Sulphur(lone pair)⋯π interactions with FAD in flavoenzymes

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Abstract. The interactions of π-systems with lone-pairs of electrons are known and have been described in biological systems, involving lone-pairs derived from metals, metalloids, sulphur, oxygen and nitrogen. This study describes a bibliographic survey of the disulphide-bound sulphur(lone-pair) interactions with π-systems residing in the flavin adenine dinucleotide (FAD) cofactor oxidoreductase enzymes (flavoenzymes). Thus, of the 172 oxidoreductase enzymes evaluated for gamma-S(lone pair)⋯π(FAD) interactions, 96 proteins (56%) exhibited these interactions corresponding; 61% of 350 the constituent monomers featured at least one gamma-S(lone pair)⋯π(FAD) interaction. Two main points of association between the S(lone pair) and the isoalloxazine moiety of FAD were identified, namely at the centroid of the bond linking the uracil and pyrazine rings (60%), and the centroid of the uracil ring (37%). Reflecting the nature of the secondary structure in three prominent classes of oxidoreductase enzymes: glutathione disulphide reductases (GR; 21 proteins), trypanothione disulphide reductases (TR, 14) and sulphydryl oxidases (SOX, 22), the approach of the gamma-S(lone pair)⋯π(FAD) residue was to the si-face of the isoalloxazine ring system, i.e. to the opposite side as the carbonyl residue, for all GR and TR examples, and to the re-face for all SOX examples. Finally, the attractive nature of the gamma-S(lone pair)⋯π(FAD) interactions was confirmed qualitatively by an examination of the non-covalent interaction plots.

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Introduction

Flavoproteins are biomolecules involved in the catalysis of a broad range of redox reactions in which a riboflavin derivative is actively engaged in the electron-transfer process [1-4]. The most common flavin cofactors in flavoproteins are flavin mononucleotide (FMN; riboflavin-5′-phosphate) and flavin adenine dinucleotide (FAD) [3, 5]. FMN and FAD each feature a heterocyclic aromatic moiety known as isoalloxazine [2, 3], with a sequence of three fused six-membered rings, that is often reported to be engaged in π–π stacking with neighbouring aromatic amino-acid residues [6]. A depiction of the oxidised and fully reduced forms of isoalloxazine is shown in Figure 1a. Isoalloxazine in its oxidised state is approximately planar [7] and may be involved in hydrogen bonding and interactions involving π-systems with surrounding molecules [8]. Deformation of the isoalloxazine molecule is also reported to occur about the N5–N10 axis, leading to deviations from planarity [7].

Fig. 1: (a) A representation of the oxidised and reduced isoalloxazine moieties present in the flavin cofactors FMN and FAD. (b) A simplified representation of the isoalloxazine framework showing atom labels following the standard numbering scheme. The ring labels refer to the uracil (U), pyrazine (P) and dimethylbenzene (B) rings.

In the quest for rational drug design, research and development, the knowing and understanding of the specific interactions between flavoproteins and their cofactors are of both structural relevance and biomedical interest [3]. Some enzymes, such as glutathione disulphide reductases (Enzyme Commission number: EC 1.8.1.7), trypanothione disulphide reductases (EC 1.8.1.12) and sulphhydryl oxidases (thiol oxidases; EC 1.8.3.2), for example, that involve FAD-mediated electron-transport in redox reactions, have a cysteine residue close to the isoalloxazine region of the cofactor [9-11]. More specifically, the disulphide bridge crucial for these processes occurs across the Cys58 and Cys63 sites with the sulphur atom of the latter, i.e. the gamma sulphur atom (SG), is proximate to the isoalloxazine moiety.

It is only when the disulphide bond is broken as a result of enzymatic activity that researchers point to a stabilising interaction between the thiolate-S atom and the isoalloxazine moiety, by way of a flavin-thiolate charge-transfer complex [12-14]. In some cases, the interaction is directed towards the C4X atom of the cofactor or in the surrounding region of this atom [15-17], see Figure 1b. In this contribution, an investigation of the putative interactions between sulphur in the neutral disulphide precursor complex, via a sulphur-bound lone-pair of electrons, hereafter referred to as S(lp), and the π-systems of isoalloxazine is reported. There is increasing awareness of the role S(lp)–π interactions play in biological systems [18].
such as, for example, their importance in providing stability to conserved domains in the D2 Dopamine Receptor protein [19] and in drug design [20].

In fact, element(lp)−π interactions were first mentioned in the literature in a biological context, namely with the cytidine-O-bound lone-pair interacting with the five-membered ring of a guanine reside to stabilise the conformation of Z-DNA [21, 22]. Subsequently, O(lp)−π contacts were noted in protein structures [23] as well as in molecular compounds [24] and their influence on supramolecular aggregation demonstrated [25-27]. A very recent study suggested S(lp)−π interactions form preferentially over O(lp)−π interactions [28]. Indeed, many post-transition metal elements, starting originally with tellurium [29], have been surveyed for element(lp)−π interactions with specific reference to influence on supramolecular association [30-40]. While at first glance such element(lp)−π interactions might appear to be repulsive, they are in fact attractive. The nature of the interaction is now thought to involve the electrostatic interaction of a polar cap or σ-hole at the tip of the lp, which is electron-deficient, and it is this which interacts with the π-electron density of the aromatic system [41-43]. While element(lp)−π interactions are inherently weak, it is noted that with the heavier elements, e.g. antimony and bismuth, interacting with electron-rich π-systems, these contacts can provide energies of stabilisation comparable to conventional hydrogen bonding interactions [44].

In this paper, the results of an extensive geometric and visual analysis of 172 structures of oxidoreductases, with crystallographic data deposited in the Protein Data Bank (PDB) [45], containing FAD as a cofactor and a disulphide bridge proximate to the isalloxazine moiety are reported. In this way, 96 examples of oxidoreductase/FAD complexes featuring attractive S(lp)−π(FAD) interactions were identified; the attractive nature of these interactions were ascertained from an analysis of the non-covalent interaction plots. This contribution presents an overview of this investigation.

Methods

The crystallographic data of 172 oxidoreductase structures deposited in the PDB [45] were interrogated for the presence of a possible interaction between a cystine-sulphur atom of a disulphide bridge close to the aromatic moieties of a FAD cofactor, i.e. the isalloxazine and adenine regions of FAD. The query parameters used for searching the PDB were: (a) the presence of the fully-oxidised FAD cofactor in the crystal, (b) the proteins were oxidoreductase enzymes (EC 1.8) only, (c) there was at least one disulphide bond in the protein structure, (d) the structure was determined by X-ray diffraction and (e) the resolution was equal to or lower than 2.50 Å.

The analysis of the structures obtained from the PDB was performed with a system developed in our laboratories, i.e. Weak Interaction Mapping (WIM; to be published and released in the near future [46]), which calculates the distances and angles between the centroids of ring moieties and sulphur atoms in a protein, according with the descriptors detailed in Figure 2. The angle in the search was constrained to be less than 30° to avoid side-on interactions and to capture all delocalised S(lp)−π(FAD) interactions whereby the sulphur atom lies approximately plumb to the ring centroid [47, 48]. The distance, d, was constrained to be approximately the sum of van der Waals radii (vdW) for sulphur (1.80 Å) [49] and π-systems (1.90 Å) [50]. In keeping with previous practice [30-40], the search limit
was increased 10\% over the sum of the vDW\(_i\) in order to capture as many S(lp)\(\rightarrow\)\(\pi\)(FAD) interactions as possible, giving a threshold limit of 4.07 Å.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{fig2.png}
\caption{An illustration of the geometric descriptors of the interactions between the S(lp) and ring moieties, highlighted by a schematic drawing with benzene. Symbols: S is the sulphur atom, Ct is the centroid of the aromatic ring, \(V_1\) is the vector normal to the aromatic ring, \(V_2\) is the vector that connects S and Ct, \(\alpha\) is the angle between \(V_1\) and \(V_2\) and \(d\) is the distance from S to Ct.}
\end{figure}

Then, each structure was analysed with a molecular visualization program (Discovery Studio Visualizer® DSV v3.5 [51]) in order to confirm the position of the centroids relative to sulphur as well as the values of distances and angles. Five centroids were defined in isoxaloxazine in order to better describe the relative orientation of \(V_2\), i.e. the ring centroids, U, P and B, Figure 1b, and centres of the C–C bonds linking the BP and PU rings, i.e. Cm12 and Cm23, respectively. Additionally, two centroids were defined in the adenine system of FAD.

Finally, identified S(lp)\(\rightarrow\)\(\pi\)(FAD) interactions were analysed using the program NCIPLOT [52, 53] in order to confirm the attractive relationship between the participating species.

**Results and discussion**

Many of the 172 oxidoreductase enzymes analysed have more than one monomer, resulting in the comprehensive analysis of 350 monomers. Evidence for S(lp)\(\rightarrow\)\(\pi\)(FAD) interactions were found in 96 proteins corresponding to 56\% of the sample, and in 214, i.e. 61\%, of the monomers. The remaining 76 proteins did not show evidence for S(lp)\(\rightarrow\)\(\pi\)(FAD) interactions. While in some of these, evidence for close approach of sulphur to FAD’s isoxaloxazine was found, the sulphur atom was not part of a disulphide, rather, being part of other ligands.

Data summarising the values of d and \(\alpha\) are given in Table 1. In is interesting to note that with the exception of one distance, i.e. \(d = 4.11\) Å (PDB code 3R7 C [54]), all other values of d were found within the sum of the vDW\(_i\) + 10\% for sulphur and \(\pi\)-systems, i.e. 4.07 Å, in 214 monomers of oxidoreductases. The mean distance d was 3.34 Å, and the mean value for the \(\alpha\) angle was 9.5\°. Thus, the mean value for d and the median of the distribution of d is very similar, while the mean value of \(\alpha\) is about 0.5\° greater than the median of the distribution. From the data in Table 1, it is concluded that the distances characterising S(lp)\(\rightarrow\)\(\pi\)(FAD) interactions in the studied oxidoreductases are remarkably short: 10\% shorter than the sum of vDW\(_i\) (3.70 Å) and 18\% shorter, on average, than the sum of vDW\(_i\) + 10\% (4.07 Å).
Tab. 1: Distances (d) and angles (α) characterising S(lp)–π(FAD) interactions

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<tr>
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<th>min.</th>
<th>max.</th>
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<tr>
<td>d (Å)</td>
<td>2.93</td>
<td>4.11</td>
<td>3.34</td>
<td>0.19</td>
<td>0.04</td>
<td>3.31</td>
</tr>
<tr>
<td>α (°)</td>
<td>2.2</td>
<td>28.7</td>
<td>9.5</td>
<td>4.38</td>
<td>19.14</td>
<td>9.0</td>
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The distribution of the distances defining the S(lp)–π(FAD) interactions are plotted in Figure 3, and follows an almost normal distribution where the majority of the recovered values are less the sum of vDW for sulphur and π-systems (3.70 Å). In almost half of the interactions, 47%, the sulphur atom and the closest centroid of the FAD-isoalloxazine group were in the range 3.20 to 3.40 Å. If the interval 3.00 to 3.60 Å is considered, the percentage of structures increases to over 90%. Interestingly, there were four cases where the distances were found shorter than 3.00 Å, which is almost 20% less than the sum of vDW, i.e. in PDB-ID: 3DK4 [55], 3DK9 [55], 3P0K [56] and 1JR8 [57].

Fig. 3: Histogram of the distribution of d in 214 monomers of oxidoreductases. Over 90% of the distances of interaction fall in the range 3.00 to 3.60 Å.

The distribution of the α angles is plotted in Figure 4 where it can be seen that more than 92% of the angles were within 15° of the V₁ normal vector to centroids in isoalloxazine interacting with the sulphur atom’s lone-pair. No correlation was found between the distances and the angles of interaction, an observation noted previously [30-40].

Fig. 4: Histogram of the distribution of α angles in the 214 monomers of oxidoreductases. Over 90% of the angles are below 15°.

The retrieved data for the S(lp)–π(FAD) interactions indicate the preferred anchorage points of the cofactor to the enzyme. The distribution of contact sites is shown in Figure 5 from which it can be noted that the most prevalent point of contact is at the centroid Cm23, i.e. the bond providing the link between uracil and pyrazine rings, which participates in 60% of the contacts, and, therefore, is termed a semi-localised interaction [47,
This is followed by the Ct(U) centroid of the uracil ring, 37% of contacts, corresponding to delocalised S(lp)·π(FAD) interaction. The preference of the Cm23 site over Ct(U) is readily explained in terms of the significant resonance delocalisation over the bond linking the two imine bonds, making the C4X–C10 bond electron-rich. The only other site revealed in the present survey to participate in S(lp)·π(FAD) interactions is the Ct(P) centroid of the pyrazine ring which occurs less than 3% of the time. The dimethylbenzene ring Ct(B) of the isoalloxazine is not involved in any of the interactions nor are any of the rings of the adenine residue.

![Pie chart showing the distribution of isoalloxazine centroids of all 214 FAD molecules involved in S(lp)·π(FAD) interactions. The preferred centroids for the interaction are the uracil ring Ct(U) and the centroid of the bond linking the uracil and the pyrazine rings (Cm23).](image)

**Fig. 5:** Pie chart showing the distribution of isoalloxazine centroids of all 214 FAD molecules involved in S(lp)·π(FAD) interactions. The preferred centroids for the interaction are the uracil ring Ct(U) and the centroid of the bond linking the uracil and the pyrazine rings (Cm23).

In addition to the geometric analysis of the interactions, the direction of approach of the sulphur atom to the isoalloxazine moiety of FAD was determined, Figure 6. This si-face/re-face positioning of a system interacting with isoalloxazine has frequently been used to discriminate between the two regions of approach towards the isoalloxazine ring [58]. In 57 of the 214 studied monomers, the S(lp)·π(FAD) interaction occurred through the re-face of the isoalloxazine, i.e. opposite the carbonyl group in the side-chain, which is just over a quarter (27%) of all observed interactions. The clear majority of the studied monomers (73%) show evidence for the interaction occurring from the si-face of the tricyclic system.

![The distribution of the 214 monomers with identified S(lp)·π interactions according to the position of disulphide bond relative to isoalloxazine of FAD. The si-face interaction shows on 73% of the monomers, while the re-face interaction was found on only 23% of the monomers. Sulphur atoms are shown in yellow, oxygen (red), nitrogen (blue) and carbon (grey). Hydrogen atoms are not represented.](image)

**Fig. 6:** The distribution of the 214 monomers with identified S(lp)·π interactions according to the position of disulphide bond relative to isoalloxazine of FAD. The si-face interaction shows on 73% of the monomers, while the re-face interaction was found on only 23% of the monomers. Sulphur atoms are shown in yellow, oxygen (red), nitrogen (blue) and carbon (grey). Hydrogen atoms are not represented.
With the above observations and trends in mind, it was thought of interest to survey three well-represented classes of oxidoreductases to determine whether specific classes of enzymes exhibit specific trends in terms of their si-re-face approach to isalloxazine compared with all 214 enzymes. The three classes chosen for further analysis, i.e. glutathione reductases (GR) and trypanothione reductases (TR), targeting of which is related to the treatment of Chagas disease [59], and sulfhydryl oxidases (SOX), for which several applications/potential applications are known [60].

Of the total of 21 GR found in the PDB [45], 26 monomers have a FAD cofactor. The presence S(lp)–π(FAD) interactions were found in 21 monomers (16 proteins). Usually the contacts involved the U ring (17 hits) with the remaining (4 hits) having the lone-pair orientated towards the C4X–C10 bond (Cm23) centroid. All of the interactions occur via the si-face of the isalloxazine.

The 14 examples of TR gave rise to 42 monomers with FAD. Evidence for S(lp)–π(FAD) interactions was found in 38 monomers (13 proteins). As for GR, the U ring was the favoured target for the interaction (29 hits), while 9 cases showed the C4X–C10 bond centroid (Cm23) as being the point of contact. Again, as for GR, all of the interactions S(lp)–π(FAD) occur via the si-face of the isalloxazine.

Finally, in the 22 SOX enzymes found in PDB, there were 45 monomers having FAD. The S(lp)–π(FAD) interactions were found in 37 of the monomers (17 proteins). By contrast to the situation with GR and TR, the S(lp) interacted mainly with the C4X–C10 bond centroid (Cm23, 31 hits), leaving four interactions with the U ring centroid and two with the P ring. Further, all of the interactions occur via the re-face of the isalloxazine.

In the present survey, it was found that in both GR and TR, an open, fully-extended conformation was noted for FAD, consistent with the literature [61] which correlates with the observed conformation of the relevant protein’s secondary structure. By contrast, a curved or bent conformation was apparent for all FAD residues in all of the evaluated SOX proteins, again consistent with the relevant protein sequence [62]. When FAD is present in the curved conformation, steric hindrance precludes the approach of the disulphide to the si-face of the cofactor [63].

As mentioned above, element(lp)–π interactions are inherently weak, especially for the lighter elements. The question remains then, whether the interactions revealed in the present survey are attractive or simply the result of the sulphur atom not having anywhere else to reside. With this in mind, the identified non-covalent S(lp)–π(FAD) interactions were also subjected to non-covalent interaction calculations, which were performed using NCIPLOT [52, 53]. Such calculations provide a qualitative indication of attraction (or repulsion) between atoms. If an interaction is attractive, the isosurface will be blue in appearance, only weakly attractive interactions appear green and repulsive interactions appear as red isosurfaces.

Results for three examples of each of GR, TR and SOX are shown in Figure 7. As can be seen from the images in Figure 7a–c, the S(lp)–π(FAD) interaction is the dominant attractive interaction of those provided by the atoms in the disulphide. Also, the S(lp)–π(FAD) interaction in the 1K4Q [64] and 300H [65] proteins show the orientation of sulphur-lp to the Ct(U) centroid, Figure 7a and b, while in 3SQP [66], the sulphur-lp is directed towards the Cm23 centroid of the C4X–C10 bond, Figure 7c.

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Title
Sulphur(lone pair)–π interactions with FAD in flavoenzymes

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Page
7 (11)

Sulphur (lone pair) – π interactions with FAD in flavoenzymes

As calculated for GR enzymes above, the S(lp) – π(FAD) interactions in the TR enzymes also appear as the most significant attractive contributor between the disulphide and isoalloxazine. Both 1AOG [67] and 1FEA [68] exhibit directionality of the sulphur-lp towards the uracil ring Ct(U) centroid, Figures 7d and e. By contrast, 2WPF [69] displays an orientation rather more towards the centroid of the C4X–C10 bond (Cm23), Figure 7f.

Representatives of the SOX enzymes are illustrated in Figures 7g-i and are consistent with the prevalence of sulphur forming the attractive S(lp) – π(FAD) interactions in the NCI plots shown in Figures 7a-f. In both 1JR8 [57] and 3TD7 [70], the directionality of the sulphur-lp is towards the centroid of the C4X–C10 bond (Cm23), Figures 7g and h, and in 3QCP [71], the interaction is directed towards the centroid of the P ring, Figure 7i. The major difference occurs in the nature of the approach of the sulphur-lp towards the isoalloxazine moiety. Whereas in the illustrated examples for the GR and TR reductases, the interactions occur via the si-face,
for the SOX enzymes, the interactions occur through the re-face (see discussion above).

Conclusions

The biological relevance of S(lp)–π(FAD) interactions has been established in an analysis of 172 oxidoreductase enzymes whereby evidence for these interactions were found in 96 proteins, 56% of the sample, and in 214, i.e. 61%, of the 350 constituent monomers. These interactions were proven to be attractive from an analysis of non-covalent interaction plots. Two primary points of association between the S(lone pair) and FAD were identified: at the centroid of the bond linking the uracil and pyrazine rings (Cm23, 60%), and the centroid of the uracil ring (U, 37%). The only other site identified was the P site (3%) indicating all interactions involved the isoalloxazine tricyclic heterocycle as no contacts with the adenine residue were identified. In the overwhelming number of structures featuring S(lp)–π(FAD) interactions, the distance between the sulphur nucleus and the centroid was at least 10% less than the sum of the vdW. Differential behaviour was noted for three prominent/well-represented oxidoreductases in that all of the glutathione reductases and trypanothione reductases identified with S(lp)–π(FAD) interactions, the approach of the sulphur atom was to the si-face of isoalloxazine, consistent with a fully-extended conformation for FAD. By contrast, for sulphhydryl oxidase proteins, the approach was to the re-face, consistent with a curved conformation for FAD which, owing to steric hindrance, precludes the close approach of the gamma-sulphur to the si-face of the cofactor.

The present comprehensive study of the oxidoreductases shows that the sulphur atoms interact with FAD via S(lp)–π(FAD) interactions that provide stability to the secondary structure and that may be related to the mechanism of catalysis operating in the active site of those enzymes by providing an anchoring point before redox activity. The data reported here highlights the presence of previously unidentified interactions in biological systems that can be of significant importance in understanding the structural stability and the reactivity of enzymes.

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