Human Plasma Monoamine Oxidase

II. KINETIC STUDIES

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(Received for publication, November 27, 1964)

The preceding paper (1) described the purification of a soluble monoamine oxidase from human plasma and showed that this enzyme catalyzes the oxidative deamination of a number of aliphatic amines according to the general equation

\[ \text{RCH}_2\text{NH}_2 + \text{O}_2 + \text{H}_2\text{O} \rightarrow \text{RCHO} + \text{NH}_3 + \text{H}_2\text{O} \]  

Although this human plasma enzyme was shown (1) to have certain characteristics in common with plasma amine oxidases from other mammals (2-6), it could be distinguished from these other plasma enzymes by its substrate specificity.

During studies relating to the substrate specificity of the human enzyme, the oxidation of aliphatic amines was noted to have a number of distinctive kinetic features. These consist chiefly of inhibition at high substrate concentration, strict dependence of apparent Michaelis and inhibitor constants upon pH, and contributions of nonpolar residues of substrates to their affinities for the enzyme. None of these features appears to be recognized in previous studies with other plasma amine oxidases. These findings indicate certain characteristics of the active center of the human plasma enzyme.

EXPERIMENTAL PROCEDURE

Materials

Purified human plasma monoamine oxidase was prepared as previously described (1). Aldehyde dehydrogenase, partially purified from yeast (7), was a gift of Dr. Simon Black. Lyophilized horseradish peroxidase (RZ > 1) was purchased from Distillation Products Industries and freed from ammonium salts before use by dialysis against 0.02 M phosphate buffer, pH 7.4. Ethylamine hydrochloride, purchased from Worthington Biochemical Corporation and was freed from ammonium salts before use by dialysis against 0.02 M phosphate buffer, pH 7.4. Ethylamine hydrochloride, purchased from Worthington Biochemical Corporation, and 1,8-diaminooctane a product of Fluka, Inc. Other aliphatic amines, purchased as the free bases from either Distillation Products Industries or The Matheson Company, Inc., were converted to the hydrochlorides by treatment with hydrogen chloride gas in absolute ethanol and then recrystallized at least twice from absolute ethanol. Kynuramine dihydrobromide was a product of Regis Chemical Company, and 1,8-diaminoctane a product of Fluka, Inc. Other aliphatic amines, purchased as the free bases from either Distillation Products Industries or The Matheson Company, Inc., were converted to the hydrochlorides by treatment with hydrogen chloride gas in absolute ethanol and then recrystallized at least twice from absolute ethanol. n-Octyl alcohol was obtained from J. T. Baker Chemical Company. 1,8-Diaminoctane was purchased from Distillation Products Industries and recrystallized twice from absolute ethanol. DPN was purchased from the Sigma Chemical Company.

Methods

Spectrophotometric Methods—Initial rates of amine oxidation, as determined by the direct and coupled assays given below, were obtained spectrophotometrically. A Cary recording spectrophotometer (model 11M) with a thermostatically controlled cuvette chamber and cuvettes with 1-cm light paths were used for this purpose.

Activity of Monoamine Oxidase Preparations—Purified preparations of the plasma monoamine oxidase were assayed by the method of Tabor, Tabor, and Rosenthal (3). The units of activity have been previously defined (1). The specific activities of the preparations used in this work represented greater than 3000-fold purifications of the enzymatic activity from crude plasma.

Measurements of Benzylamine and Kynuramine Oxidation—Rates of benzylamine oxidation were determined directly by recording the increase in absorbance at 250 nm due to the production of benzaldehyde (3). The molar absorptivity of benzaldehyde at 250 nm was assumed to be 1.2 × 10⁴ M⁻¹ cm⁻¹ (8). Oxidation of kynuramine was measured directly by recording the increase in optical density at 316 nm due to the production of 4-hydroxyquinoline (1,9). In agreement with the results of other workers (10), the molar absorptivity of 4-hydroxyquinoline at 316 nm was found to be constant between pH 7.8 and 9.8 and to be 1.2 × 10⁴ M⁻¹ cm⁻¹.

Amine Oxidation as Measured by Hydrogen Peroxide Production—Initial rates of oxidation of aliphatic amines at relatively alkaline pH values were determined by a modification (1) of the peroxidase-α-dianisidine reaction (11), which is in common use for the colorimetric estimation of glucose. As previously described (1), rates of increase in optical density at 440 nm (due to the production of a colored oxidation product resulting from the reaction of hydrogen peroxide and α-dianisidine in the presence of peroxidase) were converted to rates of hydrogen peroxide production by comparison with optical densities obtained after the addition of known amounts of hydrogen peroxide. This assay was used only under conditions in which the chromophore was demonstrated to be stable for 1 hour at 25°C.

Measurements of Butylamine Oxidation—Yeast aldehyde dehydrogenase (7) irreversibly reduces DPN in the presence of a variety of aliphatic and aromatic aldehydes. Since its \( K_m \) values with respect to aldehyde substrates are relatively low, this enzyme proved suitable for the determination of initial rates of the enzymatic conversion of butylamine to butyraldehyde. Partially purified yeast aldehyde dehydrogenase (a product of

1 Phosphate buffer was made from dibasic sodium phosphate and monobasic potassium phosphate.
RESULTS

Inhibition by High Substrate Concentration—The effect of substrate concentration upon initial velocities of amine oxidation by the plasma monoamine oxidase (Figs. 1 and 2A) obeys the Michaelis-Menten equation at relatively low concentrations of substrate. At relatively high substrate concentrations, however, the velocity of the reaction declines with increasing substrate concentration. Simultaneous binding of more than 1 substrate molecule at the active center of the enzyme with the subsequent formation of relatively inactive substrate-enzyme complexes is a possible mechanism of such substrate inhibition. The general equation derived by Haldane (17) to fit the kinetics of such an inhibitory mechanism may be written (18) as follows.

\[
\frac{V_{\text{max}}}{v} = 1 + \frac{K_m}{S} + \left(\frac{S}{K_m'}\right)
\]

It relates the velocity of the reaction (v) to the theoretical maximal velocity (V_{\text{max}}), substrate concentration (S), the apparent dissociation constant for the substrate in the activable (or optimal) position (K_m), and the apparent dissociation constant for the substrate in the inhibitory position (K_m').

In the case of benzylamine oxidation by the plasma monoamine oxidase, the experimental data were shown graphically to fit this general equation. (In the case of octylamine oxidation, the relative insolubility of this long chain monoamine prevented the use of sufficiently high substrate concentrations to make a comparable study.) In keeping with mathematical derivations of Equation 3 (17, 18), a plot (Fig. 2B) of initial velocities of benzylamine oxidation against substrate concentrations on a logarithmic scale yields a symmetrical curve. The occurrence of a maximum in such a curve is implicit in Equation 3 and may be expressed in the derived equation (17, 18).

\[
S_0 = \sqrt{K_mK_m'}
\]

where S_0 is the optimum substrate concentration. The apparent K_m for benzylamine oxidation at pH 8.2, obtained by the method of Lineweaver and Burk (13) (Fig. 2A), is 33 μM. The apparent dissociation constant for the substrate in the inhibitory position (K_m') estimated from a Dixon plot of reciprocal velocity against substrate concentration (19) is 4.7 mM. The theoretical value of S_0 (0.39 mM) computed from Equation 4 is in good agreement with the substrate concentration at which the curve of Fig. 2B is maximal. From Equations 3 and 4 it follows that the velocity (v_0) observed at the optimal substrate concentration (S_0) should be defined by the equation

\[
v_0 = \frac{V_{\text{max}}}{1 + \frac{K_m}{S_0}}
\]

The observed maximal velocity (Fig. 2B) is also in excellent agreement with the v_0 value of 140 μmoles per hour computed from Equation 5.

Since the experimental data are consistent with Equation 3, it would seem that the active center of the plasma monoamine oxidase consists of more than one site for the interaction with
Fig. 2. Benzylamine oxidation. Purified monoamine oxidase (10 units; specific activity, 113) was incubated at 25° in 3.0 ml of 0.1 M phosphate buffer, pH 8.2, containing the indicated final concentrations of benzylamine. Initial rates of benzaldehyde production, determined spectrophotometrically as described under "Methods," are presented in Lineweaver-Burk ($A$) and semilogarithmic ($B$) plots.

Fig. 3. Effect of substrate concentration upon apparent pH optima. $A$, butylamine oxidation: 12 units of monoamine oxidase (specific activity, 113) were incubated at 25° in 1.0 ml of 0.2 M phosphate buffer (pH indicated) containing 0.15 mg of yeast aldehyde dehydrogenase, 1.0 or 5.0 mM butylamine, 0.5 mM DPN, 1 mM mercaptoethanol, and 0.1 M KCl. $B$, kynuramine oxidation: substrate. Other possibilities have been proposed, however, to explain similar inhibition at high substrate concentrations (18, 20). For this reason, additional evidence for a two-site attachment of substrate to enzyme in the Michaelis complex was sought.

Effect of pH on Apparent $K_m$ of Substrate and Apparent $K_i$ of Competitive Inhibitor—During the course of investigating the substrate specificity of the plasma monoamine oxidase, it was noted that the pH for optimal oxidation differed markedly from substrate to substrate and that the apparent pH optimum for a given substrate varied with the substrate concentration used. Examples of this anomalous behavior are given in Fig. 3. With both butylamine and kynuramine, the apparent pH optima
shifted to higher pH values as the substrate concentration was reduced. Whereas benzylamine manifested an apparent pH optimum of 7.1 to 7.4 at substrate concentrations of 1 to 5 mM (1), the stronger base, butylamine, was optimally oxidized at a higher pH in this same range of substrate concentration. A possible interpretation of these findings is that saturation of the enzyme with substrate depends upon the concentration of un-ionized amine present and not on that of total or protonated amine. Further evidence was sought to support such an interpretation.

In the case of benzylamine, it could be demonstrated that the Michaelis complex must be defined in terms of un-ionized substrate. Apparent $K_m$ values ($K_m$ values with respect to total amine present) were determined for benzylamine (Fig. 4) from Lineweaver-Burk plots over a wide range of pH values and were noted to decrease logarithmically as the pH of the measurement increased. Michaelis constants defined with respect to un-ionized benzylamine ($K_m$ values) were derived from these apparent Michaelis constants and were found to be constant (2.2 $\mu M$) throughout the pH range studied. This figure also presents the incidental finding that the calculated maximal velocities ($V_{max}$ values) for benzylamine oxidation are greatest in the pH range of 7.0 to 7.5. The curve for calculated maximal velocities does not show the sharp decline in rate at both extremes of pH seen with pH curves obtained with 3.3 mM concentrations of benzylamine (1). The latter curves may now be explained by substrate inhibition effects at alkaline pH values (see below) and $K_m$ effects at relatively acid pH values.

The Michaelis complex is formed from enzyme and un-ionized amine; nitrogenous bases other than substrates of the enzyme might be found to manifest reactivity at the site of substrate attachment. The time-dependent inhibition of human plasma monoamine oxidase by the substituted hydrazine, iproniazid, has already been shown to be prevented (but not reversed) by substrates of the enzyme (1). Ammonia, a product of the enzymatic reaction, is a competitive inhibitor of benzylamine oxidation (Fig. 5A). Apparent $K_i$ values (apparent association constants for the inhibitor calculated with respect to total ammonia present) for ammonia inhibition were shown (Fig. 6) to decrease in the expected fashion with increasing pH, while the inhibitor constants ($K_i$ values) that were calculated with respect to un-ionized ammonia present remained relatively constant throughout the pH range studied.

Benzylamine oxidation at pH 7.7 and pH 8.6 is inhibited by monoalkylamines in a strictly competitive fashion. As an
example of the Lineweaver-Burk plots obtained with these aliphatic amines, the inhibition of benzaldehyde production by n-octylamine at pH 7.7 is presented in Fig. 7. (Octyl alcohol inhibition is discussed below.) Inhibitor constants derived for n-hexylamine, n-octylamine, and n-decylamine at these two pH values are presented in Table I. It may be seen that, whereas the $K_i$ values computed for each amine differ markedly with pH, the inhibitor constants ($\tilde{K}_i$ values) computed with respect to un-ionized amine are relatively constant. The $\tilde{K}_i$ values found for n-octylamine are also in good agreement with the $K_m$ derived for this amine as a substrate at pH 8.5 (Fig. 1).

These observations indicate that the substrate and inhibitor complexes are formed from enzyme and un-ionized amines. Since un-ionized ammonia, which possesses no alkyl residue, is a competitive inhibitor of the enzyme, the active center of the enzyme may be considered to include an electrophilic site for the attachment of unprotonated nitrogenous bases. The data do not permit a more precise definition of this binding site. They are compatible with a number of possible chemical interactions (e.g. the formation of complexes between amines and a protein-bound metal, or the addition of amines to a carbonyl prosthetic group). On the other hand, the constancy of the $\tilde{K}_i$ values for benzylamine (Fig. 4) and the $K_i$ values for ammonia (Fig. 6) in the pH range of 6.0 to 9.0 indicates that the electrophilic site cannot be represented by a group with a state of ionization that changes in this pH range.

![Fig. 6. Inhibitor constants for competitive inhibition by ammonia. Apparent inhibitor constants ($K_i$ values) were derived from Lineweaver-Burk plots of initial rates of benzaldehyde production at $25^\circ$. Reaction mixtures contained 14.7 mM concentrations of total ammonia in addition to the components described in Fig. 4. Inhibitor constants ($\tilde{K}_i$ values) with respect to un-ionized ammonia (NH$_3^+$) were derived from apparent inhibitor constants as described in the text.](image)

![Fig. 7. Competitive inhibition by octylamine and octyl alcohol. Monoamine oxidase (10 units; specific activity, 45) was incubated with the indicated concentrations of benzylamine in 3.0 ml of 0.2 M phosphate buffer, pH 7.7, containing 0.33 mM octyl alcohol (A), 0.33 mM octylamine (O), or no inhibitor (C). Velocities are expressed in terms of benzaldehyde production as measured spectrophotometrically.](image)

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**Effect of Chain Length of Alkyl Group on Michaelis and Inhibition Constants**—It was previously noted that, in a homologous series of aliphatic amines, a decrease in the substrate concentration from 1.0 mM to 0.05 mM markedly reduced the rate of oxidation of short chain aliphatic amines at pH 8.6, but not that of the long chain homologues (1). This observation suggested that the apparent $K_m$ values of short chain aliphatic amines are considerably greater than those of long chain homologues. Since the pH
Although inhibition by octyl alcohol was easily detected at concentrations of 0.3 mM or less, 0.2 mM methanol or 0.1 mM ethanol had no apparent effect on the oxidation of benzylamine. The $K_i$ (0.18 mM) for the inhibition by octyl alcohol at pH 7.7 was actually less than the apparent $K_i$ (0.26 mM) for the inhibition by octylamine. This finding supports the conclusion, drawn from the good agreement of $K_i$ values at different pH values, that the protonated species of octylamine (which predominates at pH 7.7) has a relatively insignificant affinity for the enzyme.

It was previously noted (1) that simple aliphatic amines with polar substituents on the alkyl side chain are relatively poor substrates of the plasma monoamine oxidase. Of the aliphatic amines tested, short chain diamines were poorly oxidized, if at all, but the oxidation of 1,8-diaminooctane was easily detected. Since polar substitution of even long chain amines should adversely affect hydrophobic bonding, the oxidation of 1,8-diaminooctane was studied under conditions identical with those used for $n$-octylamine in Fig. 1 (with the exception that octanediamine concentrations were varied from 0.10 to 1.0 mM). Comparison of the Lineweaver-Burk plots of octylamine (Fig. 1) and octanedi-amine oxidation at pH 8.5 revealed that while the maximal velocities were comparable (9.5 mmoles per hour per unit of enzyme for octylamine and 10.4 mmoles per hour per unit of enzyme for octanediamine), the apparent $K_m$ for octanedi-amine (43 $\mu$M) was markedly less than the apparent $K_m$ for octane-diamine (440 $\mu$M). Since the $pK_a$ corresponding to the second dissociation constant of 1,8-diaminooctane, 10.1 (23), is lower than the $pK_a$ of octylamine (10.65), it is apparent that the affinity of the enzyme for the monocationic species of octanedi-amine is also markedly less than its affinity for un-ionized octylamine.

There is reason to believe that the un-ionized species of monoalkylamines are primarily responsible for inhibition at high substrate concentration and that this inhibition also depends upon a hydrophobic interaction of substrate with enzyme. A comparison of Lineweaver-Burk plots of benzylamine oxidation at pH 8.2 (Fig. 24) and pH 7.2 (Fig. 5.4) shows that inhibition was not apparent at the lower pH with substrate concentrations that caused definite inhibition at the higher pH. Although the molar absorptivity of very high concentrations of benzylamine prevented a quantitative evaluation of the variation of $K_m'$ with $pH$, the data from which Fig. 4 is derived indicate that substrate inhibition is increasingly difficult to demonstrate as the pH decreases. Substrate inhibition was also more difficult to demonstrate with short chain aliphatic amines (Fig. 5B) than with long chain homologues (Fig. 1).

**DISCUSSION**

The results presented in this paper indicate that the active center of human plasma monoamine oxidase contains binding sites for both the free amino group and the hydrocarbon residue of substrates. This hypothesis is supported by three types of kinetic data, which may be summarized as follows. (a) Apparent Michaelis constants and apparent inhibitor constants for the interaction of the enzyme with amines and ammonia are directly related to the nonprotonated species of amine or ammonia present, and therefore indicate an electrophilic site within the active center. (b) Octyl alcohol is a competitive inhibitor of the enzyme, and constituents of the aliphatic residues of amines contribute to the affinity with which the amines are bound to the enzyme, in a manner that is consistent with hydrophobic binding. (c) The inhibition observed at high concentrations of substrate...
follows the Haldane equation (17) for simultaneous binding of 2 or more substrate molecules at the active center. It follows from these observations that the activatable position of substrate within the active center is one in which the substrate is bound to both the electrophilic and the hydrophobic sites.

The relation of apparent Michaelis or inhibitor constants to pH, over a wide range of hydrogen ion concentrations, has not previously been reported for other amine oxidases. It is clear from the data presented in this paper that the species of amine that predominates at the pH optimum of enzymatic activity does not necessarily correspond to the species of amine that constitutes the enzyme-substrate complex. For this reason, previous assumptions (based on pH optima) that protonated amines react with particular monoamine oxidase (24) as well as with the plasma amine oxidases of lower mammals (4) may not be valid. There are indications in the literature, moreover, that the association constants of certain preparations of mitochondrial monoamine oxidase may depend upon pH in a manner similar to that observed for the human plasma enzyme. The monoamine oxidase of guinea pig mitochondria is reported to oxidize amines optimally at pH values where the un-ionized species of the substrates predominate (25). The apparent (at a single substrate concentration) pH optima for the oxidation of monoalkylamines by rabbit liver monoamine oxidase vary with the length of the aliphatic side chain of the amine; in general, proportionately lower pH optima were found for those (longer chain) amines that have the lower apparent Michaelis constant at pH 7 (26). The weakly basic (pKₐ less than 5.0) amine, o-phenanthroline, is reported to be a competitive inhibitor of kynuramine oxidation by the mitochondrial monoamine oxidase of beef liver; at constant concentrations of substrate and inhibitor, the inhibition became increasingly less at alkaline pH values (27). Previous assertions that tissue preparations with monoamine oxidase activity contain multiple amine oxidases have often depended either on relative rates of substrate oxidation in homogenates from different tissues (26, 28, 29) or on changes in apparent substrate specificity produced by pH and nucleophilic inhibitors (26, 27). The present data on the monoamine oxidase of human plasma suggest that substrate ionization constants may be responsible for the observed effects of pH and nucleophilic inhibitors upon apparent substrate specificity.

In other respects, the findings with the human plasma monoamine oxidase are qualitatively similar to those reported for mitochondrial monoamine oxidase. The affinity of unsubstituted α-alkylamines for mitochondrial monoamine oxidase increases as the length of the hydrocarbon residue of the substrate increases; amines with polar substituents (including aliphatic diamines) are less tightly bound than the corresponding unsubstituted monoamines (26, 30). Although consistent with the postulated active center of human plasma monoamine oxidase, the inhibition observed with octyl alcohol was unexpected since this agent is usually considered to inhibit mitochondrial monoamine oxidase specifically (24), and relatively high concentrations of this alcohol are reported not to inhibit spermine oxidation by the amine oxidase of beef plasma (3). Inhibition at high substrate concentration has been reported for the oxidation of alkylamines by the mitochondrial monoamine oxidase of beef and rabbit liver; as is the case with the human plasma enzyme, substrate inhibition was more apparent with long chain than with short chain amines (31). Substrate inhibition occurring by the Haldane mechanism implies that the orientation of the substrate molecule in the active center of the enzyme is a determinant of the enzymatic rate. Although reported data are insufficient to establish that the kinetics for mitochondrial monoamine oxidase follows the Haldane equation, other kinetic evidence has been reported to support the hypothesis that the orientation of the substrate in the Michaelis complex does determine the enzymatic rate of mitochondrial monoamine oxidase (32).

While similarities may be found for the enzyme-substrate interactions of human plasma monoamine oxidase and mitochondrial monoamine oxidase, unequivocal differences have been drawn between the amine oxidases of all mammalian plasmas and mitochondrial monoamine oxidases (3-5, 33). As previously noted (1), the chief difference may be considered the inability of all plasma amine oxidases (including the human plasma monoamine oxidase) to oxidize secondary and tertiary amines. For this reason, final definition of the human plasma monoamine oxidase must await identification of its origin from tissue.

**SUMMARY**

1. The effect of pH upon apparent Michaelis and inhibitor constants for the highly purified monoamine oxidase from human plasma has been studied. The results indicate that un-ionized amines are the species of substrate or competitive inhibitor responsible for interaction with the enzyme. Ammonia, a product of the enzymatic reaction, is also inhibitory.

2. Increasing the chain length of simple alkylamines increases the affinity of these substrates for the enzyme. Octanediamine has less affinity for the enzyme than octylamine. Octyl alcohol is a competitive inhibitor.

3. The results indicate that the active center of the enzyme contains both electrophilic and hydrophobic binding sites. Inhibition at high substrate concentration is consistent with this evidence for multiple binding sites.

**Acknowledgments**—I wish to thank Drs. Herbert Tabor and Simon Black for helpful suggestions during the course of this investigation. The technical assistance of Mr. Kenneth Cullen is also acknowledged.

**REFERENCES**

11. **Keston, A., Abstracts of the American Chemical Society Meeting, Dallas, April 1966, p. 31C.
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