Inhibition of Copper Amine Oxidase by Haloamines: A Killer Product Mechanism†

Rosaria Medda,‡ Alessandra Padiglia,‡ Jens Z. Pedersen,§ Alessandro Finazzi Agrò,|| Giuseppe Rotilio,⊥ and Giovanni Floris*,‡

Institute of Biological Chemistry, University of Cagliari, Cagliari, Italy, Departments of Biology and Experimental Medicine, University of Rome "Tor Vergata", Rome, Italy, CNR Institute of Experimental Medicine, Rome, Italy, and Department of Chemistry, Odense University, Odense, Denmark

Received June 13, 1996; Revised Manuscript Received September 30, 1996⊗

ABSTRACT: The observation that the alkylamines 2-Br-ethylamine and 2-Cl-ethylamine and 1,2-diaminoethane, the shortest diamine, are irreversible inhibitors of several copper amine oxidases led to the investigation of the mechanism by which these compounds react with the highly active amine oxidase from lentil seedlings. 1,2-Diaminoethane, 2-Br-ethylamine, and 2-Cl-ethylamine were found to be both poor substrates and irreversible inhibitors of lentil amine oxidase; inactivation took place in both the presence and absence of oxygen. All three compounds strongly affected the spectrum of the enzyme, leading to the formation of a stable band at 336 nm both in anaerobiosis and in aerobiosis, consistent with an interaction with the enzyme cofactor 6-hydroxydopa. On the contrary, the corrresponding propylamine compounds 1,3-diaminopropane, 3-Br-propylamine, and 3-Cl-propylamine were reversible inhibitors of lentil amine oxidase. Inhibition was shown to be due to the aldehyde oxidation products rather than the short chain amines themselves; a reaction mechanism is presented which involves attack of the aldehyde on the 6-hydroxydopa-derived free radical catalytic intermediate. With 1,2-diaminoethane, 2-Br-ethylamine, and 2-Cl-ethylamine, the complex produced will form a stable 6-membered ring, causing irreversible inhibition of the enzyme.

Amine oxidases (amine oxygen oxidoreductase deaminating, copper containing; EC 1.4.3.6) are homodimers of 70−95 kDa subunits. Each subunit contains tightly bound Cu(II) and 6-hydroxydopa (TOPA)[1] formed from tyrosine in a post-translational event (Cai & Klinman, 1994; Tanizawa et al., 1994; Matsuzaki et al., 1994). The presence of TOPA in copper amine oxidases from various sources has been confirmed by different techniques (Plastino & Klinman, 1995; Steinebach et al., 1995). These enzymes catalyze the oxidative deamination of primary amines to the corresponding aldehydes, ammonia, and hydrogen peroxide. Recently, the complete catalytic cycle of lentil amine oxidase has been outlined using poor substrates and inhibitors (see Scheme 1) (Medda et al., 1995).

It has been reported that beef plasma and *Aspergillus niger* amine oxidases can catalyze a nonoxidative elimination reaction on some analogs of their substrates. In order to check the ability of beef plasma amine oxidase to catalyze a nonoxidative elimination reaction, Neumann et al. (1975) investigated the action of the enzyme on some 2-haloamines. These authors showed that 2-Br-ethylamine inactivated the enzyme irreversibly under aerobic conditions and that in the course of the process the enzyme became covalently modified. Two moles of product were incorporated per mole of dimeric enzyme. No significant inactivation was observed when 2-Br-ethylamine was added under anaerobic conditions. They also found that 2-Cl-ethylamine underwent a process of oxidative deamination only, not followed by inactivation of the enzyme. Later, Kumagai et al. (1979) showed that amine oxidase from *A. niger* simultaneously catalyzed the oxidative deamination and the elimination reaction of 2-Br-ethylamine to form acetaldehyde, and that the oxidase was irreversibly inactivated during the reaction. 2-Cl-ethylamine also inactivated the enzyme syncatalytically, but details of this reaction were not investigated. The inactivation mechanism proposed by Neumann and by Kumagai suggested the alkylation of an SH group located in the active site of the enzyme. On the other hand, Gacheru et al. (1989) demonstrated that 2-substituted ethylamine derivatives were suicide inhibitors of lysyl oxidase and proposed that the formation of a cyclic pyrazine adduct might explain the inactivation of the enzyme by 1,2-diaminoethane. The synthesis of such an adduct with analogs of TOPA was demonstrated by Wang et al. (1993).

Lentil amine oxidase shows peculiar properties when compared with beef plasma and *A. niger* amine oxidases. It is highly active, but its substrate specificity is restricted to diamines of critical length and it is very poorly active on monoamines. Further it is not a sulfhydryl-dependent protein (Floris et al., 1983). In this paper, the action of lentil amine oxidase on 2-Br-ethylamine, 2-Cl-ethylamine, 3-Br-propylamine, 3-Cl-propylamine, 1,2-diaminoethane, and 1,3-diaminopropane is investigated. We demonstrate that LSAO catalyzes the oxidation of 2-Br-ethylamine and 2-Cl-ethylamine to produce the corresponding aldehydes, which can

†This study was partially supported by MURST "40%" and "60%" funds and by EC funds (European Social Funds).

* Author to whom correspondence should be addressed at: Istituto di Chimica Biologica, via della Pineta 77, 09125 Cagliari, Italy. Tel: 39-70-300728. FAX: 39-70-340280.

‡ Institute of Biological Chemistry, University of Cagliari.

§ CNR Institute of Experimental Medicine, Rome, and Department of Chemistry, Odense University.

|| Department of Experimental Medicine, University of Rome "Tor Vergata".

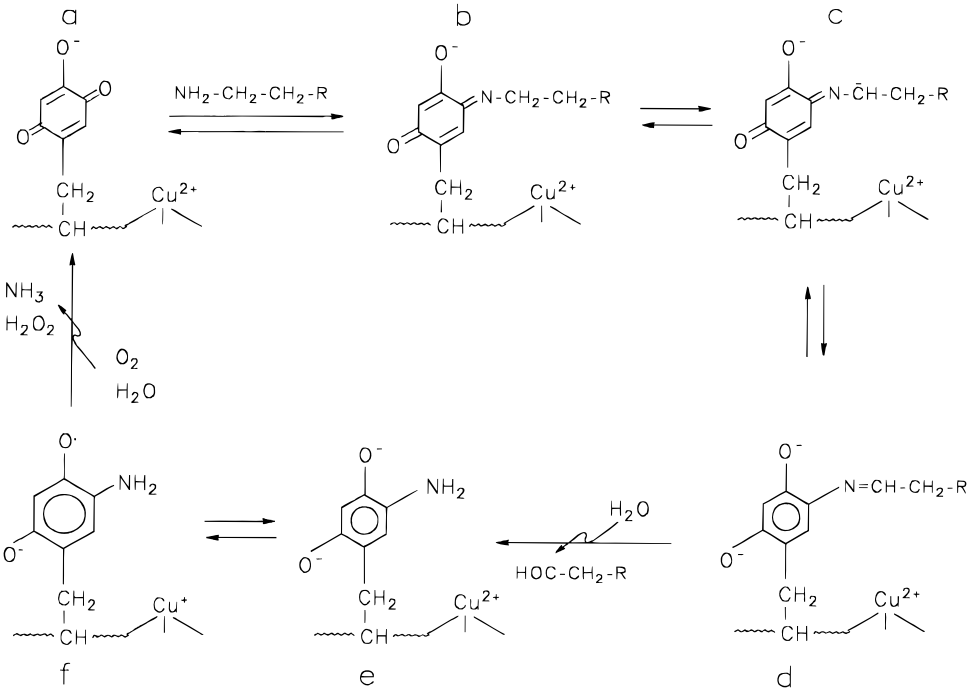
⊥ Department of Biology, University of Rome "Tor Vergata".

⊗ Abstract published in *Advance ACS Abstracts,* January 15, 1997.

[1] Abbreviations: LSAO, lentil seedling amine oxidase; TOPA, 6-hydroxydopa (2,4,5-trihydroxyphenylalanine).

S0006-2960(96)01410-9 CCC: $14.00

Scheme 1



react with the highly reactive radical species in the catalytic cycle and covalently label the enzyme. Afterward, ring closure with the elimination of Br^- and Cl^- may occur to give irreversible inhibition. On the contrary, 3-Br-propylamine and 3-Cl-propylamine are reversible inactivators of LSAO. Further we show that LSAO catalyzes the oxidation of 1,2-diaminoethane, being afterward irreversibly inactivated, while 1,3-diaminopropane is a reversible inactivator. Finally, we show that the copper-free protein is not inactivated by haloamines nor by 1,2-diaminoethane.

EXPERIMENTAL PROCEDURES

Materials. Acetaldehyde, Br-acetaldehyde, aminoacetaldehyde, 2-Br-ethylamine hydrobromide, 2-Cl-ethylamine hydrochloride, 3-Cl-propylamine hydrochloride, and 2,4-dinitrophenylhydrazine were from Fluka. 3-Br-propylamine hydrobromide, 1,2-diaminoethane hydrochloride, 1,3-diaminopropane hydrochloride, tetranitromethane, and diethyl pyrocarbonate were from Sigma. Amine oxidase from lentil (*Lens esculenta*) seedlings was purified according to Floris et al. (1983). An ϵ_{498} of 4.1×10^3 M^{-1} cm^{-1} or an ϵ_{278} of 2.45×10^5 M^{-1} cm^{-1} for the purified enzyme (2 copper ions and a M_r of 150 000) was used to estimate the enzyme concentration (Padiglia et al., 1992). All experiments were made several times at different enzyme concentrations. Copper-free lentil amine oxidase was prepared as previously described (Rinaldi et al., 1984).

Spectroscopic Methods. Absorption spectra were recorded at room temperature with a Cary 2300 spectrophotometer. Anaerobic experiments were made after several cycles of vacuum followed by flushing with O_2-free argon at 25 °C in a Thunberg-type spectrophotometer cuvette, where anaerobic additions of various reagents could be made through a rubber cap with a syringe. ESR measurements were made using a Bruker ESP 300 instrument; Cu(II) spectra were recorded at 100 K with a standard TE_{102}-type cavity, while spectra of the amine oxidase radical were recorded at room temperature with a high-sensitive TM_{110}-type cavity. Anaerobic samples were prepared in two-compartment Thunberg cells fitted with an ESR quartz tube sidearm. Radical spectra in the $g = 2.0$ region were recorded using 1.0 G modulation and 25 mW microwave power, whereas Cu(II) spectra were measured over a 1000 G range using 10 G modulation and 5 mW power.

Assays of Products. The aldehydes released were determined using a coupled enzyme assay based on alcohol dehydrogenase and NADH according to Kumagai et al. (1979), as well as by the 3-methyl-2-benzothiazolinone hydrazone test (Sawicki et al., 1961). Identification of the aldehydes was made after conversion to their phenylhydrazone derivatives through reaction with 2,4-dinitrophenylhydrazine (Kumagai et al., 1979). The hydrazones were extracted with toluene and separated by thin layer chromatography on a silica gel plate, using a benzene/ethyl acetate (95:5) mixture. Genuine aldehyde samples were used for comparison. In this system, 2,4-dinitrophenylhydrazone of acetaldehyde (R_f 0.5) was easily distinguishable from that of Br-acetaldehyde (R_f 0.72). Ammonia production was determined from the amount of NADH consumed in the presence of glutamate dehydrogenase (Berg & Abeles, 1980). Hydrogen peroxide was determined with the peroxidase/4-hydroxy-3-methoxyphenylacetic acid method (Leyton, 1981).

Activity Measurements. Oxygen uptake was determined polarographically at 37 °C using a Gilson Oxygraph equipped with a Clark electrode. The standard reaction mixture (1 mL) contained the enzyme in 100 mM KP_i buffer, pH 7.0. The reaction was started by addition of substrate solution after at least 10 min preincubation. The volume changes caused by successive additions of enzyme were negligible; typically 5 μL was added.

Other Methods. Carbanion formation in the reaction between LSAO and 2-Br-ethylamine, and the titration of histidyl residues of LSAO inactivated by 2-Br-ethylamine were determined as described by Medda et al. (1993).

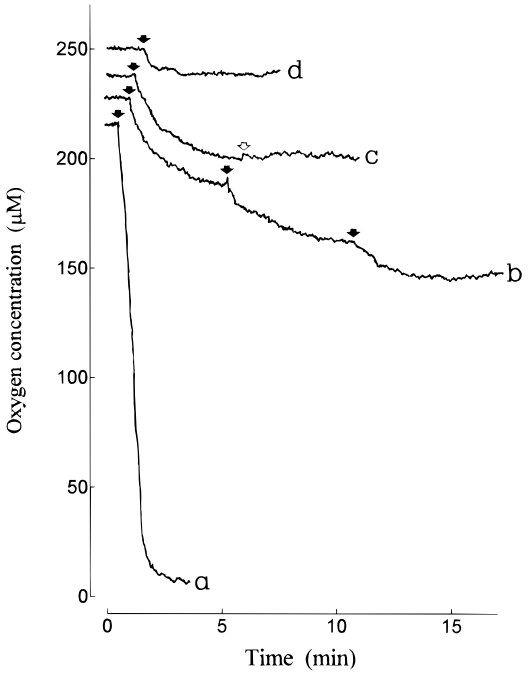


FIGURE 1: Reaction of lentil amine oxidase with 2-Br-ethylamine measured through oxygen uptake. The reaction mixture contained 2 mM putrescine (a) or 2-Br-ethylamine (b−d) in 1 mL of 100 mM KP_i buffer, pH 7. Filled arrows indicate the addition of 0.012 μM enzyme (a) or 5 μM enzyme (b−d); empty arrow indicates the addition of 2 mM putrescine. Sample (d) contained 0.1 mM Br-acetaldehyde.

Briefly, the occurrence of an enzyme carbanion intermediate was shown through its ability to induce the hydrolysis of tetranitromethane (0.25 mM) together with the substrate to generate the nitroform anion, which could be detected through the characteristic absorption at 350 nm ($\epsilon_{350} = 1.44 \times 10^4$ M^{-1} cm^{-1}). Only a small percentage of the carbanions formed during the catalytic cycle react in this way. Treatment of the native enzyme with diethyl pyrocarbonate (5 mM, 30 min incubation in 100 mM phosphate buffer, pH 7.0) was used to modify histidyl residues to their *N*-carbethoxy derivatives. The reaction was followed through the increase in absorption at 240 nm; an ϵ_{240} of 3.2×10^3 M^{-1} cm^{-1} was used to determine the number of modified residues. It is known that two essential histidines in LSAO can be titrated in this way (Medda et al., 1993).

RESULTS

The reaction of LSAO with the short chain diamines 1,2-diaminoethane and 1,3-diaminopropane and the corresponding haloamines 2-Br-ethylamine, 2-Cl-ethylamine, 3-Br-propylamine, and 3-Cl-propylamine was studied. All these compounds turned out to be very poor substrates but good inhibitors of LSAO, with I_{50} between 10^{-6} and 10^{-3} M.

Reaction of Native LSAO with 2-Br-ethylamine. (A) Mechanism of Inhibition. When a good substrate such as putrescine was added to LSAO, the reaction went on until the dissolved O_2 was completely consumed (Figure 1a). In contrast, when the enzyme reacted with 2-Br-ethylamine, oxygen uptake decreased rapidly and stopped in spite of the large amounts of substrate and oxygen still present, and the enzyme became inactive (Figure 1c). Addition of putrescine did not lead to further O_2 uptake (Figure 1c), while the addition of fresh enzyme caused a renewed brief O_2 uptake

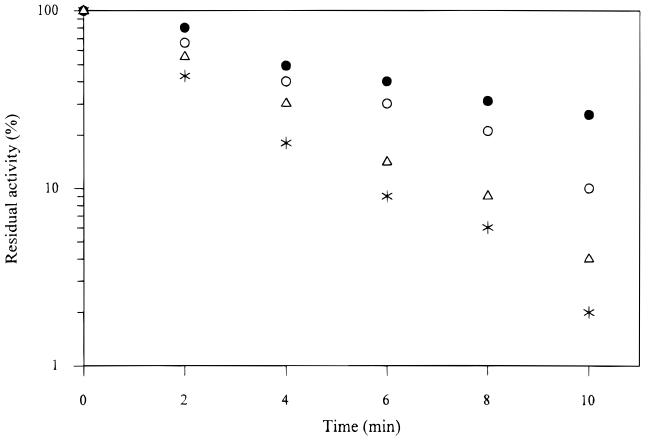


FIGURE 2: Inactivation of lentil amine oxidase by 2-Br-ethylamine. The enzyme (6 nM) was preincubated with the indicated concentrations of 2-Br-ethylamine at 37 °C in 100 mM phosphate buffer, pH 7. Concentrations of 2-Br-ethylamine were: (●) 5 μM; (○) 7 μM; (△) 10 μM; (∗) 15 μM.

(Figure 1b). The enzyme appeared to be permanently inactivated, also after removing the excess inhibitor and products by dialysis. These results, in agreement with those obtained with beef plasma amine oxidase (Neumann et al., 1975) and *A. niger* amine oxidase (Kumagai et al., 1979), confirmed that 2-Br-ethylamine was both a substrate and an irreversible inhibitor also for the lentil seedling enzyme. The time dependence of the inhibition was determined by preincubating LSAO at 37 °C in the presence of various concentrations of 2-Br-ethylamine. The rate of enzyme activity loss increased linearly with 2-Br-ethylamine concentrations, the inactivation following apparent first-order kinetics (Figure 2). A double reciprocal plot of the inactivation rate constants against 2-Br-ethylamine concentrations showed a linear concentration dependence and a positive intercept on the *y*-axis (Figure 3A). This result is consistent with the reversible formation of an enzyme−inhibitor complex [E·I], followed by an irreversible inactivation due to the generation of a stable enzyme−inhibitor complex [E−I]:

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} [E\cdot I] \xrightarrow{k_2} [E-I]$$

A limit value for the inactivation rate constant k_2 of 0.8 min^{-1} and an apparent inhibition constant K_i of 5.4×10^{-5} M were determined from the plot (Figure 3A). These findings were in agreement with the previously suggested mechanism of inhibition (Neumann et al., 1975; Kumagai et al., 1979). However, other experimental observations cannot fit this mechanism. Repeated additions of fresh enzyme to the reaction mixture resulted in lower and lower oxygen uptake, as if the enzyme were inactivated more rapidly (Figure 1b). This suggested that inhibition was actually due to the accumulation of oxidation product(s) in the sample. It has been reported that amine oxidases can deaminate 2-Br-ethylamine with the formation of acetaldehyde and ammonia, but without oxygen consumption (Neumann et al., 1975; Kumagai et al., 1979). To check this point, the reaction products were determined. Table 1 reports the stoichiometry of the products obtained after oxidation of excess 2-Br-ethylamine with 10 nmol of LSAO (active sites). A total of 1 μmol of ammonia and 1 μmol of aldehyde were released, while only 0.06 μmol of oxygen was

Table 1: Stoichiometry of the Reaction of 2-Br-ethylamine, 2-Cl-ethylamine, 1,2-Diaminoethane, and 3-Br-propylamine with Lentil Amine Oxidase[a]

substrate	O_2 consumed (nmol)	aldehyde (nmol)	ammonia (nmol)	H_2O_2 (nmol)	turnovers before inactivation
2-Br-ethylamine*	65 ± 6	1010 ± 100	1030 ± 76	1015 ± 72	100
2-Cl-ethylamine§	96 ± 6	ND	107 ± 5	100 ± 4	416
1,2-diaminoethane§	139 ± 13	130 ± 7	142 ± 8	125 ± 8	521
3-Br-propylamine§	102 ± 10	ND	98 ± 5	105 ± 3	437

[a] The reaction was carried out at 37 °C in a vessel with an oxygen electrode. The reaction mixture contained *10 nmol of LSAO active sites or §0.24 nmol of active sites, and 2 μmol of substrates, in 1 mL of 100 mM phosphate buffer, pH 7. After oxygen consumption had completely stopped (after 10 min for 3-Br-propylamine), the samples were removed and the amounts of aldehydes and ammonia were measured as described in the text. Hydrogen peroxide was determined in a separate incubation as described in Experimental Procedures. Aldehyde, ammonia, and hydrogen peroxide determinations are the mean of five different experiments. ND, not determined.

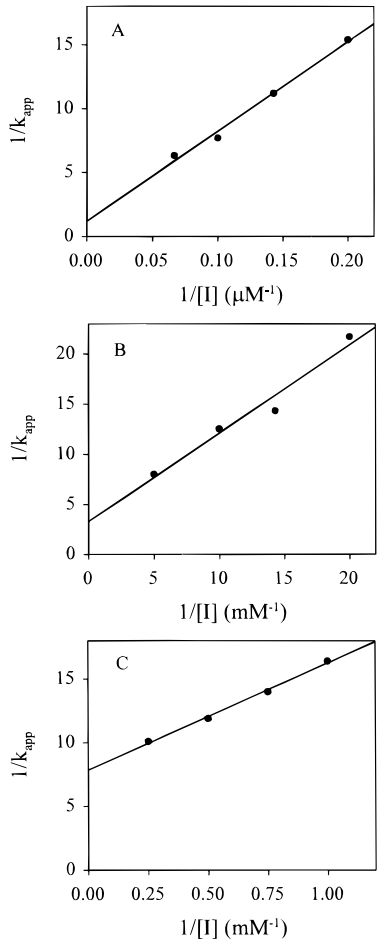


FIGURE 3: Double reciprocal plot of apparent first-order rate constants of inactivation (k_{app}) versus inhibitor concentrations: 2-Br-ethylamine (A); 2-Cl-ethylamine (B); 1,2-diaminoethane (C).

consumed, in agreement with previous reports. Furthermore, the formation of 1 μmol of hydrogen peroxide was demonstrated (Table 1). Thus the 1:1:1 stoichiometry of the reaction products was found also for this substrate, despite the low oxygen uptake measured before inactivation of LSAO. Upon closer inspection of these numbers, it is evident that the amount of H_2O_2 formed would require 4 times more oxygen than present in the reaction vessel. The same observation can be made using the values reported by Kumagai et al. (1979). It is therefore likely that spontaneous nonenzymatic oxidation of 2-Br-ethylamine took place after the samples had been removed from the reaction vessel for product analysis. In fact, when catalytic amounts of catalase were added to the reaction vessel at the end of the reaction, only approximately 50% of the oxygen consumed was released, in agreement with an O_2 to H_2O_2 ratio of 1:1.

Qualitative analysis of the aldehyde produced by thin layer chromatography of the 2,4-dinitrophenylhydrazone derivatives showed the presence of Br-acetaldehyde only (R_f = 0.72) while acetaldehyde (R_f = 0.50) could not be detected (results not shown). Since hydrogen peroxide and ammonia are also produced in the oxidation of normal substrates, the Br-acetaldehyde remained the likely candidate as the inhibitory product. To test this hypothesis, the oxidation of 2-Br-ethylamine was measured after addition of authentic Br-acetaldehyde to the sample. The enzyme was inactivated much faster (Figure 1d).

Incubation of resting LSAO with Br-acetaldehyde did not give rise to any inactivation; after dialysis the enzyme was fully active (results not shown). Irreversible inhibition only occurred in the presence of a substrate, but high concentrations of a good substrate protected the enzyme. Thus incubation with 1 mM Br-acetaldehyde resulted in 65% inactivation with 1 mM putrescine, only 16% inactivation with 4 mM putrescine, and no measurable inhibition when 17 mM putrescine was added.

Atomic absorption measurements on the inactivated enzyme showed that it did not contain bound bromide (i.e., less than 0.2 mol/mol of enzyme).

(B) Spectroscopic Features. Like other amine oxidases, LSAO has a characteristic brick-red color due to the presence of the oxidized TOPA cofactor, which has a broad absorption band around 498 nm in the visible spectrum. Addition of 2-Br-ethylamine caused the immediate disappearance of this band, indicating the rapid formation of a reduced bromoethylamine−TOPA intermediate. However, within few seconds a new band appeared in the spectrum at approximately 330 nm; after 1−2 min this absorption reached its maximum (Figure 4). No further changes were observed in the spectrum, and then the enzyme was completely inactivated. Even after exhaustive dialysis, the enzyme did not recover the original absorption nor the activity. When the same experiment was carried out under anaerobic conditions, the addition of 2-Br-ethylamine again resulted in a rapid bleaching of the broad absorption at 498 nm, but in this case new sharp bands at 434 and 464 nm appeared in the spectrum (Figure 5). These bands are diagnostic of the free radical intermediate (Scheme 1f) which accumulates in the absence of oxygen (Finazzi-Agrò et al., 1984; Dooley et al., 1987). Their appearance suggested that also the oxidation of 2-Br-ethylamine followed the pathway shown in Scheme 1. The radical species generated in the absence of air and with good substrates such as putrescine remained stable for many hours. In contrast, with 2-Br-ethylamine the radical

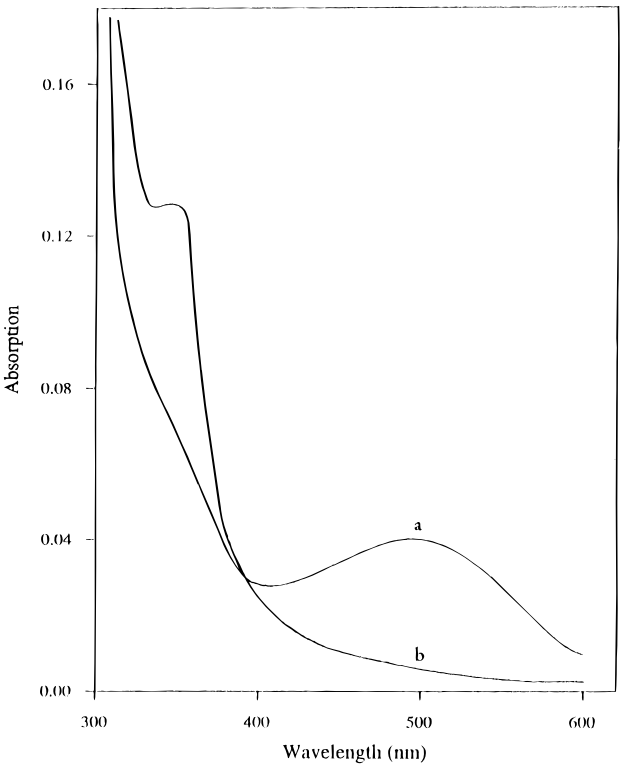


FIGURE 4: Absorption spectra of LSAO upon reaction with 2-Br-ethylamine. Spectra of 20 μM enzyme in 100 mM air-saturated KP_i buffer, pH 7 (a); 10 min after the addition of 2 mM 2-Br-ethylamine, 2-Cl-ethylamine, or 1,2-diaminoethane (b).

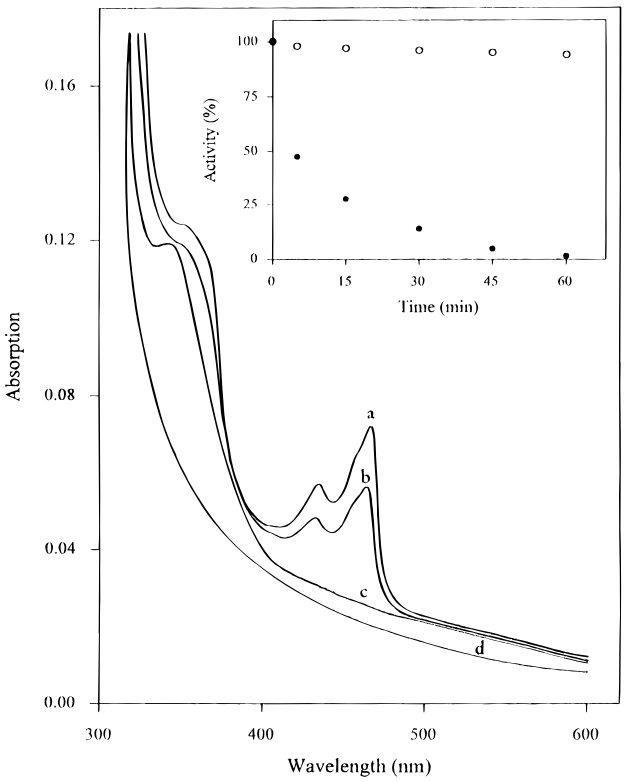


FIGURE 5: Reaction of 8.5 μM LSAO under anaerobic conditions with 2 mM 2-Br-ethylamine in 100 mM KP_i buffer, pH 7, in a Thunberg-type spectrophotometer cuvette. Different spectra were recorded: (a) immediately after addition of 2-Br-ethylamine, (b) after 5 min, and (c) after 60 min. Line (d) is the spectrum of 8.5 μM copper-free LSAO in the presence of 2 mM 2-Br-ethylamine. Inset: Inactivation of lentil amine oxidase in anaerobiosis. Aliquots were withdrawn at intervals from the Thunberg cuvette and assayed polarographically for the initial rates of putrescine oxidation: (○) LSAO alone; (●) LSAO in the presence of 2-Br-ethylamine.

decayed even under anaerobic conditions. At pH 7 the disappearance of the bands at 434 and 464 nm was fast, with $t_{1/2} = 10$ min (Figure 5). In parallel, the enzyme became inactivated (Figure 5, inset), and the absorption band at 330 nm appeared, eventually reaching the same height as in the experiment made in the presence of oxygen. Involvement of the product Br-acetaldehyde was confirmed in an experiment, where 5 mM authentic Br-acetaldehyde was included in a sample containing LSAO and putrescine in anaerobiosis. In this case, the free radical intermediate disappeared with a $t_{1/2} = 60$ min, with concomitant formation of the peak at 330 nm. After complete disappearence of the bands at 434 and 464 nm, the enzyme was found to be irreversibly inhibited (data not shown).

(C) Site of LSAO Inhibition. A series of experiments was carried out in order to determine in detail the mechanism of inhibition by 2-Br-ethylamine.

It is known that 2-Br-ethylamine can react with cysteine residues to form aminoethyl derivatives. However, treatment of LSAO with thiol reagents such as *N*-ethylmaleimide and iodoacetate did not affect the activity (Floris et al., 1983), ruling out an involvement of cysteine alkylation. Neither were the essential histidine residues (Medda et al., 1993) modified after inactivation of the enzyme by 2-Br-ethylamine (data not shown).

The catalytic cycle of LSAO involves a carbanion intermediate (Scheme 1c), that can be detected through its reaction with tetranitromethane to generate nitroform (Medda et al., 1993). Upon addition of 2-Br-ethylamine and tetranitromethane to the enzyme, the formation of nitroform was soon observed, but within 2 min it slowed down and then stopped completely. No generation of nitroform could be detected in a control sample containing all the reaction components (tetranitromethane, ammonia, hydrogen peroxide, 2-Br-ethylamine, Br-acetaldehyde, or acetaldehyde) except the enzyme, confirming that nitroform generation was indeed catalyzed by LSAO.

Copper-free LSAO has a visible spectrum very similar to that of the native enzyme, but the peak is shifted to 480 nm (Rinaldi et al., 1984). Reaction of the apoprotein with 2-Br-ethylamine resulted in the immediate and permanent reduction of TOPA, as shown by the disappearance of the 480 nm band. As expected, there was no formation of the free radical species, which requires the presence of Cu(II), nor was there formation of the 330 nm band (Figure 5). Two moles of 2-Br-ethylamine per mole of dimeric enzyme were required for complete bleaching of the apoenzyme, producing 2 mol of Br-acetaldehyde. Identical results were obtained in both the absence and presence of oxygen, even after addition of Br-acetaldehyde to the samples. The enzyme did not regain its oxidized absorption spectrum. However, after dialysis in 100 mM KP_i buffer, pH 7, and incubation with $CuSO_4$, fully active LSAO could be obtained in its oxidized form, with the characteristic absorption band at 498 nm. Thus, at variance with the holoenzyme, the apoenzyme was not irreversibly inactivated.

Kumagai et al. (1979) reported that the *A. niger* amine oxidase modified with 2-Br-ethylamine retained the ability of reacting with 2 mol of phenylhydrazine/mol of enzyme through transaldimination to give the characteristic 450 nm peak. Instead, LSAO modified with 2-Br-ethylamine did not

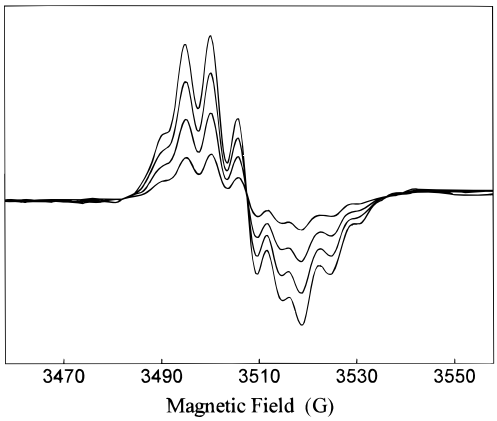


FIGURE 6: ESR spectra showing the decay of the amine oxidase free radical intermediate formed upon reaction with 1,2-diaminoethane. Spectra were recorded after 8, 70, 170, and 290 min; the sample contained 60 μM enzyme and 15 mM 1,2-diaminoethane in 100 mM phosphate buffer, pH 7.

react at any extent with phenylhydrazine, indicating the formation of a very stable complex (data not shown).

Reaction of LSAO with 1,2-Diaminoethane and 2-Cl-ethylamine. As with 2-Br-ethylamine, the oxygen uptake gradually decreased and stopped during the reaction of LSAO with 1,2-diaminoethane or 2-Cl-ethylamine and the enzyme became inactive (results not shown). It is important to note that the amounts of O_2 consumed were stoichiometric to the amounts of products (ammonia and hydrogen peroxide) formed (Table 1). The rate constant for inactivation (k_2) was 0.13 min^{-1} for 1,2-diaminoethane and 0.3 min^{-1} for 2-Cl-ethylamine (Figure 3B,C) while the apparent inhibition constants (K_i) were 1.1×10^{-3} M and 2.6×10^{-4} M, respectively. Under these conditions, each enzyme active site on average turned over about 580 times with 1,2-diaminoethane and 420 times with 2-Cl-ethylamine before becoming inactive.

1,2-Diaminoethane and 2-Cl-ethylamine gave exactly the same pattern of inhibition as 2-Br-ethylamine, although the rates of inactivation were lower. Upon addition to LSAO, these compounds also caused the disappearance of the 498 nm absorption and the formation of a new band centered at 330 nm (Figure 4). This band had the same shape and intensity for all the three ethylamine derivatives. An absorption coefficient $\epsilon_{330} = 20$ mM^{-1} cm^{-1} could be calculated, assuming the complete conversion of the enzyme into the inactive species.

The semiquinone radical intermediate was formed during oxidation of both 1,2-diaminoethane and 2-Cl-ethylamine in anaerobiosis, as into the reaction with 2-Br-ethylamine. As expected from the lower inhibitory efficiency of these two amines, the radical was much more stable. For 2-Cl-ethylamine a $t_{1/2} = 9$ h was measured. Also, in these cases the disappearance of the radical was accompanied by the gradual inactivation of the enzyme and by the formation of the 330 nm band (not shown). ESR measurements of the decay of the semiquinone species did not show the presence of any other free radical (Figure 6), indicating that the 330 nm band belonged to a diamagnetic reaction product. Incubation of apo-LSAO with 1,2-diaminoethane or 2-Cl-ethylamine resulted again in a rapid and permanent bleaching of LSAO, without any formation of the radical intermediate or the 330 nm band. After dialysis and reconstitution with

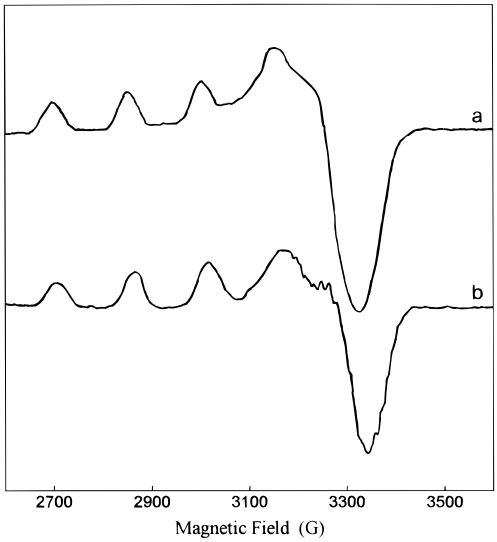


FIGURE 7: Effect of 1,2-diaminoethane on the Cu(II) ESR spectrum of LSAO. Samples containing 50 μM enzyme in phosphate buffer at pH 7.0 were measured using 5 mW power and 10 G modulation. (a) Control without additions; (b) control plus 15 mM 1,2-diaminoethane after 100 min incubation at room temperature in the presence of oxygen.

Cu(II), the enzyme was reoxidized and fully active (data not shown).

Since 1,2-diaminoethane is known to be a good copper chelator, the integrity of the Cu(II) site was checked by ESR spectroscopy (Figure 7). Incubation with 1,2-diaminoethane did not reduce the Cu(II) or remove it from the enzyme. The ligand field of the metal was slightly perturbed and nitrogen-linked hyperfine splittings became detectable in the $g_\perp$ region. This effect is often seen when substrates bind to the enzyme (Mondovì et al., 1969).

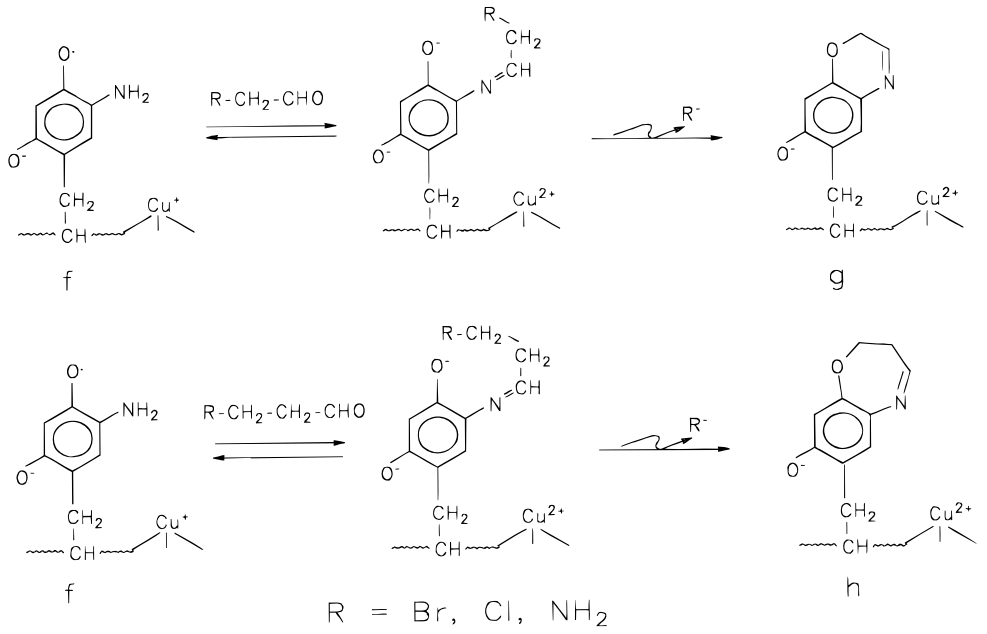
Reaction of LSAO with 3-Br-propylamine, 3-Cl-propylamine, and 1,3-Diaminopropane. When these three propylamine analogs were oxidized by LSAO, the enzyme activity decreased almost to zero within few minutes (data not shown). However, in contrast to the results obtained with the ethylamine analogs, the enzyme was never completely inactivated; a very small fraction of the activity remained, irrespective of the concentration of the propylamine used. Thus in a sample containing 2 mM 3-Br-propylamine, approximately 100 nmol was oxidized by 0.24 nmol (active sites) of LSAO in a rapid initial phase (Table 1). In spite of the inhibition of the enzyme, the reaction slowly went on, and after 22 h all 3-Br-propylamine was oxidized. At this point, the enzyme was no longer inhibited.

After addition of one of the propylamine analogs to LSAO, the absorption band at 498 nm disappeared immediately and a new absorbance band centered at 340 nm was formed. This species decayed with a $t_{1/2} = 3$ h and disappeared completely after approximately 20 h (Figure 8) concomitantly with the restoration of the oxidized enzyme, with its 498 nm band. These results are consistent with the reversible formation of an [E·I] complex followed by a stable but not irreversible complex [E·I°] that in the presence of oxygen regenerates the oxidized active enzyme with release of the products (ammonia, aldehyde, and hydrogen peroxide):

$$E + I \Leftrightarrow [E{\cdot}I] \Leftrightarrow [E{\cdot}I^\circ]$$

Reaction of the apoenzyme with 3-Br-propylamine in anaerobiosis or in aerobiosis resulted in rapid and permanent

Scheme 2



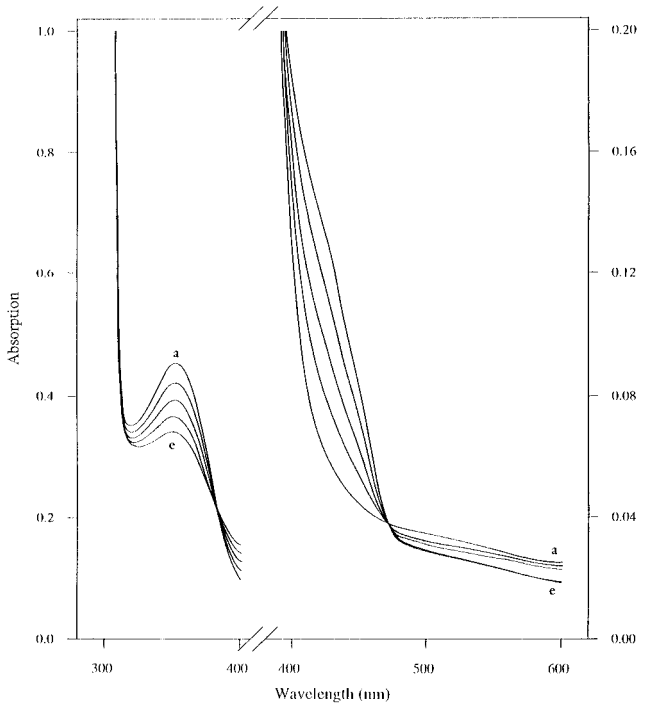


FIGURE 8: Reaction of 10 μM lentil amine oxidase in aerobic conditions with 2 mM 3-Br-propylamine in 100 mM KP_i buffer, pH 7.0. Difference spectra were recorded from 15 min (a) to 135 min (e) with intervals of 30 min.

bleaching of the 480 nm band. The resulting stable intermediate did not give any new spectral feature in the ultraviolet and visible range (data not shown). The complete absence of bands at 434 and 464 nm showed that the formation of the free radical intermediate did not occur. The enzyme stayed reduced and did not regain its absorption spectrum. After addition of 10^{-6} M $CuSO_4$, the colorless copper-free enzyme turned red and the oxidized form band at 498 nm was restored (data not shown).

The results obtained using 1,3-diaminopropane and 3-Cl-propylamine were similar to those with 3-Br-propylamine.

DISCUSSION

It is well-known that short chain amines can be inhibitors of copper amine oxidases and that 2-haloethylamines behave as suicide substrates, but until the discovery of the TOPA cofactor the details of inactivation remained elusive (Neuman et al., 1975; Kumagai et al., 1979). The results presented here, in combination with the current knowledge of the catalytic cycle, allow us to establish the mechanism of inhibition.

Two main conclusions are immediately obvious from the experimental findings. First, all 2-substituted ethylamines seem to inhibit by the same mechanism, whereas a different mechanism operates for all 3-substituted propylamines. Second, in all cases the inhibition is due to the reaction of the enzyme with the product aldehyde. The inactivation of amine oxidases by 2-haloethylamines is therefore an example of a killer product mechanism.

The experiments made with 2-Br-ethylamine under anaerobic conditions provide a key to the understanding of the mechanism. In anaerobiosis the enzyme is trapped either in the aminoresorcinol form (Scheme 1e) or in the free radical form (Scheme 1f), after having released one molecule of aldehyde per active site (Medda et al., 1995). Since the enzyme is inactivated also in the absence of oxygen (Figures 5 and 6), the attack of the aldehyde product must take place on one of these two intermediates. The copper-free enzyme also releases the aldehyde, but is not able to proceed to the free radical species and exists entirely as the aminoresorcinol form (Medda et al., 1995). However, the apoenzyme does not react with the aldehyde; if residual amounts of 2-Br-ethylamine and of Br-acetaldehyde are removed from the copper-free enzyme by dialysis, addition of Cu(II) restores the active native enzyme. This indicates that the aldehyde reacts with the free radical 1f to form a covalently modified enzyme. Attack on the radical rather than on the aminoresorcinol is consistent with the lower reactivity of the latter, which is stable in the presence of oxygen.

The inactivated enzyme did not contain bromide, indicating that the halogen is released and not responsible for the

inactivation of the enzyme. For the reaction of LSAO with 2-Cl-ethylamine or 1,2-diaminoethane, we obtained the same qualitative results as with 2-Br-ethylamine. These findings suggest a common mechanism of inactivation, as illustrated in Scheme 2. The reaction with aldehyde in the first step is thought to be reversible; there is no evidence for the generation of a second radical species. The second step, ring closure with release of the substituent, is irreversible with formation of a stable 6-membered ring. Notice that the end product 2g, absorbing at 330 nm, will be identical for all three inhibitors. According to this model, LSAO inactivation by 1,2-diaminoethane proceeds in the same way as with 2-Br- or 2-Cl-ethylamine, with elimination of ammonia at C-2, whereas the mechanism proposed for the inactivation of lysyl oxidase (Gacheru et al., 1989; Wang et al., 1993) requires the formation of a quinoxaline. Unfortunately, the extra amount of ammonia produced by elimination from C-2 is too little for exact determination. The structure of isolated complex 2g obtained after the reaction between LSAO and 2-Br-ethylamine or 1,2-diaminoethane is currently under investigation.

Following Scheme 2, the rate of the inactivation reaction will be $V_i = k_i[E^\bullet][RCHO]$, where $E^\bullet$ is the radical intermediate form of the enzyme and RCHO is the aldehyde product. It is not easy to determine the relevant concentrations; hence a detailed kinetic analysis is complicated. However, the second-order inhibition constant k_i may be estimated from the half-life of the radical species under anaerobic conditions. In LSAO, the radical species accounts for about 20% of the active enzyme in anaerobiosis, as determined from ESR measurements of the Cu(II) to Cu(I) ratio (unpublished result from our laboratories). This percentage remains constant during the reaction because the free radical form is in rapid equilibrium with the aminoresorcinol form (Turowski et al., 1993). One molecule of aldehyde is released per active site; therefore, [RCHO] = [E] and $[E^\bullet] = 0.20[E]$, where [E] is the concentration of active sites. The rate equation can thus be written as $V_i = 0.20k_i[E]^2$. The integral form of this equation is $k_i t = (0.20[E])^{-1} - (0.20[E]_0)^{-1}$; at $t = t_{1/2}$ this expression reduces to $k_i = (0.20[E]_0 t_{1/2})^{-1}$. For 2-Br-ethylamine in anaerobiosis the half-life of the radical was 10 min using 20 μM enzyme (corresponding to 40 μM active sites), giving an irreversible inhibition rate constant k_i of 1.25×10^4 M^{-1} min^{-1}. In contrast, for 2-Cl-ethylamine with $t_{1/2} = 9$ h, a k_i of 2.3×10^2 M^{-1} min^{-1} can be calculated.

In the reaction of LSAO with 1,3-diaminopropane and 3-Br- and 3-Cl-propylamine, we obtained similar results as with 2-Br-ethylamine: a complex [E•I°] is formed with an absorbance band at 340 nm. In anaerobiosis this complex is stable, but in the presence of oxygen it breaks down slowly with the release of products and reoxidation of the enzyme. In analogy with the mechanism presented above, these compounds could lead to the formation of a 7-membered ring (Scheme 2h). It is at present not known whether such a structure will be intrinsically unstable, perhaps due to motional constraints imposed by the protein matrix, or so energetically unfavorable that it is never formed at all; but it is certain that inhibition is reversible for the propylamine derivatives.

In conclusion, the inhibition of LSAO by diamines with short C chains or substituted C-2 or C-3 alkylamines is caused by reaction of the enzyme with the aldehyde products. These compounds are very good reagents to investigate the structure of the active site of copper amine oxidases.

REFERENCES

Berg, K. A., & Abeles, R. H. (1980) *Biochemistry 19*, 3186−3189.

Cai, D., & Klinman, J. P. (1994) *J. Biol. Chem. 269*, 32039−32042.

Dooley, D. M., McGuirl, M. A., Peisach, J., & McCracken, J. (1987) *FEBS Lett. 214*, 274−278.

Dooley, D. M., McGuirl, M. A., Brown, D. E., Turowski, P. N., McIntire W. S., & Knowles, P. F. (1991) *Nature 349*, 262−264.

Finazzi Agrò, A., Rinaldi, A., Floris, G., & Rotilio, G. (1984) *FEBS Lett. 176*, 378−380.

Floris, G., Giartosio, A., & Rinaldi, A. (1983) *Phytochemistry 22*, 1871−1874.

Gacheru, S. N., Trackman, P. C., Calaman, S. D., Greenaway, F. T., & Kagan, H. M. (1989) *J. Biol. Chem. 264*, 12963−12969.

Kumagai, H., Uchida, H., & Yamada, H. (1979) *J. Biol. Chem. 254*, 10913−10919.

Leyton, G. B. (1981) *Pathology 13*, 327−333.

Matsuzaki, R., Fukui, T., Sato, H., Ozaki, Y., & Tanizawa, K. (1994) *FEBS Lett. 351*, 360−364.

Medda, R., Padiglia, A., Pedersen, J. Z., & Floris, G. (1993) *Biochem. Biophys. Res. Commun. 196*, 1349−1355.

Medda, R., Padiglia, A., Pedersen, J. Z., Rotilio, G., Finazzi Agrò, A., & Floris, G. (1995) *Biochemistry 34*, 16375−16381.

Mondovì, B., Rotilio, G., Finazzi Agrò, A., Vallogini, M. P., Malmström, B. G., & Antonini, E. (1969) *FEBS Lett. 2*, 182−184.

Neumann, R., Hevey, R., & Abeles, R. H. (1975) *J. Biol. Chem. 250*, 6362−6367.

Padiglia, A., Medda, R., & Floris, G. (1992) *Biochem. Int. 28*, 1097−1107.

Plastino, J., & Klinman, J. P. (1995) *FEBS Lett*. 371, 276−278.

Rinaldi, A., Giartosio, A., Floris, G., Medda, R., & Finazzi Agrò, A. (1984) *Biochem. Biophys. Res. Commun. 120*, 242−249.

Sawicki, E., Hauser, T. R., Stanley, T. W., & Elber, W. (1961) *Anal. Chem. 33*, 93−96.

Steinebach, V., Groen, B. W., Wijmenga, S. S., Niessen, W. M. A., Jongejan, J. A., & Duine, J. A. (1995) *Anal. Biochem. 230*, 159−166.

Tang, S., Simpson, D. E., & Kagan, H. M. (1984) *J. Biol. Chem. 259*, 975−979.

Tanizawa, K., Matsuzaki, R., Shimizu, E., Yorifuji, T., & Fukui, T. (1994) *Biochem. Biophys. Res. Commun. 199*, 1096−1102.

Turowski, P. N., McGuirl, M. A., & Dooley, D. M. (1993) *J. Biol. Chem. 268*, 17680−17682.

Wang, F., Bae, J.-Y., Jacobson, A. R., Lee, Y., & Sayre, L. M. (1994) *J. Org. Chem. 59*, 2409−2417.

BI961410W