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ON THE CATALYTIC MECHANISM OF D-AMINO-ACID OXIDASE

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SUMMARY

1. Upon varying the oxygen concentration, a series of parallel lines is obtained in the plot of the reciprocal velocity of the reaction catalysed by of D-amino-acid oxidase *versus* the reciprocal D-alanine concentration. In the presence of a competitive inhibitor, however, these lines do converge to a point in the third quadrant.

2. It can be concluded that in the reaction mechanism the substrate–reduced enzyme is reoxidized before the product dissociates from the enzyme, a conclusion supported by the observation that the product of the reaction is a competitive inhibitor towards the substrate.

3. Several relations between rate constants can be derived. For a limiting case it is possible to calculate some rate constants.

INTRODUCTION

D-Amino-acid oxidase (D-amino-acid:O_2 oxidoreductase (deaminating) EC 1.4.3.3) specifically catalyses the oxidation of glycine and D-amino acids. In the plot of the reciprocal velocity *versus* the reciprocal substrate concentration, straight lines are obtained. MASSEY AND co-workers[1,2] obtained parallel lines in the $1/v$ *versus* $1/[S]$ plot at different oxygen concentrations. This was confirmed by DIXON AND KLEPPE[3], who proposed that the product dissociates from the enzyme before the reduced enzyme is reoxidized by oxygen. On the other hand the catalytic mechanism proposed by MASSEY AND GIBSON[2] is different; the reduced enzyme is reoxidized before the product dissociates from the enzyme.

The present study shows that it is possible to resolve this difference in interpretation. It can be deduced that in a catalytic mechanism such as the one proposed by MASSEY AND GIBSON[2] the lines plotted for reciprocal velocity *versus* reciprocal substrate at different acceptor concentrations converge in the presence of high concentrations of a competitive inhibitor. In the mechanism proposed by DIXON AND KLEPPE[3] the lines are parallel in the presence of a competitive inhibitor. A useful method for distinguishing between the two mechanisms is the method of SLATER[4].

With succinate dehydrogenase (EC 1.3.99.1) a similar problem was solved[5–8] in

the same way. In fact it could be shown that the maximum activity of this enzyme is independent of the nature of the electron acceptors.

METHODS

D-Amino-acid oxidase was prepared from hog kidneys by the method of MASSEY, PALMER AND BENNETT[1]. The enzyme was freed of benzoate by reduction with an excess of D-alanine followed by precipitation at half-satd. $(NH_4)_2SO_4$ (*cf.* ref. 9). This was repeated twice after which the enzyme was dialysed overnight against 0.1 M pyrophosphate (pH 8.3). To be certain that not too much FAD had dissociated from the protein, an excess of FAD was added and the enzyme precipitated at half-satd. $(NH_4)_2SO_4$. The precipitate was washed several times with 50% aq. $(NH_4)_2SO_4$ and finally dissolved in 0.1 M pyrophosphate (pH 8.3). Spectrophotometric control ensured that the enzyme was free of benzoate.

The enzymatic activity was measured in Gilson differential respirometers at 37° under the conditions described by BURTON[10]. The different oxygen concentrations were obtained by mixing nitrogen and oxygen from cylinders and flushing these mixtures through the respirometers. The reaction was started by tipping the enzyme from the side arm. D-Alanine was used as substrate.

The stopped-flow experiments were performed with a Durrum–Gibson stopped-flow spectrophotometer at 26°. The velocity was calculated as moles of oxygen consumed per sec.

The experiment with pyruvate in the presence of ammonia was performed at 37° with the Gilson Oxygraph Model KM.

MATERIALS

D-Alanine was obtained from Fluka, Switzerland; pyruvate and catalase from Boehringer; FAD from Sigma; benzoic acid and pyrophosphate (Analar) from British Drug Houses.

RESULTS

DIXON AND KLEPPE[3] proposed the following kinetic reaction mechanism on the basis of their results:

Mechanism I

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES$$

$$ES \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} E'P$$

$$E'P \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} E' + P$$

$$E' + O_2 \xrightarrow{k_{+4}} E + H_2O_2$$

in which E is the oxidized and E' the reduced enzyme. For the steady-state with $[P] = 0$ in the presence of a competitive inhibitor (*cf.* refs. 3, 11), the rate equation becomes

$$v = \frac{V}{1 + \frac{K_S}{[S]}\left(1 + \frac{[I]}{K_i}\right) + \frac{K_O}{[O_2]}}$$

$[S]$ = D-alanine concentration; $[I]$ = inhibitor concentration; K_i = the dissociation constant of the enzyme–inhibitor complex. It is clear from this formula that the lines plotted from the values of $1/v$ *versus* $1/[S]$ at different oxygen concentrations, also in the presence of a competitive inhibitor, should be parallel.

An alternative is the following mechanism, which is related to the mechanism proposed by MASSEY AND GIBSON[2]:

Mechanism II

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES$$

$$ES \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} E'P$$

$$E'P + O_2 \xrightarrow{k_{+3}} EP + H_2O_2$$

$$EP \underset{k_{-4}}{\overset{k_{+4}}{\rightleftharpoons}} E + P$$

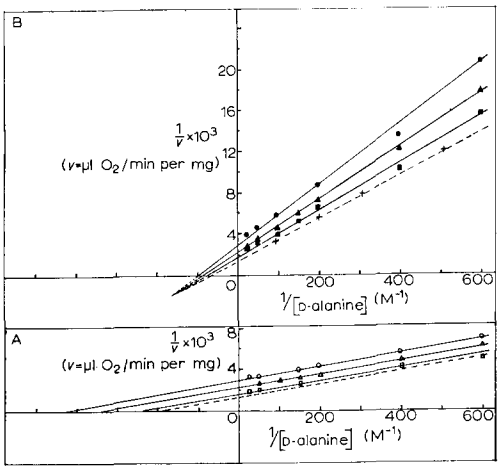
With the restriction that $[P] = 0$ in the presence of a competitive inhibitor, the following rate equation is valid for the steady state (*cf.* refs. 5, 11):

$$v = \frac{V}{1 + \frac{K_S}{[S]}\left(1 + \frac{[I]}{K_i}\right) + \frac{K_O}{[O_2]} + \frac{K_{OS}}{[S]\,[O_2]}\left(1 + \frac{[I]}{K_i}\right)}$$

From this equation it can be concluded that in the $1/v$ *versus* $1/[S]$ plot at different oxygen concentrations the lines normally converge, in the presence or absence of a competitive inhibitor.

Nevertheless, when $K_{OS}/[S]$ is small in comparison with K_O, the rate equation becomes similar to that derived for Mechanism I, with parallel lines at different oxygen concentrations. In the presence of the competitive inhibitor however, the term $K_{OS}/[S]$ is multiplied by the factor $(1 + [I]/K_i)$ and thus, depending on the ratio $[I]/K_i$, may make a considerable contribution to the rate equation, which results in converging lines. Therefore it is possible that in the absence of a competitive inhibitor the lines are parallel, but in its presence they converge.

Fig. 1A shows that our results confirm those of MASSEY and co-workers[1,2] and DIXON AND KLEPPE[3]. Parallel lines are obtained for different oxygen concentrations in the $1/v$ *versus* $1/[$D-alanine$]$ plot. Fig. 1B shows the same experiment carried out in the presence of a competitive inhibitor (benzoate, 50 μM). It is clear that under this condition the lines converge to a point in the third quadrant.



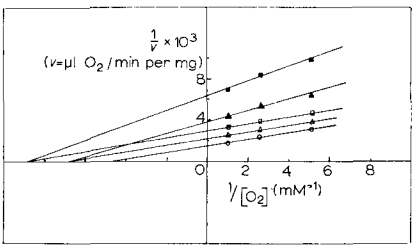
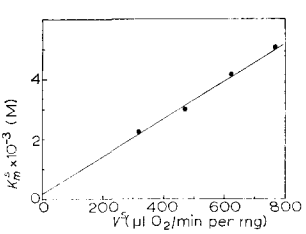


Fig. 1. A. $1/v$ *vs.* $1/$[D-alanine] plots at different oxygen concentrations. ○—○, 20% O_2; △—△, 40% O_2; □—□, 100% O_2; - - - -, extrapolated to infinite oxygen concentration at 37°. B. $1/v$ *vs.* $1/$[D-alanine] plots in the presence of 50 μM benzoate at different oxygen concentrations. ●—●, 20% O_2; ▲—▲, 40% O_2; ■—■, 100% O_2; +—+, at infinite oxygen concentration.

Fig. 2. $1/v$ *vs.* $1/[O_2]$ plots at different concentrations of D-alanine □—□, 5 mM D-alanine; △—△, 10 mM D-alanine; ○—○, at infinite D-alanine concentration. The plots ■—■ and ▲—▲ are at respectively 5 mM and 10 mM D-alanine, but in the presence of 50 μM benzoate.

From the rate equations it can be concluded that the inhibitor acts in Mechanism I uncompetitively and in Mechanism II noncompetitively towards oxygen. Fig. 2 gives the $1/v$ *versus* $1/[O_2]$ plot from which it is clear that the inhibitor acts noncompetitively towards oxygen.

For Mechanism II there are not enough relationships between the parameters to allow the calculation of the individual rate constants from the kinetic data. From



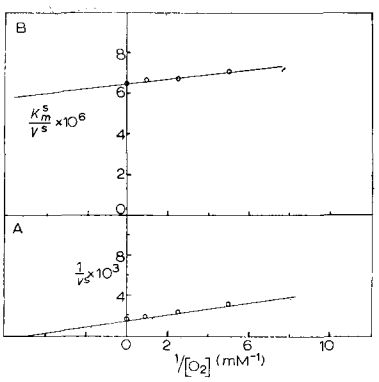


Fig. 3. The plot K_m^s *vs.* V^S. The values of K_m^s and V^S are calculated from Fig. 1A. The line is calculated by the method of the least squares.

Fig. 4. A. The plot $1/V^S$ *vs.* $1/[O_2]$. These values are calculated from Fig. 1A. B. The K_m^s/V^S *vs.* $[1/O_2]$. The values of K_m^s/V^S are calculated from Fig. 1A.

the rate equation however, the following relations between the kinetic parameters can be derived in which V^S is the velocity at an infinite concentration of D-alanine and a finite concentration of oxygen and K_m^S is the apparent Michaelis constant in the $1/v$ *versus* $1/$[D-alanine] plot at that particular oxygen concentration.

$$K_m^S = V^S\left(\frac{k_{-1}+k_{+2}}{k_{+1}k_{+2}[E_0]} - \frac{k_{-1}k_{+2}k_{-2}+k_{-1}k_{-2}k_{+4}}{k_{+1}k_{+2}k_{+4}(k_{+2}+k_{-2})\,[E_0]}\right) + \frac{k_{-1}}{k_{+1}}\left(\frac{k_{-2}}{k_{+2}+k_{-2}}\right)$$

$$1/V^S = \frac{k_{+2}+k_{-2}}{k_{+2}k_{+3}[E_0]} \cdot \frac{1}{[O_2]} + \frac{k_{+2}+k_{-4}}{k_{+2}k_{+4}[E_0]}$$

$$K_m^S/V^S = \frac{k_{-1}k_{-2}}{k_{+1}k_{+2}k_{+3}[E_0]} \cdot \frac{1}{[O_2]} + \frac{k_{-1}+k_{+2}}{k_{+1}k_{+2}[E_0]}$$

$[E_0]$ = total enzyme concentration.

When the method of SLATER[4] is applied, the plot K_m^S *versus* V^S (Fig. 3) gives a straight line which does not pass through the origin, as was calculated by the method of the least squares. The intercept with the ordinate ($V^S = 0$) is equal to $k_{-1}k_{-2}/k_{+1}(k_{+2}+k_{-2})$. In the $1/V^S$ *versus* $1/[O_2]$ plot (Fig. 4A) the intercept with the ordinate ($1/[O_2] = 0$) is

$$\frac{k_{+2}+k_{+4}}{k_{+2}k_{+4}} \cdot \frac{1}{[E_0]}$$

From the plot K_m^S/V^S *versus* $1/[O_2]$ the intercept with the abscissa ($K_m^S/V^S = 0$) is

$$\frac{k_{+3}(k_{-1}+k_{+2})}{k_{-1}k_{-2}}$$

and with the ordinate the intercept ($1/[O_2] = 0$) is

$$\frac{k_{-1}+k_{+2}}{k_{+1}k_{+2}[E_0]}$$

The relationships between the kinetic parameters are summarized in Table I.

It is possible to calculate the rate constants for the limiting case, that the enzyme–

TABLE I

THE RELATIONS BETWEEN KINETIC PARAMETERS CALCULATED FOR MECHANISM II, WITH D-ALANINE AS SUBSTRATE AT 37°

Conditions as described in the text.

$\frac{k_{+1}(k_{+2}+k_{-2})}{k_{-1}\cdot k_{-2}} = 5\cdot 10^3$ mole$^{-1}\cdot$l	$K_S = 4\cdot 10^{-3}$ mole$\cdot$l^{-1} $K_O = 4\cdot 10^{-4}$ mole$\cdot$l^{-1}
$\frac{k_{-1}+k_{+2}}{k_{+1}\cdot k_{+2}} = 8\cdot 10^{-5}$ mole$\cdot$l$^{-1}\cdot$sec	$K_{OS} = 8\cdot 10^{-8}$ mole$^2\cdot$l^{-2}
$\frac{k_{+3}(k_{-1}+k_{+2})}{k_{-1}\cdot k_{-2}} = 54\cdot 10^3$ mole$^{-1}\cdot$l	$\frac{k_{+1}k_{+2}k_{+3}}{k_{-1}k_{-2}} = 7\cdot 10^8$ mole$^{-2}\cdot$l$^2\cdot$sec^{-1}
$\frac{k_{+2}+k_{+4}}{k_{+2}\cdot k_{+4}} = 18\cdot 10^{-3}$ sec	

substrate complex is rapidly converted into either $E'P$ or the free enzyme, *e.g.* Mechanism IIA (*cf.* refs. 4, 5)

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E'P$$

$$E'P + O_2 \xrightarrow{k_{+3}} EP + H_2O_2$$

$$EP \underset{k_{-4}}{\overset{k_{+4}}{\rightleftharpoons}} E + P$$

The following kinetic relationships can be derived:

$$1/V^S = \frac{1}{k_{+1}[E_0]}\left(1 + \frac{k_{+4}}{k_{+3}[O_2]}\right)$$

Upon plotting $1/V^S$ *versus* $1/[O_2]$ a straight line is obtained (Fig. 4A), which does not pass through the origin. The intercept with the ordinate ($1/[O_2] = 0$) equals $1/k_{+4}[E_0]$ from which k_{+4} can be calculated. The intercept on the abscissa ($1/V^S = 0$) is equal to $-k_{+3}/k_{+4}$, from which k_{+3} can be calculated.

$$K_m^S = \frac{(k_{-1} + k_{+3}[O_2])\, k_{+4}}{(k_{+3}[O_2] + k_{+4})\, k_{+1}}$$

therefore

$$\frac{K_m^S}{V^S} = \frac{1}{k_{+1}[E_0]}(1 + k_{-1}/k_{+3}[O_2]).$$

The plot K_m^S/V^S *versus* $1/[O_2]$ gives a straight line (Fig. 4B), which does not pass through the origin, while the intercept with the ordinate is equal to $1/k_{+1}[E_0]$. On the abscissa ($K_m^S/V^S = 0$) the intercept is equal to $-k_{+3}/k_{-1}$. Table II summarizes the values of the different constants calculated from Fig. 4A and B.

By the stopped-flow method[12,13] more evidence for Mechanism II is provided. It has been demonstrated that in the catalysis a spectral intermediate is involved[2], which can be monitored at 550 nm, a wavelength at which neither the oxidized nor the reduced enzyme shows any absorption. By analysis of the time course of the formation and disappearance of this spectral intermediate upon mixing the enzyme with oxygen and an excess of substrate, it is possible to calculate the rate of oxygen consumption at a number of oxygen concentrations from just one experiment. Fig. 5 shows that in the absence of benzoate a series of parallel lines is obtained (*cf.* ref. 14). On the other hand the addition of benzoate (1.25 μM) changes the slopes of the lines

TABLE II

RATE CONSTANTS CALCULATED FOR MECHANISM IIA, WITH D-ALANINE AS SUBSTRATE AT 37°
Conditions as described in the text.

$k_{+1} = 1.3 \cdot 10^4$ mole$^{-1}\cdot$l$\cdot$sec^{-1}
$k_{-1} = 50$ sec^{-1}
$k_{+3} = 2.6 \cdot 10^5$ mole$^{-1}\cdot$l$\cdot$sec^{-1}
$k_{+4} = 55$ sec^{-1}

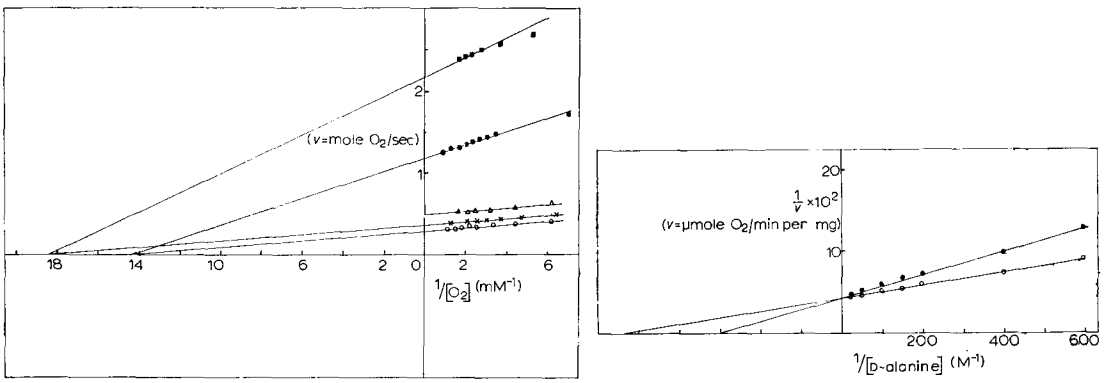


Fig. 5. The plots $1/v$ *vs.* $1/[O_2]$ at different D-alanine concentrations at 26°. These results are obtained with the stopped-flow apparatus by applying the method described in refs. 11, 12. Benzoate-free D-amino-acid oxidase (15.6 μM enzyme–flavin) is mixed with 0.64 mM O_2 at different D-alanine concentrations in the presence of an excess of FAD (0.1 mM). ○—○, 10 mM D-alanine; ×—×, 5 mM D-alanine; △—△, 2.5 mM D-alanine. The plots ●—● and ■—■ are at respectively 10 and 5 mM D-alanine, but in the presence of 1.25 μM benzoate. (All concentrations after mixing.

Fig. 6. $1/v$ *vs.* 1/[D-alanine] plot showing the competitive inhibition of pyruvate (50 mM) in the presence of NH_4^+ (50 mM) at 37°.

in the plot $1/v$ (v = moles O_2 consumed per sec) *versus* $1/[O_2]$. In agreement with Mechanism II, the inhibition is noncompetitive towards O_2.

Mechanism II also predicts that the product of the reaction has to be competitive towards the substrate. Fig. 6 shows that pyruvate in the presence of NH_4^+ acts according to this prediction. The inhibition constant is 38 mM.

DISCUSSION

From the results shown it can be concluded in agreement with MASSEY and co-workers[1,2] that in the catalytic overall reaction of D-amino-acid oxidase, *e.g.* the oxidation of D-alanine by O_2, after reduction by substrate, the enzyme is reoxidized before the product dissociates from the enzyme. This conclusion disagrees with the mechanism proposed by DIXON AND KLEPPE[3], in which the product first dissociates from the enzyme before the latter is reoxidized. The reason for this discrepancy lies in the fact that in the rate equation for Mechanism II, the term $K_{os}/[S]$ is relatively small compared with K_0, which means that this mechanism apparently changes into Mechanism I. The term $K_{os}/[S]$ in the presence of a competitive inhibitor is multiplied by a factor $(1 + [I]/K_i)$, which will therefore manifest itself in the rate equation.

As has been pointed out by DIXON[15], the rate equation for a two-substrate mechanism which does not give a term depending on both substrates, will show in the K_m^S *versus* V^S plot as introduced by SLATER[4] a straight line which passes through the origin:

$$V^S = V[S_2]/([S_2] + K_2)$$

$$K_m^s = K_1[S_2]/([S_2] + K_2)$$

However, in the case of a mechanism which gives a term in the rate equations depend-

ing on both substrates, the straight line in the K_m^S *versus* V^S plot does not pass through the origin:

$$V^S = V[S_2]/([S_2] + K_2)$$
$$K_m^S = (K_1[S_2] + K_{12})/([S_2] + K_2).$$

This is due to the contribution of that term in the equation for K_m^S. If this contribution is small, the lines obtained in the $1/v$ *versus* $1/[S]$ plots at different concentrations of the second acceptor apparently become parallel. The line in the K_m^S *versus* V^S plot, however, does not pass through the origin, although its point of intersection with the K_m^S axis may be very close to it. In such a case the use of a competitive inhibitor is recommended to distinguish clearly between the two mechanisms.

The results of RADHAKRISNAN AND MEISTER[16] showed that the catalytic reaction is reversible under anaerobic conditions. Their results are easily explained by Mechanism I and were interpreted by DIXON AND KLEPPE as a support for it. However, these results can also be explained by Mechanism II. It has been shown (ref. 17 and J. F. KOSTER AND C. VEEGER, unpublished results) that when the enzyme is mixed with the substrate a broad absorption band above 500 nm appears transiently and disappears upon full reduction of the enzyme-flavin. This band belongs to a catalytically active intermediate. When pyruvate and ammonia are added to the fully reduced enzyme this absorption band is restored. Since the spectral properties of this band depend on the product of the enzymatic reaction, it is clear that the reaction $E'P \rightleftharpoons E' + P$ exists as a slow side reaction, which becomes important under anaerobic conditions in static experiments like spectral studies. Thus it is clear that the results of RADHAKRISNAN AND MEISTER can also be obtained in case of Mechanism II.

Further support for this mechanism comes from the observation that pyruvate, in the presence of ammonia and in the process of forming the imino acid (product of the reaction), inhibits competitively towards the substrate D-alanine. This is in full agreement with Mechanism II, but quite difficult to understand if Mechanism I should be valid.From previous studies[18–20] it is known that the oxidized enzyme in the pre- pyruvate shows the same spectral effect as in the presence of the competitive inhibitor sence of benzoate.

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