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53. Redner, R.L. et al. (1999) MTA2 and MBD3 are the carbon atom involved in the active site of a flavoprotein dehydrogenase. EMBO J. 18, 6385–6395.
55. Redner, R.L. et al. (1999) MTA2 and MBD3 are the carbon atom involved in the active site of a flavoprotein dehydrogenase. EMBO J. 18, 6385–6395.
58. Redner, R.L. et al. (1999) MTA2 and MBD3 are the carbon atom involved in the active site of a flavoprotein dehydrogenase. EMBO J. 18, 6385–6395.
in this C-H bond the site of oxidative attack. The reviewed enzymes display various folding topologies and are either FMN- or FAD-dependent proteins (Table 1). Despite such a structural diversity, they have a few remarkably similar properties in their mode of binding both flavin and substrate.

The flavin-binding site

The tricyclic isoalloxazine ring system is the reactive part of the flavin (Fig. 1). It is an amphipathic molecule formed by the fusion of the hydrophobic dimethylbenzene moiety with the hydrophilic pyrimidine ring. The redox potential for the two-electron reduction of the flavin is about −200 mV. However, this value can greatly vary in flavoenzymes, spanning a range from approximately −400 mV to +60 mV in vivo. In general, the proximity of a positive charge is thought to increase the redox potential, whereas a negative charge or a hydrophobic environment are expected to lower it.

Remarkably, a few flavoenzymes have a covalently bound FAD molecule. Site-directed mutagenesis studies suggest that such a covalent interaction could enhance the oxidizing power of the flavin. These observations highlight the crucial role that is played by the protein milieu in fine-tuning the chemical properties of the flavin. The isoalloxazine can adopt conformations that deviate significantly from exact planarity (e.g. polyamine oxidase). However, independent of the degree of planarity, the ability of the flavin to form hydrogen bonds appears to be fully satisfied in all enzymes under investigation. In all the structures examined (Table 1), the N1–C2–O2 locus (Fig. 1) is always in contact (<3.5 Å distance) with a positively charged entity. This can be either fully charged, such as a Lys or Arg side chain, or partially charged, such as the N terminus of an α helix or a cluster of peptide nitrogens (Table 1). A positive charge at this location is functionally relevant because it can stabilize the anionic form of the reduced flavin (Fig. 1) and increase the co-factor’s redox potential. Moreover, in dihydroorotate dehydrogenase and trimethylamine dehydrogenase, placing the positively charged residue close to N1 with a neutral side chain resulted in an inactive enzyme that was unable to bind to FMN. Thus, the recurrent presence of a positively charged

| Table 1. Binding features of the isoalloxazine ring in flavin-dependent enzymes |
|--------------------------|----------|-----------------|-----------------|-----------------|-----------------|
| Flavoenzyme* | Ref. | Cofactor* | Folding topology | Protein residue contacting N-C2-O2 | H-bond partner | Distance (Å) | Angle with N(2)/N(5) (°) | Flavin side |
| Old yellow enzyme (10YB) | 36 | FMN | αi | R243 | N37 | 2.8 | 151 | ne |
| Neurospora chromobacter (1FCB) | 4 | FMN | αi | K349 | N419 | 3.0 | 139 | ne |
| Trimethylamine dehydrogenase (2TMD) | 16 | FMN | αi | K154 | N4143 | 3.3 | 170 | re |
| Polyamine oxidase (1B37) | 15 | FAD | PHBH fold | Helix | N449 | 3.3 | 125 | si |
| Polyamine oxidase (1B37) | 15 | FAD | PHBH fold | Helix | N6129 | 3.4 | 129 | si |
| Polyamine oxidase (1B37) | 15 | FAD | PHBH fold | Helix | H401 | 3.0 | 152 | si |
| Medium-chain acylCoA dehydrogenase (3MDE) | 3 | FAD | αi | N163 | N7136 | 2.9 | 170 | si |
| Medium-chain acylCoA dehydrogenase (3MDE) | 3 | FAD | αi | N6150 | N6149 | 3.1 | 145 | re |
| Polyamine oxidase (1B37) | 15 | FAD | PHBH fold | Helix | N449 | 3.3 | 125 | si |
| Polyamine oxidase (1B37) | 15 | FAD | PHBH fold | Helix | N6129 | 3.4 | 129 | si |
| Polyamine oxidase (1B37) | 15 | FAD | PHBH fold | Helix | H401 | 3.0 | 152 | si |
| Medium-chain acylCoA dehydrogenase (3MDE) | 3 | FAD | αi | N163 | N7136 | 2.9 | 170 | si |
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| Polyamine oxidase (1B37) | 15 | FAD | PHBH fold | Helix | H401 | 3.0 | 152 | si |

*Name and PDB entry for the flavoenzyme structures. When available, structures of enzymes in complex with active-site ligands were used. Glucose oxidase and dihydroorotate dehydrogenase are not listed, as they share considerable homology and essentially identical flavin sites with cholesterol oxidase and vanillyl-alcohol oxidase, respectively. Flavocytochrome c3 has been chosen as the representative protein of the succinate dehydrogenase family of oxidoreductases, which includes glycolate oxidase and vanillyl-alcohol oxidase. The ordered water molecule that is H-bonded to N5 of polyamine oxidase also interacts with N10, R214, and K43, respectively.

**Table 1. Binding features of the isoalloxazine ring in flavin-dependent enzymes**

- **Flavoenzyme**: The name of the flavoenzyme.
- **Ref.**: The reference number.
- **Cofactor**: The type of cofactor present in the enzyme.
- **Folding topology**: The folding topology of the enzyme.
- **Protein residue contacting N-C2-O2**: The residue of the protein that contacts the N-C2-O2 locus.
- **H-bond partner**: The residue of the flavin that forms the hydrogen bond.
- **Distance (Å)**: The distance between the H-bond partners.
- **Angle with N(2)/N(5) (°)**: The angle between the H-bond partners.
- **Flavin side**: The side of the flavin where the hydrogen bond is formed.
Upon reduction, N5 becomes protonated at N5 might have a more subtle effect. However, the particular hydrogen bond donor ranging from 116° to 117° (Table 1). The N5 hydrogen bond, exhibiting a relatively high (−25 mV) redox potential. Further studies are needed to clarify the exact role of the frequently observed hydrogen bond at the N5 locus.

The substrate-binding site

Owing to the inherent instability of enzyme-substrate complexes, their three-dimensional structures are difficult to analyze by X-ray crystallography. However, the stereochemistry of substrate binding can be inferred from the structures of the enzymes in complex with reaction products or competitive inhibitors that are sufficiently similar to the substrate. The proteins under investigation act on substrates that vary greatly in both chemical nature (amines, alcohols, lactic acids, hydroxy and amino acids) and size (ranging from bulky cholesterol to small lactate (Tables 1, 2)).

Despite this diversity, examination of the enzyme complexes reveals some common features. When the isoalloxazine rings of these structures are superimposed, a striking similarity emerges in the position of the ligand atom that mimics the substrate atom being dehydrogenated by the flavin. As inferred from the crystal structures of enzyme complexes, the site of oxidative attack (Fig. 1) typically binds in front of the flavin at 3.5 Å distance from N5, defining an angle with the N5-N10 atoms in the narrow range of 96–117° (Figs 2, 3).

To visualize this similarity, it is instructive to project the substrate atoms onto the plane defined by the isoalloxazine ring. This shows that the projected position of the site of oxidative attack falls in a well-defined location. More precisely, the projected position is offset by 0.2–1.1 Å from the line connecting N5 to N10 (distance a in Fig. 3). The offset is in the direction of the O4 locus and so, the projected position invariably falls between the two lines defined by the C4a-C10a and N5-N10 atoms, respectively. Such a detailed level of conservation is truly remarkable. It shows how carefully flavoenzyme active site engineering takes into account the geometrical constraints imposed by the isoalloxazine ring of the flavin.

Figure 1

General scheme of a flavin-dependent dehydrogenation reaction. The carbon atom that underlies the rupture of a C-H bond with transfer of two electrons to the flavin represents the site of oxidative attack (red circle). The ‘activating’ X group (blue square) facilitates the dehydrogenation reaction. The atomic positions discussed in the text are numbered. The isoalloxazine system is shown with the si side facing the viewer (see Table 1). R1 and R2 indicate unspecific substituents of the OH atom undergoing oxidation.
centres are designed and optimized to achieve a stereochemically precise coordination between the flavin and the reactive part of the substrate.

Cholesterol oxidase exhibits a binding mode of the substrate that deviates significantly from the above-described stereochemistry (Fig. 2), as inferred from the structure of the enzyme-product complex. However, this oxidase has a dual activity; it catalyses substrate oxidation followed by isomerization. The observed

### Table 2. Stereochemistry of substrate binding in flavoenzyme structures

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate activation</th>
<th>Distance (Å)</th>
<th>Angle (°)</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-amino acid oxidase</td>
<td>Deprotonated amino group</td>
<td>3.8</td>
<td>110</td>
<td>5,27</td>
</tr>
<tr>
<td>Vanillyl-alcohol oxidase</td>
<td>Phenolate anion stabilization</td>
<td>3.3</td>
<td>117</td>
<td>26</td>
</tr>
<tr>
<td>Cholesterol oxidase</td>
<td>Deprotonation of hydroxyl group by His</td>
<td>3.7</td>
<td>162</td>
<td>7</td>
</tr>
<tr>
<td>Dihydroorotate dehydrogenase</td>
<td>α-Proton abstraction by Cys</td>
<td>3.5</td>
<td>96</td>
<td>17,22</td>
</tr>
<tr>
<td>AcylCoA dehydrogenase</td>
<td>α-Proton abstraction by Glu</td>
<td>3.0</td>
<td>106</td>
<td>20,21</td>
</tr>
<tr>
<td>Flavocytochrome δ2</td>
<td>Unresolved</td>
<td>3.7</td>
<td>104</td>
<td></td>
</tr>
<tr>
<td>Old yellow enzyme</td>
<td>Proton donation by Ty side chain</td>
<td>3.5</td>
<td>101</td>
<td>25</td>
</tr>
<tr>
<td>NADPH quinone reductase</td>
<td>Unresolved</td>
<td>3.5</td>
<td>103</td>
<td></td>
</tr>
<tr>
<td>UDP-N-acetylglucosamine reductase</td>
<td>Proton donation by Cu by ser side chain</td>
<td>3.1</td>
<td>108</td>
<td>40</td>
</tr>
<tr>
<td>Flavocytochrome c₅</td>
<td>Proton donation by Arg side chain</td>
<td>3.4</td>
<td>98</td>
<td>24</td>
</tr>
</tbody>
</table>

*aThe dotted circles and squares outline the site of oxidative attack and the group facilitating the formation of the double bond in the oxidized product, respectively.*

*bThe stereocchemical parameters in substrate binding were deduced from the available structures of enzymes in complex with substrate analogues. The position of the CH group representing the site of oxidative attack were measured based on the following atoms (see Table 1 for PDB entries): Cα of imino-Trp in complex with reduced D-amino acid oxidase; C7 of isoeugenol bound to vanillyl-alcohol oxidase; C3 of dehydroisoandrosterone bound to cholesterol oxidase; C6 of orotate bound to dihydroorotate dehydrogenase; C3 of octanoylCoA bound to medium chain acylCoA dehydrogenase; C2 of p-hydroxybenzaldehyde bound to old yellow enzyme; C2 of duroquinone in complex with NADPH-quinone reductase; EC3 of enolpyruvoyl-UDP-N-acetylglucosamine in complex with UDP-N-acetylglucosamine reductase and, C3 of malic acid bound to flavocytochrome c₅. This analysis does not include the complexes of glycolate oxidase with 3-decyl-2,5-dioxo-4-hydroxy-3-pyrroline and 4-carboxy-5-(1-pentyl)hexylsulphanyl-1,2,3-triazole because these inhibitors bear little resemblance to the substrate.*

*The mechanism of the lactate dehydrogenation reaction catalysed by flavocytochrome δ2 is controversial. In the assumption of a carbanion mechanism, His373 would abstract the substrate α-proton. Alternatively, in the hypothesis of a hydride transfer mechanism, His373 is supposed to deprotonate the substrate hydroxyl group.*

*Old yellow enzyme, NADPH quinone reductase, UDP-N-acetylglucosamine reductase and flavocytochrome c₅ catalyse a 'hydrogenation' (i.e. substrate reduction) rather than a dehydrogenation reaction.*
Several flavoenzymes catalyse an \( \alpha,\beta \) dehydrogenation. In this reaction, a carbon atom in the \( \alpha \) position with respect to a carbonyl or carboxyl group is deprotonated by an active-site base to allow reformation of an \( \alpha,\beta \) double bond.

Enzymes that catalyse a reaction of this kind typically establish a network of hydrogen bonds with the substrate carbonyl or carboxylate oxygen. These interactions are crucial in that they lower the pK\(_a\) of the \( \alpha \) carbon to make proton abstraction by a base feasible. A beautiful example of this type of modulation of substrate reactivity is provided by acylCoA dehydrogenases\(^{24}\) (Table 2). These enzymes shift the pK\(_{a}\) of the acylCoA \( \alpha \) carbon by 9–13 units using hydrogen bonds between the substrate carbonyl oxygen with the \( 2' \)-hydroxyl group of FAD and a backbone nitrogen atom. Such a drastic pK\(_{a}\) change allows a Glu side chain to act as the base that abstracts the proton from the substrate \( \alpha \) carbon, facilitating the oxidation reaction\(^{27}\).

A similar mechanism is employed by dihydroorotate dehydrogenase. In this enzyme, the substrate carbonyl group interacts with two Asn side chains, which increase the acidity of the adjacent carbon, facilitating its deprotonation by a Cys residue\(^{25,26}\). An analogous catalytic strategy is exhibited by UDP-\( N\)-acetylcytosine reductase\(^{27}\) and the old yellow enzyme\(^{28}\); although these enzymes catalyse the reaction in the opposite direction (a dehydrogenation rather than a dehydrogenation reaction). In these enzymes, an active-site residue (Ser, Arg and Tyr, respectively) donates a proton to the substrate \( \alpha \) carbon, coupled to reduction of the adjacent carbon atom by the flavin.

Vanillyl-alcohol oxidase displays another mechanism of substrate activation: the active-site cavity shifts the pK\(_{a}\) of the substrate amino group, thereby facilitating deprotonation of the substrate. This oxidase probably stabilizes the deprotonated form of the substrate amino group, thereby facilitating formation of the imino acid product. There is an evident functional analogy between the cofactor- and substrate-binding sites. Neither has a passive role in ligand recognition but instead are both active in modulating the chemical properties of the bound molecule. The protein milieu is crucial in that it fine-tunes the redox properties of the flavin and enhances the reactivity of the substrate towards dehydrogenation.

**Protecting the substrate from the solvent**

Another notable feature shared by flavin-dependent dehydrogenases is the accessibility of the active site. In all the available structures of enzyme complexes, it can be seen that the reactive part of the substrate invariably hides in a buried site located underneath the protein surface. This feature implies that catalysis takes place in a solvent-protected environment. Flavoenzymes are equipped with ingenious devices to open the active centre for substrate admission. In several cases, a mobile loop (\( \omega \)-amino acid oxidase\(^2\), cholesterol oxidase\(^2\) and dihydronicotinamide reductase\(^{24}\)) acts as a gate, closing and opening the active site. Likewise, a flexible domain can be used to facilitate formation of the imino acid product.

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**Implications for the catalytic mechanism**

A comparison of the catalytic sites of flavoenzymes catalysing dehydrogenation facilitates formation of the quinone methide intermediate, produced by substrate oxidation\(^8\). The intermediate is then hydrated in subsequent steps of the reaction. Although it is less well known, \( \omega \)-amino acid oxidase\(^2\) is also thought to promote catalysis by acting on the protonation state of the substrate. This oxidase probably stabilizes the deprotonated form of the substrate amino group, thereby facilitating formation of the imino acid product.

**Shielding the substrate from solvent**

Completion of the dehydrogenation reaction requires the formation of a double bond in the oxidized product (Fig. 1). This is brought about by the group adjacent to the site of oxidative attack. Flavoenzymes are able to modulate the reactivity of this sort of ‘activating group’, facilitating the dehydrogenation reaction. In a few cases, the structural elements with this modulating function have been analysed in detail. Several flavoenzymes catalyse an \( \alpha,\beta \) dehydrogenation. In this reaction, a carbon atom in the \( \alpha \) position with respect to a carbonyl or carboxyl group is deprotonated by an active-site base to allow reformation of an \( \alpha,\beta \) double bond.

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**Implications for the catalytic mechanism**

A comparison of the catalytic sites of flavoenzymes catalysing dehydrogenation...
reactions has led to the recognition of several structural relationships. These proteins appear to have adopted a sort of ‘two-handle’ strategy to perform their catalytic function. The site of oxidative attack represents the first ‘handle’; flavoproteins bind the substrate C–H group undergoing oxidation in a precisely defined position in front of the N5–C4a locus of the isoalloxazine ring (Fig. 3). The second ‘handle’ is given by the ‘activating group’, which is involved in the formation of the double bond of the dehydrogenated product. The protein actively modulates the reactivity of this group, often by affecting its protonation state.

Three additional features augment the catalytic effectiveness of such a two-handle strategy: First, the protein–flavin interactions are effective at fine-tuning the cofactor’s redox properties. Second, the conserved hydrogen-bond interactions at the N1 and NS loci can regulate the ability of the flavin to carry out the two-electron oxidation of the substrate. Finally, the substrate and the residues directly involved in catalysis are invariably shielded from the solvent, implying that catalysis takes place in a protected and highly controlled environment.

The presence of these structural relationships raises the question of their mechanistic implications. A fundamental problem in flavoenzymology concerns the detailed mechanism of the dehydrogenation step: how do flavoenzymes catalyse the rupture of the C–H bond at the site of oxidative attack with transfer of two electrons to the flavin? Several hypotheses have been put forward:\cite{13,27,30,31} (Fig. 4):

(1) hydride transfer, which involves the direct transfer of a hydride anion from the substrate C–H group to the flavin N5 position;
(2) the radical mechanism, in which rupture of the C–H bond occurs by the removal of a hydrogen atom from the substrate carbon with formation of a radical pair that collapses to produce the reduced flavin and oxidized product;
(3) the carbanion mechanism, in which an active-site base removes a proton from the substrate C–H group, thus producing a carbanion that donates two electrons to the flavin either directly or via a covalent intermediate.

These mechanistic problems cannot be solved solely on the basis of structural data. However, it seems that all these mechanistic proposals require, at least to some extent, juxtaposition between the flavin N5–C4a locus and the reactive C1 group of the substrate. Thus, generally speaking, the stereochemically conserved proximity between the flavin and the site of oxidative attack does not necessarily indicate the existence of just one conserved mechanism for flavin-mediated dehydrogenation. Indeed, although many of the reviewed enzymes are proposed to function via hydride transfer, there is no general consensus about the exact mechanism for some of them, flavocytochrome b5 and o-amino acid oxidase being the most controversial cases:\cite{12,19}

On the other hand, the stereochemical principles underlying the mutual interactions between the substrate CH group and the flavin are surprisingly well conserved and cannot be neglected. They represent a validation test for the plausibility of any proposed mechanism, which must be compatible with the observed stereochemistry of substrate binding. An important challenge for future flavoenzymological studies will be defining the exact stereochemical requirements of each of the proposed mechanisms, to evaluate their compatibility with the three-dimensional structures. In this regard, significant insight will be gained from the examination of flavoenzyme structures refined at atomic resolution, which will become available thanks to the power of the newest synchrotron X-ray diffraction beam lines. These structures will provide the framework for quantum-mechanical calculations and molecular simulations:\cite{32}. The integration of these data with those derived from classical enzymological analyses will ultimately solve this mechanistic riddle.

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So you’re saying Y2K is NOT a new tyrosine kinase ?!

Jamie Simon, The Salk Institute, La Jolla, USA.