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INHIBITION OF BOVINE PLASMA AMINE OXIDASE BY SUPEROXIDE DISMUTASE ACTIVE CU(II) COMPLEXES

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Summary: Aqueous Cu^{2+} and Cu(II) complexes of salicylate, lysine, and tyrosine decrease the rate of benzylamine oxidation by bovine plasma amine oxidase. Bis-salicylato Cu(II) and Cu^{2+} inhibit non-competitively with respect to benzylamine. Lysine, tyrosine, $Cu(EDTA)^{2-}$, Zn^{2+}, and Co^{2+} do not inhibit, and erythrocyte Cu, Zn superoxide dismutase shows only slight inhibition of the amine oxidase. The data are most consistent with an inhibitory mechanism involving dismutation of O_2^- by the Cu(II) complexes within a site relatively inaccessible to the enzyme superoxide dismutase. Excess lysine significantly decreases inhibition by the bis-lysine complex of Cu(II).

For the past several years intensive effort has been devoted to elucidating the catalytic mechanism(s) of copper-containing amine oxidases. These enzymes catalyze the oxidative deamination of primary amines:

$$RCH_2NH_2 + O_2 + H_2O \longrightarrow RCHO + H_2O_2 + NH_3 \quad .$$

Highly purified amine oxidases from bovine plasma, pig plasma, pig kidney, pea seedlings, and the fungus Aspergillus niger have been extensively studied by physical and kinetics methods (1-3). Although similar in many respects, the enzymes display distinct differences which must be considered in any general formulation of the mechanism. Nevertheless, most steady-state and transient phase kinetics experiments are consistent with the minimum mechanism in Scheme 1 (1-5).

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Abbreviations used: sal, salicylate; PIPES, piperazine-N,N'-bis[2-ethane sulfonic acid]

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(1) $E + RCH_2NH_2 \longrightarrow E \cdot RCH_2NH_2 \longrightarrow E_{red} + RCHO$

(2) $E_{red} + O_2 \longrightarrow E + H_2O_2 + NH_3$

Scheme 1

Apparently step (1) or step (2) can be rate limiting, depending on the specific enzyme and the substrate (1-5).

Our own interests center on the activation and utilization of O_2 by the copper-containing amine oxidases and the role of copper in the reaction. A pertinent question in this regard is whether the reduction of O_2 proceeds in one-electron or two-electron steps. One-electron reduction of O_2 generates the superoxide radical anion O_2^-, which then might exist as an intermediate (probably remaining within the active site). In fact, aqueous Cu^{2+}, $Cu(lys)_2$ and $Cu(tyr)_2$ have previously been shown to inhibit crude pig kidney amine oxidase (6). We have investigated the inhibition of highly purified bovine plasma amine oxidase by superoxide dismutase active Cu(II) complexes and the results are reported herein.

MATERIALS AND METHODS: Bovine plasma amine oxidase was purified by a method developed by B. Mondovi and coworkers (7). After plasma collection, ammonium sulfate fractionation, and dialysis (8), two affinity columns are employed: first, an AH-sepharose-4B column and, second, a Concanavalin A-sepharose-4B column. Preparations of specific activity ~2000 units/mg were routinely obtained. SDS polyacrylamide gel electrophoresis indicated the enzyme to be $\geq$ 90% pure. Bovine ceruloplasmin was also purified to homogeneity (9); based on comparisons of visible absorption and CD spectra and SDS gel electrophoretic patterns, no ceruloplasmin could be detected in the amine oxidase preparations. Enzyme activity was measured at 22± 2°C using benzylamine as substrate in 0.1M phosphate buffer, pH 7.2. Some experiments were carried out in 50mM PIPES, pH 7.2. One activity unit is defined as an 0.001/min. absorbance increase at 250 nm (10). Protein was estimated from the absorbance at 280 nm using $E^{1\%}_{1cm}$ = 20.8 (1).

Inhibition studies were also carried out by monitoring the oxidation of benzylamine to benzaldehyde at 250 nm using the assay conditons above. Except where noted, the benzylamine concentration was 3.3mM in a final volume of 3 mls.

$Cu(lys)_2$ and $Cu(tyr)_2$ were synthesized by a published method (11). $Cu(sal)_2$ was prepared by the method of deAlare and coworkers (12) and recrystallized from water. $Cu(EDTA)^{2-}$ was prepared by mixing a 10% molar excess (to insure complete complexation) of Na_2EDTA with $CuSO_4$ in water.

Bovine superoxide dismutase, Concanavalin A-sepharose-4B, and AH-sepharose-4B were obtained from Sigma. Benzylamine was purchased from Eastman. All chemicals were reagent grade and were used without further purification. Kinetics measurements were made on a Perkin-Elmer 576 UV-VIS spectrometer.

TABLE I

EFFECTS OF INHIBITORS ON THE ACTIVITY OF BOVINE PLASMA AMINE OXIDASE

Inhibitor	Concentration (μM)	Conditions	Activity Exp.: 1	2	3	4	Percent Inhibition Exp.: 1	2	3	4
None	-	A	115	121	99	96	-	-	-	-
Erthyrocyte Cu,Zn	-	-	-	-	-	-	-	-	-	-
Superoxide Dismutase	1	A	-	-	92	90	-	-	7.3	5.8
$Cu(lys)_2$	100	A	97	105	-	-	15.7	13.2	-	-
$Cu(lys)_2$	200	A	93	100	79	79	19.1	17.4	20.2	17.7
$Cu(tyr)_2$	100	A	100	109	-	-	13.0	9.9	-	-
$Cu(tyr)_2$	200	A	96	101	-	-	16.5	16.5	-	-
lys	200	A	117	125	-	-	- 1.7	- 3.3	-	-
tyr	200	A	117	123	-	-	- 1.7	- 1.7	-	-
$CuSO_4$	100	A	75	82	-	-	34.8	32.2	-	-
$CuSO_4$	200	A	70	75	-	-	39.1	38.0	-	-
$ZnSO_4$	200	A	113	120	-	-	1.7	0.8	-	-
$Co(NO_3)_2$	170	A	-	-	99	-	-	-	0.0	-
$Cu(sal)_2$	100	A	67	78	58	-	41.7	35.5	41.4	-
$Cu(EDTA)^{2-}$	100	A	115	121	-	-	0.0	0.0	-	-
Cu(lys)	200	A + 400μM lys	-	-	99	90	-	-	0.0	6.0
None	-	B	81	-	-	-	-	-	-	-
$CuSO_4$	200	B	24	-	-	-	70.4	-	-	-
$Cu(sal)_2$	200	B	20	-	-	-	75.3	-	-	-
$Cu(lys)_2$	200	B	28	-	-	-	65.4	-	-	-
$Cu(lys)_2$	200	B + 400μM lys	85	-	-	-	- 4.9	-	-	-
$Co(NO_3)_2$	170	B	78	-	-	-	3.7	-	-	-

Each entry is the average of three assays. A: 0.1M phosphate buffer, pH 7.2. B: 50mM PIPES buffer, pH 7.2.

RESULTS: Effects of superoxide dismutase complexes on the rate of benzylamine oxidation are presented in Table I. Essentially identical results were obtained (T. S. Coolbaugh and D. M. Dooley, unpublished data) on plasma amine oxidase purified by another method (8). $Cu(sal)_2$ and aqueous Cu^{2+} (as $CuSO_4$) inhibit amine oxidase activity to approximately the same extent. $Cu(lys)_2$ and $Cu(tyr)_2$ also inhibit the enzyme but are about a factor of two to three less effective than $Cu(sal)_2$ or aqueous Cu^{2+}. Double-reciprocal plots of rate versus benzylamine concentration in the presence of Cu^{2+} and $Cu(sal)_2$ are shown in Figure 1.

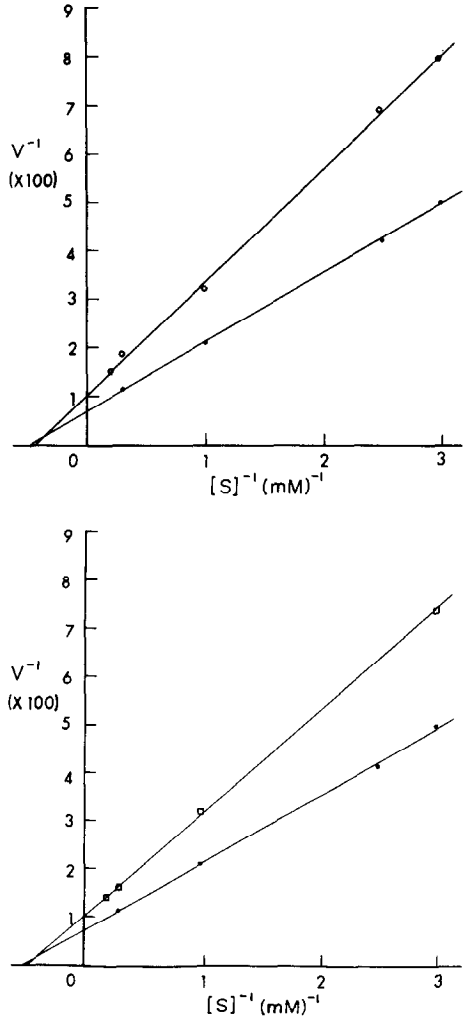

Figure 1: Inhibition of bovine plasma amine oxidase by Cu^{2+} and $Cu(sal)_2$ in 0.1M phosphate buffer pH 7.2. —●—, no inhibitor; —○—, 200 μM $Cu(sal)_2$; —□—, 200 μM $CuSO_4$.

pretreatment of E. coli RNase P (1) and that which Koski used to inactivate KB cell RNase P (7), and has been shown to be free of proteases. In addition the protease inhibitor PMSF is included in all our pretreatment mixtures (see Methods).

RNase T_1 pretreatment: RNase P pretreated with RNase T_1 loses activity (Fig. 1C, lane 2). Since RNase T_1 does not precipitate in $(NH_4)_2SO_4$ under conditions where RNase P does (8), the pretreatment mixtures were fractionated by precipitation prior to assay for RNase P. Even so, a slight amount of residual RNase T_1 activity is evident by accumulated breakdown products in Fig. 1C, lane 2). Further purification of the pretreatment mixtures by sucrose gradient centrifugation (see Methods) removed the last residue of RNase T_1 from these mixtures (not shown). The end result observed with respect to the inactivation of RNase P is the same as shown in Figure 1C, lane 2.

RNase A pretreatment: RNase A pretreated mixtures were fractionated by $(NH_4)_2SO_4$ and then further purified by velocity sedimentation as described in the Methods. Even after these steps some residual RNase A was evident in the assays of the sucrose gradient fractions (Fig. 2B). In particular, there is some degradative activity in the bottom fraction of the gradient, presumably due to unresuspended RNase A which precipitated in the $(NH_4)_2SO_4$ fractionation, and at the top of the gradient where this enzyme is expected to sediment because of its small size. Nevertheless, the presence of RNase P in the relevant fractions from the gradient containing the control treated sample (Fig. 2A, lanes 2-4) is apparent whereas no such activity is seen in the same fractions from the gradient containing the RNase A pretreated sample (Fig. 2B, lanes 2-4). These experiments were carried out with various concentrations of soluble RNase A and with RNase A bound to polyacrylamide beads and yielded the same result. To exclude the possibility that RNase P cleavage products might be digested by residual RNase A in reaction mixtures containing the pretreated samples, we assayed reconstruction mixtures, containing both control treated and RNase A pretreated samples, for RNase P activity. We detected both RNase

our data. O_2^-, produced at a site inaccessible to the Cu,Zn superoxide dismutase, has previously been proposed to participate in the catalytic cycle of crude pig kidney amine oxidase (6).

Both the slopes and the intercepts of the double-reciprocal plots are increased in the presence of $Cu(sal)_2$ or aqueous Cu^{2+}, indicating that these complexes are non-competitive inhibitors with respect to benzylamine. Other results are inconsistent with inhibitory mechanisms involving simply enzyme-inhibitor complex formation and subsequent blocking of the active site. Neither lysine nor tyrosine inhibit bovine plasma amine oxidase. Co^{2+} and Zn^{2+}, which can often be substituted for Cu^{2+} in metal binding sites on proteins (and vice versa), but do not catalyze superoxide dismutation, also show no inhibition of benzylamine oxidation.

A reasonable interpretation of these results is that the rate of reoxidation of substrate-reduced enzyme by O_2 is decreased by the copper complexes tested. Two outstanding mechanistic possibilities are: (1) dismutation of an O_2^- intermediate; and (2) oxidation of the reduced enzyme via electron transfer to the copper complex. The latter mechanism is less likely since $Cu(EDTA)^{2-}$, which could also inhibit in this manner, does not decrease the overall rate of benzylamine oxidation. Scheme 2 summarizes our suggested mechanism for inhibition by superoxide dismutase active copper complexes. The mechanism requires that the one-electron reduced enzyme, E^1_{red}, react more slowly with O_2 or other redox agents than the fully reduced enzyme, E^2_{red}. Since O_2^- (or a protonated form) would have to be released after the reaction of O_2 with E^1_{red}, the requirement above does not seem too restrictive, especially since the lack of inhibition by the Cu, Zn superoxide dismutase enzyme indicates that O_2^- may be tightly bound by bovine plasma amine oxidase. Another possibility is that O_2^- dissociation is

$$E^2_{red} \xrightarrow{O_2} E^2_{red}\cdot O_2 \longrightarrow E^1_{red}\cdot O_2^- \xrightarrow{\text{Cu complex}} E^1_{red} + O_2$$

Scheme 2: E^2_{red} and E^1_{red} represent 2 electron and 1 electron reduced enzyme, respectively.

sufficiently slow such that oxidized enzyme is regenerated via reaction with a second molecule of the copper complex.

We have not explicitly included enzyme copper in Scheme 2 as no definitive experiments on its role in the bovine plasma enzyme have been reported, and because results on other amine oxidases (15-18) are not altogether in agreement. Good evidence has been presented that copper is the dioxygen binding site in pig plasma amine oxidase (15). One-electron steps in the reduction of O_2 are not inconsistent with the involvement of copper in this process since an observable Cu(I) intermediate, which is ruled out by a variety of evidence (1-3), is not required. For example, the equilibrium E^2_{red} —— Cu(II) $\rightleftharpoons$ E^1_{red} ——Cu(I) could lie far to the left in the absence of O_2; after its addition E^1_{red} —— Cu(II) —— O_2^- could be the predominant enzyme species formed.

Finally, the effects of lysine upon the inhibition of bovine plasma amine oxidase by $Cu(lys)_2$ are worth noting. Excess lysine could decrease the superoxide dismutase activity of $Cu(lys)_2$ by forming tris- or tetrakis-complexes that would be inactive. This seems rather unlikely as only bis-complexes have been observed in potentiometric titrations (19). Another possibility is that equilibrium concentrations of Cu(lys) and Cu^{2+} contribute significantly to O_2^- dismutation in solutions containing $Cu(lys)_2$ and that lysine drives the equilibria further towards $Cu(lys)_2$, which is less active. Consideration of the formation constants (19) indicates that the total concentration of Cu(lys) + Cu^{2+} would be less than 10 μM in a solution formally containing 200 μM $Cu(lys)_2$, but it seems clear that at least part of the superoxide dismutase activity attributed to $Cu(lys)_2$ in solution may arise from Cu(lys) and Cu^{2+}. At pH 7.2 the concentration of aqueous Cu^{2+} is completely negligible in solutions of $Cu(sal)_2$. The predominant superoxide dismutase active species are probably Cu(sal) and $Cu(sal)_2^{2-}$ (20).

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