in Scheme II, the Schiff base of the adduct in the earlier work apparently was protected from hydrolysis during dialysis, apparently as a result of the method of enzyme purification.

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Mechanism for Reactivation of *N*-Cyclopropylbenzylamine-Inactivated Monoamine Oxidase by Amines†

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ABSTRACT: The effect of 18 different amines, two mercaptans, and two alcohols on the reactivation of *N*-cyclopropylbenzylamine- (N-CBA-) inactivated bovine liver monoamine oxidase (MAO) is described. All of the compounds that reactivate the enzyme produce a time-dependent pseudo-first-order return of enzyme activity and exhibit saturation kinetics. There is no direct correlation between the ability of a compound to serve as a substrate for native MAO and its ability to reactivate N-CBA-inactivated MAO. Amines containing an aromatic moiety, in general, are better reactivators than the aliphatic amines. The amine must be primary or secondary in order for reactivation to occur. The distance between the aromatic portion and the amino group is critical to the reactivation properties of the compound. The mercaptans and alcohols do not reactivate N-CBA-inactivated MAO, nor do they interfere with the reactivation reaction by benzylamine. Three mechanisms for the reactivation reaction are considered. One involves initial Schiff base formation with the active site adduct produced by N-CBA inactivation of MAO followed by base-catalyzed β-elimination to the imine of acrolein. The second mechanism is the same as the first except no prior Schiff base formation is invoked. The third mechanism is an S_N2 displacement by the amine of the active site amino acid residue attached to the adduct. Experiments are carried out to exclude the S_N2 mechanism. The results of the reactivation experiments favor the Shiff base mechanism.

Mitochondrial monoamine oxidase (MAO,[1] EC 1.4.3.4), an enzyme containing a covalently bound FAD cofactor, catalyzes the oxidative deamination of biogenic amines. It has been shown in recent years that *N*-cyclopropyl-*N*-arylalkylamines are mechanism-based inactivators of MAO (Silverman et al., 1980; Silverman & Hoffman, 1980, 1981a; Silverman & Yamasaki, 1984; Silverman, 1984; Winn et al., 1975;

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[1] Abbreviations: MAO, monoamine oxidase; N-CBA, *N*-cyclopropylbenzylamine; N-[1-^{3}H]CBA, *N*-[1-^{3}H]cyclopropylbenzylamine; N-CαMBA, *N*-cyclopropyl-α-methylbenzylamine; N-CMBA, *N*-cyclopropyl-*N*-methylbenzylamine; FAD, flavin adenine dinucleotide.

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Murphy et al., 1978; Long et al., 1976; Fuller et al., 1978). A mechanism-based inactivator is a chemically unreactive compound that is converted by an enzyme via its catalytic mechanism to a species that, without prior release from the active site, produces inactivation of that enzyme (Silverman & Hoffman, 1984; Walsh, 1984; Abeles & Maycock, 1976; Rando, 1974). The mechanism proposed for the inactivation of MAO by N-CBA was described in the preceding paper in this issue (Vazquez & Silverman, 1985). Other substituted cyclopropylamines have been proposed to inactivate MAO by a similar initial one-electron transfer followed by cyclopropyl ring opening (Silverman & Yamasaki, 1984; Silverman & Hoffman, 1981a; Silverman, 1983; Silverman & Zieske, 1985). The covalent adduct that is formed when N-CBA inactivates MAO is labile and can be released, concomitant with the return of enzyme activity, upon treatment of the inactivated enzyme with benzylamine (Silverman & Hoffman, 1980; Vazquez & Silverman, 1985). This reactivation phenomenon as a result of added substrate has been observed by others. Zeller et al. (1962) reported that 4-phenyl-1-butylamine reactivated MAO that had been inactivated by *cis*- and *trans*-2-phenylcyclopropylamines. Rando & Eigner (1977) showed that the adduct formed between MAO and allylamine was released when the inactivated enzyme was treated with benzylamine. They suggested that the benzylamine binds to the aromatic binding site of the enzyme and then attacks the postulated flavin–allylamine adduct. No experimental evidence was presented, however, in favor of this binding hypothesis or of a flavin adduct.[2] In this paper, experiments are described for the reactivation of N-CBA-inactivated MAO that show that the reactivator, an amine meeting certain structural limitations, initially binds to the enzyme prior to reactivation and that the reactivator need not be a substrate for native MAO. A mechanism for the reactivation of N-CBA-inactivated MAO is suggested on the basis of the presented data.

Materials and Methods

Enzymes and Reagents. The purification and assay of the enzyme from bovine liver mitochondria were described previously (Vazquez & Silverman, 1985). Active site concentrations were determined by [^{14}C]pargyline binding (Chuang et al., 1974).

Horseradish peroxidase (160 purpurogallin units/mg), benzylamine, *n*-heptylamine, 5-hydroxytryptamine hydrochloride, β-mercaptoethanol, (+)-α-methylbenzylamine, (−)-norepinephrine hydrochloride, sodium borohydride, and tyramine hydrochloride were bought from Sigma. (Aminomethyl)cyclohexane (cyclohexylmethylamine), benzylmercaptan, *n*-butylamine, *n*-butylmercaptan, isoamylamine, leuco crystal violet, (−)-α-methylbenzylamine, *N*-methylbenzylamine, 2-phenylethylamine, 4-phenyl-1-butylamine, 3-phenyl-1-propylamine, and sodium cyanoborohydride were purchased from Aldrich. Aniline and *N*,*N*-dimethylaniline were obtained from J. T. Baker, and benzyl alcohol and *n*-butyl alcohol were from Mallinckrodt. N-CBA was synthesized by the method of Bumgardner et al. (1972), *N*,*N*-dimethylbenzylamine was prepared as described by Clarke et al. (1933), and *N*,*N*-dimethyl-2-phenylethylamine was prepared as described by Icke et al. (1955). [7-^{14}C]Pargyline hydrochloride was a gift of Prof. Roy McCauley (Wayne State University). All amine compounds were converted to their hydrochloride salts by the addition of HCl in methanol followed by evaporation and recrystallization from methanol–ethyl acetate. N-[1-^{3}H]CBA (Silverman & Hoffman, 1981b), N-[1-^{3}H]-CαMBA (Silverman, 1984), and (±)-α-methyl[α-^{3}H]-benzylamine (Silverman, 1984) were prepared as previously reported.

Scintillation Counting. Radioactivity was measured with a Beckman LS-3133T scintillation counter using 10 mL of 3a70B scintillation fluid from Research Products International. [^{14}C]Toluene (4.7×10^5 dpm/mL) or [^{3}H]toluene (1.57×10^6 dpm/mL, corrected for first-order decay), obtained from New England Nuclear, were used as internal standards.

Preparation of N-CBA-Inactivated MAO. In a typical preparation of N-CBA-inactivated enzyme, 25 μL of 0.28 mM MAO in 50 mM potassium phosphate, pH 7.2, was mixed with 975 μL of 2 mM N-CBA·HCl in the same buffer. The reaction mixture was incubated in the dark at 25 °C for 90 min before being dialyzed exhaustively against four changes (200 mL each) of 20 mM potassium phosphate, pH 7.0, at room temperature. A control containing no N-CBA was prepared simultaneously. Aliquots from the control enzyme and inactivated enzyme were removed for activity assays. Differences in protein concentrations were determined by the method of Lowry et al. (1951), and the corrected enzyme activities are reported.

Screening for Reactivators of N-CBA-Inactivated MAO. To 90 μL of the dialyzed inactivated enzyme solution (prepared as described above) was added 10 μL of a 40 mM solution of the reactivator candidate in 100 mM potassium phosphate, pH 7.0, containing 100 mM β-mercaptoethanol. The mixture was incubated in the dark at 25 °C, and 5-μL aliquots were removed after 12 h for activity assays. The mixture was then diluted 1:1 with 4 mM benzylamine hydrochloride in 28 mM potassium phosphate, pH 7.0, containing 10 mM β-mercaptoethanol and incubated in the dark at 25 °C for another 12 h. A 5-μL aliquot was then removed and assayed for enzyme activity in the same manner. The enzyme activity was determined relative to a control enzyme that had not been inactivated previously by N-CBA.

Kinetic Studies. Compounds determined to be reactivators of N-CBA-inactivated MAO were submitted to the following kinetic experiment: To 90 μL of the dialyzed N-CBA-inactivated MAO solution, prepared as described above (see Preparation of N-CBA-Inactivated MAO), was added 10 μL of a solution of the reactivator (several different concentrations were used; see Table II) in 100 mM potassium phosphate, pH 7.0, containing 100 mM β-mercaptoethanol. The mixture was incubated in the dark at 25 °C, and 5-μL aliquots were removed periodically for enzyme activity assays. The enzyme activity was determined relative to a control enzyme that had not been inactivated previously by N-CBA.

The Michaelis constant, K_m, catalytic rate constant, k_{cat}, and inhibition constant, K_i, of the various substrates and inhibitors of MAO were determined from the following initial velocity measurements: 5 μL of 6.4 μM native MAO in 28 mM potassium phosphate, pH 7.0, was incubated with 500 μL of the substrate or substrate plus inhibitor at several different concentrations in the same buffer for 2 min at 25 °C. The amount of enzymatic oxidation, measured by the production of hydrogen peroxide, was determined by the leuco crystal violet method of Mottola et al. (1970). The incubation period selected was such that the production of hydrogen peroxide was linear with time.

Treatment of N-CBA-Inactivated MAO with (±)-α-Methyl[α-^{3}H]benzylamine in the Presence of Sodium Cyanoborohydride. To 675 μL of 2 mM N-CBA·HCl in 50 mM potassium phosphate, pH 7.2, was added 0.28 mM MAO (75

[2] We have found that allylamine does not become attached to the flavin during inactivation (Silverman et al., 1985).

μL) in the same buffer. The reaction mixture was incubated in the dark at 25 °C for 90 min before being dialyzed exhaustively against three changes (150 mL each) of 20 mM potassium phosphate, pH 7.0, at room temperature. The dialyzed mixture was divided into two equal-volume batches. One batch was dialyzed over a 12-h period against three changes (200 mL each) of 1 mM benzylamine hydrochloride in 20 mM potassium phosphate, pH 7.0, containing 10 mM β-mercaptoethanol at room temperature. The other batch was treated in the same manner, but without the presence of benzylamine. The two batches were then dialyzed exhaustively against three changes (150 mL each) of 20 mM potassium phosphate, pH 7.0, at room temperature. Aliquots were removed from both batches for enzyme activity assays. The enzyme activity was determined relative to a control enzyme that had not been inactivated previously by N-CBA. To each batch (270 μL) was added 30 μL of 20 mM (±)-α-methyl-[α-^{3}H]benzylamine hydrochloride in 20 mM potassium phosphate, pH 7.0, containing 100 mM sodium cyanoborohydride. The reaction mixtures were incubated in the dark at 25 °C for 15 h before being dialyzed exhaustively against three changes (200 mL each) of 8 M urea in 20 mM potassium phosphate, pH 7.0. Aliquots were removed for scintillation counting, and in all cases, protein contents were normalized by the method of Lowry et al. (1951). The experiment was repeated exactly as above except with a 5-fold higher concentration of (±)-α-methyl[α-^{3}H]benzylamine hydrochloride.

Sodium Borohydride Treatment of N-[1-^{3}H]CBA-Inactivated MAO: Prevention of the Reactivation by Benzylamine. To 450 μL of 2 mM N-[1-^{3}H]CBA·HCl in 50 mM potassium phosphate, pH 7.2, was added 0.28 mM MAO (50 μL) in the same buffer. The reaction mixture was incubated in the dark at 25 °C for 90 min before being dialyzed exhaustively against three changes (200 mL each) of 20 mM potassium phosphate, pH 7.0, followed by one change (200 mL) of 50 mM sodium borate, pH 9.0, at room temperature. The dialyzed enzyme was divided into two 225-μL batches. To one batch was added 50 mM sodium borate, pH 9.0 (25 μL), and to the other batch was added 10 mM sodium borohydride in 50 mM sodium borate, pH 9.0 (25 μL). The reaction mixtures were incubated in the dark at 25 °C for 3 h before being dialyzed exhaustively against three changes (200 mL each) of 20 mM potassium phosphate, pH 7.0. Aliquots were removed for enzyme activity assays and scintillation counting. The two batches were then dialyzed over a 14-h period against three changes (200 mL each) of 1 mM benzylamine hydrochloride in 20 mM potassium phosphate, pH 7.0, containing 10 mM β-mercaptoethanol at room temperature. The two batches were finally dialyzed exhaustively against three changes (200 mL each) of 20 mM potassium, pH 7.0. Aliquots were removed for enzyme activity assays and scintillation counting. The enzyme activities were determined relative to the corresponding control enzyme that had not been inactivated previously by N-[1-^{3}H]CBA. In all cases, protein contents were normalized by the method of Lowry et al. (1951). This experiment was repeated at a 5-fold higher concentration of sodium borohydride and with the reduction period extended to 10 h. A similar experiment was carried out with MAO inactivated by *N*-[1-^{3}H]cyclopropyl-α-methylbenzylamine, an analogue of N-CBA shown to be incorporated into MAO with a 1:1 stoichiometry (Vazquez & Silverman, 1985).

Release of Tritium from N-[1-^{3}H]CαMBA-Inactivated MAO by N-CBA, N-CMBA, and N-CαMBA. MAO (50 μL) was inactivated by N-[1-^{3}H]CαMBA and dialyzed as above. The inactivated enzyme containing 1.3 equiv of tritium per active site (1600 dpm) was diluted with 100 μL of 20 mM potassium pyrophosphate, pH 9.0 buffer containing β-mercaptoethanol and either 10 mM N-CBA or N-CMBA. An inactivated enzyme solution treated the same except without amine served as a control. All samples were allowed to incubate in the dark at 25 °C for 4 h and then were dialyzed against 3 × 200 mL of 20 mM potassium phosphate, pH 7.2 buffer. The same experiment was carried out with MAO containing 1.1 equiv of tritium per active site and was reactivated as above except substituting N-CαMBA for N-CBA.

Table I: Reactivation of N-CBA-Inactivated MAO by Various Compounds[a]

compound	% reactivation[b]
none	<2
benzylamine	100
N-methylbenzylamine	93
N,*N*-dimethylbenzylamine	3.5
(+)-α-methylbenzylamine	93
(−)-α-methylbenzylamine	86
2-phenylethylamine	86
tyramine	91
N,*N*-dimethyl-2-phenylethylamine	3.1
(−)-norepinephrine	<2
3-phenyl-1-propylamine	15
4-phenyl-1-butylamine	5.6
5-hydroxytryptamine (serotonin)	5.9
aniline	57
N,*N*-dimethylaniline	<2
n-butylamine	18
isoamylamine	30
n-heptylamine	64
(aminoethyl)cyclohexane	40
n-butyl alcohol	<2
n-butylmercaptan	<2
benzyl alcohol	<2
benzylmercaptan	<2

[a] The N-CBA-inactivated MAO (6.4 μM), prepared as described under Materials and Methods, was incubated individually with the compounds listed in this table (4 mM) in 28 mM potassium phosphate, pH 7.0, containing 10 mM β-mercaptoethanol at 25 °C in the dark for 12 h. Aliquots were then removed for enzyme activity assays (Tabor et al., 1954). [b] The enzyme activity, before and after treatment with the test compound, was determined relative to a control enzyme that had not been inactivated previously by N-CBA. The percent reactivation reported has been corrected for residual activity in N-CBA-inactivated MAO before treatment with the test compound.

RESULTS

Screening for Reactivators of N-CBA-Inactivated MAO. When dialyzed N-CBA-inactivated MAO was allowed to incubate with 4 mM benzylamine at pH 7.0 for 12 h, complete return of enzyme activity was observed. No reactivation occurred in the absence of benzylamine. Other compounds were tested for their effectiveness as reactivators of N-CBA-inactivated MAO, and the results are shown in Table I. None of the compounds tested prevented the reactivation of N-CBA-inactivated MAO by an equal concentration (2 mM) of benzylamine.

Kinetic Studies. When dialyzed N-CBA-inactivated MAO was allowed to incubate with benzylamine at pH 7.0, a time-dependent pseudo-first-order return of enzyme activity was observed. The rate of reactivation was dependent upon the concentration of benzylamine and reached a maximum with increasing concentration. The dissociation constant for the reactivator–N-CBA-inactivated enzyme complex, K_r, and the reactivation rate constant at saturation, k_{react}, were determined, and the results are shown in Table II. The other reactivators of N-CBA-inactivated MAO listed in Table I displayed similar behavior to benzylamine, and their kinetic parameters (K_r and k_{react}) are shown in Table II. The con-

Table II: Kinetic Parameters[a] of Reactivators of N-CBA-Inactivated MAO

compound	concn range of reactivator (mM)	K_r (mM)	k_{react} (min^{-1})	K_m (mM)[b]	k_{cat} (min^{-1})
benzylamine	0.4–4.0	0.94	0.016	0.34	270
N-methylbenzylamine	0.4–4.0	0.60	0.0041	0.22	170
N,*N*-dimethylbenzylamine		nr[c]	nr	ns	ns
(+)-α-methylbenzylamine	0.4–2.0	0.56	0.0028	ns	ns
(−)-α-methylbenzylamine	0.4–2.0	0.58	0.0033	ns	ns
2-phenylethylamine	0.4–4.0	0.58	0.0033	0.075	240
tyramine	2.5–20	2.5	0.0069	0.23	68
N,*N*-dimethyl-2-phenylethylamine		nr	nr	0.31	71
(−)-norepinephrine		nr	nr	ps	ps
3-phenyl-1-propylamine		pr	pr	0.054	161
4-phenyl-1-butylamine		nr	nr	0.068	141
5-hydroxytryptamine (serotonin)		nr	nr	ps	ps
aniline	2.0–10	3.0	0.0078	ns	ns
N,*N*-dimethylaniline		nr	nr	ns	ns
n-butylamine		pr	pr	0.26	290
isoamylamine	6.7–40	5.6	0.0020	0.12	98
n-heptylamine	0.67–4.0	1.4	0.0014	0.069	234
(aminoethyl)cyclohexane	6.7–40	45	0.0067	0.065	81

[a] K_r is the dissociation constant of the reactivator–N-CBA-inactivated enzyme complex, and k_{react} is the reactivation rate constant at saturating concentrations of reactivator. K_m is the Michaelis constant, and k_{cat} is the turnover number for the native MAO. See Materials and Methods for experimental details. [b] Concentrations of substrates used were within a factor of 10 on both sides of the K_m. [c] nr, no reactivation; pr, poor reactivation; ns, not a substrate; ps, poor substrate.

centration ranges for reactivators used also are listed in Table II. Included with the kinetic parameters for reactivation are the Michaelis constant, K_m, and the turnover number k_{cat}, of each amine compound tested with the native enzyme.

There was no difference in the rate of reactivation of N-CBA-inactivated MAO by 1 mM benzylamine in the presence or absence of 20 mM *n*-butyl alcohol, benzyl alcohol, (−)-norepinephrine, or 5-hydroxytryptamine. The presence of 4 mM benzylmercaptan (benzylmercaptan was not soluble to 10 mM) also did not affect the rate of reactivation by benzylamine. However, the presence of 10 mM *N*,*N*-dimethyl-2-phenylethylamine decreased by a factor of 4.6 the rate of reactivation of N-CBA-inactivated enzyme by 1 mM benzylamine.

With benzylamine as the substrate, benzyl alcohol and *n*-butyl alcohol were found to be reversible competitive inhibitors of MAO with inhibition constants (K_i) of 0.030 and 0.50 mM, respectively. The same K_i values were found when *n*-butylamine was used as the substrate.

Treatment of N-CBA-Inactivated MAO with (±)-α-Methyl[α-^{3}H]benzylamine in the Presence of Sodium Cyanoborohydride. When a final concentration of 2 mM of (±)-α-methyl[α-^{3}H]benzylamine was used, the sample reactivated by benzylamine contained 0.50 mol of ^{3}H/mol of enzyme and the sample not treated with benzylamine contained 1.01 mol of ^{3}H/mol of enzyme, after correction for residual enzyme activity in the original N-CBA-inactivated MAO. This difference indicates the covalent attachment of 0.51 mol of ^{3}H/mol of inactivated active site. When the final concentration of (±)-α-methyl[α-^{3}H]benzylamine was increased to 10 mM, the difference relative to the benzylamine-treated control was 0.12 mol of ^{3}H/mol of inactivated active site.

Sodium Borohydride Treatment of N-[1-^{3}H]CBA-Inactivated MAO: Prevention of the Reactivation by Benzylamine. Both the borohydride-reduced and unreduced samples contained 1.9–2.0 mol of ^{3}H/mol of enzyme, and neither sample regained any enzyme activity under the reaction conditions. After incubation with benzylamine, the sample not treated with sodium borohydride regained full enzyme activity concomitant with the release of 1.0–1.2 mol of ^{3}H/mol of enzyme. After incubation with benzylamine, the sample treated with 1 mM sodium borohydride regained 68% of the enzyme activity concomitant with the release of 0.72 mol of ^{3}H/mol of enzyme. However, when 5 mM sodium borohydride was used in the reduction, benzylamine treatment resulted in the recovery of only 17% of the enzyme activity concomitant with the release of 0.13 mol of ^{3}H/mol of enzyme. The control enzymes, not inactivated previously with N-[1-^{3}H]CBA, retained essentially full enzyme activity during the course of the experiment. Similar results were obtained when the enzyme was inactivated with N-[1-^{3}H]CαMBA.

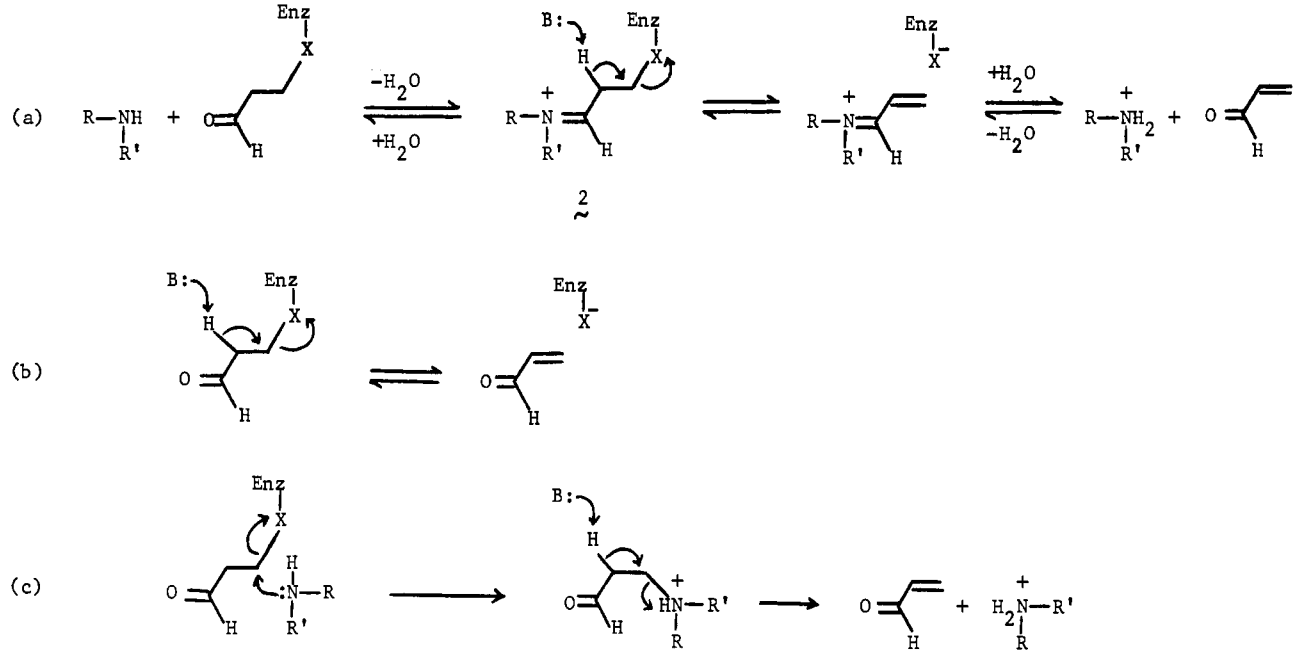
Release of Tritium from N-[1-^{3}H]CαMBA-Inactivated MAO by N-CBA, N-CMBA, and N-CαMBA. No tritium was released when N-CMBA or N-CαMBA was used as reactivator, but 37% of the tritium was lost when N-CBA was used.

Discussion

All of the reactivation experiments described here were carried out in the presence of 10 mM β-mercaptoethanol. Initial reactivation experiments were done without β-mercaptoethanol, but a significant time-dependent rate of inhibition of the control enzyme (i.e., MAO not pretreated with N-CBA) was observed for many of the reactivators. This phenomenon may be the result of product inhibition, because the control enzyme would be capable of metabolizing the reactivator and thereby causing a buildup of product with time. An alternative explanation for time-dependent inhibition by substrate was reported by Kinemuchi et al. (1982) for 2-phenylethylamine in which they suggested that inhibition was the result of a substrate–enzyme dead-end complex. Previously we reported (Silverman & Yamasaki, 1984) that β-mercaptoethanol was a reversible inhibitor of MAO; upon dilution of the β-mercaptoethanol-inhibited enzyme, full recovery of MAO activity resulted. The presence of 10 mM β-mercaptoethanol in all experiments was sufficient to block control enzyme activity and prevent inhibition; the 100-fold dilution resulting when aliquots were added to the enzyme assay mixture was sufficient to bring back full enzyme activity to the controls.

All of the compounds that reactivated N-CBA-inactivated MAO produced a time-dependent pseudo-first-order return of enzyme activity. The rate of reactivation reached a plateau with increasing reactivator concentration (i.e., saturation kinetics were observed) in all cases, suggesting that a simple bimolecular reaction was not involved. If enzyme reactivation is treated kinetically as the reverse of enzyme inactivation, then

Scheme I: Possible Mechanisms for Reactivation of N-CBA-Inactivated MAO[a]



[a] X represents an active site amino acid residue, and B: represents a base. R = aryl, arylalkyl, or alkyl; R' = H or alkyl.

a plot of the time at which 50% reactivation takes place ($t_{1/2}$, the half-death?) vs. the reciprocal of the reactivator concentration (1/[R]) should give a straight line. The intercept at the abscissa should be the negative inverse of the dissociation constant of the reactivator–N-CBA-inactivated enzyme complex (K_r), assuming that a rapid equilibrium relative to the catalytic step is operative (Segel, 1975). The reactivation rate constant at saturation for the reactivator (k_{react}) could be obtained from the intercept at the ordinate. It has been reported by Husain et al. (1982) that bovine liver MAO does, in fact, form a rapid equilibrium with its substrates. Therefore, one can compare K_r with K_m and see that a compound almost always is bound better as a substrate than as a reactivator (K_r/K_m = 2.7–11 for arylalkylamines and 20–690 for alkylamines). The difference between its affinity for the native and modified active site, however, is not great, considering the chemical and physical differences involved, namely, that the modified active site is alkylated (Scheme I) and contains a reduced flavin cofactor (Silverman & Hoffman, 1980; Vazquez & Silverman, 1985). Because of the saturation kinetics observed during reactivation, these results suggest that the reactivator initially binds to the modified active site before the enzyme-catalyzed reactivation step (k_{react}), which presumably occurs by approximation (Jencks, 1975). Since this reactivation process is not in the genealogy of MAO, the rates for amine oxidation catalyzed by native enzyme are much greater than the rates for amine reactivation of N-CBA-inactivated MAO ($k_{cat}/k_{react} \approx 10^4$–$10^5$). It should be noted, however, that the k_{react} values for all of the amines studied are quite similar; excluding benzylamine, the difference is only a factor of 5–6. This suggests that the catalytic step either does not involve the amine or that it is only partially rate-determining.

From the work described here (Tables I and II), it can be concluded that there is no direct correlation between the ability of a compound to serve as a substrate for native MAO and its ability to reactivate N-CBA-inactivated MAO. For example, *n*-butylamine, 3-phenyl-1-propylamine, 4-phenyl-1-butylamine, and *N*,*N*-dimethyl-2-phenylethylamine are very good substrates for MAO but are poor or ineffective reactivators of N-CBA-inactivated MAO, whereas aniline and (+)- or (−)-α-methylbenzylamine are essentially not substrates (Silverman, 1984) but are good reactivators. In contrast, benzylamine, *N*-methylbenzylamine, 2-phenylethylamine, tyramine, and the alkylamines (except for *n*-butylamine) are good substrates and reactivators whereas *N*,*N*-dimethylbenzylamine, *N*,*N*-dimethylaniline, (−)-norepinephrine, and 5-hydroxytryptamine (serotonin) are neither substrates nor reactivators. It is apparent from Table II that amines containing an aromatic moiety are generally better reactivators than aliphatic amines by virtue of their superior binding properties; the k_{react} values are similar for aromatic-containing amines and aliphatic amines. Another restriction for reactivation by amines is that they must be primary or secondary amines; whereas benzylamine and *N*-methylbenzylamine are very good reactivators, *N*,*N*-dimethylbenzylamine is not at all a reactivator. *N*,*N*-Dimethyl-2-phenylethylamine is not a reactivator even though a 10-fold excess of this tertiary amine decreased by 5-fold the rate of reactivation of N-CBA-inactivated MAO by benzylamine, suggesting that it is a competitive inhibitor.

A further important feature of the arylalkylamine reactivators appears to be the distance from the amino group to the aromatic ring. The ideal reactivator is one whose amino group and aromatic ring are separated by one or two methylene groups (i.e., benzylamine or 2-phenylethylamine); aniline (zero methylene groups) has a high K_r but an efficient k_{react} and 3-phenyl-1-propylamine (three methylene groups) and 4-phenyl-1-butylamine (four methylene groups) are ineffective as reactivators. Distance is not the only consideration, however, since two arylalkylamines containing an amino group and an aromatic part separated by two methylenes, namely, (−)-norepinephrine and serotonin (5-hydroxytryptamine), were not reactivators. These amines have a special significance with regard to MAO. MAO is known to exist in two major forms termed MAO A and MAO B (Johnston, 1968). Functionally, the difference in these forms is in their effectiveness to catalyze the oxidation of the various biogenic amine substrates, and they are differentiated by their susceptibility to certain inactivators (Knoll, 1978; Fowler et al., 1978). Benzylamine and 2-phenylethylamine are excellent MAO B substrates but poor

MAO A substrates, whereas (−)-norepinephrine and 5-hydroxytryptamine are excellent MAO A substrates but poor MAO B substrates. Since bovine liver MAO is the B form (Salach, 1979), it is not surprising that the MAO A substrates, (−)-norepinephrine and 5-hydroxytryptamine, are not reactivators of N-CBA-inactivated MAO if active site binding considerations also are important to the reactivation process. The interactions of these compounds with the modified active site must be rather weak since a 10-fold greater amount of them had no effect on the reactivation process by benzylamine. The other two amines listed in Table II that are not substrates for native MAO as well as being ineffective reactivators are the tertiary amines *N*,*N*-dimethylaniline and *N*,*N*-dimethylbenzylamine, but their poor substrate and reactivator properties probably are unrelated (vide infra).

The amino group is very important to the reactivation process since *n*-butyl alcohol, *n*-butylmercaptan, benzyl alcohol, and benzylmercaptan do not reactivate N-CBA-inactivated MAO. Benzyl alcohol and *n*-butyl alcohol, however, were shown to be potent competitive reversible inhibitors of the oxidation of benzylamine and of *n*-butylamine by native MAO. The aromatic and aliphatic binding sites for amine oxidation in native MAO appear to be the same since the K_i values for benzyl alcohol (0.03 mM) and *n*-butyl alcohol (0.50 mM) when benzylamine is the substrate are identical to those found when *n*-butylamine is the substrate. The alcohols and mercaptans apparently do not bind to N-CBA-inactivated MAO since a 4–20-fold excess of alcohol or mercaptan had no effect on its rate of reactivation by benzylamine.

Having noted the above observations, there are several mechanisms for the reactivation process that can be considered (Scheme I). Mechanism a involves initial Schiff base formation between the reactivator and the aldehyde group of the adduct followed by base-catalyzed β-elimination of the resulting imine to give the imine of acrolein. It has been shown that acrolein is a product of reactivation (Vazquez & Silverman, 1985). The base may be an amino acid residue located within the modified active site or a second reactivator molecule. Mechanism b is the same as a except no imine formation is invoked. Mechanism c is an S_N2 displacement of the enzyme from the adduct by the reactivator. This third mechanism was suggested by Rando & Eigner (1977) for the reactivation of allylamine-inactivated MAO. The inactivated enzyme adduct that they postulated also was a 3-oxopropyl enzyme, but allylamine is attached to a different active site moiety than is N-CBA.[2] The results of two sets of experiments that we carried out indicate that mechanism c is unlikely. First, the rate of an S_N2 reaction is proportional to the nucleophilicity of the attacking group (March, 1977). Since thiols are more powerful nucleophiles than amines (especially at pH 7 when the amine is almost completely protonated), it would be expected for an S_N2 mechanism that benzylmercaptan would be a better reactivator than benzylamine. Benzylmercaptan and benzyl alcohol, however, were completely impotent as reactivators. Also, if mechanism c were relevant to the reactivation process, the presence of the carbonyl in the adduct should be unimportant. N-[1-^{3}H]CBA-inactivated MAO was treated with 5 mM sodium borohydride to convert the aldehyde to an alcohol. Upon benzylamine treatment of the reduced enzyme adduct, only 17% of the enzyme activity was regenerated and 0.13 mol of ^{3}H was released relative to a control of labeled enzyme that was not reduced. Since the same experiment using 1 mM sodium borohydride in the reduction step resulted in a 68% regain of enzyme activity and release of 0.72 mol of ^{3}H, incomplete reduction of the carbonyl is most likely responsible for *any* rejuvenation of enzyme activity during benzylamine treatment. This prevention of reactivation as a result of carbonyl reduction is inconsistent with an S_N2 process (mechanism c) but is consistent with an elimination reaction (mechanism a or b), which is favored when there is an acidic proton β to the leaving group.

Mechanisms a and b differ only by imine formation. The reaction of an aldehyde with a primary or secondary amine to form an imine is a facile process; however, an imine cannot be formed from an aldehyde with a tertiary amine. As pointed out above, none of the three tertiary amines tested acted as a reactivator. However, *N*,*N*-dimethyl-2-phenylethylamine inhibited the reactivation of N-CBA-inactivated MAO by benzylamine and, therefore, was capable of entering the modified active site. The observation that none of the tertiary amines is a reactivator supports the Schiff base mechanism (mechanism a). Also consistent with mechanism a is the fact that the intermediate imine (**2**, Scheme I) in this mechanism is a proposed intermediate in the inactivation mechanism (Vazquez & Silverman, 1985). If the same intermediate arises during inactivation that occurs during reactivation, it would be expected that reactivation should occur concomitant with inactivation. This, in fact, is the case. It was found previously (Vazquez & Silverman, 1985) that, during inactivation of MAO by N-CBA, acrolein production (a product of reactivation) was linear with time, even though the enzyme appeared to be inactive. Considering that the k_{cat} at saturation for the inactivation of MAO by N-CBA (1.89 min^{-1}) is 461 times greater than the k_{react} at saturation for reactivation of N-CBA-inactivated MAO by *N*-methylbenzylamine (a model for N-CBA), it is not surprising that the enzyme would appear to remain inactive even though acrolein was being produced. Also, when MAO was inactivated with N-[1-^{3}H]CαMBA, tritium (acrolein) was released by N-CBA, thereby confirming the reactivation property of N-CBA. Furthermore, N-CMBA did not release tritium, consistent with the inability of tertiary amines to form Schiff bases with ketones. An experiment was carried out to trap the intermediate Schiff base during reactivation. When 2 mM (±)-α-methyl[α-^{3}H]benzylamine was added to the N-CBA-inactivated MAO in the presence of sodium cyanoborohydride, 0.51 mol of ^{3}H was incorporated per mole of N-CBA-inactivated MAO relative to active enzyme, suggesting that a Schiff base in half of the inactivated enzyme molecules was reduced to an amine. Previously it was shown (Silverman, 1984) that when MAO which was inactivated by *N*-cyclopropyl-α-methyl[α-^{3}H]benzylamine was treated with sodium cyanoborohydride, no incorporation of tritrium was observed. This was explained as an equilibrium phenomenon; the intermediate Schiff base produced during inactivation might favor the α-methyl[α-^{3}H]benzylamine plus aldehyde side of the equilibrium, and therefore, no radioactivity would be incorporated into the enzyme upon reduction. This hypothesis is supported in the converse experiment described above in which case there is a large excess of α-methyl[α-^{3}H]benzylamine relative to the amount of inactivated enzyme, so the equilibrium mixture of Schiff base is increased and radioactivity *is* incorporated by reduction. However, since the α-proton of a protonated Schiff base is more acidic than that of an aldehyde (Feeney et al., 1975), elimination also would be favored by imine formation. When the reduction experiment was repeated with an increased α-methyl[α-^{3}H]-benzylamine concentration (10 mM), only 0.12 equiv of radioactivity was incorporated into the enzyme. This *decreased* amount of radioactivity incorporated with *increased* amine

concentration could reflect an increased rate of reactivation relative to reduction as a result of a higher imine concentration or competition between the amine and sodium cyanoborohydride for the modified active site.

It is not clear whether the base (B:) shown in Scheme I is an active site residue or a second molecule of the amine. Two arguments can be made in favor of an active site residue as the essential base. As noted above, the k_{react} values are within a factor of 5–6 for all of the amines studied, which span a pK_a range of 10.5–11 for the alkylamines to 4.6 for aniline (Gordon & Ford, 1972). It seems reasonable that if the amine were the essential base, the overall rate should be affected by this large different in pK_a values. The second argument in favor of an active site residue as the essential base is that many of the reactivators are rather large molecules, and it seems unlikely that there would be a site to accommodate a second molecule of amine. However, the identity of the base cannot be elucidated by the work described here.

The observations made in this study with regard to reactivator ability can be rationalized by the following model for the reactivation of N-CBA-inactivated MAO by amines. It is clear from the data that an initial binding of the reactivator must occur before reactivation. The binding site could be a hydrophobic region of the modified active site, and this anchor point is probably situated at such a distance from the aldehyde group of the adduct that the amine and hydrophobic (anchored) portions of the reactivator are separated by a distance equivalent to one or two methylene groups. 3-Phenyl-1-propylamine and 4-phenyl-1-butylamine may not be reactivators because they are too long to be both anchored at the hydrophobic binding site and also positioned to form the requisite Schiff base for reactivation or, if the Schiff base does form, the α-proton of the adduct may be distored away from the base that is required to remove it (**2**, Scheme I). The failure of 5-hydroxytryptamine and (−)-norepinephrine to reactivate N-CBA-inactivated MAO is due to their lack of effective binding to the inactivated enzyme. Perhaps the presence of the hydrophilic hydroxyl groups weaken the binding interactions. Evidence for this notion can be seen by comparing the K_r values of 2-phenylethylamine and tyramine. If the hydrophobic region of the modified active site contains aromatic residues, then additional binding energy for those reactivators containing an aromatic moiety could come from aromatic edge-to-face interactions (Thomas et al., 1982). This might explain why, in general, reactivators containing aromatic groups have lower dissociation constants, K_r, than strictly aliphatic reactivators where no such interactions are possible. Once binding takes place, the primary or secondary amino group is properly positioned to react with the adduct aldehyde and to form a Schiff base, which then can undergo the base-catalyzed β-elimination.

The mechanism proposed for amine reactivation of N-CBA-inactivated MAO is analogous to that suggested for the reactivation of inactivated (phosphorylated) acetylcholinesterase by pyridine aldoximine methiodide (Frode & Wilson, 1971). The pyridinium moiety is believed to bind to the anionic site adjacent to the esteratic site. This, then, positions the hydroxylamine moiety appropriately for transphosphorylation and reactivation of the enzyme.

In summary, the results of this study show that there is no direct correlation between substrate and reactivator ability since the active sites in native and N-CBA-inactivated MAO are different as are the mechanisms for substrate oxidation and enzyme reactivation by amines. A reasonable mechanism for amine reactivation of N-CBA-inactivated MAO is initial Schiff base formation followed by base-catalyzed β-elimination of H–XEnz and release of acrolein imine into solution. Air oxidation of the flavin cofactor then leads to active enzyme.

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Kinetics of RNA Replication: Competition and Selection among Self-Replicating RNA Species†

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ABSTRACT: The process of Darwinian selection in the self-replication of single-stranded RNA by Qβ replicase was investigated by analytical and computer-simulation methods. For this system, the relative population change of the competing species was found to be a useful definition of selection value, calculable from measurable kinetic parameters and concentrations of each species. Critical differences in the criteria for selection were shown to pertain for replicase/RNA ratios greater than or less than 1, for the case that formation of double-stranded RNA occurs and when comparisons are made of closed with open systems. At a large excess of enzyme, RNA species grow exponentially without interfering with each other, and selection depends only on the fecundity of the species, i.e., their overall replication rates. For RNA concentrations greater than the replicase concentration, the selection of species is governed by their abilities to compete for enzyme. Under conditions where formation of double strands occurs, competition leads to a coexistence of the species; the selection values vanish, and the concentration ratios depend only on the template binding and double-strand formation rates. The approach to coexistence is rapid, because when its competitors are in a steady state, a species present in trace amount is amplified exponentially. When formation of hybrid double strands occurs at a substantial rate, coexistence of hybridizing species is essentially limited to cases where the formation rate of heterologous double strands is smaller than the geometric mean of the formation rates of the homologous double strands. At limiting cases, e.g. in the steady states, simple analytical expressions for the main aspects of the selection process were found. Experimental data support the analytical expressions and the simulations.

Darwinian behavior, usually considered to be a characteristic of life itself, arises whenever a system has the characteristics of metabolism, self-reproduction, and mutability. It results in the production of species present in populations that depend on their "Darwinian fitness". The population changes of each species are functions not only of the prevailing conditions but also of the populations of all species present. It is thus not possible to derive selection values for species in an ecosystem from known characteristics of the species, e.g., fecundity, alone.

Laboratory studies of evolution at the molecular level were introduced in a classic series of experiments by Spiegelman and co-workers [Mills et al., 1967; reviewed by Biebricher (1983)] using RNA that was self-replicating in the presence of Qβ replicase. They showed that new RNA species can arise and outgrow other species. In a previous paper (Biebricher et al., 1983) we described an investigation of RNA replication kinetics by analytical and computer-simulation methods. A critical dependence on the RNA populations was found: Exponential growth occurs when enzyme is present in excess of RNA, while linear growth can occur when RNA is in excess. In a subsequent study (Biebricher et al., 1984), we explored the implications of two important features of RNA replication, the presence of two complementary RNA strand populations and their reaction with each other to form antiparallel double helices. The same methods are extended in this paper to considerations of the processes of competition and selection as they arise in laboratory studies of short single-stranded RNA replication by the enzyme Qβ replicase.

The primary results of the theoretical development are a demonstration that different selection criteria apply for competition between RNA species when the enzyme is saturated (RNA:enzyme ratio greater than 1) and when it is not (RNA:enzyme ratio less than 1) and that still different situations pertain when formation of double-stranded RNA proceeds in competition with recycling of single-stranded RNA template. For each of these situations, it was possible to derive analytical formulas epitomizing the selection criteria. Finally, distinctions are made between competition in closed systems and in open systems such as flow reactors or their discrete realizations as serial-transfer experiments.

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