Understanding the FMN cofactor chemistry within the *Anabaena* Flavodoxin environment

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**A B S T R A C T**

The chemical versatility of flavin cofactors within the flavoprotein environment allows them to play main roles in the bioenergetics of all type of organisms, particularly in energy transformation processes such as photosynthesis or oxidative phosphorylation. Despite the large diversity of properties shown by flavoproteins and of the biological processes in which they are involved, only two flavin cofactors, FMN and FAD (both derived from the 7,8-dimethyl-10-1’-D-ribityl)-isoalloxazine), are usually found in these proteins. Using theoretical and experimental approaches we have carried out an evaluation of the effects introduced upon substituting the 7- and/or 8-methyls of the isoalloxazine ring in the chemical and oxido-reduction properties of the different atoms of the ring on free flavins and on the photosynthetic *Anabaena* Flavodoxin (a flavoprotein that replaces Ferredoxin as electron carrier from Photosystem I to Ferredoxin-NADP+ reductase). In *Anabaena* Flavodoxin both the protein environment and the redox state contribute to modulate the chemical reactivity of the isoalloxazine ring. *Anabaena* apoflavodoxin is shown to be designed to stabilise/destabilise each one of the FMN redox states (but not of the analogues produced upon substitution of the 7- and/or 8-methyls groups) in the adequate proportions to provide Flavodoxin with the particular properties required for the functions in which it is involved in vivo. The 7- and/or 8-methyl groups of the isoalloxazine can be discarded as the gate for electrons exchange in *Anabaena* Fld, but a key role in this process is envisaged for the C6 atom of the flavin and the backbone atoms of Asn58.

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1. Introduction

Flavodoxins (Flds) are flavin-dependent proteins participating in a large number of electron transfer (ET) reactions in the main energy transformation processes in prokaryotic organisms and certain algae [1,2], while in eukaryotic proteins containing Fld-like modules are implicated in key metabolic functions [3,4]. The FMN cofactor confers these electron transferases the ability to act as low-potential electron carriers in one-ET processes. This is a consequence of its isoalloxazine ring being able to exist in three different redox states within the protein environment; oxidised (ox), neutral semiquinone (sq) and anionic hydroquinone (hq), characterised by a different number of electrons and protons. The isoalloxazine establishes several non-covalent interactions with the apoflavodoxin (Apofld) moiety, and upon interaction, the FMN midpoint reduction potentials for the two semi-reactions, $E_{\text{ox/sq}}$ and $E_{\text{sq/hq}}$, result inverted and well-separated, allowing for the stabilisation of the protonated one-electron reduced state, the otherwise thermodynamically unstable neutral semiquinone [5,6]. The stabilisation of this state allows flavoproteins in general, and Fld in particular, to mediate obligatory processes of a single electron with those of two-electrons, being therefore key intermediares in the photosynthetic chain of several algae and cyanobacteria, as well as in many other metabolic processes involving energy transformation in all type of organisms. Stretches of aminoacids 56–59 and 93–95 of Apofld (*Anabaena* Fld (AnFld) numbering used throughout the text) contribute to the interaction with the isoalloxazine ring and to tune the midpoint reduction potentials [7–16]. In AnFld the Asn58-Ile59 peptide bond is hypothesised to flip between an “O-Down” conformation, in the oxidised and the hydroquinone states, and an “O-Up” one in the semiquinone, by changing its H-bond network with the flavin N5 (as shown in the highly homologous Fld from *Anacystis nidulans* (AyFld)) (Fig. 1) [17]. The fact that the semiquinone state is less stable in AnFld and AyFld than in other species correlates with: i) the presence in AnFld and AyFld of a H-bond between the
un-protonated N5 of the flavin and the amine group of residue 59 that is not observed in other Fl霸道, and ii) the H-bond established between the N5H and the carbonyl of Asn58 being weaker in AnFld霸道 and AyFld霸道 than those formed in Flds with smaller side-chains at this position[6,15,17]. However, despite the “O-Down” and “O-Up” flip provides with a versatile device to modulate midpoint reduction potentials and chemical reactivity within the flavin ring, in AnFld this structural rearrangement still needs to be structurally proved[14–16].

The 7- and 8-methyls of FMN are the only portions of the isalloxazine ring accessible to the solvent in Flдов. This confers particular mechanistic interest on these positions because of the role they might play in the interaction with protein partners, and, particularly, as possible gates for electrons entering and leaving the flavin ring[18]. Taking advantage of the reversibility of the Flm:ApoFld complex[15,19], we experimentally produced AnFld variants where the 7- and/or 8-methyls of FMN were replaced by chlorine and/or hydrogen. These variants are able to interact, and even to exchange electrons, with the AnFld physiological partners, Photosystem I (PSI) and Ferredoxin-NADP⁺ reductase (FNR), but their midpoint reduction potentials are less negative than expected (Fig. SP1)[20]. The latter observation suggests the chemical reactivity of the avin ring might also contribute to modulate the effects induced in the isalloxazine properties by the protein environment. Here, we analyse the chemical and structural changes produced by the substitutions in the different oxido-reduction states of the isalloxazine, either free or bound to AnApoFld, by combining experimental and theoretical approaches. These results contribute to better understand the flavin reactivity within the protein environment.

2. Materials and methods

2.1. Biological material and FMN analogues

Wild-type (WT) AnFld was over-expressed in E. coli and purified as described[21]. Its ApoFld was prepared by treatment with 3% trichloroacetic acid at 4 °C in the presence of dithiothreitol. The precipitated apoprotein was separated from FMN by centrifugation and dissolved in 500 mM MOPS pH 7.0 before dialysis against 50 mM MOPS pH 7.0. 8-nor-CI-FMN, 7,8-nor-7,8-CI-FMN, and 7-nor-7-CI,8-nor-FMN and 7-nor-8-nor-8-CI-FMN (herein 8-CI-FMN, 7,8-diCI-FMN, 7-CI,8-H-FMN, 7-H,8-CI-FMN) were produced from the corresponding riboflavin analogues as previously described[20].

2.2. Determination of the free binding energy for the flavin:ApoFld complexes of the FMN analogues in the different redox states

Dissociation constants (Kd) for the FMNox:ApoFld complexes were determined fluorometrically by following the quenching of the flavin fluorescence upon titration with ApoFld in 50 mM MOPS pH 7.0, at 25 °C (Fig. SP2). In a typical experiment, 1 ml of ~200 nM of the FMN analogue was titrated with aliquots of 10–20 μM ApoFld. After each addition, the system was allowed to equilibrate for 2 min. Excitation was at 445 nm and emission was monitored at 525 nm. Fitting of the experimental data to the theoretical equation for a 1:1 complex, as previously described, allowed the calculation of Kd for the FMNox:ApoFld complex and the starting concentration of flavin (Cf) [15,22]. Standard deviation between replicates during determination

Fig. 1. Details of the isalloxazine binding site in Flдов. Conformational changes associated with the reduction of AyFld. Detail of the FMN (coloured in orange CPK) environment that shows: (A) “O-Down” conformation in AyFld霸道 (pale green, PDB 1CZU). (B) “O-Up” conformation in AyFld霸道 (blue, PDB 1CZL). (C) “O-Down” conformation in AyFld霸道 (green, PDB 1D04). For the purpose of comparison (A) includes superimposed AnFld霸道 (pink, PDB 1FLV), while (B) and (C) include AyFld霸道. Arrows indicate positions of observed displacements. (D) Definition of the QM region (atoms in orange) used for the QM/MM calculations. (E) Detail of the main interactions stabilising the isalloxazine ring in the AnFld environment.
of K_{ds}, including numerical error after fitting analysis, were within 10% of the media value (Table 1). The free energy for the formation of the FMN_{ox}:ApoFld complex (ΔG_{ox}) was obtained directly from the experimental K_{ds} with an error below ±0.2 kcal/mol (in general larger than error propagation for each individual variant). Free energy values for FMN_{ox}:ApoFld (ΔG_{sq}) and FMNH_{sq}:ApoFld (ΔG_{hq}) were calculated from the equations derived from a thermodynamic cycle (Fig. S3A) [15,22,23]:

\[
\Delta G_{sq} = \Delta G_{ox} - F \left( E_{ox/sq} - E_{sq/sq} \right)
\]

(1)

\[
\Delta G_{hq} = \Delta G_{sq} - F \left( E_{sq/hq} - 2E_{m} \right)
\]

(2)

where F is the Faraday's constant, E_{ox/sq} and E_{sq/hq} are the previously reported semi-reduction midpoint potentials for the different constituted Fld variants (data from Table 1 in [20] with an error within ±5 mV of the given value), and E_{sq/sq} is the semi-reduction midpoint potentials for the free FMN form at pH 7.0. For the different FMN analogues only midpoint reduction potentials for the ox/hq couple, E_{m} are, were known [24,25], being their E_{ox/sq} and E_{sq/hq} values calculated using the equations

\[
E_{ox/sq} = E_{sq/sq} / 2 = E_{m}
\]

(3)

\[
E_{sq/hq} = 2.303 RT / F \log K
\]

(4)

In all cases the semiquinone formation constant (K) has been assumed the same as that determined for FMN using pulse radiolysis (0.000022) [26]. Determination of this value is not trivial and even for free FMN it has only been measured with low accuracy [27]. Therefore, errors in the determined E_{ox/sq} and ΔG_{sq} are, and, particularly, in E_{sq/hq}, are, difficult to quantify. ΔG_{hq} is not affected by the value of K and was calculated below ±0.3 kcal/mol for all the variants.

2.3. Production of structural models for AnFlds reconstituted with FMN analogues in the oxidised, semiquinone and hydroquinone states

Starting in silico AnFld_{ox} and AnFld_{sq} models were generated by replacing the FMN_{ox} cofactor in the three-dimensional structure of AnFld_{ox} (PDB code: 1FLV) with each of the corresponding FMN_{ox} or FMNH_{sq} analogues, since the “O-Down” conformation is expected for both states. The starting model for AnFld_{sq} was produced in the “O-Up” conformation by using the AnFld sequence, the GENO-3D platform [28] and the crystal structure of AyFld_{sq} (PDB code: 1CZL, “O-Up” conformation) as template. Then, each of the FMN_{sq} analogues was situated in the model generated for AnApofld_{sq} at the position of FMN_{sq} in AyFld_{sq}.

Quantum mechanics/molecular mechanics (QM/MM) molecular dynamics (MD) simulations were performed with AMBER 9.0 [29], using the amber94 force field and the semiemipirical method Austin Model 1 (AM1) [30]. The QM subsystem included the lumiflavin (LM) ring of each Fld. The QM/MM interface was handled by including a link atom between the C1’ and C2’ atoms of the FMN ribityl chain (Fig. 1D) [31]. Each molecular system was neutralised by the addition of sodium ions, and solvated with the TIP3P water model in a cubic box. The cut-off distance for the non-bonded interactions was 10 Å. Solvent molecules and counter ions were relaxed and allowed to redistribute around the restrained protein molecule by minimisation with 1000 steps of Steepest Descent (SD) and 2000 steps of Conjugate Gradient (CG), while the protein atoms were constrained with a harmonic force constant of 500 kcal/mol Å². A second energy minimisation was carried out with 2000 steps of SD and 3000 steps of CG also allowing protein relaxation. The resulting system was heated from 0 K to 300 K at constant volume with the protein atoms constrained with a harmonic force constant of 10 kcal/mol Å². The system was then equilibrated during 100 ps at 300 K by using a Langevin temperature equilibration algorithm at 1 atm with periodic boundary conditions, and the Particle Mesh Ewald method to treat long-range electrostatic interactions. Production runs consisted of 3–4 ns of MD with 2 fs steps. Temperature was kept at 300 K using a Berendsen constant temperature algorithm. The pressure was kept at 1 atm using a weak-coupling pressure algorithm. System coordinates were collected every 2 ps. During equilibration and simulations the leapfrog Verlet integration scheme and the SHAKE algorithm were used [32]. Three-dimensional structures and trajectories were visually inspected using PyMOL and VMD [33,34]. Interatomic distances and angles, as well as the root mean square deviation from a given structure, were monitored using PTRAJ [29].

2.4. Gas-phase calculation of the structures for the different free analogues

The LM form was chosen as a model in the investigation of the electronic properties of the free flavin ring in the different redox states. Calculations were carried out with Gaussian 03 [35]. The structures of all the LM analogues were optimised using the hybrid B3LYP functional and 6–31G* basis set [36,37].

2.5. Determination of reactivity indices

Chemical reactivity indices arising from density functional theory were calculated for the different redox states of the flavin variants [38]. These indices include: the electronic chemical potential (μ), a measure of the directions of charge transfers during a chemical reaction; the hardness (\(\eta\)), a measure of the system resistance to exchange electronic charge; the softness (S), a measure of the system polarisability; and the electrophilicity (\(\omega\)), an indicator of the stabilisation energy of the system when obtaining electronic charge from the environment. An approach using the method of finite differences allowed calculating their global values in the flavin ring in terms of the ionisation potential, I, and of the electron affinity, A, [38–40].

\[\mu \approx (I + A) / 2\]

\[\eta \approx (I - A) / 2\]

\[S \approx I / 2\eta\]

\[\omega \approx \mu^2 / 2\eta\]

Table 1: Dissociation constants for the FMN_{ox}:ApoFld complexes and free energies for the formation of the corresponding oxidised, semiquinone and hydroquinone complexes. Data obtained in 50 mM MOPS pH 7.0 at 25 °C.

<table>
<thead>
<tr>
<th>Flavin form</th>
<th>(K_d) (nM)</th>
<th>ΔG_{ox} (kcal/mol)</th>
<th>ΔG_{sq} (kcal/mol)</th>
<th>ΔG_{hq} (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FMN</td>
<td>0.7 ± 0.06</td>
<td>-12.48</td>
<td>-15.23</td>
<td>-7.62</td>
</tr>
<tr>
<td>8-Cl-FMN</td>
<td>0.69 ± 0.06</td>
<td>-12.49</td>
<td>-15.35</td>
<td>-8.71</td>
</tr>
<tr>
<td>7,8-dCl-FMN</td>
<td>0.76 ± 0.02</td>
<td>-12.43</td>
<td>-15.71</td>
<td>-11.05</td>
</tr>
<tr>
<td>7-HCl-FMN</td>
<td>0.34 ± 0.08</td>
<td>-12.91</td>
<td>-15.72</td>
<td>-8.25</td>
</tr>
<tr>
<td>7-ClH-FMN</td>
<td>0.85 ± 0.07</td>
<td>-12.37</td>
<td>-15.43</td>
<td>-9.07</td>
</tr>
</tbody>
</table>

a Determined from fluorometric titrations of the different FMN_{ox} analogues with ApoFld. Standard deviation in the experimental determination of \(K_d\) is indicated.

b Calculated from data in and \(\Delta G_{ox} = -RT \ln K_d\). Errors below ±0.2 kcal/mol, a value in general larger than error propagation for each individual variant.

c Calculated as described in Eq. (1) [22], with \(E_{ox/sq}^{\text{free}}\) and \(E_{sq/sq}^{\text{free}}\) values from [20]. Errors difficult to quantify due to assumptions in the \(K_d\) constant.

d Calculated as described in Eq. (2) [22], with errors below ±0.3 kcal/mol.
where I and A are obtained from the electronic energy calculations on the N−1, N and N+1 electron systems.

\[ I = E_{N-1} - E_N; \quad A = E_N - E_{N+1} \]  

(7)

Local reactivity of the flavin atoms was predicted using the Fukui function \( f(r) \). Their electrophilic \( f^+ \), nucleophilic \( f^- \) and radical \( f^0 \) components provide an indication of the preferred sites for an attack by an electrophilic, a nucleophilic, and a radical agent, respectively. A highly different value between the electrophilic and nucleophilic local indices is associated with a high reactivity at that point in the molecular regions. Fukui functions of free LM analogues were obtained using an algorithm described by Contreras et al. [41]. For Flds, an approximation was used and Fukui functions for their FMN molecules were determined in terms of atomic charges according to [42]:

\[ f^+_k \approx q_k(N) - q_k(N + 1) \]  

(8)

\[ f^-_k \approx q_k(N-1) - q_k(N) \]  

(9)

\[ f^0_k \approx \frac{1}{2} \left( f^+_k + f^-_k \right) \]  

(10)

where \( f_k \) is the Fukui function at atom \( k \) and \( q_k \) is the charge over atom \( k \) for the systems with \( N + 1, N \) and \( N - 1 \) electrons. Local softness and electrophilicity were calculated according to the expressions [38,42]:

\[ S_k = f^+_k f^-_k \]  

(11)

\[ \omega_k = f^0_k f^0_k \]  

(12)

3. Results

3.1. Strength of the FMN:ApoFld complexes with FMN analogues

The experimentally determined affinities of AnApoFld for all the FMN\(_{an}\) analogues were found to be similar to that for FMN (Table 1). The FMN:ApoFld interaction energy profiles as a function of the flavin redox state had the same V-shape for the different analogues as that for WT AnFld (Fig. SP3B) [14,15,22]. Uncertainties in binding energies for the semiquinone state when using the analogues make difficult to predict changes regarding Fldsq. However, an increase in the relative stabilisation of the hydroquinone complexes is detected for the Flds reconstituted with the analogues (Fig. SP3B, Table 1), particularly for 7,8-diCl-Fldsq (by 3.4 kcal/mol compared to Fldsq).

3.2. Dynamics of the FMN:ApoFld complexes

QM/MM MD simulations for all the Fldox and Fldhq variants maintain the typical “O-Down” conformation of the AnFldox crystal structure (Figs. 1 and 2, Table 2). Simulations of WT Fldsq and 8-Cl-Fldsq variants keep the starting “O-Up” conformation for this redox state. However, analysis of the evolution of the position of the nitrogen and oxygen backbone atoms of Asn58 regarding the C6 atom of the semiquinone flavin ring showed an intermediate situation between the “O-Down” and “O-Up” conformations for the rest of the variants (Fig. 3), with the Asn58-Ile59 peptide bond in a perpendicular arrangement with regard to the flavin ring. The main interactions between the oxidised isoalloxazine ring and the protein environment are conserved along the simulations in all the variants, including: i) stabilisation of the pyrimidine ring by polar contacts with backbone atoms of Gly60, Asp80, Asn97 and Cln99, ii) the weak H-bond between the N5 position of the pyrimidine ring by polar contacts with nitrogen atoms of Ile59, ii) a π–π stacking interaction of Tyr94 at the si-face of the pyrazine moiety of the flavin ring, iv) the main carbonyl of Thr56 both stacking at the re-face of the pyrimidine and pyrazine rings and H-bonding the 2’OH of the ribityl, and v) the stacking of the aromatic side-chain of Trp57 at the re-face of the benzene portion (Figs. 1E and 2, Table 2). Most of these interactions are similar to those stabilising the hydroquinone states. However, in these cases the side-chain of Trp57 slightly changes its relative position regarding both the isoalloxazine ring and its position in Fldsq, increasing the distance between the mass centres of the isoalloxazine and the Trp57 side-chain. This occurs in all the Fld variants (particularly in WT Fldsq) with the only exception of 8-Cl-Fldsq where, contrarily, it is closer...
Table 2

Table 2: Calculated average distances (Å) between selected atoms of the flavin ring and several residues of the protein environment along QM/MM MD simulations of the different Fld variants.

<table>
<thead>
<tr>
<th>Flavin</th>
<th>O2-Na</th>
<th>N3-Oa</th>
<th>N5-Na</th>
<th>N5-Oa</th>
<th>Y94-W57</th>
<th>FMNa</th>
<th>FMNb</th>
<th>Oa(T56)</th>
<th>O1-Na</th>
<th>C4a</th>
<th>N10</th>
<th>C7-NE(W57)</th>
<th>N97</th>
<th>N58</th>
<th>(G60)</th>
<th>(Q99)</th>
<th>(I59)</th>
<th>(N58)</th>
<th>(N58)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-Cl-Fldox</td>
<td>3.53</td>
<td>3.14</td>
<td>2.98</td>
<td>3.91</td>
<td>6.03</td>
<td>4.54</td>
<td>3.61</td>
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<td>5.73</td>
<td>6.75</td>
<td>4.14</td>
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<td>4.37</td>
<td>3.75</td>
<td>3.33</td>
<td>3.48</td>
<td>3.53</td>
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<tr>
<td>7-Cl,8-H-Fldox</td>
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<td>2.98</td>
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<td>3.70</td>
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<td>4.08</td>
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<td>3.48</td>
<td>3.52</td>
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<tr>
<td>7,8-diCl-Fldsq</td>
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<tr>
<td>WT Fldhq</td>
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<td>4.68</td>
<td>3.76</td>
<td>3.91</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a These N and O atoms belong to the backbone of the corresponding residue.

b Distances between the mass centre of the corresponding side-chain aromatic rings and that of the isoalloxazine.

c Values obtained from the X-ray crystal structure of flavin regarding atom would be preferred for the rest of the Fld variants.

3.3. Effects of substitutions in the chemical reactivity of free LM analogues

The molecular electrostatic potentials (MEP) calculated for the free LM analogues indicate that, independently of the redox state, substitutions at the 7- or 8-methyls produce an electron withdrawal effect on the pyrazine and pyrimidine rings (Fig. 4). Such effects are larger when the 7- and 8-methyls are simultaneously replaced, either one with a chlorine and the other one with a proton, but particularly, if both of them are simultaneously replaced with chlorine. The neutral LMsq state shows a zone of low charge density at the protonated N5H, not observed in the oxidised or hydroquinone states, which is highly sensitive to substitutions at positions 7- and 8- of the ring (Fig. 4). The entrance of the second electron to generate the anionic LMsq produces an increase of the negative charge density throughout the isoalloxazine ring, but particularly in the pyrazine and pyrimidine rings. Global reactivity indices indicate that electrophilicity increases in LMsq upon replacement of the methyl groups, while softness is hardly influenced (Table 3). This latter parameter also indicates the neutral semiquinone state as the one suffering the larger polarisation by the protein environment, being the hydroquinone state the less affected.

Fig. 5 indicates the flavin atoms with the highest Fukui function for the different free analogues (numerical values in Table S1). All LMsq analogues show considerably higher values for $f^-$ than $f^+$ at C4a and, particularly, N5. This indicates an electrophilic character at these positions, predicting preferential entrance of electrons over the N5-C4a region. The highest Fukui function in the anionic LMsq analogues are similarly ascribed to C4a and N5, being in this case $f^-$ considerably larger than $f^+$. Therefore, in the anionic LMsq variants the C4a-N5 region is the preferential site for electrons leaving the ring. The LMsq state shows some notable differences: i) N3 and C8 are predicted as the most reactive positions in LMsq for both nucleophilic or radical attacks, suggesting they are the preferred positions for the second electron entrance, and ii) substitutions at the 7- and 8-methyls modulate reactivity of the different positions turning N10 into the most reactive (with the exception of 8-Cl-LMsq, CM7). Therefore, the electron withdrawing effect introduced by the substituents in C7 and C8 will, in general, displace the LMsq isovaloxazine electrophilic character towards the N10 position of the flavin ring.

3.4. Protein influence of the electronic distribution of the FMN analogues

Fukun function were also determined for the Fld variants by using the Mulliken charges obtained from QM/MM minimisations (Fig. 5 and Table S1). Despite all the Fld variants maintain the N5 position as the preferred site for entrance of electrons, the second possible reactive site follows closely showing small differences in the magnitude of the Fukui function. In WT Fldox and 8-Cl-FMNA:Fldox the C8 position appears as highly probable for electron entrance, while the C6 atom would be preferred for the rest of the Fld variants.

The protein environment also has a particular impact on the reactivity in the semiquinone state. The first interesting observation regarding $f^+$, $f^-$ and $f^0$ is that they show quite similar values among them. This might indicate that these atoms could be involved in either accepting electrons, donating electrons or reacting with radical species, a fact probably related with the high percentage of maximum semiquinone stabilised in the apoprotein environment. Additionally, the entrance/exit of the electron is predicted to occur over different atoms of the flavin ring in the Fld variants compared to the corresponding free LMsq analogues, and also to depend on the flavin.
Fig. 3. Relative disposition of the C6 atom of the flavin ring regarding the nitrogen (red line) and oxygen (black line) backbone atoms of Asn58 in the semiquinone state for the different reconstituted variants. Snapshots at the QM/MM MD equilibrium for (A) Fld_sq and (B) 7-Cl-8-H-Fld_sq. (C) Evolution of the C6-N-Asn58 and C6-O-Asn58 distances along the QM/MM MD for the different reconstituted variants.

Fig. 4. MEP, calculated using a Hartree–Fock method and 6–31G* basis set, of the different LM analogues in the oxidised, neutral semiquinone, and anionic hydroquinone states.
Table 3
Global softness (S) and electrophilicity (\(\omega\)) of the free LM analogues.

<table>
<thead>
<tr>
<th>Flavin form</th>
<th>S (au)</th>
<th>(\omega) (au)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LMox</td>
<td>4.18</td>
<td>0.13</td>
</tr>
<tr>
<td>8-Cl-LMox</td>
<td>4.20</td>
<td>0.14</td>
</tr>
<tr>
<td>7,8-dCl-LMox</td>
<td>4.22</td>
<td>0.16</td>
</tr>
<tr>
<td>7-H,8-Cl-LMox</td>
<td>4.11</td>
<td>0.15</td>
</tr>
<tr>
<td>7-CL8-H-LMox</td>
<td>4.17</td>
<td>0.15</td>
</tr>
<tr>
<td>LMhq</td>
<td>5.38</td>
<td>0.13</td>
</tr>
<tr>
<td>8-Cl-LMhq</td>
<td>5.39</td>
<td>0.15</td>
</tr>
<tr>
<td>7,8-dCl-LMhq</td>
<td>5.41</td>
<td>0.16</td>
</tr>
<tr>
<td>7-H,8-Cl-LMhq</td>
<td>5.32</td>
<td>0.15</td>
</tr>
<tr>
<td>7-CL8-H-LMhq</td>
<td>5.30</td>
<td>0.15</td>
</tr>
<tr>
<td>LMsq</td>
<td>4.14</td>
<td>0.01</td>
</tr>
<tr>
<td>8-Cl-LMsq</td>
<td>4.16</td>
<td>0.01</td>
</tr>
<tr>
<td>7,8-dCl-LMsq</td>
<td>4.31</td>
<td>0.00</td>
</tr>
<tr>
<td>7-H,8-Cl-LMsq</td>
<td>4.12</td>
<td>0.01</td>
</tr>
<tr>
<td>7-CL8-H-LMsq</td>
<td>4.09</td>
<td>0.01</td>
</tr>
</tbody>
</table>

The dimethylbenzene moiety of FMN interacts with hydrophobic ApoFld areas, leaving the 7- and 8-methyls as the only portions accessible to the solvent \([48,49]\), in agreement with modifications at these positions only producing minor effects in the FMN binding loop. In AnFld, the 7- and 8-methyls are not predicted to modulate midpoint reduction potentials or FMN binding, while, as experimentally suggested, these parameters are rather influenced by the relative strength of the H-bond between the NS of the flavin and the Asn58-Ile59 residues \([15,22,47]\). These observations indicate that in Fld both the protein environment and the redox state of the cofactor contribute to modulate the chemical reactivity of atoms within the isalloxazine ring.

4.2. Chemical reactivity of the isalloxazine upon substitution of the 7- and 8-methyls

The dimethylbenzene moiety is predicted as preferred for electron entrance and the pyrazine–pyrimidine for its exit, and are in agreement with experimental evidences suggesting that the methyl groups on the benzene ring are not the main points for electrons exchange in Fld \([20]\). AnFld crystal structures have only been reported in the oxidised state, which adopt an “O-Down” conformation. Our QM/MM simulations support the hypothesis of the “O-Down” conformation being preferred also for AnFld-hq, while AnFld-\(\eta1\) would adopt the “O-Up” conformation. These simulations also predict a slight displacements of the Tyr94 side-chain towards the N5 position (also observed in crystal structures for AyFld-\(\eta1\) and AyFld-\(\eta2\) \([17]\)) and, particularly, of the position of Trp57 in AnFld-\(\eta1\) and AnFld-\(\eta2\) that modulates the solvent accessibility of the pyrazine–pyrimidine region (Fig. 2, Table 2). X-ray structures for Fld and ApoFld from \(Anabaena\) and \(Helicobacter pylori\) \([43,44]\) indicated that the Apo forms already present the overall protein fold. The most significant differences were confined to the 56–59 isalloxazine binding loop. Although the stabilisation in the latter ApoFld of the empty FMN binding site is not reached by an aromatic–aromatic interaction as in \(Anabaena\), in both cases the mechanism similarly involves extensive rearrangements in dihedral angles in the 56–59 loop. NMR evidences additionally support that the FMN binding loops might be more flexible than seen in the X-ray structures \([45,46]\). However, changes in these dihedral angles are not observed in our simulations in any of the redox states, in agreement with FMN binding inducing rigidity in the 56–59 binding loop. These data support the fact that the aromatic stacking of the Trp57 side-chain against the isalloxazine is not critical to modulate midpoint reduction potentials or FMN binding, while, as experimentally suggested, these parameters are rather influenced by the relative strength of the H-bond between the NS of the flavin and the Asn58-Ile59 residues \([15,22,47]\). These observations indicate that in Fld both the protein environment and the redox state of the cofactor contribute to modulate the chemical reactivity of atoms within the isalloxazine ring.

4.1. Redox state and protein environment modulate the isalloxazine chemical reactivity

Our calculations for free LM identify the N5-C4a region as the preferred for electron exchange in LMox and LMhq, while the C8 and N3 are predicted for the exchange in LMsq (Fig. 5). These data also indicate that the neutral LMsq as the state with the most homogeneous charge distribution, while LMox and anionic LMhq show, respectively, lower and higher charge density in the pyrimidine and pyrazine rings with respect to the benzene one (Figs. 4 and 5). Reactivity indices for the FMN atoms in Fldox, Fldsq and Fldhq indicate that the non-covalent interactions between the isalloxazine and ApoFld displace the most reactive positions and are modulated by the differential charge distribution among redox states (Fig. 5). These effects are observed particularly in Fldox and Fldhq, where the benzene–pyrazine moiety is predicted for electron entrance and the pyrazine–pyrimidine for its exit, and are in agreement with experimental evidences suggesting that the methyl groups on the benzene ring are not the main points for electrons exchange in Fld \([20]\). AnFld crystal structures have only been reported in the oxidised state, which adopt an “O-Down” conformation. Our QM/MM simulations support the hypothesis of the “O-Down” conformation being preferred also for AnFld-hq, while AnFld-\(\eta1\) would adopt the “O-Up” conformation. These simulations also predict a slight displacement of the Tyr94 side-chain towards the N5 position (also observed in crystal structures for AyFld-\(\eta1\) and AyFld-\(\eta2\) \([17]\)) and, particularly, of the position of Trp57 in AnFld-\(\eta1\) and AnFld-\(\eta2\) that modulates the solvent accessibility of the pyrazine–pyrimidine region (Fig. 2, Table 2). X-ray structures for Fld and ApoFld from \(Anabaena\) and \(Helicobacter pylori\) \([43,44]\) indicated that the Apo forms already present the overall protein fold. The most significant differences were confined to the 56–59 isalloxazine binding loop. Although the stabilisation in the latter ApoFld of the empty FMN binding site is not reached by an aromatic–aromatic interaction as in \(Anabaena\), in both cases the mechanism similarly involves extensive rearrangements in dihedral angles in the 56–59 loop. NMR evidences additionally support that the FMN binding loops might be more flexible than seen in the X-ray structures \([45,46]\). However, changes in these dihedral angles are not observed in our simulations in any of the redox states, in agreement with FMN binding inducing rigidity in the 56–59 binding loop. These data support the fact that the aromatic stacking of the Trp57 side-chain against the isalloxazine is not critical to modulate midpoint reduction potentials or FMN binding, while, as experimentally suggested, these parameters are rather influenced by the relative strength of the H-bond between the NS of the flavin and the Asn58-Ile59 residues \([15,22,47]\). These observations indicate that in Fld both the protein environment and the redox state of the cofactor contribute to modulate the chemical reactivity of atoms within the isalloxazine ring.

4.2. Chemical reactivity of the isalloxazine upon substitution of the 7- and 8-methyls

The dimethylbenzene moiety of FMN interacts with hydrophobic ApoFld areas, leaving the 7- and 8-methyls as the only portions accessible to the solvent \([48,49]\), in agreement with modifications at these positions only producing minor effects in the FMN-\(\eta1\):ApoFld affinity and in the network of non-covalent interactions maintaining this association (Figs. SP2, SP3B, Tables 1 and 2). However, modifications increase the FMN-\(\eta1\):ApoFld affinity (Table 1). The chloride electron-
withdrawing effect at position 7- and/or 8- causes removal of negative charge from all atoms, but especially in the pyrazine and pyrimidine rings, building-up some negative character at the edge of the benzene ring (Fig. 4). Since formation of the anionic FMNhq is energetically unfavourable in the ApoFld negative binding site, removal of negative charge from the pyrimidine in the FMNhq analogues appears as one of the main reasons contributing to the strengthen of the FMNhq:ApoFld interaction (Fig. SP3B, Table 1) [8,13,50–52].

Isosteric substitutions on the isoalloxazine ring modulate midpoint potentials of free flavins [53,54], but if substituents did not produce significant effects on the flavin-apoprotein interaction shifts in potentials upon protein binding should be predictable by a linear relationship between $E_{\text{free}}$ and $E_{\text{Fld}}$ with a slope ~1.0 [55–57]. Even considering the low accuracy in the estimation of $E_{\text{free}}$ and $E_{\text{Fld}}$ for the analogues, midpoint potentials for the reconstituted Flds appeared less shifted to negative values than in WT Fld (Fig. SP1). Moreover, the correlation between the global isoalloxazine electrophilicity of free analogues and the midpoint potential values shows that the protein influence is roughly proportional to the isoalloxazine electrophilicity (Fig. 6, Fig. SP4), suggesting that differential electronic properties of the analogues contribute to the observed effects. Thus, the stronger the substituents withdrawal effect is, the stronger the FMNhq:ApoFld interaction and the smaller the ApoFld influence in setting midpoint reduction potentials, particularly $E_{\text{sq/hq}}$. Thus, the protein environment enhances the substituents withdrawal effect, in agreement with the polarity and electrostatic properties of the flavin environment being tuned in each protein to modulate its midpoint potentials [13,50,58,59]. These data indicate that AnApoFld is optimised to destabilise the interaction with FMNhq to much larger extent than with any other of the analogues here described.

4.3. Chemical reactivity of Fld as electron transferase in photosynthesis

Previous kinetics characterisations indicated that substitutions at the 7- and 8-methyls allowed Fldox to interact with PSI and FNR, while interaction of 7-H,8-Cl-Fldsq, 7-Cl,8-H-Fldsq and 7,8-diCl-Fldsq with PSI was poor. Moreover, despite the theoretical increase in the driving force for Fld accepting electrons, drastic changes in reactivity upon reduction by PSI were observed (see Fig. 7 from [20]). Fukui function predicts that the most probable position for electron entrance for all Fldsq variants is the C6 atom of the flavin ring (Fig. 5 and Table SP1). Thus, a change in the environment of this position might alter the PSI-Fld competent complex and, therefore, influence the ET mechanism. In fact WT Fldsq and 8-Cl-Fldsq are the only Fldsq variants i) with a methyl substituent at C7, ii) showing an “O-Up” conformation for the Ans58-Ile59 backbone and iii) accepting electrons from PSI through the formation of a reorganisation complex [20]. Therefore, the methyl at position C7 and the Asn58-Ile59 backbone conformation appear as a determinant for the formation of the competent complex for ET between PSIrd and Fldsq. These results

![Fig. 6. Midpoint reduction potentials and global electrophilicity correlations as a function of the Fld redox state. Correlation between (A) $E_{\text{ox/sq}}^{\text{Fld}}$ and global electrophilicity of LMox analogues ($R^2$ 0.91) and (B) $E_{\text{sq/hq}}^{\text{Fld}}$ and global electrophilicity of LMsq analogues ($R^2$ 0.87).](image)

![Fig. 7. Correlation between the apparent rate constants, (A) $k_{ap1}$ and (B) $k_{ap2}$, for the reduction of the Fldox variants by FNR$\text{hq}$ and the local softness of the N5 flavin atom. In both cases the lines correspond to the linear fitting excluding 7-H,8-Cl-FMN:Fld.](image)
additionally support an electron exchange pathway including the C6 of the flavin ring and Asn58 backbone atoms in Flavodoxin.

A positive correlation was reported for the ET rates from FNRhq or FNRsq to the Flavodoxin variants and their midpoint potentials (see Fig. 7 from [20]). Some tendency for a reverse correlation might also have been envisaged between the N5 local softness of the LMas variants and the apparent ET rates reported for the reduction of the Flavodoxin variants by FNRhq (kap1 and kap2, corresponding to the Flavodoxin + FNRhq → Flavodoxin + FNRsq and Flavodoxin + FNRsq → Flavodoxin + FNRreox processes, respectively) (Fig. 7). This suggests contribution of the N5 atom of the flavin, as well as of the thermodynamic driving force, in the overall ET. Since reactions involving soft-soft or hard-hard reactants will be preferred over those involving soft-hard ones [60], the LM global softness might contribute as an additional factor to explain why, in general, kap1 is considerably faster than kap2: Softness of LMreox analogues is more similar to LMhq than that of the LMas (Table 3). In the WT system the initial orientation driven by the alignment of the FNR and Flavodoxin dipole moments contributes to the formation of a number of alternative binding modes competent for the ET, where the substituent at C7 has one of the largest propensities for being at the interaction surface [61]. The large divergence of 7-H,8-Ci-FMN:Flavodoxin in Fig. 7 agrees with its reactivity with FNR being slower than expected from the thermodynamics (see Fig. 7 from [20]). Changes in the direction of the molecular dipole moment and the increase in the accessible surface area of the flavin reactive region (caused by the smaller volume of the C7 substituent) might favour orientations between 7-H,8-Ci-FMN: Flavodoxin and FNR less-productive than those favoured in the WT system. Similar situations have already been reported upon mutation of some residues in the isoalloxazine environment on the Flavodoxin surface [16,62], where modulation of the ET processes by different orientations and distances between the redox centres explained different reactivities. Thus, several factors contribute to the efficiency of the reactions involving Flavodoxin, including dipole moment orientation, surface complementarity, thermodynamic driving forces or electronic factors. Among them, contribution of structural and electronic factors is key in the ET from PSI to Flavodoxin, while in general the ET between FNR and Flavodoxin has a major dependence on the thermodynamics.

Analysis along QM/MM MD simulations also indicate that independently of the redox state and of the Flavodoxin variant, the backbones of Thr56 (particularly its carbonyl group situated at the re-face of the isoalloxazine ring) and Asn58 are the closest atoms to the preferred sites for the exchange of the electrons, the C6–N5–C4a region of the isoalloxazine (Fig. 5 and Table 2). Therefore, the data here presented indicate that the Thr56–Trp57–Asn58–Ile59 backbone has a key contribution in the ET efficiency. This is in agreement with previous site-directed mutagenesis studies indicating these residues are the key to modulate the Flavodoxin ability to bind to and to exchange electrons with its physiological partners, FNR and PSI [63]. Additionally changes in the dihedral angles in this backbone region might also contribute to the slight displacement of Trp57, opening the access towards the C6–N5–C4a region for electron exchange.

5. Conclusions

Altogether, the data here presented indicate that substitutions in the isoalloxazine ring will modulate its chemical reactivity, as well of that of the flavoproteins they are part from. Both the apoprotein environment and the redox state modulate the chemical reactivity of the isoalloxazine ring within flavoproteins. Moreover, the protein portion of AmFlavodoxin is particularly optimised to stabilise the charge distribution of the neutral FMNH2 and to destabilise the negative charge of the pyrazine ring of FMNH2, critical aspects to modulate Eox/reox and Emn/reox within the values required for the physiological functions in which Flavodoxin is involved. Using Flavodoxin as model we have proven that the use of different derivatives of the isoalloxazine ring in flavoproteins might potentially increase their versatility. However, this would provide the cell with a number of isoforms of each particular flavoprotein exhibiting different redox properties. Additionally, the cell would require to increase, or to adapt, its machinery to produce, or to obtain from the environment, the different flavin derivatives. Instead, Nature has optimised its cellular resources by using a single organic molecule, riboflavin (7,8-dimethyl-10-(1-D-ribityl)-isoalloxazine or Vitamin B2), as precursor of all the flavin cofactors used by flavoproteins. The single 7,8-dimethyl-isoalloxazine ring has been selected as the reactive portion in all flavoproteins, being the protein environment in each flavoprotein (as here proven for Flavodoxin) carefully tuned to provide to the flavin ring with the chemical reactivity, midpoint reduction potentials and capacities required for the different reactions in which it is involved within each particular protein environment.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbabi.2012.08.008.

References

Tuning of the FMN binding and oxido-reduction properties by neighboring side chains in flavoan fla


