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Mini-Review

Carbanion versus hydride transfer mechanisms in flavoprotein-catalyzed dehydrogenations

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Abstract

The present understanding of the mechanisms by which flavoproteins oxidize amino acid or hydroxy acids to the respective imino or keto acids is reviewed. The observation that many of these enzymes catalyze the elimination of HBr or HCl from the appropriate β-halogenated substrate was long considered evidence for a carbanion intermediate. Recent structural and mechanistic studies are not compatible with the intermediacy of carbanions in the reactions catalyzed by D-amino acid oxidase and flavocytochrome b_2. In contrast, the data are most consistent with mechanisms involving direct hydride transfer.

1. Introduction

Flavoproteins participate in a wide range of metabolic pathways due to the ability of the isoalloxazine ring to participate in a variety of redox reactions. The oxidized flavin can undergo one or two electron reduction, allowing flavin cofactors to react with virtually any physiological electron carrier. In addition, the dihydroflavin can react with molecular oxygen to form a 4a-peroxyflavin, which is capable of hydroxylating activated aromatic rings [1]. A large number of flavoprotein-catalyzed reactions involve oxidation of a bond between carbon and a heteroatom, typically oxygen or nitrogen. These reactions include oxidation of amines, alcohols, α-amino acids, and α-hydroxy acids. Formally, these reactions can be described as hydride

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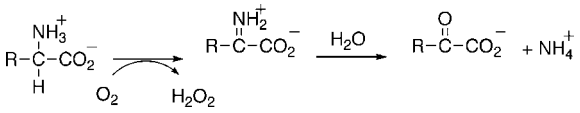
transfers, in that two electrons and a proton are transferred. Unlike the similar reactions catalyzed by pyridine nucleotide-dependent enzymes, the mechanisms of flavoprotein-catalyzed oxidations have been controversial. The mechanism of flavoprotein-catalyzed amine oxidation remains unclear; mechanisms involving adducts formed by nucleophilic attack of the amine nitrogen on the flavin [2] and one electron oxidation of the amine [3] have been proposed. Recent structural studies of monoamine oxidase do not support the involvement of a radical intermediate [4,5], but definitive evidence for the involvement of a flavin adduct is still lacking. Studies of the mechanism of oxidation of alcohols to aldehydes or ketones catalyzed by enzymes such as glucose oxidase, cholesterol oxidase, and methanol oxidase are consistent with a mechanism involving direct transfer of a hydride from the substrate to the flavin [6], but are not yet definitive. In contrast, mechanistic and structural studies of enzymes oxidizing α-amino or α-hydroxy acids have progressed to the point where definitive mechanistic conclusions can be drawn. This review addresses the present understanding of the mechanisms of substrate oxidation by these two families of flavoenzymes.

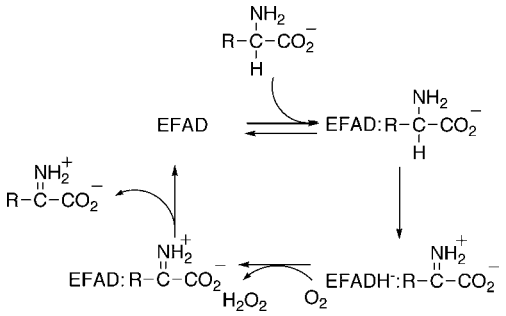
2. Amino acid oxidation

The paradigm enzyme for study of α-amino acid oxidation by flavoproteins has been D-amino acid oxidase (DAAO).[1] DAAO was one of the first flavoproteins to be described [7], and its ready availability from pig kidney has allowed extensive studies. There has been less study of the L-amino acid oxidases due to their lesser abundance and lack of success in obtaining most in a recombinant form. While these enzymes have a different protein fold from DAAO [8], critical features of the active site are common, consistent with similar mechanisms [9,10]. The physiological role of DAAO has been an unresolved question since its discovery, but there is recent evidence linking the human DAAO gene to schizophrenia [11], presumably due to its ability to metabolize D-serine [12]. The overall reaction catalyzed by DAAO, as shown in Scheme 1, is the oxidation of an amino acid to a keto acid plus ammonia, transferring the electrons removed from the amino acid to molecular oxygen to form hydrogen peroxide. The second step in Scheme 1 is nonenzymatic [13]. With the vast majority of amino acids studied as substrates, the kinetic mechanism of DAAO can be described by the mechanism of Scheme 2 [14,15]. As is common for flavoproteins, the reaction can be divided into a reductive half-reaction, in which the amino acid is oxidized and the flavin reduced, and an oxidative half-reaction, in which the reduced flavin is oxidized by molecular oxygen and the oxidized amino acid is released. The fact that amino acid oxidation occurs prior to any reaction with oxygen has several advantages: the V/K value for the amino acid substrate reports only on steps in substrate oxidation, and rapid reaction studies in the absence of oxygen can be used to study the kinetics of substrate oxidation.

[1] *Abbreviations used:* DAAO, D-amino acid oxidase; Fb_2, flavocytochrome b_2.



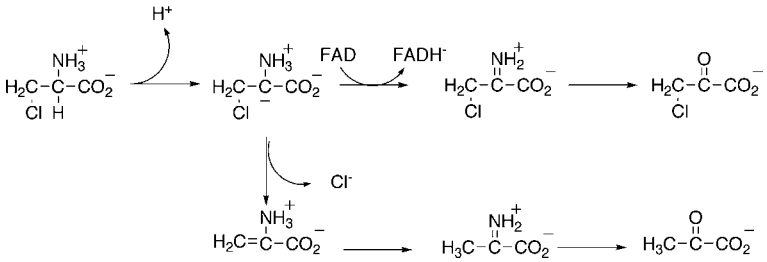
Scheme 1.



Scheme 2.

2.1. Evidence for a carbanion

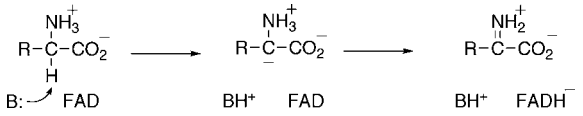
A seminal observation in developing mechanistic proposals for DAAO was the finding that the enzyme catalyzed elimination of HCl from D-3-Cl-alanine to form pyruvate in addition to 3-Cl-pyruvate [16]. Less complete analyses showed that *Crotalus adamanteus* L-amino acid oxidase catalyzed a similar reaction with L-3-Cl-alanine, consistent with a similar mechanism for both enzymes. In the case of DAAO, the rate of 3-Cl-alanine consumption decreased almost twofold with the α-deuterated substrate, but the partitioning between the two products was not affected, consistent with partitioning from an intermediate in which the substrate α-CH bond had already been cleaved [16]. Based on analogy to PLP-dependent systems, this result was interpreted as evidence for a carbanion intermediate which could undergo further oxidation or eliminate HCl (Scheme 3). Similar elimination reactions were subsequently found with other substituted amino acids, including D-2-amino-3-Cl-butyrate [17,18]. In the latter case the only product was α-ketobutyrate and the



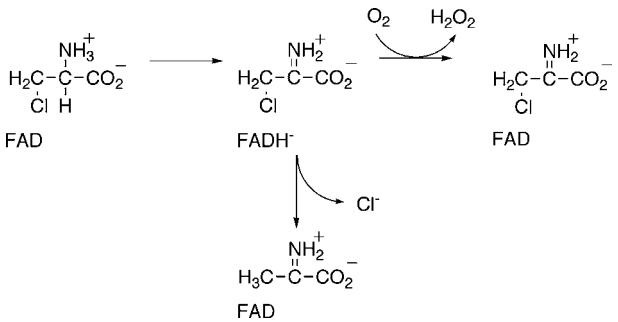
Scheme 3.

reaction did not require oxygen. The α-ketoglutarate produced upon HCl elimination from D-[2-^{3}H]2-amino-3-Cl-butyrate contained tritium, demonstrating that the hydrogen removed from the substrate could be retained on the enzyme [19]. Based upon these results, a mechanism for DAAO involving a carbanion intermediate (Scheme 4) was proposed. The mechanism of transfer of electrons from the carbanion to the flavin was not established; adducts formed by attack of the carbanion on the flavin have been proposed [20], and the susceptibility of the FAD in DAAO to attack by nitroalkane anions provided support for the involvement of such adducts in the normal catalytic reaction [21].

The mechanism of Scheme 4 was generally accepted for over two decades after its was initially proposed [15,22,23]. Still, several authors did point out that other mechanisms could account for HCl elimination [15,22]. One possibility was that HCl elimination occurred from the 3-Cl-imino acid product bound to the reduced enzyme [15]. This would account for the oxygen dependence of the product composition obtained with D-3-Cl-alanine. In the presence of oxygen equimolar amounts of pyruvate and 3-Cl-pyruvate are formed, while pyruvate is the exclusive product in the absence of oxygen [20], suggesting that chloride elimination occurs from a species which reacts with oxygen during the normal catalytic reaction. Reaction with oxygen requires that the FAD be reduced, and flavin reduction can only occur if the amino acid is oxidized. Since HCl elimination can proceed for multiple turnovers in the absence of oxygen, the reduced flavin must be reoxidized by a species other than oxygen in this case; the only possibility is the oxidized amino acid. (In the original mechanistic proposal, the reduction of the flavin by the carbanion was proposed to be freely reversible in order to couple elimination and flavin oxidation by oxygen [20]. However, subsequent proposals omitted this and resembled Scheme 3 [18].) A mechanism which accounts for these latter observations is given in Scheme 5; here,



Scheme 4.



Scheme 5.

a hydride supplied by the reduced flavin displaces the chloride to yield pyruvate directly. To be consistent with results with tritiated 2-amino-3-Cl-butyrate, the hydrogen donated by the flavin must have initially been at the α-carbon of the 2-amino-3-Cl-butyrate. Thus, while a mechanism involving a carbanion was generally accepted, the data supporting such a mechanism were in fact compatible with other mechanisms.

2.2. Mechanistic insight from structural studies

The three-dimensional structure of the pig kidney enzyme with the inhibitor benzoate bound was described nearly simultaneously by two different research groups in 1996 [24,25]. This structure showed that there is no active site residue properly placed to act as a base for carbanion formation. The only interactions of the inhibitor with active site residues involved the carboxylate of benzoate and Arg283 and Tyr228. More recent structures of DAAO from *Rhodotorula gracilis* show similar interactions between the carboxylate of D-3,3,3-F_3-alanine or L-lactate and the homologous arginine and tyrosine residues (Fig. 1) [26]. In addition, the amino group of D-3,3,3-F_3-alanine forms hydrogen bonds with a water molecule and the backbone carbonyl oxygen of Ser335. The only other active site residues with expected pK_a values less than 15 are Tyr224 in the pig kidney enzyme and Tyr238 in the *R. gracilis* enzyme. These residues are not conserved; Tyr224 in the pig kidney enzyme, which is parallel to the benzoate ring and forms a hydrogen bond with a water molecule, lies on a loop which is absent in the yeast enzyme, while *R. gracilis* Tyr238, the hydroxyl oxygen of which is 3.4 Å from the carboxylate of D-3,3,3-F_3-alanine in that enzyme, is replaced with a phenylalanine in the pig kidney enzyme. In all of the structures, the carbon corresponding to the α-carbon of the amino acid substrate is about 3.4 Å from the flavin N5, appropriate for direct hydride transfer (Scheme 6). All of the structures are more compatible with such a mechanism than with one involving a

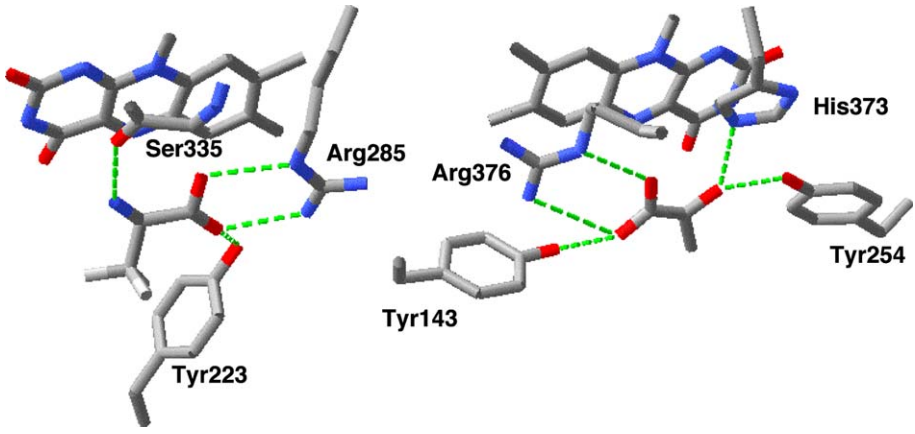
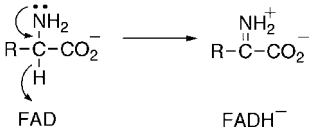


Fig. 1. Active sites of *R. gracilis* DAAO with D-3,3,3-F_3-alanine bound (left) and yeast flavocytochrome b_2 with pyruvate bound (right). The structures were drawn using the PDB files 1C0L and 1FCB.



Scheme 6.

carbanion intermediate, a fact noted immediately once the structure of the pig kidney enzyme was determined [25].

2.3. Evidence for hydride transfer

Kinetic isotope effects have been used to determine the changes in bond order of bonds to the α-carbon during oxidation of the amino acid by DAAO. Critically, in the carbanion mechanism of Scheme 4, the substrate CH bond is cleaved to form the carbanion in a step different from the one in which the CN double bond is formed. In a hydride transfer mechanism (Scheme 6), both bond changes occur in the same transition state. Cleavage of the CH bond will result in a primary deuterium kinetic isotope effect, while formation of the CN double bond will yield an inverse secondary ^{15}N kinetic isotope effect. The critical distinction between the two mechanistic possibilities is whether these isotope effects arise from the same transition step. Experimentally, the approach used was to identify conditions under which CH bond cleavage is fully rate-limiting for reduction, so that only isotope effects arising from this step are observed, and then determine whether this step also exhibits an ^{15}N kinetic isotope effect. The criterion for whether CH bond cleavage is fully rate-limiting is whether the intrinsic primary deuterium kinetic isotope effect is expressed in the kinetic parameter being measured. Heavy atom kinetic isotope effects are necessarily measured on steady state V/K values, so the $^{D}V/K$ value for the amino acid substrate must equal the intrinsic kinetic isotope effect for such measurements to allow distinction between the proposed mechanisms. This is the case with D-serine as a substrate for pig kidney DAAO [27]. The ^{15}N kinetic isotope effect on the V/K value with D-serine as substrate was pH-dependent, decreasing from a value of 1.0128 at pH 7.5 to a value of 0.9991 at pH 10.1 and increasing to 1.0175 in D_2O at pD 7.5 [28]. Since the deuterium isotope effects are pH-independent, the pH dependency of the ^{15}N effects could not be due to changes in the transition state for the CH bond cleavage step. Instead, they are due to the effect of pH on the fraction of the amino acid substrate which is correctly protonated for productive binding to the enzyme. Substitution of ^{15}N for the common ^{14}N increases the pK_a value for the substrate amino group due to an ^{15}N isotope effect of 1.1063 on the equilibrium constant for protonation; this increases to 1.0214 in D_2O [29]. In other words, at any pH, the ^{15}N-containing amino acid will have a greater mole fraction with the amino group protonated. The observed ^{15}N effects are the product of the kinetic isotope effect on the CH bond cleavage step and this equilibrium isotope effect on the concentration of the correctly protonated form of the substrate. Using the known pK_a values for the D-serine amino group, the observed ^{15}N effects can be corrected for

the contribution of the equilibrium isotope effect. If this is done by assuming that the substrate amino group must be neutral for proper binding, one obtains an ^{15}N kinetic isotope effect on the V/K value for D-serine of 0.996 which is independent of the pH or solvent. This value agrees with that expected for the secondary ^{15}N isotope effect when an sp^3 nitrogen atom is converted to sp^2 [29], establishing that the CN double bond forms at the same time the CH bond is cleaved. This result is consistent with the predictions of the hydride transfer mechanism of Scheme 6 and rules out the carbanion mechanism of Scheme 4.

The ^{15}N isotope effects also establish the protonation state of the substrate required for reaction to be the anion as indicated in Scheme 6. This is consistent with the lack of a solvent isotope effect on the V/K value for D-serine as a substrate for the pig kidney enzyme [27]. Solvent isotope effects greater than one have been reported for the enzymes from *R. gracilis* and *Triconopsis variabilis* [30,31]. The significance of these is difficult to interpret, since the effects were determined off the pH optimum in both cases and can therefore be fully accounted for by the effects of D_2O on the pK_a values for the enzyme and substrates. In the case of the *R. gracilis* enzyme, Ser335 has been proposed as the active site residue involved in removing the proton from the positively charged amino group [26], but this residue is not conserved in the pig kidney enzyme.

Thus, the conclusions drawn from analyses of isotope effects agree with the structural studies that the mechanism of CH bond cleavage is by direct hydride transfer. Recent computational studies also support the viability of a direct hydride transfer mechanism involving the anionic form of the amino acid substrate [32].

3. Hydroxy acid oxidation

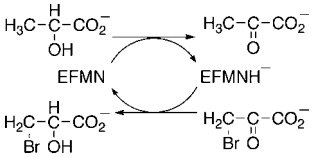
While study of the mechanism of oxidation of amino acids by flavoproteins has focused on DAAO, studies of the mechanism of α-hydroxy acid oxidation have been directed at several enzymes. Lactate monooxygenase, lactate oxidase, glycolate oxidase, mandelate dehydrogenase, flavocytochrome b_2 (Fb_2), and long chain hydroxy acid oxidase are homologous proteins with TIM-barrel structures which catalyze the oxidation of α-hydroxy acids to the respective keto acids in the reductive half-reaction [33–37], transferring the electrons either to oxygen or to a cytochrome in the oxidative half-reaction. In the case of lactate monooxygenase, the reaction with oxygen is coupled to decarboxylation of the pyruvate product to acetate [38]. In general the kinetic mechanisms resemble that of DAAO, with the exception that release of the keto acid product occurs before flavin oxidation in lactate oxidase and Fb_2 [37,39].

3.1. Evidence for a carbanion

The similarities of the reductive half-reactions reactions of the hydroxy acid oxidizing flavoproteins to that of DAAO has led to the general assumption that these enzymes have the same catalytic mechanism as DAAO [15]. Soon after the demonstration that DAAO will catalyze HCl elimination from D-3-Cl-alanine, lactate

monooxygenase was shown to catalyze a similar reaction with L-3-Cl-lactate, forming both pyruvate and Cl-acetate [16]. As was the case with DAAO, the partitioning between the two products varied with the oxygen concentration, such that the only product in the absence of oxygen was pyruvate. When [2-^{3}H]3-Cl-lactate was used, 30% of the tritium was found in the pyruvate product, providing a further similarity to the DAAO-catalyzed elimination reaction. Not surprisingly, these data were interpreted as evidence for a carbanion intermediate in the lactate monooxygenase-catalyzed reaction. Similar analyses with Fb_2, a yeast lactate dehydrogenase, and with rat hydroxy acid oxidase did not detect any HCl elimination during turnover with 3-Cl-lactate [40,41]. However, Fb_2 can use 3-Br-pyruvate as an electron acceptor for lactate oxidation, producing pyruvate and 3-Br-lactate (Scheme 7) [42]. Rat hydroxy acid oxidase will similarly catalyze pyruvate formation from 3-Br-pyruvate with lactate as the reducing substrate [43]. If [^{14}C]3-Br-pyruvate is used as the oxidizing substrate for Fb_2, the pyruvate produced contains ^{14}C, demonstrating that some of the pyruvate was formed by HBr elimination from 3-Br-pyruvate. When Fb_2 is incubated with [2-^{3}H]3-Br-pyruvate and lactate, some of the pyruvate produced contains tritium [44]. Thus, this transhydrogenation reaction with 3-Br-pyruvate resembles in many ways the elimination reactions of DAAO and lactate monooxygenase, suggesting that a similar intermediate is involved.

While the elimination reactions of lactate monooxygenase, Fb_2, and hydroxy acid oxidase were taken as evidence for a carbanion intermediate, an alternative is that elimination occurs from the reduced enzyme/3-Br-pyruvate complex, as proposed above for DAAO, and not from a carbanion. The observation that the partitioning between HBr elimination and the normal catalytic process with lactate monooxygenase depends on the concentration of oxygen, as was the case with DAAO and D-3-Cl-alanine, suggests that the partitioning involves a species which reacts with oxygen and thus is a form of the reduced enzyme in this case also. With lactate as a substrate, pyruvate dissociates extremely slowly from reduced lactate monooxygenase [45]. In contrast, pyruvate dissociates very quickly from reduced Fb_2 [46–48]. This raises the possibility that HBr elimination is not seen when 3-Br-lactate is a substrate for Fb_2 because Br-pyruvate dissociation from the reduced enzyme is much more rapid than HBr elimination, while the product/reduced enzyme complex has a sufficiently long lifetime in the case of lactate monooxygenase for elimination to occur. When Br-pyruvate is used as the oxidizing substrate, a large fraction of the enzyme is trapped as the Br-pyruvate/reduced enzyme complex, so that elimination is much more likely under these conditions. Consistent with elimination occurring from a

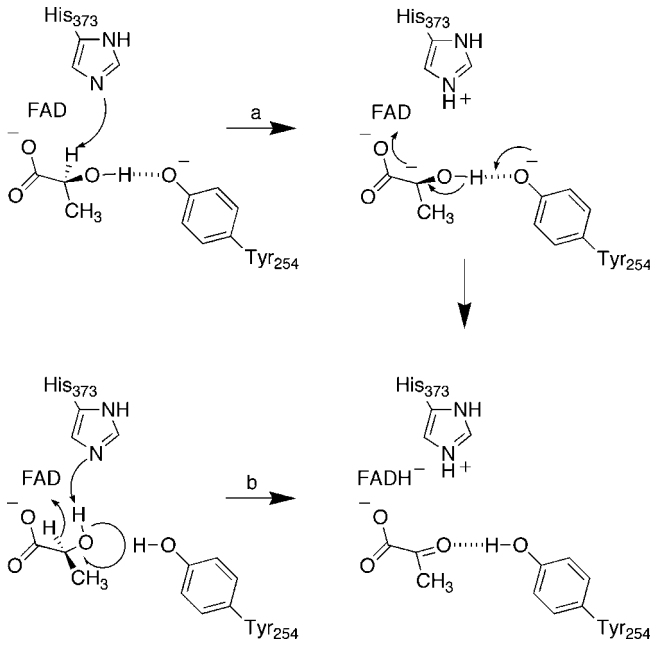


Scheme 7.

reduced enzyme complex, the relative amount of pyruvate versus Br-lactate formed from Br-pyruvate by Fb_2 is much greater when Br-pyruvate is used in the transhydrogenation reaction, conditions which would favor accumulation of the reduced enzyme/Br-pyruvate complex, than when Br-lactate is used as a reducing substrate, conditions in which any Br-pyruvate produced would rapidly dissociate from the reduced enzyme [44,49]. Thus, as was the case with DAAO, the enzyme-catalyzed elimination reactions, while frequently interpreted as supporting a carbanion mechanism, are also consistent with other mechanistic possibilities.

3.2. Mechanistic insight from structural studies

In contrast to the case with DAAO, knowledge of the structures of Fb_2 [50], glycolate oxidase [51], and mandelate dehydrogenase [52] did not resolve questions regarding the mechanism of substrate oxidation. The active sites of DAAO and Fb_2 are compared in Fig. 1. The substrates bind on opposite sides of the flavin in the two enzymes. In both active sites the carboxylate of the bound inhibitor or substrate interacts with a conserved arginine and tyrosine. While the amino group of the substrate bound to DAAO only interacts with a backbone carbonyl, the active site of Fb_2 contains Tyr254 and His373, both of which form hydrogen bonds with the carbonyl oxygen of the bound pyruvate. Both of these residues have the potential to act as active site bases in the formation of a carbanion, and both are conserved in all family members. It is possible to model lactate into the Fb_2 active site in



Scheme 8.

two orientations which lead to different conclusions regarding the mechanism (Scheme 8) [53,54]. In one (path a), Tyr254 forms a hydrogen bond with the lactate hydroxyl, and His373 is appropriately placed to abstract the α-proton from lactate. Alternatively (path b), lactate can be placed such that His373 would abstract the substrate hydroxyl proton as a hydride is transferred to the flavin. Mutagenesis of His373 results in a Fb_2 with no detectable activity [55], consistent with an essential role for this residue, while mutagenesis of Tyr254 decreases the rate of flavin reduction by about two orders of magnitude [53,56]. Similar results have been obtained with other members of this family [57–59]. Thus, a critical difference between the active sites of DAAO and of the hydroxy acid oxidizing flavoproteins is that the latter contain essential active site residues which could be involved in carbanion formation. Defining the roles of these residues is obviously crucial to understanding the mechanisms of these enzymes.

Given the conserved active sites of the hydroxy acid oxidizing flavoenzymes, efforts have been made to use rational mutagenesis to interconvert their substrate specificities. In the structure of Fb_2, the methyl group of the bound pyruvate contacts the side chains of Ala198 and Leu230; this suggested that the inability of this enzyme to use mandelate as a substrate is due to steric clash of its aromatic ring with these two residues [54]. Indeed, A198G/L230A Fb_2 prefers mandelate to lactate as a substrate [60], although the mutant enzyme is still less active as a mandelate dehydrogenase than the native mandelate dehydrogenase [61]. In their modeling of the binding of mandelate to wild type Fb_2, Gondry et al. [54] were able to find a binding mode for mandelate in which there is no steric clash with Ala198 or Leu230; in this model mandelate is positioned incorrectly for carbanion formation but properly for hydride transfer. Gondry et al. [54] therefore concluded that mandelate is such a poor substrate for Fb_2 because it could not adopt the proper orientation for carbanion formation and the mutageneses of Ala198 and Leu230 allowed mandelate to adopt the required conformation. This proposal has been addressed by determining the structure of the A198G/L230A enzyme with phenylglyoxylate bound. The binding mode of mandelate is infact incompatible with formation of a carbanion, instead exhibiting the binding mode predicted for hydride transfer, with the pyruvate carbonyl oxygen His373 hydrogen bonding with both His373 and Tyr254 [62]. Thus, this mutant enzyme appears to provide a clear example of a hydroxy acid oxidizing flavoprotein which utilizes a hydride transfer mechanism.

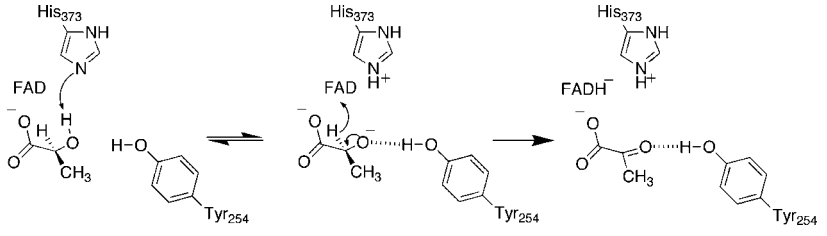
3.3. Evidence for hydride transfer

Irrespective of the mechanism, during the oxidation of lactate to pyruvate the lactate α-CH and OH bonds are broken and a double bond is formed between the α-carbon and the attached oxygen. The relative timing of the bond cleavage steps has been probed using kinetic isotope effects, measuring primary deuterium isotope effects with [^{2}H]lactate to follow the CH bond cleavage step and solvent isotope effects to follow OH bond cleavage. With wild type Fb_2, the CH bond cleavage step is not rate-limiting in the steady state, in that the primary deuterium isotope effect on the rate constant for flavin reduction measured in the stopped flow spectrophotometer

is about twice the isotope effect on the V_{max} value or the V/K value for lactate when ferricyanide is used as the oxidizing substrate [48]. The isotope effect on the rate constant for flavin reduction at saturating concentrations of lactate is sufficiently large to be considered the intrinsic value [48,63]. Thus, isotope effects on flavin reduction report on the transition state for CH bond cleavage. The solvent isotope effect on this step is 1.0 ± 0.1, establishing that the OH bond is not being cleaved in the same transition state as the CH bond [63]. An alternative approach, using a site directed mutant to slow CH bond cleavage, yields a similar result; D282N Fb_2 exhibits a deuterium isotope effect on the V_{max} value which is equivalent to the value obtained in single turnover studies and has a ^{D_2O}V value of 0.97 ± 0.04 [63]. These results are not consistent with the predictions of a hydride transfer reaction in which the OH and CH bands are broken in the same step, but they are consistent with the predictions of a carbanion mechanism.

Further analyses of the solvent isotope effects with wild type Fb_2 and mutant enzymes suggested an additional mechanistic possibility. Surprisingly, the V/K value for lactate showed an inverse solvent isotope effect in the case of the wild type enzyme and a mutant protein lacking the heme domain [63]. This required that a D_2O-sensitive step precede CH bond cleavage. The V/K value for lactate also increased in the presence of glycerol, and both D_2O and glycerol stabilize proteins [64,65]. These results suggested that a conformational change precedes CH bond cleavage, and that this conformational change is more favorable in D_2O or glycerol. Solvent isotope effects are inherently ambiguous in that one cannot know the number of sites at which deuterium replaces hydrogen. In the case of the solvent isotope effect on the V/K value for lactate, the data could not distinguish between an inverse isotope effect due exclusively to a deuteration at a single site favoring the reaction or a combination of a favorable effect on a conformational change and an unfavorable effect on the transfer of the hydroxyl proton. Thus, the data could also be explained of the mechanism of Scheme 9, in which the removal of the hydroxyl proton is coupled to a conformational change which precedes transfer of a hydride to the flavin.

Given the critical role of Tyr254 in the proposed mechanisms, several studies have analyzed the effects of mutating Tyr254. Reid et al. [56] reported that Y254F Fb_2 exhibited a decrease in V_{max} of about 50-fold, but no change in the K_m value for lactate. This result was interpreted as evidence for an interaction of Tyr254 with lactate only in the transition state. Propionate binds 50-fold less tightly to Fb_2 than lactate [54],



Scheme 9.

suggesting that the lactate hydroxyl does form a hydrogen bond with an active site residue prior to formation of the transition state. Dubois et al. [53], based on modeling studies, proposed that this residue was His373 in the mutant enzyme and Tyr254 in the wild type enzyme. Since an interaction between His373 and the lactate hydroxyl was most consistent with a hydride transfer mechanism (Scheme 8), these authors determined whether the Y254F enzyme could still carry out the reactions attributed to the carbanion mechanism. Critically, the mutant enzyme could still catalyze the formation of pyruvate from Br-pyruvate; during this reaction tritium was transferred from [2-^{3}H]lactate to pyruvate, although less efficiently than with the wild type enzyme. These results were taken as evidence that the Y254F enzyme also utilized a carbanion mechanism and that a solvent water molecule replaced Tyr254 in removing the lactate hydroxyl proton. An alternative explanation is that the modeling studies of the mutant enzyme were correct and that HBr elimination can be carried out by an enzyme which catalyzes lactate oxidation by hydride transfer.

pH and isotope effects have also been used to study the mechanism of the Y254F enzyme [66]. For the mutant enzyme, the primary deuterium kinetic isotope effect on the V/K value for lactate and the V_{max} value are both equal to the intrinsic isotope effect for the wild type enzyme, establishing that CH bond cleavage has become rate-limiting in the mutant protein. In stark contrast to the wild type enzyme, both of these kinetic parameters also exhibit a significant solvent isotope effect, suggesting that the lactate hydroxyl proton is being transferred in the same transition state that the CH bond is cleaved. This conclusion was confirmed by measuring the solvent isotope effect using deuterated lactate and the primary deuterium isotope effect in D_2O. Measurement of multiple isotope effects in this manner provides a powerful method of determining whether the two effects arise from the same step, since the effect will increase or remain the same if the reaction is concerted, but decrease if it is stepwise [67]. In the case of Fb_2, the results showed that the OH and CH bonds were cleaved in the same transition state. The mechanism most consistent with these results is the concerted hydride transfer mechanism of path a in Scheme 8. The pH dependence of the mutant enzyme was also consistent with the Tyr254 phenol being protonated in the wild type enzyme, as indicated in Scheme 8. Thus, the isotope effects on the mutant enzyme concur with the conclusion drawn from the modeling studies by Dubois et al. [53].

In light of the evidence that Y254F Fb_2 utilizes a hydride transfer reaction, the obvious question is whether the mutation results in a change in the mechanism or whether the wild type enzyme also utilizes a hydride transfer mechanism. The observation that the Y254F enzyme will catalyze HBr elimination from Br-pyruvate establishes that this reaction can be catalyzed by an enzyme which does not form a carbanion as an intermediate, as does the DAAO catalysis of HCl elimination from D-3-Cl-alanine. The switch from stepwise to concerted cleavage of the lactate OH and CH bonds can be rationalized for a hydride transfer reaction but is difficult to reconcile with the carbanion mechanism of Scheme 8. If, as indicated in Scheme 9, removal of the lactate hydroxyl proton by His373 precedes hydride transfer to the flavin, mutagenesis of Tyr254 would destabilize the lactate alkoxide so that it is no longer a discrete intermediate and the reaction becomes concerted. If removal of the

lactate hydroxyl by Tyr254 occurs after transfer of the lactate α-hydrogen to His373 as a proton, mutagenesis of Tyr254 could result in a detectable solvent isotope effect but would not result in a concerted reaction.

The proposed role of Tyr254 to bind the lactate alkoxide intermediate (Scheme 9) is consistent with the conclusion that this residue is not required for formation of the Michaelis complex with lactate, but only for catalysis [56]. It is also supported by studies of the role of Tyr254 in binding inhibitors. The K_i values for propionate, D-lactate, L-mandelate, and pyruvate change less than twofold when Tyr254 is mutated to phenylalanine [54]. In contrast, the K_i values for sulfite and oxalate, both of which have negative-charged oxygen atoms, increase substantially in the mutant protein. The structure of wild type Fb_2 with sulfite bound shows a hydrogen bond between Tyr254 and a sulfite oxygen [68], providing direct structural support for an analogous interaction of Tyr254 with lactate. A similar decrease in the affinity for oxalate is seen when the homologous residue in glycolate oxidase, Tyr129, is mutated to phenylalanine [69]. Finally, oxalate has been shown to be a tight-binding inhibitor of lactate monooxygenase, where it was proposed to be a transition state analog for a carbanion intermediate [70]. An alternative possibility is that oxalate is a mimic of the lactate alkoxide and the tight binding is due to the interactions which normally stabilize this intermediate.

Dewanti and Mitra [71] have recently reported the detection of a transient intermediate in the reductive half-reaction of mandelate dehydrogenase. The properties of this intermediate were consistent with a complex of an ionic species and oxidized flavin. While this species was assumed to be a carbanion by the authors, the conservation of active sites in this family of enzymes and the strong evidence against a carbanion mechanism suggests that it is the mandelate alkoxide.

4. Conclusion

While the finding that flavin amino acid and hydroxy acid oxidizing enzymes will catalyze HBr or HCl elimination from the appropriate β-substituted substrates has long been interpreted as evidence for a carbanion intermediate in catalysis, recent structural and mechanistic studies do not support such a model. In the case of DAAO, the lack of an active site base in the structures and the solution studies supporting a hydride transfer mechanism clearly agree. The presence of potential active site bases in Fb_2 and related enzymes has made resolution of their mechanisms more complex, but the combination of structural and mechanistic data which have accumulated strongly support a stepwise hydride transfer mechanism and are difficult to reconcile with the involvement of a carbanion in catalysis.

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