HYDRAZINES AS SUBSTRATES FOR BOVINE PLASMA AMINE OXIDASE (PAO)

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Summary: Various hydrazines were found to form complexes with bovine plasma amine oxidase (PAO). These enzymically-inactive complexes, when isolated by ultrafiltration, decomposed to active PAO and a product(s) incapable of inhibiting PAO. The recovery of activity followed first order kinetics with respect to the enzyme-hydrazine complex concentration. Half lives of the complexes ranged from 1.5 hours for hydrazine to about 10 hours for benzylhydrazine. After the hydrazine-inhibited enzyme recovered full activity, it could be retitrated at least twice. A ^{14}C,1-dimethylhydrazine-PAO complex was isolated by gel filtration.

Certain amine oxidases, which are dependent upon tightly-bound copper and pyridoxal phosphate (PLP) prosthetic groups, are extremely sensitive to inhibition by various carbonyl reagents (1,2,3,4,&5). In our attempts to utilize hydrazines as active site reagents for PAO, we observed that hydrazines reacted specifically and stoichiometrically with PAO even when the enzyme was of varying degrees of purity. Higher concentrations of hydrazines (several fold excesss) did not completely inhibit PAO even though the mole equivalent of the 50% inhibition value for hydrazine corresponded almost exactly to one-half a mole of PAO. Experiments reported here indicate that hydrazines may function as PAO substrates, albeit poor substrates, with a high affinity for the active site of the enzyme.

PAO was purified from fresh bovine blood by the procedure of Yamada and Yasunobu (6) as modified by Achee (7). Purified PAO preparations had specific activities up to 488 S. U. units/mg according to the assay method

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of Tabor, Tabor and Rosenthal (1). Protein concentration was determined by the method of Warburg and Christian (8). Calculation of moles of PAO was based upon a specific activity of 444 S. U. units/mg and a molecular weight of 170,000 (9). PAO-hydrazine complexes were isolated by either Sephadex (G-25) column chromatography or by ultrafiltration with XM-50 membranes (Amicon Corp.). Hydrazines were obtained from commercial sources and used without further purification.

Hydrazines reacted rapidly and specifically with PAO even when present in crude plasma preparations. PAO of purity varying from 1.4% to essentially 100% was incubated with various hydrazines for 30 minutes prior to addition of amine substrate. The ratio of moles of inhibitor to moles of enzyme at 50% inhibition $(I/E)_{50}$ ranged from 0.53 for hydrazine to 1.96 for 1,1-dimethylhydrazine (Table I). Varying the amine substrate (benzylamine) concentration from 0.3 to 5.0 mM did not alter the $(I/E)_{50}$ ratios. When PAO was added to mixtures of benzylamine and any one of these hydrazines, inhibition was delayed but not prevented. Therefore, the high affinity between PAO and hydrazine appears to give a titration of the active site of PAO. Yamada and Yasunobu (5) reported 1.07 moles of PLP per mole of PAO. The amount of hydrazine bound, as calculated from the $(I/E)_{50}$ value, (1.06 moles of hydrazine/mole of PAO) agrees with the PLP content

Table I. K_i' and $(I/E)_{50}$ Values for the Inhibition of PAO by Hydrazines

Inhibitor		$(I/E)_{50}$	K_i' (muM)
Hydrazine	NH_2NH_2	0.53	1.9
Benzylhydrazine	$\emptyset CH_2NHNH_2$	0.59	5.7
β-Hydroxyethylhydrazine	$HOCH_2CH_2NHNH_2$	0.63	8.6
Monomethylhydrazine	CH_3NHNH_2	0.71	14.1
1,1-Dimethylhydrazine	$(CH_3)_2NHNH_2$	1.96	98.8

PAO preparations of varying degrees of purity were titrated with hydrazine inhibitors by procedures described in the text. PAO concentration was constant at 67.5 muM in the 3.0 ml assay. The values listed represent an average of five to eleven determinations on the different PAO fractions. K_i' values were calculated for a mutual depletion system (10).

of PAO. Thus, these data reconfirm the proposal (1) that PLP is involved in the active site of PAO.

Nearly complete inhibition of PAO required hydrazine concentrations much greater than those required for 50% inhibition, indicating possible decomposition of the inhibitor. In addition, partially-inhibited PAO began to show recovery of activity after approximately 30 to 40 minutes (Fig. 1). When the inhibitor, β-hydroxyethylhydrazine, was added in three-fold excess of PAO, recovery of activity was delayed for nearly four hours. This behavior is typical of a system in which enzyme-inhibitor complex formation is occurring and then decomposition of the complex followed by retitration of regenerated enzyme. β-Hydroxyethylhydrazine solutions incubated in the absence of PAO remained capable of inhibiting PAO during the entire period of the experiment even at the low concentrations employed (Fig. 1).

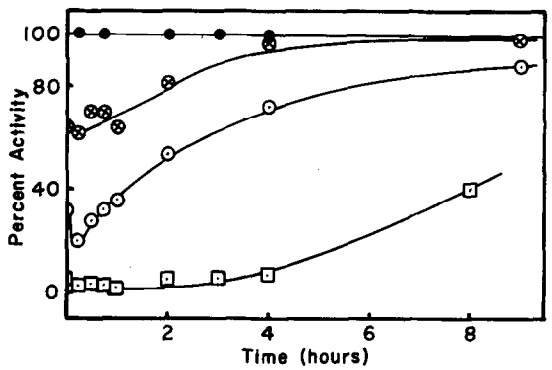

Fig. 1. Effect of incubation time on PAO inhibition by β-hydroxyethylhydrazine at different I/E ratios. Percent activity is referred to the activity of the enzyme incubated in the absence of inhibitor for the same interval of time and at the same temperature, 25°. Inhibitor incubated in buffer during the incubation period did not change in its capacity to inhibit PAO. ●, control; ⊗, I/E = 0.3; ⊙, I/E = 0.6, and ⊡, I/E = 2.9.

Further evidence that the complexes between PAO and hydrazines were decomposing came from their isolation by ultrafiltration and then following the recovery of PAO activity. Reaction of PAO with excess hydrazine (20 fold) followed by ultrafiltration permitted the isolation of fully

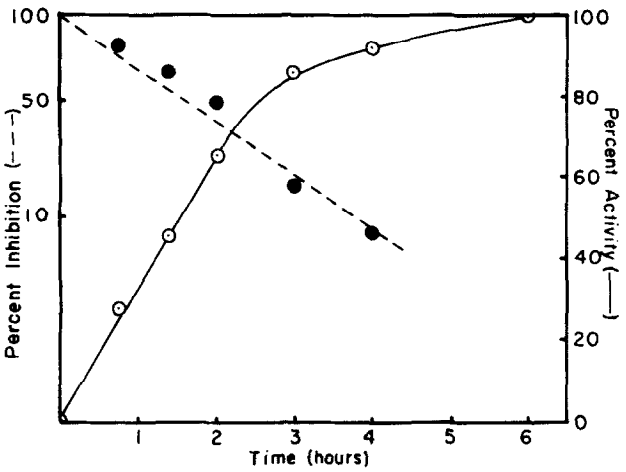

Fig. 2. Kinetics of the recovery of PAO activity during the decomposition of isolated PAO-hydrazine complex. Hydrazine (20 fold excess on a mole basis) was reacted with PAO at 25° and the PAO-hydrazine complex isolated by ultrafiltration and washing with 0.01 M phosphate buffer, pH 7.0.

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inhibited PAO-hydrazine complex free of excess hydrazine. Addition of PAO to the isolated complex without loss of activity of the added enzyme was used as evidence that complete removal of excess hydrazine was achieved by filtration. The rate of recovery of PAO activity was found to be first order with respect to the hydrazine-PAO complex concentration (Fig. 2). Addition of substoichiometric quantities of hydrazine to the isolated complex at various time intervals caused retitration of equivalent amounts of the recovered PAO. Therefore, decomposition of the complex gave products which are either not inhibitors of PAO or much less effective inhibitors. Half live values for several different hydrazine-PAO complexes ranged from 1.5 hours for hydrazine, 2.5 hours for β-hydroxyethylhydrazine and monomethylhydrazine to about 10 hours for benzylhydrazine at 25°. Full PAO activity recovery was achieved with hydrazine even after three cycles of inhibition and recovery of PAO activity. Proof that PAO inhibition was due to binding of hydrazines was obtained by reacting ^{14}C-1,1-dimethylhydrazine (^{14}C-UDMH) with PAO followed by isolation of the ^{14}C-UDMH-protein complex

by gel filtration. The level of radioactivity found in the protein fraction (0.42 moles of UDMH were bound per mole of PAO) corresponded with the degree of inhibition of PAO (45%) remaining after gel filtration. The total time elapsed during the experiment was three hours.

In conclusion, hydrazines appear to be capable of reacting specifically and rapidly at the active site of PAO to form complexes which can be isolated in a fully inactive form. These complexes subsequently decompose to active PAO with first order kinetics with respect to the complex concentration. Investigations are currently underway to determine the nature of the products of the reaction and if oxygen plays a role in the formation and decomposition of the complexes.

References

1. Tabor, C. W., Tabor, H., and Rosenthal, S. M., J. Biol. Chem. 208, 645 (1954).
2. Blaschko, H., and Buffoni, F., Proceed. Roy. Soc. London Ser. B 163, 45 (1965).
3. Goryachenkova, E. V., Scherbatiuk, L. I., and Zamarev, C. I., In: Pyridoxal Catalysis: Enzymes and Model Systems. Proceed. International Union of Biochem. Moscow, Sept. 1966, Eds. E. E. Snell et al. New York, Interscience, 1968, p. 391.
4. Mondovi, B., et al. Arch Biochem. Biophys. 119, 373 (1967).
5. Yamada, H., and Yasunobu, K., J. Biol. Chem. 238, 2669 (1963).
6. Yamada, H., and Yasunobu, K., J. Biol. Chem. 237, 1511 (1962).
7. Achee, Francis, Private communication 1968.
8. Warburg, O., and Christian, W. Biochem. Zeitschrift 310, 385 (1942).
9. Wang, T. M., Achee, F. M., and Yasunobu, K. T., Arch. Biochem. Biophys. 128, 106 (1968).
10. Webb, J. Leyden, In: Enzyme and Metabolic Inhibitors, Vol. I, Academic Press 1963. Chapter 3, p. 66.