_{int,i}}^{charges}$ refers to change of the i^{th} residue due to the interaction with other ionizable residues. The intrinsic pK_a of the i^{th} residue can be represented as Eqn. (5):

$$pK_{a_{int,i}}^p = pK_{a_i}^w - \frac{\bar{q}_i}{2.3RT} \Delta\Delta G_{self,i}^{w \rightarrow p} \quad (5)$$

Where $pK_{a_i}^w$ refers to the $pK_{a_i}^w$ of the i^{th} residue in water; \bar{q}_i refers to the charge of the residue in its ionized form (+1 for cation, -1 for anion); $\Delta\Delta G_{self,i}^{w \rightarrow p}$ refers to the self-energy by moving the residue from water to the protein site. The second term in Eqn. (4) can be obtained by Eqn. (6):

$$\Delta pK_{a_i}^{charges} = -\frac{\bar{q}_i}{2.3RT} \sum_{j \neq i} \Delta G_{ij}^p \quad (6)$$

Where ΔG_{ij}^p refers to the interaction between the i^{th} and j^{th} residue in protein. The total energetics of the m^{th} combination of protonation state can be expressed by Eqn. (7):

$$\Delta G^m = -\sum_i 1.38q_i^m (pK_{a_{int,i}}^p - \text{pH}) + 1.66 \sum_{i,j \neq i} \frac{q_i^m q_j^m}{r_{ij} \epsilon_{ij}} \quad (7)$$

Where q_i^m and q_j^m refer to the i^{th} and j^{th} charge in the m^{th} charge configuration of the protein; r_{ij} refers to the distance between the centers of the i^{th} and j^{th} residues; ϵ_{ij} is the effective dielectric constant defined as Eqn. (8):

$$\epsilon_{ij} = 1 + 60(1 - e^{-0.1r_{ij}}) \quad (8)$$

Eqn. (7) can be used to calculate the partition function of the protein system, and hence the average charge of i^{th} residue as shown in Eqn. (9):

$$\langle q_i \rangle = \frac{\sum_m q_i^m \exp(-\beta \Delta G^m)}{\sum_m \exp(-\beta \Delta G^m)} \quad (9)$$

The average charge of all ionizable residues at certain pH is a good description of IC of a protein. To make the calculation practical and reliable, only ionizable residues within 15 Å of the binding pocket were included in the search of combinations of protonation

states of the protein. In the calculation of the total energetics, intrinsic pK_a 's of the most ionizable residues were assumed to be equal to their pK_a 's in water. For ionizable residue clusters containing the Asp120, Asp158 and Asp162, intrinsic pK_a calculations were done when the emitter was in the neutral form and applied to the following average charge calculations. The ionization configurations of enzyme-emitter complex were calculated separately for the 3 ionization forms of the emitter (i.e., phenolate, amide and pyrazine forms), assuming in each calculation only the neutral form and one of the charged forms of the emitter was considered in the calculations. The pK_a 's of aspartic acid and different forms of the emitter, both in the ground and excited states, were obtained by DFT calculations as described below.

The proton transfer (PT) mechanism involved in the bioluminescence process were obtained by pK_a calculations using the PDL method. The pK_a calculations for specific PT pairs should be considered together, which means in most of the calculations, pK_a of the PT residue was calculated while the emitter was in the neutral form. The exception was the PT correction for the RLuc8, in which pK_a (Asp162) was calculated while the emitter is in the charged phenolate form. In these calculations, the corresponding ionization configuration were used because the protonation states of nearby residues can affect the pK_a of interest significantly. In each calculation, pK_a value was obtained by averaging over 10 configurations sampled over 2 ps MD simulation with a 0.1 fs time step at 300K with a 0.3 kcal/(mol·Å²) constraints with respect to the corresponding initial structure.

For the reference pK_a in water (the first term in Eqn. (5)), we utilized DFT to determine the pK_a of the ionizable group in the emitter and the acid species in both the ground and first excited singlet states using Eqn. (10) shown below. For the ground state, all geometries, whether in the gas or solvent phase, were optimized using the CAM-B3LYP³⁹/Def2-SVP level of theory. For the excited state, TD-CAM-B3LYP was employed. The SMD solvation model⁴⁰ was applied for solvent phase calculations, and the GD3BJ method⁴¹ was used for dispersion correction. Electronic and thermal energies, calculated at 298.15 K, were determined using standard statistical thermodynamic methods, incorporating unscaled frequencies and the ideal gas, rigid rotor, and harmonic oscillator

approximations. The Gaussian 16 software suite⁴² was used to carry out all electronic structure calculations. The charges of the chromophore in the ground and excited state used in PDL and QCFF/PI calculations were obtained using the DFT method described above. From the resulting charges, we generated the restrained electrostatic potential (RESP)-fitted charges^{43–45} using AmberTools17.⁴⁶

The free energy of deprotonation (ΔG_{aq}) for the acid species in solution, given by, $HA + H_2O \rightleftharpoons H_3O^+ + A^-$ can be calculated using:

$$\Delta G_{aq} = \Delta G_g + \Delta G_{sol}(A^-) + \Delta G_{sol}(H_3O^+) - \Delta G_{sol}(HA) - \Delta G_{sol}(H_2O) \quad (10)$$

and the corresponding pK_a is given by,

$$pK_a = \frac{\Delta G_{aq}}{RT \ln 10} - \log[H_2O] \quad (11)$$

The concentration of bulk water is taken as 55.4 M. It has been shown that a potential source of error in Eqn. (10) is the solvation free energy of hydronium. To address this, a value of 4.54⁴⁷ is subtracted from the pK_a derived from Eqn. (11).

The DFT-calculated pK_a values for the ionizable amide, phenol, and acetic acid (which serves as a truncated model for aspartic acid) in both the ground and excited states are presented in Table S4 of the Supporting Information.

3. Results and Discussion

In our study, we utilized the high-resolution X-ray structure of RLuc8, which was complexed with the coelenteramide chromophore, to perform a QCFF/PI MD simulation on the first excited singlet state surface. It is important to note that in the X-ray crystal structure of RLuc8, the residue at position 162 is an alanine, resulting from a mutation of the native aspartate (referred to as RLuc8-D162A). Given that the emission spectra for the phenolate forms of the emitter have been experimentally reported in RLuc8, we reverted this alanine to its original aspartate (Asp) using a rotamer library within the Chimera software suite. Prior to analyzing the emission energy and spectra of the studied systems, we first examined rigorously the ionization states of the protein residues.

Emission Energy of Phenolate Anion

We began by identifying the ionizable protein residues in the proximity of the chromophore in its phenolate form within 15 Å, calculating their ionization states (pK_a) employing the PDL/D/S-LRA method. At a pH of 7, our calculations revealed 11 ionized residues, that are depicted in Figure 2. Ion pairs within the protein were ionized, while distant residues remained neutral. It is crucial to mention that for the phenolate form of the emitter in RLuc8, the proximate Asp162 residue is presumed to be protonated. This is under the assumption of a proton transfer (PT) between them on the first excited state surface, given that the distance between the oxygen atom of the 6-para hydroxyphenyl group of the emitter and the protonated Asp162 residue is 2.9 Å. This close proximity suggests the likelihood of a PT process and is discussed below.

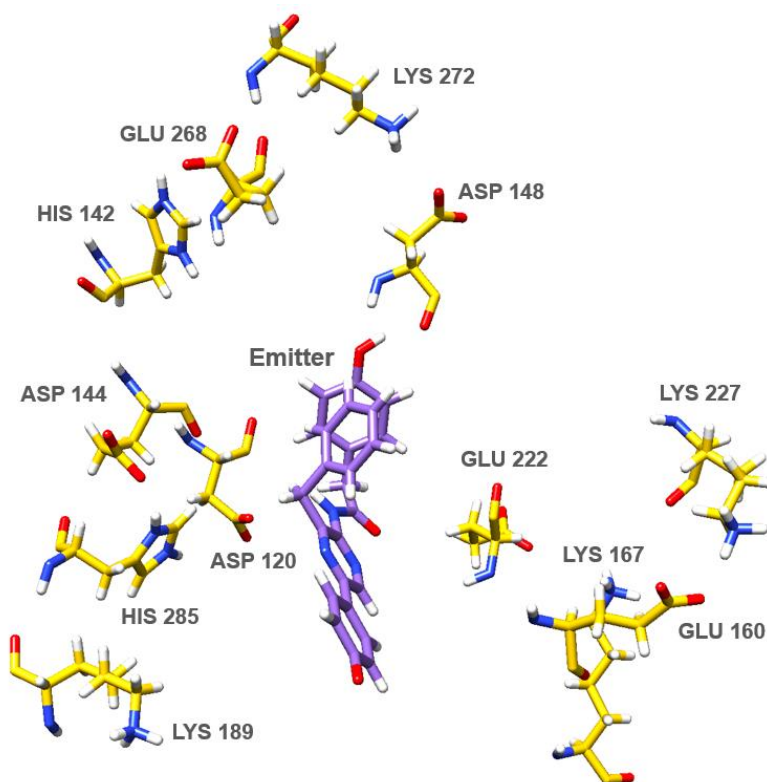


Figure 2. Assignment of ionization states to nearby ionizable residues of the chromophore in the phenolate form in RLuc8 at pH=7.

Upon establishing the ionization states, we proceeded with QM/MM MD simulations. The quantum mechanical (QM) component was characterized using the QCFF/PI approach to define the chromophore in excited states. The MD simulations consisted of 20,000 steps with a timestep of 0.5 fs at a temperature of 300K. We recorded the emission energies of both the isolated chromophore and the chromophore influenced by MM-derived charges. The collected data points (2000 in total) were distributed across 20 bins, ranging from the lowest to the highest emission wavelengths. We then calculated the emission energies (λ) and their corresponding relative intensities as a fraction of occurrences per bin, normalized against the total number of data points. The emission peak (λ_{max}) for the phenolate form in RLuc8, after correcting for the water reference value using Eqn. (3) (also refer to Table S6 for the reference emission energy in water), was determined to be 467 nm. This closely matches the experimentally observed value of 480 nm. Figure 3 displays the QCFF/PI vertical emission spectra. Additionally, we examined the neutral chromophore while keeping Asp162 unionized. Our calculations using the QCFF/PI approach yielded an emission energy of 395 nm. This finding is in good accordance with the reported emission energy of neutral coelenteramide (400 nm) observed in other bioluminescent proteins such as Ca^{2+} regulated photoprotein.^{14,16,17} However, this value is significantly different from the observed emission in RLuc8. This discrepancy, along with the strong agreement between the experimental and calculated emission maximum of the phenolate anion, emphasizes the importance of Asp162 protonation state. It confirms that in RLuc8, the coelenteramide emitter is indeed in the phenolate form during its photochemical and photophysical processes.

PT Mechanism

Using gas-phase TD-DFT calculations, we carried out a preliminary investigation to study the proton transfer (PT) process using the neutral emitter and acetate as a model system. We computed the emission energy at several fixed points along the PT reaction coordinate, which is defined by the transfer of a proton from the OH group of the phenolic fragment to the oxygen atom of the acetate (See Fig. 4). A significant red shift was

observed as the neutral emitter is converted to the phenolate form, indicating that the deprotonation process has a considerable impact on the emission properties.

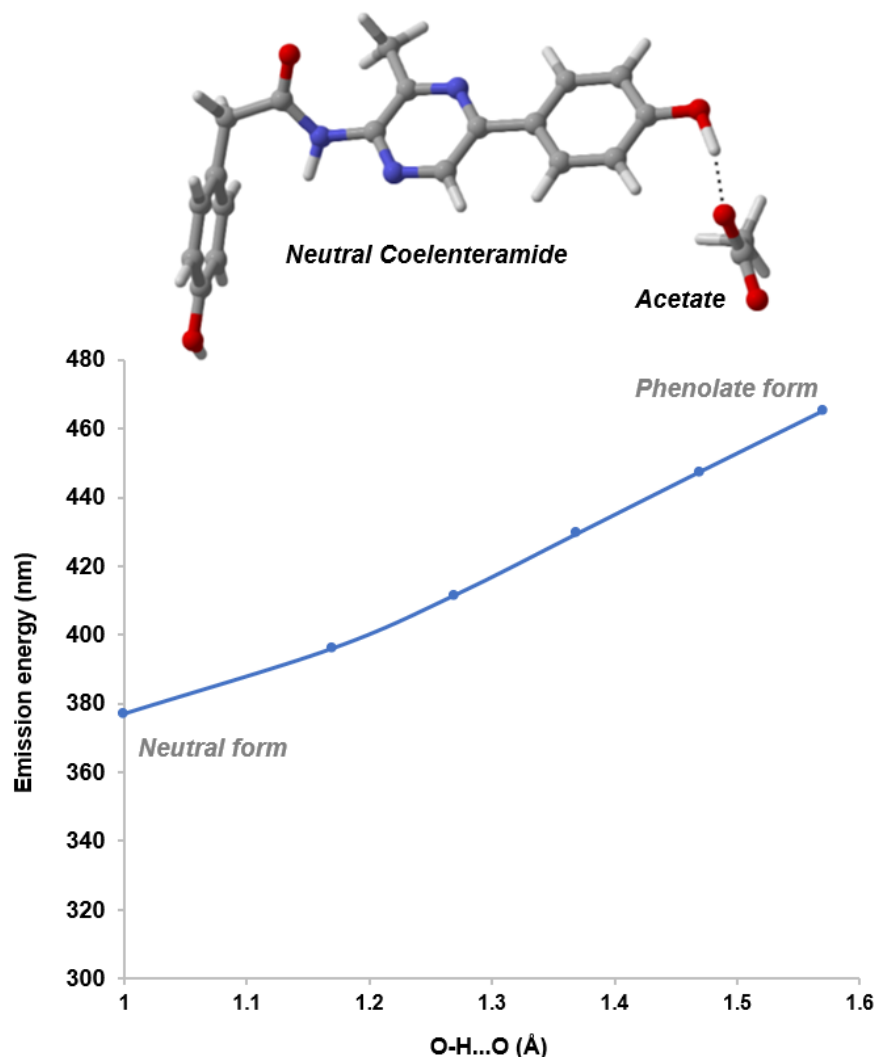


Figure 4. TD-DFT calculated emission maxima (nm) of the emitter transitioning from neutral to phenolate form at specific fixed points along the O-H bond breaking process.

Subsequently, we focused on evaluating the primary influence of the protein on the emission energy and the PT process between the neutral emitter and Asp162 by employing the PDL/D/S-LRA method. This required a meticulous analysis of the PT process across both the ground and excited state free energy surfaces (ΔG), and the

integration of the free energy change ($\Delta\Delta G$) between these surfaces into the QCFF/PI emission energy calculations, as illustrated in Figure 4. To delineate these free energy surfaces, we estimated the deprotonation free energy using the calculated pK_a values of the neutral emitter and Asp162 in the ground state (S_0) and the first excited singlet state (S_1), applying Equation (12):

$$\Delta\Delta G_{ES-GS} = 1.38 * [(pK_a(HA)^* - pK_a(B^{*-})) - (pK_a(HA) - pK_a(B^-))] \quad (12)$$

In this equation, $(HA)^*$ and HA represent the phenolic -OH group of the neutral emitter in the excited state (ES) and ground state (GS), respectively, while B^- and (B^{*-}) denote the proton acceptor Asp162 in the GS and ES, correspondingly.

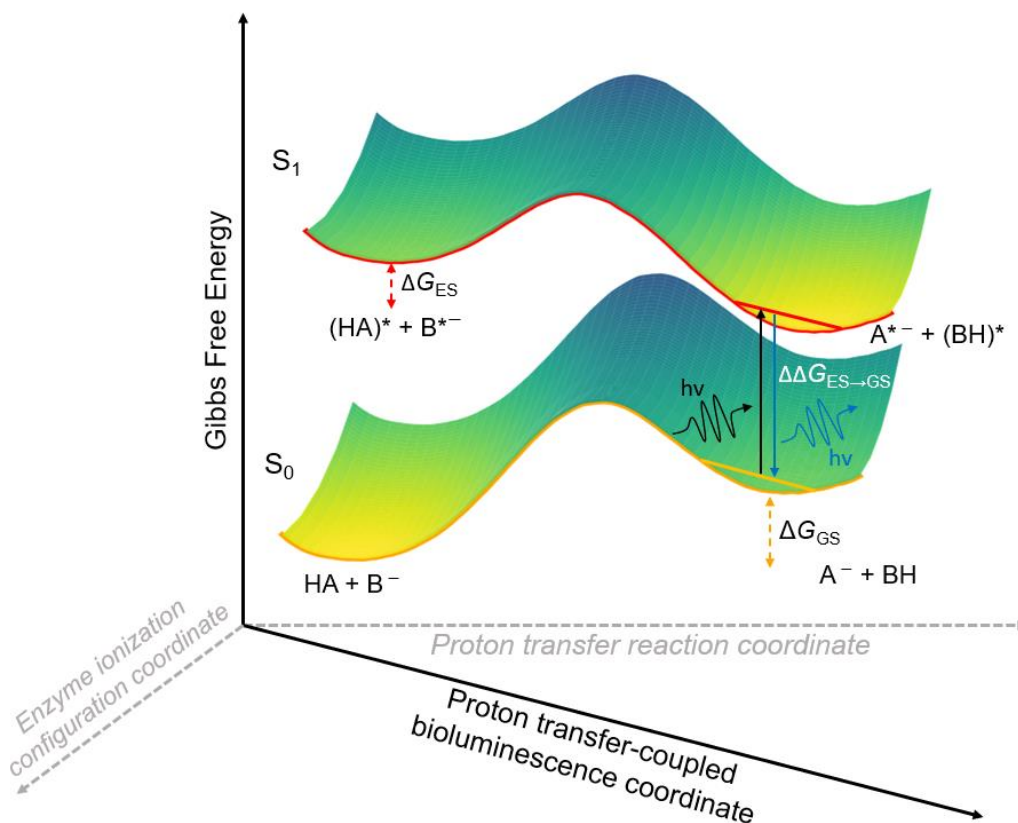


Figure 5. Proton transfer reaction profile in the ground (S_0) and excited (S_1) state. HA and B^- represent ionizable group of the emitter and proton acceptor (Aspartic acid in our case) in the ground state. The asterisk sign indicates species in the excited state (S_1). The PT process is based on ΔpK_a difference calculated using PDL/D/S-LRA (see Section

2.2). The $\Delta\Delta G$ term here represent the PT correction that is added to the emission energy in Eqn. 3. The Z-axis shows the ionization configuration of the protein environment, while the diagonal axis indicates the overall incorporation of PT reaction coordinate during the bioluminescence.

Our consensus PDL/D/S-LRA calculated values for the emitter and D162 at pH 7 in RLuc8 are 7.6 and 4.9, respectively, in the GS, and -0.8 and 5.1 in the ES. Utilizing Eqn. 12, this translates to reaction free energies (ΔG) of +3.7 kcal/mol in the GS and -8.1 kcalmol⁻¹ in the ES, signifying that the PT reaction is energetically more favored in the ES. It should be noted that our analysis focuses solely on thermodynamic aspects, and the depicted barrier in Figure 5 serves an illustrative purpose only. Nevertheless, the PT process in the ES is typically associated with an energy barrier of a few kcal/mol, as observed in other bioluminescent proteins.^{48,49} To estimate the emission energy during the PT reaction, we incorporated the free energy change ($\Delta\Delta G$) into our calculated QCFF/PI energy for the neutral emitter. This yielded an emission value of 472 nm, which is in excellent agreement with the observed value of 480 nm, and closely matches with our calculated emission energy of 467 nm obtained for the phenolate ion, thus providing strong evidence of the excited-state PT reaction in RLuc8 and that the emitter exclusively is in phenolate form.

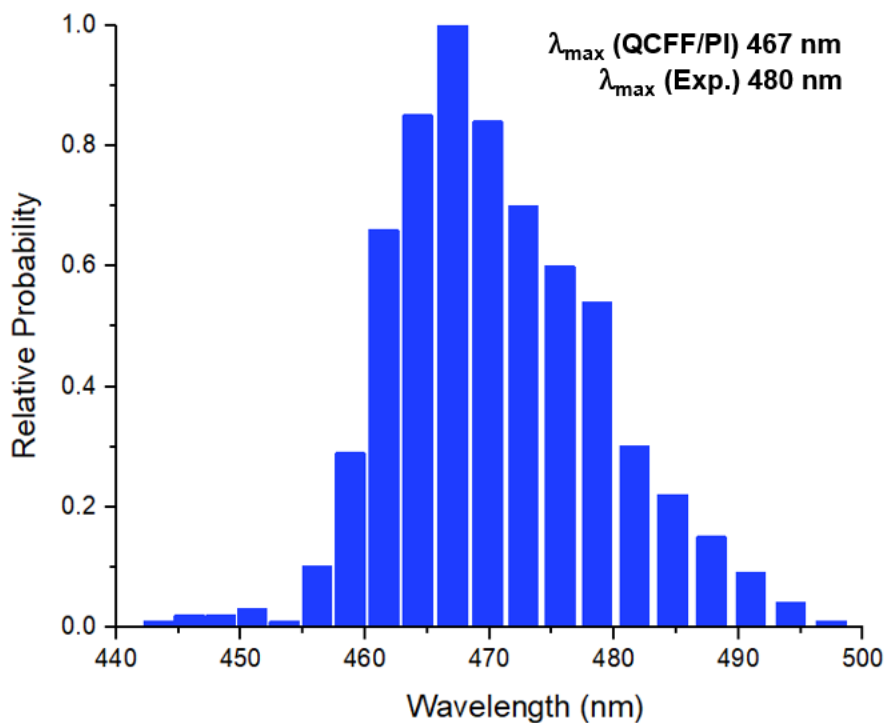


Figure 6. Calculated probability profiles for the emission energies transition from first excited singlet state to the ground state. The profile was obtained by running trajectories on the excited state surfaces of the phenolate as emitter (when Asp162 is protonated) and evaluating the histograms of the calculated emission energies.

Emission Energy of the Amide anion

In the chemiluminescence mechanism, the excited state of the amide anion is transiently produced and is favored over the neutral or phenolate forms, as outlined in the established reaction pathway depicted in Scheme 1. This particular emitting species is crucial for chemiluminescence, where the excitation is driven by chemical energy. Specifically, in the RLuc8-D120A mutant of *Renilla* Luciferase, an emission peak corresponding to this amide anion species has been observed.

Complementing these findings, our PDLT titration calculations indicate that the ionization states of the amino acids in the RLuc8-D120A variant closely resemble those in the RLuc8, with the notable exceptions being the un-ionized state of His142 and the

ionized state of Asp162 (refer to Table S2 in the Supporting Information for full ionization configurations). This suggests that, preceding the transition of the amide ion to a neutral emitter via a PT process (see Scheme 1), Asp162 remains in its ionized form. The un-ionized state of His142 implies a more intricate network of PT processes potentially involving nearby residues or the bulk solvent, as the emitter transitions from the amide to the neutral or phenolate form.

Moving on to our findings for the emission energy of RLuc8-D120A mutant, the calculated emission spectra (Figure 7) has a maximum at 456 nm. The position of this maximum represents a modest red shift of 26 nm, which aligns well with the experimentally observed value of 430 nm. Notably, our calculated value is consistent with the luminescence results obtained by Shimomura and Teranishi,¹⁹ who reported an emission maximum for the amide-deprotonated emitter in the range of 435–458 nm. The correlation between the calculated emission energy and experimental data strongly suggests that the amide ion is the principal luminescent species in this mutant. This is attributed to the Asp120 position being unoccupied by aspartate, which precludes the usual proton transfer, making the formation of neutral or phenolate emitters unfavorable.

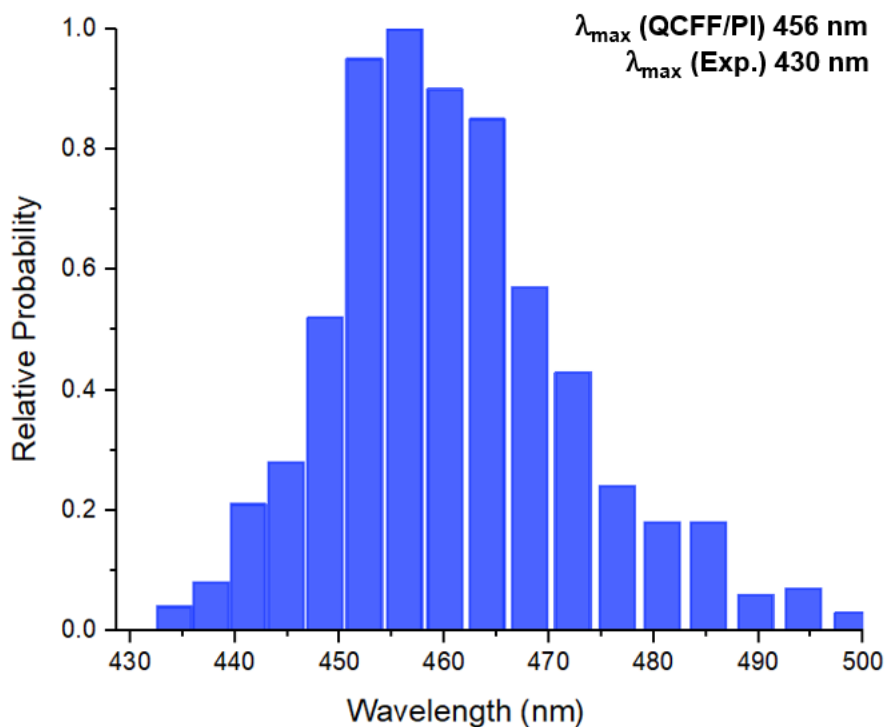
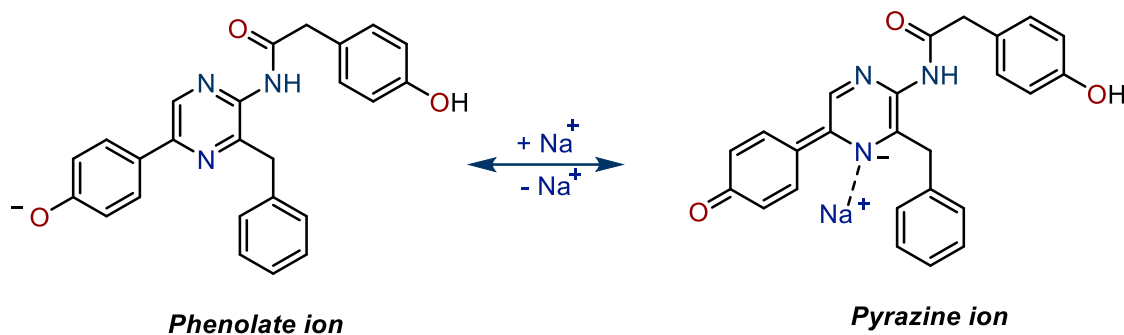


Figure 7. Calculated probability profiles for the emission from the first excited singlet state to the ground state in RLuc8-D120A. The profile was obtained by running trajectories on the excited state surfaces of the amide as emitter and evaluating the histograms of the calculated emission energies.

The emission Energy of The Pyrazine anion

We next explored the pyrazine anion as a potential emitter, as identified for RLuc8-D162A. Previous research by Shimomura and colleagues¹⁹ demonstrated that in various solvents and with alkali metals, the phenolate ion can transition to a pyrazine ion, a resonance structure of phenolate. However, this experiment was observed in the absence of protein environment. In the recent study of RLuc8-D162A,¹¹ the experiment was conducted in 100 mM potassium phosphate buffer, providing a high concentration of potassium ions and therefore the resonance structure of phenolate emitter might form due to the presence of this alkali metal ion. The potassium ion thus acts as a counter ion near the pyrazine ring, creating a polarized N⁻...K⁺ bond. This interaction subsequently

leads to the phenolate oxygen transitioning to a keto form, a process driven by charge delocalization (refer to Scheme 2).



Scheme 2. Resonance form between phenolate and pyrazine ion. For our system we employed Na^+ as counter ion in the formation of pyrazine anion.

We modeled the pyrazine ion with an explicit Na^+ positioned 2.3 Å from the pyrazine ring's nitrogen. The formation of pyrazine ion necessitates the deprotonation of the OH group, initially yielding a phenolate ion, which then undergoes resonance shifting to the pyrazine ion (as depicted in Scheme 2). Examining the X-ray crystal structure of RLuc8-D162A reveals that the nearest proton accepting residue Asp158 is located 4.9 Å from the oxygen of phenolic group of the emitter. This donor-acceptor gap is considerably lengthy for efficient proton transfer, hinting at an unfavorable process. The deprotonation of the phenolic group could potentially occur to the bulk solvent, or through a Grotthuss-type water-mediated proton transfer involving the phenolic group of the emitter and Asp158. However, these possibilities are not within the purview of the current study and warrant further investigation.

Moving on to the QCFF/PI emission energy of the pyrazine ion in RLuc8-D162A, our calculated emission maximum yield a value of 552 nm, slightly red-shifted by 32 nm, but in good agreement with experimental value of 520 nm, thus indicating the existence of pyrazine ion in this variant. While our emission maximum reproduces closely the corresponding observed value, the experimental study did also report a smaller peak at 400 nm, which was absent in our simulated spectra (refer to Fig. 8). This discrepancy

may be attributed to additional structural changes in the emitter within the protein environment, possibly involving another π to π^* transitions. Prior experimental studies¹⁹ conducted in the absence of protein environment have demonstrated that the presence of a counter ion can toggle the biomolecular state of the emitter, resulting in two distinct peaks. The higher peak corresponds to the pyrazine anion, while the lower peak is linked to the amide anion. Further investigation is necessary to explore the significance of these intriguing findings within the protein environment. Additionally, considering that the experiment used a higher concentration of potassium phosphate buffer solution as mentioned above, further experimental studies focusing on the impact of buffer solution concentration effect on the emission spectra could provide insights into the biomolecular state of the emitter in RLuc8-D162A.

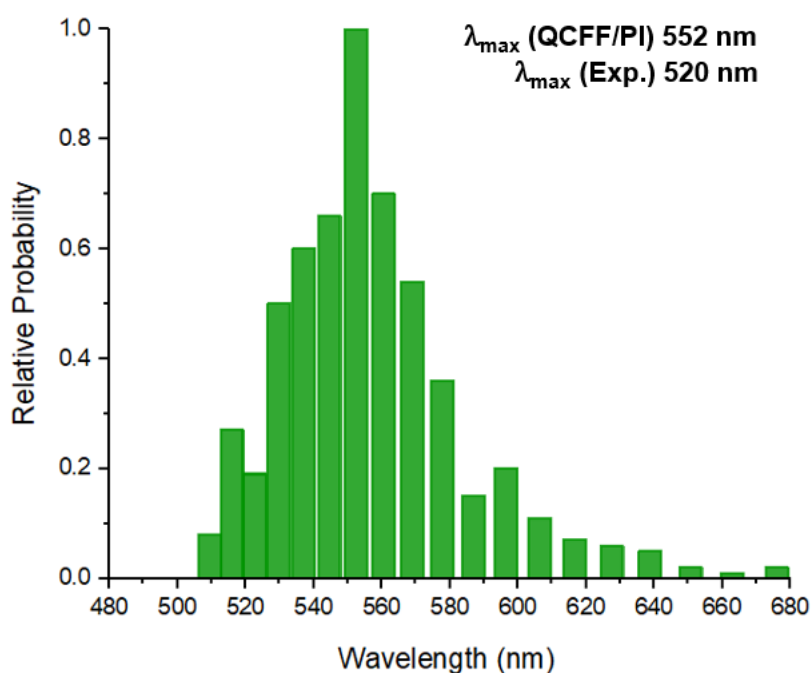


Figure 8. Calculated probability profiles for the emission energies of pyrazine anion transition from first excited singlet state to the ground state for RLuc8-D162A.

4. Conclusion

In this study, we investigated the origins of bioluminescence and chemiluminescence in *Renilla* Luciferase mutants. Our findings reveal that in RLuc8, the chromophore (coelenteramide) predominantly exists in its phenolate form when the nearby Asp162 is protonated. This conclusion is supported by our QCFF/PI calculations, which matches closely the experimental values of the emission maxima. Furthermore, we studied the proton transfer (PT) process that leads to the formation of the primary emitter in the RLUC8's bioluminescent reaction, utilizing both PDL/D/S-LRA and QCFF/PI methods. Our calculations demonstrate that incorporating the deprotonation reaction energy between the neutral emitter and Asp162 in both the excited and ground states into the emission energy calculations accurately replicates the emission maximum of the phenolate ion. This indicates strongly that Asp162 serves as a potential proton acceptor, making the phenolate ion the dominant emitting species in RLUC8.

This study also sheds light on an important aspect of the PT process i.e., its dependency on the proton transfer rate. In certain bioluminescent organisms like D-luciferin,^{48,49} the PT is known to take place in picoseconds, while the fluorescence emission takes place over nanoseconds. Considering that Asp162 in RLuc8 is merely 2.9 Å away from the hydroxyl group of the emitter, it provides the shorter pathway for PT. This proximity results in the neutral emitter being a transient species, leading to the formation of the phenolate anion. Consequently, this might explain why the emission maximum of the neutral emitter in RLuc8 was not experimentally detected.

Additionally, our calculations also provide theoretical evidence supporting the chemiluminescence mechanism in the RLuc8-D120A mutant, where the light emitter is in its amide form. The emission energy of this form aligns well with experimental findings. Moreover, our investigation into the bioluminescence of the RLuc8-D162A mutant reveals that the presence of a counter ion (Na⁺) is essential for the formation of the pyrazine anion, with its maximum emission energy closely matching observed values. However, our method did not replicate the second, lower emission energy peak reported for RLuc8-D162A. This peak might be associated with another biomolecular state of the emitter that our approach did not capture, suggesting a need for further investigation. More

sophisticated theoretical approaches will be required to fully understand and assess the significance of these intriguing results. Overall, our methods offer an efficient means to study the bioluminescence and chemiluminescence properties, as well as the proton transfer (PT) mechanism, in a manner that is considerably less resource-intensive than conventional quantum mechanics/molecular mechanics (QM/MM) methods and proves to be a valuable tool for studying PT processes in the luminescent reactions of photoproteins.

Notes

The authors declare no competing financial interest.

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