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STUDIES ON L-AMINO-ACID OXIDASE

I. EFFECTS OF PH AND COMPETITIVE INHIBITORS

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SUMMARY

1. Aromatic carboxylates are found to be competitive inhibitors of L-amino-acid oxidase (L-amino-acid:O_2 oxidoreductase (deaminating), EC 1.4.3.2). The inhibitor constants for a number of ring-substituted benzoates and some other compounds have been determined. They do not follow a linear Hammett relationship.

2. L-Amino-acid oxidase reacts immediately with these inhibitors to form spectrally detectable enzyme–inhibitor complexes. Almost all inhibitors cause a blue shift of either one or both visible absorption bands of the flavoprotein and have influences on the extinction coefficients of their absorption maxima. These effects may be explained by indirect changes in the environment of the isoalloxazine rather than by direct complex formation of the inhibitor with this ring system. The enzyme complex with *o*-aminobenzoate gives an additional broad band, extending from 540 to 750 nm. *o*-Mercaptobenzoate causes a red shift of the long-wavelength band of the enzyme, immediately followed by a slow and incomplete reduction of the enzyme-bound FAD.

3. Kinetically determined inhibition constants are in good agreement with the dissociation constants of the enzyme–inhibitor complexes, determined by titration of the enzyme with the inhibitor at the same pH and temperature.

4. The dissociation constants, thus determined, were found to be dependent on the pH of the reaction mixture. For the L-amino-acid oxidase-*o*-aminobenzoate complex, the dissociation constant had a minimum value of 0.24 mM at pH 8.6. From curves relating pH to absorbance differences, a group in the free enzyme was found with an apparent pK_a value of 7.80, which participates in the binding of the inhibitor.

5. The spectrum of the free enzyme is also dependent on the pH. An instantaneous and reversible blue shift is observed when going from pH 5.0 to pH 7.0, with a midpoint near pH 6.1. As a result, the unresolved 460-nm band at pH 5.0 becomes slightly resolved at higher pH. Above pH 8.2 an additional slow and apparently irreversible blue shift occurs, which is also found on inactivation of the enzyme in the presence of 0.1 M phosphate, at lower pH and higher temperature, as described previously.

INTRODUCTION

It has been reported that L-amino-acid oxidase (L-amino-acid:O_2 oxidoreductase (deaminating), EC 1.4.3.2) is a glycoprotein, containing two FAD groups per molecular weight unit of 130 000 (refs. 1, 2). As with other flavoproteins, the mechanism of action of this enzyme is explained in terms of identical and independently reacting active sites[1,4].

With both the L- and D-amino-acid oxidases, it has been concluded from various studies[5–10] that charged groups in the active center participate in the binding of charged substrates and competitive inhibitors, such as benzoates, to the enzyme. SINGER AND KEARNEY[7] concluded from their study of the reversible inactivation of L-amino-acid oxidase, that a histidine, in conjunction with a charged amino group, might be involved in the binding process. HELLERMAN AND COFFEY[10] were able to show the participation of an ε-amino group of lysine in catalysis with both D- and L-amino-acid oxidases. Studies of DIXON AND KLEPPE[11] with D-amino-acid oxidase have shown the participation of a group with a pK value of 8.5 in the binding of substrate and inhibitor.

Competitive inhibitors have been used successfully in the study of the active sites of enzymes, especially when the reaction of an enzyme with inhibitor could be observed directly by some means. This is the case with flavoproteins, where it has been shown that competitive inhibitors may cause changes in the absorption spectra of the enzymes[9,12,13]. In the case of L-amino-acid oxidase, these effects are discussed in the present paper, together with the influence of pH on the spectrum of the enzyme and on the binding of inhibitors. A preliminary report of part of this work has been presented elsewhere[13].

METHODS AND MATERIALS

Preparation of the enzyme

L-Amino-acid oxidase was purified from dried *Crotalus adamanteus* venom, purchased from Sigma and Koch Light. The purification procedure was identical to that described by WELLNER AND MEISTER[2]. After one crystallization step, the enzyme was dissolved in 0.2 M KCl and stored at 0° in the dark. When not used within 1 month, the enzyme preparation was stored as a suspension in saturated $(NH_4)_2SO_4$, in which case it was stable for months. Freezing of the purified enzyme led to inactivation (*cf.* ref. 14). The purified enzyme had a specific activity at 38°, as measured manometrically under the conditions specified by WELLNER AND MEISTER[2] of 16–21 μmoles L-leucine oxidized/min per mg protein.

Spectrophotometry and spectrophotometric determination of dissociation constants (K_D values)

Absorption spectra were recorded in a Cary model 14 recording spectrophotometer, thermostated at 25°, in cells with a 1-cm light-path. The difference spectra were recorded with the 0–0.1 absorbance indicating slidewire. For experiments which were carried out under anaerobic conditions, Thunberg spectrophotometer cuvettes were used. On these cuvettes a titration assembly could be attached. Anaerobic conditions were obtained by evacuation and refilling of the cuvettes with N_2, freed from oxygen

by washing with alkaline pyrogallol solution. This procedure was repeated at least 5 times.

The enzyme concentration was calculated from its absorption at 464 nm, using a value of $11.3 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$ for the extinction coefficient of the enzyme-bound FAD[15] and assuming 2 moles of FAD bound per mole of enzyme (*cf.* ref. 2).

For the determination of K_D values, increasing amounts of inhibitor in 0.2 M Tris buffer were added to a constant amount of enzyme in the same buffer. Buffer solutions were added to the blank cuvette, containing the same amount of enzyme in order to correct for differences in the enzyme concentrations. After each addition the difference spectrum was recorded. Corrections were made for absorption of the inhibitor alone, if necessary, and for dilution caused by additions to the cuvettes. Constant values of K_D were obtained, assuming a 1:1 complex with respect to FAD. The dissociation constants were calculated from

$$K_D = \frac{([\mathrm{FAD}] - [\mathrm{FAD}I])([I] - [\mathrm{FAD}I])}{[\mathrm{FAD}I]}$$

in which [FAD] is total FAD concentration, [I] total inhibitor concentration and [FADI] the complex concentration calculated from $\Delta A/\Delta A_{max} \times$ [FAD]. ΔA_{max} is the maximum difference obtained upon saturation of the enzyme with inhibitor.

Most K_D values, thus obtained, were much higher than the FAD concentration; thus the inhibitor concentration which gave half maximal changes in the absorption difference spectrum was also equal to the K_D value.

The pH studies were done in a slightly different way. Titrations were performed with a series of 0.2 M Tris–maleate buffers, ranging from pH 5.0 to pH 9.4. At each pH increasing amounts of the inhibitor were added as neutralized unbuffered solutions of the acid in water. Equivalent amounts of water were added to the blank cuvette, containing the enzyme in the same buffer. After the experiment the pH was checked with a Corning micro glass electrode. Small changes in the pH occurred only at the pH extremes and with the highest inhibitor concentrations. The pH effect on the spectrum of the enzyme was studied with the same buffers.

Manometric determination of inhibitor constants (K_i values)

Inhibitor constants were determined manometrically at 25°. The reaction mixture contained 150 μmoles KCl, 250 μmoles Tris–HCl buffer (pH 7.4), L-leucine and inhibitor. After an equilibration period of 10 min, the enzyme solution, containing 50 μmoles of KCl, was tipped in from the side arm. The enzyme concentration was chosen to give an O_2 uptake between 2 and 6 μl/min. The center well contained NaOH. Using varying amounts of L-leucine in concentrations below those where substrate inhibition is observed[16] and one fixed concentration of inhibitor, the K_i values were calculated from double reciprocal plots, relating the initial velocity to the substrate concentration in the presence and in the absence of the inhibitor. All measurements were done in duplicate.

Reagents

The *o*-, *m*- and *p*-substituted benzoates, DL-, D- and L-mandelic acid, *p*-toluenesulfonic acid, benzoic acid, *p*-chlorobenzenesulfonic acid, L-leucine and sodium maleate were obtained from the British Drug Houses. The substituted benzoates,

when not analytical grade, were recrystallized from water. The aminobenzoic acids were purified with active charcoal. L-Leucine was recrystallized from water. α-Keto-isocaproic acid, DL-α-hydroxyisocaproic acid and orthanilic acid were used as supplied by Fluka. Tris, β-phenylpyruvic acid and sodium pyruvate were from Sigma. The water used in preparing solutions was twice distilled in glass.

RESULTS

The reaction of L-*amino-acid oxidase with competitive inhibitors*

ZELLER AND MARITZ[5] showed that several aromatic carboxylates and sulfonates were inhibitors of the oxidative deamination of L-amino acids, catalyzed by unpurified snake venom preparations. They could not find a Hammett relationship upon using ring-substituted phenylalanines as substrate[6], while RADDA[8] found a non-linear Hammett plot upon using ring-substituted phenylglycines at one concentration.

With the purified oxidase from *Crotalus adamanteus* the inhibitor constants of a number of ring-substituted benzoates and some other compounds have been measured. These results are summarized in Table I.

TABLE I

INHIBITOR CONSTANTS DETERMINED KINETICALLY WITH PURIFIED L-AMINO-ACID OXIDASE AT 25° AND pH 7.4

These values, for some of which we thank H. HUTTINGA, were determined as described in METHODS.

Inhibitor	K_i *(mM)*	*Inhibitor*	K_i *(mM)*
Benzoate	1.7	*o*-Fluorobenzoate	1.4
o-Aminobenzoate	0.42	*m*-Fluorobenzoate	0.37
m-Aminobenzoate	1.6	*p*-Fluorobenzoate	1.3
p-Aminobenzoate	1.4	*o*-Nitrobenzoate	0.86
o-Hydroxybenzoate	2.2	*m*-Nitrobenzoate	0.34
m-Hydroxybenzoate	2.1	*p*-Nitrobenzoate	2.3
p-Hydroxybenzoate	2.3	*o*-Mercaptobenzoate	1.0
o-Chlorobenzoate	1.0	D-Mandelate	11.0
m-Chlorobenzoate	0.15	L-Mandelate	11.0
p-Chlorobenzoate	0.70	Orthanilic acid	1.2

If the variation of the K_i values is entirely due to a different degree of interaction between a positive charge on the enzyme and the inhibitor anion, neglecting complications due to steric effects, a linear relationship between the K_i values and σ for meta- and para- substituted benzoates, with a positive ρ-value, would be expected, according to the Hammett relation[17]. A Hammett plot for 10 *m*- and *p*-substituted benzoate derivatives is shown in Fig. 1. Clearly the picture is more complicated than presented above. The general tendency is rather the opposite: a stronger binding occurs at higher σ values, the nitro compounds behaving differently. The same results were recently obtained at 37° with the oxygen electrode system[18], both with D-amino-acid oxidase and L-amino-acid oxidase.

Changes in the absorption spectrum of the enzyme, caused by the addition of these inhibitors also suggest that other interactions are introduced. Fig. 2A shows the

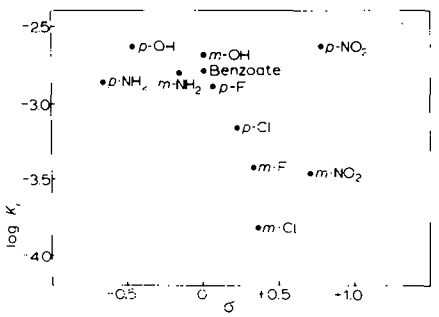
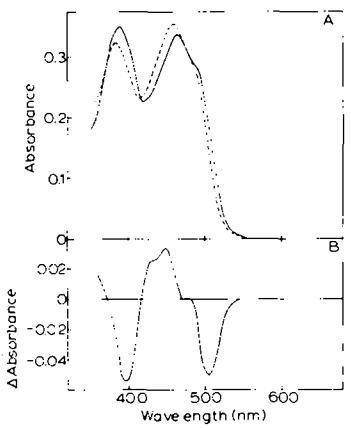


Fig. 1. Test of the validity of the Hammett equation for the reaction of ring-substituted benzoates with L-amino-acid oxidase. The σ values were taken from ref. 17. The K_i values were taken from Table I.

effects of DL-mandelate on the absorption spectrum of the enzyme. This compound causes a blue shift and a disappearance of the shoulder at 490 nm. An increase in the extinction coefficient of the absorption maximum at 464 nm and a decrease at 390 nm are also observed. Thus the absorption difference spectrum shows two minima (Fig. 2B), one at about 500 nm and one at 400 nm. It is of particular interest that no difference was found between the effects of D- and L-mandelate on the absorption spectrum, nor on the K_i value. This may indicate that there is no interaction of the α-hydroxyl group with the enzyme.

The effects on the absorption spectrum of the enzyme, described here in detail



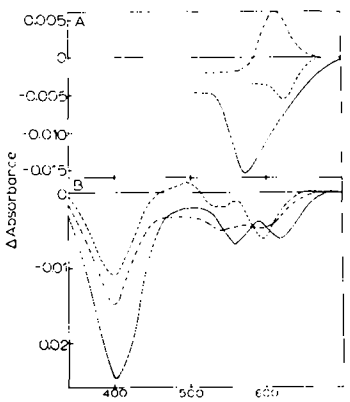


Fig. 2. The effect of DL-mandelate on the absorption spectrum of L-amino-acid oxidase. The enzyme concentration was 15 μM, the inhibitor concentration was 0.1 M. Solutions were in 0.2 M Tris–HCl buffer (pH 7.4). A. ——, no addition; - - - - -, after addition of DL-mandelate. B. Difference spectrum from A. —·— –, enzyme with mandelate *minus* enzyme.

Fig. 3. The effect of competitive inhibitors on the absorption difference spectrum of L-amino-acid oxidase. The enzyme concentration was 15 μM, the inhibitor concentrations were 0.1 M. All solutions were in 0.2 M Tris–HCl buffer (pH 7.4). A. · · · –, enzyme with *p*-nitrobenzoate *minus* enzyme; - - - - -, enzyme with *o*-nitrobenzoate *minus* enzyme; - · · - · · - , enzyme with *m*-nitrobenzoate *minus* enzyme. B. — · —, enzyme with *p*-chlorobenzoate *minus* enzyme; - - - - -, enzyme with *m*-chlorobenzoate *minus* enzyme; · - · - · - , enzyme with *o*-chlorobenzoate *minus* enzyme.

for mandelate, are found in general with most of the aromatic carboxylates studied. However, in many cases the changes in the absorption spectrum are quite small. Most of the inhibitors showed a somewhat larger effect on the 390-nm band than on the 460-nm band, while some (*e.g.* the chlorobenzoates) showed almost no effect on the 460-nm band but influenced the 390-nm band markedly (Fig. 3A). A very small red shift was observed with *m*-nitrobenzoate (Fig. 3B). No relation could be found between the magnitude of the K_i value and the magnitude of the spectral effect.

Two inhibitors showed exceptional difference spectra. In addition to the blue shift in the spectrum, the largest observed, *o*-aminobenzoate caused an absorption increase at wavelengths between 520 and 750 nm (Figs. 4A and B). This increase in absorption was not observed with any of the other substituted benzoates, listed in Table I. *o*-Mercaptobenzoate caused a red shift directly after addition with a maximum in the difference spectrum at 510 nm and extending to 600 nm (Fig. 5). This difference spectrum changed, however, on standing, due to the slow reduction of the FAD. This is in contrast to the results obtained with *o*-hydroxybenzoate, which gives the usual stable blue shift. A comparatively fast initial reduction was observed after the addition of 10 μmoles of *o*-mercaptobenzoate to 0.055 μmole of enzyme under anaerobic conditions. After 16 h an apparent equilibrium was reached at 42% reduction. The further addition of another 10 μmoles of *o*-mercaptobenzoate did not appreciably affect the spectrum. The subsequent addition of leucine reduced the enzyme completely, while after reoxidation the original spectrum of the complex was restored. It is not known what kind of mechanism this reaction may represent. The red shift may be due to the formation of a semiquinoid species. A similar type of reaction has been observed with D-amino-acid oxidase[19].

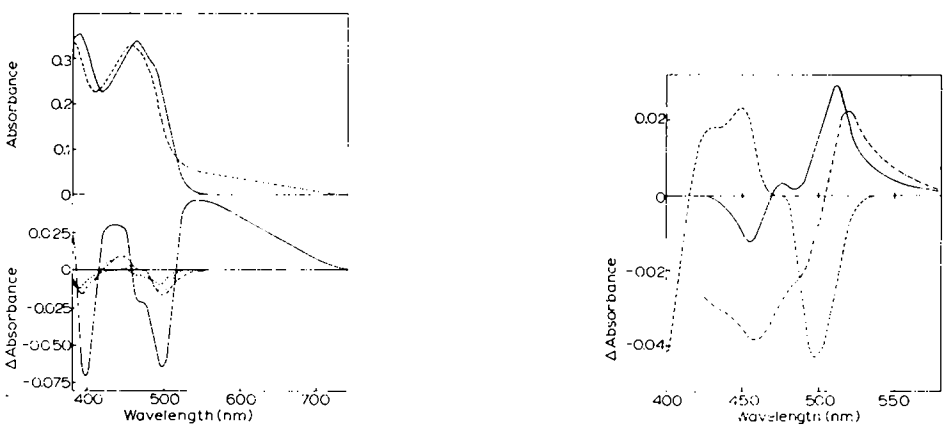


Fig. 4. The effect of aminobenzoate on the absorption spectrum of L-amino-acid oxidase. The enzyme concentration was 15 μM, the inhibitor concentrations were 0.1 M. All solutions were in 0.2 M Tris–HCl (pH 7.4). A. ——, no addition; - - - - -, after addition of *o*-aminobenzoate. B. Difference spectra. – –, enzyme with *o*-aminobenzoate *minus* enzyme; - - - - -, enzyme with *m*-aminobenzoate *minus* enzyme; — · — · —, enzyme with *p*-aminobenzoate *minus* enzyme; · · · · ·, enzyme with benzoate *minus* enzyme.

Fig. 5. The effect of competitive inhibitors on the absorption spectrum of L-amino-acid oxidase. The enzyme concentration was 15 μM, the inhibitor concentrations were 0.1 M. The solutions were in 0.1 M Tris-HCl (pH 7.4). – – —, enzyme with *o*-mercaptobenzoate *minus* enzyme, directly after the addition of inhibitor; — · — · — · , same after 15 min (aerobic conditions); - - - - -, enzyme with *o*-hydroxybenzoate *minus* enzyme.

In contrast with D-amino-acid oxidase[9], no spectrally detectable complexes were found with aliphatic carboxylates, including α-oxo and α-hydroxy acids. No spectral effects were found with D-amino acids and aromatic sulfonates such as *p*-toluenesulfonate and *p*-chlorobenzenesulfonate, though it has been reported that sulfonates inhibit the enzyme[5]. *o*-Aminobenzenesulfonate, however, caused the usual blue shift of the spectrum and is a relatively good inhibitor (K_i = 1.2 mM).

Though there is no correlation between the magnitudes of spectral effects and inhibitor constants, a good correspondence was found between the K_i values, determined kinetically and the K_D values, determined by spectral titration of the enzyme with inhibitor (Table II). The determination of K_i and K_D for *o*-aminobenzoate is illustrated in Fig. 6. The time-dependent changes, observed with *o*-mercaptobenzoate, together with some turbidity, observed in this case at the highest inhibitor concentrations, are responsible for inconsistent values of K_D. With many of the other inhibitors the spectral changes were either too small to give meaningful results or saturation of the enzyme could not be achieved.

TABLE II

COMPARISON OF INHIBITOR CONSTANTS, DETERMINED KINETICALLY, AND DISSOCIATION CONSTANTS DETERMINED SPECTROPHOTOMETRICALLY

Inhibitor	K_i *(mM)*	K_D *(mM)*
o-Aminobenzoate	0.42	0.80
o-Hydroxybenzoate	2.2	1.2
Orthanilic acid	1.2	0.90
o-Mercaptobenzoate	1.0	4.9
DL-Mandelate	11.0	4.3

If it is assumed that only one molecule of inhibitor reacts reversibly with each site on the enzyme, the sites reacting independently and identically, the equation:

$$FAD + I \rightleftharpoons FADI$$

may be used to calculate the dissociation constant. The use of this equation led to the most constant values at the different inhibitor concentrations. Because of the high dissociation constants found, making equilibrium dialysis impractical, and the difficulty of working at much higher enzyme concentrations, no information could be obtained about the actual number of inhibitor molecules bound per enzyme molecule.

Effect of pH on the visible absorption spectrum of the enzyme and on the reaction with inhibitors

A small but reproducible effect of the pH on the absorption spectrum of the enzyme was found (Fig. 7). From pH 5.0 to pH 7.4, the long-wavelength edge of the 460-nm band shifted to the blue, while some increase in absorption occurred near 475 nm, resulting in the formation of a slight shoulder at neutral pH. The effect of pH on the 390-nm band was negligible. The midpoint of this reversible shift was near pH 6.1. Above pH 8.0 a slow additional blue shift took place, which was complete after about 2 h at pH 8.6 and 25° and after about 20 min at pH 9.3 at the same temper-

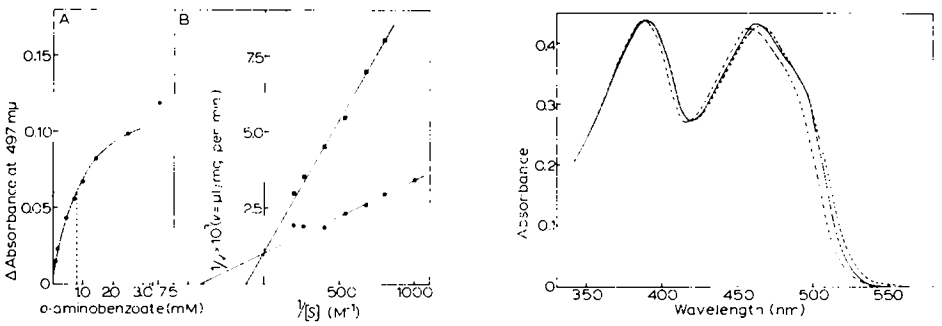


Fig. 6. A. Determination of the dissociation constant (K_D) of the L-amino-acid oxidase-*o*-aminobenzoate complex by spectral titration. The enzyme concentration was 22 μM. Solutions were in 0.2 M Tris–HCl (pH 7.4). Temp., 25°. B. Determination of the inhibitor constant (K_i) of *o*-aminobenzoate at 25° and pH 7.4 by the Lineweaver Burk plot. The enzyme concentration was 0.2 μM, the inhibitor concentration was 1 mM. ●—●, without inhibitor added; ■—■, with inhibitor.

Fig. 7. The effect of pH on the visible absorption spectrum of L-amino-acid oxidase. The enzyme concentration was 19 μM. Solutions were in 0.2 M Tris-maleate buffers. Temp., 25°. ——, spectrum of the enzyme at pH 7.4; - - - - -, spectrum at pH 5.0; —· · ·—, spectrum at pH 9.3 after 20 min incubation.

ature. The effect on the 390-nm band was again rather small, but the long-wavelength side of the 460-nm band shifted 5–6 nm, with loss of the shoulder (Fig. 7). This shift resulted in the inactivation of the enzyme, which at this stage appears irreversible. The FAD remained bound to the enzyme as judged from the absorbance at 390 nm and the absence of fluorescence. The enzyme was almost completely protected in the presence of 1 mM *o*-aminobenzoate. These effects, as described here for Tris–maleate buffers, were also found in pyrophosphate and in the presence of Cl^-. Upon reversible inactivation of the enzyme as described by SINGER AND KEARNEY[20], the same blue shift was observed.

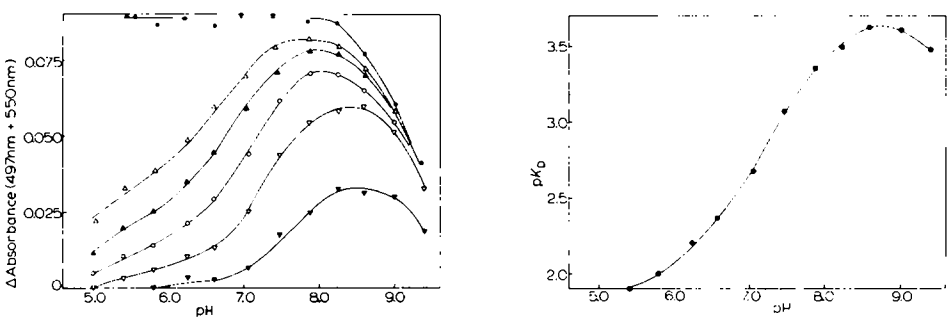


Fig. 8. The effect of pH on the concentration of the L-amino-acid oxidase–*o*-aminobenzoate complex. The enzyme concentration was 13 μM. 0.2 M Tris maleate buffers were used. ▼—▼, with 0.20 mM *o*-aminobenzoate; ▽—▽, with 0.78 mM *o*-aminobenzoate; ○—○, with 2.1 mM *o*-aminobenzoate; ▲—▲, with 4.2 mM *o*-aminobenzoate; △—△, with 7.8 mM *o*-aminobenzoate; ●—●, with 54 mM *o*-aminobenzoate. Temp., 25°. The experiment was done twice, the values given are averages.

Fig. 9. The effect of pH on the dissociation constant of the L-amino-acid oxidase *o*-aminobenzoate complex. The values were calculated from Fig. 8.

The concentration of the enzyme–inhibitor complexes was found to be dependent on the pH of the reaction mixture. At high inhibitor concentrations the enzyme remained saturated with inhibitor down to pH 5.0. Curves which show the change in concentration of the L-amino-acid oxidase–*o*-aminobenzoate complex with pH at six different *o*-aminobenzoate concentrations are shown in Fig. 8. The decline at high pH is caused by the inactivation reaction, mentioned above: a slow decrease in the absorption near 500 nm in the blank, which is prevented by the presence of the inhibitor in the reaction cuvette, resulting in an apparent decrease in the concentration of enzyme–inhibitor complex. It was impossible to make adequate corrections for this effect, because it is determined by two inactivation rates, one in the blank and the other in the reaction cuvette, which is slowed down as inhibitor is added. However, from the curve at the highest inhibitor concentration it can be concluded, that this process is only important above pH 8.2. Therefore only the maxima of the curves with 0.20 and 0.78 mM inhibitor might be too low. On the other hand, the points of these curves were the first to be determined after mixing the enzyme with buffer, Due to the slow reaction below pH 8.6 (smaller than 0.01 absorption unit per 30 min, the time of one complete titration), we do not expect a large effect on these maxima either. This is substantiated by the fact that the pK_a values, calculated from the curves (see below) are nearly the same for all curves.

On the acid side, the titration curves show rather long tails. A reflection of this behaviour is also found with pH curves at different leucine concentrations (*cf.* ref. 21) and may be related to the shift in the spectrum observed between pH 5.0 and pH 7.4 (see DISCUSSION). Though less precisely determined, the same effects were observed with DL-mandelate and *o*-hydroxybenzoate.

A plot of the dissociation constants *vs.* pH is shown in Fig. 9. A minimum K_D of 0.24 mM was found at pH 8.6. When it is assumed that the decline in the concentration of the enzyme–inhibitor complex is due to the protonation of a group in the free enzyme (the inhibitors have pK_a values below 5.0), the apparent pK_a of this group can be calculated from the pH at the midpoint of the titration curves. Furthermore, it was assumed in the derivation of the equation that, due to the fact that two identical FAD groups are present per molecule, two protonations on identical sites are involved. From the equilibria

$$E + I \overset{K_D}{\leftrightharpoons} EI$$

$$E + H^+ \overset{K_a}{\leftrightharpoons} EH^+$$

in which $[E]$ is the site concentration, it is clear that

$$\frac{K'_D}{[I]} = \frac{[E]}{[EI]} \qquad (1)$$

At the midpoint (mp) of the titration curve defined by Eqn. 1, half of the total EI and half of E are converted into EH^+, thus

$$[EH^+]_{mp} = [E]_{mp} + [EI]_{mp}$$

$$[EH^+]_{mp} = [E]_{mp}\left(1 + \frac{[I]}{K'_D}\right)$$

or

$$\frac{[E]_{mp}}{[EH^+]_{mp}} = \frac{1}{1 + \frac{[I]}{K'_D}}$$

Substituting in Eqn. 2

$$K_a = \frac{[E][H^+]}{[EH^+]}$$

leads *via*

$$K_a = \frac{[H^+]_{mp}}{1 + \frac{[I]}{K'_D}}$$

to equation:

$$pK_a = pH_{mp} + \log\left(1 + \frac{[I]}{K'_D}\right)$$

in which K'_D is the dissociation constant for the unprotonated enzyme. Using the value of 0.24 mM for K'_D, the pK_a values calculated were 7.79, 7.79, 7.83, 7.77 and 7.54 (increasing inhibitor concentrations). The existence of species like EIH^+ cannot be excluded by these experiments. On the other hand we have no evidence for the presence of such a form, since the positions of the maxima and minima in difference spectra are not pH dependent (see DISCUSSION).

DISCUSSION

Inhibition studies with L-amino-acid oxidase[5] have shown that an aromatic ring, coupled with an anionic or cationic group is a requirement for inhibition of this enzyme. It was found that this charged group could be a carboxylate, a sulfonate or an amino acid ester. The present studies show that aromatic carboxylates, but not sulfonates and aliphatic carboxylates, influence the spectrum of the enzyme.

With D-amino-acid oxidase, similar, but less stringent, requirements for spectral effects were found[9,13]. With this enzyme, aromatic carboxylates also give the most effective inhibition, with large spectral effects, while sulfonates failed to bring about such effects. Aliphatic carboxylates, though less effective, also gave pronounced changes in the spectrum. Acid or basic amino acids as well as dicarboxylate inhibitors failed to combine with either enzyme[5,9], unless the charges are separated by a large number of atoms[9] or one of the charges is neutralized[5].

These effects have been explained as due to interactions between dissimilar charges, or, with the higher charged molecules, to repulsion of similar charges. The requirement for aromaticity suggests the presence of a second binding center with inhibitors, while substrates must have at least three binding centers to meet the requirements for stereospecifity. We would like to call all these interactions primary interactions to distinguish them from possible secondary changes brought about by these primary interactions in the protein (*i.e.* conformational changes[18], breakage or formation of hydrogen bonds[9], or changes in the structure of the internal complex of FAD[22]).

With reactions of aromatic systems, the Hammett relation is used to relate the effect of charge density on the reaction center at hand to the rate or equilibrium constant of such reactions[17,23]. This relation may be expressed for this particular application as:

$$\log K_i = \sigma\varrho + \log K_i^0$$

in which K_i and K^0_i are the inhibition constants, respectively, for the substituted and unsubstituted benzoate. σ represents the ability of the ring substituent to attract or repel electrons and ϱ is a measure of the sensitivity of the reaction series at hand to a change in charge density at the reaction center.

A linear relationship is only obtained with a single reaction center in the molecule with the assumption that no steric effects are introduced by the ring substituents. This presents a serious limitation in the application of this relation to biological systems (*cf.* ref. 23). Nevertheless this relation may be used to give an indication of such multiple interactions.

With D-amino-acid oxidase a good linear relationship was reported with substituted benzoates up to $\sigma = +0.5$, whereas the nitro-compounds showed a completely different behaviour[18]. With L-amino-acid oxidase the plot is much less ideal, but the same tendency is observed: the binding becomes stronger at higher σ values (negative ϱ value), while again the nitro-compounds behave differently. For an interaction of a negatively charged inhibitor molecule with a positive group on the enzyme, a positive ϱ value is expected: an electron-donating group facilitates the binding. We would conclude therefore that another interaction with a negative ϱ value plays a more important role in the binding process. This may well be an interaction of the nucleus of the aromatic inhibitor with an electropositive group in the enzyme, such as isoalloxazine or histidine. Histidine has been suggested as a good candidate for the binding of the charged amino group of the substrate[7]. It may well be that the imidazole ring system as a whole is also concerned in the binding of the inhibitors. The linearity of the Hammett plot suggests that such an interaction is more important in D-amino-acid oxidase than in L-amino-acid oxidase, where both interactions may have the same order of magnitude. An explanation of the different behavior of the nitro-compounds may be due to the strong electron-withdrawing properties of the nitro-group, resulting in a di-anionic structure. The enzymes would have less affinity for such a structure (*cf.* the reaction with dicarboxylates).

Because there is evidence that secondary reactions in the proteins also take place[9,18], there may be some doubt as to the validity of these assumptions. The measured K_i values represent the total change in the free energy of the system on addition of the inhibitor. The Hammett relation would still be valid if the change in free energy, caused by these other reactions is constant or proportional to the σ values.

The spectral changes observed here cannot be related directly to changes in ground state interactions. Our knowledge about the transitions which occur in the visible region of the absorption spectrum of FAD is inadequate for this. Some quantum mechanical calculations have been made for the isoalloxazine system[24,25], but these are still to approximate to account even for the λ_{max} of the lowest energy absorption bands.

At this time, however, the spectral changes, observed when the FAD is bound to the apoprotein or when inhibitors are added to the holoenzyme, can be related to similar changes observed when a model compound is subjected to known environmental changes. This work has been initiated by HARBURY *et al.*[26], who studied the effect of solvent polarity on the spectrum of 3-methyllumiflavin. However, their results cannot be related directly to protein-bound FAD, because the FAD in a protein may be involved in both non-polar and polar interactions (H-bond formation) at the same time. Another interaction, which may be of importance here, as pointed out by

WEBER[22], is a change in the structure of the internal complex of adenine and isoalloxazine, in which water seems to play an important role.

Inhibitors with a completely different electronic structure cause the same spectral shifts in each enzyme, while inhibitors with the same electronic arrangement cause different spectral effects in the two enzymes. It is unlikely therefore that a direct interaction occurs between the inhibitor and FAD, at least to the extent that the observed spectral changes are entirely due to such interaction. However, the spectral changes found with *o*-aminobenzoate which are similar in both enzymes, may be caused by such direct interaction and probably reflect a partial electron transfer, either in the ground state or in the exited state (charge–transfer complex).

MASSEY AND GANTHER[9] interpreted the spectral effects of inhibitors in D-amino-acid oxidase as being due to the abolition of hydrogen bonding between a charged amino-group and one of the carbonyl oxygens of FAD. However, a binding constant of 10^{-6} M^{-1} requires a decrease in free energy of about 8 kcal/mole at room temperature, whereas the abolition of hydrogen bonding increases this amount by about 4 kcal/mole. A decrease of about 12 kcal/mole would then be required from other interactions. The question is therefore left open as to what extent the abolition of hydrogen bonding to the flavin contributes directly to the overall change in the spectrum, whereas it is reasonable to assume that the major effects are due to the influence of conformational changes in the protein (*cf.* ref. 18).

It has been shown recently (*cf.* refs. 27, 28) that the catalytic mechanisms of D-amino-acid oxidase and L-amino-acid oxidase are closely related. Furthermore the phenomenon of substrate inhibition could be explained in terms of temperature-dependent conformational changes in both enzymes[18]. These studies show that the spectral effects observed upon the addition of certain competitive inhibitors, although different in magnitude and direction of the shifts, are probably based on the same phenomenon, *e.g.* changes in environment upon binding (*cf.* refs. 9, 13). Again the close relationship between these enzymes, isolated from completely different species is obvious.

The effect of pH on the spectrum of L-amino-acid oxidase will need further study. The reversible shift, found at low pH may be related to the tailing, observed in the titrations with inhibitors. This tailing effect is also observed in the pH *vs.* activity curves[21]. It may reflect a conformational change in the enzyme and also be related to the reversible inactivation process, whose pH dependence is in this region[7]. A direct effect (*i.e.* a red shift occurring in the free enzyme but not in the complex, thus causing a relatively increased absorption when going to lower pH) is unlikely due to the linearity of the curve with excess inhibitor (Fig. 8).

The group necessary in the free enzyme for the binding of the inhibitor has an apparent pK_a value of 7.8. This may be the pK value of the electropositive group to which the inhibitor nucleus is thought to be bound or any other group in the protein whose ionization indirectly affects the binding. The pK_a of histidine in this region is very sensitive to environmental changes, as has been pointed out by SINGER AND KEARNEY[7]. It may well be that a conformational change causes the relatively higher binding constants at low pH. Although no evidence was obtained in these studies for the existence of EIH^+, the presence of such a species with a much higher dissociation constant may provide another explanation for the tailing observed at the acid side of the titration curve.

The irreversible shift at high pH may be related to the ionization of the N-3 of isoalloxazine, as described by MASSEY AND GANTHER[9]. The slow, irreversible reaction may then be caused by the abolition of a previously existing hydrogen bond between N-3 and the enzyme. However, spectral changes in the near ultraviolet, as found by MASSEY AND GANTHER[9] were not observed here. Recent studies with lipoamide dehydrogenase have shown[29] that it is possible to bind N-3-methyl FAD and N-3-carboxymethyl FAD to the apoenzyme to obtain a catalytically active complex. Although attempts to bind these compounds to D- and L-amino-acid oxidase were unsuccessful, a direct interaction between N-3 and the protein seems rather doubtful.

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