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THE RELATION BETWEEN TEMPERATURE-INDUCIBLE ALLOSTERIC EFFECTS AND THE ACTIVATION ENERGIES OF AMINO-ACID OXIDASES

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SUMMARY

1. In the catalytic reaction at 37° of D- and L-amino-acid oxidases with D-alanine and L-leucine, respectively, as substrate, benzoate and its derivatives act as competitive inhibitors. The Hammett plots show no straight-line relationship between the K_i and the σ values of the different substitutents.

2. With D-amino-acid oxidase (D-amino acid:O_2 oxidoreductase (deaminating), EC 1.4.3.3) at 10° in the $1/v$ *vs.* $1/[\text{D-alanine}]$ plot, the lines obtained in the presence and in the absence of inhibitor (benzoate or its derivatives, with the exception of the nitro-substituents), intercept at finite [D-alanine]. This was also the case with D-methionine as substrate. At 25° with D-methionine substrate inhibition is found at relatively high concentrations, which is not observed with D-alanine. This substrate inhibition can be abolished by benzoate and ATP, but not by *m*- or *p*-nitrobenzoate. ATP acts as non-competitive inhibitor, but only inhibits the reaction partially. At 37° the inhibition by ATP is abolished by benzoate. Titration of D-amino-acid oxidase with benzoate showed that, to obtain 50% saturation, 2–3 times more benzoate is necessary in the presence of an excess of ATP than in its absence. ATP itself does not induce spectral shifts.

It is concluded that two forms of D-amino-acid oxidase are present, a low-temperature and a high-temperature conformation, which have about the same activation energies, but differ in activity, due to different transition probabilities (ΔS^* = 0.7 e.u.). ΔH for this conformational change is 55 000 cal·mole^{-1}; ΔS 185 e.u. It can be shown that benzoate and its derivatives have more affinity for the high-temperature conformation, while ATP has more affinity for the low-temperature form; *m*- and *p*-nitrobenzoate have equal affinity for both forms.

3. With L-amino-acid oxidase (L-amino-acid:O_2 oxidoreductase (deaminating), EC 1.4.3.2) it was found that ATP and pyrophosphate influence the catalytic oxidation reaction. The linear Arrhenius plot obtained with L-leucine as substrate is converted by the addition of pyrophosphate into a Z-shaped curve. The substrate inhibition in the presence of L-leucine is abolished by pyrophosphate at low temperature. In the presence of L-valine as substrate a Z-shaped curve is obtained, of which, as in the case of L-leucine, the high-temperature and low-temperature parts are parallel. In the presence of pyrophosphate the curve shows one break. As in the case with D-amino-

acid oxidase, this enzyme also exists in two conformations with different activities and entropies of activation ($\Delta S^* = 1.8$–2.3 e.u.), but with the same activation energies. From the transition it can be calculated that $\Delta H = 46\,000$ cal·mole^{-1} and $\Delta S = 145$ e.u. for the conformational transition from the low- to the high-temperature form.

4. The general phenomenon of non-linear Arrhenius plots of enzyme-catalyzed reaction is discussed in connection with these observations.

INTRODUCTION

NEIMS, DELUCA AND HELLERMAN[1] investigated with D-amino-acid oxidase (D-amino-acid:O_2 oxidoreductase (deaminating), EC 1.4.3.3) the relationship between the logarithm of the maximum velocity and the σ values for *m*- and *p*-substituted phenylalanine and *C*-phenylglycine. They obtained an asymmetrical, biphasic plot with a maximum at $\sigma = + 0.04$ for *C*-phenylglycine and at $\sigma = + 0.23$ for phenylalanine. Although it is known that benzoate[2] and some substituted benzoates[3] are competitive inhibitors of this enzyme, no attempt was made to investigate a correlation between the inhibition constants and the corresponding σ values.

With L-amino-acid oxidase (L-amino-acid:O_2 oxidoreductase (deaminating), EC 1.4.3.2), RADDA[4] investigated the correlation between the velocity and the σ value of ring-substituted phenylglycines as substrates in one concentration. As with D-amino-acid oxidase, benzoate is a competitive inhibitor for L-amino-acid oxidase[5]. ZELLER AND CLAUS[6] reported, however, that they could not find a correlation between the v_{max}, K_m and K_i determined for ring-substituted phenylalanine as substrate, the various benzoic acids as inhibitors and the σ values.

MASSEY, CURTI AND GANTHER[7] reported that D-amino-acid oxidase shows a discontinuous Arrhenius plot, from which it was concluded that the enzyme exists in two forms, a low-temperature form and a high-temperature form, in equilibrium with each other, having different activation energies. The temperature at which the break in the plot occurs, is dependent on the substrate used. With D-alanine it is at about 14°, while with D-methionine it is at 24°. In connection with fluorescence and ultraviolet-spectral studies, it was concluded that D-alanine has equal affinities for both forms, while D-methionine has more affinity for the low-temperature form than for the high-temperature form. With other enzymes a different explanation has been given for this phenomenon, *e.g.* an equilibrium between two or more forms differing in their catalytic activities[8] or a temperature-induced conformational change of the active site[9].

In this paper we present evidence that benzoate, substituted benzoates and ATP have different affinities for both forms, which differ in catalytic activity. Furthermore it will be shown that L-amino-acid oxidase can undergo temperature-dependent conformational changes, which depend on the substrate used and are influenced by ATP and by pyrophosphate.

Preliminary data of this work have been published elsewhere[10,11].

METHODS

D-Amino-acid oxidase (EC 1.4.3.3) was prepared from hog kidneys according to the method of MASSEY, PALMER AND BENNETT[12] and was made benzoate-free by treatment with excess of D-alanine[13]. L-Amino acid oxidase (EC 1.4.3.2) was isolated

from snake venom (*Crotalus adamanteus*) according to the method of WELLNER AND MEISTER[14].

The enzymatic activities were measured with the Gilson Oxygraph Model KM. A polarizing voltage of 0.63 V was applied to the electrode. The oscillation frequency of the electrode was 120 cycles/sec and the greatest amplitude was used. The amount of O_2 consumed was calculated from the slope of the recorded line. The conditions described by BURTON[2], excepting catalase were used for D-amino-acid oxidase; for L-amino-acid oxidase, those described by WELLNER AND MEISTER[14] were used.

Agla micrometer syringes (Burroughs Wellcome and Co., London) were used for the spectrophotometric titration experiments. The optical differences were recorded with the 0–0.1 absorbance slide wire of the Cary model 14 recording spectrophotometer. The differences were recorded at 497.5 nm.

MATERIALS

D-Alanine and D-methionine were obtained from Fluka, Switzerland; ATP and GTP from Boehringer and Söhne; L-leucine, benzoate and the substituted benzoates from British Drug Houses. L-Leucine was recrystallized; some of substituted benzoates (B.D.H.) were first treated with charcoal after which they were recrystallized.

RESULTS AND DISCUSSION

The relation between the inhibition constants and the σ values of the substituted benzoates

Benzoate and the substituted benzoates inhibit D-amino-acid oxidase competitively. Table I summarizes the inhibition constants of benzoate and the ring-substituted benzoate derivatives for D-amino-acid oxidase, measured at 37° with D-alanine as substrate. In general the *o*-substituted benzoates give a lower inhibition than the *m*- and *p*-substituted derivatives, the *m*-substituents giving the highest inhibition. Exceptions are the hydroxy- and amino-substituents, of which the *o*-

TABLE I

THE INHIBITION CONSTANTS (μM) OF BENZOATE AND BENZOATE DERIVATIVES IN THE CATALYTIC OVERALL REACTION OF D-AMINO-ACID OXIDASE

The inhibition constants were determined by means of the Lineweaver Burk plots. These experiments were performed at 37° in 0.1 M pyrophosphate buffer (pH 8.3) with D-alanine as substrate.

Substituent	K_i (μM) *of*		
	o-	*p*-	*m*-
H	20.4		—
NO_2	1370	38	11.1
OCH_3	1130	110	208
OH	76	330	254
F	294	3.2	2.9
Cl	361	10.6	2.9
Br	385	10.2	2.9
I	648	20.5	12.6
$N(CH_3)_2$	—	1000	1000
NH_2	103	313	704

substituted derivatives give the highest inhibition, probably due to the formation of hydrogen bridges. The lower inhibition with the other *o*-substituted benzoates could be due to steric hindrance, but *o*-fluorobenzoate (fluoro is in size comparable with hydrogen) has an inhibition constant of the same order as the other halogen-substituted benzoates. The results confirm those of BARLETT[15] and the spectrophotometric experiments of MASSEY AND GANTHER[16].

Fig. 1 shows the logarithm of the inhibition constants of the benzoate-derivatives for D-amino-acid oxidase obtained at 37°, plotted against the σ value. The line drawn

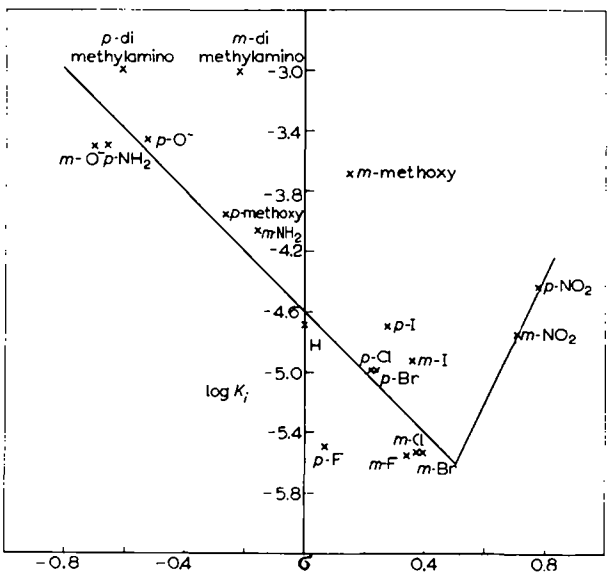


Fig. 1. Hammett plot of the action of ring-substituted benzoate derivatives with D-amino-acid oxidase. The logarithms of the inhibition constants at 37° are plotted against their σ-values. The K_i's were determined from Lineweaver–Burk plots; D-alanine was used as substrate. The line drawn was calculated by the method of the least squares; in this calculation *m*-dimethylamino-benzoate and *m*-methoxybenzoate were not taken into account for reasons of steric hindrance.

is calculated by the method of the least squares. The σ value is experimentally obtained for the individual substituents[17]; the σ value of hydrogen is arbitrarily chosen as zero, and a positive value of σ means a stronger electron-attracting character than hydrogen. The plot is biphasic with a minimum at $\sigma = +0.5$. The slope of the line of the substituents with a σ value lower than 0.5 has a ϱ value of -2.0, with a correlation coefficient $r = -0.6$.

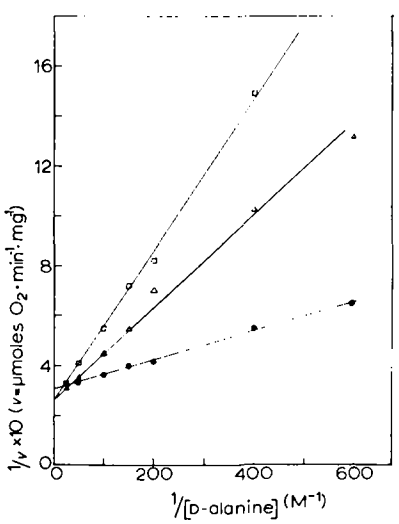
From the plot it can be concluded that the inhibition of the catalytic reaction increases with increasing electron-attracting character (increasing σ value) of the substituents. The other part of the plot is formed by the *m*- and *p*-nitrobenzoates. The slope of this part has a ϱ value of about $+4.9$. The substituents with a σ value smaller than 0.5 have a $-I$ and $+R$ effect, while the nitro-derivatives have a $-I$ and $-R$ effect. NEIMS, DELUCA AND HELLERMAN[1], working with ring-substituted substrates, found that increasing σ values lead to an increasing maximum velocity up to

a σ value depending on the substrate used. At a higher σ value, a decline in activity is observed.

A similar plot made for L-amino-acid oxidase differs in some respects from that of D-amino-acid oxidase, but the behavior of *m*- and *p*-nitrobenzoate is similar to both enzymes. Other substituted benzoates with negative σ values have approximately the same inhibition constants with L-amino-acid oxidase. The *m*-halogen-substituted derivatives, on the other hand, show a sharp increase in inhibition. This behavior at 37° differs only quantitatively from that at 25°; binding of these derivatives induce a shift of the flavin absorption band to shorter wavelengths[18]. The results show that there is a correlation between σ value and the inhibition constant in agreement with the results of RADDA[4] and in contrast to the results of ZELLER and co-workers[5,6].

The effect of benzoate, substituted benzoates and ATP on D-amino-acid oxidase

The $1/v$ *vs.* $1/$[D-alanine] plot at 10° of Fig. 2 shows that the line obtained in the presence of benzoate (similar phenomena are found with substituted benzoates) intersects the line obtained in the absence of inhibitor at finite concentration of D-alanine. At temperatures of 15° and higher benzoate and derivatives are competitive inhibitors (*cf.* ref. 19); on the other hand, *m*- and *p*-nitrobenzoate exhibit strictly competitive behavior over the temperature range 5–40°. The same phenomenon is evident at 10° with D-methionine as substrate (Fig. 3). In this case the effect disappears as the temperature is raised above 24° and the inhibition becomes competitive. The most striking phenomenon in these experiments is the observation that, at high concentration of both D-alanine and D-methionine, the inhibitory effect of benzoate is replaced by an activating effect. In this respect benzoate and its derivatives behave like allo-



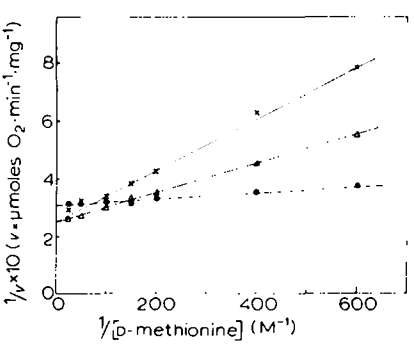


Fig. 2. Effect of benzoate on D-amino-acid oxidase with D-alanine as substrate. Conditions described under METHODS. Temp., 10°. ●—●, no benzoate; △ △, [benzoate] = 5 μM; □ □, [benzoate] = 10 μM.

Fig. 3. Effect of benzoate on D-amino-acid oxidase with D-methionine as substrate. Conditions described under METHODS. Temp., 10°. ●—●, control; △ △, [benzoate] = 7.5 μM; ×—×, [benzoate] = 25 μM.

steric inhibitors (*cf.* refs. 20, 21), with the difference that the activating effect is not visible at low substrate concentrations. The model of MONOD, WYMAN AND CHANGEUX[21], which approaches this phenomenon mathematically, predicts that a concerted transition mechanism will show substrate inhibition in case the substrate has a higher affinity to a catalytically inactive than to a catalytically active state.

If the discontinuity of the Arrhenius plot[7] is due to the presence of an inactive form at lower temperature, it can be expected that substrate inhibition will be observed in case the substrate has a higher affinity for the inactive form. When the low temperature form is less active rather than inactive, as assumed in the allosteric theory, only under certain conditions can substrate inhibition be observed. By assuming that the enzyme concentration is much smaller than the substrate concentration, one can derive that, for the steady-state approximation, the general equation for the initial rate of the reaction becomes:

$$v = \frac{n\{(1+\alpha)^n v^{R}_{max}[S]/([S]+K_m^{R}) + L(1+c\alpha)^n v^{T}_{max}[S]/([S]+K_m^{T})\}}{(1+\alpha)^n + L(1+c\alpha)^n} \quad (1)$$

n is the number of substrate binding sites; L is the equilibrium constant between the R-form and T-form free of ligand, defined as $[T] = L[R]$; α is the ratio of the free substrate concentration and the dissociation constant for the binding to the R-form; c is the ratio of the dissociation constants for the R- and the T-form, *i.e.* $c = K^{R}_{D}/K^{T}_{D}$; v^{T}_{max} and v^{R}_{max} are the maximum velocities for the T-state and R-state, respectively, per catalytic site; K^{T}_{m} and K^{R}_{m} are the Michaelis constants for the T-state and the R-state, respectively. It must be pointed out that v^{T}_{max}, v^{R}_{max}, K^{T}_{m} and K^{R}_{m} vary with the concentration of the second substrate[19]. Furthermore it is assumed that this second substrate does not influence the equilibrium between R and T. This equation which is derived from the fraction of protein in the R- and T-states is valid, provided the

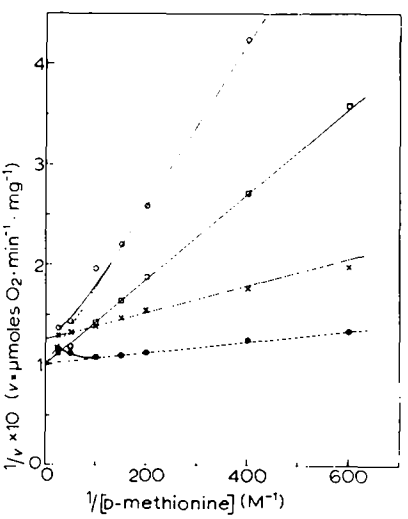


Fig. 4. Effects of effectors on the substrate inhibition of D-amino-acid oxidase with D-methionine as substrate. Conditions described under METHODS. $1/v$ *vs.* $1/[\text{D-methionine}]$ plot with different additions at 25°. ●—●, control; □—□, [benzoate] = 75 μM; ○—○, [*p*-nitrobenzoate] = 0.3 mM; ×—×, [ATP] = 20 mM.

equilibrium between these forms is established at the time of the first rate measurement. This equation is, in our opinion, preferable to the relation of the ligand saturation function with the velocity (*cf.* ref. 22), since this binding function does not take into consideration other possible intermediates in the conversion of substrate to product. The rate is determined by the normal kinetic parameters. Since the K_m for most enzymes is not related to the dissociation constant of the enzyme–substrate complex, it is better not to use the latter in these equations unless a special mechanism is involved (*cf.* refs. 23, 24).

It can be seen from Eqn. 1 that in the case when the equilibrium constant L is small (but the substrate has more affinity for the less active T-form) at high concentration substrate inhibition will be observed. Fig. 4 shows that this is the case at high concentration of D-methionine. At higher or lower temperatures, this inhibition disappears. With D-alanine no substrate inhibition is observed at any temperature, indicating that this substrate has about equal affinities for both forms (*cf.* ref. 7). The observation (Figs. 2 and 3) that the activating effect of benzoate with D-methionine is more pronounced than with D-alanine is in agreement with the above-mentioned conclusion, provided benzoate has a higher affinity for the R-conformation. Furthermore these figures show that the plots in the presence of inhibitor remain straight at lower concentrations of benzoate. At higher levels of benzoate, deviation from linearity is observed at high concentrations of D-methionine. Extrapolation of the initial slope to infinite substrate concentration gives the same interception on the $1/v$ axis as obtained with the line at low level of benzoate. These results indicate that benzoate has a higher affinity for the high-temperature form than for the low-temperature state.

The presence of a non-reacting ligand binding to both states with different affinities modifies Eqn. 1 into:

$$v = \frac{n\{(1+\alpha)^n(1+\beta)^n v^{R}_{max}[S]/(K_s + K_m^{R}) + L(1+c\alpha)^n(1+d\beta)^n v^{T}_{max}[S]/(K_s + K_m^{T})\}}{(1+\alpha)^n(1+\beta)^n + L(1+c\alpha)^n(1+d\beta)^n} \quad (2)$$

In case the ligand is a competitive inhibitor Eqn. 2 changes to:

$$v = \frac{n\{(1+\alpha+\beta)^n v^{R}_{max}[S]/([S] + K_m^{R}(1+\beta)) + L(1+c\alpha+d\beta)^n v^{T}_{max}[S]/([S] + K_m^{T}(1+d\beta))\}}{(1+\alpha+\beta)^n + L(1+c\alpha+d\beta)^n} \quad (3)$$

In these equations β is the ratio of the free-ligand concentration and the dissociation constant of the R-form; d is the ratio of the dissociation constants of the binding of the ligand to the R- and T-form.

The main factors which determine the effects to be observed are L, c and d. Thus it can be expected that the substrate inhibition observed without benzoate (Fig. 4) is abolished upon benzoate addition. At the temperature used in these experiments, L is small and $c > 1$, which exerts its influence only at very high D-methionine concentrations. By adding benzoate $d < 1$, this effect is delayed and substrate inhibition is apparently abolished. The results obtained are those for true competitive inhibition.

Nitrobenzoates (*m*- and *p*-) act as true competitive inhibitors over the temperature range 5–40° with D-alanine as substrate. With D-methionine as substrate, however, although extrapolation on the initial slope indicates a true competitive inhibition (Fig. 4), at high concentrations of substrate deviation from the linearity and substrate inhibition occurs. It must be pointed out, however, that this only occurs

at about 25°. At higher or lower temperatures the inhibition is competitive. In connection with the ideas developed, it is clear that these inhibitors have about equal affinities for both forms ($d \approx 1$).

With D-alanine as substrate ATP is a non-competitive inhibitor. The concentration of ATP which inhibits the activity is strongly temperature dependent, for instance at 5° hardly any inhibition is observed in the presence of 200 μM ATP and a small inhibition (less than 8%) with 20 mM ATP; at 37° 300 μM ATP gives an inhibition of 26% (Figs. 5A, B). Furthermore it must be remarked that GTP in about the same concentration gives similar results. Fig. 4 shows that, in the presence of a large concentration of ATP, no substrate inhibition is obtained with high [D-methionine]. ATP inhibits the enzyme only partially, since a 100-fold increase in concentration does not affect the inhibition very much (Fig. 6). Furthermore at that high ATP concentration, in contrast to the low, substrate inhibition is observed. Although only at very high concentration of D-methionine is a deviation from the linearity observed, it must be remarked that three different experiments show this effect.

These results indicate that ATP has a higher affinity for the low-temperature conformation than for the high-temperature form. This non-competitive inhibition is due to a shift of the equilibrium between the two states. This equilibrium lies at 37°

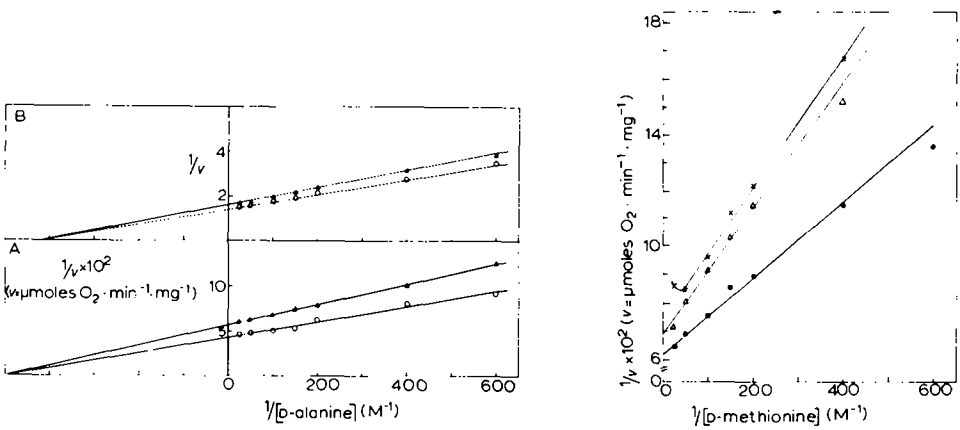


Fig. 5. Effect of temperature on the inhibition of D-amino-acid oxidase by ATP with D-alanine as substrate. Conditions described under METHODS. A. Temp., 37°, ○—○, control; △—△, [ATP] = 200 μM. B. Temp., 5°, ○—○, control; △—△, [ATP] = 20 mM.

Fig. 6. Effect of ATP on D-amino-acid oxidase with D-methionine as substrate at 37°. Conditions described under METHODS. ○—○, control; △—△, [ATP] = 200 μM; × ··· ×, [ATP] = 20 mM.

toward the R-form, at 5° toward the T-form. It is the cooperation between D-methionine and ATP, both having higher affinity to the T-form, which at 37°, where the allosteric constant is small, causes substrate inhibition only at very high concentrations of both compounds.

Figs. 7 and 8 provide further evidence for the idea that benzoate and ATP have their highest affinity to different forms of the enzyme. Fig. 7 shows that the non-competitive inhibition of ATP at 37° is abolished by the addition of benzoate. The fact that the curve in the presence of benzoate is convex towards the abscissa is in

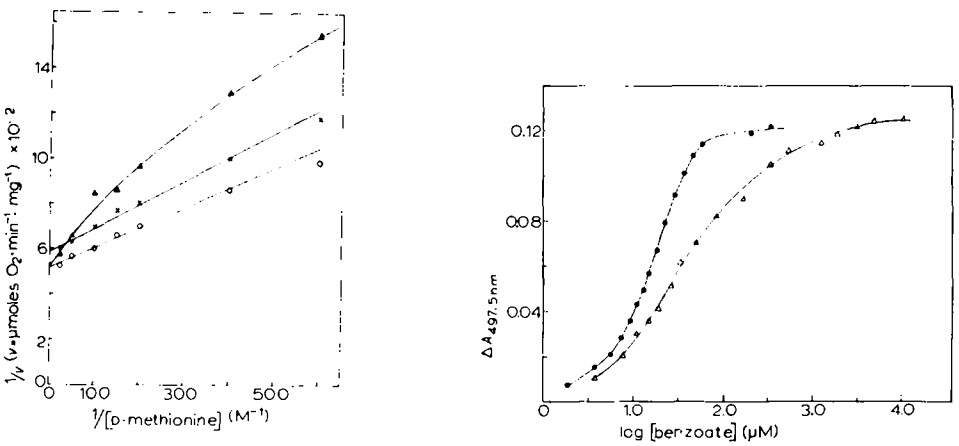


Fig. 7. Effect of ATP and benzoate on D-amino-acid oxidase with D-methionine as substrate at 37°. Conditions described under METHODS. ○—○, control; △—△, [ATP] = 200 μM + 10 μM benzoate; ×—×, [ATP] = 200 μM.

Fig. 8. Effect of ATP on the formation of the spectral enzyme–benzoate complex of D-amino-acid oxidase. Differences in $A_{497.5\ nm}$ obtained by titration with benzoate are plotted. The values are corrected for dilution. ●—● without ATP; △—△, in the presence of 20 mM ATP. Temp., 4.9°. [Enzyme–flavin] = 32 μM in 0.1 M pyrophosphate buffer (pH 8.3).

agreement with the ideas outlined here. At low concentrations of D-methionine the activating effect of benzoate is larger than at higher concentrations, since it can more easily overcome the synergistic action between ATP and the substrate.

The binding of benzoate induces a red shift in the flavin absorption with a maximum at 497.5 nm (refs. 16, 25–27). ATP itself does not induce a spectral shift. When the enzyme is titrated with benzoate a 2–3-fold higher concentration of benzoate is needed for 50% saturation in the presence of ATP (Fig. 8). The non-competitive inhibition of ATP at 37° and the small inhibition at 5° indicates that the counteraction by ATP of the benzoate binding is not due to binding of the nucleotide to the active site. The titration curves do not show signs for cooperative interactions. On the other hand, the curve in the presence of ATP is very asymmetrical; that in the absence of ATP is not completely symmetrical. MONOD, WYMAN AND CHANGEUX[21] have pointed out that the S-shape of the titration curves is dependent on the value of the allosteric constant L and the ratio of the dissociation constants c. The fact that, in the presence of 20 mM ATP, no S-shaped curve is observed is due to the binding of benzoate to both forms, provided that benzoate also gives the same spectral complex with the low-temperature form.

Further evidence for the presence of two conformations with different velocities and affinities comes from the following experiments. The Arrhenius plot (Fig. 9) of the maximum velocity with D-methionine shows a break point at 24° (*cf.* ref. 7). In the presence of benzoate however, the break becomes less sharp, when the extrapolation of the linear part of the Lineweaver–Burk plot is taken as maximum velocity (*cf.* Figs. 3–5). In the presence of ATP, a fairly good straight-line relationship is obtained, but with lower activities at temperatures higher than 5°. The plot in the presence of ATP (ΔE = 9900 cal·mole^{-1}) is almost parallel with the upper part of

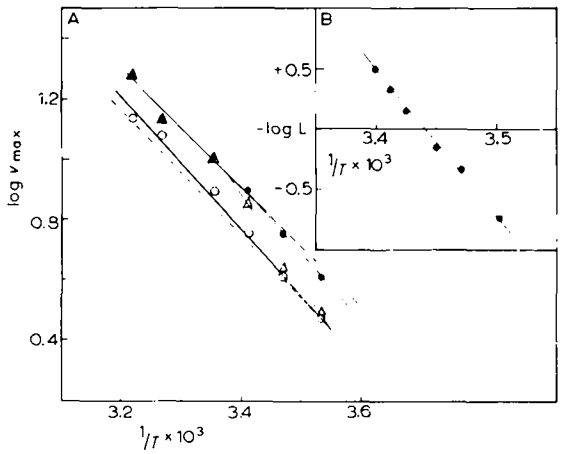


Fig. 9. A. Effect of effectors on the Arrhenius plot of D-amino-acid oxidase. The log v_{max} (μmoles $O_2 \cdot min^{-1} \cdot mg^{-1}$) with D-methionine as substrate, obtained by extrapolating the linear part of the Lineweaver-Burk plot, is plotted against $1/T$. △—△, control; ●—●, in the presence of 25 μM benzoate; ○—○, in the presence of 20 mM ATP. B. Van 't Hoff plot of the temperature-dependent equilibrium between the low- and high-temperature forms of D-amino-acid oxidase. The equilibrium constant L was calculated as described in the text.

the Arrhenius plot without any effector present ($\Delta E = 9300$ cal$\cdot$mole^{-1}). The fact that the plots are not completely parallel is partly due to the fact that we were working at rather low ATP concentrations to avoid substrate inhibition (*cf.* Fig. 6). This gives a small increase in maximum velocity due to the presence of some R-form, which results in a small increase of the activation energy. A close examination of the experimental points reveals that the tendency of a break is still present. Affinity for both conformations in the case of benzoate binding leads to the Arrhenius plot not being completely straight. The presence of the T-form with lower activity leads to a smaller value of the maximum velocity. Therefore it is concluded that the two forms have the same activation energy but differ in maximum rate, indicating that identical transition states have different probabilities. From the difference in rates it can be calculated that the difference in entropy of activation between the R- and T-forms $\Delta S^{\ddagger} = 0.7$ e.u.

If for a certain condition v_{max} is the experimentally obtained maximum velocity, v^{T}_{max} the maximum velocity of the T-form, v^{R}_{max} the maximum velocity of the R-form, f_R the molar fraction of the R-form, and $1 - f_R$ the molar fraction of the T-form, then for the equilibrium between the two forms the following relations are valid:

$$v_{max} = f_R v^{R}_{max} + (1 - f_R) v^{T}_{max} \qquad f_R = \frac{v_{max} - v^{T}_{max}}{v^{R}_{max} - v^{T}_{max}}$$

and

$$L = \frac{[T]}{[R]} = \frac{1 - f_R}{f_R}$$

Since the activation energies are the same, the f_R value can be calculated for the temperature traject where the transition occurs and connected with this the allosteric

constant L. From the Van 't Hoff plot of the temperature-dependent variation of L (Fig. 9B), it can be calculated that $\Delta H = 55\,000$ cal·mole^{-1} and $\Delta S = 185$ e.u..

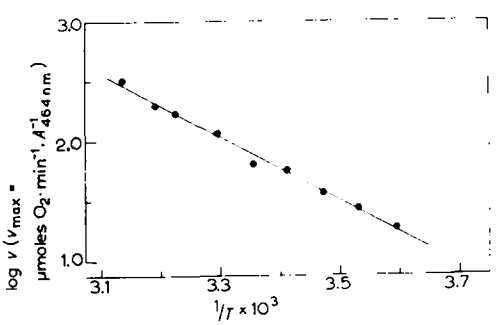
It is rather difficult to explain the kinetic results with the temperature-dependent association[28] observed with this enzyme. In that case the high-temperature form is the polymer of the low-temperature form. The results indicate that benzoate has a higher affinity for the R-form, but it has been reported that in the presence of benzoate the enzyme is less able to polymerize (*cf.* ref. 28). It is thus more likely that the results obtained are more due to conformational changes of the protein rather than to polymerization.

Temperature-dependent conformational changes of L-*amino-acid oxidase and the effect of pyrophosphate and ATP*

Comparison of the kinetic properties of L-amino-acid oxidase with those of D-amino-acid oxidase (*cf.* ref. 29) indicates the possibility that the large substrate inhibition observed with L-leucine as substrate and the fact that hardly any substrate inhibition is found with L-valine (*cf.* ref. 30) might be due to an equilibrium between two forms of the enzyme. Similarly these two forms must have different maximum rates and different affinities for the substrate. From the large inhibition with L-leucine, it could thus be concluded that this substrate must have a higher affinity for the less active low-temperature form; therefore L-valine must have about equal affinity for both forms.

The application of the results obtained with D-amino-acid oxidase to the Arrhenius plots of L-amino-acid oxidase provides evidence that the latter enzyme also exists in two temperature-dependent conformations with different activities.

In Fig. 10 where the Arrhenius plot with L-leucine as substrate is presented, a straight line is obtained. In the presence of 6.7 mM pyrophosphate (Fig. 11A) the Arrhenius plot changes considerably; the plot becomes Z-shaped by showing two



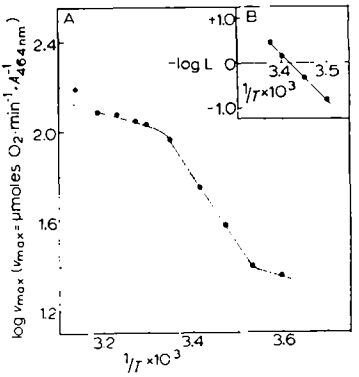


Fig. 10. The Arrhenius plot of L-amino-acid oxidase with L-leucine as substrate in 0.05 M Tris–KCl buffer (pH 7.4). Conditions described under METHODS. v_{max} obtained as described in Fig. 9A.

Fig. 11. A. Effect of pyrophosphate on the Arrhenius plot of L-amino-acid oxidase with L-leucine as substrate. Conditions the same as Fig. 10 but in the presence of 6.7 mM pyrophosphate (pH 7.4). v_{max} was obtained as described in Fig. 9A. B. Van 't Hoff plot of the temperature-dependent equilibrium between the two forms of L-amino-acid oxidase in the presence of 6.7 mM pyrophosphate. The equilibrium constant L was calculated as described in the text.

breaks at 26° and 10°. The high-temperature part is parallel to the low-temperature part, the three points at the lowest temperatures being averages of two independent determinations. From this plot it can be concluded that L-amino-acid oxidase exists in two temperature-dependent forms. The appearance of two breaks indicates that pyrophosphate shifts the equilibrium between the two forms towards the high-temperature form. Furthermore, it is clear that L-leucine must have a high affinity to the less active form, the conclusion strengthened by the fact that substrate inhibition occurs over the whole temperature range (5–45°). On the other hand, at low temperature in the presence of pyrophosphate there is practically no substrate inhibition. The activation energy calculated from the plot of Fig. 10 is about the same as the activation energy calculated form the transition range of Fig. 11A in the presence of pyrophosphate (Table II).

This indicates that the linear plot in the absence of pyrophosphate represents the transition traject. These results are a warning against the conclusion that a linear Arrhenius plot is the reflection of one enzyme conformation.

With D-amino-acid oxidase the Arrhenius plot was dependent on the substrate used; the same is the case with L-amino-acid oxidase. With L-valine as substrate, in

TABLE II

THE ACTIVATION ENERGIES ΔE CALCULATED FROM THE ARRHENIUS PLOTS OF THE CATALYTIC REACTION OF L-AMINO-ACID OXIDASE WITH L-LEUCINE AND L-VALINE AS SUBSTRATES AND THE INFLUENCE OF ATP (20 mM) AND PYROPHOSPHATE (6.7 mM)

Substrate	*Addition*	ΔE *(cal·mole^{-1})*	*Temp. range (°C)*
L-Leucine	No	13 300	5–45
L-Leucine	Pyrophosphate	2 760	>26
		13 800	10–26
		2 760	<10
L-Valine	No	2 300	>40
		10 600	20–40
		2 300	<20
L-Valine	Pyrophosphate	2 300	>33
		11 500	<33
L-Valine	ATP	2 300	>40
		10 900	20–40
		2 300	<20

contrast with L-leucine, a discontinuous plot with two breaks (at 40° and 20°) is obtained (Fig. 12A). In the presence of 6.7 mM pyrophosphate just one break occurs at 33°, the second one at low temperature has disappeared. Again the shift of the break towards a lower temperature is an indication that pyrophosphate shifts the equilibrium towards the high-temperature form (*cf.* L-leucine with 6.7 mM pyrophosphate).

With D-amino-acid oxidase ATP has a higher affinity to the low-temperature form; in the case of L-amino-acid oxidase ATP shifts the equilibrium slightly to the high-temperature form with little influence on the temperature of the break (Fig. 13A).

Table II summarizes the values of the activation energies for the different temperature ranges with L-leucine and L-valine as substrates in the presence and absence of pyrophosphate and ATP. The enzyme used in the experiments of Figs. 12A and 13A are from different preparations; every experimental point is the average of two independent determinations.

In the same way as has been done with D-amino-acid oxidase, the enthalpy and entropy changes of the conformational transition can be calculated from Figs.

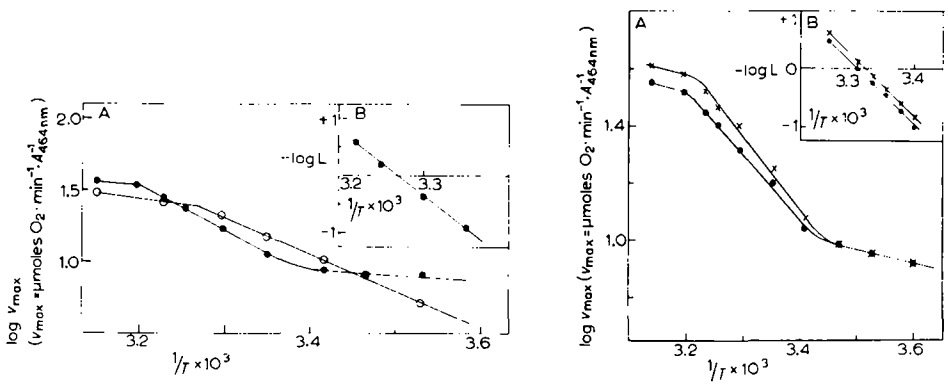


Fig. 12. A. The Arrhenius plot of L-amino-acid oxidase with L-valine as substrate in the absence and presence of pyrophosphate. Conditions as in Fig. 10. ●—●, no pyrophosphate; ○···○, with 6.7 mM pyrophosphate (pH 7.4). v_{max} was obtained as described in Fig. 9A. B. Van 't Hoff plot of the temperature-dependent equilibrium between the two forms of L-amino-acid oxidase. The equilibrium constant L was calculated as described in the text.

Fig. 13. A. Effect of ATP on the Arrhenius plot of L-amino-acid oxidase with L-valine as substrate. Conditions as in Fig. 10. ●···●, no ATP; ×- ×, with 20 mM ATP. v_{max} was obtained as described in Fig. 9A. B. Van 't Hoff plots of the temperature-dependent equilibrium between the two forms of L-amino-acid oxidase in the presence and absence of ATP. The equilibrium constant was calculated as described in the text. ●- ●, no ATP; ×- ×, $[ATP]_f$ = 20 mM.

11B, 12B and 13B. For L-leucine in the presence of 6.7 mM pyrophosphate the value is 46 000 cal·mole^{-1}; L-valine alone 47 000 cal·mole^{-1} and L-valine with 20 mM ATP 46 000 cal·mole^{-1}; ΔS varies from 140–150 e.u.. From these values it seems that the ΔH is rather independent of the substrate and additions made, indicating that the differences between the two protein conformations are the main factor involved. Another conclusion is that conformational changes which occur upon substrate binding induce about the same changes in thermodynamic parameters of both the low- and high-temperature forms.

From the differences in rates the difference in entropy of activation for both forms under each condition is calculated; $\Delta S^* = 2.3$ e.u. for L-leucine with 6.7 mM pyrophosphate; $\Delta S^* = 1.8$ e.u. for L-valine and for L-valine with 20 mM ATP $\Delta S^* = 2.1$ e.u.

The great difference between the two amino acid oxidases is the substrate inhibition, occurring with L-amino-acid oxidase. In the literature different explanations are given for this inhibition[29,30]. It was assumed by WELLNER AND MEISTER[30] that the half reduced enzyme is reoxidized faster than the total reduced enzyme. This explanation was ruled out by the experiments of MASSEY AND CURTI[29]. They proved

that the reoxidation of the total reduced enzyme cannot be the rate-limiting step in the catalytic overall reaction and showed that the reaction mechanism was quite similar to that of D-amino-acid oxidase (*cf.* ref. 19). To explain the inhibition they assumed the formation of a complex between the fully reduced enzyme and the substrate. This complex should be unreactive or at least react slowly with oxygen. Our explanation of the substrate inhibition is similar to the explanation of the substrate inhibition occurring with D-amino-acid oxidase with D-methionine as substrate. The results shown above indicate that the behavior of the two oxidases is basically the same in spite of the apparent differences.

The phenomenon of non-linear Arrhenius plots is well known in enzymology. MASSEY, CURTI AND GANTHER[7] have recently given a list of enzymes showing these effects. Many explanations have been given, the majority rejected on theoretical grounds (*cf.* refs. 8, 9). Recent explanations deal with the existence of different protein conformations, either each with similar activity, but differing in energy of activation[7,31], or with different activities[8,9].

Our results strongly support the latter idea. They show that a non-linear Arrhenius plot is to be expected in case the enzyme can undergo a reversible temperature-dependent transition between two conformations with the same activation energies (*e.g.*, the same active complex) but differing in the probabilities of the transitions. Such a mechanism can only be explained in terms of conformational changes around the active site of the enzyme (*cf.* ref. 9). The results show clearly that cooperative effects[21] are observable in such a case, which leads to the question whether similar phenomena occur in other so-called allosteric enzymes. This is especially important in relation to very complex enzymes like glutamate dehydrogenase. Although explanations have been given for the marked substrate inhibition by the assumption of multiple binding sites for NADH (ref. 32), the results presented here show that other explanations are possible (*cf.* ref. 33). Furthermore, it must be considered that in two-substrate reactions, in the absence or presence of modifiers, all reagents can have different affinities for two or more conformations. This leads to a role of one substrate as an allosteric effector on the kinetic parameters of the second substrate. Variation of the concentrations of both substrates can lead to apparent contradictions. All these phenomena will be dependent on the kinetic mechanism, *e.g.* the case of either a modified enzyme mechanism, an ordered or a random mechanism.

These and other studies[34] show the importance of varying the temperature in testing involvement of different conformations. Furthermore, the results show that it is possible to observe two breaks, which according to these hypotheses are to be expected. Even the observation of a linear Arrhenius plot does not allow the conclusion that one conformation is involved. The results also provide an explanation for the exchange in the Arrhenius plot upon the addition of effectors to the enzyme-catalyzed reaction[8,9].

From the data obtained it is clear that the slope in the Arrhenius plot does not always represent the activation energy of the reaction. For determining the activation energy, the largest possible temperature range should be taken, because by using a limited temperature range there is a possibility, even upon finding a straight-line relationship, that one is dealing with a transition between two conformations. In the transition range the slope represents, in addition to the activation energy, the change

in activation entropy of the reaction due to the conversion of the low-temperature conformation into the high-temperature one, both having different activities. In fact it is possible to distinguish two cases:

(1) The high-temperature form has a higher activity than the low-temperature conformation; in this case the part of the Arrhenius plot connected with the transition will have a larger slope than the parts of the plot representing either the pure high-temperature or the pure low-temperature conformation. Examples are the data presented here.

(2) The high-temperature form has a lower activity than the low-temperature conformation; in this case the part of the Arrhenius plot connected with the transition will have a smaller slope than the parts of the plot representing either the pure low-temperature or pure high-temperature conformation. An example might be the results obtained with fumarase (*cf.* ref. 31).

Whether these ideas are applicable to those obtained with other enzymes is an open question. However, many of the interpretations in enzyme catalysis based on activation energies have become doubtful in the light of these results and this interpretation.

Although a more physical explanation has been given for cooperative binding phenomena[35] in terms of the relation between sequential changes and ligand binding, our interpretations are more related to the concerted transition model of MONOD, WYMAN AND CHANGEUX[21]. However, it must be emphasized that an involvement of the induced-fit mechanism can not be ruled out by these experiments.

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