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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 flavinylation in vanillyl-alcohol oxidase, the functional role of the histidyl-FAD bond was studied. Three His^{422} mutants (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 analysis 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 reduction 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-mediated flavin reduction limiting the turnover rate.

Elucidation of the crystal structure of the H422A mutant 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 cofactor, thereby facilitating redox catalysis. Thus, formation of a histidyl-FAD bond in specific flavoenzymes might have evolved as a way to contribute to the enhancement of their oxidative power.

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 flavinylation 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 dehydrogenase, was already identified in 1955 (3), the rationale for covalent flavinylation 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 flavinylation might be advantageous for catalysis, it should be mentioned that several covalent flavoenzymes have homologous flavin-dissociable counterparts that display similar enzyme activities (13–15). Therefore, the rationale for this atypical 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 compartmentalized (16). Therefore, the motive for covalent flavinylation might also reside in the physiological function and environment of these flavoenzymes. Further, covalent flavinylation can also be of structural benefit. Except for cofactor saturation of the active site, the introduction of a covalent flavin-protein bond may well

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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: 1qlt and r1qltsf; PDB codes for isoeugenol-complexed: 1qlu and r1qlusf).

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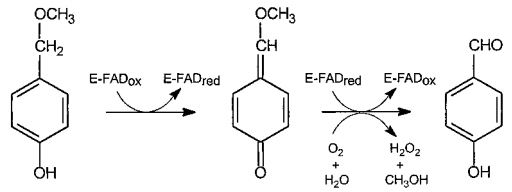

SCHEME 1

result in an improved protein stability.

In this study, we have addressed the function of covalent flavinylation in vanillyl-alcohol oxidase (VAO),[1] which is a covalent flavoprotein containing 8α-(N^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-γ-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^{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—Escherichia coli strain DH5αF′ (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 *vaoA* gene. T4 DNA ligase, restriction enzymes, isopropyl-β-D-thiogalactopyranoside, yeast extract, and tryptone extract were from Life Technologies, Inc. *Pwo* 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.

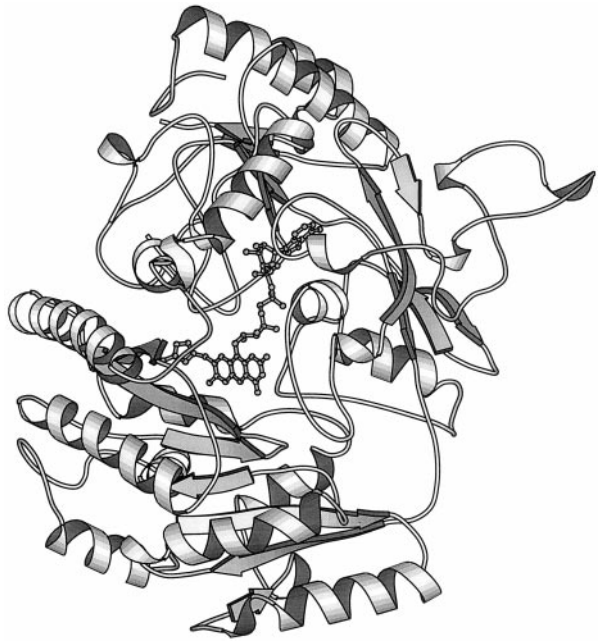

FIG. 1. ***Ribbon representation*** **of a vanillyl-alcohol oxidase monomer.** The histidyl-bound FAD cofactor is shown in a *ball-and-stick model*. This *figure* was prepared with MOLSCRIPT (55).

Mutagenesis—To simplify the site-directed mutagenesis procedure, a *Sal*I restriction site was created by a silent mutation at position 882 in the original expression plasmid pIM3972 (27). For this, the *Nco*I–*Nsi*I fragment of pIM3972 was ligated into pGEM-5Zf(+). Subsequently, the silent mutation was introduced by polymerase chain reaction-based mutagenesis using the oligonucleotide 5′-CAAGCCGTCGACATTAT-TCGTCC-3′ (where C denotes the mutated base). The mutated *Nco*I–*Nsi*I fragment was ligated into pIM3972. The resulting pBC11 plasmid was used for polymerase chain reaction-based mutagenesis. For the His^{422} replacements, the *Sal*I–*Kpn*I fragment of pBC11 was ligated into pUCBM20. This construct was used for polymerase chain reaction-based mutagenesis with the oligonucleotide 5′-CCCTAATGGTGCG*XX*-TCTGTTCTTCTCTCC-3′ (where *XX* denotes the replacement for GC (H422A), AC (H422T), and TG (H422C), respectively). The mutated *Sal*I–*Kpn*I 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 histidyl-FAD fluorescence was detected as described earlier (29). Analytical gel filtration was performed on a Superdex 200 HR 10/30 column using an Äkta 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 Aminco DW-2000 spectrophotometer. Flavin fluorescence emission spectra (excitation at 450 nm) were obtained using an Aminco SPF-500 fluorometer. Redox potentials were determined by the method described by Massey (31). For this, a cuvette containing enzyme (2–12 μM), benzyl viologen (2.0 μM), redox dye (2–10 μM), and xanthine (400 μM) was made anaerobic by flushing with argon, after which 0.2–0.5 nM xanthine oxidase was added (total volume was 750 μl). 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 M300 monochromator diode-array

[1] The abbreviation used is: VAO, vanillyl-alcohol oxidase.

detector (Salisbury, United Kingdom) as described previously (20). Deconvolution of spectral data was performed using the Specfit Global Analysis program version 2.10 (Spectrum Software Associates, Chapel Hill, NC).

Crystallization, Data Collection, and Processing—Bright yellow crystals of all His^{422} mutants were obtained using the hanging drop vapor diffusion method at similar conditions (5% (w/v) polyethylene glycol 4000, 100 mM sodium acetate/HCl, pH 5.1) that were used for crystallizing wild type VAO (32). Crystallization of H422A resulted in the most regularly shaped crystals. The crystal structure of H422A was determined in the absence and presence of the substrate analog isoeugenol using crystals of about $0.2 \times 0.2 \times 0.3$ mm^3. Binding of isoeugenol was achieved by soaking the crystal for 3 h in a solution containing a 1 mM concentration of the inhibitor.

Data sets were collected at the x-ray diffraction beam line of ELETTRA (Trieste, Italy) at 100 K. Before freezing, the crystals were briefly transferred to a solution containing 20% (w/v) polyethylene glycol 400, 5% (w/v) polyethylene glycol 4000, 20% (v/v) glycerol, and 100 mM acetate/HCl, pH 5.1. A complete data set was collected at 100 K using a single crystal. The data were processed using MOSFLM (written by A. G. W. Leslie). Crystals of the H422A mutant were isomorphous to those of wild type enzyme, and they belong to space group I4. Crystallographic refinement was performed with the REFMAC program (33). A bulk solvent correction was applied using programs of the CCP4 program package (34). Model building was carried out using the program O (35), whereas positions of ordered water were identified using the ARP program (36). The free *R*-factor was calculated employing the same reflections used for the free *R*-factor calculations in the refinement of the wild type structure (21). The free *R*-factor was used to monitor the progress of refinement. Table II gives a summary of the final refinement statistics.

RESULTS

Purification and General Characterization—The three His^{422} 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^{422} 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% 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^{422} 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 oligomeric forms could be demonstrated. In both the octameric and dimeric state, the ratio of protein (280 nm) absorbance and flavin (450 nm) absorbance was identical. These results indicate that the purified mutants display a similar oligomerization behavior with respect to wild type VAO (30).

Spectral Properties—The ratio of absorbance between 280 and 439 nm for all mutants was 11.5, which is similar to the value reported for wild type VAO (17). Further, the flavin absorption spectra of all three mutants were virtually identical with maxima at 390 and 439 nm (Fig. 2). When comparing the flavin spectra of the mutants and wild type enzyme, it can be clearly seen that the spectral characteristics at lower wavelengths have drastically changed, while the spectral characteristics at around 439 nm are hardly affected by the mutation. These results are in line with the fact that the mutants do not form a covalent FAD bond, since 8α-substituted histidyl-FAD can be identified by a typical hypsochromic shift of the near-UV absorbance maximum with respect to unmodified FAD (37). In addition to histidyl-FAD, cysteinyl-bound FAD has been observed in several covalent flavoproteins (2). The observation that the H422C mutant does not contain any detectable covalent FAD indicates that formation of the covalent histidyl-FAD linkage in VAO is a very specific mechanism. Further, although the mutations prevent covalent linkage of FAD, all mutants contain tightly bound FAD, since they were purified in the holo form. Attempts to generate apoenzyme using established methods (38) failed. The noncovalent His^{422} mutants displayed similar flavin fuorescence properties when compared with wild type VAO. 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^{422} 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.

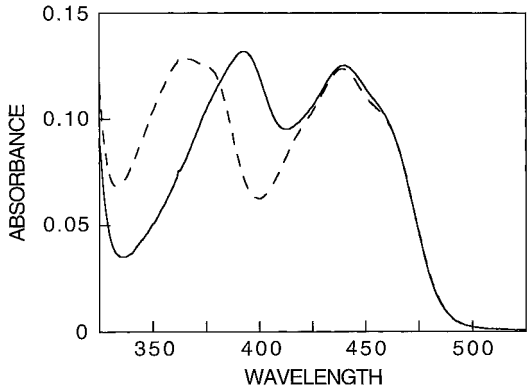

FIG. 2. **Flavin absorption spectra of 10 μM wild type VAO (*dashed line*) and the H422A mutant (*solid line*) in 50 mM potassium phosphate, pH 7.5.**

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 k_{cat} values of the three mutants decreased by about 1 order of magnitude, while the K_m 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 K_d 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^{422} mutants and wild type VAO with 4-(methoxymethyl)phenol (pH 7.5, 25 °C)

Parameter	Wild type[a]	H422A	H422C	H422T
K_m (μM)	55	34	37	41
k_{cat} (s^{-1})	3.1	0.27	0.32	0.28
K_d (μM)	48	18	ND[b]	ND
k_{red} (s^{-1})	3.3	0.30	ND	ND
k_{ox} (M^{-1} s^{-1})	1.5×10^5	2.5×10^5	ND	ND

[a] Data taken from Ref. 20.
[b] ND, not determined.

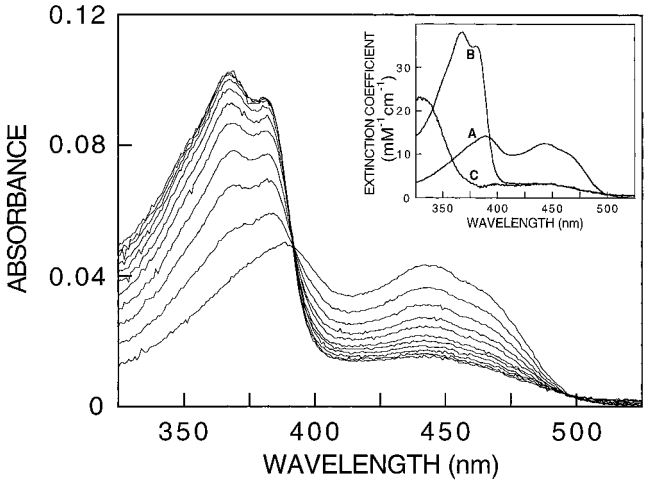

FIG. 3. **Spectral changes observed upon anaerobic reaction of H422A (4.0 μM) with 4-(methoxymethyl)phenol (500 μM, 25 °C, pH 7.5).** Original spectra are shown from 5.6 ms to 10 s with intervals of 1 s. The *inset* shows normalized spectra obtained after deconvolution of the original 96 spectral scans 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 fit with a consecutive irreversible model ($A \rightarrow B \rightarrow C$).

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, by 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 methide product intermediate. Decay of this complex, resulting in formation of 4-hydroxybenzaldehyde, 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 pK_a of the one-electron-reduced form of the flavin cofactor in VAO. Such a pK_a shift of the flavin semiquinone has also been observed in flash photolysis studies on 8α-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 (E_1) and the semiquinone/hydroquinone couple (E_2) 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 E_1 and E_2 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 ($E_m = (E_1 + E_2)/2$; 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 noncovalent 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, as 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

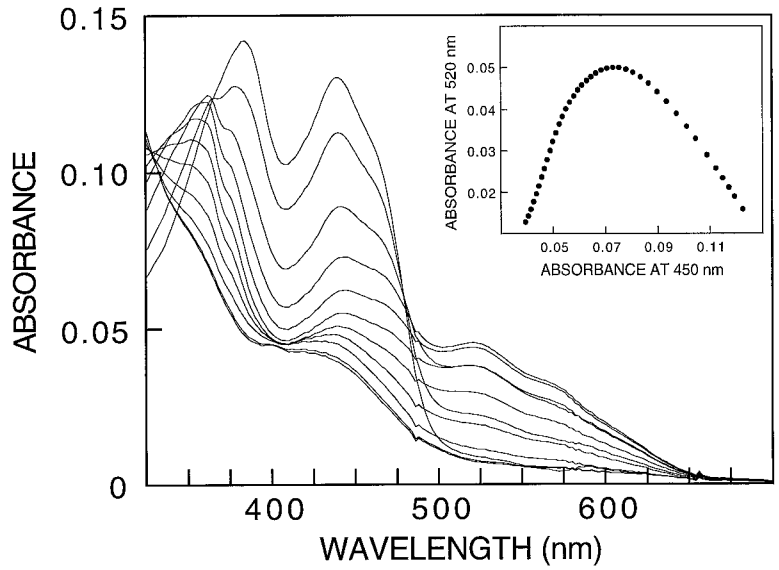

FIG. 4. **Spectral changes during the anaerobic xanthine oxidase-mediated reduction of 12.0 μM H422A (see "Experimental Procedures").** Spectra were obtained at regular time intervals (12 min), while full reduction was achieved after 120 min. *Inset*, by plotting the absorbance at 520 nm *versus* the absorbance at 450 nm, the amount of transiently formed flavin semiquinone can be estimated (data are shown with 3-min intervals).

TABLE II
Summary of crystallographic analysis of H422A VAO

	H422A	H422A with isoeugenol
Resolution (Å)	20–2.2	20–2.4
Observed reflections	285,553	216,054
Unique reflections	55,414	43,343
Completeness of data (%)[a]	93.1 (78.8)	94.8 (88.4)
Multiplicity[a]	2.1 (1.8)	2.1 (1.9)
Intensities (I/σ)[a]	6.0 (2.8)	4.9 (2.5)
R_{sym} (%)[a]	8.9 (22.6)	11.9 (32.1)
Cell dimensions (Å)	$a = b = 129.66$, $c = 132.30$	$a = b = 129.84$, $c = 133.90$
R_{factor} (%)	21.0	21.9
R_{free} (%) (2000 reflections)	26.4	27.3
Number of protein atoms	8692	8692
Number of water atoms	373	257
Number of FAD atoms	106	106
Number of ligand atoms	8 (acetate)	22 (isoeugenol)
r.m.s.d. from ideality[b]		
Bond lengths (Å)	0.013	0.012
Bond angles (°)	2.3	2.3
Trigonal groups (Å)	0.023	0.022
Planar groups (Å)	0.011	0.011
Ramachandran plot (%)[c]	88.3/11.6/0.1/0	89.0/10.8/0.2/0

[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).

change significantly, resulting in a distance between the C-8 methyl group of the flavin and the C-β atom of Ala^{422} 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 Ile^{414} (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 His^{422}. Furthermore, the imidazole ring of His^{61} rotates by about 50°, pointing to the C-8α 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 C-α 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 His^{422} 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). From a thorough comparison of the mutant structure with that of wild type VAO it can be concluded that the two structures are virtually identical as shown by a root mean square deviation for all C-α atoms of 0.27 Å. Inspection of the

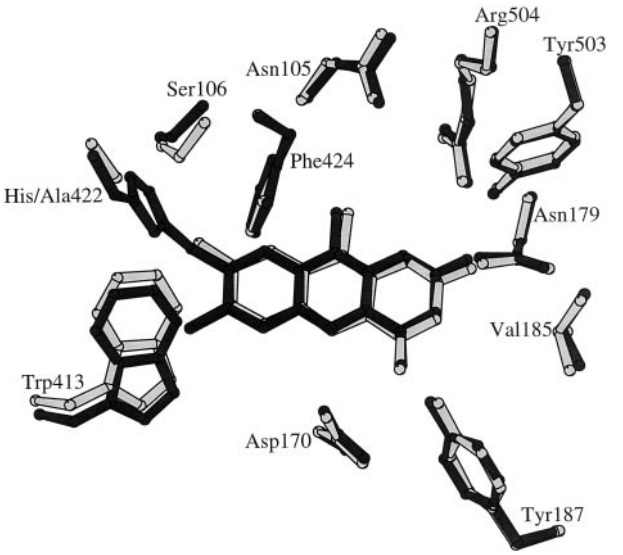

FIG. 5. **Superposition of active site residues in the unliganded H422A (*shaded*) and wild type VAO structures (*black*).** This *figure* was prepared with MOLSCRIPT (55).

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 His^{422} 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*-quinone 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 His^{422} mutants still display appreciable activity.

Determination of the redox potential of several flavin derivatives has revealed that covalent aminoacyl modifications at the 8α-position of the isoalloxazine ring can result in an increase of the redox potential by 50–60 mV (7, 9). Furthermore, studies on 8α-*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 structural changes but is merely caused by the covalent bond deletion. Apparently, the drastic reduced reactivity of the flavin is directly reflected in a decreased substrate-mediated flavin reduction rate. Further, opposite to wild type VAO, the H422A mutant displays a significant stabilization of the one-electron-reduced state. This indicates that the covalent histidyl-FAD linkage and the resulting interaction with the protein environment modulates the redox properties of the flavin cofactor in such a way that it can efficiently be reduced by a direct two-electron transfer mechanism at a relatively high potential. As suggested by Parsonage *et al.* (49), this may well reflect the ability of the enzyme to be tuned to accept simultaneously two electrons from the reducing substrate during catalysis, which is in agreement with the proposed hydride transfer mechanism (20).

Mutagenesis of the target residue for flavinylation and subsequent kinetic characterization of the resulting mutant protein has previously been performed with only a few other covalent flavoenzymes: 6-hydroxy-D-nicotine oxidase (39), monoamine oxidase A (40), fumarate reductase (10), succinate dehydrogenase (12), and trimethylamine dehydrogenase (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 oxidase (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 8α-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

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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