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[17] Redox Properties of Vanillyl-Alcohol Oxidase

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Redox Properties of Flavoproteins

Flavins are a ubiquitous class of redox-active coenzymes that are able to catalyze a number of different chemical reactions when bound to apoproteins. They play an important role in (de)hydrogenation and hydroxylation reactions, in oxygen activation, and in one- and two-electron transfer processes from and to redox centers.[1,2] Because of their chemical versatility, flavins are involved in a wide range of biological processes. They have been shown to be involved in programmed cell death by signal transduction[3] and in detoxification of a wide variety of aromatic compounds.[4] They also have a function in regulating biological clocks,[5] in DNA damage repair,[6] and plant phototropism.[7] These unique properties of flavins are always controlled by specific noncovalent or covalent interactions with the apoproteins to which they are bound.

The first flavin-containing protein was isolated in 1933 by Warburg and Christian.[8] Since then, a large number of flavoproteins have been purified and characterized. Most of these proteins contain either a tightly but noncovalently bound flavin mononucleotide (FMN) or flavin adenine dinucleotide (FAD) prosthetic group as redox-active center. However, a significant number of flavoproteins contain a covalently linked flavin.[9] Mammalian succinate dehydrogenase was the first protein recognized to have such a covalent protein–flavin linkage.[10] At present, about 30 covalent flavoproteins have been reported. The precise function of the covalent linkage in most flavoproteins is unknown, but it has been suggested that the anchoring prevents flavin dissociation, increases protein stability, and improves

[1] V. Massey, *FASEB J.* **9,** 473 (1995).
[2] V. Massey, *Biochem. Soc. Trans.* **28,** 283 (2000).
[3] S. A. Susin, H. K. Lorenzo, N. Zamzami, I. Marzo, B. E. Snow, G. M. Brothers, J. Mangion, E. Jacotot, P. Costantini, M. Loeffler, N. Larochette, D. R. Goodlett, R. Aebersold, D. P. Siderovski, J. M. Penninger, and G. Kroemer, *Nature (London)* **397,** 441 (1999).
[4] B. Entsch and W. J. H. van Berkel, *FASEB J.* **9,** 476 (1995).
[5] A. R. Cashmore, J. A. Jarillo, Y. J. Wu, and D. Liu, *Science* **284,** 760 (1999).
[6] M. S. Jorns, B. Wang, and S. P. Jordan, *Biochemistry* **26,** 6810 (1987).
[7] M. Salomon, J. M. Christie, E. Knieb, U. Lempert, and W. R. Briggs, *Biochemistry* **39,** 9401 (2000).
[8] O. Warburg and W. Christian, *Biochem. Z.* **266,** 377 (1933).
[9] M. Mewies, W. S. McIntire, and N. S. Scrutton, *Protein Sci.* **7,** 7 (1998).
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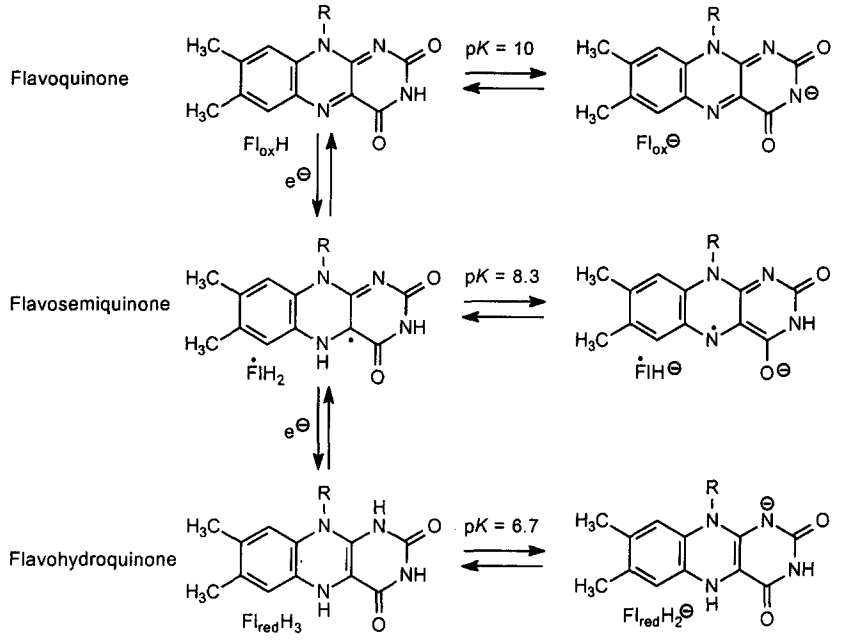

FIG. 1. The structures of biologically relevant flavin species in three different redox states.

resistance against proteolysis. Moreover, we have demonstrated that the covalent bond can raise the oxidative power of the flavin.[11]

Flavins are capable of performing both one- and two-electron transfer processes. The redox potentials for the two-electron reductions of FAD and FMN free in solution at pH 7.0 are −219 mV[12] and −205 mV,[13] respectively. Because a small percentage of the flavin semiquinone (one-electron reduced) is formed in thermodynamic equilibrium with the quinone (oxidized) and hydroquinone (two-electron reduced) (Fig. 1), the overall two-electron reduction can be analyzed for the two single-electron transfer steps, and a redox potential for both one-electron reductions can be determined. The semiquinone species can exist in either the blue neutral form (absorbance maximum in the 500- to 600-nm region) or the red anionic form (absorbance maximum in the 370- to 400-nm region), depending on the pH (pK = 8.3) (Fig. 1). When bound to apoprotein the flavin redox characteristics can change dramatically. Some flavoenzymes show essentially no stabilization of semiquinone on reduction, whereas others give nearly 100% stabilization. In the latter case, the protein may stabilize the neutral semiquinone over a wide pH range,

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[13] R. D. Draper and L. L. Ingraham, *Arch. Biochem. Biophys.* **125,** 802 (1968).

indicative of a high pK value, whereas others stabilize only the anionic form, indicative of a low pK. When the semiquinone is stabilized the redox potentials of the two steps in reduction are more separated.[14]

At present, the redox potentials of about 40 flavoenzymes are known, varying from −367 mV for nitroalkane oxidase[15] to +55 mV for vanillyl-alcohol oxidase (VAO).[11] For a selected number of flavoproteins the effects of substrate binding and pH have been studied[16] to obtain a better understanding of the catalytic mechanism. Especially when a crystal structure and active site mutant enzymes are available, such studies can reveal important information about the regulation of the flavin redox potential. As an example, we describe the redox properties of VAO, a covalent flavoprotein with an exceptionally high redox potential. However, we first briefly review the most common methods of flavin redox potential determination.

Determination of Flavin Redox Potentials

Electrochemical Methods

Electrochemical methods have proved to be effective approaches for studying the redox properties of flavoenzymes.[17,18] In these methods the oxidation state of the enzyme is changed electrochemically, using an electrode, while measuring the change in some property of the enzyme-bound flavin responsive to its redox state. The redox state of the flavin can be monitored, for example, by absorption spectroscopy, cyclic voltammetry, circular dichroism, or electron spin resonance. However, as the three different redox states of flavoenzymes have distinct spectral properties, absorption spectroscopy is most often used.

The electrochemical technique also allows the determination of the total number of electrons transferred from the electrode to the flavin. The power of this method lies in the fact that it gives quantitative information, which is especially useful when analyzing multi-redox-center enzymes.

Spectrophotometric Method

In the spectrophotometric method the equilibrium of partially reduced enzyme-bound flavin and a redox reference dye is analyzed optically. This is perhaps the simplest method for determining the redox potential of a flavoprotein because

[14] W. M. Clark, *in* "Oxidation–Reduction Potentials of Organic Systems." Williams & Wilkins, Baltimore, MD, 1960.

[15] G. Gadda and P. F. Fitzpatrick, *Biochemistry* **37,** 6154 (1998).

[16] M. T. Stankovich, *in* "Chemistry and Biochemistry of Flavoenzymes" (F. Mueller, ed.), Vol. I, p. 401. CRC Press, Boca Raton, FL, 1991.

[17] W. R. Heineman, *Anal. Chem.* **50,** 390A (1978).

[18] M. T. Stankovich, *Anal. Biochem.* **109,** 295 (1980).

there is no need for any specialized equipment. This optical method only requires a procedure to introduce reducing equivalents into the solution, a system to make a cuvette anaerobic, and a thermostatted scanning spectrophotometer.

In addition to the discussed electrochemical technique, several other procedures are available for introducing reducing equivalents into the reaction system. Chemical reduction by sodium dithionite is widely used for this purpose, as dithionite is a strong reducing agent.[19] Major disadvantages of dithionite, however, are its high reactivity with molecular oxygen, its changes in redox potential with pH, and its instability at low pH values (pH < 6.5). Moreover, the oxidized form of dithionite can form an adduct with several flavoproteins.[20] Photoreduction with 5-deazaflavin is another procedure for introducing reducing equivalents in a system. Catalysis of 5-deazaflavin in photoreduction is due to the formation of the strongly reducing 5-deazaflavosemiquinone species, which is generated by photolysis of the preformed 5-deazaflavin dimer.[21] Photoreduction is technically less complex to use than chemical reduction, because after the initial addition of 5-deazaflavin, reducing equivalents will be introduced continuously after exposing the system to light. A major drawback is that 5-deazaflavin is not commercially available and other photocatalysts do not always reduce the enzyme-bound flavins completely. A third commonly used method for introducing reducing equivalents into a system is the xanthine–xanthine oxidase method.[22] This method is extremely convenient for the determination of redox potentials of many flavoproteins. It uses xanthine oxidase, a low-potential reference dye, and xanthine to introduce reducing equivalents. We discuss this procedure in more detail, as we used it to determine the redox properties of VAO.

Xanthine–Xanthine Oxidase Method

Xanthine oxidase is a flavin-containing enzyme, which oxidizes xanthine to urate with the subsequent reduction of molecular oxygen to hydrogen peroxide. Xanthine oxidase can use, besides molecular oxygen, a number of low-potential dyes as electron acceptors, such as benzyl viologen ($E_m = -359$ mV at pH 7.0) and methyl viologen ($E_m = -449$ mV at pH 7.0). These dyes can react with other electron acceptors and also with most enzyme-bound flavins.

The assay described here is based on the method described by Massey.[22] Equal concentrations of the flavoprotein and the reference dye, typically 8–10 μM, 400 μM xanthine, and 2.5 μM methyl viologen or benzyl viologen, are placed

[19] S. G. Mayhew, *Eur. J. Biochem.* **85,** 535 (1978).

[20] V. Massey, F. Muller, R. Feldberg, M. Schuman, P. A. Sullivan, L. G. Howell, S. G. Mayhew, R. G. Matthews, and G. P. Foust, *J. Biol. Chem.* **244,** 3999 (1969).

[21] V. Massey, M. Stankovich, and P. Hemmerich, *Biochemistry* **17,** 1 (1978).

[22] V. Massey, *in* "Flavins, and Flavoproteins 1990" (B. Curti, S. Ronchi, and G. Zanetti, eds.), p. 59. Walter de Gruyter, Berlin, 1991.

in an anaerobic cuvette (total volume, 800 μl). The use of a Hellma (Plainview, NY) QS-117-104 cuvette sealed with a Subaseal 13 septum gives excellent results. A 10 m*M* xanthine stock solution is prepared by mixing xanthine with water and adjusting the pH to pH 11 with potassium hydroxide. The mixture of flavoprotein, reference dye, and xanthine is made anaerobic by either continuous flushing with oxygen-free argon gas or repeated cycles of evacuation and flushing with oxygen-free argon. The reduction of the flavoprotein and the reference dye is initiated by the anaerobic addition of xanthine oxidase, using a Hamilton syringe. Anaerobic conditions during the reductive process are maintained by continuously flushing the headspace of the cuvette with oxygen-free argon. To ensure equilibration between the oxidized and reduced species of enzyme and reference dye, the reduction must be sufficiently slow. The rate of reduction can be regulated by the concentration of xanthine oxidase. The final concentration needed depends on the redox potentials of the enzyme-bound flavin and dye, but is generally within 0.5–2.0 μg/ml. During the xanthine oxidase-mediated reduction, typically lasting 1–2 hr for full reduction of both enzyme and dye, spectra are recorded every 30 sec with a scanning diode-array spectrophotometer at 25°. Ideally, the concentrations of the oxidized and reduced forms of the flavoprotein and the reference dye are determined at a wavelength at which the other redox partner has an isosbestic point between the oxidized and reduced forms or has no absorbance at all. It is important to note that the redox potential of the reference dye should be within 30 mV of the flavoprotein to obtain reliable data. A number of useful redox reference dyes are given in Durst *et al.*[23] and Mayhew.[24] By using this method we could estimate the redox potentials of VAO variants, usually within 5 mV.

The potentials at 50% reduction of the flavoenzyme can be calculated by the Nernst equation[14]:

$$E_h(\text{dye}) = E_m(\text{dye}) + 2.303(RT/n_{\text{dye}}F)\log(\text{dye}_{ox}/\text{dye}_{red})$$
$$E_h(\text{E}) = E_m(\text{E}) + 2.303(RT/n_{\text{E}}F)\log(E_{ox}/E_{red})$$
$$E_h(\text{dye}) = E_h(\text{E}) \qquad \text{(at equilibrium)}$$

where E_h is the observed potential, E_m is the potential when the concentrations of oxidized and reduced forms are equal, R is the gas constant (8.31 J K^{-1} mol^{-1}), T is the temperature in degrees Kelvin, n is the number of electrons needed to convert the oxidized form to the reduced form, and F is the Faraday constant (96,496 J V^{-1} mol^{-1}). Thus, at 25°, 2.303(RT/nF) is equal to 0.059/n V.

[23] R. A. Durst, E. A. Blubaugh, K. A. Bunding, M. L. Fultz, W. A. MacCrehan, and W. T. Yap, *Clin. Chem.* **28,** 1922 (1982).

[24] S. G. Mayhew, *in* "Flavoprotein Protocols" (S. K. Chapman and G. A. Reid, eds.), Vol. 131, p. 49. Humana Press, Totowa, NJ, 1999.

Redox Properties of Vanillyl-Alcohol Oxidase

Vanillyl-Alcohol Oxidase

Vanillyl-alcohol oxidase (VAO; EC 1.1.3.38) is a homooctameric flavoenzyme[25] that catalyzes the oxidative demethylation of methyl ethers of *p*-cresol, providing the ascomycete *Penicillium simplicissimum* with a tool for metabolizing these lignin-derived aromatic compounds.[26] Each VAO subunit contains an 8α-N^3-histidyl-FAD as covalently bound prosthetic group.[25] The crystal structure of VAO has revealed that each VAO monomer consists of two domains: the larger domain comprises residues 1–270 and 500–560 and forms the FAD-binding domain, whereas residues 271–499 form the smaller cap domain.[27] The catalytic center of VAO is located at the interface of the two domains in the core of the protein. From sequence alignments it was recognized that VAO is a representative of a widespread family of structurally related oxidoreductases sharing a conserved flavin-binding domain.[28] A similarly folded domain is, for example, also present in the peripheral membrane respiratory flavoenzyme D-lactate dehydrogenase[29] and in UDP-*N*-acetylenolpyruvylglucosamine reductase (MurB), a flavoenzyme involved in peptidoglycan biosynthesis.[30]

Redox catalysis of VAO involves two half-reactions, in which the flavin is reduced by the substrate (reduction) and subsequently the reduced flavin is reoxidized by molecular oxygen (oxidation). The reductive half-reaction involves the initial transfer of a hydride from the substrate to the flavin, resulting in a binary complex between the two-electron reduced enzyme and the *p*-quinone methide of the substrate. In the oxidative part of the reaction, the reduced flavin is reoxidized by molecular oxygen with the concomitant hydration of the quinone methide intermediate (Fig. 2). For the reaction with the physiological substrate 4-(methoxymethyl)phenol, the reductive half-reaction is rate limiting in overall catalysis.[31]

Redox Properties

The midpoint redox potential of ligand-free VAO was determined by the xanthine–xanthine oxidase method in the presence of the redox reference dye

[25] E. de Jong, W. J. H. van Berkel, R. P. van der Zwan, and J. A. M. de Bont, *Eur. J. Biochem.* **208,** 651 (1992).

[26] M. W. Fraaije, M. Pikkemaat, and W. J. H. van Berkel, *Appl. Environ. Microbiol.* **63,** 435 (1997).

[27] A. Mattevi, M. W. Fraaije, A. Mozzarelli, L. Olivi, A. Coda, and W. J. H. van Berkel, *Struct. Fold. Des.* **5,** 907 (1997).

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[31] M. W. Fraaije and W. J. H. van Berkel, *J. Biol. Chem.* **272,** 18111 (1997).

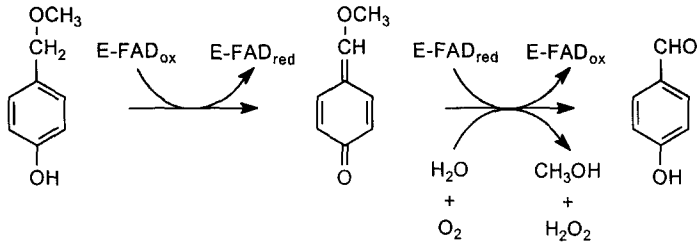

FIG. 2. Oxidative demethylation of 4-(methoxymethyl)phenol by VAO.

thionin (E_m = +60 mV) at pH 7.5. Figure 3 clearly shows that the enzyme-bound flavin and the dye reduce simultaneously via a single two-electron process, indicating that the dye and the flavin have a similar redox potential. The observed two-electron process fits well with the catalytic function of VAO, which involves the initial hydride transfer from the substrate to the flavin. When $\log(VAO_{ox}/VAO_{red})$ versus $\log(dye_{ox}/dye_{red})$ was plotted according to Minnaert[32] a midpoint redox potential of +55 mV was estimated (see inset, Fig. 3).[33] This redox potential is exceptionally high compared with other flavin-dependent enzymes. Only thiamine oxidase, also containing an 8α-N^3-histidyl-FAD, has an equally high redox potential (+55 mV).[34] The redox potential of VAO complexed with the substrate analog isoeugenol was determined in the presence of methylene blue (E_m = +11 mV). The enzyme reduced in a single two-electron reduction process and the redox potential was estimated to be +15 mV. Thus, when the active site cavity is saturated with the phenolic ligand isoeugenol the midpoint redox potential decreases by 40 mV.

In our studies we mainly focused on the rationale for the exceptionally high redox potential of VAO. A recurrent feature on analyzing flavin-containing oxidoreductases is the presence of a hydrogen bond donor near the N5 atom of the flavin prosthetic group. In most oxidoreductases this hydrogen bond donor is a backbone or a side-chain nitrogen atom.[35] However, in VAO such a hydrogen bond donor is absent. Instead, a negatively charged residue, Asp-170, is located near the flavin N5 atom (distance, 3.5 Å) (Fig. 4).[27] The side chain of Asp-170 is positioned in such a way that during catalysis it might interact with the reduced FAD cofactor, thereby stabilizing the reduced form. Intriguingly, glycolate oxidase also lacks a hydrogen bond donor to flavin N5 and, like VAO, this enzyme has a relatively high redox potential (E_m = −21 mV).[36]

[32] K. Minnaert, *Biochim. Biophys. Acta* **110,** 42 (1965).

[33] R. H. H. van den Heuvel, M. W. Fraaije, A. Mattevi, and W. J. H. van Berkel, *J. Biol. Chem.* **275,** 14799 (2000).

[34] C. Gomez-Moreno, M. Choy, and D. E. Edmondson, *J. Biol. Chem.* **254,** 7630 (1979).

[35] M. W. Fraaije and A. Mattevi, *Trends Biochem. Sci.* **25,** 126 (2000).

[36] C. Pace and M. Stankovich, *Biochemistry* **25,** 2516 (1986).

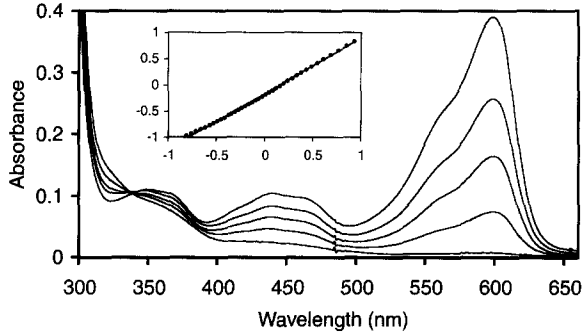

FIG. 3. Determination of the redox potential of wild-type VAO. VAO, 9 μM in potassium phosphate buffer, pH 7.5 at 25°, was reduced in the presence of 9 μM thionin by the xanthine–xanthine oxidase method. The reduction was finished after 100 min. *Inset:* $\log(VAO_{ox}/VAO_{red})$ (measured at 439 nm after correction for thionin) versus $\log(dye_{ox}/dye_{red})$ (measured at 600 nm). [Data from R. H. H. van den Heuvel, M. W. Fraaije, A. Mattevi, and W. J. H. van Berkel, *J. Biol. Chem.* **275,** 14799 (2000).]

The role of Asp-170 in VAO has been studied in detail by site-directed mutagenesis. When Asp-170 is replaced by glutamate or serine the flavin redox potential decreases 50 and 160 mV, respectively.[33] Kinetic characterization of the mutants revealed that Asp-170 is required for rapid substrate-mediated flavin reduction. D170E and D170S are 50-fold and 1000-fold less active, respectively.[33] Moreover, structural analysis of D170S suggested that the mutation does not induce any significant structural perturbations. These results indicate that both the presence

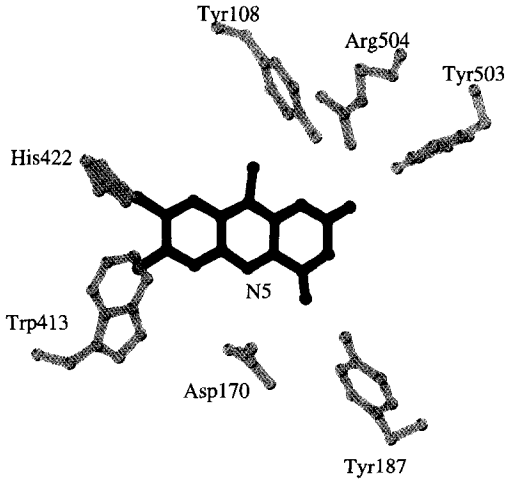

FIG. 4. The active site cavity of VAO. Distance from the flavin N5 atom to Asp-170 is 3.5 Å.

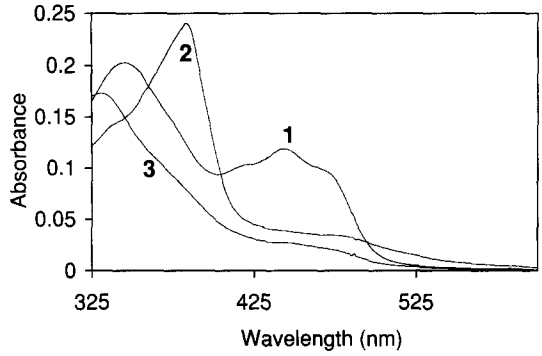

FIG. 5. Reduction of D170S by the xanthine–xanthine oxidase method. Curve 1, quinone form; curve 2, semiquinone anionic form; curve 3, hydroquinone form of D170S. [Data from R. H. H. van den Heuvel, M. W. Fraaije, A. Mattevi, and W. J. H. van Berkel, *J. Biol. Chem.* **275,** 14799 (2000).]

and the orientation of the negative charge near flavin N5 are crucial for the high redox potential of the flavin in VAO, and thus for the oxidative power of the enzyme. The behavior of the D170S mutant in the xanthine oxidase-mediated reduction is different from wild-type VAO, as this mutant highly stabilizes the red flavin semiquinone anion with a typical absorption maximum at 385 nm (Fig. 5). This thermodynamic stabilization of the semiquinone is different from kinetic substrate-mediated reduction experiments, in which no semiquinone formation is observed in the stopped-flow time scale.[33]

Redox potential determinations of flavin derivatives have revealed that modifications at the 8α position of the isoalloxazine ring result in increased redox potentials by 50–60 mV[37,38] and that ionization of the imidazole of 8α-*N*-imidazolylflavins results in changed redox properties.[39] To investigate the effect of the 8α-(N^3-histidyl)-flavin linkage in VAO, we substituted His-422, the residue to which the flavin is bound to the protein, for alanine.[11] The produced VAO mutant H422A binds the flavin tightly but noncovalently. The H422A mutation does not have any significant effects on the structure of the enzyme, but lowers the turnover rate with the physiological substrate 4-(methoxymethyl)phenol by one order of magnitude. The estimation of the redox potential of H422A revealed a 120-mV lower potential than for wild-type VAO. As Ala-422 is relatively far from the catalytic center of VAO, additional effects on the kinetics of the enzyme are unlikely. Therefore, the lower activity of the enzyme must be fully attributed to the decreased redox potential of the flavin.[11] This is the first report in which it has been

[37] D. E. Edmondson and T. P. Singer, *J. Biol. Chem.* **248,** 8144 (1973).

[38] D. E. Edmondson and R. De Francesco, *in* "Chemistry and Biochemistry of Flavoenzymes" (F. Mueller, ed.), Vol. I, p. 73. CRC Press, Boca Raton, FL, 1991.

[39] G. Williamson and D. E. Edmondson, *Biochemistry* **24,** 7918 (1985).

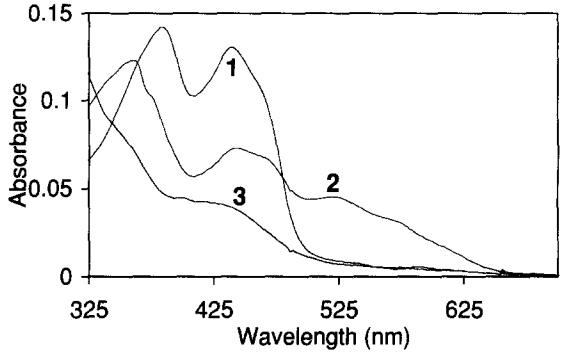

FIG. 6. Reduction of H422A by the xanthine–xanthine oxidase method. Curve 1, quinone form; curve 2, semiquinone neutral form; curve 3, hydroquinone form of H422A. [Data from M. W. Fraaije, R. H. H. van den Heuvel, W. J. H. van Berkel, and A. Mattevi, *J. Biol. Chem.* **274**, 35514 (1999).]

demonstrated that a covalent protein–flavin bond raises the redox potential of the flavin, and, therefore, the oxidative power of the flavoenzyme. Interestingly, the xanthine oxidase-mediated reduction of H422A occurs in two discrete one-electron reduction steps. First, a blue neutral flavin semiquinone species is formed, which is further reduced to the flavin hydroquinone (Fig. 6). Thus, the H422A mutation shifts the p*K* of the flavin semiquinone to a higher value compared with the D170S mutation, resulting in the neutral form of the flavin semiquinone at pH 7.5.

Conclusions

In the described research we have investigated the redox properties of the flavin prosthetic group in VAO. Site-directed mutagenesis studies have revealed that the exceptional redox properties of VAO are, at least in part, due to two rare structural determinants: the negative charge of Asp-170 near the flavin N5 atom and the covalent histidyl linkage between the protein and the flavin. The negative charge at hydrogen bond distance from the flavin N5 atom (3.5 Å) stabilizes the reduced form of the cofactor, thus facilitating the oxidation of substrates. Except for glycolate oxidase,[36] none of the other flavoenzymes with known three-dimensional structure harbor a hydrogen bond acceptor near flavin N5.[35] In agreement with earlier suggestions,[9] a covalent linkage between the protein and the isoalloxazine ring of the flavin can markedly increase the redox potential of the flavin prosthetic group.[11] This indicates that the histidyl–flavin bond in certain flavoproteins has evolved as a tool for raising the oxidative power of the flavin cofactor.