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EFFECTOR BINDING SITES IN PLASMA AMINE OXIDASE

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Summary

The interaction between plasma amine oxidase (EC 1.4.3.6) and chlorpromazine was studied by spectroscopic methods. The binding of chlorpromazine to the enzyme is paralleled by an increase in the emission anisotropy of the ligand. Titration of the enzyme with chlorpromazine using fluorescence methods revealed the presence of an heterogeneous population of binding sites characterized by the dissociation constants K_1 = 7.4 μM and K_2 = 35 μM. A kinetic analysis showed that a change in V and K_m is induced by chlorpromazine. The increase in V (5.7 fold) is significant and cannot be attributed to modifications in the chemical structure of the effector chlorpromazine. The results are consistent with the concept that the binding of the effector induces a change in the conformation of the enzyme which is transmitted to the catalytic site.

Introduction

The enzyme plasma amine oxidase (EC 1.4.3.6) has been extensively investigated in several laboratories [1—4]. The presence of cupric atoms has been firmly established and extensive progress has been made in elucidating the role of Cu^{2+} in the catalytic mechanism [4—6]. Despite extensive kinetic studies on this enzyme, the presence of effector binding sites on plasma amine oxidase has not been reported. It is the aim of this paper to report the binding of chlorpromazine to plasma amine oxidase and to examine its effect on the catalytic function of the enzyme. It is shown that chlorpromazine is an efficient activator of kynuramine oxidase activity.

Methods

Fluorescence emission spectra were obtained with the use of a spectrofluorimeter designed in our laboratory [7]. Polarization of fluorescence measurements were performed in an apparatus similar to that described by Weber [8].

Illumination was provided by a xenon-mercury lamp, (250 W, Hanovia) with wavelengths selected by a quartz prism monochromator. The excitation wavelength was 315 nm. Fluorescence polarized light was passed through a CS-3-72 Corning glass filter. The detector system consisted of an EMI 9502 B photomultiplier and digital voltmeter. Emission anisotropy measurements were performed with a precision of ±0.02. Absorption spectra were recorded in a Cary model 15 spectrophotometer.

Preparation of the enzyme. The enzyme plasma amine oxidase from pig blood was purified by the procedure of Buffoni and Blaschko [1] as modified by Lindstrom and Petterson [2]. The preparation used throughout this work is homogeneous as determined by the criteria of polyacrylamide gel electrophoresis, isoelectric focusing and sedimentation in the ultracentrifuge. The specific activity of this preparation is 0.12 unit/mg of enzyme when benzylamine is used as a substrate according to the spectrophotometric method of Tabor et al. [9]. Enzymatic assays using kynuramine as a substrate were monitored by spectrophotometric [10] and fluorimetric methods. The spectrophotometric method consists in following the consumption of substrate (kynuramine) (ϵ = 4200 at 360 nm) or the formation of the product 4-hydroxyquinoline ($\epsilon = 11 \cdot 10^3$ at 317 nm). In the fluorimetric method, the reaction mixture containing enzyme and substrate is allowed to incubate at 37°C for 30 min. Aliquots (0.1 ml) withdrawn from the incubation mixture at several time intervals are diluted with 1 ml of 0.1 M NaOH and the fluorescence intensity recorded at 380 nm (excitation 317 nm). The fluorescence quantum yield is Q = 0.40 at alkaline pH. This sensitive method permits the detection of a concentration of 4-hydroxyquinoline of 0.1 μM. Protein concentrations were determined using an extinction coefficient of 250 $mM^{-1} \cdot cm^{-1}$ at 280 nm [2].

Materials

Pig blood was obtained from East Tennessee Packing Co. Kynuramine, benzylamine and horse radish peroxidase were purchased from Sigma. H_2O_2 was from Fisher. Chlorpromazine was a gift from Smith, Kline and French Laboratories, Philadelphia, Pa., U.S.A. Other chemicals used in this research were of reagent grade.

Results

The interaction between plasma amine oxidase and several phenothiazine derivatives were studied by fluorescence spectroscopy. Fig. 1 shows the emission spectra of chlorpromazine in the absence and presence of enzyme at pH 7.4 in 0.05 M phosphate. At a protein concentration of 10 μM and chlorpromazine concentration of 5 μM, the fluorescence emission band of the ligand is shifted towards shorter wavelengths. Similar spectral shifts are observed when chlorpromazine is dissolved in solvents characterized by dielectric constants lower than water (methanol, dioxane). The interaction of chlorpromazine with plasma amine oxidase is also accompanied by a dramatic increase in the emission anisotropy without any significant change in the fluorescence yield. The emission anisotropy of a mixture containing enzyme (20 μM) and chlorpro-

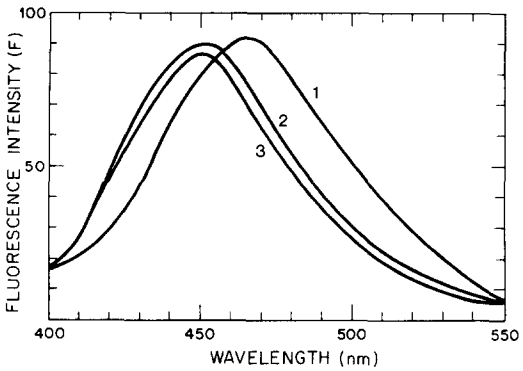

Fig. 1. Emission spectra of chlorpromazine (5 μM) in 0.05 M phosphate (pH 7.4) (1) and in dioxane (2). Emission spectra of chlorpromazine (5 μM) + plasma amine oxidase (10 μM) in 0.05 M phosphate (pH 7.4) (3). Excitation wavelength 320 nm.

mazine (5 μM) is considerably larger (A = 0.30) than the emission anisotropy of free chlorpromazine (A = 0.06). The fluorescence lifetimes of either free or bound chlorpromazine are shorter than 2 ns. The change in anisotropy associated with the interaction of the ligand chlorpromazine with the enzyme was used to determine the stoichiometry of binding. To this end, the fluorescence intensities (F) and emission anisotropy values (A) of samples containing a fixed concentration of enzyme (10 μM) and varying concentrations of ligand were measured at 25°C (Fig. 2). The emission anisotropy values ($\bar{A}$) recorded as a function of increasing concentrations of ligand reflect the relative contribution of both free and bound ligand to the total fluorescence intensity of the system. When chlorpromazine is the only component of the system, the emission anisotropy A_F is related to the Brownian rotation of the free ligand. Upon addition of enzyme, a fraction of the population of chlorpromazine molecules

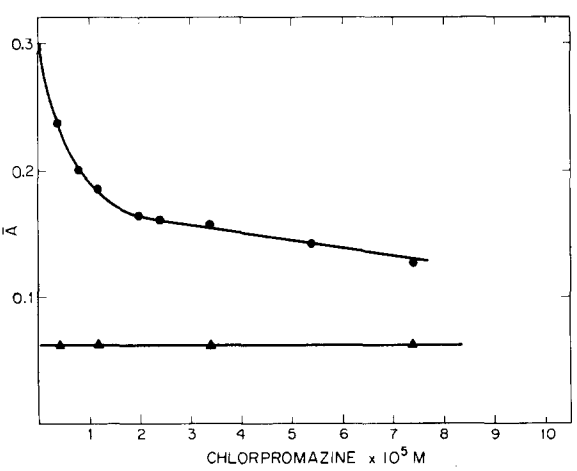

Fig. 2. Changes in emission anisotropy (A) upon addition of chlorpromazine to a fixed concentration of plasma amine oxidase (10 μM) in 0.05 M phosphate (pH 7.4) at 25°C (●). The emission anisotropy of chlorpromazine in the absence of enzyme is also given in the figure (▲).

is bound to the enzyme and their Brownian motion is restricted between excitation and emission of light. Consequently, an increase in the emission (A) is detected. When the protein is saturated with chlorpromazine, the emission anisotropy approaches the value $A_B = 0.30$. This situation is encountered when excess of enzyme (20 μM) is added to chlorpromazine (5 μM).

The results obtained by titrating plasma amine oxidase with chlorpromazine were used to determine the fraction of bound ligand (α) (Eqn. 1) [11]. Thus

$$\alpha = \frac{(A - A_F)}{(A_B - \bar{A})\beta + \bar{A} - A_F} \quad (1)$$

where $\beta = qB/qF$ is the ratio of fluorescence yields for bound and free chlorpromazine, respectively. Since the fluorescence quantum yield of bound chlorpromazine is practically identical to that of free chlorpromazine, the fraction bound (α) can be determined by using Eqn. 2

$$\alpha = \frac{\bar{A} - A_F}{A_B - A_F} \quad (2)$$

The emission anisotropy A is related to the fluorescence polarization by Eqn. 3

$$\bar{A} = \frac{2\bar{P}}{3 - P} \quad (3)$$

The average number of ligand molecules bound per mol of enzyme (M_r = 180 000) $\bar{V}$ was determined (Eqn. 4)

$$\bar{V} = \alpha \frac{[L_0]}{[P_0]} \quad (4)$$

Where $[L_0]$ is total ligand concentration and $[P_0]$ total protein concentration. The titration results were analyzed by plotting $\bar{V}/[L]$ vs. $\bar{V}$, where $[L]$ is the concentration of free ligand (chlorpromazine). The results are included in Fig. 3. It is evident that the titration results indicate that the binding process is not consistent with the results expected for one class of binding sites. If this were the case, the plot of $\bar{V}/[L]$ vs. $\bar{V}$ should give a straight line intersecting the abscissa at the number of equivalent binding sites of identical dissociation constant. The simplest model found to fit the titration results is based on two classes of binding sites characterized by the dissociation constants K_1 and K_2. Judging from the results included in Fig. 3, it appears that approximately two sites have a dissociation constant of 7.1 μM (K_1), whereas the remaining binding sites are characterized by a dissociation constant of approx. 35 μM (K_2). Thus, the titration results indicate that chlorpromazine binds to the enzyme plasma amine oxidase at concentrations far below the critical micelle concentration (1 mM). The binding of chlorpromazine to plasma amine oxidase is a reversible process as indicated by two independent lines of experimental evidence. Firstly, dilution of samples containing enzyme and chlorpromazine resulted in a decrease in the observed emission anisotropy (A) due to dissociation of the ligand from the protein. Secondly, gel filtration through Sephadex G-25 brings about complete separation of the ligand from the enzyme. The absorption properties of chlorpromazine remain essentially invariant after addition of the enzyme. Neither the substrate kynuramine nor the product 4-

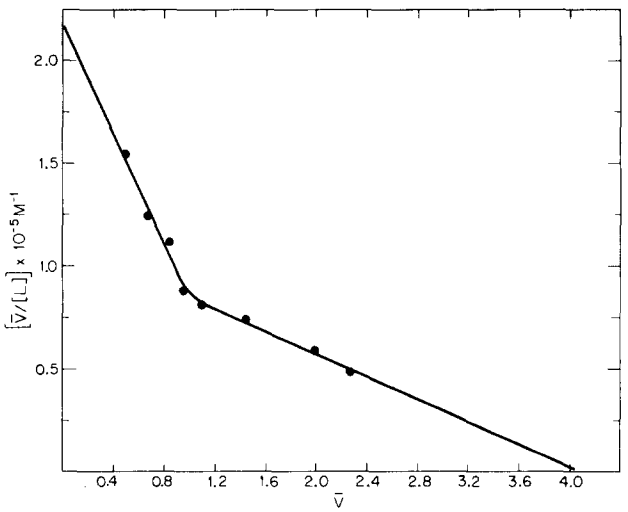

Fig. 3. Analysis of the binding results obtained by emission anisotropy measurements from the Scatchard's plot of $V/[L]$ vs. V. Two dissociation constants, K_1 = 7.1 μM and K_2 = 35 μM were determined for the binding of chlorpromazine to plasma amine oxidase.

hydroxyquinoline tend to form charge-transfer complexes with chlorpromazine under the experimental conditions used for the binding studies. It should be noted that the chemical structure of chlorpromazine is not altered by the addition of the enzyme plasma amine oxidase even in the presence of substrate. This is in marked contrast to the effect exerted by other enzymes, i.e. peroxidase [12] and ceruloplasmin [13] on the chemical structure of chlorpromazine. It has been shown that the enzyme peroxidase catalyzes the formation of chlorpromazine free radicals which display a strong absorption band at around 520 nm [12]. As shown in Fig. 4, no absorption band corresponding to a chlorpromazine free radical was detected at 520 nm after mixing concentrated solutions of the enzyme with chlorpromazine.

Effect of the enzymatic activity

Kynuramine oxidase activity was monitored as a function of substrate con-

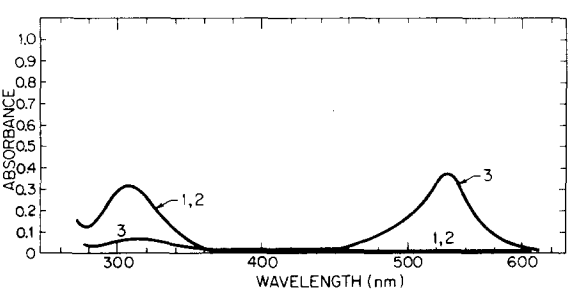

Fig. 4. Absorption spectra of chlorpromazine (0.1 mM) in the absence (1) and presence of enzyme (10 μM) (2). The differential spectra of the mixture chlorpromazine plus enzyme was obtained using a solution of enzyme (10 μM) as reference. The differential spectra was recorded 15 min after mixing chlorpromazine and enzyme. The absorption spectrum of chlorpromazine (0.1 mM) plus 1 mM H_2O_2 plus horse radish peroxidase (1 μM), recorded immediately after addition of peroxidase (3) is also given in the figure.

centration in the absence and presence of fixed concentrations of chlorpromazine. Plots of initial velocity versus kynuramine concentrations at fixed chlorpromazine concentrations yielded hyperbolic curves. The double reciprocal plots of $1/v$ vs. $1/[S]$ are shown in Fig. 5. It is immediately apparent that both V and K_m for kynuramine as a substrate are influenced by the presence of chlorpromazine. The increase in V, 5.7-fold, indicates that chlorpromazine is an efficient activator of the enzyme. A kinetic model which describes the activation of the enzyme plasma amine oxidase by chlorpromazine is given:

$$\begin{array}{ccccc} E + S & \overset{K_S}{\rightleftharpoons} & ES & \xrightarrow{K_{cat}} & E + P \\ + & & + & & \\ A & & A & \searrow^{\alpha K_A} & \\ K_A \updownarrow & & & & \\ EA + S & \overset{\alpha K_S}{\rightleftharpoons} & EAS & \xrightarrow{\beta K_{cat}} & EA + P \end{array}$$

This model is one of non-essential activation [14], in which the reaction can occur in the absence of external ligand. In this scheme, S is the substrate, A the activator, K_A and K_s are the dissociation constants for binding of activator and substrate to the enzyme, respectively. K_{cat} is the catalytic rate constant. α is the factor by which K_s changes when A occupies the enzyme, β is a factor by which V changes when A occupies the enzyme. The rate of reaction can be expressed by the following equation (eqn. 5)

$$v = \frac{\dfrac{V[S]}{K_S + \beta V} + \dfrac{\beta V[A][S]}{\alpha K_A K_S}}{1 + \dfrac{[S]}{K_S} + \dfrac{[A]}{K_A} + \dfrac{[A][S]}{\alpha K_A K_S}} \qquad (5)$$

An analysis of the kinetic results included in Fig. 5 permit the determination of α, β, K_A and K_S (see ref. 14 for further details). The most significant aspect of

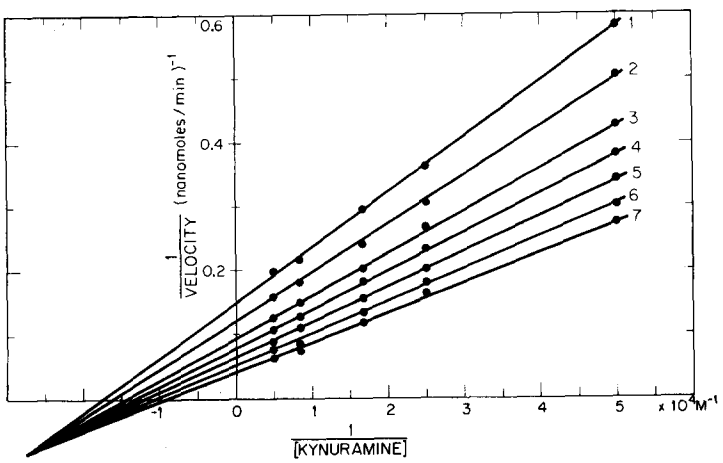

Fig. 5. Double reciprocal plots obtained at fixed concentrations of chlorpromazine. Enzymatic assays performed at 37°C. Chlorpromazine concentrations are as follows 1, 2, 3, 4, 5, 6, 7 correspond to 0, 6, 12, 20, 40, 80 and 120 μM, respectively. The analysis of these kinetic results yielded the following values for the parameters K_A = 32 μM; K_S = 34 μM, V = 7 nmol/min, α = 3.1 and β = 5.7.

the steady-state kinetic measurements in the finding that binding of chlorpromazine induces a 5.7-fold increase in the V. This change in V is accompanied by an increase in the K_m for the substrate. Thus, in the absence of chlorpromazine the K_m for L-kynuramine is 34 μM, whereas in the presence of chlorpromazine the K_m for the substrate is increased to 0.1 mM.

Conclusion

The phenothiazine derivative, chlorpromazine, binds to the enzyme plasma amine oxidase and induces a significant effect on the catalytic activity. The titration experiments designed to determine the affinity of chlorpromazine for the enzyme revealed the presence of an heterogeneous population of effector binding sites which can be characterized by the dissociation constants K_1 = 7.4 μM and K_2 = 35 μM. Since the apparent binding constant determined from kinetic measurements is 32 μM, it appears that all the binding sites available for the effector chlorpromazine are involved in the mechanism of enzyme activation. A model designed to explain the effect of chlorpromazine on plasma amine oxidase must take into account the following experimental facts, (a) the effector binds to more than two sites on the enzyme, (b) the activation effect exerted by chlorpromazine is detected over a wide range of pH values, from pH 5.7 to pH 9, and (c) the effector influences the K_m and V values. The simplest model found to describe the effect of chlorpromazine on plasma amine oxidase is as follows: Molecules of chlorpromazine bind rapidly to the enzyme inducing or stabilizing a change in conformation which is transmitted to the catalytic site of the enzyme and renders it more reactive to the substrate kynuramine. The nature of the change occuring at the catalytic site remains unknown, but it is interesting to note that as a result of this change in conformation, the V is 5.7-fold increased.

Acknowledgements

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