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Substrate Dehydrogenation by Flavoproteins

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ABSTRACT

Enzymes with tightly bound FMN or FAD as cofactor catalyze the oxidation of a wide range of substrates. The chemical versatility of the isoalloxazine ring provides these enzymes with a range of potential mechanisms. Recent progress in elucidating the mechanisms of oxidation of organic substrates by flavoenzymes is described, focusing on the oxidation of alcohols, amino and hydroxy acids, amines, and nitroalkanes. With each family of enzymes, an attempt is made to integrate mechanistic, structural, and biomimetic data into a common catalytic mechanism.

Enzymes which contain riboflavin derivatives as tightly or covalently bound cofactors are found throughout cellular metabolism. While the cofactor can be found at both the FAD and FMN levels, it is the heteroaromatic isoalloxazine ring which is involved in catalysis. A major reason for the ubiquity of flavin-dependent enzymes in biological systems is the chemical versatility of the isoalloxazine ring. The fully oxidized cofactor can readily and reversibly be reduced by either one or two electrons, so that flavoproteins have the potential for transfer of single electrons, of hydrogen atoms, and of hydride ions. In addition, the oxidized flavin molecule is susceptible to nucleophilic attack, especially at N(5) and C(4a), allowing for a variety of covalent intermediates in the enzyme-catalyzed reactions.[1] The fully reduced dihydroflavin is an effective reductant, with an E_m value at pH 7 of about −200 mV.[1] In addition, it reacts readily with molecular oxygen, unlike the reduced pyridine nucleotides; among the possible products, the most important for biological systems is the flavin peroxide, which serves as an oxygen donor in flavoprotein hydroxylases.[2,3] This plethora of possible reactions must be considered in analyzing the enzyme-catalyzed reactions. One of the most critical roles the protein component of a flavoprotein plays is to limit this range of possible reactions to those beneficial to the reaction to be catalyzed; how this is done is still poorly understood. Recent structural studies of a number of flavoproteins have resulted in increased insight into the role that the protein moiety plays in catalysis. These results can be combined with mechanistic studies in allowing one to draw general conclusions about the specific mechanisms of classes of flavoenzymes. Rather than attempt to encompass the complete range of flavoprotein mechanisms, the present Account will focus on the enzymes which catalyze the dehydrogenation of bonds between carbon and either nitrogen or oxygen.

Alcohol Oxidation

A number of flavoproteins catalyze the oxidation of primary alcohols to aldehydes. The best understood are glucose oxidase, cholesterol oxidase, and methanol oxidase, all of which belong to the GMC oxidoreductase enzyme superfamily.[4−6] This designation was initially based on comparison of amino acid sequences; the subsequent demonstration that glucose oxidase and cholesterol oxidase have similar three-dimensional structures[7] suggests that all members of this family share a common fold and similar or identical mechanisms.

Studies of the nonenzymatic oxidation of alcohols by flavins are consistent with the radical mechanism of Scheme 1,[8,9] although a hydride transfer mechanism cannot be ruled out.[10] Substrate analogues containing a cyclopropyl moiety have been utilized as probes for similar radical intermediates in the reactions of both methanol oxidase and cholesterol oxidase. Incubation of methanol oxidase with cyclopropanol results in inactivation of the enzyme and covalent attachment of the ring-opened inhibitor to the flavin.[11] An analogous reaction occurs upon inactivation of cholesterol oxidase by 2α,3α-cyclopropano-5α-cholestan-3β-ol.[12] In neither case does catalytic turnover occur, and no free ring-opened product is detectable. Two alternative mechanisms were initially proposed for inactivation of methanol oxidase, one involving ring opening of a cyclopropyl radical and the other an ionic mechanism[11] (Scheme 2). No inactivation of methanol oxidase is seen with enzyme containing 5-deaza-FAD in place of the native FAD, consistent with a radical mechanism.[11] The proposed radical mechanism in Scheme 2 for inactivation by cyclopropyl alcohol differs from the oxidation mechanism derived from model studies (Scheme 1) in that the former involves an oxygen radical while the latter proposes a carbon-centered radical. No inactivation of methanol oxidase is seen with cyclopropyl methanol as a substrate;[11] catalysis in this case would involve a carbon-centered radical α to the cyclopropyl ring, with subsequent ring opening, if the mechanism of Scheme 1 applied. One resolution to this apparent contradiction is that inactivation by these cyclopropyl compounds may involve intermediates which are not along the normal catalytic pathway, while still involving reactions within the flavin repertoire.

When the active site histidine of cholesterol oxidase (vide infra) is mutated to glutamine, the rate of inactivation by 2α,3α-cyclopropano-5α-cholestan-3β-ol decreases

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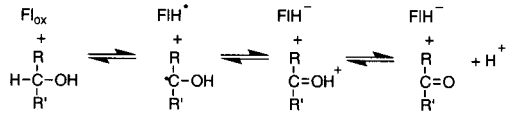
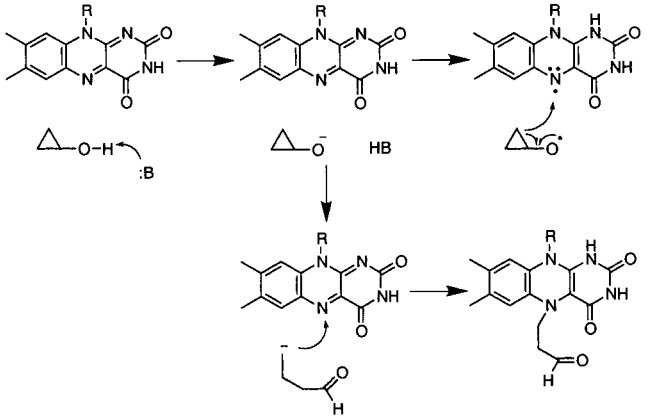
Scheme 1



Scheme 2

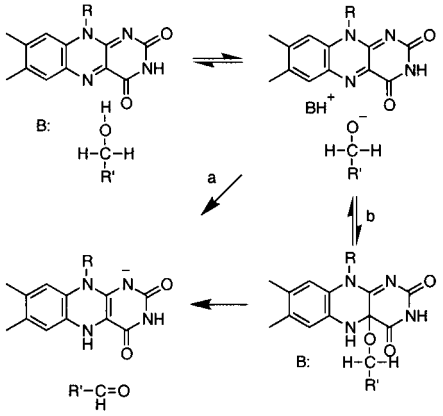


10-fold, to the rate at which the cyclopropyl ring opens nonenzymatically.[12] Thus, the ionic pathway of Scheme 2 can account for the inactivation of cholesterol oxidase by the cyclopropyl compound without the need to invoke a radical intermediate.

Kinetic isotope effects provide a more direct probe of the catalytic mechanism of the members of this superfamily. Among the critical issues which are amenable to study by isotope effects is the relative timing of cleavage of the substrate OH and CH bonds. In the case of glucose oxidase with glucose as substrate, there is a primary deuterium isotope effect of 2−3 on the steady-state kinetic parameter $V/K_{glucose}$ when 1-[^{2}H]glucose is used as substrate, but no change in rates is reported when the reaction is run in D_2O.[13] This suggests that the OH and CH bonds may be cleaved in different steps in catalysis. However, chemical steps are not fully rate-limiting in this case, so that the observed isotope effects are significantly decreased from their intrinsic values. Analogous isotope effects for the cholesterol oxidase reaction are even more difficult to interpret because that enzyme catalyzes not only alcohol oxidation but also the subsequent isomerization of the ketone. The primary deuterium isotope effects that have been reported for cholesterol oxidase are smaller than expected for fully rate-limiting CH bond cleavage, ranging from 1 to 2.2, consistent with masking of the isotope-sensitive steps by other slower steps.[14,15] Solvent isotope effects have also been measured with this enzyme, but these are even smaller.[14,15] Thus, the kinetic complexities of both glucose and cholesterol oxidase do not, at present, allow unambiguous determination of the relative timing of the bond cleavage steps.

The most extensive studies utilizing isotope effects have been carried out with methanol oxidase from *Hansenula polymorpha*.[16] With benzyl alcohol as substrate, the V/K value for the alcohol has a solvent isotope effect of 2 and a primary deuterium isotope effect very close to 1. Characterization of the kinetics with a series of substituted

Scheme 3

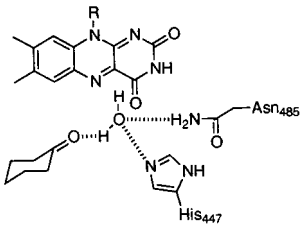


benzyl alcohols yields a ρ value of 1.9. Both results are consistent with cleavage of the hydroxyl OH bond rather than cleavage of the CH bond being at least partially rate-limiting in oxidation of the alcohol to the aldehyde. In contrast, with 2-substituted ethyl alcohols as substrates, the ρ value is −1.2, and the relative values of the solvent and primary isotope effects show a reciprocal relationship. For example, 2-chloroethanol and 2-bromoethanol give primary deuterium isotope effects on the V/K value of about 5 and solvent isotope effects very close to unity, while ethanol yields a primary isotope effect of 1.5 and a solvent isotope effect of 1.9. These results are consistent with a mechanism in which OH bond cleavage precedes CH bond cleavage, either in separate steps or in a highly asynchronous concerted reaction. The simplest such mechanism is direct hydride transfer (Scheme 3, path a). This mechanism is analogous to the reaction catalyzed by pyridine nucleotide-dependent liver alcohol dehydrogenase, in which initial formation of the alkoxide occurs in a step prior to hydride transfer.[17,18] In that case a metal ion is used to replace the hydroxyl proton, while in the flavoprotein oxidases deprotonation of the hydroxyl proton must necessarily involve only amino acid side chains. Facile deprotonation of the alcohol upon binding would be consistent with the inactivation of members of this flavoprotein family by cyclopropyl-containing inhibitors discussed above, with the inactivation being due to opening of the alkoxide form of the inhibitor.

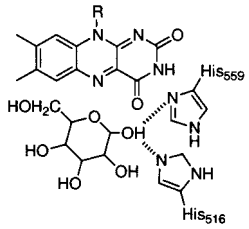
An alternative catalytic mechanism consistent with the reactivity of the isoalloxazine ring is nucleophilic attack of the alkoxide on the flavin N5 or C4a position (Scheme 3, path b). This possibility requires that all steps up to CH bond cleavage be reversible to account for the isotope effect data. No such intermediate has been detected in rapid reaction studies of these enzymes, although it is not clear that the absorbance spectrum of such an adduct would be readily distinguished from that of the reduced flavin. In addition, the structure of the flavin adduct formed upon inactivation of methanol oxidase by cyclopropanol[11] is not consistent with formation of such a complex during inactivation, although catalysis and inactivation could involve different mechanisms.

Removal of the hydroxyl proton from the alcohol requires an active site base. Superposition of the structures

Scheme 4



Scheme 5

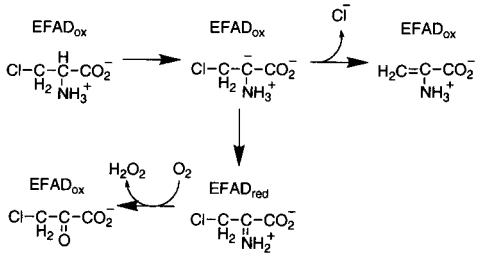


of cholesterol oxidase and glucose oxidase shows that there is a single histidine residue in equivalent positions in the active sites of each.[6] This histidine is one of the few residues conserved throughout the GMC superfamily. In the structure of cholesterol oxidase crystallized in the presence of the steroid dehydroisoandrosterone, this histidine, residue 447, interacts with the oxygen of the substrate through an intervening water molecule[5] (Scheme 4). (Whether the active site contained dehydroisoandrosterone or the product androstenedione could not be determined.) When glucose is modeled into the structure of glucose oxidase, the glucose O1 is 2.8 Å from the homologous histidine in that enzyme[19] (Scheme 5). Mutation to alanine of this residue in glucose oxidase decreases the enzyme activity 4 orders of magnitude,[19] consistent with this histidine being the active site base. Mutation of the conserved histidine in cholesterol oxidase to glutamine only decreases the rate of substrate oxidation 140-fold,[14] but, as noted above, chemical steps do not appear to be fully rate limiting with that enzyme.

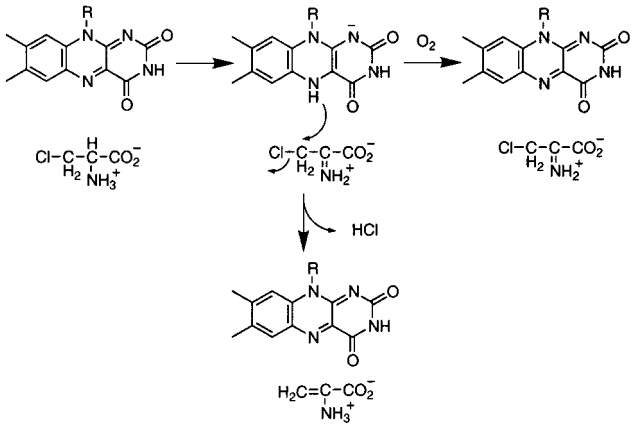
Amino Acid Oxidation

D-Amino acid oxidase was one of the first flavoenzymes to be purified and has remained the archetypal flavoenzyme for mechanistic studies of oxidation of amino acids and of the energetically similar oxidation of α-hydroxy acids. A seminal contribution to any discussion of the mechanism of D-amino acid oxidase is the series of reports by Walsh and co-workers which describe the ability of this enzyme to catalyze the elimination of HCl from β-Cl-substituted D-amino acids to form the respective keto acids.[20,21] With α-^{2}H-chloroalanine, the isotope effects are the same for formation of chloroalanine and of pyruvate,[20] consistent with cleavage of the CH bond occurring in an intermediate common to both pathways. These results were taken as evidence for the ability of the enzyme to remove the α-hydrogen as a proton; the effects of oxygen concentration and isotopic substitution are consistent with partitioning between HCl elimination and

Scheme 6



Scheme 7



oxidation to the Cl-substituted α-keto acid from a common carbanion intermediate along the catalytic pathway (Scheme 6). The mechanism of subsequent electron transfer to the FAD was not addressed by these experiments, although both radical and nucleophilic mechanisms have been proposed by others.[22,23]

Hydride transfer from the amino acid directly to the flavin is an alternative possibility for the mechanism of D-amino acid oxidase. When D-amino acid oxidase in which the native FAD has been replaced with 5-deaza-FAD is incubated with 2-^{3}H-labeled amino acids, tritium is transferred to the cofactor,[24,25] demonstrating the viability of hydride transfer with this flavin analogue. To reconcile amino acid oxidation by direct hydride transfer with HCl elimination from β-chlorinated amino acids, one must propose that the HCl elimination occurs from a species which is not along the direct catalytic path between reduced and oxidized substrate. A possibility that is consistent with the data is that HCl elimination is due to attack of the reduced flavin on chloropyruvate (Scheme 7). This would re-form oxidized FAD as well. Such a mechanism predicts that the hydrogen removed from the β-chloroamino acid will be found on the β carbon of the keto acid product. This is, indeed, the case with both β-chloroalanine and β-chloroaminobutyrate, although the transfer is not stoichiometric.[20,21] This mechanism also predicts that higher levels of HCl elimination will be seen at decreased oxygen concentrations, as is found.[20]

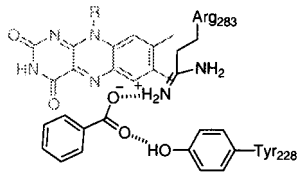
Kinetic isotope effects have been used as probes of the catalytic mechanism of D-amino acid oxidase. One of the complicating factors with the commonly studied pig enzyme is that substrate binding and product release are rate-limiting with the most commonly utilized substrates,

such as D-alanine.[26,27] However, with D-serine, the rate-limiting step is cleavage of the substrate CH bond, based on the pH independence of the deuterium isotope effect on the V/K value for the amino acid, comparison of primary deuterium and tritium isotope effects, and direct measurement of the deuterium isotope effect on flavin reduction by stopped-flow methods.[28,29] The bond cleavage step also becomes rate-limiting at pH extremes with glycine and D-alanine as substrates.[29] This has made it possible to measure the secondary tritium isotope on the V/K value for glycine under conditions where the intrinsic primary isotope effect is seen. The $^{\alpha T}(V/K_{gly})$ is small, 1.03 $\pm$ 0.02, suggesting that there is only a small amount of rehybridization of the bonds to the carbon in the transition state. While this result would be in line with a carbanion mechanism in which there is some contribution from the *aci* form of the substrate, the intrinsic primary deuterium isotope effect with glycine is only 3.6, compared to values of 4.5 with D-serine and 5.7 with D-alanine.[29] This suggests that the transition state with glycine may be quite early, with a structure close to that of the substrate.

An alternative approach to determining the structure of the transition state is to measure ^{15}N isotope effects. D-Serine was chosen for these analyses because the CH bond cleavage is rate-limiting. At pH 7.5, the measured $^{15}(V/K_{ser})$ value is 1.0128. This increases to 1.0175 in D_2O and decreases to 0.9991 at pH 10.1.[30] The effects of pH and D_2O cannot be due to CH bond cleavage becoming more rate-limiting under these conditions, since the primary deuterium isotope effect is unchanged. Instead, the ^{15}N effect changes because the amino group of the substrate must be unprotonated for productive binding, and there is an ^{15}N effect on the pK_a value for the amino group. This $^{15}K_{eq}$ effect is 1.016, increasing to 1.022 in D_2O.[31] As a result, the amount of the amino acid which is correctly protonated for catalysis changes with pH and D_2O content. If this is corrected for, the ^{15}N effect on the V/K value is 0.997 under all conditions.[30] This is the expected value for concerted formation of an imino acid from an unprotonated amino acid.[32]

The assignment of the amino acid with the amino group unprotonated as the active form of the substrate is supported by other analyses. By following the reaction of D-amino acid oxidase in a stopped-flow spectrophotometer in the presence of the pH-sensitive dye phenol red, it can be shown that a proton is lost during the reduction of the enzyme to the enzyme−imino acid complex.[33] This proton can be assigned to the amino group of the substrate. The pH dependence of the V/K value for D-serine shows the involvement of two groups, with pK_a values of 8.5 and 9.3, one of which must be protonated and one unprotonated. Because these values are so close to one another, it is not possible, a priori, to tell which is which.[34] The pK_a value of 9.3 matches the pK_a value of the amino group of D-serine. The pK_a value of 8.5 can then be assigned to a protein residue which must be protonated for binding the amino acid carboxylate. Finally, the solvent isotope effect on the reductive half-reaction with D-serine as substrate has been determined. This required that the pH dependence of the V/K value for this amino acid be determined in D_2O, to correct for the change in the pH dependence in that solvent.[28] The pK_a values are shifted to higher values in D_2O, with the increase in the higher pK_a value matching the change in the pK_a value of the amino group of D-serine found in D_2O. More importantly, if one compares the V/K values at the pH optimum in D_2O versus H_2O, there is no effect on the activity. This establishes that there is no exchangeable proton in flight in the transition state for CH bond cleavage, as expected if the amino group of the amino acid is already unprotonated.

Scheme 8



A clear difference between carbanion and hydride transfer mechanisms is the need for a catalytic base to remove the proton from the α-carbon in order to form the carbanion. The structure of hog D-amino acid oxidase has been determined by two separate groups,[35,36] in each case with a bound inhibitor. The carboxylate of the inhibitor, benzoate or anthranilate, is bound by Arg283 and Tyr228 (Scheme 8). Mutagenesis to lysine of the conserved arginine in the *Rhodotorula gracilis* enzyme decreases the affinity for amino acids and the rate of amino acid oxidation by 2−3 orders of magnitude,[37] clearly implicating this residue in binding/catalysis. Mutagenesis of Tyr228 in the pig enzyme to phenylalanine decreases the rate of flavin reduction 2 orders of magnitude;[38] similar mutagenesis of the homologous residue in the *Rhodotorula gracilis* enzyme has only a minor effect on the rate of reduction but decreases the affinity for the amino acid substrate by 1−2 orders of magnitude.[39] Tyr228 is a reasonable candidate for the residue responsible for the pK_a value of 8.5 seen in the V/K pH profile. While Tyr224 is found in the active site of the hog enzyme and is a potential base, this residue is not conserved in D-amino acid oxidase from nonmammalian sources. This leaves no identifiable residue in the active site to act as the catalytic base in formation of a carbanion.

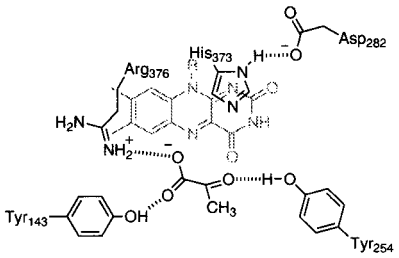
Unlike the situation with the GMC oxidoreductases, there is no other enzyme from the same family as D-amino acid oxidase which has seen comparable mechanistic study. D-Aspartate oxidase is homologous, with both the active site tyrosine and the active site arginine conserved,[40] but there has been relatively little study of its mechanism to date. Monomeric sarcosine oxidase has the same fold as D-amino acid oxidase; however, the active site residues are not conserved between the two enzymes.[41] There are a number of flavoproteins which oxidize L-amino acids, but they do not show any structural similarity to D-amino acid oxidase, and their mechanistic study has lagged that of D-amino acid oxidase.

Hydroxy Acid Oxidation

A number of flavoproteins have been identified which oxidize short-chain α-hydroxy acids to ketones. At present this family includes flavocytochrome b_2 (L-lactate dehydrogenase), L-mandelate dehydrogenase, glycolate oxidase, long-chain hydroxy acid oxidase, lactate oxidase, and lactate monooxygenase.[42] The similarity of the oxidation of a hydroxy acid to that of an amino acid has meant that many of the experiments initially carried out with D-amino acid oxidase were soon done with one or more of the hydroxy acid oxidizing flavoenzymes. When β-chlorolactate is utilized as substrate for lactate monooxygenase, both HCl elimination and normal oxidation occur, with the partitioning depending on the oxygen concentration, and tritium is transferred from the α-carbon to the β-carbon during elimination.[43] These results are identical to those found with D-amino oxidase (see Scheme 6) and have been similarly interpreted as favoring a carbanion mechanism. In contrast, neither flavocytochrome b_2 nor rat kidney hydroxy acid oxidase will catalyze formation of pyruvate from β-chlorolactate,[44,45] although the former enzyme will catalyze HBr elimination from β-bromolactate.[46] Flavocytochrome b_2 will also catalyze a transhalogenation reaction in which enzyme which has been reduced by lactate can utilize β-chloro- or β-bromopyruvate as an electron acceptor, forming the respective β-substituted lactate plus unsubstituted lactate.[46,47] When 2-^{3}H-lactate is used as substrate in the transhalogenation reaction with β-bromopyruvate, both 3-^{3}H-lactate and 3-bromo2-^{3}H-lactate are formed.[48] This is reminiscent of the formation of 3-^{3}H-aminobutyrate from 3-chloroaminobutyrate by D-amino acid oxidase.[49] The formation of bromolactate from β-bromopyruvate shows a larger isotope effect with both 2-^{2}H-lactate and 2-^{3}H-lactate than does the formation of pyruvate;[50] this has been taken as evidence that these two species do not arise by hydride attack on bromopyruvate.[51] However, they need not have the same isotope effect, since HBr elimination is irreversible while reduction to bromolactate is readily reversible.

The mechanistic interpretation of the elimination of HCl or HBr catalyzed by flavocytochrome b_2 and the other members of the hydroxy acid oxidase family suffers from the same ambiguities found with D-amino acid oxidase. While these results have been presented as evidence for the intermediacy of a carbanion, the mechanism of Scheme 7 could also apply. Indeed, at least in the case of flavocytochrome b_2, pyruvate dissociation from the reduced enzyme is relatively rapid, while it is quite slow with D-amino acid oxidase and lactate oxidase.[52−54] The lack of elimination seen with substituted lactates and flavocytochrome b_2 may simply reflect the short lifetime of the complex of reduced enzyme and pyruvate. In addition, just as with D-amino acid oxidase, when ^{3}H-lactate is incubated with lactate monooxygenase or flavocytochrome b_2 in which the native FMN has been replaced with 5-deaza-FMN, tritium is found in the reduced cofactor.[55,56]

Scheme 9

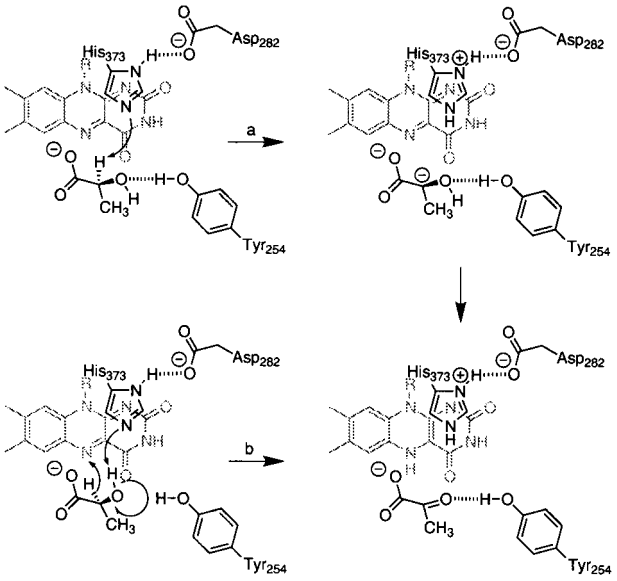


Structural analyses of these enzymes have not provided clear evidence for a carbanion or hydride transfer mechanism. Three-dimensional structures have been determined for spinach glycolate oxidase[57,58] and for yeast flavocytochrome b_2.[59] The structure of the active site of flavocytochrome b_2 with pyruvate bound is shown in Scheme 9. All of the amino acid residues shown are conserved throughout this family of enzymes. The substrate binds on the opposite side of the flavin from that in D-amino acid oxidase; adjusting for this allows the active sites of the two enzymes to be overlaid.[35] In both D-amino acid oxidase and the hydroxy acid oxidizing flavoproteins, the carboxylate of the bound inhibitor interacts with conserved arginine and tyrosine residues. Mutagenesis of the conserved arginine or of Tyr143 in the hydroxy acid oxidizing enzymes has effects similar to those seen when the analogous residues in D-amino acid oxidase are mutated,[60−64] consistent with similar roles in binding the substrate carboxylate.

There are several conserved active site residues found in the hydroxy acid oxidizing enzymes which are not present in D-amino acid oxidase, His373, Asp282, and Tyr454 in flavocytochrome b_2. The structure of this enzyme with pyruvate bound has been used to model the structure of the enzyme−lactate complex,[50] in order to gain insight into the roles of these residues. Two different orientations for the substrate arise from such an analysis. In the first (Scheme 10, path a), His373 is properly placed to abstract the proton from the α-carbon to form a carbanion. In the second (Scheme 10, path b), His373 is positioned to abstract the hydroxyl proton, and the α-hydrogen is positioned for hydride transfer to the flavin. The results of site-directed mutagenesis are consistent with either model. Mutagenesis of the histidine results in enzymes with no measurable ability to oxidize hydroxy acid substrates,[65−67] while a decrease of 100-fold occurs when Asp282 is mutated to asparagine in flavocytochrome b_2.[68] Mutagenesis of Tyr254 to phenylalanine decreases the rate of hydroxy acid oxidation by 1−2 orders of magnitude.[50,69]

The debilitating effect of mutagenesis of the conserved histidine in this family of enzymes is fully consistent with a role as the active site base in a carbanion mechanism. If hydroxy acid oxidation instead proceeds via hydride transfer, one must justify this result. D-Amino acid oxidase does not require a base to assist with hydride transfer because the substrate binds with the amino group already unprotonated. The much higher pK_a value of the hydroxyl proton results in far too little of the hydroxy acid existing

Scheme 10



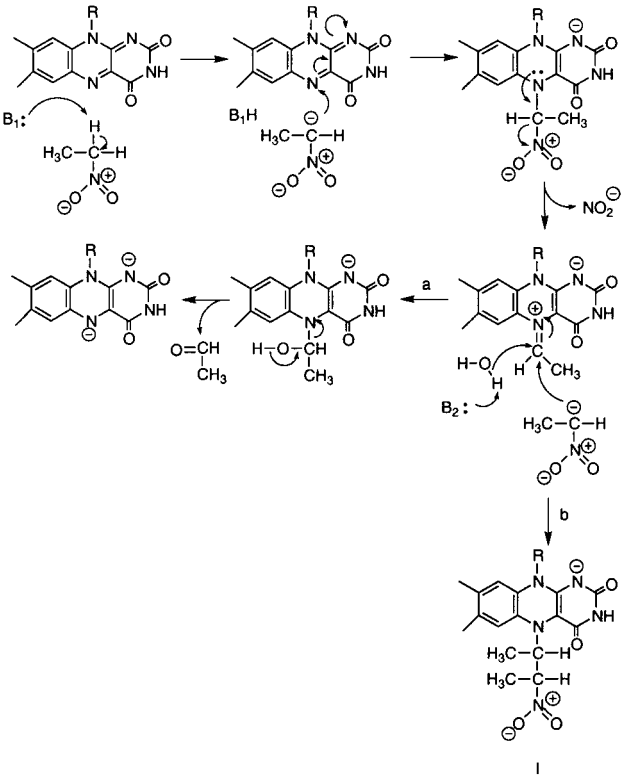
as the alkoxide at neutral pH for this to be the active form of the substrate. Deprotonation of the substrate hydroxyl on the enzyme surface would require the presence of an active site base even for a hydride transfer mechanism and would explain the decrease in activity seen upon mutation of the histidine.

Nitroalkane Oxidation

Inasmuch as the evidence supports a hydride transfer mechanism for D-amino acid oxidase and such a possibility cannot be ruled out for the hydroxy acid oxidases, the question arises whether any flavoprotein has been definitively demonstrated to oxidize its substrate via a carbanion intermediate. There is one example where the evidence is strong for a carbanion, nitroalkane oxidase from *Fusarium oxysporum*. This enzyme catalyzes the oxidation of primary and secondary nitroalkanes to the respective aldehydes or ketones, liberating nitrite in the process. Catalysis of the oxidation of nitroalkane anions is a nonphysiological property of a number of glucose and D-amino acid oxidases.[70−73] In contrast, the oxidation of nitroalkanes by nitroalkane oxidase appears to be physiologically relevant, in that the enzyme is induced to high levels when the fungus is grown on nitroethane as a carbon source,[74] and the neutral nitroalkane is the substrate rather than the anion.

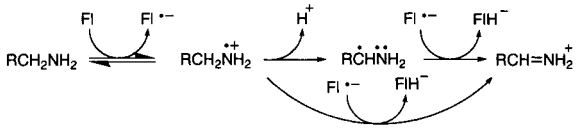
When nitroalkane oxidase is isolated from cells induced with nitroethane, the enzyme has low activity and an unusual visible absorbance spectrum. In contrast to typical flavin peaks at 450 and 380 nm, there is a single maximum at 336 nm.[75] Mass spectral analysis of the cofactor isolated from freshly purified enzyme showed that it is a 5-(3-nitrobut-2-yl)-1,5-dihydroflavin (I in Scheme 11). Incubation of the native form of the enzyme at 37 °C for 1−2 days results in the conversion of this species to FAD and a concomitant increase in enzyme activity, demonstrating that the active form of the enzyme contains FAD as cofactor. This has been confirmed by reconstitution of the apo-enzyme with FAD.[76] The FAD-containing enzyme can be converted to the butylflavin by incubation with a mixture of nitroethane and preformed nitroethane anion.[75] This result is consistent with formation of the flavin adduct by path b in Scheme 11. Catalysis would occur by path a.

Scheme 11



That it is the neutral rather than the anionic form of nitroalkanes that is the true substrate for nitroalkane oxidase has been demonstrated in two ways. Nitroalkanes are unusual acids in that deprotonation occurs relatively slowly. Thus, it is possible to test the neutral species and the anion separately at neutral pH. The pH dependence of nitroalkane oxidase determined with neutral nitroalkanes as substrates shows that that two groups must be correctly protonated for productive binding of the substrate to the free enzyme, a group with a pK_a value of 6−7 which must be unprotonated and one with a pK_a value of 9.9 which must be protonated.[77,78] If the nitroalkane is allowed to reach protonic equilibrium at each pH, the pH dependence still shows the effects of the group with the lower pK_a value, but the upper pK_a value is 9.3 with nitropropane and 6.7 with phenylnitromethane.[79] These values agree well with the pK_a values of these nitroalkanes, as expected if the protonated form is the substrate. In addition, if the neutral nitroalkane is added to nitroalkane oxidase in the absence of oxygen, the enzyme is rapidly reduced. Readmission of oxygen then regenerates the active FAD-containing enzyme. In contrast, if the experiment is done with the anionic form, the inactive nitrobutyl flavin form results.[75] Nitroalkane oxidase thus appears to be a promising system for study of the oxidation of substrates by flavoproteins via a carbanion. In addition,

Scheme 12



the extensive literature on the properties of nitroalkane anions[80] provides nonenzymatic data for comparison.

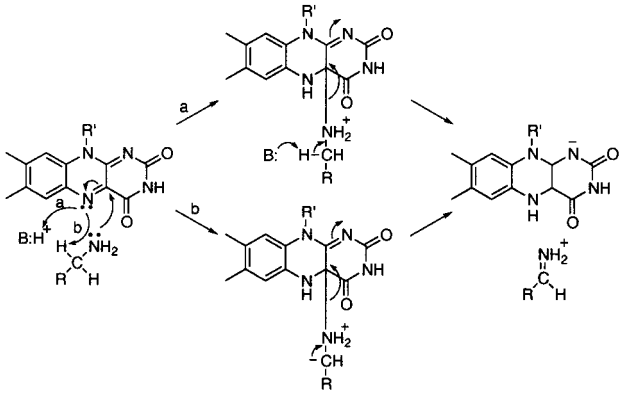
Neither the three-dimensional structure nor the complete primary structure is known for nitroalkane oxidase, although enough of the sequence is known to establish that the enzyme is not homologous to a known protein.[81] To date, both a tyrosine and an arginine have been shown to be essential for catalysis.[81,82] These residues may play roles similar to those in both D-amino acid oxidase and the hydroxy acid oxidizing enzymes, binding to the oxygens of the substrate.

Amine Oxidation

Monoamine oxidases oxidize primary or secondary amines to the corresponding imines, which are subsequently hydrolyzed nonenzymatically to the corresponding aldehydes or ketones. There are two isozymes, monoamines A and B, which are 70% identical in sequence but differ in their substrate specficities.[83] Because of the physiological importance of this enzyme in the catabolism of amine neurotransmitters, monoamine oxidase has been extensively studied for several decades. As this topic has been reviewed in this journal relatively recently,[84] only a very brief summary of the present understanding of its mechanism will be given here. Silverman and colleagues have characterized the inactivation of monoamine oxidase by a series of mechanism-based inhibitors, many of which contain cyclopropyl groups or other moieties which rapidly rearrange as radicals. Based upon these studies, they have proposed the mechanism of Scheme 12.[84] The initial step is transfer of an electron from the amine nitrogen to the flavin. This is then followed either by loss of a proton to form the carbon-centered radical which transfers a second electron to the flavin or by transfer of a hydrogen atom. The involvement of an amino acid residue in this process has also been suggested.[84] Similarly to the situation with methanol oxidase, which is inactivated by cyclopropyl alcohol but not cyclopropyl methanol,[11] monoamine oxidase is inactivated by cyclopropylamines but not cyclopropyl methylamines.[84] While the latter class of compounds would be expected to be inhibitors if the mechanism of Scheme 6 is correct, monoamine oxidase is inactivated by aminomethylcubane,[85] consistent with formation of a carbon-centered radical as an intermediate.

In an alternative mechanism, initially proposed by Brown and Hamilton,[23] the amine adds to the 4a carbon of the flavin, forming an adduct which can collapse to the products (Scheme 13, path a). This mechanism has recently received support from model studies which show that the reaction of 2-phenylcyclopropylamine with 5-ethyl-3-methyllumiflavin produces a flavin adduct which decays

Scheme 13



to the ring-opened aldehyde.[86] Miller and Edmondson studied the oxidation of a series of ring-substituted benzylamines by both the A and B isozymes and concluded that the kinetics were consistent with a nucleophilic mechanism.[87] To rationalize the large primary deuterium kinetic isotope effects (6−12) seen with benzylamines, CH bond cleavage and adduct formation were proposed to be concerted, with the flavin N5 acting as the base (Scheme 13, path b).[87]

The ρ value for the effect of the substituent in the aromatic ring on the rate of flavin reduction is different for the two isozymes, with monoamine oxidase A giving a value of 2 and monoamine oxidase B showing no effect.[87,88] While a ρ value of 2 is larger than one would expect for formation of a radical, the reason for the differences between the two isozymes is not clear. It may simply be that relatively subtle differences in the energetics of the different transitions states result in different steps being rate-limiting. In the case of monoamine oxidase A, there is a correlation of the K_d values of the substrates with the van der Waals volume of the para substituents; this necessitated the assumption that the unprotonated amine is the substrate,[87] as would be the case for either a radical or a nucleophilic mechanism.

Conclusion

The goal of the present Account has been to synthesize the results of mechanistic and structural studies of the different families of flavoprotein oxidases in order to develop unified mechanisms for each. As is clear, in most if not all cases, multiple mechanisms still remain viable despite intensive study. This remaining uncertainty can be attributed to the range of reaction mechanisms potentially available to the flavin cofactor noted in the Introduction. The reactivity of the isoalloxazine ring means that one must consider radical and nucleophilic mechanisms in addition to more straightforward hydride transfer. In addition, one must consider the possibility that reactions seen with substrate analogues may diverge from the normal catalytic mechanism. The cases of the alcohol and monoamine oxidases may well be examples of this, in that results with mechanism-based inhibitors lead to different mechanistic conclusions than do studies with more normal substrates. While the growing amount of

structural data has provided mechanistic insight in some cases, such as D-amino acid oxidase, in others it has not. Still, significant progress has been made in recent years, and it seems likely that the remaining mechanistic questions described here will be resolved soon.

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