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MECHANISM OF INHIBITION OF D-AMINO ACID OXIDASE

IV. INHIBITORY ACTION OF CHLORPROMAZINE

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SUMMARY

1. The interaction of chlorpromazine and FAD was studied in aqueous solution and in a reaction mixture containing D-amino acid oxidase. The formation of a complex between chlorpromazine and FAD and the competition of these compounds for the apo-enzyme were demonstrated.

2. The formation of complexes of chlorpromazine and flavins was demonstrated by means of spectrophotometry and fluorimetry. The dissociation constant of the complex of chlorpromazine with FAD at pH 7.0 and 20°, measured by fluorimetry, appeared to be $1.0 \cdot 10^{-3}$ *M*. The same dissociation constant was found for the chlorpromazine complexes with riboflavin, FMN or riboflavin 5′-monosulfate.

3. The competition of chlorpromazine with FAD for the apo-enzyme was demonstrated by means of kinetic analysis. The dissociation constant of the complex of chlorpromazine with the apo-enzyme at pH 8.3 was calculated to be $2.3 \cdot 10^{-5}$ *M*.

4. The binding site of the apo-enzyme with chlorpromazine was determined by using riboflavin 5′-monosulfate and adenosine 5′-monosulfate as indicators. The results showed that chlorpromazine competes with adenosine 5′-monosulfate for the apo-enzyme, which means that chlorpromazine binds at the same site as the AMP moiety of FAD.

Abbreviations: FAD, flavin adenine dinucleotide; FMN, flavin monophosphate; AMP, adenosine monophosphate; FMS, riboflavin 5′-monosulfate; AMS, adenosine 5′-monosulfate.

INTRODUCTION

In our series of reports[1-3] on the mechanism of the inhibition of D-amino acid oxidase, the complex formation of a certain substance with FAD which resulted in the inhibition of the oxidase was demonstrated; it was also shown that this substance competes with FAD.

As previously reported, chlorpromazine competes with FAD in a reaction mixture containing D-amino acid apo-oxidase[4], and also forms a complex with FAD in aqueous solutions[5]. This complicated inhibitory action was of interest in the study of the mechanism of inhibition of D-amino acid oxidase.

However, the inhibitory action of chlorpromazine on flavin enzyme had been assumed to have some connection with the pharmacological effects of chlorpromazine in the brain[4]. Moreover, LÖW[6] reported recently that this action may be related to the uncoupling action of chlorpromazine in the oxidative phosphorylation of rat liver mitochondria.

Consequently, it was considered that the elucidation of the mechanism of the inhibition of flavin enzyme by chlorpromazine would provide valuable suggestions for the study of the actions of chlorpromazine in the living body.

Hence the precise mechanism of the inhibitory action of chlorpromazine was studied from this point of view.

MATERIALS

D-amino acid oxidase apo-protein, free from FAD, was prepared by the method of NEGELEIN AND BRÖMEL[7]. FAD was prepared by the method of YAGI *et al.*[8]. Chlorpromazine–HCl, N-(3′-dimethylaminopropyl)-3-chlorophenothiazine hydrochloride, was supplied by Rhône-Poulenc-Specia. FMS and AMS were synthesized by the method of TAKAHASHI, YAGI AND EGAMI[9] and TAKAHASHI AND EGAMI[10], respectively.

METHOD

Investigation of the complex formation of chlorpromazine with FAD

The complex formation of chlorpromazine with FAD was examined by spectrophotometry and fluorimetry.

The shift of the absorption spectrum of FAD by chlorpromazine was measured by a Beckman type DU spectrophotometer. The quenching of the fluorescence of an aqueous solution of FAD by chlorpromazine was analysed by using a fluorimeter designed by YAGI *et al.*[11]. Assuming that the reaction of chlorpromazine and FAD is bimolecular and that the complex is non-fluorescent, the relation between the fluorescence intensity of FAD and the concentration of chlorpromazine (C) can be shown to be

$$\frac{f}{f'} = 1 + \frac{C}{K} \qquad (1)$$

where f and f' are the fluorescence intensities of FAD in the absence and presence of chlorpromazine respectively and K is the dissociation constant of chlorpromazine from its complex with FAD. By plotting f/f' against C, a straight line with the intercept 1 can be obtained, and K can be calculated from the slope of this line.

Spectrophotometric and fluorimetric analyses were also applied to examine the interaction between chlorpromazine and riboflavin, FMN or FMS.

Kinetic study of the competition of chlorpromazine with FAD

The oxidase activity was measured as described previously[1-3]. The dissociation constant of FAD with the apo-protein (K_f) was calculated to be $1.1 \cdot 10^{-7}\ M$ using the equation of MICHAELIS-MENTEN.

Assuming that chlorpromazine forms a complex with the apo-protein in competition with FAD, the enzyme activity can be represented as,

$$v = \frac{Vf}{K_f(1 + i/K_{ip}) + f} \tag{2}$$

where v is the reaction velocity in the presence of concentrations of f and i FAD and chlorpromazine respectively, V is the maximum velocity obtained in the presence of a large excess of FAD, and K_{ip} is the dissociation constant of chlorpromazine with the apo-protein in competition with FAD.

Using the LINEWEAVER-BURK form of eqn. (2), K_{ip} can be calculated from the slope of the straight line obtained by plotting $1/v$ against $1/f$ (Method I).

From the eqn. of MICHAELIS-MENTEN and eqn. (2), the following can be deduced,

$$\frac{v_0}{v} = 1 + \left(1 - \frac{v_0}{V}\right)\frac{i}{K_{ip}} \tag{3}$$

where v_0 and v are the reaction velocities in the absence and presence of chlorpromazine respectively. If the values of v_0/v plotted against i give a straight line with an intercept of 1, the inhibitory action of chlorpromazine can be considered to have only one mechanism as previously reported[2]. K_{ip} can also be calculated from the slope of the straight line thus obtained (Method II).

The elucidation of the binding site of the apo-protein with chlorpromazine was carried out by the method previously reported using FMS and AMS as indicators[3]. Assuming that a substance combines with the protein in competition with both FAD and one indicator, v_0/v can be shown as,

$$\frac{v_0}{v} = 1 + \left(1 - \frac{v_0}{V}\right)\left(\frac{i_1}{K_1} + \frac{i_2}{K_2}\right) \tag{4}$$

where K_1 is the dissociation constant of the complex of the substance with the protein, and K_2 is that of the complex of the indicator with the protein. i_1 and i_2 are the concentrations of the unknown substance and the indicator respectively. When the substance combines with the protein in competition with FAD but not with the indicator, v_0/v can be shown to be

$$\frac{v_0}{v} = 1 + \left(1 - \frac{v_0}{V}\right)\left(\frac{i_1}{K_1} + \frac{i_2}{K_2} + \frac{i_1 i_2}{K_1 K_3}\right)$$

or,

$$\frac{v_0}{v} = 1 + \left(1 - \frac{v_0}{V}\right)\left(\frac{i_1}{K_1} + \frac{i_2}{K_2} + \frac{i_1 i_2}{K_2 K_4}\right) \tag{5}$$

where K_3 is the dissociation constant of (apo-protein-i_1) + $i_2 \rightleftharpoons$ (apo-protein-i_1-i_2) and K_4 is that of (apo-protein-i_2) + $i_1 \rightleftharpoons$ (apo-protein-i_1-i_2). When the position of

the binding site of the protein with one inhibitor does not influence the binding site of the protein with the other inhibitor, *i.e.*, no interaction takes place between the two binding sites of the protein, $K_1 = K_4$ and $K_2 = K_3$, and v_0/v can be shown to be

$$\frac{v_0}{v} = 1 + \left(1 - \frac{v_0}{V}\right)\left(\frac{i_1}{K_1} + \frac{i_2}{K_2} + \frac{i_1 i_2}{K_1 K_2}\right) \tag{6}$$

Plotting v_0/v against the concentrations of the inhibitors, a straight line can be obtained in the case of eqn. (4), and a curve in the case of eqn. (5) or (6). Thus it can be determined from the form of the curve whether the substance competes with the indicator or not, and, consequently, the part of the FAD which competes with the substance may be deduced (see previous report[3]).

Since, as mentioned before, chlorpromazine forms a complex with FMS, this complex formation should also be considered in the kinetic analysis shown above. The decrease of the effective concentrations of both chlorpromazine and FMS caused by their complex formation must be observed when the complex does not act as a competitive inhibitor.

In this case, the concentration of two inhibitors (i_1, i_2) in eqns. (4), (5) and (6) should be replaced by the concentration of the free inhibitors ($i_1 - x$), ($i_2 - x$), which can be approximately calculated from the following equation:

$$K = \frac{(i_1 - x)(i_2 - x)}{x} \tag{7}$$

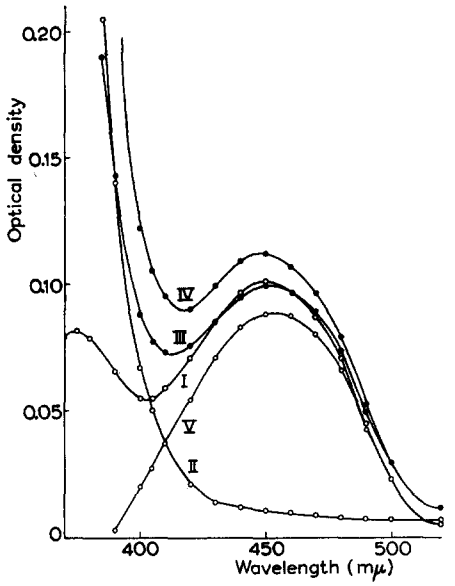
where K is the dissociation constant of the complex formed between two inhibitors in an aqueous solution, obtained by using eqn. (1). x is the concentration of the complex of chlorpromazine with FMS.

RESULTS

Complex formation of chlorpromazine with flavins

In the preliminary experiments, it was shown that the addition of a large excess of chlorpromazine to FAD solution (pH 6.5, 20°) changes the colour of the latter from yellow to brownish-yellow and also diminishes the fluorescence of the solution. So, it was expected that a change in the absorption spectrum of FAD would occur due to the formation of a complex with chlorpromazine. As shown in Fig. 1, the measured optical density of the chlorpromazine–FAD mixture in the visual wave-length region was considerably suppressed compared with its theoretical value (see curves III and IV). The peak in the difference absorption spectrum of FAD was shifted slightly to the longer wave-length region on the addition of chlorpromazine (see curves I and V). Similar changes were also observed in the cases of riboflavin, FMN and FMS.

The quenching of the fluorescence of FAD was also demonstrated by the addition of chlorpromazine. The plot of f/f' against the concentration of chlorpromazine gave a straight line with an intercept of 1, as shown in Fig. 2. From the slope of this line, K was calculated to be $1.0 \cdot 10^{-3}\,M$. The same dissociation constant was obtained for the complex of chlorpromazine with riboflavin, FMN or FMS using the same procedure.



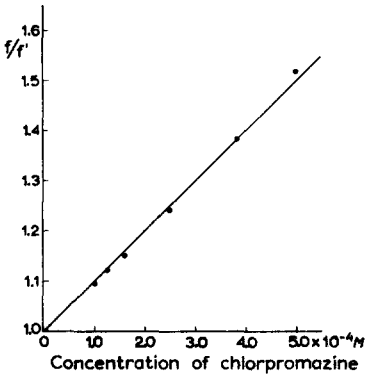


Fig. 2. Quenching action of chlorpromazine on the fluorescence of FAD: f and f' correspond to the fluorescence intensities of FAD ($1.0 \cdot 10^{-6}$ M) in the absence and presence of chlorpromazine in phosphate buffer (M/10, pH 7.0) at 20°.

Fig. 1. Spectra of FAD and chlorpromazine: I, FAD ($9.0 \cdot 10^{-6}$ M) in phosphate buffer (M/10, pH 6.5); II, chlorpromazine ($2.0 \cdot 10^{-3}$ M) in the same buffer; III, mixture of FAD ($9.0 \cdot 10^{-6}$ M) and chlorpromazine ($2.0 \cdot 10^{-3}$ M) in the same buffer; IV, theoretical spectrum of FAD + chlorpromazine; V, the difference spectrum of FAD (III–II).

Competitive inhibition of chlorpromazine with FAD

In the presence of $2.0 \cdot 10^{-5}$ M chlorpromazine in the enzyme reaction mixture, a plot of $1/v$ against $1/f$ gave a straight line with an intercept of $1/V$, as shown in Fig. 3 (Method I). From the slope of this line, K_{ip} was calculated to be $2.3 \cdot 10^{-5}$ M.

A plot of v_0/v against the concentration of chlorpromazine also gave a straight line with an intercept of 1, as shown by curve I in Fig. 4 (Method II). From the slope of this line, K_{ip} was also calculated to be $2.3 \cdot 10^{-5}$ M in good agreement with the value obtained by Method I.

Binding site of the apo-protein with chlorpromazine

Kinetic analysis of the binding site of the apo-protein with chlorpromazine was carried out by using FMS and AMS as the indicators. The plots of v_0/v against the concentrations of both chlorpromazine and AMS gave a straight line, as shown by curve II in Fig. 4. In the presence of both chlorpromazine and FMS, plots of v_0/v against the concentration of both inhibitors gave a second order curve, as illustrated by curve III in Fig. 4.

DISCUSSION

In the present study it was found that chlorpromazine inhibits D-amino acid oxidase in competition with FAD (dissociation constant, $2.3 \cdot 10^{-5}$ M) and at the same time forms a complex with flavin (dissociation constant, $1.0 \cdot 10^{-3}$ M).

In the previous papers[2,12], similar cases were found *i.e.*, *p*-aminosalicylic acid or *p*-aminophenol forms a complex with and, at the same time, competes with FAD

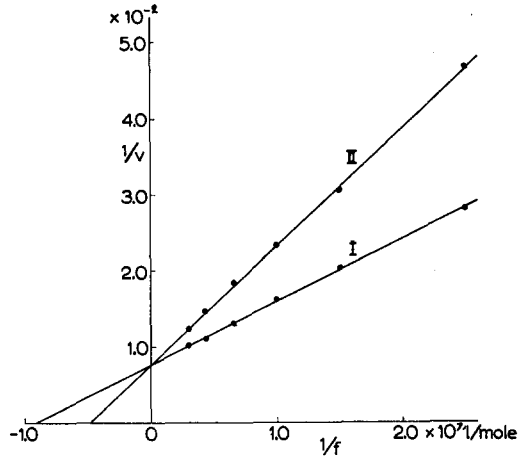


Fig. 3. Inhibitory action of chlorpromazine on D-amino acid oxidase in competition with FAD (Method I). Reaction mixture contained 15 μg of the oxidase protein, 0.1 M (final concn.) of DL-alanine and indicated concentration of FAD. After the temperature equilibrium had been reached, $2.0 \cdot 10^{-5}$ M (final concn.) of chlorpromazine or water was added to the main chamber from the side bulb. Curve I: in absence of chlorpromazine. Curve II: in presence of chlorpromazine.

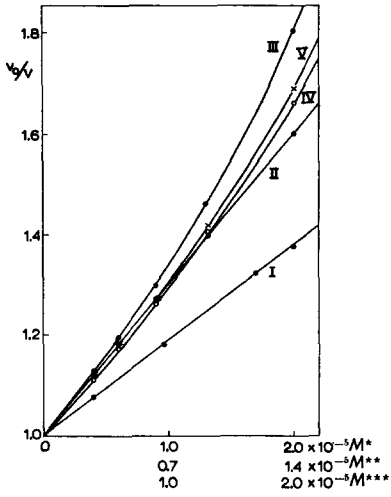


Fig. 4. Competitive inhibition of chlorpromazine with FAD (Method II) and analysis of the binding sites of the protein with chlorpromazine using kinetic indicators. v_0 and v represent the reaction velocities in the absence and presence of inhibitors. Reaction mixture contained 15 μg of the oxidase protein, 0.1 M (final concn.) of DL-alanine and $1.7 \cdot 10^{-7}$ M (final concn.) of FAD in the absence and presence of i concentration of inhibitors: * chlorpromazine concentration; ** FMS concentration; *** AMS concentration. Curve I: in the presence of chlorpromazine. Curve II: in the presence of chlorpromazine and AMS. Curve III: in the presence of chlorpromazine and FMS. Curves IV and V: theoretical values of v_0/v (see the text). V was 132 μl uptake of oxygen per 30 min and v_0 was 80 μl uptake of oxygen per 30 min.

for the protein in D-amino acid oxidase reaction mixture. In the case of p-aminosalicylic acid, the plot of v_0/v against the concentration of p-aminosalicylic acid gave a second order curve, which indicated that two different mechanisms were involved in the inhibitory action. In this case, v_0/v was expressed by the following equation[2,12],

$$\frac{v_0}{v} = 1 + \left(1 - \frac{v_0}{V}\right)\left\{\left(\frac{1}{K_{ip}} + \frac{1}{K_{if}}\right)i + \frac{i^2}{K_{ip}K_{if}}\right\} \qquad (8)$$

where K_{if} is the dissociation constant of the inhibitor–FAD complex in the oxidase reaction mixture. K_{if} and K_{ip} of p-aminosalicylic acid were reported as $7.5 \cdot 10^{-2}$ M and $1.6 \cdot 10^{-2}$ M respectively.

In the present study, however, a plot of v_0/v against the concentration of chlorpromazine practically gave a straight line, which shows that a single mechanism dominates the inhibitory action.

This phenomena can be attributed to the difference between the order of the dissociation constant of the chlorpromazine–FAD complex and that of the concentration of chlorpromazine. Considering that the former is 100 times larger than the latter, the complex formation of chlorpromazine with FAD is negligible compared with the competition of chlorpromazine with FAD. Actually, the theoretical error

caused by omitting the complex formation in the present experiment may be calculated from eqns. (3) and (8) as,

$$\left|\frac{\Delta v_0/v}{v_0/v}\right| = \frac{i\,(K_{ip}+i)\,(1-v_0/V)}{K_{if}\{K_{ip}+i\,(1-v_0/V)\}} \leqq \frac{i}{K_{if}} \qquad (9)$$

Such a treatment makes the relative error less than 2.0 %. Thus, a plot of v_0/v against the concentration of chlorpromazine practically gave a straight line when the concentration was of the order of 10^{-5} M.

The above results are sufficiently accurate to permit the kinetic method using indicators to be applied to the analysis of the behaviour of chlorpromazine in its competition with FAD.

When the kinetic analysis was applied to chlorpromazine, it was found that v_0/v plots gave a straight line during the coexistence of chlorpromazine and AMS, and that they gave a curve during the coexistence of chlorpromazine and FMS.

To interpret these results, the nature of curve III was examined by comparison with curve IV. The latter was obtained from the theoretical values calculated as follows: assuming that no interaction takes place between the two binding sites of the apo-protein, eqn. (6) was adopted. In this case, the complex formation of chlorpromazine with FMS has to be considered. Then, assuming that the complex of the two inhibitors can not act as an inhibitor, the concentration of free inhibitors was calculated approximately from eqn. (7). Substituting the concentration of free inhibitors calculated from eqn. (7) for i_1 and i_2 in eqn. (6), the theoretical values of v_0/v were calculated as shown by curve IV in Fig. 4.

The fact that curve III is more convex against the abscissa than curve IV shows that there exists an interaction between the two binding sites of the apo-protein with chlorpromazine and FMS.

Curve III was examined further by comparing it with another theoretical curve, curve V, which was deduced using the assumption that neither interaction between the two binding sites of the apo-protein nor complex formation of chlorpromazine with FMS occurred. Curve III was found to be even more convex than curve V.

The results mentioned above were interpreted as follows: Chlorpromazine combines with apo-protein in competition with the AMP part of FAD, and interaction takes place between the binding sites of the apo-protein with chlorpromazine and with FMS.

Complex formation of chlorpromazine with flavins is considered to be significant *in vivo*. The fact that the modification of the electroencephalogram by the administration of chlorpromazine is reversed by FAD may be partly explained by the complex formation of these two compounds *in vivo*[13].

Recently, Löw reported that the uncoupling action of chlorpromazine on oxidative phosphorylation is connected with FAD or FMN. The present results concerning the complex formation of chlorpromazine with flavins may furnish actual evidence for the participation of FAD in oxidative phosphorylation reported by GRABE[14]. It is noteworthy that substances known to be uncouplers of oxidative phosphorylation, namely, 2,4-dinitrophenol[2], chlortetracycline[1] and chlorpromazine[4,5] are all equally found to form complexes with flavins.

In concluding this series of reports, the following results emphasized throughout the four papers may be mentioned:

1. Some substances can inhibit D-amino acid oxidase by combining with the FAD in competition with the apo-protein.

2. A new kinetic method using indicators has been applied for the analysis of the behaviour of an inhibitor in its competition with FAD.

3. Positive interaction has been demonstrated between the binding sites of the apo-protein.

These results may be useful not only for the elucidation of the mechanism of action of several drugs reported in this series, but also for the demonstration of the actual method of complex formation involved in the enzyme action of D-amino acid oxidase.

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A RAPID ASSAY METHOD FOR TRITIUM IN BACTERIAL CELLS

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SUMMARY

1. The use of a simple bacterial filtration technique involving collodion membranes has permitted a precise means of assaying tritiated compounds. Radioassays at infinite thickness were readily converted to relative total count values. It is suggested that the method has general applicability.

2. Bacterial cultures rapidly converted exogenous tritiated thymidine to thymine. [^{3}H]diaminopimelic acid served as a more stable biochemical precursor, enabling accurate studies on the incorporation of tritium into bacterial cells.

Abbreviation: TCA, trichloroacetic acid.

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