Flavoenzymes: diverse catalysts with recurrent features

Marco W. Fraaije and Andrea Mattevi

Many biochemical processes exploit the extraordinary versatility of flavoenzymes and their flavin cofactors. Flavoproteins are now known to have a variety of folding topologies but a careful examination of their structures suggests they share recurrent features in their catalytic apparatus. The flavoenzymes that catalyse dehydrogenation reactions share a few invariant features in the hydrogen-bond interactions between their protein and flavin constituents. Similarly, the positioning of the reactive part of the substrate with respect to the cofactor is generally conserved. Modulation of substrate and cofactor reactivity and exact positioning of the substrate are key elements in the mode of action of these enzymes.

FLAVOENZYMES HAVE THE unique abil-
ity to catalyse a wide range of biochemi-
cal reactions. They are involved in the
dehydrogenation of a variety of metabo-
lates, in one- and two-electron transfer from and to redox centres, in light
emission, in the activation of oxygen for
oxidation and hydrogenation reactions7.
Because of the extraordinary spectro-
copic properties of the flavin co-factor, flavoproteins are perfectly suited to
detailed enzymological studies1. As a
result, they have emerged as one of the
best-studied enzyme families. Our
understanding of flavin chemistry has been augmented in the recent years by the wealth of information ob-
tained from structural studies of these proteins. At present, the Protein Data
Bank (PDB) contains about 300 entries for flavin adenine dinucleotide (FAD)-
dependent and flavin mononucleotide (FMN)-dependent proteins. These struc-
tures display a number of folding archi-
tectures, ranging from the frequent (β/α)-barrel to unique topologies, such as the α/β structure of acylCoA dehy-
drogenases1.

The distribution of these folds does not correlate with function. Topologically similar flavoenzymes can catalyse different reactions, whereas proteins performing similar functions can have dissimilar folding architectures. For example, flavocystochromes b3 (Ref. 4) and i-o amino acid oxidase11,12 display completely different topologies, even though they catalyse a similar chemical reaction (Table 1). Conversely, functionally dis-
similar enzymes, such as oxidases (i-
o amino acid oxidase12 and cholesterol oxidase1), hydroxylases (p-hydroxy-
benzoate hydroxylase14) and oxidoreduc-
tases (fumarate reductase and related enzymes15,16), share a topologically simi-
lar FAD-binding domain. In this respect, flavoproteins are perfectly in line with the finding of Martin et al.17 that there is little relationship between folding topol-
y and function.

The catalytic power of an enzyme lies in its active site, in which the atoms are positioned to allow substrate binding and to stabilize reaction intermediates. It is well known that structurally dis-
similar proteins can employ the same active site geometry and catalytic de-
ces to perform similar functions. This notion is beautifully exemplified by the
so-called catalytic triad present in a vast number of otherwise unrelated families of
hydroxide enzymes (recently re-
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PDB and searched for recurrent features in their catalytic apparatus. Our analy-
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2 The catalytic power of an enzyme lies in its active site, in which the atoms are positioned to allow substrate binding and to stabilize reaction intermediates. It is well known that structurally dissimilar proteins can employ the same active site geometry and catalytic devices to perform similar functions. This notion is beautifully exemplified by the so-called catalytic triad present in a vast number of otherwise unrelated families of hydroxide enzymes (recently reviewed in Ref. 12). On this basis, we have examined the three-dimensional structures of flavoenzymes that are available through the PDB and searched for recurrent features in their catalytic apparatus. Our analysis is focused on the group of flavoproteins that perform a dehydrogenation reaction. This involves the rupture of a kinetically stable C–H bond, coupled to the transfer of two electrons to the flavin (Fig. 1). Throughout this article, we shall call the carbon atom involved...
of the flavin is about potential for the two-electron reduction hydrophilic pyrimidine ring. The redox bic dimethylbenzene moiety with the formed by the fusion of the hydrophobic temper is the reactive part of the flavin approximately enzimes, spanning a range from approxi-
mately this value can greatly vary in flavo-
}
favourable in the reduced than in the oxidized state. Noticeably, vanillyl-alcohol oxidase and glycolate oxidase, which are unusual because they lack the N5 hydrogen bond, exhibit 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, fatty 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; Table 2).

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 geometries are conserved across the flavin-dependent dehydrogenase family.
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</th>
<th>Product</th>
<th>Substrate activation</th>
<th>Distance (d) ,(\text{Å})</th>
<th>Angle (\beta ), (\text{°})</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>
<td></td>
<td></td>
</tr>
<tr>
<td>Vanillyl-alcohol oxidase</td>
<td>Phenolate anion stabilization</td>
<td>3.3</td>
<td>117</td>
<td>26</td>
<td></td>
<td></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>
<td></td>
<td></td>
</tr>
<tr>
<td>Dihydroorotate dehydrogenase</td>
<td>(\alpha)-Proton abstraction by Cys</td>
<td>3.5</td>
<td>96</td>
<td>17,22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AcylCoA dehydrogenase</td>
<td>(\alpha)-Proton abstraction by Glu</td>
<td>3.0</td>
<td>106</td>
<td>20,21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flavocytochrome (\delta)</td>
<td>Unresolved</td>
<td>3.7</td>
<td>104</td>
<td>24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Old yellow enzyme(a)</td>
<td>Proton donation by Tyr side chain</td>
<td>3.5</td>
<td>101</td>
<td>25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NADPH quinone reductase(a)</td>
<td>Unresolved</td>
<td>3.5</td>
<td>103</td>
<td>40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UDP-N-acetylenolpyruvoylglucosamine reductase(a)</td>
<td>Proton donation to Cys by Ser side chain</td>
<td>3.1</td>
<td>108</td>
<td>40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flavocytochrome (c)_2</td>
<td>Proton donation by Arg side chain</td>
<td>3.4</td>
<td>98</td>
<td>24</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(a\)The 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.

\(b\)The stereochemical 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): C of imino-Trp in complex with reduced D-amino acid oxidase; C7 of isoegenol bound to vanillyl-alcohol oxidase; C3 of dehydroisoandrosterone bound to cholesterol oxidase; C2 of acetate bound to dihydroorotate dehydrogenase; C3 of dehydroisoandrosterone bound to cholesterol oxidase; C2 of orotate bound to dihydroorotate dehydrogenase; C3 of octanoylCoA bound to medium chain acylCoA dehydrogenase; C3 of carboxylate bound to flavocytochrome \(\delta\); C2 of phenylpyruvaldehyde bound to old yellow enzyme; C2 of quinonoid in complex with NADPH-quinone reductase; EC3 of aminoacyl-UDP-N-acetylglucosamine complex with UDP-N-acetylenolpyruvoylglucosamine reductase and, C3 of malonic acid bound to flavocytochrome \(c\)_2. 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. Similarly, the complex between polyamine oxidase and MDL72527 (Ref. 15) was not considered, because it does not allow an unambiguous assignment of the position of the site of oxidative attack.

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

\(d\)Old yellow enzyme, NADPH quinone reductase, UDP-N-acetylenolpyruvoylglucosamine reductase and flavocytochrome \(c\)_2 catalyse a ‘hydrogenation’ (i.e. substrate reduction) rather than a 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 carbonate or carboxyl group is deprotonated by an active-site base to allow formation of an $\alpha$,$\beta$ double bond. Enzymes that catalyse a reaction of this kind typically establish a network of hydrogen bonds with the substrate carboxyl or carbonate 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 dehydrogenases30 (Table 2). These enzymes shift the $pK_a$ of the acylCoA $\alpha$ carbon by 9–13 units using hydrogen bonds between the substrate carboxyl 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 reaction21.

A similar mechanism is employed by dihydrolipoate dehydrogenase. In this enzyme, the substrate carbon group interacts with two Asn side chains, which increase the acidity of the adjacent carbon, facilitating its deprotonation by a Cys residue22,23. An analogous catalytic strategy is exhibited by UDP-$N$-acyetylornithine:ornithine decarboxylase22 and the old yellow enzyme22; although these proteins catalyse the reaction in the opposite direction (a hydration 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, which is preferentially bound by the protein in the phenolate anionic form. In this way, the enzyme facilitates formation of the quinone methide intermediate, produced by substrate oxidation26. The intermediate is then hydrated in subsequent steps of the reaction. Although it is less well known, $\beta$-amino acid oxidase24 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.

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 binds 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 ($\beta$-amino acid oxidase25, cholesterol oxidase25 and dihydrolipoate dehydrogenase26) or side chain (UDP-$N$-acyetylornithine:ornithine decarboxylase25) acts as a gate, closing and opening the active site. Likewise, a flexible domain can be used to fulfill this role, as observed in flavocytochrome $c_6$ and related enzymes of the succinate-dehydrogenase family25. Conversely, polyamine oxidase25 and acylCoA dehydrogenases2 use a different technique to shield the substrate. In both cases, the exact match of the substrate with a narrow active-site channel causes the solvent to be expelled upon substrate binding.

**Shielding the substrate from solvent** is also a common phenomenon among the NAD(PH)-dependent dehydrogenases25. Protection from solvent seems to be a prerequisite for catalysing a dehydrogenation reaction, regardless of the type of cofactor employed by the enzyme.

**Implications for the catalytic mechanism**

A comparison of the catalytic sites of flavoenzymes catalysing dehydrogenation
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 catalyze 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:

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 direct transfer of two electrons to the flavin.

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 CH 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-aminoc acid oxidase being the most controversial cases.

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.

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Figure 4

Schematic view of different mechanisms for flavin-catalysed substrate dehydrogenation reactions – direct hydride transfer (1), the carbanion mechanism (2,3) and the radical mechanism (4,5). R1 and R2 indicate unspecific substituents of the CH atom undergoing oxidation, and X is the activating group.
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