

University of Groningen

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

Heuvel, Robert H.H. van den; Fraaije, Marco; Mattevi, Andrea; Berkel, Willem J.H. van

*Published in:*  
The Journal of Biological Chemistry

*DOI:*  
[10.1074/jbc.275.20.14799](https://doi.org/10.1074/jbc.275.20.14799)

**IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.**

*Document Version*  
Publisher's PDF, also known as Version of record

*Publication date:*  
2000

[Link to publication in University of Groningen/UMCG research database](#)

*Citation for published version (APA):*

Heuvel, R. H. H. V. D., Fraaije, M. W., Mattevi, A., & Berkel, W. J. H. V. (2000). Asp-170 Is Crucial for the Redox Properties of Vanillyl-alcohol Oxidase. *The Journal of Biological Chemistry*, 275(20). DOI: 10.1074/jbc.275.20.14799

**Copyright**

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

**Take-down policy**

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

*Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.*

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

Received for publication, December 6, 1999, and in revised form, February 24, 2000

Robert H. H. van den Heuvel‡§, Marco W. Fraaije¶, Andrea Mattevi¶, and Willem J. H. van Berkel‡\*\*

From the ‡Department of Biomolecular Sciences, Laboratory of Biochemistry, Wageningen University, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands and the ¶Department of Genetics and Microbiology, University of Pavia, Via Abbategrasso 207, 27100 Pavia, Italy

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 10<sup>3</sup>-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 flavinylation and is crucial for efficient redox catalysis.

Vanillyl-alcohol oxidase (VAO)<sup>1</sup> (EC 1.1.3.38) from *Penicillium simplicissimum* is a homooctameric covalent flavoenzyme

\* This work was performed within the framework of the Innovation Oriented Research Program Catalysis of the Dutch Ministry of Economy Affairs (Project IKA 96005). This work was supported by the Ministero dell'Università e Ricerca Scientifica e Tecnologica (Project Biosintesi del NAD: Studi Biochimici, Biologia, Strutturale e Sviluppo Razionale di Farmaci) and the Consiglio Nazionale delle Ricerche (Target Project on Biotechnology). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The atomic coordinates and structure factors (code 1DZN) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (<http://www.rcsb.org/>).

§ Supported by a short term fellowship from the Federation of European Biochemical Societies.

¶ Supported by a long term fellowship from the European Molecular Biology Organization.

\*\* To whom correspondence should be addressed. Tel.: 31-317-482861; Fax: 31-317-484801; E-mail: willem.vanberkel@fad.bc.wau.nl.

<sup>1</sup> The abbreviation used is: VAO, vanillyl-alcohol oxidase.

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 C $\alpha$ -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 C8 $\alpha$ -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

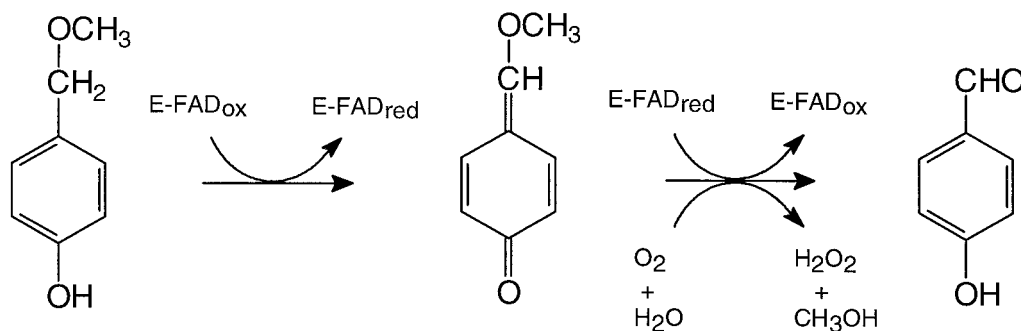


FIG. 1. Oxidative demethylation of 4-(methoxymethyl)phenol as catalyzed by VAO.

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 DH5 $\alpha$ F' (10) and the plasmids pUCBM20 (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 *vaoA* gene. Oligonucleotides, T4 DNA ligase, restriction enzymes, isopropyl  $\beta$ -D-thiogalactopyranoside, yeast extract, and tryptone 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 Super *Taq* DNA polymerase was purchased from HT Biotechnology. Ampicillin, guanidinium/HCl, benzyl viologen, methyl viologen, safranin 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 Chimica. 4-(Methoxymethyl)phenol, eugenol, isoeugenol, vanillyl alcohol, and phenosafranin were obtained from Aldrich. All other chemicals were from Merck and were of the purest grade available.

**Site-directed Mutagenesis**—The *vaoA*-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 *vaoA* gene by a silent mutation at position 882. The *NcoI*-*NsiI* fragment of pIM3972 was ligated into pGEM-5Zf(+), 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'-CAAGCCGTCGACATTATTTCGTC-3', where C denotes the mutated base. The mutated *NcoI*-*NsiI* fragment was ligated into pIM3972, yielding pBC11. 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'-CTTGATGTACCXXXCTTGGTGGCGGT-3' (where XXX denotes the replacement of GAT for GAG (D170E), TCT (D170S), GCT (D170A), and AAC (D170N), respectively). The mutated *PstI*-*SalI* fragments were ligated into pBC11, yielding pBC12 (D170N), pBC14 (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  $\mu$ g/ml ampicillin and 0.25 mM isopropyl  $\beta$ -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 PG 50/1000 gel filtration column running in 50 mM potassium phosphate buffer, pH 7.2, was introduced as final chromatography step. Enzyme purity was checked by SDS-polyacrylamide gel electrophoresis and by analytical gel filtration using a Superdex 200 HR 10/30 column.

**Analytical Methods**—All experiments were performed in air-satu-

TABLE I  
Data collection and refinement statistics for D170S VAO in complex with isoeugenol

Resolution (Å)	20–2.8
Observed reflections	23,315
Unique reflections	16,730
Completeness of data (%) <sup>a</sup>	83.7 (83.8)
Multiplicity <sup>a</sup>	2.4 (2.0)
Intensities $I/\sigma$	2.5 (1.9)
$R_{\text{sym}}$ (%) <sup>a,b</sup>	15.8 (32.3)
Cell dimensions (Å)	$a = b = 131.33, c = 134.66$
$R_{\text{factor}}$ (%)	22.6
$R_{\text{free}}$ (%)	29.1
Number of ligand atoms	22 (isoeugenol)
r.m.s.d. for ideal value <sup>c</sup>	
Bond lengths (Å)	0.013
Bond angles (°)	2.9
Trigonal atoms (Å)	0.023
Planar groups (Å)	0.011
Ramachandran plot <sup>d</sup>	85.1/14.9/0

<sup>a</sup> The values relating to the highest resolution shell (2.9–2.8 Å) are given in parentheses.

<sup>b</sup>  $R_{\text{sym}} = \sum |I_j - \langle I_j \rangle| / \sum I_j$ , where  $I_j$  is the intensity of an observation of reflection  $j$  and  $\langle I_j \rangle$  is the average intensity for reflection  $j$ .

<sup>c</sup> The root mean square deviations (r.m.s.d.) were calculated using the program REFMAC (21).

<sup>d</sup> Percentage of residues in most favored, allowed, and disallowed regions of the Ramachandran plot as checked with the program PROCHECK (24).

TABLE II  
Flavin spectral properties of wild type VAO and Asp-170 variants in the oxidized form in 50 mM potassium phosphate buffer, pH 7.5, at 25 °C

VAO	$\lambda_{\text{max}}$		$\epsilon_{\text{ox}}$		
	nm		$\text{mM}^{-1} \text{cm}^{-1}$		
	I	II	*I	*II	*I/*II
Wild type	356	439	13.6	12.5	1.09
D170E	351	437	13.9	12.7	1.09
D170S	345	443	19.7	11.9	1.66
D170A	348	441	16.6	13.3	1.25
D170N	344	441	27.3	13.1	2.08

rated 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 M 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% (w/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

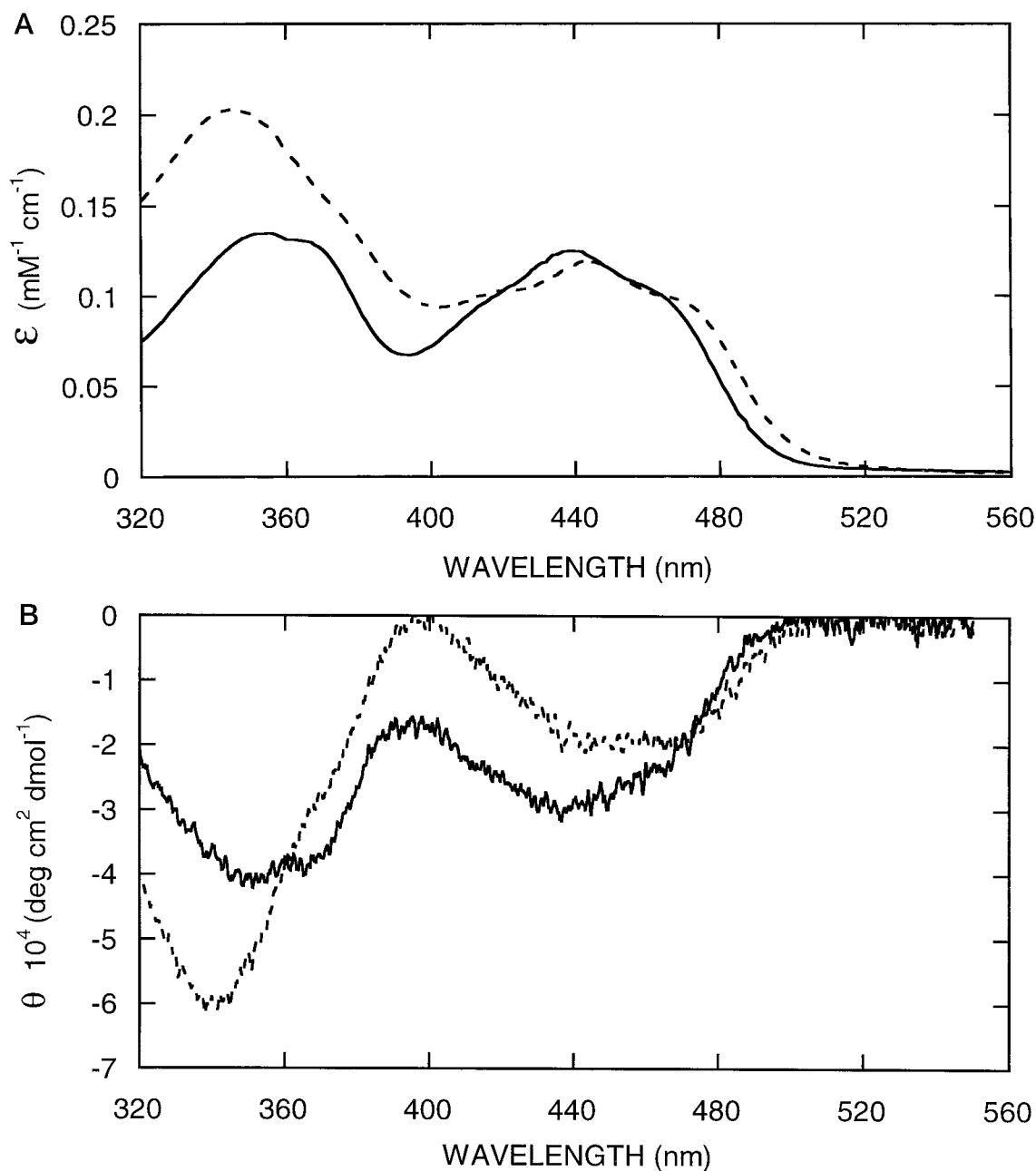


FIG. 2. **Spectral properties of Asp-170 variants of VAO.** Shown are absorption (A) and circular dichroism (B) spectra of oxidized wild type VAO (—) and D170S (---) in 50 mM potassium phosphate buffer, pH 7.5, at 25 °C. The spectral properties of wild type VAO are identical to those of D170E. D170S displayed spectral properties similar to those of D170A and D170N.

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  $3.9 \times 100$ -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  $\mu$ M), benzyl viologen or methyl viologen (2  $\mu$ M), and xanthine (400  $\mu$ M) 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 ( $E_m = +60$  mV), methylene blue ( $E_m = +11$  mV), FAD ( $E_m = -219$  mV), phenosafranin ( $E_m = -266$  mV), and safranin O ( $E_m = -280$  mV). The oxidation-reduction potentials of the VAO variants were calculated according to the method of Clark (17).

$$E_h(\text{dye}) = E_m(\text{dye}) + (59/n_{\text{dye}}) \cdot \log(\text{dye}_{\text{ox}}/\text{dye}_{\text{red}})$$

$$E_h(E) = E_m(E) + (59/n_E) \cdot \log(E_{\text{ox}}/E_{\text{red}})$$

$$\text{At equilibrium, } E_h(\text{dye}) = E_h(E) \quad (\text{Eq. 1})$$

**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

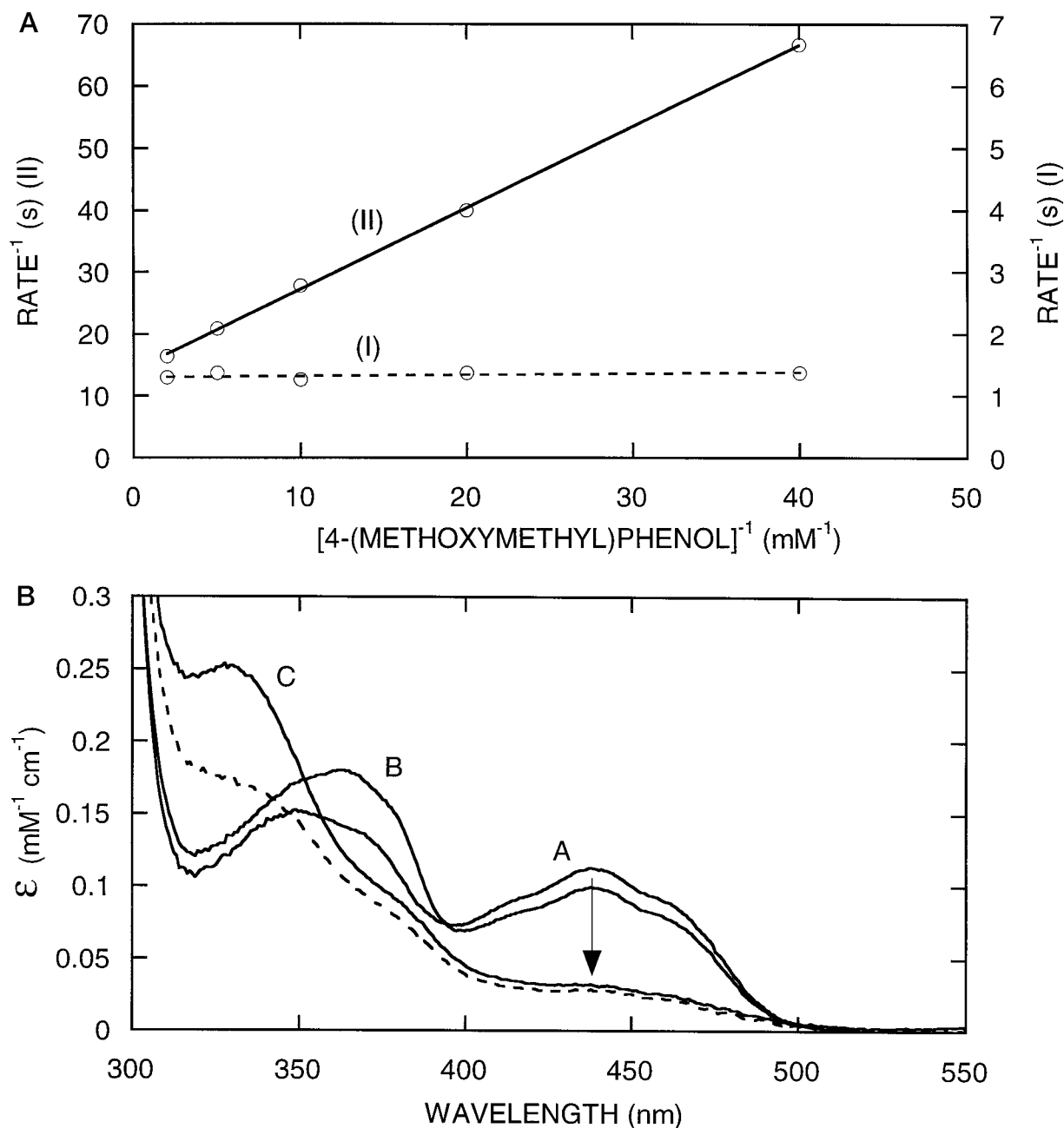


FIG. 3. Reductive half-reaction of D170E in 50 mM potassium phosphate buffer, pH 7.5, at 25 °C. A, double reciprocal plots of the two reduction rates observed during the anaerobic reductive half-reaction of 2.5  $\mu\text{M}$  D170E with 4-(methoxymethyl)phenol at 439 nm. Shown are  $k_{\text{obs1}}$  (----) and  $k_{\text{obs2}}$  (—). B, spectra obtained from deconvolution of spectral changes caused by the anaerobic reduction of 5  $\mu\text{M}$  D170E by 500  $\mu\text{M}$  4-(methoxymethyl)phenol. The data were fitted with the consecutive model A  $\rightarrow$  B  $\rightarrow$  C. When the reaction was performed at pH 6.5, only the final spectrum changed (----). The arrow indicates the direction of absorbance changes.

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 I4 with unit cell parameters  $a = b = 131.33$ ,  $c =$



TABLE III  
Kinetic parameters of wild type VAO and Asp-170 variants in air-saturated 50 mM potassium phosphate buffer, pH 7.5, at 25 °C

Substrate	Wild type		D170E		D170S		D170A		D170N	
	$K_m$	$k_{cat}$	$K_m$	$k_{cat}$	$K_m$	$k_{cat}$	$K_m$	$k_{cat}$	$K_m$	$k_{cat}$
	$\mu\text{M}$	$\text{s}^{-1}$	$\mu\text{M}$	$\text{s}^{-1}$	$\mu\text{M}$	$\text{s}^{-1}$	$\mu\text{M}$	$\text{s}^{-1}$	$\mu\text{M}$	$\text{s}^{-1}$
4-(Methoxymethyl)phenol	55 <sup>a</sup>	3.1 <sup>a</sup>	113	0.07	— <sup>b</sup>	0.004	—	0.001	—	0.002
Vanillyl alcohol	160 <sup>a</sup>	3.3 <sup>a</sup>	340	1.3	—	0.004	—	—	—	—
Eugenol	4	17.5	2	0.34	2	0.01	—	—	—	—

<sup>a</sup> Data from Ref. 4.

<sup>b</sup> —, not determined.

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  $R_{free}$ . 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.

**Spectral Properties**—The flavin spectral properties of the oxidized VAO variants are summarized in Table II. The absorption characteristics of wild type enzyme and D170E are similar. However, D170S, D170A, and D170N display significantly different spectral properties (Fig. 2A). Unfolding the D170S mutant with 0.5% (w/v) SDS or 5 M guanidinium/HCl changed the absorbance 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.

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 introduce 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,  $K_d = 3 \pm 1$  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  $13 \pm 4$ ,  $18 \pm 5$ ,  $15 \pm 10$ , and  $6 \pm 2$   $\mu\text{M}$  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  $pK_a$  of isoeugenol free in solution is 9.8, binding to wild type VAO, D170S, D170E, and D170A decreases the  $pK_a$  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  $3.1 \text{ s}^{-1}$  (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).

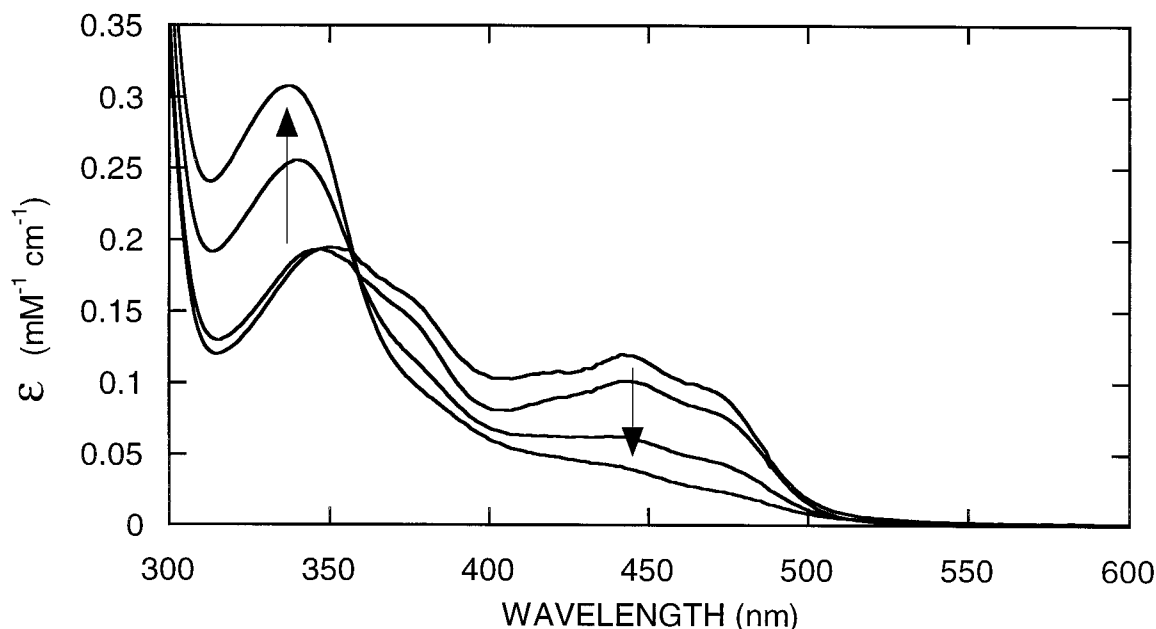


FIG. 4. **Reductive half-reaction of D170S.** Spectral changes observed upon anaerobic mixing of  $10 \mu\text{M}$  D170S with  $500 \mu\text{M}$  4-(methoxymethyl)phenol in  $50 \text{ mM}$  potassium phosphate buffer, pH 7.5, at  $25^\circ\text{C}$ . Spectra were taken after 0, 1, 12, and 29 min. The arrows indicate the direction of the absorbance changes.

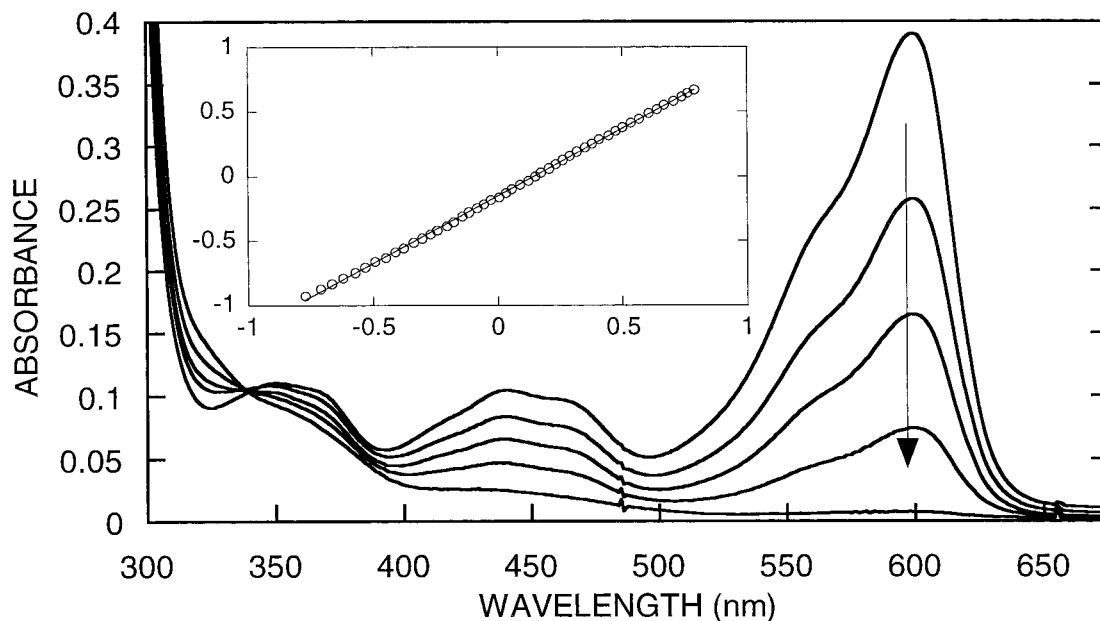


FIG. 5. **Determination of the redox potential of wild type VAO.**  $9 \mu\text{M}$  VAO in  $50 \text{ mM}$  potassium phosphate buffer, pH 7.5, at  $25^\circ\text{C}$  was reduced in the presence of  $9 \mu\text{M}$  thionin by the xanthine/xanthine oxidase system. The reduction was finished after 100 min. The arrow indicates the direction of the absorbance changes. The inset shows the  $\log(E_{\text{ox}}/E_{\text{red}})$  (measured at  $439 \text{ nm}$  after correction for thionin) versus  $\log(\text{dye}_{\text{ox}}/\text{dye}_{\text{red}})$  (measured at  $600 \text{ nm}$ ) plot.

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 ( $k_2 = 3.3 \text{ s}^{-1}$ ), 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  $364 \text{ nm}$  ( $\epsilon_{364 \text{ nm}} = 46 \text{ mM}^{-1} \text{ cm}^{-1}$ ), and its decay is too slow to be of catalytic importance ( $k_3 = 0.01 \text{ s}^{-1}$ ) (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  $364 \text{ nm}$ . During this process, the

enzyme became fully reduced. The first relatively rapid phase was virtually independent of the substrate concentration ( $k_{\text{obs1}} = 0.70 \pm 0.04 \text{ s}^{-1}$ ) (Fig. 3A). The second phase was an order of magnitude slower than the first phase and was dependent on the 4-(methoxymethyl)phenol concentration ( $k_{\text{obs2}} = 0.07 \pm 0.01 \text{ s}^{-1}$  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  $364 \text{ nm}$  during the first phase of reduction ( $k_{\text{obs1}}$ ) (Fig. 3B). We estimated a molar absorption coefficient of  $\epsilon_{364 \text{ nm}} = 50 \pm 15 \text{ mM}^{-1} \text{ cm}^{-1}$ , taking into account that the enzyme was only partially reduced in the first phase of reduction (15%). pH-dependent analysis of the

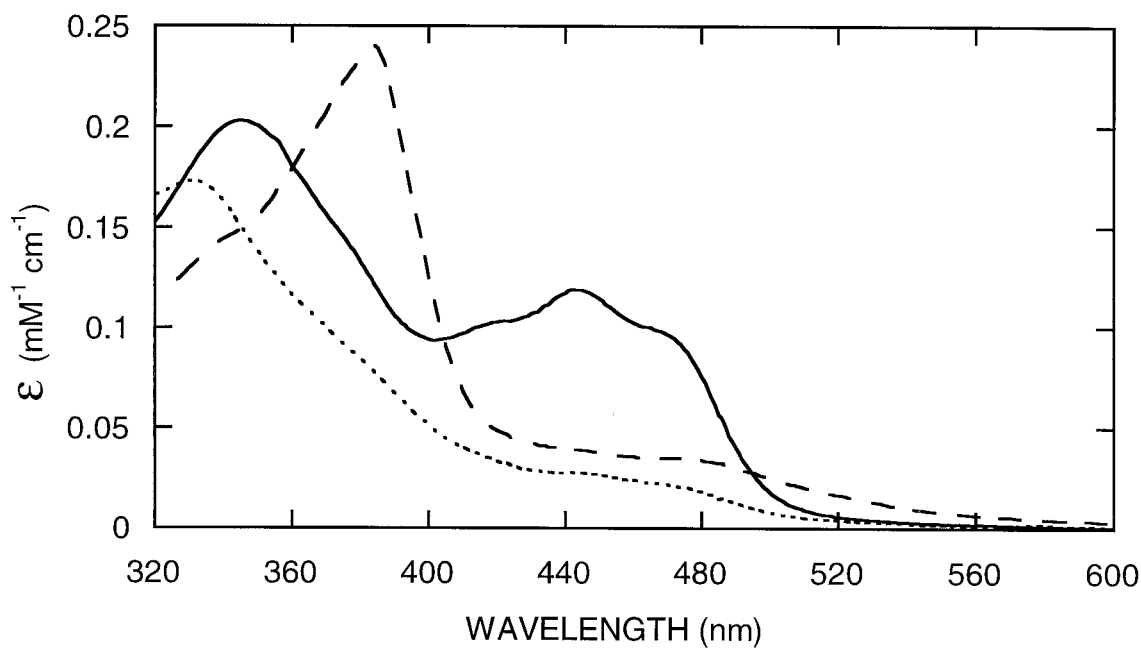


FIG. 6. Spectral properties of D170S upon reduction with the xanthine/xanthine oxidase system. D170S in 50 mM potassium phosphate buffer, pH 7.5, at 25 °C was reduced by the xanthine/xanthine oxidase system under anaerobic conditions. Shown are the oxidized enzyme (—), semiquinone form (---), and hydroquinone form (····).

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 ( $k_{\text{obs}2}$ ) and shows that the reductive half-reaction of D170E can be defined by the same equation as described for wild type enzyme. However, it should be stressed here that with wild type enzyme, the reduced enzyme-*p*-quinone methide intermediate complex is highly stabilized under anaerobic conditions.

The first phase in the reductive half-reaction of D170E leads to an equilibrium in which 15% of the flavin is in the reduced state. This suggests that the first step in flavin reduction by 4-(methoxymethyl)phenol is reversible ( $k_2 > 0$ ). 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  $k_{-2}$  ( $k_{-2}/(k_2 + k_{-2}) = (E_{\text{ox}}/E_{\text{total}})$ ) (27, 30). The calculated reduction rate ( $k_2$ ) and the rate of the reverse reaction ( $k_{-2}$ ) were 0.1 and 0.6  $\text{s}^{-1}$ , respectively. Reversible reduction was also observed for the reaction of wild type VAO with short chain 4-alkylphenols (27) and gives a rationale 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 ( $k_3$ ). 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 ( $k_{\text{obs}} = 0.005 \pm 0.002 \text{ s}^{-1}$ ), and its rate was in the same range as the turnover rate ( $k_{\text{cat}} = 0.004 \text{ s}^{-1}$ ). During this process, the flavin became almost completely reduced, with the concomitant formation of the product 4-hydroxybenzaldehyde (Fig. 4). Using diode array analysis, we could not detect the formation of the *p*-quinone methide intermediate-reduced enzyme complex, indicating that with this

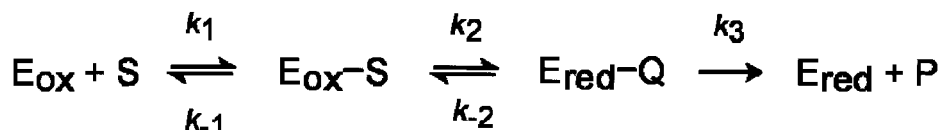
mutant enzyme the complex is not stabilized. Similar to D170S, reduction of D170A and D170N was extremely slow, and no *p*-quinone methide intermediate product was observed.

Reoxidation of free reduced wild type VAO is a fast bimolecular process ( $3.1 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ ) (4). Analysis of the reoxidation of the VAO variants with varying concentrations of molecular oxygen showed that the mutants also reoxidized in a monophasic reaction. However, the reoxidation rates of D170E and D170S were significantly lower as compared with wild type enzyme ( $1.2 \times 10^5$  and  $3.2 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ , respectively). Moreover, the reduced D170A and D170N variants reoxidized with the same rate as D170S in a monophasic fashion.

**Redox Potentials**—Because the side chain of Asp-170 is close to flavin N5, it is conceivable that this residue influences the redox potential of the flavin. Upon reduction of wild type VAO with the xanthine/xanthine oxidase system in the presence of thionin ( $E_m = +60 \text{ mV}$ ) as reference dye, the enzyme and thionin reduced simultaneously via a single two-electron reduction process (Fig. 5). From plotting  $\log(E_{\text{ox}}/E_{\text{red}})$  versus  $\log(\text{dye}_{\text{ox}}/\text{dye}_{\text{red}})$  according to the method of Minnaert (33) a redox potential of  $+55 \pm 2 \text{ mV}$  was estimated. When D170E was reduced in the presence of methylene blue ( $E_m = +11 \text{ mV}$ ), we also observed a two-electron reduction with a midpoint redox potential of  $+6 \pm 5 \text{ mV}$  (not shown).

In contrast to wild type VAO and D170E, the xanthine/xanthine oxidase-mediated reduction of D170S initially gave rise to the formation of a one-electron reduced flavin semiquinone anion with a typical absorbance maximum at 385 nm (13) (Fig. 6). This 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 ( $E_m = +60 \text{ mV}$ ) and phenosafranin ( $E_m = -266 \text{ mV}$ ), respectively. The  $\log(E_{\text{ox}}/E_{\text{red}})$  versus  $\log(\text{dye}_{\text{ox}}/\text{dye}_{\text{red}})$  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 ( $E_m = -219$





EQUATION 2

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 C $\alpha$ -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

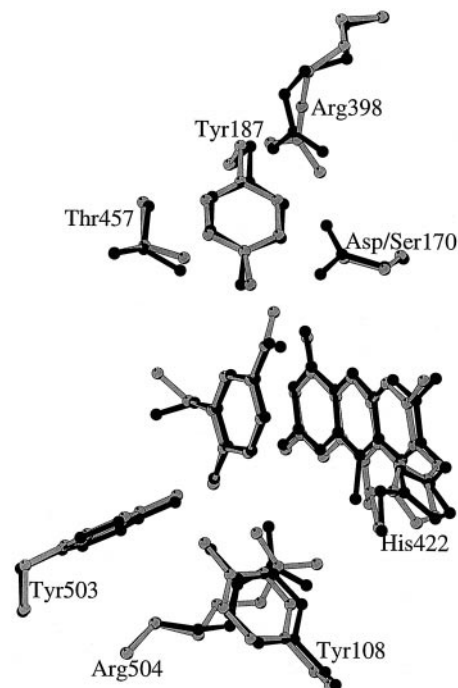


FIG. 7. Drawing of the superposition of wild type VAO (black bonds) and D170S (gray bonds) in complex with isoeugenol. The side chain of Asp-170 in wild type enzyme is 3.5 Å from flavin N5, and the side chain of Ser-170 in D170S is 4.0 Å from flavin N5. This figure was prepared with MOLSCRIPT (46).

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 the enzyme as this residue is of importance for the covalent attachment of the flavin to His-422. Recently, we have shown that the covalent histidyl-flavin bond increases 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 8 $\alpha$  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

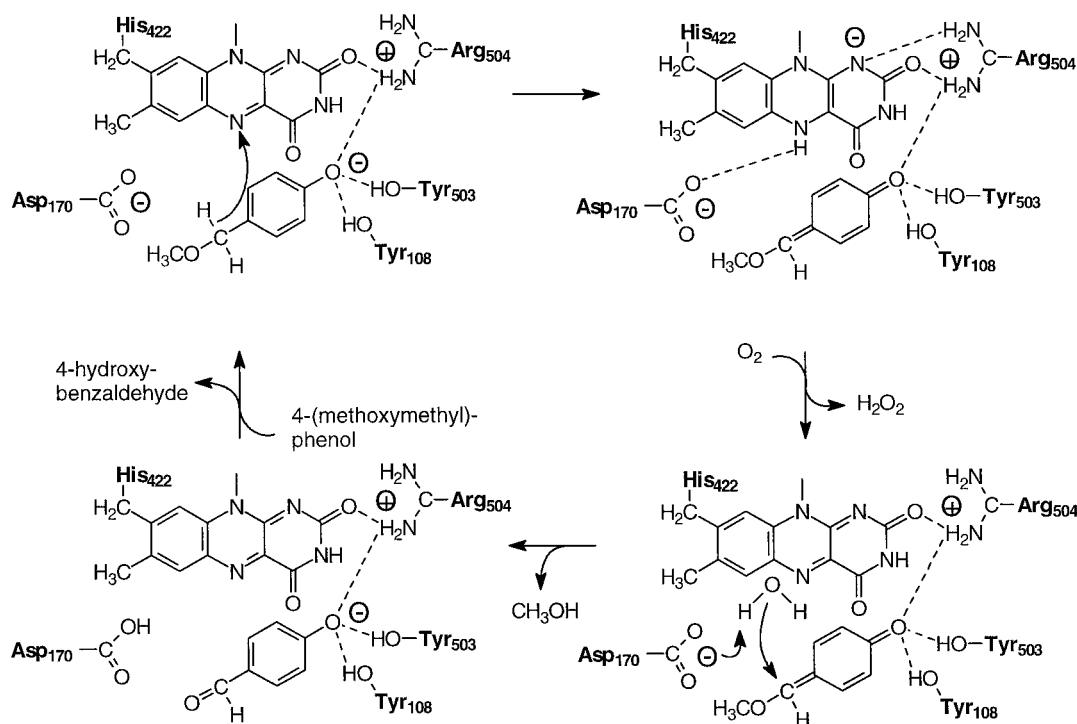


FIG. 8. Proposed role of Asp-170 in the VAO-mediated conversion of 4-(methoxymethyl)phenol (4, 7, 44).

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 *p*-quinone methide intermediate is not as stabilized as in wild type enzyme and reacts with water to form the final products 4-hydroxybenzaldehyde and methanol. On the basis of these observations, we propose that the introduction of Glu-170 changes the electrostatic interactions between the side chain of residue 170 and flavin N5 such that the complex between the reduced enzyme and the *p*-quinone methide intermediate is less stabilized (Fig. 8). The 4-(methoxymethyl)phenol-mediated flavin reduction in D170S was extremely slow. As a result, the mechanism for oxidative demethylation of 4-(methoxymethyl)phenol by D170S may also follow a ping-pong mechanism, whereas wild type enzyme reacts through a true ternary complex mechanism.

We have proposed before that Asp-170 might act as an active site base by activating the water involved in hydration of the *p*-quinone methide intermediate (Fig. 8) (7). However, D170S, D170A, and D170A also efficiently hydrate the *p*-quinone methide intermediate, as 4-(methoxymethyl)phenol is quantitatively converted to 4-hydroxybenzaldehyde. As with the wild

type enzyme (4), the formation of 4-hydroxybenzaldehyde by the mutant enzymes is not influenced in the presence of 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.

#### REFERENCES

1. Fraaije, M. W., Pikkemaat, M., and van Berkel, W. J. H. (1997) *Appl. Environ. Microbiol.* **63**, 435–439
2. Fraaije, M. W., van Berkel, W. J. H., Benen, J. A. E., Visser, J., and Mattevi, A. (1998) *Trends Biochem. Sci.* **23**, 206–207
3. Fraaije, M. W., Veeger, C., and van Berkel, W. J. H. (1995) *Eur. J. Biochem.* **234**, 271–277
4. Fraaije, M. W., and van Berkel, W. J. H. (1997) *J. Biol. Chem.* **272**, 18111–18116
5. Hopper, D. J. (1976) *Biochem. Biophys. Res. Commun.* **69**, 462–468
6. McIntire, W. S., Hopper, D. J., Craig, J. C., Everthart, E. T., Webster, R. V., Causer, M. J., and Singer, T. P. (1984) *Biochem. J.* **224**, 617–621
7. Mattevi, A., Fraaije, M. W., Mozzarelli, A., Olivi, L., Coda, A., and van Berkel, W. J. H. (1997) *Structure* **5**, 907–920
8. Fraaije, M. W., van den Heuvel, R. H. H., van Berkel, W. J. H., and Mattevi, A. (1999) *J. Biol. Chem.* **274**, 35514–35520
9. Fox, K. M., and Karplus, P. A. (1994) *Structure* **2**, 1089–1105
10. Woodcock, D. M., Crowther, P. J., Doherty, J., Jefferson, S., DeCruz, E.,

- Noyer-Weidner, M., Smith, S. S., Michael, M. Z., and Graham, M. W. (1989) *Nucleic Acids Res.* **17**, 3469–3478
11. Gibson, T. G. (1984) *Studies on the Epstein-Barr Virus Genome*. Ph.D. thesis. University of Cambridge, United Kingdom
12. Benen, J. A. E., Sánchez-Torres, P., Wagemaker, M., Fraaije, M. W., van Berkel, W. J. H., and Visser, J. (1998) *J. Biol. Chem.* **273**, 7865–7872
13. De Jong, E., van Berkel, W. J. H., van der Zwan, R. P., and de Bont, J. A. M. (1992) *Eur. J. Biochem.* **208**, 651–657
14. Laemmli, U. K. (1970) *Nature* **227**, 680–685
15. Van den Heuvel, R. H. H., Fraaije, M. W., and van Berkel, W. J. H. (1998) *J. Bacteriol.* **180**, 5646–5651
16. Massey, V. (1991) in *Flavins and Flavoproteins 1990* (Curti, B., Ronchi, S., and Zannetti, G., eds) pp. 59–66, Walter De Gruyter & Co, New York
17. Clark, W. M. (1960) *Oxidation-Reduction Potentials of Organic Systems*, pp. 184–203, Williams & Wilkins, Baltimore
18. Gibson, Q. H., Swoboda, B. E. P., and Massey, V. (1964) *J. Biol. Chem.* **239**, 3927–3934
19. Abrahams, J. P., and Leslie, A. G. W. (1996) *Acta Crystallogr. Sec. D Biol. Crystallogr.* **52**, 30–42
20. Collaborative Computational Project Number 4 (1994) *Acta Crystallogr. Sec. D Biol. Crystallogr.* **50**, 760–767
21. Murshudov, G. N., Vagin, A. A., and Dodson, E. J. (1997) *Acta Crystallogr. Sec. D Biol. Crystallogr.* **53**, 240–255
22. Lamzin, V. S., and Wilson, K. S. (1993) *Acta Crystallogr. Sec. D Biol. Crystallogr.* **49**, 129–147
23. Jones, T. A., Zou, J. Y., Cowan, S. W., and Kjeldgaard, M. (1991) *Acta Crystallogr. Sec. A* **47**, 110–119
24. Morris, A. L., MacArthur, M. W., Hutchinson, E. G., and Thornton, J. M. (1992) *Proteins* **12**, 345–364
25. Fraaije, M. W., Mattevi, A., and van Berkel, W. J. H. (1997) *FEBS Lett.* **402**, 33–35
26. Ghisla, S., Massey, V., and Choong, Y. S. (1979) *J. Biol. Chem.* **254**, 10662–10669
27. Fraaije, M. W., van den Heuvel, R. H. H., Roelofs, J. C. A. A., and van Berkel, W. J. H. (1998) *Eur. J. Biochem.* **253**, 712–719
28. Visser, A. J. W. G., van Berkel, W. J. H., and de Kok, A. (1995) *Biochim. Biophys. Acta* **1229**, 381–385
29. Massey, V., Müller, F., Feldberg, R., Schuman, M., Sullivan, P. A., Howell, L. G., Mayhew, S. G., Mathews, R. G., and Foust, G. P. (1969) *J. Biol. Chem.* **244**, 3999–4006
30. Strickland, S., Palmer, G., and Massey, V. (1975) *J. Biol. Chem.* **250**, 4048–4052
31. Porter, D. J. T., Voet, J. G., and Bright, H. J. (1977) *J. Biol. Chem.* **252**, 4464–4473
32. Pollegioni, L., Blodig, W., and Ghisla, S. (1994) *J. Biol. Chem.* **272**, 4924–4934
33. Minnaert, K. (1965) *Biochim. Biophys. Acta* **110**, 42–56
34. Palfey, B. A., Entsch, B., Ballou, D. P., and Massey, V. (1994) *Biochemistry* **33**, 1545–1554
35. Mancini-Samuels, G. J., Kieweg, V., Sabaj, K. M., Ghisla, S., and Stankovich, M. T. (1998) *Biochemistry* **37**, 14605–14612
36. Chang, F.-C., and Swenson, R. P. (1999) *Biochemistry* **38**, 7168–7176
37. Müller, F. (1991) in *Chemistry and Biochemistry of Flavoenzymes* (Müller, F., ed) Vol. III, pp. 557–595, CRC Press Inc., Boca Raton, FL
38. Mewies, M., McIntire, W. S., and Scrutton, N. S. (1998) *Protein Sci.* **7**, 7–20
39. Miller, J. R., and Edmondson, D. E. (1999) *J. Biol. Chem.* **274**, 23515–23525
40. Frost, J. W., and Rastetter, W. H. (1980) *J. Am. Chem. Soc.* **102**, 7157–7159
41. Trickey, P., Wagner, M. A., Schuman Jorns, M., and Mathews, F. S. (1999) *Structure* **7**, 331–345
42. Bolton, J. L., Turnipseed, S. B., and Thompson, J. A. (1997) *Chem. Biol. Interact.* **107**, 185–200
43. Bodell, W. J., Ye, Q., Pathak, D. N., and Pongracz, K. (1998) *Carcinogenesis* **19**, 437–443
44. Cunane, L. M., Chen, Z.-W., Shamala, N., Mathews, F. S., Cronin, C., and McIntire, W. S. (2000) *J. Mol. Biol.* **295**, 357–374
45. Mathews, F. S., Chen, Z.-W., Bellamy, H. D., and McIntire, W. S. (1991) *Biochemistry* **30**, 238–247
46. Kraulis, P. J. (1991) *J. Appl. Crystallogr.* **24**, 946–950