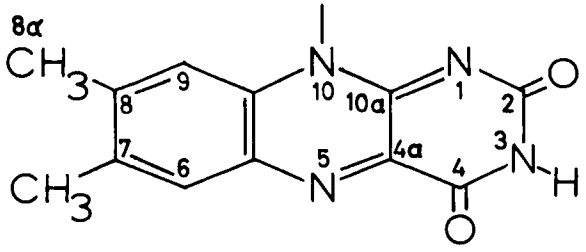
ROLE OF CHARGE-TRANSFER INTERACTIONS IN FLAVOPROTEIN CATALYSIS

Vincent Massey and Sandro Ghisla

Department of Biological Chemistry
The University of Michigan
Ann Arbor, Michigan 48104

The last decade has produced a veritable explosion of knowledge on the chemical reactivity of the isoalloxazine ring system of the flavin coenzymes, largely because of a side-by-side development with knowledge of flavoenzymes and model flavin studies. In this period the visible absorption spectra and epr spectra of the flavin semiquinone in its cationic, neutral, and anionic forms have been defined[1-6] (FIGURE 1). In the same period a large number of flavoenzymes have been investigated by rapid reaction spectrophotometric techniques, and in nearly all cases distinctive spectra characterized by long-wavelength-absorption bands have been detected as catalytic intermediates. With few exceptions, the spectra of the intermediates are unequivocally different from those of flavin semiquinone in either its neutral or its anionic form. As will be discussed in more detail below, these intermediates are in general readily thought to contain the flavin in the oxidized or reduced state, on the basis of their spectral properties.

During this period the great chemical reactivity of the flavin system has been uncovered.[7,8] This model work has led to the synthesis of well-defined substituted isoalloxazines, with substituents at almost every atom of the isoalloxazine ring system. The recognition of this great reactivity has led to the obvious suggestion that intermediates in flavoenzyme catalysis involve covalent linkage of substrate moieties with the flavin.[7-11] While the formation of such intermediates cannot be excluded rigorously on the basis of present evidence, the known spectral properties of such model substituted flavins fail to account for the intermediates observed in flavoenzyme catalysis.



Substitution of the oxidized isoalloxazine at positions N(3), C(7), and C(8α) is found to produce only minor modification of the absorption properties of the flavin.[12-14] Substitution of oxidized flavin at N(1) or N(5) leads to cationic forms, with loss of the 450 and 370 nm bands typical of the normal, unmodified flavins. In the case of substitution at N(1), no long-wavelength absorbance is found; with substitution at N(5), a new band is found at around 550 nm.[12,15] Known substitutions of oxidized flavin at C(2), C(4), or C(8)

lead to drastic changes in the absorption spectrum, in which no long-wavelength band has been detected so far.[12, 16–18] The only known substitutions of oxidized flavin functions that give rise to long-wavelength absorbance are hydroxyl substitutions at positions C(6) and C(9).[19, 20] In these cases, however, the appearance of the long-wavelength band is accompanied by the loss of the typical double-banded spectrum of unsubstituted flavin, which indicates that there is a drastic alteration in the electronic structure of the isoalloxazine ring system.

The known substituted *reduced* flavin model compounds can be classified

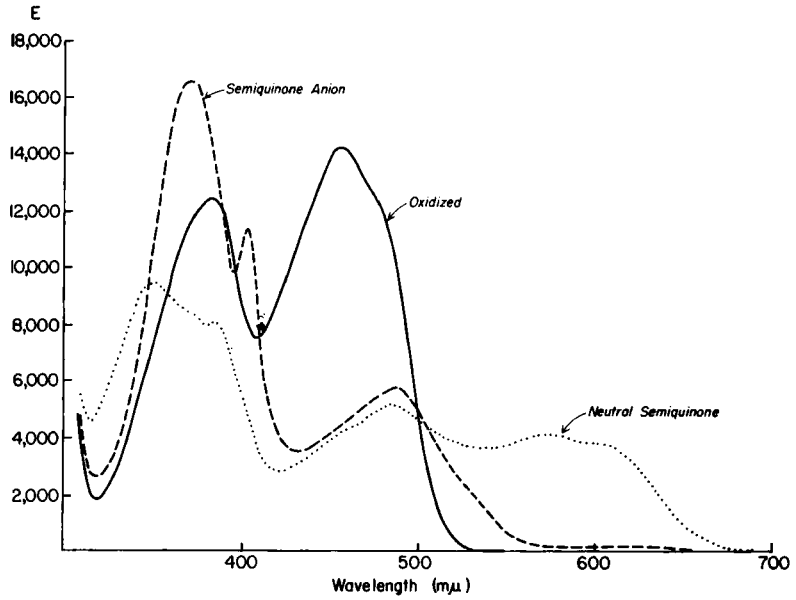
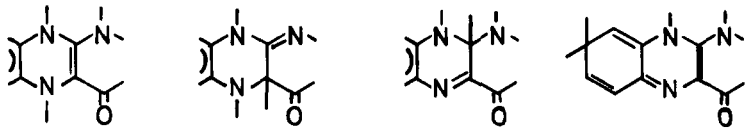


FIGURE 1. Spectra of the two semiquinoid forms of glucose oxidase. Enzyme (3.0 ml) in 0.025 M pyrophosphate (pH 9.2), which contained 0.023 M EDTA, was irradiated with visible light in an anaerobic cuvette to produce the red anionic semiquinone. The blue neutral semiquinone was produced by tipping from a side arm 0.08 ml 1 M citric acid, which lowered the pH to 5.85. Spectra similar to either the neutral or anionic form are produced with a wide variety of flavoproteins.[1] (From Massey *et al.*[54] Reprinted by permission of Springer-Verlag.)

in four classes, which have different spectral characteristics according to their electronic structure (or the position of substitution). The 1,5-dihydroflavin (at position A in the diagram below) has a poorly resolved spectrum, with shoulders at 310 and ~400 nm in the free system.[12] Substitution at the positions N(1), N(3), C(4),[12] N(5),[15] C(6),[20] C(8), and C(8α) has either no effect or a hypsochromic effect on the absorption spectrum; this effect is especially pronounced in the case of N(5) substitution.[15] Only substituents at position C(2) were found to cause a slight red shift of the absorption spectrum.[12] The absorption spectra of derivatives of C(4a),N(5)-dihydro-

flavins (position B) show maxima at values below 370 nm,[15] and the isomeric, substituted C(10a),N(1)-dihydroflavins (position C) have been reported to absorb around 420 nm.[21] Finally, derivatives of the 1,8-dihydroflavin (position D) have been assigned absorption maxima at 420 and 460 nm.[22, 23]



Clearly none of the above model compounds can even approximately simulate the spectral phenomena observed in the biological systems. Furthermore, covalent intermediates should exhibit spectral properties that should be relatively independent of the nature of the substituent attached to the flavin, if we assume that only a single species of intermediates occurs during catalysis. From these comparisons we conclude that the observed long-wavelength bands probably cannot be attributed to any covalent intermediate.

An alternative, more attractive explanation, is that many of the observed catalytic intermediates arise from charge-transfer donor-acceptor interaction[24] of the flavin with small molecules. In such complexes, in agreement with theoretical expectations, the absorption of the basic flavin chromophore is only slightly perturbed and the energy of the observed additional charge-transfer transition is highly dependent on the chemical nature of the partner molecule. In only a few cases has a correlation of the energy of the charge-transfer band with the ionization energy of the "substrate" been successfully attempted. But the energy and/or intensity of this transition is expected to be dependent on the orientation of the donor-acceptor partners in the complex, and this orientation can obviously be influenced by the protein.

The ability of the flavin to act as an acceptor in its oxidized state and as a donor in its reduced state is not surprising. The (iso)alloxazine as a *p*-quinoid molecule is electron-deficient in its oxidized state. This property is enhanced by protonation or alkylation in the model system, and could be enhanced by H-bonds in the protein. On the other hand, 2-electron reduction yields the 1,5-dihydroflavin, the chemical properties of which indicate that it is a good donor. Consideration of its middle pyrazine moiety shows that 1,5-dihydroflavin is closely related to the "antiaromatic" (8π-el) dihydropyrazines, which in turn are extremely labile as electron excess systems and behave chemically in the expected fashion.[25] 1,5-Dihydroflavins have been found to be bent at their N(5)-N(10) axis,[26] and their reactivity and nucleophilicity have been shown to be dependent on the degree of bending in the model system. Therefore the protein could govern the donor properties of the reduced flavin by affecting its planarity.

The ability of the (iso)alloxazine molecule to form molecular complexes in its oxidized (reduced) form with a variety of donor (acceptor) molecules, or with itself in the reduced (oxidized) form, is well documented; some representative examples are listed in TABLE 1. These interactions are generally reflected in an increased absorption in the 490 and 390 nm region and a decreased absorption around 440 nm of the oxidized flavin chromophore. In addition, a long-tail absorption that extends up to 700 nm may be observed.

In only a few cases has a charge-transfer complex been established unambiguously in the free system. The complexes of N(1)-substituted flavoquinonium cations with iodide [43] and of flavins with naphthalene diols and hydroquinone have been crystallized and investigated by x-ray crystallography.[39, 44] A review of the chemical characteristics of the molecules that have been found to give spectrally detectable complexes (which may reasonably be ascribed to charge-transfer interaction) with flavins in the oxidized state reveals that good donor properties and unsaturated character are common features. It is therefore reasonable to assume that in these cases a (π-π) donor-acceptor interaction plays a role in producing the observed optical transitions. The same considerations apply to the complexes of the reduced flavin with potential acceptors.

In contrast to the situation with model flavins, where demonstration of intermolecular complex formation that results in charge-transfer absorption often requires high concentrations of the reactants, many flavoprotein com-

TABLE 1

EXAMPLES OF MOLECULAR COMPLEXES OF FLAVINS WITH DIFFERENT CLASSES OF MOLECULES OF BIOLOGICAL INTERSET OBSERVED IN THE FREE SYSTEM *

Interactants	References
$Fl_{ox}/Fl_{red}H_2$	31, 32, 33
$Fl_{red}H_2$/pyridine nucleotides	32, 34
Fl_{ox}/heteroaromatic molecules (for example, indoles, pyrroles, imidazoles)	28, 35, 36, 37
Fl_{ox}/phenols, catechols, naphthalene diols	28, 35, 38, 39
Fl_{ox}/purines, pyrimidines	36, 40, 41
Fl_{ox}/barbituric acid	42

* For a complete review of this field, we refer you to References 28–30 and the literature cited therein.

plexes that exhibit charge-transfer absorption bands have been described in the literature in which the complete visible spectral changes can be readily observed. A representative list of such complexes is shown in TABLE 2. Historically, the first such charge-transfer complex to be recognized as such was that between the reduced FAD of lipoyl dehydrogenase (donor) and NAD^+ (acceptor).[32] Complexes with similar composition ($FADH_2 \rightarrow NAD(P)^+$) and basically similar spectral characteristics (medium to intense long-wavelength band, centered in the region 600–800 nm) have since been observed with glutathione reductase,[59] *p*-hydroxybenzoate hydroxylase,[54] NADH-rubredoxin reductase,[62] and melilotate hydroxylase.[61] In the cases of *p*-hydroxybenzoate hydroxylase and melilotate hydroxylase, these charge-transfer complexes have been demonstrated to be intermediates in the catalytic reaction. Other known or likely charge-transfer complexes that involve the fully reduced flavin coenzyme as donor are the catalytic intermediates observed with D- and L-amino acid oxidases [46, 63–65] and with lactate monooxygenase,[66] in which the same spectral species can be generated in an equilibrium situation by the addition to reduced

TABLE 2

EXAMPLES OF CHARGE-TRANSFER COMPLEXES WITH FLAVOPROTEINS

Enzyme	Donor or Acceptor	References *
With oxidized enzyme as acceptor:	Donor:	
D-Amino acid oxidase	amino- and hydroxy-benzoates, thioproline, Δ^1-pyrroline-2-carboxylate, Δ^1-piperidine-2-carboxylate, indole-2-carboxylate, pyrrole-2-carboxylate	45–47
D-Amino acid oxidase	enamines, carbanions	48, 49
L-Amino acid oxidase	amino- and hydroxy-benzoates	46, 50
Old Yellow enzyme	aromatic and heteroaromatic molecules with hydroxyl functions	51–53
Old Yellow enzyme	NADPH, NADH	52, 54
Butyryl CoA dehydrogenase	unsaturated acyl CoA's	55
Ferredoxin-$NADP^+$ reductase	NADPH	54
p-Hydroxybenzoate hydroxylase	NADPH	54
Thioredoxin reductase	NADPH	54
Lipoyl dehydrogenase	active site thiolate anion	56–58
Glutathione reductase	active site thiolate anion	59
With reduced enzyme as donor:	Acceptor:	
Lipoyl dehydrogenase	NAD^+	32
Glutathione reductase	$NADP^+$	59
Thioredoxin reductase	$NADP^+$	60
Ferredoxin $NADP^+$ reductase	$NADP^+$	54
p-OH-benzoate hydroxylase	$NADP^+$	54
Melilotate hydroxylase	NAD^+	61
NADH-rubredoxin reductase	NAD^+	62
D-Amino acid oxidase	imino acids	63, 64
L-Amino acid oxidase	imino acids	46, 65
Lactate monooxygenase	keto acids	66
Palmitoyl CoA dehydrogenase	unsaturated acyl CoA's	67
Butyryl CoA dehydrogenase	unsaturated acyl CoA's	55
Arginine monooxygenase	oxidation product of arginine	68
Lysine monooxygenase	oxidation product of lysine	69
D-2-Hydroxy acid dehydrogenase	keto acids	70

* It should be stated that in many cases the references are merely to spectral properties of the enzyme complexes, and that the description of these as charge-transfer complexes is ours.

enzyme of imino acids (in the case of D-amino acid oxidase [63]) or keto acids (in the case of lactate monooxygenase [66]).

Turning to cases in which charge-transfer complexes have been observed, or are the most likely explanation, but in which the oxidized flavin coenzyme acts as the acceptor, we may cite the very transient spectral species observed when NADPH is mixed with Old Yellow enzyme, ferredoxin-$NADP^+$ reductase, thioredoxin reductase, and *p*-hydroxybenzoate hydroxylase.[54] Other possible examples are the very characteristic absorption bands observed on two-electron reduction of lipoyl dehydrogenase and glutathione reductase. These enzymes

are known to contain a redox-active disulfide as part of the active center.[56, 71, 59] From the observed spectra, it seems reasonable to ascribe the species to internal charge-transfer complexes between active center thiolate anion (donor) and enzyme-bound FAD (acceptor). This suggestion was first made by Sanadi and colleagues,[56] and later by Kosower.[58] The most dramatic examples of charge-transfer complexes that involve oxidized flavin as acceptor have come from D-amino acid oxidase, Old Yellow enzyme, and butyryl CoA dehydrogenase. The latter two enzymes had both been isolated or observed in green forms, with spectra that are strongly suggestive of charge-transfer interaction.[51, 72-74] Recent work has demonstrated that the green form of the Old Yellow enzyme is due to charge-transfer interaction between oxidized flavin (acceptor) and various small molecule donors.[51-53] Similarly, the green form of butyryl CoA dehydrogenase has been demonstrated to be due to interaction between the oxidized flavin and a rather tightly bound, unsaturated acyl CoA.[55] Perhaps the most instructive case of flavoenzyme charge-transfer complexes is provided by D-amino acid oxidase. This enzyme in the oxidized form is known to bind a wide variety of carboxylic acids tightly; the resulting complexes show varying degrees of spectral perturbation, some of which exhibit typical charge-transfer absorption characteristics.[15] FIGURE 2 illustrates the spectral perturbation produced on formation of a complex with benzoate; it is evident in this case that the fairly pronounced spectral alterations produced on complex formation exhibit none of the properties common to typical charge-transfer interaction; that is, there is no sign of long wavelength absorption. In contrast, when substituted benzoates that contain electron-rich groups (such as amino or

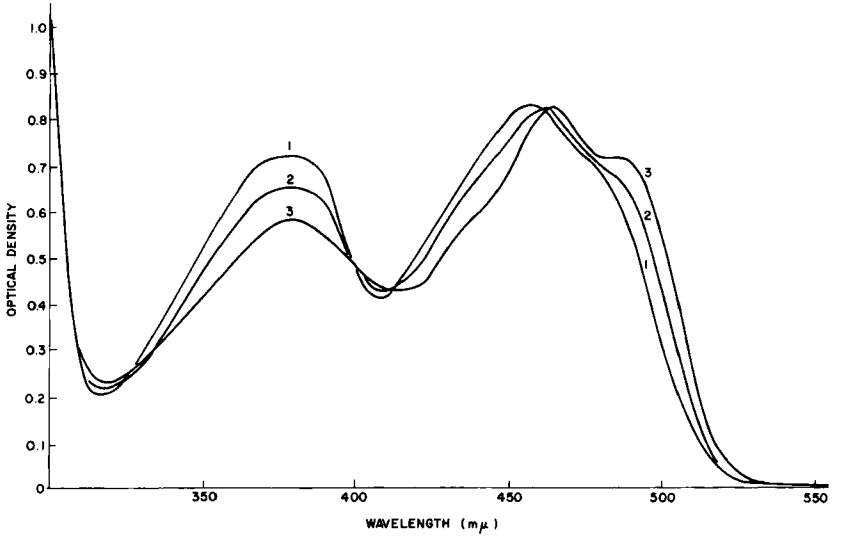


FIGURE 2. Effect of benzoate on the spectrum of D-amino acid oxidase. Curve 1 is the spectrum of benzoate-free enzyme (4.85 mg/ml) in 0.1 M pyrophosphate, (pH 8.5); curve 2, the spectrum after the addition of 0.46 mol benzoate/mol enzyme FAD; and curve 3, after the addition of 4.33 mol benzoate. (From Massey and Ganther.[15] Reprinted by permission of Biochemistry.)

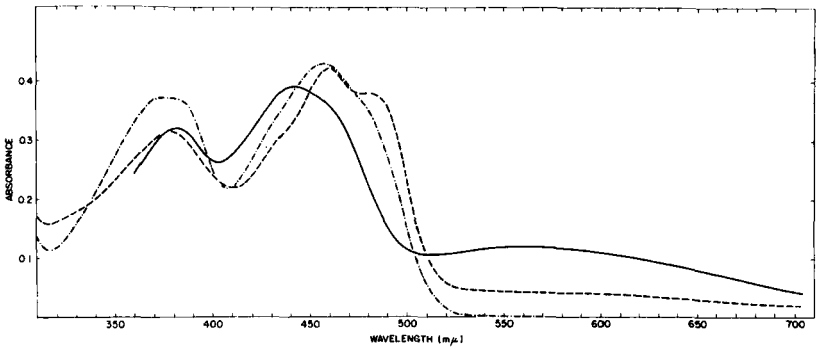


FIGURE 3. Comparison of the spectra of the complexes of D-amino acid oxidase with *o*- and *m*-amino benzoates. -·-·-·-=No additions; ———, plus 300 mol *o*-amino benzoate/mol enzyme FAD; - - -, plus 300 mol *m*-amino benzoate. (From Massey and Ganther.[45] Reprinted by permission of Biochemistry.)

hydroxyl groups) are reacted with D-amino acid oxidase, long-wavelength-absorption bands are observed. FIGURE 3 illustrates this phenomenon with ortho- and meta-amino benzoates. A particularly interesting case of charge-transfer interaction is displayed when the enzyme is complexed with indole-2-carboxylate or pyrrole-2-carboxylate (FIGURE 4). These compounds have electron-rich π clouds, and are somewhat analogous structurally to the enamine forms of imino acids. FIGURE 5 shows that a compound such as piperidine-2-carboxylate can act either as a charge-transfer donor (with E·FAD as acceptor) or as an acceptor (with E·$FADH_2$ as donor).

The spectral properties of transient intermediates in the catalytic reaction of D-amino acid oxidase with various substrates bear marked resemblances to the stable species described above, and lead us to a reinterpretation of these intermediates as charge-transfer complexes of the enzyme and the substrate

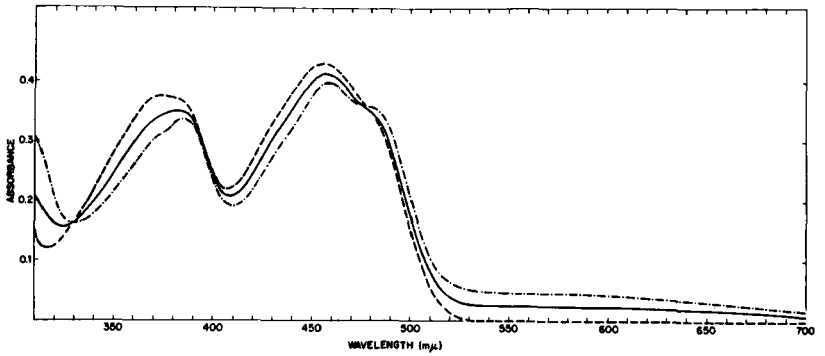


FIGURE 4. Effect of indole-2-carboxylate on the spectrum of D-amino acid oxidase. - - - -=No additions; ——, plus 0.5 mol indole-2-carboxylate; -·-·-, 1.5 or 115 mol indole-2-carboxylate/mol enzyme FAD. (From Massey and Ganther.[45] Reprinted by permission of Biochemistry.)

(or product) in various stabilized states. Our understanding of the reaction mechanism of D-amino acid oxidase (and other flavoprotein oxidases) has been much enhanced recently by the work of Walsh and colleagues,[11] who showed that a primary step in catalysis must be the abstraction of a proton from the α-carbon atom of the substrate by an enzyme base. This conclusion was reached largely on the basis of the discovery that β-chloroalanine served as a substrate for the enzyme under anaerobic conditions, with Cl^-, NH_4^+, and pyruvate as reaction products. It was found that under aerobic conditions, β-chloroalanine served also as a typical oxidase substrate (with H_2O_2,NH_4^+, and chloropyruvate as products), and that a clear competition between the two pathways of catalysis existed, dependent on the concentration of O_2. These results indicated that a carbanion species of the substrate probably exists, at least transiently, in the catalytic pathway. In more recent work carried out collaboratively between Dr. Abeles' and this laboratory, we have examined in some detail the spectral changes that occur in the Cl^- elimination pathway.[48]

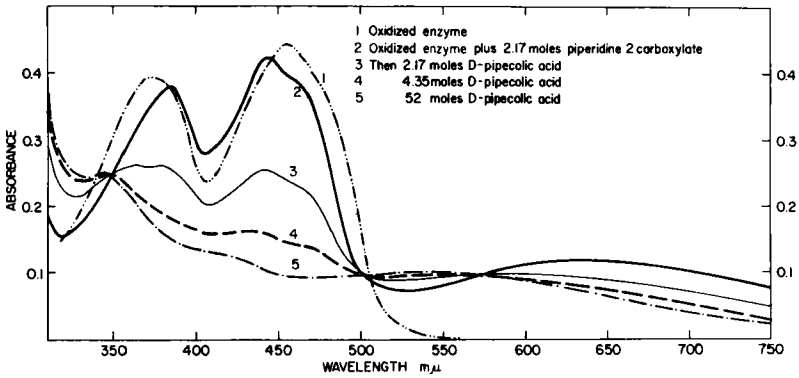


FIGURE 5. Charge-transfer complexes between piperidine-2-carboxylate and D-amino acid oxidase in either its oxidized or reduced form. The conditions were anerobic, in 0.1 M pyrophosphate (pH 8.5). Adapted from Massey *et al.*[75]

For this work we have followed mainly the reaction of the enzyme with β-chloro-α-aminobutyrate, a substrate that is found to be effective only in the elimination reaction, and not to serve as an oxidase substrate. On mixing enzyme and β-chloro-α-aminobutyrate, there is a rapid biphasic development of long wavelength absorbance, which demonstrates the existence of at least two spectroscopically distinguishable intermediates in the enzymic reaction. The kinetics of the formation of these intermediates is the same at all wavelengths, but may be most conveniently followed in the 505 nm region, where the first-formed intermediate has a greater extinction coefficient than the second. FIGURE 6 shows the time course of the formation of these two intermediates, and the observed first-order rate constants for the particular concentration of substrate used. FIGURE 7 shows the spectra of the two intermediates, constructed from experiments such as those shown in FIGURE 6. It should be noted that the rapidly produced intermediate has spectral properties very similar to those of the oxidized enzyme-indole-2-carboxylate complex (see

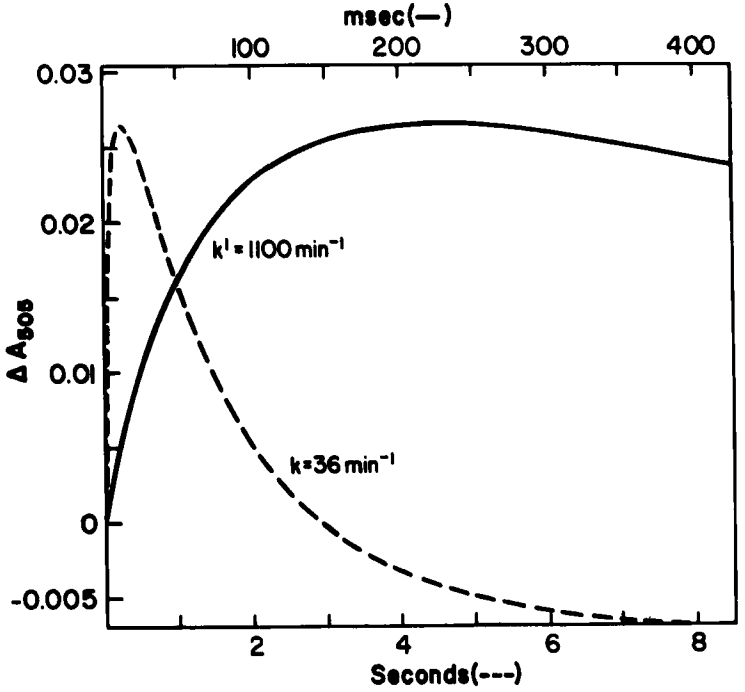


FIGURE 6. Kinetic traces at 505 nm of the reaction between D-amino acid oxidase and β-chloro-α-aminobutyrate. The conditions were identical to those described in the legend to FIGURE 7. The reaction was recorded on different time scales to illustrate the biphasic nature of the spectral changes (that is, that two spectrally distinctive intermediates are produced). (From Massey *et al.*[48] Reprinted by permission of The Journal of Biological Chemistry.)

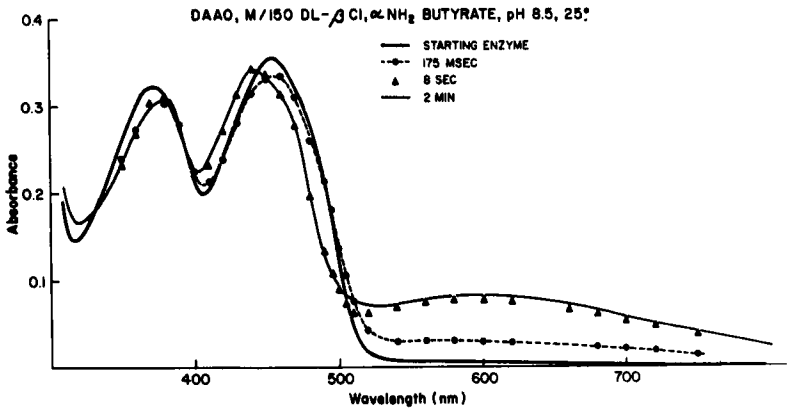


FIGURE 7. Changes in the absorption spectrum on the reaction of D-amino acid oxidase with β-chloro-α-aminobutyrate. Enzyme, 2.86×10^{-5} M with respect to bound FAD, in 0.1 M pyrophosphate (pH 8.5) was mixed with an equal volume of M/75 DL-β-chloro-α-aminobutyrate, and the reaction was followed in a Gibson-Milnes stopped-flow spectrophotometer. (From Massey *et al.*[48] Reprinted by permission of The Journal of Biological Chemistry.)

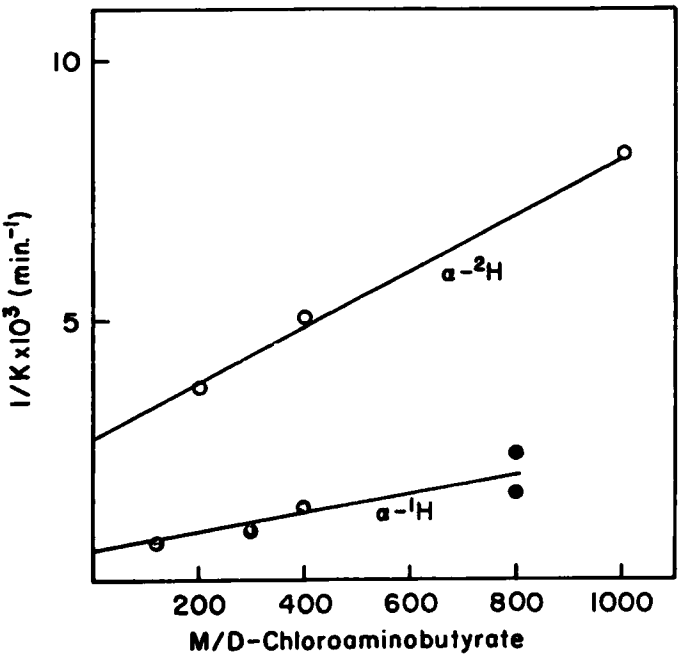
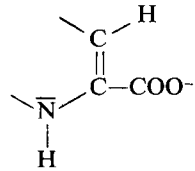


FIGURE 8. Deuterium isotope effect on the rate of formation of the first intermediate in the reaction of D-amino acid oxidase with β-chloro-α-aminobutyrate. The conditions were as in FIGURES 6 and 7. The apparent first-order rate constants for the formation of the rapidly formed intermediate were obtained from data analogous to those shown in FIGURE 6. (From Massey *et al.*[48] Reprinted by permission of The Journal of Biological Chemistry.)

would appear to be reasonably effective, while with β-chloro-α-aminobutyrate the reaction with O_2 must be slow compared to the rate of elimination.

Thus we would propose that the first observed spectral intermediate is a charge-transfer complex between oxidized enzyme and the corresponding enamine that is produced after the elimination of Cl^-. This interpretation is supported by the similarity of its spectral properties to those of the oxidized enzyme-indole-2-carboxylate or -pyrrole-2-carboxylate complexes. These forms should be good donors, and should contain the same structural elements around the α-carbon atom:



The second intermediate, formed at a rate of 40 min^{-1}, could be attributed to the formation of either of the imine species shown. We consider it an attractive possibility that the intense long-wavelength band of this intermediate

FIGURE 4), while the second intermediate has spectral properties remarkably similar to those of the oxidized enzyme-piperidine-2-carboxylate complex (see FIGURE 5).

Because of the low turnover number of the elimination reaction with this substrate, ~8 min^{-1},[48] the enzyme remains in the steady state form of the second intermediate for considerable times (depending on the concentration of β-chloro-α-aminobutyrate) until the substrate becomes exhausted, whereupon the enzyme spectrum reverts to that of the original (but slightly perturbed through equilibrium) complex formation with the products (α-ketobutyrate + NH_4^+). When the enzyme is reacted with different concentrations of β-chloro-α-aminobutyrate, it is found that only the rate of formation of the first intermediate is affected; that of the second is virtually independent of substrate concentration. Furthermore, the concentration-rate dependence of the first intermediate shows saturation behavior, as illustrated in FIGURE 8. These results enable us to conclude that the first intermediate is formed from a primary enzyme substrate complex, with a K_{diss} of 2.8×10^{-3} M and with a limiting rate of 2,000 min^{-1}. The subsequent step or steps that lead to the formation of the second intermediate proceed with a rate constant of 40 min^{-1}. When β-chloro-α-aminobutyrate was replaced by deuterium at the α-carbon atom, exactly the same overall spectral changes were observed, but a very pronounced deuterium isotope effect was found in the rate of formation of the first intermediate. The rate of formation of the second intermediate (40 min^{-1}) was the same as that found when the normal H-form of the substrate was used. The results are shown in FIGURE 8. With the α-deutero substrate, the limiting rate of formation of the first intermediate is only 400 min^{-1}; that is, there is a fivefold deuterium isotope effect. The K_{diss} of the primary enzyme deutero substrate complex (2.1×10^{-3} M) is not significantly different from that of the normal substrate.

In FIGURE 9 we offer a possible interpretation of these results. From the initial study of Walsh and colleagues[11] with β-chloroalanine it is clear that a carbanion species of the substrate could be formed, at least transiently. This conclusion is substantiated by the finding of a fivefold deuterium rate effect in the formation of the first long-wavelength intermediate observed with the analogous β-chloro-α-aminobutyrate. The nature of this intermediate is of obvious interest. One possibility consistent with the kinetic results would be that it is due to a charge-transfer interaction between the oxidized enzyme and the carbanion. This possibility, however, appears unlikely for the following reasons. Firstly, the carbanion would be expected to react very rapidly with the flavin by intermolecular electron transfer, to give flavin and the corresponding imine. Alternatively the carbanion could be expected to eliminate Cl^- rapidly, to produce the corresponding enamine (the pathway shown by dotted arrows in FIGURE 9). It seems reasonable to conclude that the event more likely to follow the production of carbanion would be the rapid formation of a reduced enzyme-imino acid complex, as shown. This formulation would then offer a logical explanation of β-chloroalanine's being a normal oxidase substrate as well as undergoing the Cl^- elimination reaction, since the formation of reduced enzyme would be necessary in order for the normal oxidase reaction to be exhibited. Competing with O_2 for the reduced enzyme-chloroimino acid complex would be the reductive elimination reaction, to produce the oxidized enzyme-enamine complex. With β-chloroalanine this competition

TABLE 3

INCORPORATION OF ^3H AT THE β-POSITION OF PYRUVATE CATALYZED BY D-AMINO ACID OXIDASE *

	Incorporation of Radioactivity, as a Percentage of the Theoretical Full Amount, at the Given Time	
Incubation time	6 hours	12 hours
Experimental sample	25%	41%
Control sample	3%	3%

* A solution that contained ^3H_2O ($\sim 1.3 \times 10^9$ dpm/ml), sodium pyruvate (0.1 M), pyrophosphate buffer (0.1 M, pH 8.3), ammonium sulfate (8×10^{-2} M), and D-amino acid oxidase (1.34×10^{-4} M), was incubated at 24° C. The control experiment had the same composition but contained no L-amino acid oxidase. 1.0 ml Aliquots were heated at 95° C for 4 min, the protein precipitate was filtered, and the filtrate was applied to a 4.5 ml Dowex AG 1-X8 column equilibrated with water. The column was washed with water until no radioactivity was detected in the eluate ($\sim$ 70 ml H_2O), and then the pyruvate was eluted with 0.1 N HCl. The pyruvate content in the eluate was measured as described elsewhere,[70] and the radioactivity was measured with a Packard Tri-Carb Model 3320 scintillation counter.

is due to the ionized form of the imine. There are several lines of evidence that support this conclusion. Firstly, when α-ketobutyrate and NH_4^+ are mixed with oxidized enzyme, the spectral changes found are minor and have no similarities to the spectrum of the second intermediate. Therefore it is unlikely that the complex of enzyme and imino acid is responsible for the observed intense long-wavelength absorption. Secondly, as will be described below, the probable formation of such carbanion species as are illustrated has been shown to occur in tritium incorporation experiments. Thirdly, studies with model flavins (also to be detailed below) have demonstrated charge-transfer interactions between carbanion (donor) and oxidized flavin (acceptor). Fourthly, the similarity between the spectral properties of the second intermediate and those of the complex between oxidized enzyme and piperidine-2-carboxylate is striking. This compound is a weak C-H acid, which on deprotonation would yield a carbanion with elements structurally similar to the form shown in the scheme. The fact that α-keto acid and NH_4^+ does not produce this intense absorption band could be due to an unfavorable equilibrium, as indicated by the slowness of the incorporation of tritium into keto acid (TABLE 3). This scheme is also consistent with the finding[48] that when an α-tritium-labeled substrate is employed, a substantial amount of tritium is found to be incorporated at the β-carbon position of the product.

The scheme presented above to offer an explanation of the elimination reaction also suggests a reinterpretation of the mechanism of the normal oxidase reaction with D-amino acids. Previous kinetic studies with this system[63] demonstrated that long-wavelength-absorbing species are rapidly produced on reaction with D-amino acids, and this is followed by the rapid reaction of these species with O_2 and the rate-limiting dissociation of imino acid products. At the time, it was suggested that the long-wavelength-absorbing species were biradical complexes of flavin semiquinone and amino acid radical; it now seems much more reasonable to ascribe them to charge-transfer complexes of

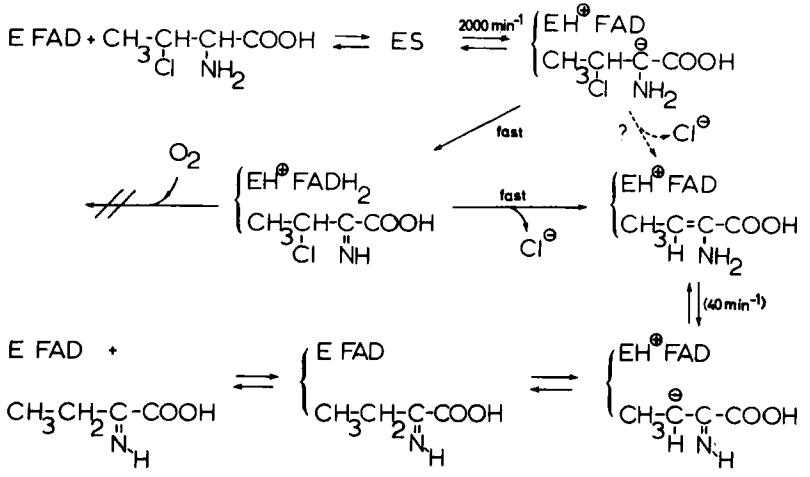


FIGURE 9.

reduced enzyme (donor) and imino acid (acceptor). In view of the work of Walsh and colleagues,[11] it is also necessary to include the transient occurrence of a carbanion species of the substrate. Accordingly, FIGURE 10 offers a possible reinterpretation of the pathway of the normal oxidase reaction. In this scheme the initial formation of enzyme substrate complex would be followed by very rapid proton abstraction from the α-carbon atom by an enzyme base to yield a carbanion, which by rapid intramolecular electron transfer would yield a complex of reduced enzyme and imino acid. (This

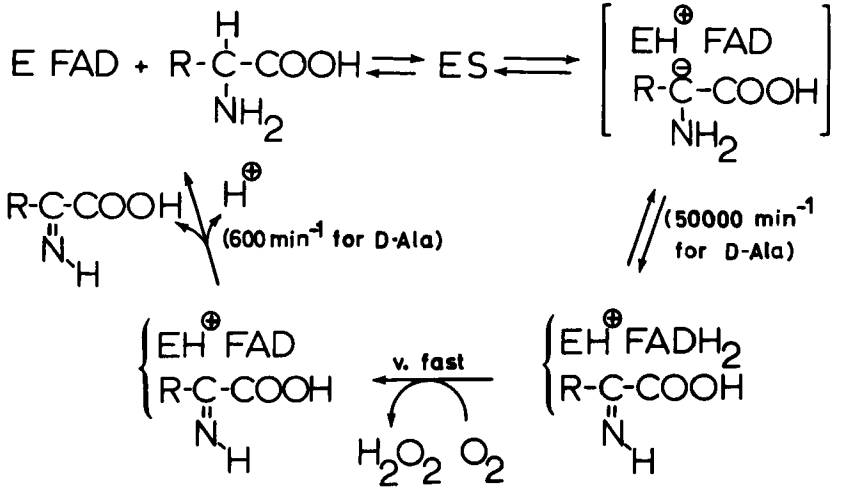


FIGURE 10.

reaction could proceed via a transient covalent intermediate; experimental evidence of such an intermediate has been given by Porter and colleagues.[49]) By virtue of the acceptor nature of the imine function, charge-transfer interaction could be expected, and could account for the spectral characteristics of the observed intermediate. This intermediate would then react rapidly with O_2 to yield H_2O_2 and a complex of oxidized enzyme and imino acid, and the catalytic cycle would be completed by the slow dissociation of the imino acid from this complex. FIGURE 11 shows the spectra of the catalytic intermediates produced with D-alanine, D-methionine, and D-proline as substrates. It is evident that the complexes formed have the characteristics of reduced enzyme charge-transfer complexes. FIGURE 12 shows that a very similar complex can be formed

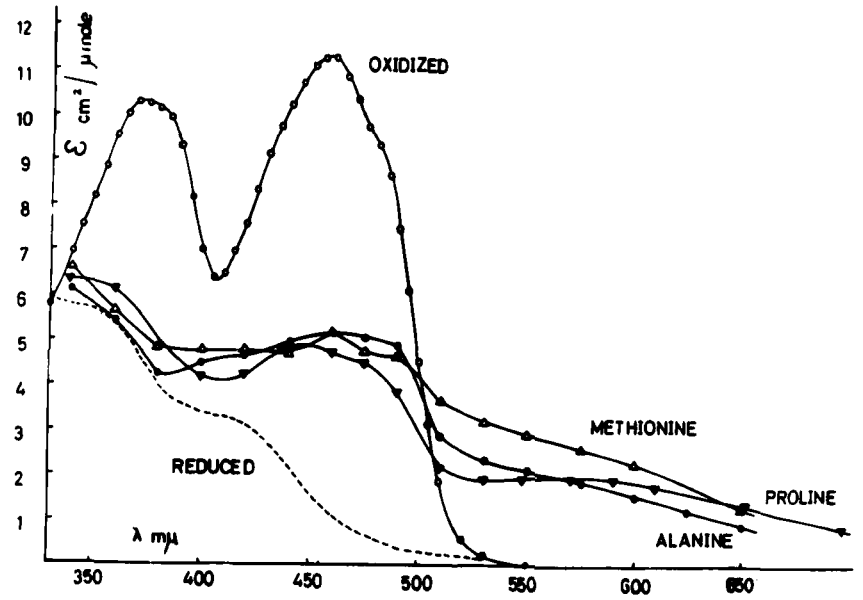


FIGURE 11. Spectra of the intermediates produced on the reaction of D-amino acid oxidase with D-alanine, D-methionine, and D-proline. The spectra of the intermediates were determined by stopped-flow spectrophotometry. (From Massey and Gibson.[63] Reprinted by permission of Federation Proceedings.)

under equilibrium conditions by the addition to reduced enzyme of high concentrations of keto acid + NH_4^+ ($\rightleftharpoons$imino acid). This is the celebrated "purple complex" of Yagi.[64] Similar spectral characteristics are shown in FIGURE 5 for the charge-transfer complex between enzyme $FADH_2$ (donor) and piperidine-2-carboxylate (acceptor).

Further experimental evidence in support of the schemes outlined above has been obtained in studies with model flavins and in tritium incorporation studies with D-amino acid oxidase. In FIGURE 9, starting with free oxidized enzyme and keto acid + NH_4^+ ($\rightleftharpoons$imino acid), we envisage a series of reversible equilibria that imply proton addition and abstraction at the β-carbon of the imino acid. TABLE 3 shows that a slow incorporation of tritium does indeed

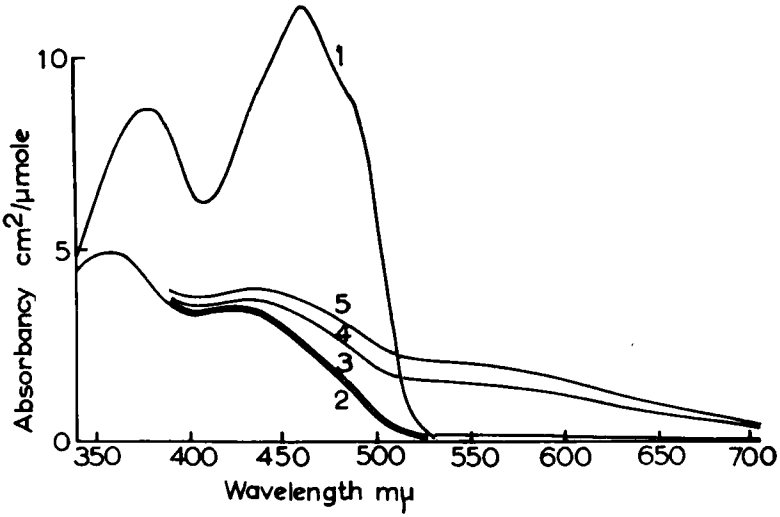
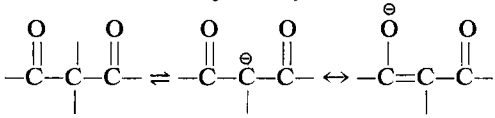


FIGURE 12. Effect of pyruvate and NH_4^+ on the spectrum of D-amino acid oxidase reduced with D-alanine. Curve 1 indicates oxidized enzyme; curve 2, the enzyme after the addition of 130 mol D-alanine/mol enzyme FAD; curve 3, then after the addition of 326 mol sodium pyruvate; curve 4, then after the addition of 1,300 mol NH_4^+, as $(NH_4)_2SO_4$; and curve 5, after the addition of 3,900 mol NH_4^+. The conditions were anerobic, in 0.1 M pyrophosphate (pH 8.5); the temperature was 10° C. (From Massey and Gibson.[63] Reprinted by permission of Federation Proceedings.)

occur at the β-carbon atom of pyruvate when it is incubated with D-amino acid oxidase in the presence of NH_4^+. This incorporation is clearly dependent on the enzyme, and could be catalyzed by the same enzyme base as is responsible for the initial proton abstraction from the α-carbon atom of the substrate; and it is consistent with the migration of tritium from the α-carbon of β-chloroalanine to the β-carbon of the final elimination product (pyruvate) observed by Walsh and colleagues.[48]

The observed intense long-wavelength-absorption bands with D-amino acid oxidase and piperidine-2-carboxylate, and with butyryl CoA dehydrogenase and acetoacetyl CoA,[55, 75] are at first sight rather surprising, since such compounds would not be expected to be good π-donors, as are most of the compounds that exhibit such charge-transfer absorption spectra (for example, aminobenzoate, phenolates). On the other hand, both molecules are weak C-H acids that on deprotonation would yield delocalized carbanion species, the enamine and enolate anions respectively. Here is an example:



Such unsaturated systems would be expected to be good π-donors and could therefore account for the observed charge-transfer bands. A long-wavelength band has also been observed in the reaction of D-amino acid oxidase with the stable anions of nitroalkanes.[49]

Charge-transfer interactions have also been observed between free flavins and a variety of anions of substituted 1,3-diketones. One example is illustrated in FIGURE 13; it shows the interaction between the anion of acetyl acetone (donor) and 3-carboxymethyl lumiflavin (acceptor). Clearly the spectral behavior shown is not identical to the typical spectral behavior of a flavoenzyme, but it does show absorption that extends out to 700 nm, which is typical of charge-transfer interaction. FIGURE 11 also shows the interaction between *o*-aminobenzoate and 3-carboxymethyl lumiflavin. Again the spectral changes are not identical to those observed on complex formation of D-amino acid oxidase with *o*-amino benzoate (FIGURE 3), but again the long-wavelength band extends out to at least 700 nm. In addition, the complex between *o*-amino benzoate and lumiflavin can be isolated in a deep red crystalline form with an equimolar composition of the two reactants. The structure of this complex is under investigation by x-ray diffraction techniques.

As was pointed out by Slifkin,[30] the characteristics of charge-transfer transitions are highly dependent on the molecular orientation of the donor and acceptor, and on the contribution of other weak binding forces such as van der Waals forces to the total potential energy of the system. Thus it is not surprising that the spectral characteristics of the charge-transfer interaction of a donor and free flavin may differ from those of the same pair with a flavoenzyme, since in the latter case the protein can be expected to exert a highly specific orienting effect on the donor molecule, so that only one or a few of the large number of possible orientations are displayed. For example,

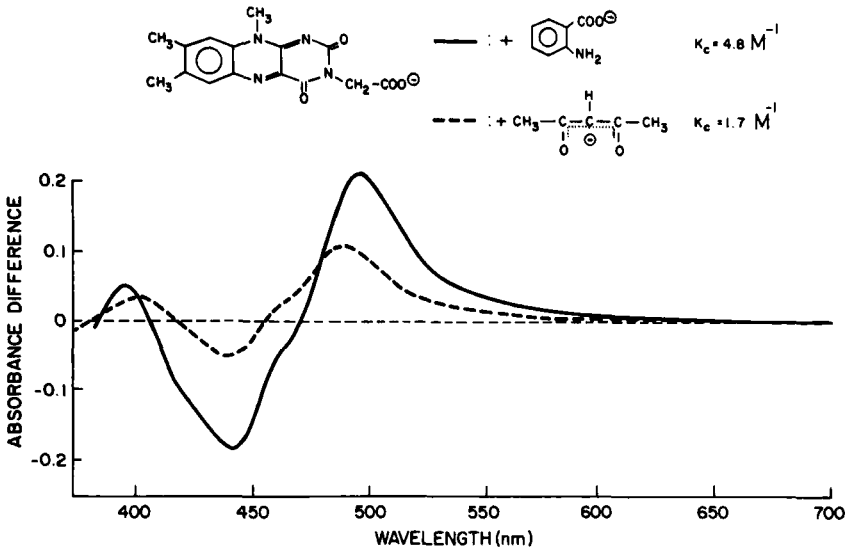


FIGURE 13. Effect of *o*-amino benzoate (pKs=2.05, 4.95; curve=———) and of acetonylacetonate (pK=9.0; curve=- - - -) on the spectrum of N(3)-carbomethoxy-lumiflavin. The association constants were determined spectrophotometrically (by measuring the difference spectra) by variation of the *o*-amino benzoate and acetonylacetonate concentrations, in the range 10^{-2}–1.0 M, at pH 7.2 and 10.0 respectively. The ionic strength was kept constant by the addition of KCl to a total of 1.0 M.

with D-amino acid oxidase, ortho- and para-amino benzoate, which would be expected to have similar donor properties, both bind to the enzyme, but with very different spectral effects. Thus *o*-amino benzoate displays a strong charge-transfer interaction, while *p*-amino benzoate shows only weak charge-transfer interaction.[45]

Finally, we may examine the role, if any, of charge-transfer complexes in enzymic catalysis. From the foregoing discussion, it is evident that many examples have now been accumulated of charge-transfer complexes of flavins that act as intermediates in enzyme catalysis. For example, the charge-transfer complexes between various enamine and imine forms as donor, and oxidized flavin as acceptor, are clearly intermediates in the Cl^- elimination pathway of D-amino acid oxidase when reacting with β-chloroamino acids. Similarly, charge-transfer complexes with the reduced flavin as donor and imino acid as acceptor are intermediates in the "normal" oxidase reactions of D-amino acid oxidase, and by analogy, also of L-amino acid oxidase.[65] A similar situation exists with lactate monooxygenase, in which a central intermediate appears to be a charge-transfer complex between reduced enzyme and α-keto acid product.[66] In addition, charge-transfer complexes between reduced flavin (donor) and oxidized pyridine nucleotide (acceptor) have been found to be intermediates in the catalytic mechanisms of melilotate hydroxylase[61] and *p*-hydroxybenzoate hydroxylase.[77] Entirely similar charge-transfer complexes between enzyme $FADH_2$ and oxidized pyridine nucleotide have been found under some conditions, however, with lipoyl dehydrogenase[32, 71] and glutathione reductase,[59] and in the case of lipoyl dehydrogenase, at least, they have been demonstrated not to be catalytic intermediates.[32, 78] Furthermore, many examples are known in which small-molecule inhibitors are bound to flavoproteins, which by spectral perturbation must clearly interact with the isoalloxazine ring system; yet relatively few of these produce the typical long-wavelength charge-transfer absorption bands. Thus, despite the impressive list of flavin charge-transfer complexes, it might appear that the only important criterion for either catalysis or the inhibition of catalysis is complex formation *per se*, and that the frequent occurrence of charge-transfer absorption bands is merely an accompanying phenomenon that is dictated by the chemical characteristics of the two partners in the complex. This phenomenon is obviously an important one from an experimental point of view, in that it offers us a convenient spectroscopic handle for the investigation of reaction pathways. But aside from this desirable property, do charge-transfer complexes contribute in any fundamental fashion to the course of flavoenzyme catalysis?

It is generally considered that the energy involved in a molecular complex interaction is of the order of only a few thousand calories per mole,[29, 30] and that the donor-acceptor interaction *per se* contributes only to a minor extent to the overall stabilization of a complex. A stabilization energy of 3,000 cal/mol would be equivalent to a hundredfold increase in the stability of a given complex. It is not hard to imagine that such an increased stability could have very important functions in the regulation or direction of a catalytic reaction. For example, recent work from this laboratory with lactate monooxygenase[66] has shown that the rate of reaction of O_2 with the reduced enzyme pyruvate complex (a charge-transfer complex) is some 200-fold greater than with uncomplexed reduced enzyme.

While more examples need to be accumulated, one possible function of charge-transfer interaction between reduced flavin and small-molecule acceptors

may be to speed up the reaction of molecular oxygen with reduced flavin. Perhaps in such cases these molecular interactions result in changed geometry of the reduced flavin (which in the free state is bent, or in a butterfly conformation) to a more planar state, in which O_2 attack may be more favored.

In the case of lactate monooxygenase the products of the reaction are different, depending on the conditions. With free reduced enzyme the products are oxidized enzyme and H_2O_2; with the reduced enzyme pyruvate complex the products are acetate, CO_2, and H_2O. The latter decarboxylation reaction probably proceeds through the primary production of a complex of oxidized enzyme, pyruvate, and H_2O_2. The dissociation constant of the reduced enzyme pyruvate complex is only 2×10^{-3} M, corresponding to a ΔF^0 of only 3,700 cal/mol; most of this could well be contributed by the charge-transfer interaction. It is clear, therefore, that in this particular case the energy contributed by charge-transfer interaction may well be the crucial factor in determining that this enzyme catalyzes the oxidative decarboxylation of lactate, rather than merely the production of H_2O_2 and pyruvate. Similar considerations may well be very important in controlling the rate at which enzyme reactions that involve charge-transfer complexes proceed. While understandable emphasis has been placed on the remarkable phenomenon of the greatly increased rates at which enzyme-catalyzed reactions occur, as compared to uncatalyzed reactions, only in recent years has the importance of the control or damping of such catalysis received attention. Stabilization of intermediates in flavoprotein catalysis through charge-transfer interaction may well be an important feature in the control mechanisms of these enzymes.

References

1. Massey, V. & G. Palmer. 1966. Biochemistry **5:** 3181–3189.
2. Ehrenberg, A., F. Müller & P. Hemmerich. 1967. European J. Biochem. **2:** 286–293.
3. Müller, F., Hemmerich, A. Ehrenberg, G. Palmer & V. Massey. 1970. European J. Biochem. **14:** 185–196.
4. Müller, F., P. Hemmerich & A. Ehrenberg. 1971. *In* Flavins and Flavoproteins. H. Kamin, Ed. : 107–122. University Park Press. Baltimore, Md.
5. Palmer, G., F. Müller & V. Massey. 1971. *In* Flavins and Flavoproteins. H. Kamin, Ed. : 123–140. University Park Press. Baltimore, Md.
6. Müller, F., M. Brüstein, P. Hemmerich, V. Massey & W. H. Walker. 1972. European J. Biochem. **25:** 573–580.
7. Hemmerich, P., G. Nagelschneider & C. Veeger. 1970. FEBS Letters **8:** 69–83.
8. Numerous articles. 1971. *In* Flavins and Flavoproteins. H. Kamin, Ed. University Park Press. Baltimore, Md.
9. Palmer, G. & V. Massey. 1968. *In* Biological Oxidations. T. P. Singer, Ed. : 263–300. John Wiley & Sons, Inc. New York, N.Y.
10. Hamilton, G. A. 1971. *In* Progress in Bioorganic Chemistry. Vol. 1. E. T. Kaiser & F. J. Kezdy, Eds. : 83–157. John Wiley & Sons, Inc. New York, N.Y.
11. Walsh, C. T., A. Schonbrunn & R. H. Abeles. 1971. J. Biol. Chem. **246:** 6855–6866.
12. Dudley, K. H., A. Ehrenberg, P. Hemmerich & F. Müller. 1964. Helv. Chim. Acta **47:** 1354–1383.
13. Ghisla, S. & P. Hemmerich. 1971. FEBS Letters **16:** 229–232.

14. WALKER, W. H., T. P. SINGER, S. GHISLA & P. HEMMERICH. 1972. European J. Biochem. **26:** 279–289.
15. HEMMERICH, P., S. GHISLA, U. HARTMANN & F. MÜLLER. 1971. *In* Flavins and Flavoproteins. H. Kamin, Ed. : 83–105. University Park Press. Baltimore, Md.
16. MÜLLER, F., W. H. WALKER & P. HEMMERICH. 1966. Helv. Chim. Acta **49:** 2365–2369.
17. GHISLA, S. & S. G. MAYHEW. 1973. J. Biol. Chem. In press.
18. HEMMERICH, P., B. PRIJS & H. ERLENMEYER. 1959. Helv. Chim. Acta **42:** 2164–2177.
19. SCHÖLLNHAMMER, G. & P. HEMMERICH. 1972. Z. Naturforsch. **27b:** 1030–1031.
20. MAYHEW, S. G., C. WHITFIELD, S. GHISLA & M. SCHUMAN-JORNS. Submitted for publication.
21. MÜLLER, F. 1971. *In* Flavins and Flavoproteins. H. Kamin, Ed. : 363–373. University Park Press. Baltimore, Md.
22. BRÜSTLEIN, M., W. R. KNAPPE & P. HEMMERICH. 1971. Angew. Chem. Intern. Ed. Engl. **10:** 804–806.
23. HEMMERICH, P. & F. MÜLLER. 1973. Ann. N.Y. Acad. Sci. **212:** 13.
24. For a definition of the term charge-transfer complex and a comprehensive review of this subject we refer to: MULLIKEN, R. S. & W. B. PERSON. 1969. Molecular Complexes. John Wiley & Sons, Inc. New York, N.Y.
25. CHEN, S. J. & F. W. FOWLER. 1971. J. Org. Chem. **26:** 4025–4028.
26. KIERKEGAARD, P., R. NORRESTAM, P. E. WERNER, I. CSOEREGH, M. VON GLEHN, F. KARLSSON, M. LEIJONMARCK, O. ROENQUIST, B. STENSLAND, O. TILLBERG & L. TORBJOERNSSON. 1971. *In* Flavins and Flavoproteins. H. Kamin, Ed. : 1–22. University Park Press. Baltimore, Md.
27. TAUSCHER, L., S. GHISLA & P. HEMMERICH. 1973. Helv. Chim. Acta **56:** 630–644.
28. TOLLIN, G. '1968. *In* Molecular Associations in Biology. B. Pullman, Ed. : 393–409. Academic Press Inc. New York, N.Y.
29. FOSTER, R. 1969. *In* Organic Charge-Transfer Complexes. A. T. Blomquist, Ed.: 344–346. Academic Press Inc. New York, N.Y.
30. SLIFKIN, M. A. 1971. Charge-Transfer Interactions of Biomolecules : 132–171. Academic Press Inc. New York, N.Y.
31. BEINERT, H. 1956. J. Amer. Chem. Soc. **78:** 5323–5328.
32. MASSEY, V. & G. PALMER. 1962. J. Biol. Chem. **237:** 2347–2358.
33. GIBSON, Q. H., V. MASSEY & N. M. ATHERTON. 1962. Biochem. J. **85:** 369–383.
34. SAKURAI, T. & H. HOSOYA. 1966. Biochim. Biophys. Acta **112:** 459–468.
35. HARBURY, H., K. F. LANOUE, P. A. LOACH & R. M. AMICK. 1959. Proc. Nat. Acad. Sci. U.S. **45:** 1708–1717.
36. WILSON, J. E. 1966. Biochemistry **5:** 1351–1359.
37. DRAPER, R. D. & L. L. INGRAHAM. 1970. Arch. Biochem. Biophys. **139:** 265–268.
38. HARBURY, H. A. & K. A. FOLEY. 1958. Proc. Nat. Acad. Sci. U.S. **44:** 662–668.
39. TRUS, B. L., J. L. WELLS, R. M. JOHNSTON, C. J. FRITCHIE & R. E. MARSH. 1971. Chem. Commun. 751–752.
40. TSIBRIS, J. C., D. B. MCCORMICK & L. D. WRIGHT. 1965. Biochemistry **4:** 504–510.
41. ROTH, J. A. & D. B. MCCORMICK. 1967. Photochem. Photobiol. **6:** 657–664.
42. GORODETSKY, M., R. F. WEINER & W. R. FRISELL. 1972. Biochim. Biophys. Acta **256:** 582–585.
43. LEIJONMARK, M. & P. E. WERNER. 1970. Acta Chem. Scand. **24:** 2916–2928, 3701–3710.
44. BEAR, C. A., J. M. WATERS & T. N. WATERS. 1970. Chem. Commun. 702.
45. MASSEY, V. & H. GANTHER. 1965. Biochemistry **4:** 1161–1173.

46. VEEGER, C., D. V. DER VARTANIAN, J. F. KALSE, A. DE KOK & J. F. KOSTER. 1966. *In* Flavins and Flavoproteins. E. C. Slater, Ed. : 242–262. Elsevier Publishing Company, Amsterdam, The Netherlands.
47. YAGI, K., M. NAOI, M. NISHIKIMI & A. KOTAKI. 1970. J. Biochem. **68:** 293–301.
48. WALSH, C. T., E. KRODEL, V. MASSEY & R. H. ABELES. 1973. J. Biol. Chem. **248:** 1946–1955.
49. PORTER, D. J. T., J. G. VOET & H. J. BRIGHT. 1973. J. Biol. Chem. **248:** 4400–4416.
50. DE KOK, A. & C. VEEGER. 1968. Biochim. Biophys. Acta **117:** 35–47.
51. MATTHEWS, R. G. & V. MASSEY. 1969. J. Biol. Chem. **244:** 1779–1786.
52. MATTHEWS, R. G. & V. MASSEY. 1971. *In* Flavins and Flavoproteins. H. Kamin, Ed. : 329–348. University Park Press. Baltimore, Md.
53. ABRAMOVITZ, A. & V. MASSEY. Unpublished data.
54. MASSEY, V., R. G. MATTHEWS, G. P. FOUST, L. G. HOWELL, C. H. WILLIAMS, G. ZANETTI & S. RONCHI. 1970. *In* Pyridine Nucleotide-Dependent Dehydrogenases. H. Sund, Ed. : 393–411. Springer-Verlag. Berlin, West Germany.
55. ENGEL, P. C. & V. MASSEY. 1971. Biochem. J. **125:** 889–902.
56. SEARLS, R. L., J. M. PETERS & D. R. SANADI. 1961. J. Biol. Chem. **236:** 2317–2321.
57. MASSEY, V., Q. H. GIBSON & C. VEEGER. 1960. Biochem. J. **77:** 341–351.
58. KOSOWER, E. M. 1966. *In* Flavins and Flavoproteins. E. C. Slater, Ed. : 1–14. Elsevier Publishing Company. Amsterdam, The Netherlands.
59. MASSEY, V. & C. H. WILLIAMS. 1965. J. Biol. Chem. **240:** 4470–4480.
61. STRICKLAND, S. & V. MASSEY. 1973. J. Biol. Chem. **248:** 2953–2962.
61. STRICKLAND, S. & MASSEY, V. 1973. J. Biol. Chem. In press.
62. UEDA, T. & M. J. COON. 1972. J. Biol. Chem. **247:** 5010–5016.
63. MASSEY, V. & Q. H. GIBSON. 1964. Federation Proc. **23:** 18–29.
64. YAGI, K., K. OKAMURA, M. NAOI, N. SUGIURA & A. KOTAKI. 1967. Biochim. Biophys. Acta **146:** 77–90.
65. MASSEY, V. & B. CURTI. 1967. J. Biol. Chem. **242:** 1259–1264.
66. LOCKRIDGE, O., V. MASSEY & P. A. SULLIVAN. 1972. J. Biol. Chem. **247:** 8097–8106.
67. HAUGE, J. G., F. L. CRANE & H. BEINERT. 1956. J. Biol. Chem. **219:** 727–733.
68. OLOMUCKI, A., D. B. PHO, R. LEBAR, L. DELCAMBE & N. V. THOAI. 1968. Biophys. Acta **151:** 353–366.
69. YAMAMOTO, S., F. HIRATA, T. YAMAUCHI, M. NOZAKI, K. HIROMI & O. HAYAISHI. 1971. J. Biol. Chem. **246:** 5540–5542.
70. CAMMACK, R. 1969. Biochem. J. **115:** 55–64.
71. MASSEY, V. & C. VEEGER. 1961. Biochim. Biophys. Acta **48:** 33–47.
72. MAHLER, H. R. 1954. J. Biol. Chem. **206:** 13–26.
73. ENGEL, P. C. & V. MASSEY. 1971. Biochem. J. **125:** 879–887.
74. RUTTER, J. W. & B. ROLANDER. 1957. Acta Chem. Scand. **11:** 1663–1670.
75. MASSEY, V., G. PALMER, C. H. WILLIAMS, B. E. P. SWOBODA & R. H. SANDS. 1966. *In* Flavins and Flavoproteins. E. C. Slater, Ed. : 133–158. Elsevier Publishing Company. Amsterdam, The Netherlands.
76. HOHORST, H. J. 1963. *In* Methods of Enzymatic Analysis. H. U. Bergmeyer, Ed. : 266–270. Academic Press Inc. New York, N.Y.
77. HOWELL, L. J., V. MASSEY & S. STRICKLAND. 1972. *In* Structure and Function of Oxidation Reduction Enzymes. Å. Akeson & Å. Ehrenberg, Eds. : 445–452. Pergamon Press, Inc. London, England.
78. MASSEY, V. & Q. H. GIBSON. 1963. *In* Proceedings of the Fifth International Congress of Biochemistry. Vol. **5:** 157–173. Pergamon Press, Inc. Oxford, England.