Biochimica et Biophysica Acta, 429 (1976) 331—341

BBA 67785

MECHANISM OF ACTION OF PUTRESCINE OXIDASE

BINDING CHARACTERISTICS OF THE ACTIVE SITE OF PUTRESCINE OXIDASE FROM *MICROCOCCUS RUBENS*

WILLIAM F. SWAIN and RICHARD J. DESA

Department of Biochemistry, University of Georgia, Athens, Ga. 30602 (U.S.A.)

(Received July 29th, 1975)

Summary

Putrescine oxidase (EC 1.4.3.4), putrescine: oxygen oxidoreductase (deaminating) (flavin containing), has been found to form complexes with a variety of amines. With few exceptions these compounds competitively inhibit putrescine oxidation and also perturb the visible absorption spectrum of the enzyme (i.e., the spectrum due to FAD).

Inhibition constants are reported for a number of amines; the presence of a cationic amino group in the inhibitors appears to be the structural feature essential for competitive inhibition. Inhibition constants for amino acids are larger than those for the analogous simple amines and the inhibition constants for alkyl mono- and diamines in a homologous series are inversely related to the length of the hydrocarbon chain. Amines containing unsaturated and aromatic substituents yield relatively low inhibition constants.

The spectral changes observed upon complex formation are interpreted as indicating a less polar environment for FAD in the enzyme-inhibitor complex than in the uncomplexed enzyme.

On the basis of the enzyme's substrate specificity and comparisons among inhibitor structures and the corresponding inhibition constants, a schematic model of the enzyme's active site is proposed.

Introduction

The putrescine oxidase (EC 1.4.3.4), putrescine : oxygen oxidoreductase (deaminating) (flavin contaning), from *Micrococcus rubens* (IFO 3768, Japan) is a simple flavoprotein oxidase with a molecular weight of 88 000. It is composed of two subunits and contains one non-covalently linked FAD molecule. The visible absorption spectrum of the holoenzyme displays bands cen-

tered at 370 and 458 nm. Steady-state kinetic analysis of oxygen-dependent putrescine oxidation by the enzyme yields results consistent with a Ping Pong Bi Bi mechanism. The anionic semiquinone form of the enzyme can be produced by reduction with EDTA and light or dithionite. The enzyme does not form an adduct with sulfite [1—3].

Early experiments demonstrated that the oxidation of putrescine by the enzyme could be strongly inhibited by amines and diamines (substrate analogs) and that these compounds induced marked perturbations of the visible absorption spectrum of the enzyme [4]. In this paper we document these phenomena more fully and present quantitative data for a large variety of amine compounds. From these data we are able to describe some of the binding features of the active site.

Materials and Methods

Putrescine oxidase (95% pure) was prepared from cultures of *Micrococcus rubens* (IFO 3768, Japan) as previously described [3]. The concentration of purified preparations was determined spectrophotometrically using the molar extinction coefficients reported by DeSa, i.e., $\epsilon_{458\,nm} = 10.8 \cdot 10^3\ M^{-1}\ cm^{-1}$ and $E^{1\%}_{cm,280\,nm} = 12.3$. L-amino acid oxidase (E.C. 1.4.3.2) was prepared from *Crotalus adamanteus* venom (Ross Allen Reptile Institute, Inc., Silver Springs, Fla.) using the methods of Wellner and Meister [5]. Chemicals used were of the highest grade commercially available. All amines employed were used in the form of their hydrochlorides; in some cases the hydrochloride was obtained by cautious titration of ethanolic solutions of the free amine with HCl. The hydrochlorides were then recrystallized before use. Solutions were prepared in 0.2 M Tris · HCl buffer, pH 8.6.

Putrescine oxidase activity was assayed by monitoring the disappearance of oxygen from assay mixtures. Oxygen levels were measured continuously using a Clarke oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio) fitted to a water jacketed reaction vessel equipped with a magnetic stirrer. Solutions used in the reaction mixture were air equilibrated at 20°C ($[O_2]$ = 0.283 mM). Electrode calibration was checked periodically using standard solutions of putrescine and putrescine oxidase or standard solutions of L-leucine and L-amino acid oxidase. A unit of enzyme activity is defined as the amount of enzyme that catalyzes the utilization of one nmol of O_2 per second.

Spectral studies were carried out at room temperature (approx. 22°C) using a computer controlled Bausch and Lomb 505 spectrophotometer [6]. Difference spectra were generated by the computer by substraction of absolute absorbance spectra.

Kinetic data were fit to the Michaelis-Menton equation with the computer program of Cleland [7]. Inhibition constants for the various compounds tested were obtained from weighted (1/variance) least-squares analyses of secondary plots. Data from spectrophotometrically assayed titrations were fit to an expression of the form $K_d = [E][I]/[EI]$, where K_d is the dissociation constant for the equilibrium $EI \rightleftharpoons E + I$; [EI] is equal to the magnitude of the absorbance change divided by the difference molar extinction coefficient (i.e., $\epsilon_{EI} - \epsilon_E$), and [E] and [I] are calculated by subtracting the concentration of

enzyme-inhibitor complex from the total enzyme concentration and total ligand concentration respectively. The best fit was determined by the curve-fitting program of Faini (unpublished) using K_d and the difference molar extinction coefficient as variable coefficients.

Results

A large number of amines have been found to inhibit putrescine oxidation by putrescine oxidase. Fig. 1 is an example of the steady-state kinetic behavior of the enzyme in the presence of 1,10-diaminodecane. The pattern of lines obtained demonstrates that the inhibition observed is competitive with respect to putrescine. The inhibition is linear with respect to inhibitor concentration as shown by the data of Fig. 2, in which the slopes of the lines from Fig. 1 have been plotted as a function of inhibitor concentration. The inhibition constant (K_i) can be determined from the data of Fig. 2 by extrapolation of the curve to its intercept with the abscissa.

The inhibition constants for a variety of compounds, obtained in this way, are listed in Table I. The ammonium ion is unique among the compounds included in Table I, in that it stimulates putrescine oxidase at low concentrations but inhibits competitively at higher concentrations.

Competitive inhibition is usually interpreted as indicating that the inhibitor and substrate are competing for the same (active) site on the enzyme (e.g. [8]). Compounds which can bind effectively to the active site but are incapable of being altered by the enzyme will be potent inhibitors of substrate oxidation; a comparison, then, of the degree of inhibition exhibited by various compounds should indicate features of the active site responsible for the binding and alteration of the substrate. In order to provide further evidence that binding of the

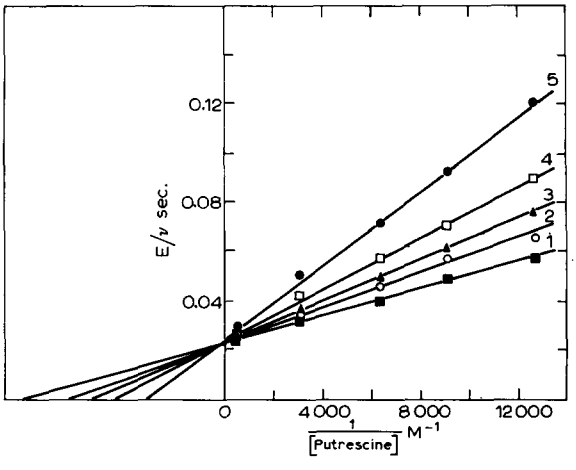

Fig. 1. Inhibition of putrescine oxidase by 1,10-diaminodecane presented in the form of Lineweaver-Burke plots. Each line represents the reciprocal of the turnover number as a function of the reciprocal of the substrate concentration at a fixed concentration of 1,10-diaminodecane. The concentrations of 1,10-diaminodecane corresponding to each of the lines are: (1) none; (2) $6.25 \cdot 10^{-7}$ M; (3) $1.25 \cdot 10^{-6}$ M; (4) $2.5 \cdot 10^{-6}$ M; (5) $5 \cdot 10^{-6}$ M.

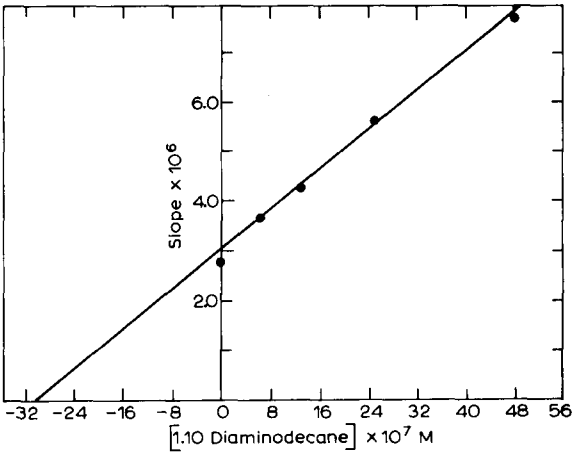

Fig. 2. Replot of the inhibition depicted in Fig. 1. The slopes of lines 1 through 5 of Fig. 1 are plotted as a function of 1,10-diaminodecane concentration.

various competitive inhibitors occurs at a single site (the active site), double inhibitor studies were carried out. In these experiments, assay mixtures contain two representative inhibitors selected from Table I (e.g., benzylamine and 5-amino-1-propanol in Fig. 3); the parallel pattern of lines obtained indicates mutually exclusive binding of the two inhibitors and lends support to the con-

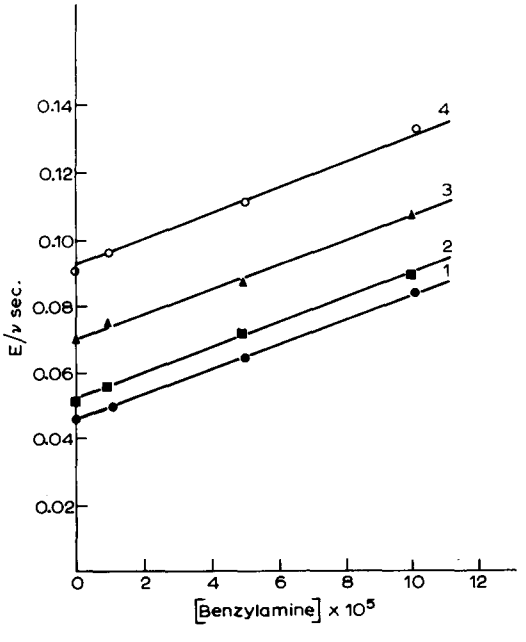

Fig. 3. Results of a double inhibitor experiment with benzylamine and 5-amino-1-pentanol (see text for details). Enzyme activity was measured in assay mixtures containing $1.09 \cdot 10^{-4}$ M putrescine, over a range of concentration of benzylamine at various fixed concentrations of 5-amino-1-pentanol. The concentrations of 5-amino-1-pentanol corresponding to each of the lines are: (1) none; (2) $2 \cdot 10^{-5}$ M; (3) $1 \cdot 10^{-4}$ M; (4) $2 \cdot 10^{-4}$ M.

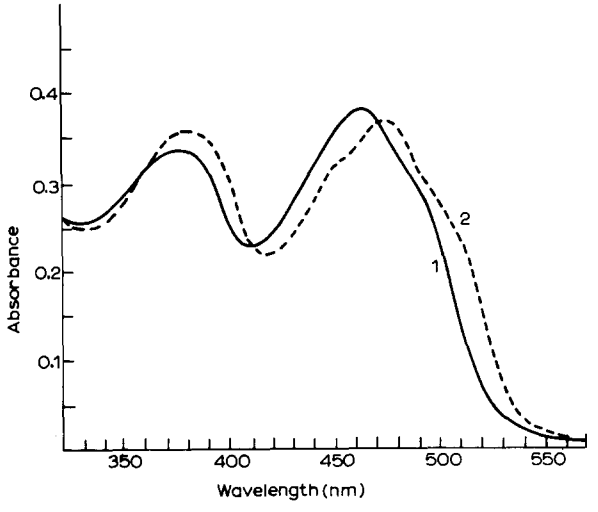

Fig. 4. Perturbation of the visible spectrum of putrescine oxidase by 1,10-diaminodecane. Spectrum 1 is that of a $3.5 \cdot 10^{-5}$ M solution of putrescine oxidase. After the addition of 1,10-diaminodecane (final concentration, $9.9 \cdot 10^{-4}$ M) spectrum 2 was recorded. Spectrum 2 has been corrected for dilution.

clusion that all the inhibitors bind at a single site [8]. Results qualitatively similar to those presented in Fig. 3 have been obtained with 1,3-diaminopropane and hexylamine.

Many of the inhibitors listed in Table I, in addition to affecting kinetic behavior, perturb the enzyme's visible absorbance spectrum, i.e. the visible spectrum due to the FAD moiety. An example is presented in Fig. 4. Although different inhibitors produce slightly different perturbations, in all cases the

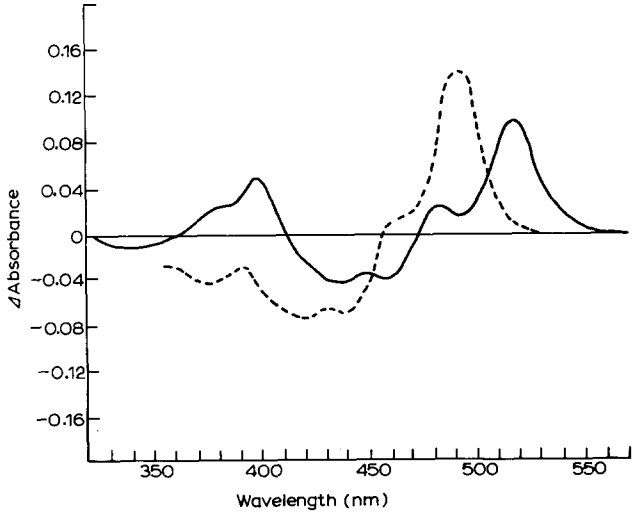

Fig. 5. Comparisons of perturbations of the flavin spectrum produced by binding of FAD to apoenzyme and by binding of inhibitors to holoenzyme. The solid-line difference spectrum was recorded after adding 1,12-diaminododecane (final concentration, $1 \cdot 10^{-3}$ M) to a $3.7 \cdot 10^{-5}$ M solution of putrescine oxidase. The broken-line spectrum was obtained from a holoenzyme reconstitution experiment by DeSa [3].

changes are characterized by a bathochromic shift and increase in structure of the 458 nm band. Changes in the 370 nm absorption band are varied, depending on the inhibitor used. The most frequently observed change in the 370 nm band is a small bathochromic shift (e.g. with alkylamines, benzylamine), but a more dramatic bathochromic shift with hyperchromism (e.g. with 1,10-diaminodecane and 1,12-diaminododecane), a hypsochromic shift with hyperchromism (e.g. with 1,7-diaminoheptane and 1,8-diaminooctane), and an absence of detectable change in the 370 nm band (phenylethylamine) have also been observed. None of the compounds tested produced long wavelength (>550 nm) absorption bands.

The spectral changes due to binding of inhibitors to holoenzyme are strikingly similar to those produced when FAD binds to apoenzyme, as shown by the difference spectra presented in Fig. 5. These difference spectra have nearly identical shapes, though the maxima and minima for inhibitor binding occur at longer wavelengths.

Titration of putrescine oxidase with benzylamine yields the difference spectra shown in Fig. 6. The data demonstrate that the spectral changes observed during the titration are isobestic at 408 and 470 nm. If an equilibrium of the form $E + I \rightleftharpoons EI$ is assumed to be responsible for the observed spectral perturbation, then the magnitude of the perturbation provides a measure of enzyme-inhibitor complex formation and allows estimation of the apparent dissociation constant for the complex. The titration data do not permit determination of the stoichiometry of complex formation. In Fig. 7a dissociation constant has been evaluated from the titration presented in Fig. 6. Upon close examination, data in Fig. 7 appear biphasic. However, the standard deviation from theoretical values is within the uncertainty of the measured value (σ = 0.003A) and biphasic character is not apparent in titrations of the enzyme with either butylamine or 3-amino-1-propanol, nor is it apparent in determinations

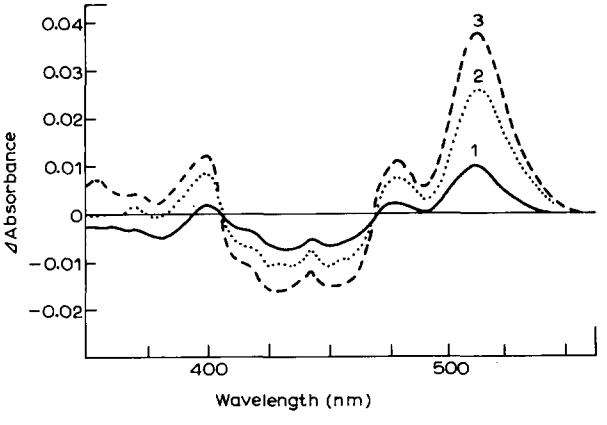

Fig. 6. Difference spectra resulting from titration of putrescine oxidase with benzylamine. The absorbance spectrum of a $2.3 \cdot 10^{-5}$ M enzyme solution was recorded before the addition of benzylamine. Subsequent spectra were recorded after successive additions of benzylamine; the spectra were corrected for dilution, and difference spectra corresponding to each concentration of benzylamine were calculated by subtraction. The benzylamine concentrations for the spectra shown are (1) $1 \cdot 10^{-5}$ M; (2) $5.8 \cdot 10^{-5}$ M; (3) $1.8 \cdot 10^{-4}$ M. Intermediate spectra have been omitted for clarity.

TABLE I

INHIBITION CONSTANTS FROM STEADY STATE KINETIC MEASUREMENTS

Compound	K_i
Monoamines	
Ammonium	$2.0 \cdot 10^{-2}$ M
Methylamine	$3.4 \cdot 10^{-3}$ M
Ethylamine	$2.3 \cdot 10^{-3}$ M
Propylamine	$6.2 \cdot 10^{-4}$ M
Butylamine	$4.6 \cdot 10^{-4}$ M
Pentylamine	$2.4 \cdot 10^{-4}$ M
Hexylamine	$3.9 \cdot 10^{-5}$ M
Heptylamine	$1.4 \cdot 10^{-5}$ M
Dodecylamine	$8.2 \cdot 10^{-6}$ M
Diamines	
Ethylenediamine	$9.5 \cdot 10^{-4}$ M
1,3-diaminopropane	$1.2 \cdot 10^{-4}$ M
1,6-diaminohexane	$2.3 \cdot 10^{-5}$ M
1,7-diaminoheptane	$8.2 \cdot 10^{-6}$ M
1,8-diaminooctane	$3.2 \cdot 10^{-6}$ M
1,10-diaminodecane	$3.0 \cdot 10^{-6}$ M
1,12-diaminododecane	$2.0 \cdot 10^{-6}$ M
N,N,N',N',-tetramethyl-1,4-diaminobutane	$1.4 \cdot 10^{-4}$ M
1,1,4,4-tetramethyl-1,4-diaminobutane	$1.9 \cdot 10^{-4}$ M
Agmatine	$3.6 \cdot 10^{-5}$ M
Amino alcohols	
2-aminoethanol	$3.0 \cdot 10^{-3}$ M
3-amino-1-propanol	$6.7 \cdot 10^{-4}$ M
5-amino-1-pentanol	$8.8 \cdot 10^{-5}$ M
6-amino-1-hexanol	$3.1 \cdot 10^{-4}$ M
ω-Amino acids	
4-amino-1-butanoic acid	$4.4 \cdot 10^{-1}$ M
5-amino-1-pentanoic acid	$5.9 \cdot 10^{-2}$ M
6-amino-1-hexanoic acid	$3.0 \cdot 10^{-1}$ M
8-amino-1-octanoic acid	$1.6 \cdot 10^{-1}$ M
Amines with unsaturated groups	
Allylamine	$1.3 \cdot 10^{-5}$ M
Benzylamine	$4.7 \cdot 10^{-5}$ M
Tyramine	$3.9 \cdot 10^{-5}$ M
Phenylethylamine	$8.7 \cdot 10^{-5}$ M
Aniline	>0.1 M
Non-amine	
n-Butanol	no inhibition

of the inhibition constants for these compounds or for benzylamine. We thus conclude that the assumed equilibrium function adequately describes the titration data and that the apparent biphasic character of Fig. 7 is purely fortuitous.

Dissociation constants obtained from spectral titrations are presented in Table II together with the corresponding inhibition constants. The values of K_d and K_i are in reasonable agreement, suggesting that the spectral perturbations result from formation of the same enzyme-inhibitor complex that is

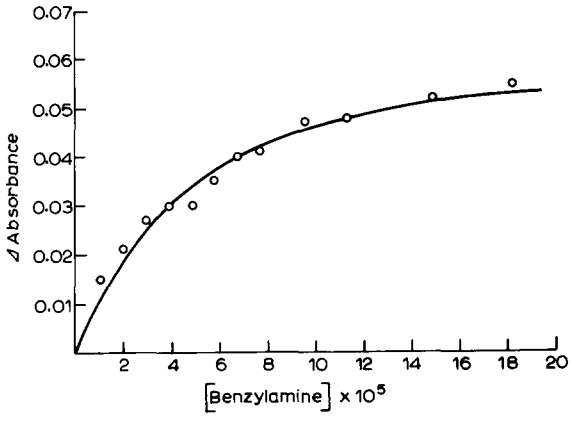

Fig. 7. Estimation of the dissociation constant from spectrally assayed titrations. The magnitude of the absorbance changes observed during the titration are plotted as a function of benzylamine concentration. The solid line is the curve defined by the equilibrium expression, K_d = [E][I]/[EI], with K_d, the dissociation constant, equal to $3.1 \cdot 10^{-5}$ M and with [$\Delta - \epsilon_{512}$ minus $\Delta - \epsilon_{450}$] for [EI] minus [E] + [I] equal to $2.7 \cdot 10^{3}$ M^{-1} cm^{-1}.

responsible for competitive inhibition of enzyme turnover; that is, binding of the inhibitor at the enzyme's active site.

During the course of these experiments we noted that 1,6-diaminohexane is also capable of being oxidized by putrescine oxidase. Table III presents the relative rates of oxidation of the four compounds now known to be oxidized by the enzyme. Since the rate of oxidation of 1,6-diaminohexane is so small, the question arises as to whether the observed rate is due to a contamination of the 1,6-diaminohexane with putrescine or cadaverine. Two points can be cited which argue against this explanation of the observed oxidation. First, the 1,6-diaminohexane (m.p. = 256—258°C) was of high purity. Second, and more significant, is the fact that 1,6-diaminohexane is a potent inhibitor of putrescine oxidation ($K_i = 2.3 \cdot 10^{-5}$ M, Table I) and, at the concentrations used to detect its oxidation, completely suppresses putrescine oxidation. Thus, when concentrations of putrescine similar to that used in normal assays are added to reaction mixtures of 1,6-diaminohexane and putrescine oxidase, no detectable change in reaction velocity occurs.

TABLE II

COMPARISONS OF K_d AND K_i

K_d was determined from spectrally assayed titrations as described in the text. K_i values are from Table I.

	K_d	K_i
Butylamine	$5.8 \cdot 10^{-4}$ M	$4.6 \cdot 10^{-4}$ M
3-Amino-1-Propanol	$6.7 \cdot 10^{-4}$ M	$5.0 \cdot 10^{-4}$ M
Benzylamine	$3.1 \cdot 10^{-5}$ M	$4.7 \cdot 10^{-5}$ M

TABLE III

COMPARISON OF REACTION RATES

Enzyme activity was determined as described in Methods. The maximum rate of putrescine oxidation under these conditions is 45—50 nmol · sec^{-1} · $nmol^{-1}$.

Substrate	*V* relative
Putrescine	100
Spermidine	10 [a]
Cadaverine	7 [a]
1,6-Diaminohexane	1

[a] Values of *V* relative for spermidine and cadaverine are from Yamada et al. [1].

Discussion

A comparison of the ionic properties of the inhibitors with the values of their inhibition constants listed in Table I reveals that compounds possessing cationic amino groups bind to the active site of the enzyme whereas compounds not possessing a cationic amino group, but otherwise structurally similar, do not bind. The presence of a negatively charged carboxyl group in the inhibitor molecule, as in the case of the amino acids, dramatically decreases the enzyme's affinity for the compound. These comparisons strongly indicate that an anion in the active site binds the amine cations of competitive inhibitors and substrates. In support of this hypothesis it has been observed that putrescine oxidase is irreversibly inactivated by several carbodiimides, and that putrescine and competitive inhibitors protect the enzyme from carbodiimide inactivation (Cook, L. and DeSa, R.J., unpublished), thus suggesting that the active site anionic charge is due to a carboxylate function.

Consideration of alkylamine and alkyldiamine inhibitors in a homologous series reveals that as the length of the hydrocarbon chain increases, the inhibition constant decreases. Amines which contain unsaturated and aromatic groups (e.g., allylamine, benzylamine, tyramine, phenylethylamine) also have relatively low inhibition constants. Thus, it appears that the enzyme active site contains a hydrophobic binding region that binds the saturated and unsaturated hydrocarbon groups. The low inhibition constant obtained for allylamine rela-

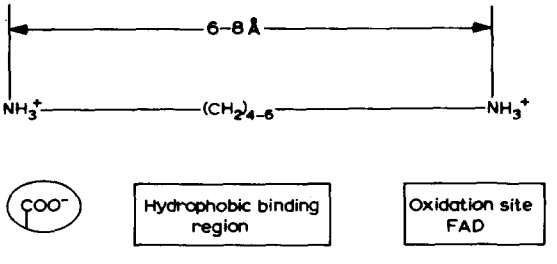

Fig. 8. Diagrammatic model of the active site of putrescine oxidase as deduced from inhibitor binding and substrate specificity.

tive to that obtained for propylamine suggests that the hydrophobic binding region may contain a pi bonding system(s).

From the conclusions discussed above it is possible to develop the active site model presented in Fig. 8. The cation binding site is proposed to be distinct from the oxidation site, since alkylmonoamines, though bound at the active site, are not oxidized by putrescine oxidase. The 6—8 Å separation of the cation binding site and oxidation site shown in Fig. 8 is based on the internitrogen distances for substrates in their extended configurations as given by Bardsley et al. [9]. The configuration of the enzyme bound substrate is not known, so the 6—8 Å distance indicated in the model is simply a convenient estimate.

The indication of a negative charge in the active site of putrescine oxidase offers an explanation of the enzyme's inertness toward sulfite. Since sulfite is also negatively charged, one would expect it to be repulsed from the active site, preventing access to the FAD moiety. This explanation does not preclude the proposal of Massey et al. that a positive charge in the active site of flavoprotein oxidases stabilized the anionic form of the semiquinone [10]. However, if a cationic group functions to stabilize the anionic semiquinone in putrescine oxidase it is not sufficient for sulfite reactivity and it does not appear to influence the binding of cations in the active site. In this regard it is interesting to note that a number of the flavoenzymes found to form flavin sulfite adducts also react with substrates which contain a negatively charged group (e.g. D-amino acid oxidase, L-amino acid oxidase, lactate oxidase, glycollate oxidase and oxynitrilase) [10]. In putrescine oxidase the inverse situation is observed, i.e., the enzyme reacts with cationic substrates, an anionic group is indicated in the active site, and flavin-sulfite adducts are not formed.

The presence of a hydrophobic binding region may explain the inactivity of the enzyme toward long chain diamines ($n > 6$). It can be argued that the long chain diamines, with proper folding, should be able to achieve an acceptable internitrogen distance, and thus should serve as substrates. A region which binds the alkyl chain may preclude the required folding, however, and render the long chain diamines unable to react at the oxidation site.

Perturbation of the enzyme's visible absorbance spectrum upon binding inhibitors indicates that inhibitor binding changes the environment of the FAD. We have not determined if the change in environment is due to direct interaction between the inhibitor molecule and FAD or if it results from conformational changes in the protein induced by inhibitor binding.

The qualitative aspects of the inhibitor-induced spectral perturbations are intriguing, since a number of flavoproteins have been observed to yield similarly perturbed spectra in the presence of appropriate ligands [11—17]. Studies by Harbury et al. [18] and Kotaki et al. [19] with model flavins in various solvents suggest that the position of the near ultraviolet absorbance band (370 nm) and the presence of structure in the visible absorbance band of uncomplexed putrescine oxidase indicates that the FAD moiety resides in a somewhat apolar, but hydrogen-bond donating environment in the holoenzyme. Binding of most of the competitive inhibitors causes a change in the environment of the FAD in the direction of decreasing polarity. Additionally, binding of those inhibitors which produce a hypsochromic shift of the near ultraviolet absorption band (e.g. 1,7-diaminoheptane and 1,8-diaminooctane) may disrupt hydrogen bonding between the apoenzyme and FAD.

Acknowledgements

We wish to thank Ms. Rita V. Brantner for her valuable technical assistance.

This work was supported by grant GM 16834-07 from the National Institutes of Health, United States Public Health Service.

References

1 Yamada, H., Tanaka, A., and Ogata, K. (1965) Agric. Biol. Chem. 29, 260—261
2 Adachi, O., Yamada, H., and Ogata, K. (1966) Agric. Biol. Chem. 30, 1202—1210
3 DeSa, R.J. (1972) J. Biol. Chem. 247, 5527—5534
4 Swain, W., Cook, L., Brantner, R., and DeSa, R.J. (1974) Fed. Proc. 33, 1312
5 Wellner, D., and Meister, A. (1960) J. Biol. Chem. 235, 2013—2018
6 DeSa, R.J. and Wampler, J.E. (1973) Appl. Spec. 27, 279—284
7 Cleland, W.W. (1967) in Advances in Enzymology (Nord, F.F., ed.) Vol. 29, pp. 1—32, Interscience, New York
8 Cleland, W.W. (1970) in The Enzymes (Boyer, P.D., ed.) 3rd edn., Vol. II, pp. 1—66, Academic Press, New York
9 Bardsley, W.G., Hill, C.M. and Lobley, R.W. (1970) Biochem. J. 117, 169—176
10 Massey, V., Muller, F., Feldberg, R., Schuman, M., Sullivan, P., Howell, L.G., Mayhew, S.G., Matthews, R.G. and Foust, G.P. (1969) J. Biol. Chem. 244, 3999—4006
11 Massey, V. and Ganther, H. (1965) Biochemistry 4, 1161—1173
12 Nakmura, S. and Ogura, Y. (1965) in Flavins and Flavoproteins (Yagi, K., ed.), pp. 164—176, University of Tokyo Press, Tokyo
13 Rodgers, M.J. and Brandt, K.G. (1971) Biochemistry 10, 4624—4630
14 Veeger, C., DerVartanian, D.V., Kalse, J.F., DeKok, A. and Koster, J.F. (1966) in Flavins and Flavoproteins (Slater, E.C., ed.) pp. 242—262, Elsevier, Amsterdam
15 Veeger, C., Voetberg, H., Visser, J., Staal, G. and Koster, J. (1971) in Flavins and Flavoproteins, (Kamin, H., ed.) pp. 261—288, University Park Press, Baltimore
16 Tagi, K., Naoi, M., Nishikimi, M. and Kotaki, A. (1970) J. Biochem. 68, 293—301
17 Yagi, K., Ozawa, T., and Naoi, M. (1969) Biochim. Biophys. Acta 185, 31—38
18 Harbury, H.A., LaNoue, K.F., Loach, P.A. and Amick, R.M. (1959) Proc. Natl. Acad. Sci. U.S. 45, 1708—1717
19 Kotaki, A., Naoi, M. and Yagi, K. (1970) J. Biochem. 68, 287—292