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L-Amino Acid Oxidase. III. Substrate Substituent Effects Upon the Reaction of L-Amino Acid Oxidase with Phenylalanines[1]

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A study was made of the effect of electron withdrawing and donating substituents of a series of phenylalanine substrates on the component steps of the reduction of L-amino acid oxidase (L-amino acid: O_2 oxidoreductase (deaminating), EC I.4.3.2) by substrate. As reported earlier, the reduction process involves a very rapid initial formation of a transient, spectrophotometrically distinct intermediate followed by the rapid decay of this intermediate into the fully reduced enzyme. Also observed was the slow intermediate decay reported earlier. The reaction was studied anaerobically by the combination of enzyme and substrate by stopped-flow techniques, and in turnover using a Clark oxygen electrode. The overall second-order rate constant for the reduction process in turnover gave a linear Hammett plot with a ρ value of +0.2 for the phenylalanine substituents having σ values between −0.66 and +0.78. A similar ρ value of +0.25 was obtained from a Hammett plot for the rapid decay of the transient intermediate into the fully reduced enzyme. The Hammett plot for the formation of the transient intermediate was biphasic in nature. The ascending segment of this Hammett plot gave a ρ value of +0.7, whereas a negative curvature of the plot was observed for substituents with σ values greater than zero. The experimental results are consistent with the formation of the transient intermediate involving a proton transfer from the substrate α-position and electron transfer to the flavin. Earlier proposed mechanisms for flavin reduction processes are reviewed and discussed and a detailed mechanism for the enzymatic reduction process is proposed which is consistent with available experimental data.

INTRODUCTION

As part of a detailed study of the elementary processes involved in the reduction of the oxidized form of L-amino acid oxidase (L-amino acid: O_2 oxidoreductase (deaminating) EC I.4.3.2) by substrate, we have previously reported the results of studies on the pH and pD-dependent deuterium isotope effects in this system (*1*, *2*). The results of this previous work suggested the possibility that during the course of the enzyme-catalyzed reaction, the amino acid α-hydrogen is removed as a proton. In order to gain understanding of the mode of α-hydrogen transfer from the amino acid, a correlation of the kinetic properties of the elementary steps of the reduction process with respect to the Hammett $\rho\sigma$ relationship (*3*) was undertaken using a series of *meta-* and *para-*substituted phenylalanines as substrates. The results of this study are the subject of this report.

Previous studies by Massey and Curti (*4*) and by Beinert (*5*) have shown that during the course of the anaerobic reduction of the oxidized form of L-amino acid oxidase by

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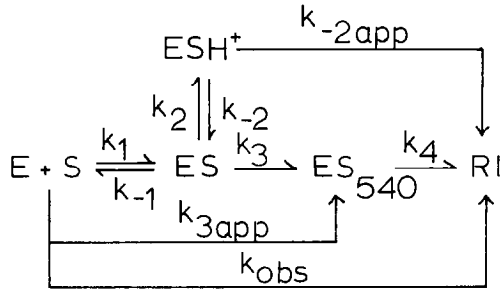
substrate, a spectrophotometrically distinct transient intermediate is formed, which unlike either the oxidized or fully reduced forms of the enzyme, has a significant absorption in the region of 540 nm. We have found (*2*) that the kinetic scheme describing the kinetically observable elementary processes of the reduction step which is most consistent with the available data is that shown in Scheme 1.



SCHEME 1. Overall kinetic scheme for the kinetically accessible steps of the reduction of L-amino acid oxidase by substrate at any pH value (*2*).

In Scheme 1, E is the oxidized form of the enzyme, RI is the complex between fully reduced enzyme and imino acid product in accord with the work of Massey and Curti (*4*), ES is the initial enzyme–substrate complex, ES_{540} is the spectrophotometrically distinct transient intermediate, and ESH^+ is the protonated, inactive charge form of ES (*2*). The kinetically accessible rate constants shown in Scheme 1 are: k_{3app}, the apparent first-order rate constant for the overall transformation of E + S into ES_{540}; k_4 the first-order rate constant for the rapid decay of ES_{540} into RI; k_{-2app}, the apparent first-order rate constant for the overall transformation of ESH^+ into RI. These rate constants are defined in greater detail elsewhere (*2*).

Previous studies of the applicability of the Hammett $\rho\sigma$ relationship to the flavin amino acid oxidases have been described by Radda (*6*) for L-amino acid oxidase and by Hellerman *et al.* (*7*) for the D-amino acid oxidase system. Radda (*6*) obtained a biphasic Hammett plot with a ρ value of 1.8 for σ values of –0.3–0.3 and a negative ρ value for substituents having σ values greater than 0.3 by studying the system manometrically using a series of *meta*- and *para*-substituted phenylglycines (α-aminophenylacetic acids) as substrates. Hellermann and coworkers (*7*) obtained similar biphasic plots for the D-amino acid oxidase system using a series of substituted phenylalanines and phenylglycines as substrates.

The purpose of the present work is to examine the effects of electron donating and withdrawing substituents of a series of phenylalanine substrates on the isolated reduction reaction between oxidized L-amino acid oxidase and substrate. This was considered important for the following reasons. It was anticipated that studies of the electronic effects on the component reactions of the reduction process would yield information concerning the manner in which electron transfer from the substrate to the flavin occurs. However, in order to obtain meaningful results in such Hammett $\rho\sigma$ correlations it is essential to study the individual component rate processes of the reduction reaction. Our previous studies on the pH-dependent primary isotope effects conducted both by turnover and stopped-flow techniques allowed us to identify rate-determining steps in the reaction sequence of L-amino acid oxidase. Utilizing this information, we therefore examined the electronic effects of substrate substituents in the L-amino acid oxidase system using both turnover and stopped-flow techniques.

MATERIALS AND METHODS

L-Amino acid oxidase was purified by the method of Wellner and Meister (*8*) from *Crotalus adamanteus* venom obtained from Sigma Chemical Corp. (type I). After crystallization, the purified enzyme crystals were redissolved in 0.1 *M* KCl. The clear solution of purified enzyme was stored at 4° and used within 10 days. The determination of active enzyme concentration was carried out as described earlier (*9*). Purified L-phenylalanine, L-tyrosine, and DL-*p*-chlorophenylalanine were obtained from Mann Biochemical Corp. Purified DL-*p*-amino phenylalanine, DL-*m*-hydroxyphenylalanine, and DL-*p*-nitrophenylalanine were obtained from Sigma Chemical Corp. DL-*m*-fluorophenylalanine was obtained from PCR Inc. and was twice recrystallized from water before use. L-*p*-Nitrophenylalanine was purchased from Cyclo Chemical Co. and was recrystallized from H_2O–30% ethanol before use. No D-isomer impurity could be detected as solutions prepared from this L-*p*-nitrophenylalanine consumed within 1% of the expected stoichiometric amount of oxygen in turnover, which was well within the experimental error of the measuring technique employed. DL-Phenylalanine was obtained from Merck Inc. and twice recrystallized from H_2O before use. The phenylglycine series was not used for study as preliminary determinations in this laboratory found the turnover rates too low to permit sufficiently accurate measurements. All experiments were performed in 0.05 *M* Tris–maleate buffer, pH 7.8, 0.1 *M* in KCl prepared using glass-distilled water.

Turnover experiments were performed at 25° by measuring the rate of oxygen uptake of enzyme and substrate solutions in buffer saturated with oxygen before the addition of enzyme. Dissolved oxygen concentration was followed polarographically using a Yellow Springs Instrument Co. Biological Oxygen Monitor. All rate measurements were made from the primary oxygen uptake data at a percentage of oxygen saturation corresponding to 8.4×10^{-4} *M* oxygen. The values of K_m were determined from Lineweaver-Burk plots. K_m was found to be independent of oxygen concentration under the conditions employed in this study in agreement with the same observation by Massey and Curti (*4*). The values of K_m were determined using substrate concentrations below that at which substrate inhibition is encountered. The method of determining k_{obs}, the overall specific rate of the reduction process, has been described elsewhere (*2*, *7*).

Stopped-flow studies were performed at 15° and 540 nm using a Durrum-Gibson stopped-flow spectrophotometer. The enzyme and substrate solutions were deoxygenated and prepared for the stopped-flow studies as described earlier (*2*). In addition, all substrate solutions were filtered through a Millipore VCWP ultrafiltration membrane before deoxygenation in order to obtain maximum clarity. The concentration of active enzyme used in the stopped-flow experiments ranged from 5.5×10^{-6} *M* to 6.8×10^{-6} *M*. The pH of the pooled reaction mixture was determined after mixing using a Sargent model DR pH meter.

RESULTS

The results of the turnover experiments will be given first. Table I lists the k_{obs} and K_m values corresponding to each of the ring-substituted phenylalanines given. The values of the substituent constant σ are taken from Ref. 10.

The k_{obs} values in Table 1 show a small but reproducible increase with increasing σ values (with the exception of DL-*p*-chlorophenylalanine). Furthermore, the values of K_m are approximately constant (within experimental error) with the exception of the anomalously low K_m value for *p*-chlorophenylalanine. It may be that the anomalous

TABLE 1

VALUES OF k_{obs} AND K_m FOR SUBSTITUTED PHENYLALANINES AS L-AMINO ACID OXIDASE SUBSTRATES AT pH 7.8 AND 25°

Phenylalanine substituent	σ	k_{obs} ($10^6\ M^{-1}\ min^{-1}$)	K_m ($10^{-4}\ M$)
p-NH_2	−0.66	1.5	1.8
p-OH	−0.36	2.0	1.8
m-OH	−0.002	1.9	1.5
H	0	2.0	1.5
p-F	0.06	2.1	3.0
p-Cl	0.23	1.2	0.78
m-F	0.34	2.1	2.1
p-NO_2	0.78	2.9	2.5

position of *p*-chlorophenylalanine is somehow related to the known interaction of chloride with L-amino acid oxidase (*11*).

The Hammett plot of log (k_{obs}) *vs.* σ for the series of phenylalanines used (excluding DL-*p*-chlorophenylalanine) is shown in Fig. 1. The linear correlation shown in Fig. 1 yields a slope $\rho = 0.2$.

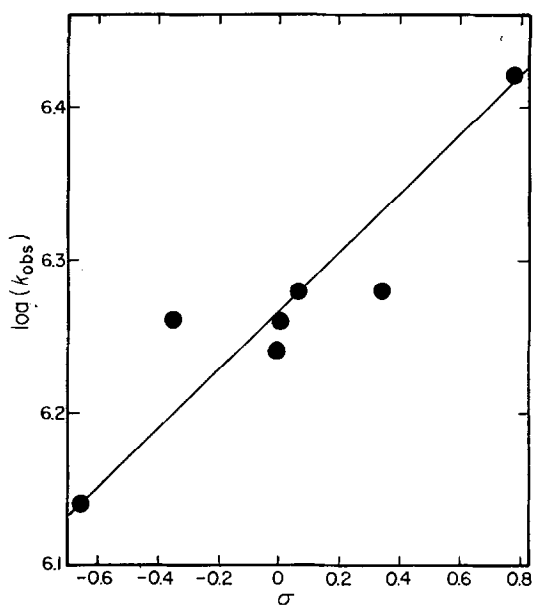


FIG. 1. Plot of log (k_{obs}) *vs.* σ for the series of phenylalanine substrates given in Table 1 (with the exception of DL-*p*-chlorophenylalanine). All points correspond to experiments performed at pH 7.8 and 25°, where k_{obs} is the overall second-order rat constant for the reduction of L-amino acid oxidase by substrate. Each point was determined from the slope of a plot of $\ln[S]_t$ *vs.* *t* determined from oxygen uptake data using $1.2 \times 10^{-3}\ M$ substrate in buffer initially saturated with oxygen at 25° ($[O_2]_0 = 1.2 \times 10^{-3}\ M$).

The results of the stopped-flow experiments were characterized by a very rapid initial formation of the transient intermediate ES_{540}, followed by a subsequent rapid decay of this species into the fully reduced enzyme form. In addition to the rapid decay,

as reported earlier for the case of leucine substrates (*2*), there was observed a slow decay process occurring concurrently with the rapid decay of ES_{540}. A first-order plot of the data in the time range of the decay reactions gave two linear segments. The segment corresponding to the slow-decay process yielded the value of k_{-2app} directly (Scheme I). The slow-decay first-order plot was extrapolated to time zero and the slow component of the overall absorbance value at each time point in the range of the decay reaction was subtracted out, leaving only the component of the total absorbance corresponding to the rapid-decay reaction. Data treated in this manner gave good linear first-order plots from which slopes the values of k_4 were calculated. The apparent first-order rate constant for the formation of ES_{540}, k_{3app}, was evaluated from the first 30% of the data corresponding to the formation of this intermediate using a Hewlett-Packard 2115A digital computer by an iterative method described earlier (*2*). In the case of phenylalanine the initial estimate for the infinite absorbance value corresponding to 100% formation of ES_{540} (i.e., no decay processes) was estimated from the initial enzyme concentration and a molar absorptivity of $1.1 \times 10^4\ M^{-1}\ cm^{-1}$ for phenylalanine ES_{540} as estimated from the data of Massey and Curti (*4*). Some concentrations of substrate caused the formation of ES_{540} to occur too rapidly to permit the use of the iterative method for calculation of k_{3app} from the primary data. Under such circumstances, the value of k_{3app} was calculated from the half-time of the reaction using the final (computer-derived) estimate of the infinite absorbance value for the ES_{540} formation

TABLE 2

THE VALUES OF THE KINETICALLY ACCESSIBLE RATE CONSTANTS OF SCHEME I[a]

Substrate	Concentration ($10^{-3}\ M$)	k_{3app} (sec^{-1})	k_4 (sec^{-1})	k_{-2app} (sec^{-1})
DL-*p*-Aminophenylalanine	2.5	21	3.9	0.18
	5.0	45	3.6	0.24
L-Phenylalanine	2.5	67	5.2	0.15
	2.5	58		
	5.0	129	4.6	0.24
	5.0	119		
	10.0	200		
DL-Phenylalanine	2.5	63		
	5.0	110		
DL-*m*-Fluorophenylalanine	1.25	35		
	2.5	65	5.5	0.17
	2.5	69		
	5.0	141	5.8	0.24
	5.0	140		
L-*p*-Nitrophenylalanine	2.5	63		
	2.5	60		
	5.0	100		
	5.0	100		
DL-*p*-Nitrophenylalanine	2.5	63	10.3	0.2
	5.0	100	10.6	0.2
	5.0	100		

[a] The methods of evaluating the rate constants and the definitions of the rate constants are given in the text (see Results). All values were determined at pH 7.8 and 15°. For those entries for which only k_{3app} is given, k_{3app} was calculated using the relation $t_{1/2} = 0.693/k_{3app}$.

reaction. Both methods of calculating k_{3app} were checked against each other and good agreement was obtained. Table 2 gives the values of the kinetically accessible rate constants of Scheme I for the series of substituted phenylalanines studied by stopped-flow techniques.

The series of phenylalanines shown in Table 2 was chosen to give the widest range of σ values over which rate differences could be unequivocally ascribed to the inherent properties of the system and not to the experimental uncertainty of the stopped-flow method, which is on the order of $\pm 10\%$. The values of k_{-2app} compare very favorably with the values reported earlier for the leucine system (*2*). This consistency lends support to the interpretation of the slow decay process as involving, as an obligatory first step, the conversion of ESH^+ (the inactive charge form of ES) into ES and thence to the fully reduced enzyme form. The values of k_4 in Table 2 show an independence of substrate concentration which is in good agreement with the work reported by Massey and Curti (*4*).

As derived in an earlier report (*2*), the three rate-constants given in Tables 1 and 2 have the following definitions with respect to Scheme I.

$$1/k_{3app} = 1/k_3 + (k_{-1} + k_3)/(k_1 k_3\ [S])$$

$$k_{obs} = k_1 k_3/(k_{-1} + k_3)$$

$$1/k_{-2app} = (1/k_{-2})\,(1 + k_2/k_3)$$

The definitions for k_{3app} and k_{obs} are exact; that for k_{-2app} is approximate, where the exact definition is given elsewhere (*2*). According to the definitions given above, a plot of $1/k_{3app}$ *vs.* 1/[S] should be linear, with an intercept of $1/k_3$ and slope of $(k_{-1}k_3)/k_1k_3 = 1/k_{obs}$. Figure 2 shows such a plot for L-phenylalanine and for DL-*m*-fluorophenylalanine substrates. The intercepts in both cases are indistinguishable from zero, in

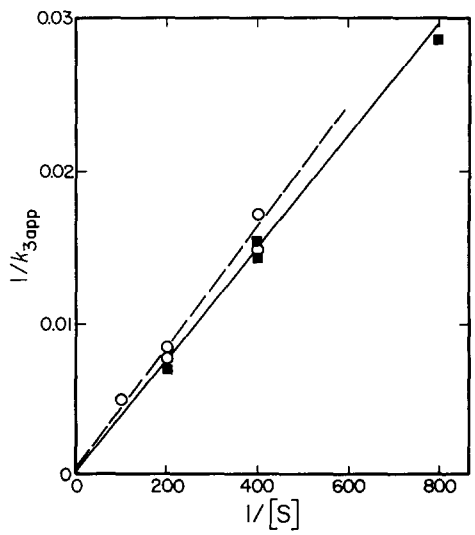


FIG. 2. Plot of $1/k_{3app}$ *vs.* 1/[substrate] where k_{3app} is the apparent first-order rate constant for the formation of the transient intermediate in the reduction of L-amino acid oxidase by L-phenylalanine (open circles) and DL-*m*-fluorophenylalanine (filled squares) at 15° and pH 7.8, as studied at 540 nm by stopped-flow technique.

accord with the earlier work of Massey and Curti (*4*), who estimate that k_3 for phenylalanine (at 0° and pH 7.8) is at least 2×10^4 min^{-1}. The slopes of the two lines in Fig. 2 give a k_{obs} value for DL-*m*-fluorophenylalanine of 1.4×10^6 M^{-1} min^{-1} and a k_{obs} value of

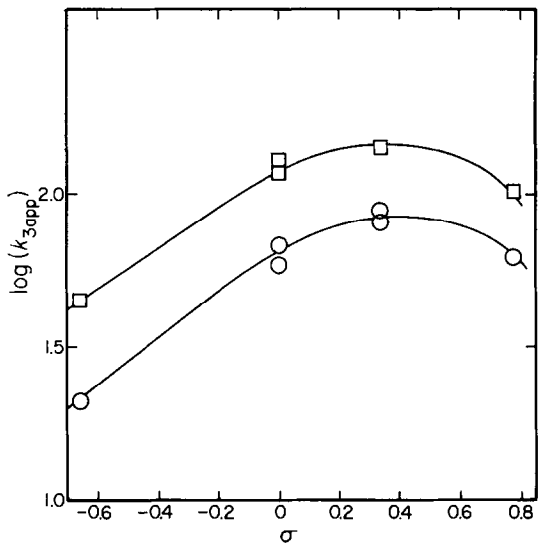


FIG. 3. Plot of log (k_{3app}) *vs.* σ for the overall formation of the transient intermediate in the reduction of L-amino acid oxidase by substrate. The points correspond to substituted phenylalanine substrates, 2.5×10^{-3} *M* (circles) and 5.0×10^{-3} *M* (squares), at 15° and pH 7.8, and were obtained from kinetic data obtained by following the anaerobic combination of oxidized enzyme and substrate at 540 nm using stopped-flow technique.

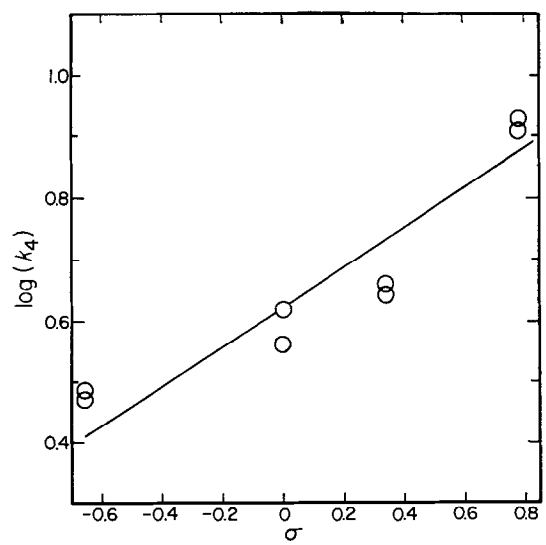


FIG. 4. Plot of log (k_4) *vs.* σ for the rapid decay of the transient intermediate, formed during the interaction of L-amino acid oxidase and substrate, into the fully reduced enzyme form. The points correspond to the values of k_4 for the corresponding substituted phenylalanine substrates reported in Table 2. All points were obtained at 15° and pH 7.8 by following the anaerobic combination of oxidized enzyme and substrate at 540 nm by stopped-flow technique.

$1.2 \times 10^6\ M^{-1}\ \text{min}^{-1}$ for L-phenylalanine. These two values, when account is taken of the differing conditions, are in excellent agreement with the values of k_{obs} (at 25°) given in Table 1 and serve to confirm the derivations presented earlier (*2*).

Figures 3 and 4 show Hammett plots for k_{3app} and k_4, respectively. The ρ value for the ascending segment of the k_{3app} Hammett plot in Fig. 3 is 0.7 for both substrate concentrations. A ρ value of 0.25 is observed for the Hammett plot of k_4 shown in Fig. 4.

It will be noted that both DL- and L-forms of phenylalanine and *p*-nitrophenylalanine were used as substrates in the stopped-flow studies. The fact that the values of k_{3app} for both the L- and DL- forms of either substrate are equal within experimental error effectively rules out the possibility of D-amino acid binding as an explanation of the curved nature of the k_{3app} Hammett plots in Fig. 3. The nature of these Hammett correlations will be discussed below.

DISCUSSION

As shown in Fig. 1, a linear Hammett plot results from the overall second-order rate constant of the reduction step as a function of σ. This linear correlation is in contrast to the biphasic Hammett plot obtained by Radda (*6*). The ρ value of 1.8 observed by Radda for the positive segment of the Hammett plot using phenylglycine substrates agrees well with the ρ value for Fig. 1 of 0.2 for phenylalanine substrates in view of the attenuative effect of the phenylalanine methylene group (*10*). Under the experimental conditions employed by Radda, it is probable that the biphasic nature of the Hammett plot obtained was the result of a shift in rate determining step from the reduction reaction at low σ values to the oxidation step at higher σ values. Such results illustrate the difficulties of attempting to draw mechanistic conclusions from overall turnover numbers or other complex rate quantities. In addition, we consider that the phenylglycine substrates react too slowly with L-amino acid oxidase to permit very accurate rate measurements.

The studies of Hellerman *et al.* (*7*) on D-amino acid oxidase also yielded biphasic Hammett plots. These workers followed the appearance of α-keto acid product, by quenching the reaction mixture and spectrophotometrically analyzing the 2,4-dinitrophenylhydrazone derivative of the α-ketoacid. The biphasic Hammett plots obtained from such data can be explained in a manner similar to that for the results of Radda (*6*). In addition the measured rates of appearance of α-ketoacid product could be in error because of the reported predominance of the enol form of phenylpyruvate species in solution (*12*), together with the fact that the interconversion of the keto and enol forms of phenylpyruvate species is relatively slow (*13*).

The low ρ value of 0.2 obtained from the k_{obs} Hammett plot in Fig. 1 compares very well with the ρ value of 0.25 for the k_4 process (Fig. 4). In this regard it is important to note that the derivation of the expression for k_{obs} with respect to Scheme 1 is based on the assumption that the k_4 process is rate-limiting in the overall conversion of oxidized enzyme and substrate to the fully reduced enzyme form.

The relatively large positive ρ value of 0.7 for the ascending segment of the Hammett plot for k_{3app} shown in Fig. 3 provides support for our earlier proposal that during the formation of the transient intermediate, ES_{540}, the amino acid α-hydrogen transferred to an acceptor group as a proton (*1*). That α-hydrogen transfer occurs during the formation of ES_{540} has been shown by the primary deuterium kinetic isotope effect studies of Porter and Bright (*14*) and of Page and VanEtten (*1*, *2*). The biphasic character of the Hammett plot of k_{3app} permits an extension of our earlier proposal that the formation

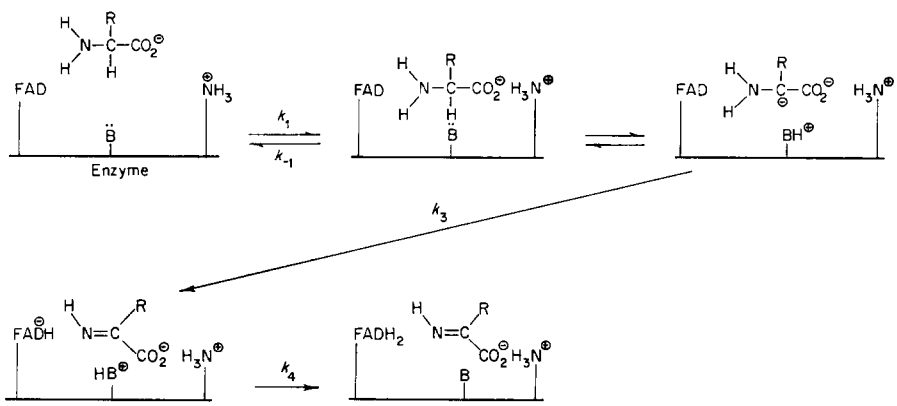
of ES_{540} is a concerted process involving a general base-catalyzed removal of the substrate α-hydrogen as a proton with electron pair transfer to the flavin either directly or as a hydride ion (*2*). Inductive electron withdrawal would facilitate proton transfer from the substrate alpha position, but would hinder electron transfer from the amino nitrogen to the flavin by stabilization of the negative charge center at the amino acid alpha position. Thus, the biphasic character of the Hammett plot resulting from such a situation must be determined by the relative ease with which proton and electron transfer (presumably hydride ion transfer) can occur.

There have been detailed mechanisms for the reduction of flavin dehydrogenases by substrate proposed by Coffey *et al.* (*15*) for the amino acid oxidases and by Brown and Hamilton (*16*) for flavin dehydrogenases in general. Coffey *et al.* (*15*) proposed that electron transfer from amino acid to flavin proceeds *via* a Schiff's base intermediate formed from a nucleophilic attack by the amino nitrogen of the substrate at a flavin isoalloxazine carbonyl group. The basis for this proposed mechanism was the formation of a reduced Schiff's base between the ε-amino group of a protein lysyl residue and the substrate, after treatment of the enzyme–substrate system in turnover with borohydride ion. Massey *et al.* (*17*) subsequently showed that catalytic activity is retained by the borohydride-reduced enzyme for both D-amino acid oxidase and L-amino acid oxidase. Massey's results suggested that a Schiff's base was not an important intermediate in the overall catalytic sequence for the amino acid oxidases. This was confirmed in an important investigation by Hafner and Wellner, who established that the reduced "intermediate" was in fact an artifact resulting from the nonenzymatic reaction of the initially produced imino acid product with various side-chain lysyl groups of the enzyme. The same reduction product could also be obtained from reaction mixtures in which free lysine or bovine serum albumin was present (*18*).

Recently, Brown and Hamilton (*16*) proposed a general mechanism for flavoenzyme-catalyzed dehydrogenations based on studies of the nonenzymic, nonaqueous flavin-catalyzed oxidation of methylphenylglycine and of methyl mandelate under anaerobic conditions in the presence of *t*-butoxide ion in the dark. This proposed mechanism has subsequently been advanced by Weibel and Bright (*19*) for the interaction between substrate and glucose oxidase. The general mechanism of Brown *et al.* involves the addition of substrate to the 4a flavin ring position *via* a nucleophilic attack on this ring position by, in the case of the amino acid oxidases, the amino nitrogen to yield a covalent flavin–substrate intermediate. In the second step α-proton removal coupled with electron pair transfer to the flavin occurs to complete the formal oxidation of the substrate. Concurrently with oxidation the covalent intermediate dissociates to give product and reduced flavin. Cited in support of this mechanism is evidence presented by Hemmerich *et al.* (*20*) that nucleophilic addition to the 4a position of the flavin isoalloxazine ring is favorable if the adjacent ring nitrogen at position 5 is protonated. However, such evidence is by no means relevant to the system studied in the dark by Brown and Hamilton, because the reactions studied by Hemmerich *et al.* involved oxidations by photoexcited flavins of a variety of aromatic carboxylic acids where the addition to the flavin apparently occurred through the methylene carbon alpha to the carboxylate function which was subsequently released as CO_2. Moreover, Hemmerich *et al.* did not report any long wavelength absorption maxima in the visible region for the type of intermediate proposed by Brown *et al.* If the covalent intermediate proposed by Brown and Hamilton were present in appreciable steady-state concentration (i.e., if the breakdown of this intermediate were rate-determining in the reaction sequence) then such a system should exhibit the spectral properties observed in the L-amino acid oxidase system, where an intermediate having an absorption in the region of 550 nm is observed (*5*). There is no

convincing evidence that the 4a position of the isoalloxazine ring is uniquely susceptible toward nucleophilic attack. Recently, Song (*21*) reported the detailed results of calculations of the sigma and pi electronic structure of isoalloxazine using sophisticated Pople–Segal SCF-LCAO-MO-CNDO methods. The sigma and pi charge densities calculated at each ring position in this manner suggest that the 4a position is, electronically, no more susceptible to attack by a nucleophile than are positions 2 or 4. Admittedly, reactivity cannot be accurately predicted by such calculations. Perhaps more importantly, data presented here and in earlier work (*1*, *2*) indicates that α-proton transfer occurs during the formation of the transient intermediate species, in contrast to the mechanism of Brown and Hamilton which proposes that α-proton transfer takes place during the decay of an intermediate species. Their proposal seems not to fit data observed for the enzymic system L-amino acid oxidase, although it may be correct for nonenzymatic reactions involving anhydrous dimethylformamide and potassium *t*-butoxide.

A proposed mechanism for the reduction of L-amino acid oxidase by substrate is presented in Scheme 2. This mechanism represents an extension of one proposed earlier (*2*, *9*). In Scheme 2 the initial binding interaction, characterized by k_1/k_{-1},



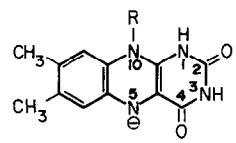
SCHEME 2. Proposed mechanism for the reduction of L-amino acid oxidase by substrate.

leads to the formation of an enzyme-substrate complex. This process must be very rapid because equilibrium is apparently reached within the dead-time of the stopped-flow apparatus, which is of the order of 2–3 msec. A positively charged group is postulated to be involved in the formation of an ionic bond to the carboxylate anion of the substrate. This proposal is consistent with the fact that a variety of anionic molecules are known to bind to the amino oxidases (*22–24*).

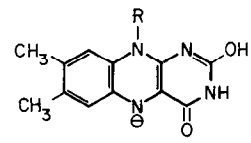
An important feature of the mechanism presented in Scheme 2 is the involvement of general-base catalysis of α-hydrogen removal from the bound amino acid leading to a carbanionic intermediate (*1*). This proposal is consistent with a study of the primary deuterium kinetic isotope effect with phenylalanine (*14*) as well as a detailed study of the pH dependence of the deuterium primary and solvent isotope effects observed with leucine as a substrate (*1*, *2*). Several model systems have subsequently been described which suggest the possible involvement of carbanionic species in flavin-catalyzed dehydrogenations proceeding in the dark (*16*, *25*, *26*). On the basis of their studies of the nonenzymatic oxidation of carbanions and enolate anions by flavin derivatives,

Rynd and Gibian have proposed (*25*) a general mode of flavin redox function which is similar to that shown in Scheme 2.

We propose that the kinetic step k_3 of Scheme 2 which leads to the colored intermediate ES_{540} (Scheme 1) involves electron transfer, either as a hydride ion directly, or as electron pair transfer coupled with proton transfer. The product of the k_3 step can reasonably be considered to be an anionic form of a fully reduced flavin tautomer. Hydride transfer could occur to N5, consistent with a recent proposal by Wu, MacKenzie, and McCormick (*27*). The structure of the resulting flavin tautomer would thus be that shown in (**1**). However, there are no data available as yet which allow us to distinguish between hydride transfer and an alternative, namely, electron pair transfer from the amino nitrogen to C2 of the flavin ring coupled with proton transfer to the carbonyl oxygen O2, resulting



(**1**)

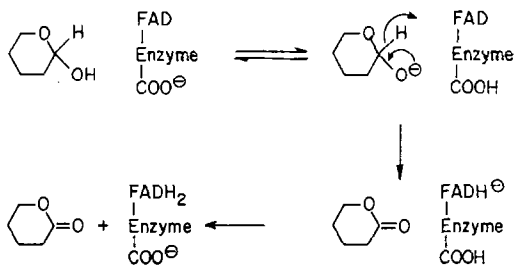


(**2**)

in the formation of a tautomer such as (**2**). It is unlikely that the tautomeric equilibria which apply to free flavins in solution are directly applicable to protein-bound flavins, particularly in cases like L-amino acid oxidase and glucose oxidase where the flavin effectively cannot be dissociated from the enzyme. The proposed nature of ES_{540} provides an alternative to the invocation of charge-transfer complexes as a means of rationalizing the fact that such transient intermediate species as are observed for the amino acid oxidase systems are not observed for all flavin oxidases (*28*). The presence of a steady-state concentration of such anionic forms in sufficient concentration to be experimentally detected would depend primarily on the ease with which protonation of such anionic intermediates could occur. This in turn depends largely on the physical situation of the flavin isoalloxazine system at the active center of a given flavoenzyme. Such a proposal would bear further study.

There are a number of examples of organic reactions which are considered to go by way of hydride transfers and which have reasonably good analogies in flavoenzymes such as amino acid oxidases, glucose oxidase, and succinate dehydrogenase. On the basis of their careful studies of the nonenzymatic oxidation of nicotinamide derivatives by riboflavin in aqueous solution, Suelter and Metzler concluded that the reaction proceeded *via* hydride transfer to the flavin. The pH dependence, effects of less polar solvents, and ionic strength dependence were all consistent with such an interpretation (*29*). We would like to call attention to the striking similarity between some flavin-catalyzed dehydrogenations and a wide variety of dehydrogenation reactions involving quinones. Quinones having electron-withdrawing substituents such as 2,3,5,6-tetrachloro-1,4-benzoquinone (Chloranil) are widely used as oxidants. The rates of quinone-catalyzed oxidations are not simple functions of the oxidation-reduction potential of the quinone, and it is often observed that *ortho* quinones are more reactive than the corresponding *para* quinones (*30*). The dehydrogenation of a variety of aromatic compounds by 3,4,5,6-tetrachloro-1,2-benzoquinone has been studied by Jackman and Thompson. They have established that the rate-determining step involves hydride transfer to the quinone (*32*). Acyclic amines can also be dehydrogenated by quinones (*33*). Recognizing that ketones can react to accept hydride ions donated by alkoxide ions (*34*), Jackman has pointed out that quinones should also be suitable acceptors (*33*). Such an

analogy between quinones and the flavins as dehydrogenating agents leads immediately to a proposed mechanism for glucose oxidase as outlined in Scheme 3. In this case the general base catalyst involved in the equilibrium leading to glucoxide ion formation may possibly be a carboxylate ion. Such a proposal has recently been advanced by Weibel and Bright (*19*).



SCHEME 3. Analogous mechanism for the reduction of glucose oxidase by substrate (see also Ref. 19).

Finally, in some step k_4 the equilibrium tautomer of the fully reduced flavin must be formed. Accompanying or preceding this must be a significant structural movement of the enzyme. This conclusion necessarily follows upon recognition of the substantial structural difference between oxidized and reduced flavin derivatives (*35*, *36*). The fully reduced flavin assumes a butterfly-like form having a large dihedral angle between the two halves of the molecule, unlike the oxidized form which is nearly planar.

The proposed fully reduced enzyme form represented in Scheme 2 need not immediately lead to a free imino acid (*18*) product. Massey and Curti suggested (*4*) that the imino acid remains bonded to the active center of the fully reduced enzyme. One reasonable possibility is that dissociation of the imino acid from the active site is blocked by the flavin as a consequence of the dihedral conformation of the fully reduced flavin form. Upon reoxidation of the reduced enzyme, the assumption of the planar oxidized flavin conformation by the prosthetic group would permit the imino acid to dissociate freely. This is consistent with the suggestion by Massey and Curti that the oxidized substrate is usually retained at the active center until reoxidation of the reduced flavin occurs (*4*). The small positive ρ value of 0.25 observed for the k_4 process (see Results) is also consistent with there being some type of interaction between the oxidized substrate and the flavin, although the effect might also be ascribed to a dissociation of the imino acid carboxylate from the active center cationic group.

The observed effects of substituents upon the rate of formation of ES_{540} for L-amino acid oxidase leads to a ρ value of +0.7 for the ascending portion of the Hammett plot. This is far beyond the ρ value of 0.14 determined for the ionization of the amino group (*7*). Our stopped-flow studies are in good agreement with the turnover results obtained by Neims, DeLuca, and Hellerman for the case of D-amino acid oxidase (*7*), suggesting a substantial similarity in the mechanisms of these two groups of enzymes. In agreement with their interpretation (*7*), we conclude that these results must mean that there is a substantial negative charge associated with the α-carbon atom of the substrate during the course of the reduction of the enzyme. Our results show that this occurs during the formation of the colored intermediate ES_{540}, a process which we have previously shown to be consistent with the involvement of general base catalysis and a rate-determining hydrogen transfer step (*2*, *9*). It appears that studies on the identity of specific groups in the active site of amino acid oxidases and on the nature of ES_{540} are particularly promising areas of research at this time.

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REFERENCES

1. D. S. PAGE AND R. L. VANETTEN, *Biochim. Biophys. Acta* **191**, 190 (1969).
2. D. S. PAGE AND R. L. VANETTEN, *Biochim. Biophys. Acta* **227**, 16 (1971).
3. L. P. HAMMETT, "Physical Organic Chemistry." McGraw-Hill, New York (1940).
4. V. MASSEY AND B. CURTI, *J. Biol. Chem.* **242**, 1259 (1967).
5. H. BEINERT, *J. Biol. Chem.* **225**, 465 (1957).
6. G. K. RADDA, *Nature London* **203**, 936 (1964).
7. A. H. NEIMS, D. C. DELUCA, AND L. HELLERMAN, *Biochemistry* **5**, 203 (1966).
8. D. WELLNER AND A. MEISTER, *J. Biol. Chem.* **235**, 2013 (1960).
9. D. S. PAGE AND R. L. VANETTEN, *Biochim. Biophys. Acta* **191**, 38 (1969).
10. H. H. JAFFE, *Chem. Rev.* **53**, 191 (1953).
11. E. B. KEARNEY AND T. P. SINGER, *Arch. Biochem. Biophys.* **33**, 397 (1969).
12. M. JOSIEN, M. JOUSSOT-DUBIEN, AND J. VIZET, *Bull. Soc. Chim. France* **1957**, 1148 (1957).
13. A. M. STOCK, W. E. DONAHUE, AND E. D. AMSTUTZ, *J. Org. Chem.* **23**, 1840 (1958).
14. D. J. T. PORTER AND H. BRIGHT, *Biochem. Biophys. Res. Commun.* **36**, 209 (1969).
15. D. S. COFFEY, A. H. NEIMS, AND L. HELLERMAN, *J. Biol. Chem.* **240**, 4058 (1965).
16. L. E. BROWN AND G. A. HAMILTON, *J. Amer. Chem. Soc.* **92**, 7225 (1970).
17. V. MASSEY, B. CURTI, F. MULLER, AND S. G. MAYHEW, *J. Biol. Chem.* **243**, 1329 (1968).
18. E. W. HAFNER AND D. WELLNER, *Proc. Nat. Acad. Sci. U.S.A.* **68**, 987 (1971).
19. M. K. WEIBEL AND H. J. BRIGHT, *J. Biol. Chem.* **246**, 2734 (1971).
20. P. HEMMERICH, V. MASSEY, AND G. WEBER, *Nature London* **213**, 728 (1967).
21. PILL-SOON SONG, *Ann. New York Acad. Sci.* **158**, 410 (1969).
22. M. DIXON AND K. KLEPPE, *Biochim. Biophys. Acta* **96**, 368 (1965).
23. M. L. FONDA AND B. M. ANDERSON, *J. Biol. Chem.* **243**, 1931 (1968).
24. K. YAGI, M. NAOI, M. NISHIKIMI, AND A. KOTAKI, *J. Biochem. Tokyo* **68**, 293 (1970).
25. J. A. RYND AND M. J. GIBIAN, *Biochem. Biophys. Res. Commun.* **41**, 1097 (1970).
26. G. D. WEATHERBY AND D. O. CARR, *Biochemistry* **9**, 351 (1970).
27. P. Y.-H. WU, R. E. MACKENZIE, AND D. B. MCCORMICK, *Biochemistry* **9**, 2219 (1970).
28. P. HEMMERICH, G. NAGELSCHNEIDER, AND C. VEEGER, *Fed. Eur. Biol. Soc. Lett.* **8**, 69 (1970).
29. C. H. SUELTER AND D. E. METZLER, *Biochim. Biophys. Acta* **44**, 23 (1960).
30. R. STEWART, "Oxidation Mechanisms," p. 126. Benjamin, New York, 1964.
31. E. A. BRAUDE, L. M. JACKMAN, AND R. P. LINSTEAD, *J. Chem. Soc.* 3548 (1954).
32. L. M. JACKMAN AND D. T. THOMPSON, *J. Chem. Soc.* 4794 (1961).
33. L. M. JACKMAN, *Advan. Org. Chem.* **2**, 329 (1960).
34. E. CLAR, *Chem. Ber.* **69**, 1686 (1936).
35. M. VON GLEHN, P. KIERKEGAARD, R. NORRESTAM, O. RONNQUIST, AND P.-E. WERNER, *Acta Chem. Scand.* **24**, 3701 (1970).
36. G. PALMER AND V. MASSEY, *in* "Biological Oxidations," (T. P. Singer, ed.), p. 263. Interscience, New York, 1968.