The Reaction Mechanism of Copper Amine Oxidase: Detection of Intermediates by the Use of Substrates and Inhibitors†

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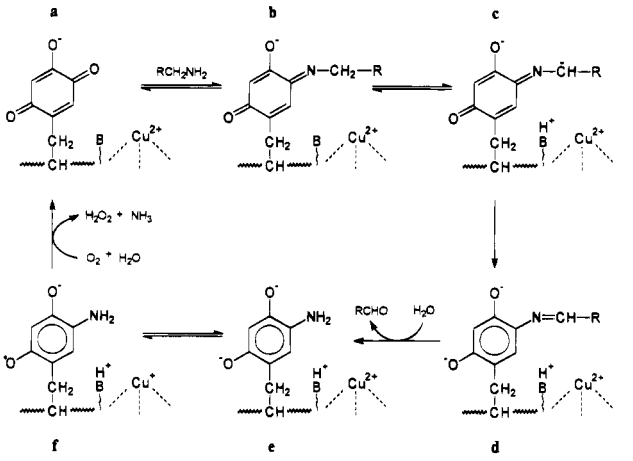
Received June 26, 1995; Revised Manuscript Received October 2, 1995⊗

ABSTRACT: Intermediate states in the catalytic mechanism of lentil copper amine oxidase have been investigated by ESR and optical spectroscopy. Using highly purified apo- and holoenzyme in combination with a poor substrate and a range of inhibitors, under both aerobic and anaerobic conditions, the single steps of the reaction mechanism can be slowed down or 'frozen' completely. In this way, a sequence of six intermediate species in the catalytic cycle has been established. Oxidative deamination of *p*-(dimethylamino)benzylamine is 5×10^5 times slower than for putrescine; the rate-limiting step is shown to be release of the aldehyde product. This process is not affected in the apoenzyme, but subsequent intramolecular electron transfer to form the characteristic free radical intermediate is completely blocked, and the apoenzyme is trapped as an aminoresorcinol species. Classic hydrazine and hydrazide inhibitors bind to the 6-hydroxydopa cofactor in the same way as active substrates, but rearrangements lead to formation of stable intermediate adducts at the step preceding release of aldehyde. The semicarbazide−6-hydroxydopa adduct is shown to bind simultaneously to Cu(II), providing the first direct evidence for localization of 6-hydroxydopa close to the copper site.

Copper amine oxidases (EC 1.4.3.6) are a family of soluble enzymes containing Cu(II) and an unusual organic prosthetic group capable of interacting strongly with carbonyl reagents. The nature of the covalently bound cofactor has been the subject of much speculation and intensive investigation. Initially, the "active carbonyl" cofactor was identified as pyridoxal phosphate in porcine plasma amine oxidase (Malmström et al., 1975). Following the discovery of the dissociable cofactor pyrroloquinoline quinone (PQQ)[1] in several bacterial dehydrogenases, it was reported that bovine plasma amine oxidase contained covalently bound PQQ (Ameyama et al., 1984; Lobenstein-Verbeek et al., 1984), but the presence of PQQ in this enzyme was later excluded (Kumazawa et al., 1990). Finally Klinman and co-workers demonstrated that 6-hydroxydopa (TOPA) was the redox factor in mammalian amine oxidases, and that PQQ-like intermediates could be formed by cyclization of TOPA derivatives during protein hydrolysis (Janes et al., 1990). The presence of TOPA in copper amine oxidase from various sources has since been confirmed by different spectroscopic techniques (Brown et al., 1991; Janes et al., 1992; Mu et al., 1992; Pedersen et al., 1992).

Scheme 1



Many studies on the reaction mechanism of copper amine oxidase have been published, and many different intermediate states in the catalytic cycle have been suggested, but little firm evidence for the structure of most of these states is available. Our current understanding of the enzyme mechanism is outlined in Scheme 1. Upon addition of a substrate, the resting oxidized enzyme **1a** is rapidly bleached due to disappearance of the characteristic broad absorption at 498 nm, presumably because of the formation of the reduced form **1d**. In the absence of oxygen, release of the aldehyde occurs, and a relatively stable yellow intermediate **1f** accumulates (Rinaldi et al., 1984; Bellelli et al., 1985) which is a free radical species containing covalently bound substrate-derived nitrogen (Finazzi Agrò et al., 1984; Dooley et al., 1987, 1990; Pedersen et al., 1992). This radical rapidly reacts with oxygen to regenerate the oxidized enzyme **1a** with the simultaneous release of the products ammonia and hydrogen

† This study was partially supported by MURST "40%" and "60%" funds and by the CNR project "ACRO".

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⊗ Abstract published in *Advance ACS Abstracts,* November 15, 1995.

[1] Abbreviations: LSAO, lentil seedling amine oxidase; MBTH, 2-methylbenzothiazolinone hydrazone; PQQ, pyrroloquinoline quinone; TOPA, 6-hydroxydopa (2,4,5-trihydroxyphenethylamine).

0006-2960/95/0434-16375$09.00/0

peroxide; no intermediate states have been detected during this process. The intermediate **1f** was identified as an amino-TOPA radical on the basis of its characteristic optical and ESR spectra, although the definitive assignment is not yet possible. In contrast, evidence for the structures of the unstable intermediates **1b**−**1e** is mainly circumstantial since they have been detected only optically by rapid kinetics tecniques. The transient bands at 310 and 340 nm were recently detected and attributed respectively to the reduced TOPA adduct **1d** and to the iminoquinone complex **1b** (Hartmann et al., 1993). Evidence for the carbanion species **1c** was obtained by electrophilic trapping with tetranitromethane (Medda et al., 1993). So far there is no spectroscopic evidence for the aminoresorcinol species **1e**, but the electron transfer rate between **1e** and **1f** is known to be extremely fast (Turowski et al., 1993) and involves a Cu(II) → Cu(I) transition (Dooley et al., 1991). The copper site must therefore be located very close to TOPA in the enzyme, but the exact configuration is not known. There is strong evidence that the substrate amine is protonated (Hartmann & Klinman, 1991), but little is known about the degree of protonation of the various intermediates.

A large number of substrates and inhibitors of amine oxidases have been described in the literature, but most studies were done before the enzyme mechanism was outlined, and there is generally little information available as to the way these compounds interact with the enzyme. We have studied the effects of different inhibitors and poor substrates to obtain further information on the intermediates of the catalytic cycle. We demonstrate that inhibitors can "freeze" the enzyme by forming stable **1b** and **1d** intermediates. Further, we show that the formation of **1e** with release of aldehyde is the rate-limiting step, and that the semiquinone **1f** is not formed in the apoprotein, leading to accumulation of **1e**. Finally, we have found that semicarbazide-derived inhibitors may bind simultaneously both to the TOPA cofactor and to Cu(II).

EXPERIMENTAL PROCEDURES

1,4-Diaminobutane dihydrochloride (putrescine), *p*-(dimethylamino)benzylamine hydrochloride, semicarbazide hydrochloride, and MBTH hydrochloride were purchased from Sigma and used without further purification. 4-(Dimethylamino)benzaldehyde and diethyldithiocarbamate were from Fluka. Phenylhydrazine hydrochloride and 4-phenylsemicarbazide obtained from Janssen and thiosemicarbazide from Aldrich were recrystallized from ethanol. *p*-[(Dimethylamino)methyl]benzylamine was prepared according to the method of Bardsley et al. (1972). Amine oxidase was purified from lentil (*Lens esculenta*) seedlings as previously described (Floris et al., 1983). An ϵ_{498} of 4100 M^{-1} cm^{-1} or an ϵ_{278} of 2.45 × 10^5 for the purified enzyme (2 copper ions and a M_r of 150 000) was used to estimate enzyme concentration (Padiglia et al., 1992). The value A_{498}/A_{410} of the purified enzyme was 1.6. One unit of enzyme activity corresponded to 1 mol of substrate oxidized per minute. All experiments were reproduced several times at different enzyme concentrations. Copper-free lentil amine oxidase was prepared as previously described (Rinaldi et al., 1984). Absorption spectra were recorded at room temperature with a Cary 2300 spectrophotometer. Anaerobic experiments were made after several cycles of evacuation 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 with a syringe through a rubber cap. 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. Hydrogen peroxide was determined by the peroxidase:4-hydroxy-3-methoxyphenylacetic acid method (Leyton, 1981), and ammonia was measured using a coupled glutamate dehydrogenase assay (Rinaldi et al., 1984). The aldehyde production from putrescine (Δ^1-pyrroline) was checked by the ninhydrin method (Naik et al., 1981). The aldehyde production from *p*-(dimethylamino)benzylamine was measured by the absorbance increase at 350 nm; an ϵ_{350} of 31 200 M^{-1} cm^{-1} for *p*-(dimethylamino)benzaldehyde (Ehrlich's reagent) was obtained by a standard curve using a pure reagent. The aromatic aldehyde production from *p*-[(dimethylamino)-methyl]benzylamine was measured by the absorbance increase at 250 nm, using an ϵ_{250} of 11 000 M^{-1} cm^{-1} for *p*-[(dimethylamino)methyl]benzaldehyde (Bardsley et al., 1972). The amount of reaction products formed was calculated as the mean of at least three different measurements.

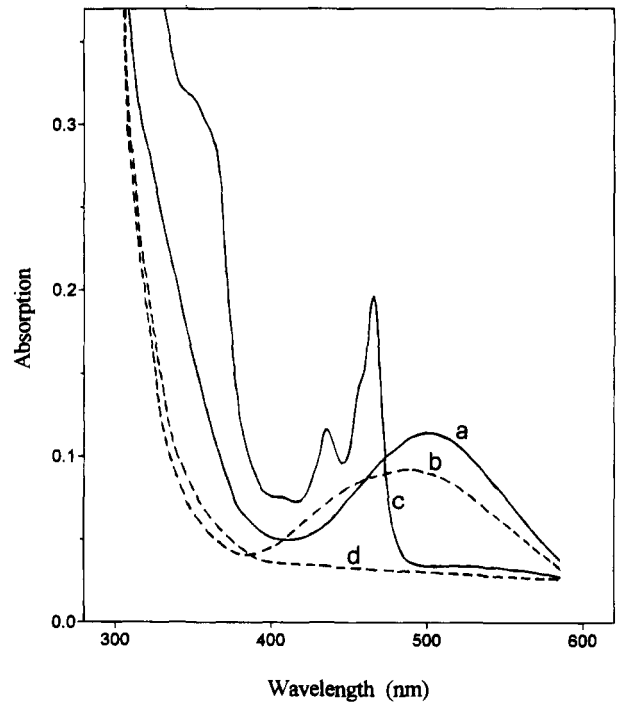


FIGURE 1: Reaction of *Lens* amine oxidase with an active substrate. Absorption spectra of 28 μM holoenzyme (a, c) and 22 μM apoenzyme (b, d) in 100 mM phosphate buffer, pH 7, in anaerobic conditions were recorded before (a, b) and after (c, d) addition of 17 mM putrescine. The reaction was completed within the time necessary for mixing the sample.

RESULTS

Intermediates Observed with Active Substrates. The visible spectrum of resting LSAO is similar to that of other copper amine oxidases, with a typical broad absorption centered around 498 nm which disappeared immediately upon reaction with a substrate. Due to the high activity of this enzyme toward good substrates such as putrescine (100 units/mg) and the low K_m for oxygen, even samples containing low concentrations of LSAO rapidly became anaerobic, after which the characteristic spectrum of the free radical intermediate appeared (Figure 1).

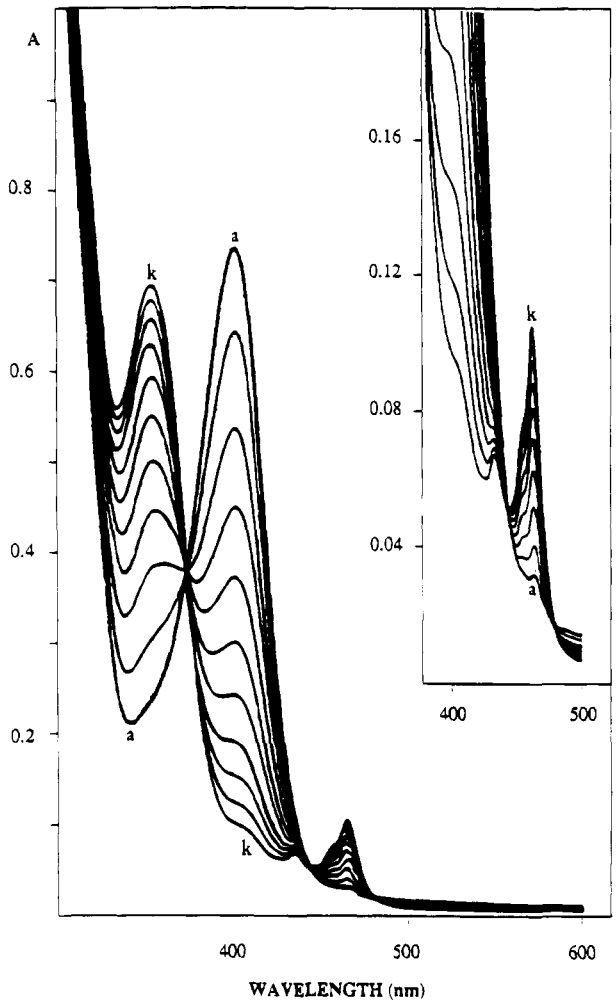
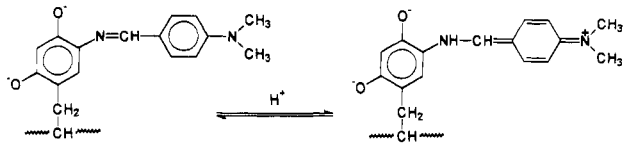


FIGURE 2: Spectral change during the reaction of 11 μM LSAO with 1 mM *p*-(dimethylamino)benzylamine in 100 mM phosphate buffer, pH 7, in anaerobic conditions. Spectra were recorded from 1 min (a) to 300 min (k) with intervals of 30 min. Inset: The spectral region with the radical intermediate absorption amplified to show isosbestic points.

Scheme 2



Copper could be removed from native LSAO by treatment with diethyldithiocarbamate. The residual copper, determined by atomic absorption spectroscopy, was 0.4 ± 0.2% of the original content. Copper-free LSAO was pink, showing a broad absorption peak in the visible region at 480 nm, shifted toward shorter wavelengths with respect to the native enzyme, but with similar intensity. Reaction of the apoenzyme with putrescine or *p*-[(dimethylamino)methyl]-benzylamine in anaerobiosis as well as in aerobiosis resulted in rapid and permanent bleaching of the 480 nm band with the concomitant release of aldehyde (2.03 ± 0.05 mol produced per mole of enzyme), without consumption of oxygen and without detectable formation of NH_3 and H_2O_2 (less than 0.1 mol/mol of enzyme). The resulting stable intermediate did not give rise to any spectral feature in the ultraviolet and visible range (Figure 1).

Catalysis in the Presence of a Poor Substrate. *p*-(Dimethylamino)benzylamine is a very poor substrate for

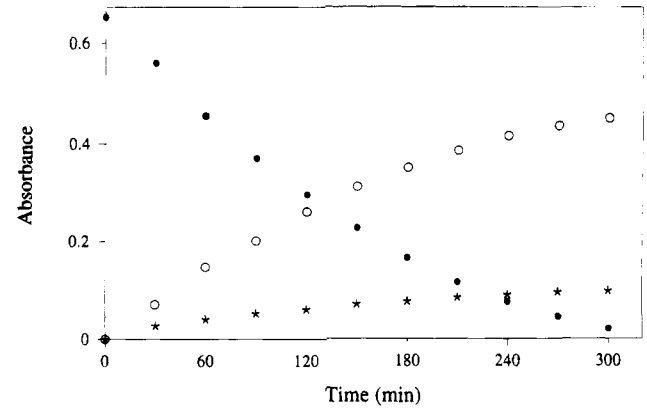


FIGURE 3: Time course of the reaction of 7 μM amine oxidase with *p*-(dimethylamino)benzylamine measured at 464 nm (★), 400 nm (●), and 350 nm (○). Experimental conditions as in Figure 2.

LSAO. The turnover number with *p*-(dimethylamino)-benzylamine was measured to 0.014 min^{-1}, a very small value compared with the corresponding number of 9300 min^{-1} found for putrescine. However, when *p*-(dimethylamino)benzylamine was added to LSAO in anaerobiosis, the broad absorption band at 498 nm disappeared instantaneously, indicating the rapid conversion of the TOPA cofactor to a bleached species, presumably the quinolaldimine (Scheme **1d**). As with effective substrates, this reaction probably proceeds through a quinoketimine intermediate, **1b**. The formation of the quinoketimine carbanion **1c** with this substrate was shown through the nitroform anion generation from tetranitromethane. More nitroform anion appeared to be generated in this case with respect to the results obtained using good substrates (Medda et al., 1993); however, this turned out to be due to reactions between *p*-(dimethylamino)-benzylamine and tetranitromethane (results not shown). Together with the formation of the quinolaldimine, a new band centered at 400 nm appeared (Figure 2). This band was assigned to the protonated tautomeric form of the quinolaldimine (a "quino"-imine, Scheme 2), based on the analogy with the findings reported for *p*-(dimethylamino)-benzylamine oxidation by monoamine oxidase (Edmonson et al., 1993). Under anaerobic conditions, this species decayed slowly, in parallel with formation of the yellow radical intermediate (Figure 2). In this process, isosbestic points at 372, 440, and 478 nm were observed. These spectral changes occurred simultaneously with the liberation of the corresponding aldehyde; in fact, formation of the aldehyde was directly observable by the absorbance increase at 350 nm and confirmed by thin-layer chromatography; 1.95 ± 0.12 mol of aldehyde was released per mole of enzyme dimer.

At pH 7, the disappearance of the "quino"-imine intermediate was very slow, with a $t_{1/2}$ = 120 min. The rate of this process was clearly reflected in the kinetics of both aldehyde production and formation of the radical intermediate (Figure 3). At pH values 8−9, the rates of aldehyde production and "quino"-imine decay were slightly decreased, as expected from the pH dependence of the enzyme activity (data not shown).

In the presence of air, the radical species reacted rapidly with oxygen to restore the oxidized enzyme and liberate NH_3 and H_2O_2 (data not shown). The bands at 434 and 464 nm were no longer present in the spectrum; during the steady-state phase, the 400 nm peak remained constant, whereas the absorption at 350 nm increased at the same initial rate

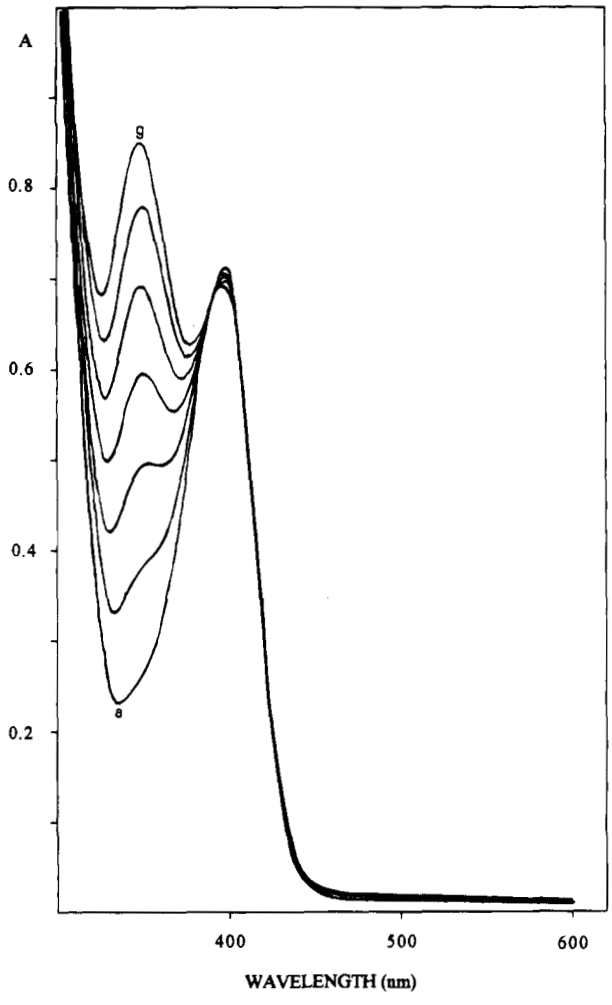


FIGURE 4: Reaction of 11 μM LSAO with 1 mM *p*-(dimethylamino)benzylamine in 100 mM phosphate buffer, pH 7, in aerobic conditions. Spectra were recorded from 1 min (a) to 180 min (g) with 30 min intervals.

as in anaerobiosis (Figure 4). After inhibition of the enzyme by MBTH, phenylhydrazine, or semicarbazide, the subsequent addition of *p*-(dimethylamino)benzylamine did not produce any spectral changes at all.

When the copper-free enzyme was used, addition of *p*-(dimethylamino)benzylamine caused the formation of the peak at 400 nm which afterward decayed in parallel with the release of aldehyde (Figure 5). Within experimental error, the absorption changes observed at 350 and 400 nm were identical to those seen for the native enzyme in anaerobiosis. However, the complete absence of bands at 434 and 464 nm showed that no formation of the free radical intermediate occurred, as confirmed also by ESR measurements (data not shown).

Effects of Inhibitors. MBTH is a hydrazone standard reagent for the staining and detection of catecholic residues. When MBTH was added to LSAO at pH 7, the intensity of the broad band around 500 nm increased while the maximum shifted to 510 nm (Figure 6). The spectral variations were seen both in anaerobiosis and in air-saturated samples. Similar results were obtained with copper-free enzyme (not shown). Since MBTH itself does not absorb in that range, the spectral variation was attributed to the direct reaction between MBTH and TOPA. MBTH was found to be a competitive inhibitor of LSAO; the K_i obtained from a Dixon's plot was 2×10^{-4} M (not shown). These results indicated the formation of an azino adduct (Scheme 3), in

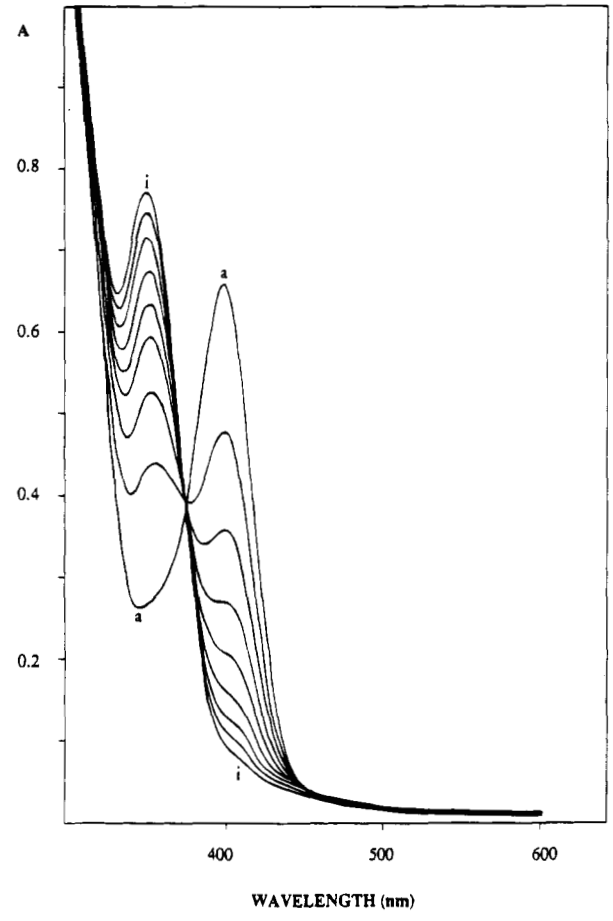


FIGURE 5: Reaction of 11 μM LSAO apoenzyme with 1 mM *p*-(dimethylamino)benzylamine in 100 mM phosphate buffer, pH 7, in anaerobic conditions. Spectra were recorded from 1 min (a) to 240 min (i) with 30 min intervals.

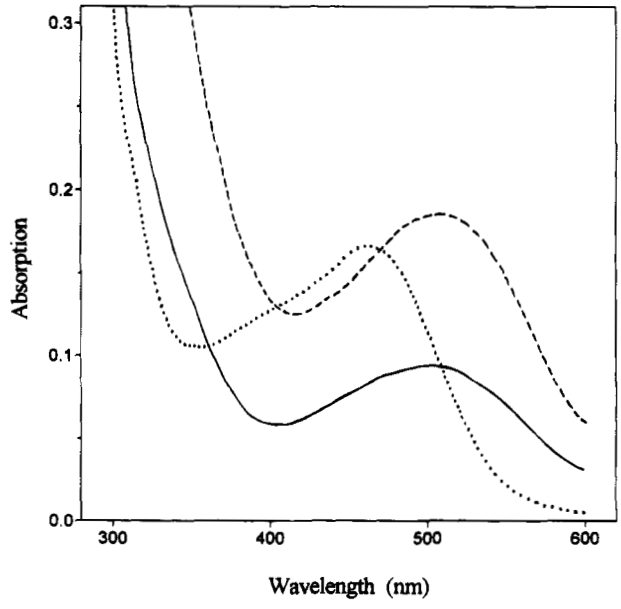
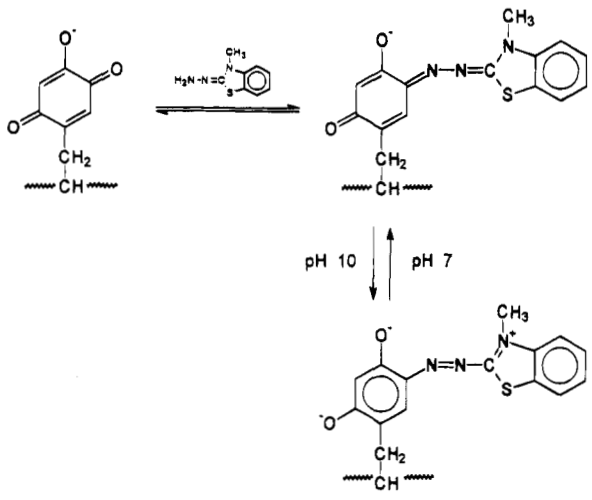


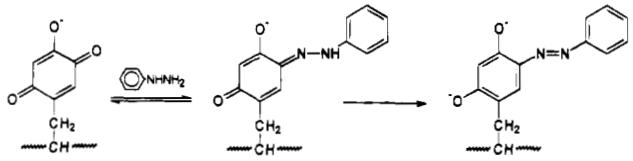
FIGURE 6: Inhibition of amine oxidase with 2-methylbenzothiazolone hydrazone. Absorption spectra of 20 μM LSAO in aerobic conditions are shown without additions (full line), after addition of 50 μM MBTH in 100 mM carbonate buffer, pH 7 (dashed line), and after addition of 50 μM MBTH in 100 mM carbonate buffer, pH 10 (dotted line). Same results were obtained using phosphate buffer.

analogy with the product formed in the reaction between MBTH and PQQ (Michaud-Soret et al., 1990). When the pH of the solution was increased to 10, the color of the

Scheme 3



Scheme 4



MBTH−enzyme complex changed from red to yellow as the absorption maximum shifted to 470 nm (Figure 6); this effect was completely reversible and probably reflected the formation of a reduced TOPA−azo adduct through tautomerization. Upon prolonged incubation with MBTH, the enzyme gradually lost activity in an irreversible way, different from the competitive inhibition seen at short times of incubation (results not shown).

It is worth recalling that other hydrazines and hydrazides are classical copper amine oxidase inhibitors which cause irreversible inactivation of the enzyme (Rinaldi et al., 1983). The addition of phenylhydrazine to LSAO led to the formation of a characteristic strong absorption band at 445 nm, with an ϵ_{445} of 64 000 M^{-1} cm^{-1} (Padiglia et al., 1992). The addition of hydrazine instead gave rise to a band at 330 nm, of rather low intensity (ϵ_{330} of 12 700 M^{-1} cm^{-1}). In both cases, the formation of these bands was concomitant with the disappearance of the TOPA absorption band at 498 nm. The reaction with phenylhydrazine was completed in 10 min when 2 mol/mol of enzyme was added (Padiglia et al., 1992). Similar results were obtained after the addition of phenylhydrazine and hydrazine to the copper-free enzyme, but the absorption changes were much slower with approximately 8 h being necessary for a complete end point titration (results not shown). The adducts obtained with hydrazines and hydrazides were very stable, and the reactions were essentially irreversible. This was compatible with the assumption that the initially formed iminoquinone complex gave rise to stable azo derivatives (Scheme 4). Hence, the reaction of TOPA with phenylhydrazine would result in a highly conjugated complex, which could account for the higher λ_{max} and extinction coefficient for phenylhydrazine when compared to hydrazine.

For structural reasons, the reaction of semicarbazides could be expected to be similar to the reaction with hydrazine compounds, producing stable azo products through rearrangement of an initially formed semicarbazono complex. However, the addition of semicarbazide to the LSAO was

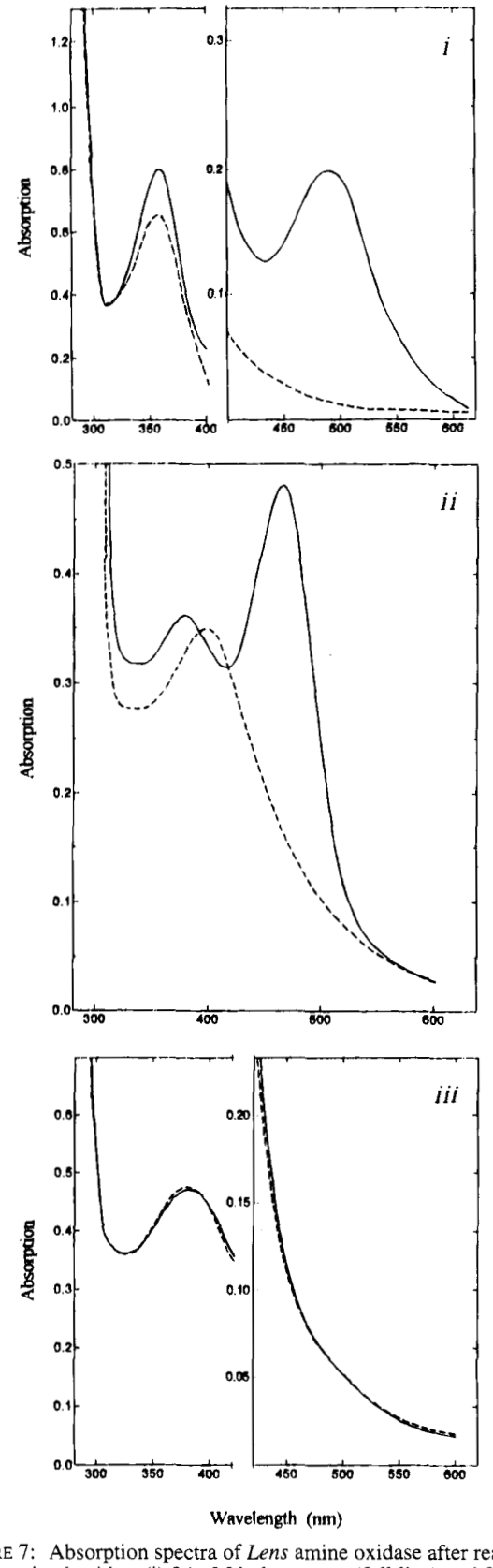


FIGURE 7: Absorption spectra of *Lens* amine oxidase after reaction with semicarbazides. (i) 24 μM holoenzyme (full line) and 20 μM apoenzyme (dashed line) after addition of 0.6 mM semicarbazide. (ii) 11 μM holoenzyme (full line) and 10 μM apoenzyme (dashed line) after addition of 0.6 mM thiosemicarbazide. (iii) 14 μM holoenzyme (full line) and 14 μM apoenzyme (dashed line) after addition of 0.6 mM phenylsemicarbazide. All measurements were made in air-saturated 100 mM phosphate buffer, pH 7.

followed by the formation of **two** absorption bands, at 492 and 360 nm, with the simultaneous disappearance of the band at 498 nm (Figure 7i). When semicarbazide was added to

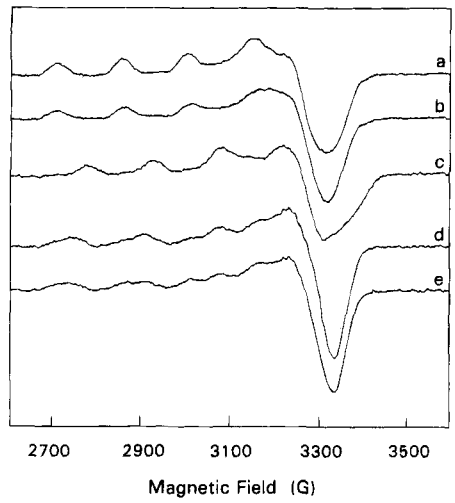


FIGURE 8: Effects of inhibitors on the Cu(II) ESR spectrum of amine oxidase. Samples of 50 μM LSAO in 100 mM phosphate buffer, pH 7, were measured in standard 3 mm quartz EPR tubes at 100 K using 5 mW power and 10 G modulation. (a) Control without additions; (b) control plus a 5-fold excess of semicarbazide after 1 min incubation at room temperature; (c) final spectrum of sample (b) after 16 h; (d) control plus 1 mM MBTH measured 1 min after mixing; (e) sample (d) measured after 90 min at room temperature.

copper-free enzyme, only the absorption at 360 nm appeared, with an intensity similar to that observed with the native enzyme (Figure 7i). The latter band was attributed to the reaction between TOPA and semicarbazide, which can be expected to take place for both apoenzyme and holoenzyme. The complete absence of the peak at 492 nm in the apoenzyme suggested that this absorption might be due to a ligand to metal charge transfer band. In the TOPA−semicarbazide adduct, the ligand available for binding of copper could be either the carbonyl oxygen or, more likely, the terminal free amino group. To test this hypothesis, the spectral changes induced by thiosemicarbazide and 4-phenylsemicarbazide were also studied (Figure 7ii−7iii). The addition of thiosemicarbazide, where the carbonyl oxygen is substituted by sulfur, produced two bands at 460 and 374 nm, while the addition of 4-phenylsemicarbazide, where the terminal amino group is blocked by a benzyl ring, gave only one peak at 380 nm. When the same experiments were carried out using the apoprotein, addition of thiosemicarbazide only resulted in the formation of the peak at 390 nm. In contrast, 4-phenylsemicarbazide gave rise to a spectrum identical to the one seen with the native enzyme. These results are consistent with interaction between the terminal amino group of the TOPA−semicarbazide adduct and the copper.

ESR Spectra of the LSAO Adducts. The addition of semicarbazide to LSAO gave rise to a perturbation of the copper ligand field which was reflected in the Cu(II) ESR spectrum. Untreated LSAO had the typical type 2 copper spectrum, characterized by $g_{\|} = 2.32$ and $A_{\|} = 153$ G and by the absence of superhyperfine structure (Rotilio et al., 1985). Only minor changes were observed immediately after addition of semicarbazide to LSAO, but after incubation at room temperature a different spectrum appeared, having $g_{\|} = 2.20$ and $A_{\|} = 155$ G (Figure 8). The reaction appeared to be complete and showed a single component present. The perturbation induced by semicarbazide was much stronger than that observed with substrates and phenylhydrazine (Rinaldi et al., 1983; Rotilio, 1985) and indicated inner sphere coordination to the copper.

Addition of MBTH initially modified the ESR spectrum of LSAO in much the same way as putrescine and phenylhydrazine, with the appearance of the characteristic nitrogen superhyperfine splitting in the $g_{\perp}$ region (Figure 8). Upon prolonged incubation with MBTH, a second component appeared in the spectrum, suggesting the involvement of a slower secondary reaction mechanism.

DISCUSSION

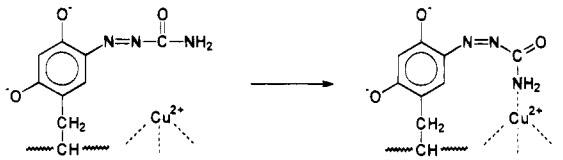
Like many other plant copper amine oxidases the enzyme from lentil seedlings has a very high specific activity, approximately 100 units/mg, compared to the values of 0.1−0.4 unit/mg typically found for the mammalian enzymes (Blanschko & Buffoni, 1965; Turini et al., 1982; Janes & Klinman, 1991; Janes et al., 1992). It can be obtained with a high degree of purity allowing an accurate titration of inhibitor binding and determination of cofactor:enzyme stoichiometries. Finally, this enzyme forms a free radical intermediate that can be observed without addition of cyanide or azide, normally used in order to detect this intermediate in other amine oxidases (Pedersen et al., 1992). LSAO is therefore very suitable for studying the copper amine oxidase reaction mechanism.

After the discovery of TOPA as the organic cofactor in these enzymes, various models of the catalytic mechanism have been suggested (Bellelli et al., 1991; Greenaway et al., 1991; Janes & Klinman, 1991; Hartman & Klinman, 1991; Pedersen et al., 1992; Turowski et al., 1993). However, detection of the postulated intermediates has turned out to be difficult. A series of para-substituted benzylamines, including *p*-(dimethylamino)benzylamine, has been used as substrates to study the reaction mechanism of bovine serum amine oxidase (Hartmann et al., 1993). Stopped-flow experiments gave evidence for the transient formation of two intermediates, corresponding to **1b** and **1d** in Scheme 1. The use of inhibitors in the present study allows the trapping of these intermediates in a different way. The sensivity of amine oxidases to carbonyl reactive inhibitors has been known for decades, and binding is known to be irreversible (Medda et al., 1993). The results presented here for different hydrazines and hydrazides point to the formation of stable adducts analogous to the **1d** intermediate. It should be emphasized that the reaction of LSAO with these inhibitors invariably causes the disappearance of the broad absorption at 498 nm, which is indicative of oxidized TOPA.

Also, MBTH reacts with LSAO with formation of an iminoquinone as the other inhibitors tested, but in this case the reaction does not proceed further. Evidently the equilibrium between the tautomers in Scheme 3 is shifted completely toward the iminoquinone form, at least at neutral pH, and therefore MBTH seems to trap the enzyme into the intermediate **1b**. It should be mentioned that MBTH previously has been claimed to identify the presence of PQQ in quinoproteins (Gallop et al., 1989), but clearly it is not specific for this cofactor.

Further details of the reaction mechanism can be obtained from the experiments with the poor substrate *p*-(dimethylamino)benzylamine. Although the overall oxidation of this compound is extremely slow, the initial steps leading to the reduced intermediate **1d** are very fast and cannot be resolved with manual mixing techniques. The subsequent formation of the intermediate **1e** with release of the aldehyde is clearly the rate-limiting step. It seems reasonable that this will be

Scheme 5



the rate-limiting step also for good substrates like putrescine. In the native enzyme, the aminoresorcinol form **1e** is known to be in rapid equilibrium with the radical species (Turowski et al., 1993). Interestingly, reaction of the apoenzyme with *p*-(dimethylamino)benzylamine gives the same kinetics of aldehyde release, but in this case there is no formation of the **1f** intermediate. It can be concluded that, when reacted with a substrate, the apoenzyme is transformed into the **1e** form which is stable in the presence of oxygen. The spectrum of this intermediate obtained with apo-LSAO and putrescine is shown in Figure 1. Unfortunately, it has no unique spectral characteristics and thus can hardly be distinguished from **1d**. This is not altogether surprising as it is known that the aminoresorcinol itself only has a weak absorption at 315 nm (Mure & Klinman, 1993).

It has previously been reported for amine oxidase from bovine serum, that addition of *p*-(dimethylamino)benzylamine led to the formation of a very broad band with λ_{max} = 460 nm, together with a transient species absorbing at 360 nm; the latter was attributed to the formation of a Schiff base with the substrate (Hartmann et al., 1993). Our results with *p*-(dimethylamino)benzylamine are rather different. We find a narrow peak at 400 nm, with spectral characteristics and pH dependence similar to those previously reported for *p*-(*N*,*N*-dimethylamino)benzylimine derivatives, in agreement with the results recently obtained with monoamine oxidase (Edmonson et al., 1993). Furthermore, the product formed, *p*-(dimethylamino)benzaldehyde, is the well-known Ehrlich's reagent which has a characteristic absorption at 350 nm. Identical results were obtained using *p*-(dimethylamino)-benzylamine purchased from various sources; the reason why LSAO and the bovine serum enzyme gave different spectral changes is not clear.

Finally, the formation of a ligand to metal charge transfer band in the LSAO−semicarbazide adduct provides the first direct evidence for the actual location of the copper atom with respect to the organic cofactor. The proposed TOPA−N=N−CO−NH_2···Cu^{II} structure (Scheme 5) is compatible with the distance of approximately 3.0 Å predicted by Turowski et al. (1993). It is possible that a similar but transient binding of substrates may also occur, but as yet no evidence for such a mechanism has been obtained, even though the binding of substrates is known to affect the Cu(II) ligand field (Rotilio, 1985). Oddly enough, all models of the copper amine oxidase active site proposed so far show copper and substrate on opposite sides of the TOPA. The definitive determination of the geometry of the active site can be expected from crystal diffration studies within short time.

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BI951440J