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Covalent Flavinylation Is Essential for Efficient Redox Catalysis in Vanillyl-alcohol Oxidase*

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By mutating the target residue of covalent flavinyla-

tion in vanillyl-alcohol oxidase, the functional role of the histidyl-FAD bond was studied. Three His422 mu-

tants (H422A, H422T, and H422C) were purified, which all contained tightly but noncovalently bound FAD. Steady state kinetics revealed that the mutants have retained enzyme activity, although the turnover rates have decreased by 1 order of magnitude. Stopped-flow anal-

ysis showed that the H422A mutant is still able to form a stable binary complex of reduced enzyme and a quinone methide product intermediate, a crucial step during vanillyl-alcohol oxidase-mediated catalysis. The only significant change in the catalytic cycle of the H422A mutant is a marked decrease in reduction rate. Redox potentials of both wild type and H422A vanillyl-

alcohol oxidase have been determined. During reduc-

tion of H422A, a large portion of the neutral flavin semiquinone is observed. Using suitable reference dyes, the redox potentials for the two one-electron couples have been determined: −17 and −113 mV. Reduction of wild type enzyme did not result in any formation of flavin semiquinone and revealed a remarkably high redox potential of +55 mV. The marked decrease in redox potential caused by the missing covalent histidyl-FAD bond is reflected in the reduced rate of substrate-medi-

ated flavin reduction limiting the turnover rate.

Elucidation of the crystal structure of the H422A mu-

tant established that deletion of the histidyl-FAD bond did not result in any significant structural changes. These results clearly indicate that covalent interaction of the isoalloxazine ring with the protein moiety can markedly increase the redox potential of the flavin co-

factor, thereby facilitating redox catalysis. Thus, forma-

tion of a histidyl-FAD bond in specific flavoenzymes might have evolved as a way to contribute to the enhancement of their oxidative power.

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The atomic coordinates and structure factors for the mutant VAO (H422A) structures have been deposited in the Macromolecular Structure Database of the European Bioinformatics Institute (EBI), Hinxton, UK (PDB codes for native: 1qtl and r1qtl; PDB codes for isoeugenol-

complexed: 1qlu and r1qlu).

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Until now, several hundred flavin-containing enzymes have been described. Most of these enzymes contain a dissociable FAD or FMN cofactor. However, it has been shown that in several cases the flavin is covalently linked to an amino acid of the polypeptide chain. In fact, in humans 10% of the cellular FAD is covalently bound to enzymes like e.g. succinate dehydrogenase and monoamine oxidase (1). Within the group of covalent flavoproteins, five different types of covalent flavinyla-

tion have been identified. Except for a few examples of cysteinyl- or tyrosyl-linked flavins, tethering to a histidine is by far the most favored binding mode, since it has been observed in about 20 isolated flavoenzymes (for a recent review, see Ref. 2).

Although the first covalent flavoprotein, succinate dehydro-

genase, was already identified in 1955 (3), the rationale for covalent flavinylaition is still unresolved. Only recently, a clear influence of the covalent bond on the reactivity of the cofactor has been observed in trimethylamine dehydrogenase. Unlike the wild type enzyme, mutants of trimethylamine dehydrogenase containing dissociable FMN (4, 5) are inactivated by hydroxylation of the cofactor. Apparently, covalent tethering of the cofactor can prevent inactivation of the cofactor. Another striking role of the covalent flavin bond has been suggested for p-cresol methylhydroxylase. Inspection of the crystal structure of this flavocytochrome indicates that the tyrosyl-FAD bond facilitates electron transfer from the reduced FAD to the heme of the cytochrome c subunit (6). It has also been shown that introduction of a covalent bond at the 8α-position of the isoalloxazine ring can result in an increase of the redox potential (7–9). A reduced oxidative activity of mutants of succinate dehydrogenase and fumarate dehydrogenase was rationalized by a decrease of the flavin redox potential due to the missing covalent bond (10–12).

Although the above mentioned results indicate that covalent flavinylaition might be advantageous for catalysis, it should be mentioned that several covalent flavoenzymes have homolo-

gous flavin-dissociable counterparts that display similar en-

zyme activities (13–15). Therefore, the rationale for this atyp-

ical protein modification might not be uniform, and also other factors may have attributed to the formation and conservation of covalent flavoproteins throughout evolution. For example, covalent binding of flavins can be favorable for flavoenzymes that are localized in a flavin-deficient environment. In this respect, it is noteworthy to mention that almost all eucaryotic covalent flavoproteins have been found to be compartmental-

ized (16). Therefore, the motive for covalent flavinylaition might also reside in the physiological function and environment of these flavoenzymes. Further, covalent flavinylaition can also be of structural benefit. Except for cofactor saturation of the active site, the introduction of a covalent flavin-protein bond may well
result in an improved protein stability.

In this study, we have addressed the function of covalent flavinylation in vanillyl-alcohol oxidase (VAO), which is a covalent flavoprotein containing 8a-(N\text{3}-histidyl)-FAD (17). VAO is a fungal enzyme of 560 residues that can efficiently oxidize a broad range of phenolic compounds (18, 19). Kinetic analysis of the conversion of the physiological substrate 4-(methoxymethyl)phenol has revealed that the enzyme operates in a ternary complex mechanism (20) (Scheme 1).

During the first half-reaction, the substrate reduces the flavin cofactor, resulting in the formation of a stable binary complex of reduced enzyme and a p-quinone methide intermediate of the substrate. Subsequent reoxidation of the cofactor by molecular oxygen completes the catalytic cycle, yielding the final products and oxidized enzyme.

Recently, we have solved the crystal structure of VAO (21), representing the first crystal structure of a flavoenzyme with a histidyl-bound FAD. The VAO monomer comprises two domains, with the larger domain forming a FAD binding module while the cap domain, containing the histidine linking the FAD, covers the active site (Fig. 1). By sequence homology analysis and inspection of the crystal structure of VAO, we have identified a novel flavoprotein family sharing a conserved FAD binding domain (22). Members of this family that have been characterized catalyze a variety of oxidation/reduction reactions and include several well studied covalent flavoproteins: e.g. 6-hydroxy-d-nicotine oxidase (23), p-cresol methylhydroxylase (6), and l-gulono-\gamma-lactone oxidase (24). Interestingly, in about 35% of the sequences of these VAO homologs, a conserved histidine residue is found, which is predicted to be flavinylated (22). Apparently, covalent flavinylation within this flavoprotein family is a relatively frequent event, indicating that the conserved topology is particularly suited for covalent flavinylation or that this flavoprotein family originates from an ancestral covalent flavoprotein.

To study the effect of covalent flavinylation on the structural and kinetic properties of VAO, we have mutated the target residue for flavinylation, His\text{422}, thereby preventing formation of the histidyl-FAD bond. Here we present the crystal structure of the H422A mutant containing a dissociable FAD. Furthermore, the effects of the covalent bond deletion on the kinetic and redox properties of VAO are discussed.

**EXPERIMENTAL PROCEDURES**

**Materials—**E. coli strain DH5\text{a} (25) and the plasmids pUCBM20 (Roche Molecular Biochemicals) and pGEM-5Zf(+) (Promega) were used for cloning, whereas E. coli strain TG2 (26) and the plasmid pEMBL19(–) (Roche Molecular Biochemicals) were used for expression of the vanA gene. T4 DNA ligase, restriction enzymes, isopropyl-\beta-D-thiogalactopyranoside, yeast extract, and tryptone extract were from Life Technologies, Inc. Pcoe DNA polymerase and dNTPs were purchased from Roche Molecular Biochemicals, and Super Taq DNA polymerase was from HT Biotechnology. Ampicillin and SDS were from BDH Chemicals. Forward M13 and reverse M13 sequencing primers were from Amersham Pharmacia Biotech. Oligonucleotides were synthesized by Life Technologies.

Glucose oxidase was from Roche Molecular Biochemicals, and benzyl viologen, indigo disulfonate, indigo tetrasulfonate, methylene blue, xanthine, and xanthine oxidase were from Sigma. Resorufin, isoeugenol, and 4-(methoxymethyl)phenol were products from Aldrich, and thionin was from Eastman Kodak Co. All other chemicals were from Merck and of the purest grade available.

**Mutagenesis—**To simplify the site-directed mutagenesis procedure, a Sfi restriction site was created by a silent mutation at position 882 in the original expression plasmid pIM3972 (27). For this, the Ncol–NsiI fragment of pIM3972 was ligated into pGEM-Zf(+). Subsequently, the silent mutation was introduced by polymerase chain reaction-based mutagenesis using the oligonucleotide 5’-CAAGCCGTCGACATTATCGTCC-3’ (where C denotes the mutated base). The mutated Ncol–NsiI fragment was ligated into pIM3972. The resulting pBC11 plasmid was used for polymerase chain reaction-based mutagenesis. For the His\text{422} replacements, the Ssal–KpnI fragment of pBC11 was ligated into pUCBM20. This construct was used for polymerase chain reaction-based mutagenesis with the oligonucleotide 5’-CCCTAATGGTGCGXXTCGTGCTTCTCC-3’ (where XX denotes the replacement for GC (H422A), AC (H422T), and TG (H422C), respectively). The mutated Ssal–KpnI fragments were ligated into pBC11, yielding pBC20 (H422A), pBC21 (H422T), and pBC22 (H422C). Successful mutagenesis was confirmed by plasmid sequencing.

**Analytical Methods—**Mutant proteins were expressed and purified as described for wild type VAO (27). SDS-polyacrylamide gel electrophoresis was carried out according to Laemmli (28). Protein staining of the gels was achieved using Coomassie Brilliant Blue R-250, while protein staining of the analytical gel filtration was performed on a Superdex 200 HR 10/30 column using an Akta system (Amersham Pharmacia Biotech) under conditions described elsewhere (30).

All analytical experiments were performed at 25 °C in 50 mM potassium phosphate buffer, pH 7.5. Flavin absorption spectra were recorded on an automated Amino DW-2000 spectrophotometer. Flavin fluorescence emission spectra (excitation at 450 nm) were obtained using an Amino SPF-500 fluorometer. Redox potentials were determined by the method described by Massey (31). For this, a cuvette containing enzyme (2–12 \mu M), benzyl viologen (2.0 \mu M), redox dye (2–10 \mu M), and xanthine (400 \mu M) was made anaerobic by flushing with argon, after which 0.2–0.5 mM xanthine oxidase was added (total volume was 750 \mu L). To maintain anaerobic conditions during the reductive titration, the head space of the septum-closed cuvette was continuously flushed with argon. During the xanthine oxidase-mediated reduction (lasting typically 1–2 h), spectra were recorded automatically every 30 s using a Hewlett-Packard 8453A diode array spectrophotometer.

Steady state kinetic experiments were performed as described earlier (18). Stopped-flow kinetics were carried out with a Hi-Tech SF-51 apparatus equipped with a Hi-Tech M390 monochromator diode-array

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The abbreviation used is: VAO, vanillyl-alcohol oxidase.
Role of Covalent Flavinylation in Vanillyl-alcohol Oxidase

Purification and General Characterization—The three His<sup>422</sup> mutant enzymes were purified using the same protocol as has been developed for wild type VAO, resulting in bright yellow protein preparations. SDS-polyacrylamide gel electrophoresis combined with protein staining showed that all three mutant preparations contained protein with a molecular weight similar to that of wild type VAO. Further, fluorescence analysis of the same gel revealed that the His<sup>422</sup> mutants do not contain covalent histidyl-FAD, since, in contrast to the wild type enzyme, no flavin fluorescence could be observed. This finding was confirmed by precipitating the mutant enzymes with 5% (w/v) trichloroacetic acid, resulting in a yellow supernatant containing FAD and a colorless pellet of aggregated protein. Treating wild type VAO in the same way resulted in a colorless supernatant, while a yellow pellet was formed (17).

Analytical gel filtration experiments showed that the His<sup>422</sup> mutants are mainly in the octameric form with a small portion being present as a dimer. By simultaneously monitoring the absorbance at 280 and 450 nm, the presence of FAD in both forms was monitored by the absorbance decrease at 439 nm (Fig. 2). When exciting at 450 nm, emission fluorescence spectra were observed with maxima at 535 nm. Similar to wild type VAO, the flavin fluorescence quantum yields were very low, indicating that the covalent histidyl-FAD bond is not a major factor in flavin fluorescence quenching in VAO. From these experiments, it can also be concluded that the His<sup>422</sup> mutants have a high affinity for FAD, since no significant amounts of free FAD could be detected. These results indicate that the covalent histidyl-FAD bond is not essential for FAD binding.

Kinetic Characterization—To examine the effect of the mutations on enzyme kinetics, the steady state kinetic parameters were determined using 4-(methoxymethyl)phenol as substrate (Table I). It was revealed that all purified mutants display significant enzyme activity. The <i>k<sub>cat</sub></i> values of the three mutants decreased by about 1 order of magnitude, while the <i>K<sub>m</sub></i> values were significantly lower with respect to wild type enzyme. In contrast to observations with some other mutated covalent flavoenzymes (5, 39, 40), the activity remained constant during prolonged incubations (10 min) with substrate. This indicates that the FAD cofactor remains bound to the enzyme and is not inactivated during catalysis. Since all three mutants showed similar spectral and steady state kinetic properties, we decided to perform a more detailed study of the H422A mutant.

The apparent increased affinity for phenolic ligands was studied in some more detail by determining the dissociation constant for the competitive inhibitor isoeugenol. Titration experiments with the H422A mutant revealed that the dissociation constant for isoeugenol was also significantly lower when compared with the corresponding <i>K<sub>d</sub></i> value for wild type enzyme (18; 4.6 versus 22 μM).

To identify the rate-limiting step in catalysis by the H422A mutant, stopped-flow experiments were performed. For this, the kinetics of the individual half-reactions were studied. The reductive half-reaction of H422A appeared to be a monophasic process as has been found for wild type VAO (Fig. 3). Anaerobic reduction by 4-(methoxymethyl)phenol resulted in fully reduced enzyme as monitored by the absorbance decrease at 439 nm. This indicates that, as in wild type enzyme, the substrate-
**TABLE I**

Kinetic constants for the reaction of the His^{222} mutants and wild type VAO with 4-(methoxymethyl)phenol (pH 7.5, 25 °C)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild type ( ^a )</th>
<th>H422A</th>
<th>H422C</th>
<th>H422T</th>
</tr>
</thead>
<tbody>
<tr>
<td>( K_{m} ) (μM)</td>
<td>55</td>
<td>34</td>
<td>37</td>
<td>41</td>
</tr>
<tr>
<td>( k_{cat} ) (s⁻¹)</td>
<td>3.1</td>
<td>0.27</td>
<td>0.32</td>
<td>0.28</td>
</tr>
<tr>
<td>( K_{d} ) (μM)</td>
<td>48</td>
<td>18</td>
<td>ND (^b)</td>
<td>ND</td>
</tr>
<tr>
<td>( k_{red} ) (s⁻¹)</td>
<td>3.3</td>
<td>0.30</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>( k_{ox} ) (M⁻¹ s⁻¹)</td>
<td>( 1.5 \times 10^6 )</td>
<td>( 2.5 \times 10^5 )</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

\(^a\) Data taken from Ref. 20.

\(^b\) ND, not determined.

**Fig. 3.** Spectral changes observed upon anaerobic reaction of H422A (4.0 μM) with 4-(methoxymethyl)phenol (500 μM, 25 °C, pH 7.5). Original spectra are shown from 5.6 ms to 10 s with intervals of 1 s. The inset shows normalized spectra obtained after deconvolution of the original 96 spectral scans ranging from 5.6 ms to 20 s, where A represents the initial spectrum, spectrum B the intermediate species, and spectrum C the finally obtained spectrum at indefinite time. The data were fitted with a consecutive irreversible model (A → B → C).

Induced reduction of the flavin cofactor is an irreversible process (41). The maximal reduction rate of 0.30 s⁻¹ approaches the maximal turnover rate, indicating that the rate of turnover is mainly determined by the rate of flavin reduction. This finding was confirmed by an enzyme-monitored turnover experiment that showed that, during turnover, more than 90% of H422A is in the oxidized state. In line with the decreased \( K_{m} \) value, it was found that the dissociation constant for 4-(methoxymethyl)phenol is also relatively low (Table I). Furthermore, using diode-array detection, it could be shown that upon anaerobic reduction with 4-(methoxymethyl)phenol, H422A is able to form and stabilize a binary complex between reduced enzyme and the p-quinone methane product intermediate. Decay of this complex, resulting in formation of 4-hydroxymethylaldehyde, was only observed when the anaerobic reduction reaction was monitored for a relatively long period of time (>10 s) (see Fig. 3, inset). Apparently, the mutant enzyme has still retained the remarkable ability to shield the formed quinone methide intermediate from solvent (20). Upon reoxidation of the reduced complex by molecular oxygen, the quinone methide intermediate was efficiently hydrated as has been found for the wild type enzyme. Analysis of the oxidative half-reaction showed that the rate of reoxidation of the binary complex is also similar to that of the wild type enzyme (Table I). From these kinetic measurements it can be concluded that except for a decrease in the rate of flavin reduction, the catalytic mechanism by which substrate is converted has essentially been conserved in the H422A mutant.

**Redox Potential Determination**—For both wild type VAO and the H422A mutant, the flavin redox potential was determined using the method described by Massey (31) (see “Experimental Procedures”). When the xanthine oxidase-mediated reduction of the mutant was monitored in the absence of a reference dye, the initial formation of a one-electron-reduced flavin species was observed (Fig. 4). Based on the typical absorbance properties in the 500–600-nm region, the radical intermediate could be identified as the blue neutral flavin semiquinone (42, 43). Previously, it has been observed that in wild type VAO the red anionic flavin radical is transiently formed upon light-induced reduction (17). Apparently, the H422A mutation results in an increase of the pKₐ of the one-electron-reduced form of the flavin cofactor in VAO. Such a pKₐ shift of the flavin semiquinone has also been observed in flash photolysis studies on 8a-histidyl flavins (44). From estimation of the maximal amount of flavin radical formed during the reduction process (70–75%) (see Fig. 4, inset), it can be deduced that the redox potentials for the oxidized/semiquinone couple \( (E₁) \) and the semiquinone/hydroquinone couple \( (E₂) \) are separated by about 85 ± 7 mV (45). By using methylene blue (+11 mV) and indigo disulfonate (−118 mV) as reference dyes, the two redox potentials could be accurately determined. The redox potential difference with respect to the dye could be calculated by plotting the log([ox]/[red]) ratio of the enzyme versus the corresponding log([ox]/[red]) ratio of the dye (46). The slopes for the two respective plots were 0.52 and 0.55, approaching the theoretical value of 0.5, indicating that equilibrium between the redox components during the reduction experiment was established. The two redox potentials \( E₁ \) and \( E₂ \) were −17 and −113 mV, respectively. These values are in line with the above mentioned estimated separation of the two redox potentials. Furthermore, the data obtained by reducing the mutant in the presence of resorufin (−51 mV) also corroborated with these results. Reduction of this dye only occurred after formation of flavin semiquinone, while full reduction of the flavin only started when most of the dye was already reduced. The midpoint redox potential \( (E_m) \) of H422A is −65 mV \( (E_m = (E₁ + E₂)/2) \); see Ref. 45). The \( E_m \) of wild type VAO could be similarly determined by using thionin (+60 mV) as reference dye. In contrast with H422A, the reduction of wild type VAO did not result in any observable formation of a radical species. Plotting the data according to Minnaert (46) resulted in a slope of 0.97, which is close to the theoretical value of 1.0 for a two-electron reduction process and a midpoint redox potential of +55 mV. The \( E_m \) for wild type enzyme (+55 mV) is markedly higher when compared with the \( E_m \) for the H422A mutant (−65 mV). This indicates that the histidyl-FAD covalent bond together with specific non-covalent cofactor-protein interactions results in an exceptional high \( E_m \) that is about 250 mV higher than the \( E_m \) of free FAD (31). To our best knowledge, only one other example of a flavoenzyme exhibiting an equally high \( E_m \) (+55 mV) has been reported, thiamine oxidase (47). Interestingly, this bacterial enzyme also contains a histidyl-bound FAD.

**Structure of H422A**—The crystal structure of unliganded H422A was determined at 2.2 Å (Table II). The resolution obtained for the mutant structure is an improvement when compared with the originally determined structure of wild type VAO (2.5 Å) (21). The x-ray analysis clearly shows that there is no covalent bond between the FAD cofactor and any residue of the polypeptide chain. A comparison between the mutant and the wild type models showed that the H422A replacement did not cause any large conformational change. Superposition of the two structures produced a root mean square deviation for all C-α atoms of only 0.27 Å. Particularly, in the wild type structure, the flavin ring is fully planar, and, except for the covalent bond, all other interactions with the isoalloxazine ring are conserved. The position of the C-α atom of Ala^{422} did not
change significantly, resulting in a distance between the C-8 methyl group of the flavin and the C-β atom of Ala422 of 5.3 Å. Further, all active site residues appear to have essentially retained their positions (Fig. 5), and, as in the wild type enzyme structure, an acetate molecule is found in the active site. The largest variations concern residues 410–417, which interact with the edge of the dimethylbenzene moiety of the flavin. The largest movement is that of C-δ1 of Ile414 (1.3 Å), although all other atomic shifts in these residues do not exceed 1.0 Å. Movements of these atoms accommodate the missing side chain of His422. Furthermore, the imidazole ring of His61 rotates by about 50°, pointing to the C-8a methyl group of the flavin ring.

The crystal structure of the H422A mutant in complex with isoeugenol was determined at 2.4 Å. The crystal soaked with isoeugenol shows clear density for the substrate analog isoeugenol in proximity of the flavin ring. The two structures of the H422A mutant are virtually identical, as indicated by a root mean square deviation of 0.13 Å for all Cα atoms.

The deletion of the covalent histidyl-FAD bond does not result in any evident structural perturbations upon ligand binding, which could explain the reduced enzyme activity of the H422A mutant. Further, the H422A-isoeugenol complex structure revealed an apparent identical binding mode for the phenolic inhibitor when compared with the wild type inhibitor complex structure (21).

**DISCUSSION**

The results presented in this paper demonstrate that covalent flavinylation is not a prerequisite for efficient FAD binding in VAO. All three His422 mutants were purified in the holo form, while no FAD dissociation could be detected in any of the performed experiments and no influence on the hydrodynamic properties was observed. These results are perfectly in line with the determined crystal structure of the H422A mutant. Analysis of the three-dimensional structure of this noncovalent FAD containing VAO variant revealed that except for the replacement of the histidine no significant structural perturbations can be observed with respect to the wild type VAO structure (21).

**TABLE II**

<table>
<thead>
<tr>
<th></th>
<th>H422A</th>
<th>H422A with isoeugenol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resolution (Å)</td>
<td>20–2.2</td>
<td>20–2.4</td>
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<tr>
<td>Observed reflections</td>
<td>285,553</td>
<td>216,054</td>
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<tr>
<td>Unique reflections</td>
<td>55,414</td>
<td>43,343</td>
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<td>Completeness of data (%)</td>
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<td>94.8 (88.4)</td>
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<tr>
<td>Multiplicity*</td>
<td>2.1 (1.8)</td>
<td>2.1 (2.5)</td>
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<tr>
<td>Intensities (I/E0)*</td>
<td>6.0 (2.8)</td>
<td>4.9 (2.5)</td>
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<tr>
<td>Rmerge (%)*</td>
<td>8.9 (22.6)</td>
<td>11.9 (22.1)</td>
</tr>
<tr>
<td>a = b = 129.66, c = 132.30</td>
<td></td>
<td>a = b = 129.84, c = 133.90</td>
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<td>Rfactor (%)</td>
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<td>Rfree (%) (2000 reflections)</td>
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<td>Number of protein atoms</td>
<td>8692</td>
<td>8692</td>
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<tr>
<td>Number of water atoms</td>
<td>373</td>
<td>257</td>
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<tr>
<td>Number of FAD atoms</td>
<td>106</td>
<td>106</td>
</tr>
<tr>
<td>Number of ligand atoms</td>
<td>8 (acetate)</td>
<td>22 (isoeugenol)</td>
</tr>
<tr>
<td>r.m.s.d. from ideality*</td>
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<td></td>
</tr>
<tr>
<td>Bond lengths (Å)</td>
<td>0.013</td>
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</tr>
<tr>
<td>Bond angles (°)</td>
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<td>Planar groups (Å)</td>
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<tr>
<td>Ramachandran plot (%)*</td>
<td>88.3/11.6/0.1/0</td>
<td>89.0/10.8/0.2/0</td>
</tr>
</tbody>
</table>

a The values relating to the highest resolution shell are given in parentheses.

b The root mean square deviations (r.m.s.d.) were calculated using the program REFMAC (33).

c Percentage of residues in most favored, allowed, generously allowed, and disallowed regions of the Ramachandran plot as checked with the program PROCHECK (54).
was prepared with MOLSCRIPT (55).

The oxidative power of specific flavoproteins.

Covalent flavinylation is a widely used mechanism to enhance the redox properties of the flavin cofactor in VAO (4). With most of these noncovalent mutant proteins, enzyme activity was retained to some extent. Only for succinate dehydrogenase, it was found that the noncovalent variants had lost the capacity of oxidizing succinate. In the case of fumarate reductase and succinate dehydrogenase, the decrease in oxidation rate of these mutant enzymes was tentatively assigned to the redox properties of the flavin cofactor (12). However, no comparative redox potentials were determined to support this hypothesis. Nevertheless, it is striking to notice, that when comparing flavoenzymes of which the redox potential has been determined, flavoproteins displaying a relatively high redox potential often contain covalent FAD or FMN. For example, the covalent flavoenzymes succinate dehydrogenase (50) (~3 mV), trimethylamine dehydrogenase (51) (+40 mV), thiamine oxi-

dase (47) (+55 mV), and vanillyl-alcohol oxidase (+55 mV) all have redox potentials near the upper limit of redox potentials that have been determined for flavoprotein oxidoreductases (52). Apparently, an increase of the flavin redox potential by covalent flavinylation is a widely used mechanism to enhance the oxidative power of specific flavoproteins.

Recent studies on trimethylamine dehydrogenase have indicated that covalent bond formation may play a major role in preventing inactivation of the enzyme by flavin modification (5). Trimethylamine dehydrogenase mutants, which are unable to form the 6-S-cysteinyl-FMN bond, readily are inactivated by hydroxylation of the C-6 of the flavin ring. It was proposed that this protective effect might also be the rationale for the occurrence of 8a-methyl flavinylated enzymes. However, from the results described in this study, it can be concluded that this proposed self-protecting function of covalent flavinylation is not generally valid. Spectral analysis of the VAO mutants following extensive incubations with or without substrate did not result in any detection of modified FAD. Further, in all experiments performed with the mutant enzymes, no appreciable inactivation could be observed. A plausible reason for the chemical stability of the isoalloxazine ring in VAO comes from inspection of the crystal structure. In both the mutant structure and that of wild type VAO, the benzyl moiety of the cofactor is found to be protected from solvent, preventing flavin hydroxylation reactions. Apparently, the susceptibility of flavin modification within the active site of flavoenzymes is highly dependent on the active site architecture. This might also hint at the fact that the evolutionary pressure toward covalent flavinylation is not necessarily driven by a single specific motive but can be multifold. However, the recently discovered

FIG. 5. Superposition of active site residues in the unliganded H422A (shaded) and wild type VAO structures (black). This figure was prepared with MOLSCRIPT (55).

Active site also showed that the active site architecture has been fully conserved. This was confirmed by the crystal structure of the H422A variant in complex with the substrate analog isoeugenol. The binding of this phenolic ligand was identical to the binding mode in wild type enzyme (21). Except for the deletion of the covalent histidyl-FAD bond, no significant change in cofactor-protein interactions could be observed. This clearly indicates that the covalent FAD linkage does not necessarily induce a specific structural feature or is a prerequisite for proper protein folding. Evidently, the noncovalent interactions of the FAD binding domain in VAO are competent in tight binding of the cofactor.

Steady state kinetic analysis showed that with respect to wild type enzyme the turnover rate for all His422 mutants has markedly decreased (Table I). The stopped-flow technique was used to identify the reaction step that limits the turnover rate. Using the H422A mutant, it was found that the rate of flavin reduction was drastically reduced, while the rate and nature of the other kinetic processes are almost unaffected by the mutation. Also, enzyme-monitored turnover experiments with the H422A mutant indicated that the kinetic mechanism is similar to that of wild type VAO (20). Spectral analysis of the reductive half-reaction also showed that the mutant is still able to stabilize the p-quinoxine methide product intermediate. This is indicative of a conserved active site architecture (Scheme 1), which was confirmed by the crystal structure. Since the active site architecture is fully conserved, the decrease in the flavin reduction rate directly reflects an apparent decrease in flavin reactivity. Nevertheless, these results also clearly show that the covalent histidyl-FAD bond in VAO is not a prerequisite for catalysis, since the His422 mutants still display appreciable activity.

Determination of the redox potential of several flavin derivatives has revealed that covalent aminoacyl modifications at the 8a-position of the isoalloxazine ring can result in an increase of the redox potential by 50–60 mV (7, 9). Furthermore, studies on 8a-N-imidazolylflavins have shown that ionization of the imidazole substituent can have significant effects on the flavin redox potential (48). In the H422A mutant, a markedly lower midpoint redox potential was found when compared with wild type VAO (~65 mV versus ~55 mV). As mentioned above, analysis of the H422A structure shows that the decrease in midpoint redox potential is not caused by any evident struc-
flavoprotein family for which VAO is the prototype shows a relative high frequency of covalent flavinylation via a histidyl bond (22). Strikingly, all characterized VAO homologs containing a histidyl-bound FAD represent oxidases, while all noncovalent VAO homologs represent dehydrogenases. This suggests that a relatively high redox potential caused by covalent flavinylation directs the enzyme to accept oxygen as electron acceptor as other physiological electron acceptors, e.g. NAD+, have relatively low redox potentials.

In conclusion, these results clearly show that the covalent interaction of the isoalloxazine ring with the protein moiety can markedly increase the redox potential of the flavin cofactor. This increase in redox potential facilitates redox catalysis by VAO. From this, it is tempting to conclude that formation of a histidyl-FAD bond in specific flavoenzymes has evolved as a way to contribute to the enhancement of their oxidative power. Moreover, the markedly high redox potential of VAO is a good illustration of the wide range of redox potentials achieved in flavin-dependent oxidases, ranging from ~367 mV in nitroalkane oxidase (53) to +55 mV in VAO.

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