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Asp-170 Is Crucial for the Redox Properties of Vanillyl-alcohol Oxidase*

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Vanillyl-alcohol oxidase is a flavoprotein containing a covalent flavin that catalyzes the oxidation of 4-(methoxymethyl)phenol to 4-hydroxybenzaldehyde. The reaction proceeds through the formation of a p-quinone methide intermediate, after which, water addition takes place. Asp-170, located near the N5-atom of the flavin, has been proposed to act as an active site base. To test this hypothesis, we have addressed the properties of D170E, D170S, D170A, and D170N variants. Spectral and fluorescence analysis, together with the crystal structure of D170S, suggests that the Asp-170 replacements do not induce major structural changes. However, in D170A and D170N, 50 and 100%, respectively, of the flavin is non-covalently bound. Kinetic characterization of the vanillyl-alcohol oxidase variants revealed that Asp-170 is required for catalysis. D170E is 50-fold less active, and the other Asp-170 variants are about 103-fold less active than wild type enzyme. Impaired catalysis of the Asp-170 variants is caused by slow flavin reduction. Furthermore, the mutant proteins have lost the capability of forming a stable complex between reduced enzyme and the p-quinone methide intermediate. The redox midpoint potentials in D170E (+6 mV) and D170S (−91 mV) are considerably decreased compared with wild type vanillyl-alcohol oxidase (+55 mV). This supports the idea that Asp-170 interacts with the protonated N5-atom of the reduced cofactor, thus increasing the FAD redox potential. Taken together, we conclude that Asp-170 is involved in the process of autocatalytic flavinylatation and is crucial for efficient redox catalysis.

Vanillyl-alcohol oxidase (VAO)1 (EC 1.1.3.38) from Penicillium simplicissimum is a homoeotameric covalent flavoenzyme involved in the biodegradation of lignin-derived aromatic compounds (1). The enzyme is the prototype of a novel family of structurally related oxidoreductases sharing a conserved FAD-binding domain (2). VAO oxidizes its physiological substrate 4-(methoxymethyl)phenol to 4-hydroxybenzaldehyde with the concomitant reduction of molecular oxygen to hydrogen peroxide (3, 4). The enzymatic reaction is initiated by the transfer of a hydride equivalent from the Cα-atom of the substrate to flavin N5. The resulting binary complex between the reduced enzyme and the p-quinone methide intermediate then reacts with molecular oxygen, reoxidizing the FAD. The p-quinone methide product subsequently reacts with water in the enzyme active site to generate the final products 4-hydroxybenzaldehyde and methanol (4) (Fig. 1). A similar reaction mechanism has been proposed for the structurally related flavocytochrome p-cresol methylhydroxylase (5, 6).

Recently, we determined the three-dimensional structures of native VAO and several enzyme-ligand complexes (7). Each VAO 64-kDa monomer consists of two domains: the cap domain covers the active site, whereas the larger domain creates a binding site for the ADP-ribityl part of the FAD cofactor. The Cα-atom of the isoalloxazine ring of the FAD is covalently linked to the Ne3-atom of His-422 of the cap domain. A detailed study toward the non-covalent H422A mutant has shown that the covalent flavin linkage is not essential for tight binding of the flavin cofactor (8). However, the covalent bond raises the redox potential of the flavin, thereby increasing the rate of substrate oxidation. The structure of VAO in complex with 4-(1-heptenyl)phenol has revealed that the shape of the active site cavity controls the substrate specificity by providing a size-exclusion mechanism. Inside the cavity, the aromatic substrate is ideally positioned for hydride transfer to flavin N5. Substrate oxidation is facilitated by ionization of the phenol, as induced by hydrogen bonding with the side chains of Tyr-108, Tyr-503, and Arg-504. Another interesting residue in the VAO active site is Asp-170. The carboxylic moiety of this residue is close to flavin N5 (3.5 Å) and Arg-398 (3.1 Å) and likely to be deprotonated. The presence of an acidic residue in the vicinity of flavin N5 is intriguing, because in most flavin-dependent oxidoreductases of known structure the N5-atom contacts a hydrogen bond donor rather than an acceptor (9). The side chain of Asp-170 is positioned in a way that, during catalysis, it might interact with the protonated N5-atom of the reduced cofactor. Moreover, we have proposed that Asp-170 might act as an active site base, activating the water involved in substrate conversion (7).

In this paper, we have addressed the functional importance of Asp-170 in VAO through the kinetic, spectroscopic and crystallographic analysis of D170E, D170S, D170A, and D170N. It is shown that the Asp-170 replacements do not introduce major
structural changes but have a profound effect on the redox properties of the enzyme. In addition, our studies indicate that Asp-170 is involved in covalently tethering the flavin prosthetic group.

**EXPERIMENTAL PROCEDURES**

**Chemicals, Bacterial Strains, and Plasmids—** *Escherichia coli* strain DH5αF’ (10) and the plasmids pUCMB20 (Roche Molecular Biochemicals) and pGEM-5Zf(+) (Promega) were used for cloning throughout, whereas *E. coli* strain TG2 (11) and the plasmid pEMBL19(+) (Roche Molecular Biochemicals) were used for expression of the *vao* gene. Oligonucleotides, T4 DNA ligase, restriction enzymes, isopropyl β-D-thiogalactopyranoside, yeast extract, and trypotide extract were from Life Technologies, Inc. Forward M13 and reverse M13 sequencing primers were from Amersham Pharmacia Biotech. Pwo DNA polymerase, dNTPs, and glucose oxidase (grade II) were purchased from Roche Molecular Biochemicals, and *Salmonella typhimurium* DNA polymerase was purchased from HT Biotechnology. Ampicillin, guanidinium/HCl, benzyl viologen, methyl viologen, safarinol O, xanthine, and buttermilk xanthine oxidase (grade III) were from Sigma. SDS was from BDH Chemicals. Thionin was purchased from Eastman Kodak Co., and methylene blue was from Across Chemica. 4-(Methoxymethyl)phenol, eugenol, viologen, methyl viologen, safranin O, xanthine, and buttermilk xanthine oxidase (grade available).

**Site-directed Mutagenesis—** The *vao*-cDNA has been cloned into pEMBL19(+) to give pIM3972 (12). In order to simplify the site-directed mutagenesis procedure, a SalI restriction site was created in the *vao* gene by a silent mutation at position 882. The *NcoI*-SalI fragment of pIM3972 was ligated into pGEM-SZf(+), and the resulting construct was used as starting frame for polymerase chain reaction-based mutagenesis. The polymerase chain reaction was performed with the oligonucleotide 5'-CAACGGCTGCAATTATTCC-3', where C denotes the mutated base. The mutated *NcoI*-SalI fragment was ligated into pUCBM20 yielding pUCBM21. Successful mutagenesis was confirmed by plasmid sequencing. For the replacement of Asp-170, the *PstI*-SalI fragment of pBC11 was ligated into pUCBM20. This construct was used for polymerase chain reaction-based mutagenesis with the oligonucleotide 5'-CTTGAATGCCGGGXXCTTGTTGCXGG-3' (where XXX denotes the replacement of GAT for GAG (D170E), TCT (D170S), GCT (D170E), pBC15 (D170S), and pBC16 (D170A). Again, successful mutagenesis was confirmed by plasmid sequencing.

**Enzyme Purification—** Transformed *E. coli* cells were grown in Luria Bertani-medium supplemented with 75 μg/ml ampicillin and 0.25 mM isopropyl β-D-thiogalactopyranoside, essentially as described before (12). The VAO variants were purified as described previously (12), with the following modifications. After phenyl-Sepharose chromatography, the enzyme was transferred into 20 mM potassium phosphate buffer, pH 7.2, and loaded onto a hydroxyapatite column equilibrated with 20 mM potassium phosphate buffer, pH 7.2. After washing, the enzyme was eluted with a linear gradient of 20–1000 mM potassium phosphate buffer, pH 7.2. Next, the enzyme was transferred into 50 mM potassium phosphate buffer and concentrated by ultrafiltration. To obtain highly pure Asp-170 variants, a Superdex 200 HR 10/30 column was used for polymerase chain reaction-based mutagenesis with the oligonucleotide 5'-CAACGGCTGCAATTATTCC-3', where C denotes the mutated base. The mutated *NcoI*-SalI fragment of pIM3972 was ligated into pGEM-SZf(+), and the resulting construct was used as starting frame for polymerase chain reaction-based mutagenesis with the oligonucleotide 5'-CAACGGCTGCAATTATTCC-3', where C denotes the mutated base. The mutated *NcoI*-SalI fragment was ligated into pUCBM20 yielding pUCBM21. Successful mutagenesis was confirmed by plasmid sequencing. For the replacement of Asp-170, the *PstI*-SalI fragment of pBC11 was ligated into pUCBM20. This construct was used for polymerase chain reaction-based mutagenesis with the oligonucleotide 5'-CTTGAATGCCGGGXXCTTGTTGCXGG-3' (where XXX denotes the replacement of GAT for GAG (D170E), TCT (D170S), GCT (D170E), and AAC (D170N), respectively). The mutated *PstI*-SalI fragments were ligated into pUCBM21 yielding pUCBM22 (D170N), pUCBM23 (D170E), pUCBM24 (D170S), and pUCBM25 (D170A). Again, successful mutagenesis was confirmed by plasmid sequencing.

**Analytical Methods—** All experiments were performed in air-saturated 50 mM potassium phosphate buffer, pH 7.5, at 25 °C unless stated otherwise. Molar absorption coefficients for protein bound flavin were determined in 100 mM sodium phosphate buffer, pH 7.0, by unfolding the enzyme with 0.5% (w/v) SDS or 5 mM guanidinium/HCl (13). Absorption spectra were recorded using a Hewlett Packard HP 8453 diode array spectrophotometer or an Aminco DW-2000 double beam spectrophotometer. The fraction of covalently bound FAD in the VAO variants was determined by the following procedure: first, the protein was treated with 5% (v/v) ice-cold trichloroacetic acid. After centrifugation, the protein precipitate was dissolved in 100 mM potassium phosphate buffer, pH 7.5, containing 6 M guanidinium/HCl. Next, the absorption spectral properties of supernatant and redissolved precipitate were compared with that of native enzyme. SDS-polyacrylamide gel electrophoresis was carried out in 12.5% slab gels as described (14). Coomassie Brilliant Blue R-250 was used for protein staining. Before protein staining, gels were incubated in 5% (v/v) acetic acid for fluorescence detection of covalently bound FAD in VAO (1). Dissociation constants of

**Table I**

| Resolution (Å) | 20–2.8 |
|---------------|--------|
| Observed reflections | 23,315 |
| Unique reflections | 16,750 |
| Completeness of data (%) | 83.7 (83.8) |
| Multiplicity | 2.4 (2.0) |
| Intensities I/σ | 25.5 (1.9) |
| R_{e}ms (%) | 15.8 (32.3) |
| Cell dimensions (Å) | a = b = 131.33, c = 134.66 |
| R_{e}ms (%) | 22.6 |
| Number of ligand atoms | 22 (isoegenol) |
| r.m.s.d. for ideal value | 0.013 |
| Bond lengths (Å) | 2.9 |
| Trigonal atoms (°) | 0.023 |
| Planar groups (Å) | 0.011 |
| Ramachandran plot | 85.1/14.9/0 |

* The values relating to the highest resolution shell (2.9–2.8 Å) are given in parentheses.

* R_{e}ms = \sqrt{I_{obs} - I_{calc}} \bigg/ I_{calc}, where I_{calc} is the intensity of an observation of reflection j and I_{calc} is the average intensity for reflection j.

* The root mean square deviations (r.m.s.d.) were calculated using the program REFMAC (21).

* Percentage of residues in most favored, allowed, and disallowed regions of the Ramachandran plot as checked with the program PROCHECK (24).
enzyme-inhibitor complexes were determined from flavin absorption perturbation spectra by titration of the enzyme with a known concentration of inhibitor (3). The dissociation constants of enzyme-sulfite complexes were determined from recording optical spectra as a function of sulfite concentration. Circular dichroism spectra were measured at 20 °C on a Jasco J-715 spectropolarimeter in a 1-cm path length cuvette. High performance liquid chromatography experiments were performed with an Applied Biosystems pump equipped with a Waters 996 photodiode array detector and a 3.9 × 100-mm Waters Novapak C18 column, essentially as described earlier (15).

**Redox Potential Determinations**—The redox potentials of the VAO variants were determined in 50 mM potassium phosphate buffer, pH 7.5, at 25 °C by the xanthine/xanthine oxidase method of Massey (16), essentially as described before (8). Equal concentrations of VAO and reference dye (7–10 mM), benzyl viologen or methyl viologen (2 mM), and xanthine (400 mM) were made anaerobic by flushing with oxygen-free argon. The reduction of VAO and reference dye was initiated by adding a catalytic amount of xanthine oxidase. To ensure equilibration between the oxidized and reduced species of enzyme and reference dye, the concentration of xanthine oxidase must be sufficiently low. Typically, the reduction of VAO and dye lasted 1–2 h. The dyes used were thionin \((E_m = +60 \text{ mV})\), methylene blue \((E_m = +11 \text{ mV})\), FAD \((E_m = -219 \text{ mV})\), phenosafranin \((E_m = -266 \text{ mV})\), and safranin O \((E_m = -280 \text{ mV})\). The oxidation-reduction potentials of the VAO variants were calculated according to the method of Clark (17).

\[
E_{\text{h}}(\text{dye}) = E_{\text{h}}(\text{dye}) + (59/n_{\text{dye}}) \cdot \log(\text{dye}_{\text{ox}}/\text{dye}_{\text{red}})
\]

At equilibrium, \(E_{\text{h}}(\text{dye}) = E_{\text{h}}(\text{E})\) (Eq. 1)

**Kinetic Studies**—All kinetic experiments were performed in 50 mM potassium phosphate buffer, pH 7.5, at 25 °C unless stated otherwise. VAO activity was routinely determined by following absorption spectral changes of aromatic substrates or by oxygen consumption experiments using a Clark electrode (15). For enzyme-monitored-turnover experiments, air-saturated enzyme and substrate were mixed, and the redox

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**Fig. 2. Spectral properties of Asp-170 variants of VAO.** Shown are absorption (A) and circular dichroism (B) spectra of oxidized wild type VAO (− − −) and D170S (- - - -) in 50 mM potassium phosphate buffer, pH 7.5, at 25 °C. The spectral properties of wild type VAO are identical to those of D170E. D170S displayed spectral properties similar to those of D170A and D170N.
state of the FAD cofactor was measured (18). Stopped-flow kinetics were performed essentially as described (4). All concentrations mentioned in the context of stopped-flow experiments are those after mixing. In anaerobic reduction experiments, glucose-containing enzyme solutions were flushed with oxygen-free argon. Glucose oxidase was added to eliminate final traces of oxygen. Reductive half-reactions were followed with a Hi-Tech M300 monochromator diode array detector (Salisbury, United Kingdom). Deconvolution of spectral data was done with the Specfit Global Analysis program version 2.10 (Spectrum Software Associates, Chapel Hill, NC). Rate constants of single wavelength kinetic traces were recorded using a Hi-Tech SU-40 spectrophotometer. The detection wavelength differed depending on the VAO mutant examined. For the generation of two-electron reduced enzyme, argon-flushed oxidized enzyme was treated with a small excess of argon-flushed dithionite. Reoxidation was measured by monitoring the redox state of the flavin after mixing the reduced enzyme with varying concentrations of molecular oxygen.

Crystallization, Data Collection, and Structure Determination—Crystals of D170S VAO were grown using the hanging-drop vapor diffusion method, in conditions essentially identical to those for wild type VAO (7). Briefly, protein solutions containing 10 mg of protein/ml in 50 mM potassium phosphate buffer, pH 7.5, were equilibrated against a reservoir solution containing 50 mM sodium acetate/hydrochloride, pH 5.1, and 5% (w/v) polyethylene glycol 4000. For data collection, the crystals were soaked in a solution containing 1 mM isoeugenol for 12 h. Diffraction data were measured from a single crystal at the ID14-EH3 beam line of the European Synchrotron Radiation Facility (Grenoble, France) using a MarCCD detector at 100 K. Prior to data collection, the crystal was exposed for a few seconds to a cryoprotecting solution containing 20% (v/v) polyethylene glycol 400, 20% (v/v) glycerol, 10% (w/v) polyethylene glycol 4000, 10 mM isoeugenol, and 50 mM sodium acetate/hydrochloride, pH 5.1. The data were processed using MOSFLM (19) and programs of the CCP4 package (20). The mutant crystals belong to space group P4 with unit cell parameters a = b = 131.33, c =

![Graph](image_url)
The functional role of Asp-170 in vanillyl-alcohol oxidase was studied by examining the properties of Asp-170 variants. The wild type enzyme binds the FAD cofactor covalently via His-422. When D170E was precipitated with 5% (w/v) ice-cold trichloroacetic acid, no flavin was present in the supernatant, indicating that all FAD is covalently bound. D170S, only a trace amount (3%) of free FAD was found. However, trichloroacetic acid precipitated D170A and D170N were mainly present in the octameric form. This hydrodynamic behavior is similar to that of wild type enzyme.

Wild type VAO binds the FAD cofactor covalently via His-422. When D170E was precipitated with 5% (w/v) ice-cold trichloroacetic acid, no flavin was present in the supernatant, indicating that all FAD is covalently bound. With D170S, only a trace amount (3%) of free FAD was found. However, trichloroacetic acid precipitation of D170A and D170N showed that in these mutants, 50% and 100%, respectively, of the flavin cofactor was non-covalently bound. These data were confirmed by SDS-polyacrylamide gel electrophoresis using fluorescence analysis. The unstained SDS gel in 5% (v/v) acetic acid showed that D170E and D170S were as fluorescent as wild type enzyme. However, the fluorescent band of D170A was less intense, and D170N was not fluorescent.

Spectral Properties—The flavin spectral properties of the oxidized VAO variants are summarized in Table I. The absorption spectrum of the covalently bound flavin to that of oxidized FAD (13). Upon this treatment, the absorbance at 450 nm remained nearly constant, revealing that the enzyme was isolated in the oxidized form. In agreement with this, native D170S displayed no fluorescence upon excitation at 360 nm, indicating that the enzyme was not isolated as a flavin N5 adduct (26, 27). The flavin in D170S was not present in the flavin iminoquinone methide form, as the cofactor was covalently attached to the enzyme. Moreover, the absorption characteristics of native D170S did not change after a single turnover experiment or after incubation with the substrate analog isoeugenol and subsequent elution over Biogel P6. On the basis of these results, we conclude that the unusual spectral features of D170S, D170A, and D170N are an intrinsic property of the mutant protein and the result of a changed flavin microenvironment.

134.66 Å and are isomorphous to the wild type crystals. The mutant structure was refined using the maximum likelihood refinement program REFMAC (21). A bulk solvent correction was applied using the programs of the CCP4 package, whereas the positions of ordered solvent molecules were located using ARP (22). The progress of the refinement was monitored by means of R_mis. Electron density maps were visually inspected using the program O (23). The isoeugenol atoms were well defined in the electron density map, except for the terminal carbon atom of the propenyl substituent. This probably reflects the presence in the soaking solution of a mixture of cis- and trans-isomers, which both bind to the enzyme. Analysis of the refined structure revealed that 85.1% of the amino acid residues of the isoeugenol complexed D170S structure fall in the most favored regions of the Ramachandran plot, whereas none fall in the disallowed regions as defined by PROCHECK (24). Data collection and refinement statistics for the structure are displayed in Table I.

RESULTS

General Properties—The Asp-170 variants D170E, D170S, D170A, and D170N were purified from E. coli in about the same yield as wild type VAO (12). Analytical gel filtration revealed that all VAO variants were mainly present in the octameric form. This hydrodynamic behavior is similar to that of wild type enzyme (25).

Wild type VAO binds the FAD cofactor covalently via His-422 (7). When D170E was precipitated with 5% (w/v) ice-cold trichloroacetic acid, no flavin was present in the supernatant, indicating that all FAD is covalently bound. With D170S, only a trace amount (3%) of free FAD was found. However, trichloroacetic acid precipitation of D170A and D170N showed that in these mutants, 50 and 100%, respectively, of the flavin cofactor was non-covalently bound. These data were confirmed by SDS-polyacrylamide gel electrophoresis using fluorescence analysis. The unstained SDS gel in 5% (v/v) acetic acid showed that D170E and D170S were as fluorescent as wild type enzyme. However, the fluorescent band of D170A was less intense, and D170N was not fluorescent.

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Circular dichroism spectra can yield valuable information about the flavin microenvironment (28). The flavin circular dichroism spectra of the four VAO variants were mirror images of the absorbance spectra (Fig. 2B). Thus, wild type VAO and D170E showed similar circular dichroism spectra, whereas D170S, D170A, and D170N displayed strong negative peaks in the near-UV region. These results corroborate that the Asp-170 replacements induce a change in the flavin microenvironment.

Flavoprotein oxidases generally form flavin N5 adducts with sulfite (29). The absorbance spectra of these complexes resemble those of the reduced enzymes. However, wild type VAO does not react with sulfite (13), and we have suggested that this is due to the presence of Asp-170 in the active site (7). Binding studies with D170E and D170S showed that only the latter enzyme interacts with sulfite. In the presence of 20 mM sulfite, bleaching of the flavin in D170S was completed after 60 min, and the reactivity did not significantly change with pH. From titration experiments, a dissociation constant for the D170S-sulfite complex, K_D = 3 ± 1 mM at pH 7.5 was estimated. These results are in line with the proposal that a negatively charged residue at position 170 in VAO prohibits the reaction between flavin N5 and sulfite through electrostatic repulsion.

Replacement of Asp-170 by Glu, Ser, and Ala did not strongly affect the binding of the competitive inhibitor isoeugenol. The dissociation constants of the isoeugenol-enzyme complexes were 13 ± 4, 18 ± 5, 15 ± 10, and 6 ± 2 μM for wild type VAO, D170E, D170S, and D170A, respectively, as determined by flavin perturbation difference spectroscopy (3). At pH 7.5, binding of isoeugenol to the VAO variants resulted in a strong increase in absorbance at 320 nm. This absorbance increase is due to the formation of the phenolate form of the inhibitor (3). As the pKa of isoeugenol free in solution is 9.8, binding to wild type VAO, D170S, D170E, and D170A decreases the pKa value of isoeugenol significantly. These data indicate that Asp-170 is not directly involved in substrate activation. D170N does not strongly interact with isoeugenol. This is concluded from the fact that no significant absorbance changes were observed in titration experiments.

Catalytic Properties—Wild type VAO oxidizes the physiological substrate 4-(methoxymethyl)phenol with a k_cat of 3.1 s⁻¹ (4). In contrast, turnover of D170E with 4-(methoxymethyl)phenol was rather slow, and the other three mutant enzymes were nearly inactive. A similar behavior was observed with the substrates eugenol and vanillyl alcohol (Table III). In the following experiments and for obvious reasons, the catalytic properties of D170E and D170S were studied in further detail.

By measuring the redox state of the FAD cofactor during turnover, information can be obtained about the rate-limiting step in catalysis (18). When oxidized D170E and D170S were mixed with excess 4-(methoxymethyl)phenol in the stopped-flow spectrophotometer, the fractions of oxidized enzyme during the steady-state phase of the reaction were 0.90 and 0.75, respectively. This indicates that, similar to wild type VAO, a step in the reductive half-reaction is limiting the rate of overall catalysis (4).
The reductive half-reaction of wild type VAO with 4-(methoxymethyl)phenol is described by Equation 2. Under anaerobic conditions, wild type VAO is reduced by 4-(methoxymethyl)phenol in a single irreversible step (k_2 = 3.3 s^-1), with the concomitant formation of a stable complex between the p-quinone methide of the product and the reduced enzyme. This complex displays a typical absorbance maximum at 364 nm (ε_{364 nm} = 46 mM^{-1} cm^{-1}), and its decay is too slow to be of catalytic importance (k_3 = 0.01 s^-1) (4). When the rate of anaerobic flavin reduction of D170E was determined by stopped-flow spectroscopy as a function of the substrate concentration, the reduction appeared to be a biphasic process when monitored at 437 and 364 nm. During this process, the enzyme became fully reduced. The first relatively rapid phase was virtually independent of the substrate concentration (k_{obs1} = 0.70 ± 0.04 s^-1) (Fig. 3A). The second phase was an order of magnitude slower than the first phase and was dependent on the 4-(methoxymethyl)phenol concentration (k_{obs2} = 0.07 ± 0.01 s^-1 at saturating substrate conditions).

Diode array spectral analysis of the anaerobic flavin reduction in D170E revealed the formation of an intermediate spectrum with an absorbance maximum at 364 nm during the first phase of reduction (k_{obs1}) (Fig. 3B). We estimated a molar absorption coefficient of ε_{364 nm} = 50 ± 15 mM^{-1} cm^{-1}, taking into account that the enzyme was only partially reduced in the first phase of reduction (15%). pH-dependent analysis of the
reaction showed that the spectral properties of the intermediate did not change between pH 6.5 and 8.0. These data suggest that the intermediate spectrum reflects the p-quinone methide intermediate-reduced enzyme complex. The final spectrum upon reduction was pH-dependent and showed an absorbance maximum at 335 nm. This points to the formation of 4-hydroxybenzaldehyde during the second phase of reduction (Fig. 4). Using diode array analysis, we estimated the rate of reduction, with the concomitant formation of the product 4-hydroxybenzaldehyde (Fig. 5). By measuring the fraction of oxidized enzyme present at the end of the first reductive step and the apparent reduction rate at saturating conditions ($k_{\text{app}} = k_2 + k_{-2}$), we could estimate $k_2$ and $k_{-2}$ ($k_2/k_{-2} = (E_{\text{ox}}/E_{\text{total}})$). The calculated reduction rate ($k_2$) and the rate of the reverse reaction ($k_{-2}$) were 0.1 and 0.6 s$^{-1}$, respectively. Reversible reduction was also observed for the reaction of wild type VAO with short chain 4-alkylphenols (27) and gives a rational for the apparent substrate concentration-independent reduction in the first phase. The reversible reduction of the flavin in D170E is followed by a substrate concentration-dependent second step in the reductive half-reaction, which reflects the decomposition of the p-quinone methide intermediate-reduced enzyme complex ($k_{-2}$). This substrate-dependent phenomenon was also reported for D-amino acid oxidase in case of $k_{-2}$ being an important term in the reductive half-reaction (31, 32).

Anaerobic reduction of D170S by 4-(methoxymethyl)phenol was an extremely slow process ($k_{\text{obs}} = 0.005 \pm 0.002$ s$^{-1}$), and its rate was in the same range as the turnover rate ($k_{\text{cat}} = 0.004$ s$^{-1}$). During this process, the flavin became almost completely reduced in the presence of methylene blue (Fig. 6). This red flavin semiquinone anion with a typical absorbance maximum at 385 nm (13) rose to the formation of a one-electron reduced flavin semiquinone anion with a typical absorbance maximum at 385 nm (13) (Fig. 6). The red flavin semiquinone was formed for more than 95% during reduction, indicating that the redox potentials of the two couples (oxidized/semiquinone and semiquinone/hydroquinone) are separated by at least 200 mV (17). The one-electron redox potentials for both couples were determined using thionin ($E_m = +60$ mV) and phenosafranin ($E_m = -266$ mV), respectively. The log($E_{\text{ox}}/E_{\text{red}}$) versus log($dye_{\text{ox}}/dye_{\text{red}}$) plots for the two one-electron reductions revealed slopes of 1.81 and 1.21, whereas both plots should give 2-unit slopes (33), indicating that there might be a kinetic barrier for reduction of the FAD in D170S. We verified the data of the second one-electron reduction using the reference dyes FAD ($E_m = -219$ mV) and methylene blue ($E_m = +11$ mV).
mV) and safranin O ($E_m = -280$ mV). These reduction experiments resulted in similar redox potential values. The redox potentials of the two couples were $+76 \pm 10$ and $-257 \pm 8$ mV, leading to a midpoint redox potential for D170S of $-91 \pm 9$ mV. These results establish that Asp-170 in VAO is essential for stabilization of the two-electron reduced form of the FAD.

**Structural Properties of D170S**—Crystals of D170S in complex with isoeugenol diffracted to 2.8 Å resolution. The structure of this mutant enzyme-inhibitor complex is highly similar to that of the corresponding complex of wild type enzyme (7). The root mean square deviation for the two structures for all Co-atoms is 0.30 Å. The active site of D170S is virtually identical to wild type enzyme with the side chain of Ser-170, pointing in the same direction as the side chain of Asp-170 in wild type VAO (Fig. 7). The O$\gamma$-atom of Ser-170 is engaged in two weak hydrogen bonds with NH1 of Arg-398 (3.2 Å) and O4 of the flavin (3.3 Å), whereas the distance from the cofactor N5 is 4.0 Å. The isoeugenol inhibitor binds in the same position as in wild type enzyme. The only noticeable difference concerns the orientation of the propenyl substituent, which is directed toward Ser-170 (Fig. 7). From these crystallographic data, we conclude that the D170S replacement does not induce significant protein structural changes, which could explain the low reactivity of D170S.

**DISCUSSION**

VAO is the prototype of a novel family of structurally related flavoproteins (2). On the basis of crystallographic data (7), we have proposed that Asp-170, located near the flavin N5-atom, is a key residue for VAO catalysis. The presence of an acidic residue near flavin N5 is rather unusual in flavoproteins and prompted us to address the role of Asp-170 in VAO via site-directed mutagenesis.

The conservative replacement of Asp-170 by Glu did not lead to structural changes in the active site. This is concluded from the flavin spectral properties of D170E, including absorption and circular dichroism data and inhibitor binding. However, the D170E replacement significantly reduced the rate of conversion of aromatic substrates. Replacement of Asp-170 by Ser, Asn, and Ala induced severe changes in the flavin spectral properties and almost completely blocked enzyme activity. The crystal structure of D170S showed that the atypical spectral characteristics of this mutant enzyme are not caused by significant structural changes. At present, we have no clear explanation for the unusual high absorbance of the oxidized flavin in D170S, D170A, and D170N around 345 nm. Nevertheless, the crystal structure of D170S and additional spectroscopic studies showed that this high absorbance is not caused by binding of a compound in the active site or a chemical modification of the flavin. Another interesting feature of D170S is the formation of a flavin-sulfite complex. This finding strongly supports that unlike many other flavoprotein oxidases, VAO does not interact with sulfite because of the electrostatic repulsion of sulfite by the acidic side chain at position 170. To our best knowledge, this is the first direct evidence that a charged residue near flavin N5 is of importance for the reaction with sulfite.

Charged residues located near the isoalloxazine ring of protein-bound flavin can have a major impact on the redox potential of the flavin and thus its reactivity (34–36). In general, the presence of a positive charge near the N1-C2 locus stabilizes the anionic reduced form of the flavin cofactor (37). This positive charge (Arg-504 in VAO) also influences flavin N5; however, this influence is more subtle as N5 is protonated in the reduced form. In most flavin-dependent oxidoreductases, a main chain nitrogen is hydrogen-bonded to flavin N5 (9), limiting the possibilities for rational mutagenesis. The presence in VAO of an acidic residue near flavin N5 offers the unique opportunity to evaluate the role of N5 interactions for flavin catalysis. The present study clearly shows that a negative charge near flavin N5 drastically increases the redox potential of the flavin cofactor, suggesting that the ionized carboxylic moiety of Asp-170 counterbalances the protonated flavin N5. This shift in redox potential is reflected in the low oxidative power of the mutant enzymes. Apart from this direct effect on catalysis, Asp-170 is also indirectly involved in modulating the redox potential of VAO by 110 mV, thereby increasing the rate of substrate-mediated enzyme reduction by a factor of 10 (8).

The precise mechanism for covalent flavinylation is still unclear, but all the evidence presently available suggests that this process is autocatalytic (8, 38, 39). The proposed linkage mechanism involves the initial removal of a proton from the $\alpha$-position of the flavin, yielding the electrophilic iminoquinone methide form (40). In the following step, the reduced covalent cofactor is formed through donation of a proton by an acidic side chain or a water molecule (41). Our studies show that...
covalent flavinylation of VAO is strongly dependent on the identity of residue 170, which is located more than 8 Å from the target residue of flavinylation, His-422. In agreement with the proposed linkage mechanism, D170E might retain the ability to donate a proton to N5, thus promoting covalent bond formation, whereas D170N is unable to act as a proton donor, preventing formation of the covalent bond. The partial flavinylation of D170A and the almost fully covalent binding of the flavin in D170S suggest that in these mutants, water might substitute for Asp-170 in activating the flavin. However, it cannot be excluded that the impairment of covalent bond formation in D170A and D170N is caused by steric constraints.

Detailed kinetic studies of D170E with 4-(methoxymethyl)phenol revealed that with this substrate, and similar to wild type VAO, a step in the reductive half-reaction is rate-limiting in overall catalysis. However, the 4-(methoxymethyl)phenol-mediated reduction of D170E appeared to be highly reversible, whereas reduction of the flavin in wild type enzyme is irreversible (4). Under anaerobic conditions, the complex between the reduced D170E and the reduced glutathione, indicating that the reduced glutathione, indicating that the p-quinone methide intermediate reacts with water in the enzyme active site. Earlier studies have shown that quinone methides are highly electrophilic and toxic, as they can form covalent adducts with proteins and DNA (42, 43). This strongly suggests that the p-quinone methide of 4-(methoxymethyl)phenol reacts readily with water in the active site of the VAO mutants, forming the final products 4-hydroxybenzaldehyde and methanol. These findings, together with the observed similarity between the wild type and D170S structures, suggest that the low redox potential is the main factor limiting the catalytic activity of the D170S mutant.

In conclusion, the present study has clearly unraveled a dual role for Asp-170 in VAO. This active site residue is involved in both catalysis and covalent flavinylation. Furthermore, we have obtained insight into the mechanisms of fine-tuning of flavin redox properties and reactivity. Interestingly, the structurally related covalent flavocytochrome p-cresol methylhydroxylase contains a Glu (Glu-380) at the position of Asp-170 in VAO (44, 45). Possibly, Glu-380 in p-cresol methylhydroxylase fulfills a role similar to that of Asp-170 in VAO, resulting in efficient covalent flavinylation and redox catalysis.

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