Messing, J. (1983) *Methods Enzymol. 101*, 20-89.
Perry, L. J., & Wetzel, R. (1984) *Science (Washington, D.C.) 226*, 555-557.
Roggenkamp, R., Dargatz, H., & Hollenberg, C. (1985) *J. Biol. Chem. 260*, 1508-1512.
Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463-5467.
Sigal, I. S., Harwood, B. G., & Arentzen, R. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 7157-7160.
Sigal, I. S., DeGrado, W. F., Thomas, B. J., & Petteway, S. R., Jr. (1984) *J. Biol. Chem. 259*, 5327-5332.
Sutcliffe, J. G. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3737-3741.
Villafranca, J. E., Howell, E. E., Voet, D. H., Strobel, M. S., Ogden, R. C., Abelson, J. N., & Kraut, J. (1983) *Science (Washington, D.C.) 222*, 782-788.
Vlasuk, G. P., Inouye, S., Ito, H., Itakura, K., & Inouye, M. (1983) *J. Biol. Chem. 258*, 7141-7148.
Wallace, R. B., Shaffer, J., Murphy, R. F., Bonner, J., Hirose, T., & Itakura, K. (1979) *Nucleic Acids Res. 6*, 3543-3557.
Wallace, R. B., Schold, M., Johnson, M. J., Dembek, P., & Itakura, K. (1981) *Nucleic Acids Res. 9*, 3647-3656.
Wilkinson, A. J., Fersht, A. R., Blow, D. M., Carter, P., & Winter, G. (1984) *Nature (London) 307*, 187-188.
Winter, G., Fersht, A. R., Wilkinson, A. J., Zoller, M., & Smith, M. (1982) *Nature (London) 299*, 756-758.
Zoller, M. J., & Smith, M. (1982) *Nucleic Acids Res. 10*, 6487-6499.

Roles of the Two Copper Ions in Bovine Serum Amine Oxidase†

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ABSTRACT: With a view to obtaining information on the roles of the two copper ions in bovine serum amine oxidase (BSAO), spectroscopic and magnetic studies on several BSAO derivatives have been carried out. Cu-depleted BSAO (Cu-depBSAO) exhibits no enzyme activity and only a low absorption intensity at ca. 475 nm, which is the characteristic absorption maximum of the chromophore in BSAO. The binding of 1 mol of Cu to 1 mol of Cu-depBSAO slightly but definitely increases the enzyme activity and the absorptivity, although they are much lower than those of native BSAO. The incorporation of 2 mol of Cu into Cu-depBSAO gives rise to a similar high activity and absorptivity as those of the native enzyme. Electron paramagnetic resonance (EPR) spectra of the BSAO derivatives reveal that two copper ions in the enzyme molecule are environmentally identical. Titrations of BSAO, Cu-depBSAO, and Cu-half-depleted BSAO (Cu-half-depBSAO), containing 1 mol of copper per mole of protein, with phenylhydrazine (an inhibitor of BSAO) indicate that only 1 mol of phenylhydrazine reacts with 1 mol of the enzyme. In other words the enzyme possesses only one chromophore or one active site, though the molecule is composed of two electrophoretically identical subunits. The binding constants between phenylhydrazine and BSAO, Cu-depBSAO, or Cu-half-depBSAO were estimated to be 5×10^6, 5×10^4, and 1×10^5 M^{-1}, respectively. The binding of phenylhydrazine to the chromophore is assisted by the presence of two copper ions by a factor of 100. One of the most important roles of the copper ions in BSAO is likely to retain the chromophore in a favorable geometric and electronic structure for binding the substrate.

Copper-containing amine oxidases [amine:oxygen oxidoreductase (deaminating) (copper-containing), EC 1.4.3.6] catalyze the oxidative deamination of amines by accepting two electrons from amines and transferring them to molecular oxygen, as expressed by the equation (Malmström et al., 1975)

$$RCH_2NH_2 + O_2 + H_2O \rightarrow RCHO + NH_3 + H_2O_2$$

They are known to contain nonblue and electron paramagnetic resonance (EPR)[1] detectable copper (Yamada et al., 1963, 1969; Mondovi et al., 1967; Lindström et al., 1974; Suzuki et al., 1980, 1983) and an organic chromophore responsible for their yellowish pink color (Yamada & Yasunobu, 1963; Adachi & Yamada, 1969; Lindström & Pettersson, 1973; Ishizaki & Yasunobu, 1976; Finazzi-Agrò et al., 1977; Suzuki et al., 1981, 1982, 1983). Reactions of the copper with complexing agents (Yamada & Yasunobu, 1962; Lindström & Pettersson, 1974; Lindström et al., 1974) or of the chromophore with carbonyl reagents (Yamada & Yasunobu, 1963; Falk, 1983) lead to an inactivation of the enzyme. Therefore, the presence of both the copper and the chromophore seems to be essential for the activity of amine oxidases. Our recent investigation on metal substitution and depletion of bovine serum amine oxidase (BSAO) established that there are two forms of the chromophore, namely, yellowish pink (oxidized) and pale yellow (reduced) forms. The chromophore and

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[1] Abbreviations: BSAO, bovine serum amine oxidase; Cu-depBSAO, Cu-depleted BSAO; Cu-excessBSAO, BSAO containing excess Cu; Cu-half-depBSAO, Cu-half-depleted BSAO; Cu-recBSAO, Cu-reconstituted BSAO; EPR, electron paramagnetic resonance; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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coppers were suggested to behave as a set in the catalytic reaction (Suzuki et al., 1983).

The molecular weight of Cu amine oxidases has been reported to be 170 000–200 000. The enzymes are composed of two identical subunits and two copper ions (Malmström et al., 1975). However, titrimetric experiments of the enzyme with substrate under anaerobic conditions (Massey & Churchich, 1977; Lindström & Pettersson, 1978) or with inhibitors such as phenylhydrazine and catran (Lindström & Pettersson, 1973; Falk, 1983) exhibited that the reaction of enzyme occurred in a 1:1 molar ratio of reagent to enzyme molecules, suggesting the existence of one active site or one chromophore per two subunits. A question thus arises as to the mode of distribution of the two coppers in the two subunits. Mondovi et al. (1984a,b) demonstrated that only one of the two coppers in bovine plasma amine oxidase is required to exhibit the full enzyme activity. On the other hand, two research groups reported that 3 or 2 mol of phenylhydrazine react with 1 mol of pig plasma amine oxidase (Buffoni & Igenesti, 1975) or *Aspergillus niger* amine oxidase (Suzuki et al., 1971), respectively.

This study was undertaken to explore the disputed number and roles of copper in the enzymatic process of BSAO.

Experimental Procedures

Materials. BSAO isolated from bovine serum was purified by the method described previously (Suzuki et al., 1983). The highly purified BSAO displayed a specific activity of 610 units/mg at 25 °C according to the method of Tabor et al. (1954). The enzyme has a molecular weight of 190 000 and contains 2 mol of copper per mole of protein which contains two electrophoretically equivalent subunits. The protein concentration was estimated by measuring the absorptivity at 280 nm ($E^{1\%}_{1cm}$ = 17.4). All reagents used were of the highest grade commercially available.

Preparations of Cu-Depleted BSAO, Cu-Half-Depleted BSAO, and Cu-Reconstituted BSAO. Copper-depleted BSAO (Cu-depBSAO) was prepared by dialyzing colorless BSAO that had been reduced with sodium dithionite against 0.2 M sodium/potassium phosphate buffer (pH 7.2) containing 10 mM KCN under nitrogen atmosphere. To obtain fully colored Cu-depBSAO, the sample was treated with Ni(II) ion in 0.2 M Tris-HCl buffer (pH 7.2), being dialyzed against complexing agents, dimethylglyoxime, and CN^- ion to remove the Ni(II) ion (Suzuki et al., 1983). The resulting Cu-depBSAO [yellowish pink oxidized form described in the previous paper (Suzuki et al., 1983)] contained 3% of residual copper for the total copper sites. The copper concentration was determined by means of atomic absorption spectroscopy. Copper-half-depleted BSAO (Cu-half-depBSAO) was prepared by incubation of about 1 mL of Cu-depBSAO with 1 equiv of copper per mole of protein in 0.2 M Tris-HCl buffer (pH 7.2) for 20 h. Further, the dialysis of the sample was continued for 50 h with four changes of 0.2 M phosphate buffer (200 mL), pH 7.2. The concentration of Cu in Cu-half-depBSAO was determined to be 0.9 mol per mole of protein. The reconstitution of holoenzyme was carried out by dialyzing about 1.5 mL of Cu-depBSAO against 200 mL of 0.2 M Tris-HCl buffer (pH 7.2) containing 2 mM Cu(II) ion for 40 h. The excess Cu ion bound to the resulting sample was removed by treatment with Chelex 100 resin (50–100-mesh resin preequilibrated with the Tris-HCl buffer). The resulting Cu-reconstituted BSAO (Cu-recBSAO) contained 1.9 mol of copper per mole of protein. All the procedures described above were performed at 4 °C.

Titrations of Native BSAO, Cu-half-depBSAO, and Cu-depBSAO with Phenylhydrazine. Because solutions of phenylhydrazine are usually unstable, a stock solution was freshly prepared for each titration experiment by dissolving phenylhydrazine or its hydrochloride in 0.2 M phosphate buffer, pH 7.2. The buffer solution (0.8 mL) containing (1–1.5) × 10^{-8} mol each of BSAO, Cu-half-depBSAO, or Cu-depBSAO was continuously titrated with the phenylhydrazine solution at room temperature. The BSAO solutions were allowed to incubate for 5 min each before reading the increase in optical density at 450 nm. In the cases of Cu-depBSAO and Cu-half-depBSAO, the absorbance at 440 nm was measured after the enzyme solutions had been allowed to stand with phenylhydrazine for 15 min each. The reaction of the chromophore with phenylhydrazine was almost completed within the above period.

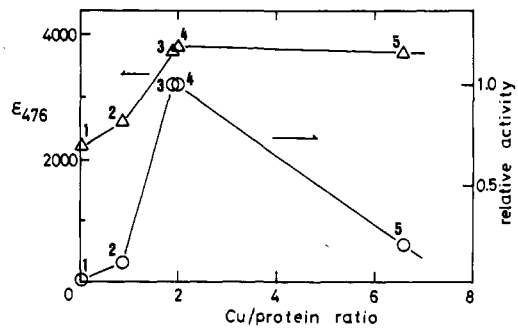

FIGURE 1: Relationship between Cu/protein ratio and absorption coefficient at 476 nm (△) or relative enzyme activity (○) in BSAO. The ϵ_{476} value is given per mole of protein, and the relative activity of native BSAO is defined as 1.0. Plots: 1, Cu-depBSAO; 2, Cu-half-depBSAO; 3, Cu-recBSAO; 4, native BSAO; 5, Cu-excessBSAO.

Instruments. The absorption, EPR, and atomic absorption spectra were measured with a Union SM-401 spectrophotometer, a JEOL JES-FE1X EPR spectrometer, and a Nippon Jarrell-Ash AA-1 spectrometer, respectively. The measurements of absorption and EPR spectra were carried out at room temperature and 77 K, respectively.

Results and Discussion

Characterizations of Cu-depBSAO, Cu-half-depBSAO, Cu-recBSAO, and Cu-excessBSAO. Figure 1 represents the variation of intensities of the characteristic absorption band (λ_{max}, 472–476 nm) against the molar ratio of copper to the protein. The enzyme activity–copper content profile is also given in the same figure. Cu-depBSAO (plots 1 in Figure 1) is almost inactive, exhibiting a smaller absorptivity as compared with that of Cu-half-depBSAO (plots 2). This implies that incorporation of only 1 mol of copper per mole of protein gives rise to a definite increase in the absorptivity and activity. The increment is, however, not so great as that of the Cu-recBSAO (plots 3) or the native BSAO (plots 4) containing 2 mol of copper per mole of protein. Of special interest is the fact that the relative activity of Cu-half-depBSAO corresponds to only one-tenth of that of the Cu-recBSAO (plots 3) or the native BSAO (plots 4) and not to half. This finding suggests that 2 mol of copper ions per mole of protein are required for BSAO to display the full activity, which contradicts the result obtained by Mondovi et al. (1984a,b). They demonstrated that plasma amine oxidase containing only one copper was as catalytically active as the enzyme having two coppers. The incorporation of an excess copper into the protein [Cu-excessBSAO (plots 5)] affords a sufficiently colored but much less active derivative as compared with the native enzyme. This suggests that 2 mol of copper per mole of protein is sufficient to keep the catalytically active conformation of the chromo-

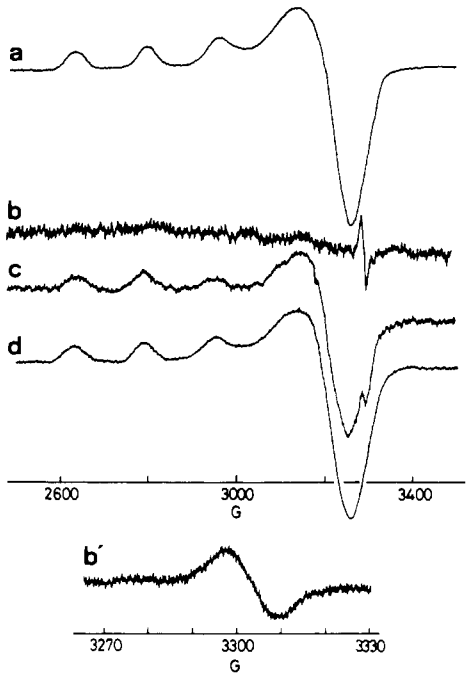

FIGURE 2: EPR signals of BSAO (a), Cu-depBSAO (b and b'), Cu-half-depBSAO (c), and Cu-recBSAO (d) at 77 K.

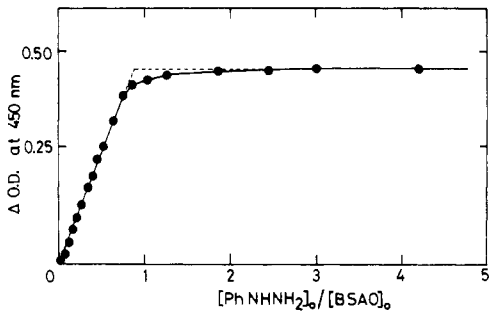

FIGURE 3: Titration curve of BSAO with phenylhydrazine. The abscissa indicates the ratio of the initial concentrations of phenylhydrazine ($PhNHNH_2$) and BSAO.

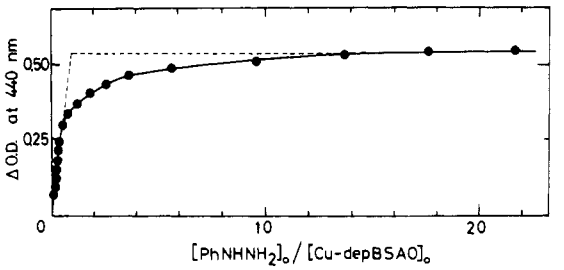

FIGURE 4: Titration curve of Cu-depBSAO with phenylhydrazine. The abscissa indicates the ratio of the initial concentrations of phenylhydrazine ($PhNHNH_2$) and Cu-depBSAO.

phore and the excess copper exceeding 2 mol acts as an inhibitor in the catalytic process.

The X-band EPR spectra of BSAO and its derivatives at 77 K are illustrated in Figure 2. The Cu signals of native BSAO (a), Cu-half-depBSAO (c), and Cu-recBSAO (d) indicate the axial symmetry of Cu(II) with the same spin-Hamiltonian parameters, $g_{\parallel} = 2.29$, $g_{\perp} = 2.06$, and $A_{\parallel} = 164$ G. In the case of Cu-depBSAO (b), only an isotropic signal characteristic of an organic radical was recorded at $g = 2.004$. The appearance of the radical signal seems to depend on the molar ratio of copper to the protein. For example, the signal is also present in the spectrum of Cu-half-depBSAO (c) while it is absent in those of native BSAO (a) and Cu-recBSAO (d). The source of the $g = 2$ signal is still unknown at the present stage. The copper signal of Cu-half-depBSAO is substantially identical with that of Cu-recBSAO or native BSAO, being considered as a monomeric Cu species. Thus the present X-band EPR data probably exclude the possibility that two copper ions are located in different environments. In other words the two copper ions in BSAO are considered to be structurally identical, in constrast to the conclusion that the two copper ions are magnetically inequivalent in pig amine oxidase (Barker et al., 1979; Kluetz & Schmidt, 1980).

Titrations of BSAO, Cu-depBSAO, and Cu-half-depBSAO with Phenylhydrazine. Titrations of BSAO and Cu-depBSAO with phenylhydrazine were monitored by increasing absorbance at 450 and 440 nm, respectively. The results are depicted in Figures 3 and 4, respectively. Inspection of Figure 3 reveals that the increase in the absorbance is directly proportional to the increase in the molar ratio of phenylhydrazine to BSAO until the latter reaches 0.9. This can be explained by assuming the existence of a single chromophore (active site) in BSAO as reported for the pig plasma amine oxidase (Lindström & Pettersson, 1973; Falk, 1983) and bovine plasma amine oxidase (Ishizaki & Yasunobu, 1984). The enzyme activity of BSAO is completely inhibited with an equimolar amount of phenylhydrazine (Horigome, T., and Okuyama, T., unpublished experiments). In the reaction of Cu-depBSAO with phenylhydrazine the addition of more than 10 equiv of phenylhydrazine is necessary before attaining the full yellow coloration, as visualized in Figure 4. Further, it should be added that the rate of reaction of phenylhydrazine with Cu-depBSAO is significantly slower than that with the native BSAO.

Titration of Cu-half-depBSAO with phenylhydrazine was also carried out in a similar fashion. The equivalence points of all the titration experiments, which can be reliably estimated by extrapolation of the linear portions of each titration curve, indicate that all the reactions of phenylhydrazine with BSAO, Cu-depBSAO, or Cu-half-depBSAO occur in an equimolar ratio. The formation constants of the chromophore–phenylhydrazine complex were calculated by use of the titration data in the unsaturated region where the molar ratio of phenylhydrazine to copper is, for example, 1–2 in Figure 3. The complex formation between phenylhydrazine and BSAO is regarded as completed at 2.5 mol of the former. The average values for BSAO, Cu-half-depBSAO, and Cu-depBSAO were thus determined to be 5×10^6, 1×10^5, and 5×10^4 M^{-1}, respectively. These are indicative of the effective assistance (by a factor of 100) of the two coppers of BSAO in the complex formation between the chromophore and phenylhydrazine. On the other hand, the single copper as in Cu-half-depBSAO does not effectively assist complex formation.

The same kind of titration was not attained for the determination of the number of active sites for benzylamine because of very weak binding ability of the chromophore with benzylamine in the absence of copper. Thus the number of chromophore and active sites for substrate remains unknown. However, the titrimetric experiments of BSAO with phenylhydrazine (this work; Ishizaki & Yasunobu, 1984) and pig amine oxidase with substrates (Massey & Churchich, 1977; Lindström & Pettersson, 1978) or inhibitors (Lindström & Pettersson, 1973; Falk, 1983) afford the grounds to assume the presence of a single active site or chromophore also for substrate in Cu amine oxidases. We previously reported that one of the most important roles of the copper ions in BSAO is likely their ability to retain the chromophore in a favorable geometric and electronic structure for substrate binding (Suzuki et al., 1983). This work offers evidence that copper

effectively assists the binding of chromophore with substrates or inhibitors.

Registry No. BSAO, 9059-11-4; Cu, 7440-50-8; $PhNHNH_2$, 100-63-0.

References

Adachi, O., & Yamada, H. (1969) *J. Biochem. (Tokyo) 65*, 639–640.

Barker, R., Boden, N., Cayley, G., Charlton, S. C., Henson, R., Holmes, M. C., Kelly, I. D., & Knowles, P. F. (1979) *Biochem. J. 177*, 289–302.

Buffoni, F., & Ignesti, G. (1975) *Biochem. J. 145*, 369–372.

Falk, M. C. (1983) *Biochemistry 22*, 3740–3745.

Finazzi-Agrô, A., Guerrieri, P., Costa, M. T., & Mondovi, B. (1977) *Eur. J. Biochem. 74*, 435–440.

Ishizaki, H., & Yasunobu, K. T. (1976) *Adv. Exp. Med. Biol. 74*, 575–588.

Ishizaki, H., & Yasunobu, K. T. (1984) *Biochem. Biophys. Stud. Proteins Nucleic Acids [Proc. Int. Symp.], 3rd, 1982 (1984)*, 317–329.

Kluetz, M. D., & Schmidt, P. G. (1980) *Biophys. J. 29*, 283–292.

Lindström, A., & Pettersson, G. (1973) *Eur. J. Biochem. 34*, 564–568.

Lindström, A., & Pettersson, G. (1974) *Eur. J. Biochem. 48*, 229–236.

Lindström, A., & Pettersson, G. (1978) *Eur. J. Biochem. 83*, 131–135.

Lindström, A., Olsson, B., & Pettersson, G. (1974) *Eur. J. Biochem. 48*, 237–243.

Malmström, B. G., Andreasson, L. E., & Reinhammer, B. (1975) *Enzymes (3rd Ed.) 12*, 507–579.

Massey, J. B., & Churchich, J. E. (1977) *J. Biol. Chem. 252*, 8081–8084.

Mondovi, B., Rotilio, G., Costa, M. T., Finazzi-Agrô, A., Chiancone, E., Hansen, R. E., & Beinert, H. (1967) *J. Biol. Chem. 242*, 1160–1167.

Mondovi, B., Befani, O., & Sabatini, S. (1984a) *Agents Actions 14*, 356–357.

Mondovi, B., Sabatini, S., & Befani, O. (1984b) *J. Mol. Catal. 23*, 325–330.

Suzuki, H., Ogura, Y., & Yamada, H. (1971) *J. Biochem. (Tokyo) 69*, 1065–1074.

Suzuki, S., Sakurai, T., Nakahara, A., Oda, O., Manabe, T., & Okuyama, T. (1980) *FEBS Lett. 116*, 17–20.

Suzuki, S., Sakurai, T., Nakahara, A., Oda, O., Manabe, T., & Okuyama, T. (1981) *J. Biochem. (Tokyo) 90*, 905–908.

Suzuki, S., Sakurai, T., Nakahara, A., Oda, O., Manabe, T., & Okuyama, T. (1982) *Chem. Lett.*, 487–490.

Suzuki, S., Sakurai, T., Nakahara, A., Manabe, T., & Okuyama, T. (1983) *Biochemistry 22*, 1630–1635.

Tabor, C. W., Tabor, H., & Rosenthal, S. M. (1954) *J. Biol. Chem. 208*, 645–661.

Yamada, H., & Yasunobu, K. T. (1962) *J. Biol. Chem. 237*, 3077–3082.

Yamada, H., & Yasunobu, K. T. (1963) *J. Biol. Chem. 238*, 2669–2675.

Yamada, H., Yasunobu, K. T., Yamano, T., & Mason, H. S. (1963) *Nature (London) 198*, 1092–1093.

Yamada, H., Adachi, O., & Yamano, T. (1969) *Biochim. Biophys. Acta 191*, 751–752.

1-Phenylcyclobutylamine, the First in a New Class of Monoamine Oxidase Inactivators. Further Evidence for a Radical Intermediate†

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ABSTRACT: 1-Phenylcyclobutylamine (PCBA) is shown to be both a substrate and a time-dependent irreversible inactivator of monoamine oxidase (MAO). Inactivation results in attachment to the flavin cofactor. For every molecule of PCBA leading to inactivation, 325 molecules are converted to product. The first metabolite formed is identified as 2-phenyl-1-pyrroline; then after a lag time, 3-benzoylpropanal and 3-benzoylpropionic acid are generated. The 3-benzoylpropanal is a product of MAO-catalyzed oxidation of 2-phenyl-1-pyrroline (presumably, of its hydrolysis product, γ-aminobutyrophenone). The aldehyde is nonenzymatically oxidized by nascent hydrogen peroxide to the carboxylic acid. These results are consistent with a one-electron oxidation of PCBA to the amine radical cation followed by homolytic cyclobutane ring cleavage. The resulting radical can partition between cyclization (an intramolecular radical trap) to the 2-phenylpyrrolinyl radical and attachment to the flavin. The cyclic radical can be further oxidized by one electron to 2-phenyl-1-pyrroline. PCBA represents the first in the cyclobutylamine class of MAO inactivators and strongly supports involvement of a radical mechanism for MAO-catalyzed amine oxidations.

Mitochondrial monoamine oxidase (MAO)[1] was discovered almost 60 years ago, yet its mechanism for oxidation of biogenic amines is unknown. On the basis of studies of the mechanism of inactivation of MAO by various cyclopropylamine analogues (Scheme I) (Silverman et al., 1980; Silverman & Hoffman, 1981; Silverman, 1983, 1984; Silverman & Ya-

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[1] Abbreviations: MAO, mitochondrial monoamine oxidase (EC 1.4.3.4); PCBA, 1-phenylcyclobutylamine.

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