Intermediates in the catalytic cycle of lentil (*Lens esculenta*) seedling copper-containing amine oxidase¹

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Spectrophotometry and rapid-scanning stopped-flow spectroscopy have been used to investigate the visible absorbance changes that occur in the course of the reduction of lentil (*Lens esculenta*) seedling amine oxidase by substrate. The catalytic cycle of the enzyme employs several intermediates but, owing to kinetic limitations, some of them were not identified in previous studies. In this study we have examined several substrates, either rapidly reacting (e.g. putrescine) or slowly reacting (e.g. γ -aminobutanoic acid). Two forms of the enzyme, namely the Cu(I)-aminoresorcinol and quinone ketimine derivatives, whose

characterization was elusive in previous studies, have been identified and assigned an optical spectrum. Moreover the reduced form of the enzyme is shown to be an equilibrium mixture of two species, the Cu(I)-semiquinolamine radical and Cu(II)-aminoresorcinol; these have been resolved by pH dependence and assigned spectra as well as a second-order rate constant for the reaction with oxygen. Thus the results presented here identify all the catalytic intermediates suggested by the chemical nature of the coenzyme and define their spectroscopic and reactivity properties.

INTRODUCTION

Amine oxidases [amine:oxygen oxidoreductase (deaminating) (copper-containing); EC 1.4.3.6] catalyse the oxidation of primary amines with the formation of the corresponding aldehyde, ammonia and H_2O_2 . These enzymes are ubiquitous, occurring in micro-organisms (fungi and bacteria) [1], plants [2] and mammals [3]. The crystal structures of copper-containing amine oxidases from *Escherichia coli* [4] and from pea seedlings [5] have recently been determined. Plant amine oxidases are soluble dimeric enzymes whose subunit molecular mass is approx. 70 kDa. Each monomer contains one Cu(II) centre that is essential for the enzyme's redox activity [6–8] and one organic prosthetic group identified as 6-hydroxydopaquinone (TPQ) [9]. The redox-dependent spectroscopic changes of TPQ can be followed at suitable wavelengths. Further information can be obtained by means of chromogenic substrates such as benzylamine, whose aldehyde presents a strong absorption band centred on 250 nm.

The catalytic mechanism of lentil amine oxidase is reported in Scheme 1: the amine substrate binds to the organic cofactor of resting oxidized enzyme [Cu(II)-TPQ, **a**] to form a Schiff base [the Cu(II)-quinone ketimine, **b**]; both these intermediates are thought to have a 498 nm absorption band. Transformation of the Cu(II)-quinone ketimine into the Cu(II)-quinolalldimine **c** is associated with the bleaching of the 498 nm absorption band; because this reaction is first-order and relatively fast the time course of the bleaching usually seems to follow second-order kinetics [10,11]. Experimental results suggest that both the above reactions are fully reversible under physiological experimental conditions (e.g. 100 mM potassium phosphate buffer, pH 7, at

20–25 °C). Oxidation of the bound substrate (followed by hydrolysis) releases the aldehyde product, yielding the Cu(II)-aminoresorcinol derivative **d**, which has one bound ammonia molecule. This species is still bleached, but the absorption of the aldehyde provides a spectroscopic marker for the reaction. In plant amine oxidases the Cu(II)-aminoresorcinol equilibrates to a variable extent with the yellow, EPR-detectable Cu(I)-semiquinolamine radical **e**, characterized by absorption bands at 464, 434 and 360 nm [12–15]. Interestingly, in mammalian amine oxidases the radical species is not present to a significant extent [16]. It is reasonable to assume that both forms of the reduced enzyme can react with O_2 to release H_2O_2 and ammonia, thereby regenerating the Cu(II)-quinone species, but at least in plant enzymes the radical seems to be much more reactive than the Cu(II)-aminoresorcinol.

The identification of as many intermediates as possible is required to support the above (or any alternative) kinetic mechanism; in a previous paper we reported the identification of three of these: the oxidized species [Cu(II)-quinone, **a**], the reduced radical [Cu(I)-semiquinolamine, **e**] and the bleached Michaelis complex [Cu(II)-quinolalldimine, **d**] [10]. The results presented here provide a partial correction of previous data because they show that the reduced enzyme is not a single species. In addition the transient Cu(II)-quinone ketimine species **b**, although strongly suggested by the chemistry of the cofactor and the substrate, could not be identified with certainty until now. The experiments presented in this paper, by using γ -aminobutanoic acid (GABA) as a substrate, provide unequivocal evidence for this species. One further intermediate is identified, i.e. the Cu(I)-aminoresorcinol **f**, a bleached species observed on

Abbreviations used: GABA, γ -aminobutanoic acid; LSAO, lentil seedling amine oxidase; SVD, singular value decomposition; TPQ, 6-hydroxydopaquinone.

¹ This paper is dedicated to Alessia Paglia, prematurely deceased on 10 January 1997.

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Scheme 1 Catalytic mechanism of lentil amine oxidase

Top panel: catalytic cycle of LSAO, showing resting oxidized enzyme [a]; Cu(II)-quinone ketimine [b]; Cu(II)-quinonolaldimine [c]; Cu(II)-aminoresorcinol [d]; Cu(I)-semiquinolamine radical [e]; Cu(I)-aminoresorcinol [f] (present in anaerobiosis only in the presence of dithionite). Bottom panel: spectra of LSAO derivatives. (i) Species [a] and [b]; (ii) species [c], [d] and [f]; (iii) species [e].

the reaction of the Cu(I)-semiquinolamine radical **e** with reductants (e.g. sodium dithionite).

EXPERIMENTAL

Materials

1,4-Diaminobutane dihydrochloride (putrescine), benzylamine hydrochloride and GABA were from Sigma. Amine oxidase from lentil (*Lens esculenta*) seedlings was purified in accordance with the procedure described previously [17]. An ϵ_{498} of $4100 \text{ M}^{-1} \cdot \text{cm}^{-1}$ or an ϵ_{278} of $2.45 \times 10^5 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for the purified enzyme (2 copper ions and a molecular mass of 150 kDa) was

used to estimate enzyme concentration [18]. All other chemicals were of analytical grade.

Spectroscopic methods

Absorption spectra were recorded at 25°C with a Cary 2300 spectrophotometer. Anaerobic experiments were made in a Thunberg-type spectrophotometer cuvette after several cycles of evacuation followed by flushing with O_2 -free argon at 25°C ; anaerobic additions of various reagents to the cuvette were made through a rubber cap with a syringe.

Stopped-flow experiments

Stopped-flow experiments were performed with an Applied Photophysics MV 17 apparatus equipped with an observation chamber with a 1 cm light path. Transient spectroscopy experiments were performed with a Tracor Northern TN6500 photodiode-array spectrophotometer coupled to the observation chamber of a Gibson–Durum stopped-flow apparatus, as described elsewhere [11].

Assays of products

The aromatic aldehyde production from benzylamine was measured by absorbance at 250 nm, with ϵ_{250} taken as 12800 M⁻¹·cm⁻¹ for benzaldehyde [19].

Data analysis

Deconvolution of the spectral contributions with singular value decomposition (SVD) was performed with the program MATLAB (The Math Works, Natick, MA, U.S.A.). This analysis requires that the time-resolved spectra be arranged into matrix **A**, each column being a spectrum and each row a time course or a titration profile at a single wavelength; application of SVD yields three matrices, **U**, **S** and **V**, such that **A** = **U** × **S** × **V**^T. **U** and **V** are orthogonal matrices, whereas **S** is diagonal, its non-zero elements arranged in decreasing order. Each column of matrix **U** × **S** represents a pseudospectrum, i.e. the absorption of one of the spectroscopic components required to approximate the original data and only the first few columns are significant, the last ones having negligibly small values. Each column of matrix **V** represents the time course or titration profile of the corresponding column of **U** × **S** and was analysed by ordinary non-linear minimization routines (by using either MATLAB or dedicated programs developed with the Borland Pascal Compiler, version 7.0), as already described [20], to obtain the pertinent equilibrium or kinetic constants and the amplitudes of the **V** elements. The fitted **V** amplitudes (**V**_{fit}) were used to calculate the spectra of each chemical species involved in the reaction in accordance with the formula:

$$\mathbf{A}_{\text{fit}} = \mathbf{U} \times \mathbf{S} \times \mathbf{V}_{\text{fit}}^T$$

RESULTS

pH dependence of the optical spectra and catalytic activity of lentil seedling amine oxidase (LSAO)

The kinetics of the reaction of LSAO with substrates was followed at pH 6 and 7; the corresponding Michaelis parameters are reported in Table 1. The *V*_{max} values were higher at pH 7 for all substrates, whereas *K*_m values were not modified.

Table 1 Substrate specificity of LSAO

The values of *K*_m were the same at pH 6 and 7; catalytic-centre activity is defined as (mol of substrate consumed)/(mol of active sites) in 1 s.

Compound	<i>K</i> _m (mM)	Catalytic-centre activity	
		pH 6	pH 7
Putrescine	0.24	101	155
Benzylamine	0.4	0.2	1.0
GABA	3.0	3.3 × 10 ⁻⁴	8.3 × 10 ⁻⁴

Figure 1 Absorption spectra of 22 μM LSAO monomer in 100 mM potassium phosphate buffer at pH 5 (trace a), pH 6 (trace b), pH 7 (trace c), pH 8 (trace d) and pH 9 (trace e)

In addition to absorption by amino acids in the UV region, LSAO absorbs specifically in the visible region (400–500 nm) owing to the TPQ cofactor. Addition of the amine substrate in the presence of excess oxygen induces a bleaching of the 498 nm absorption band, indicative of the reduction of the TPQ cofactor [21]. After the substrate has been exhausted the 498 nm band is restored. When the same experiment is performed under anaerobic conditions, the 498 nm band disappears immediately and the Cu(I)-semiquinolamine radical, with absorption peaks at 464, 434 and 360 nm, is observed.

The visible absorbance of both the oxidized and substrate-reduced enzyme is sensitive to pH and displays characteristic changes on varying the pH from 5 to 9 (Figures 1 and 2). The pH-induced absorbance changes observed in the oxidized enzyme are at present difficult to attribute; in contrast, those of the substrate-reduced LSAO indicate a rise-and-fall behaviour of the radical species, with a maximum absorbance of the 464 and 434 nm peaks at pH 7 and minima at acidic or alkaline pH values (Figure 2 and Table 2). The pH dependence of the activity is also marked, but LSAO is active even at extreme pH (*V*_{max} at pH 5 and 10 is approx. 25 % of that at pH 7; results not shown). Thus the absence of spectroscopic features of the Cu(I)-semiquinolamine at extreme pH values cannot be ascribed to inactivation of the enzyme and reflects an equilibrium between this species and the bleached Cu(II)-aminoresorcinol.

The optical spectra of the reduced enzyme at different pH values were collected into a matrix and submitted to SVD as described in the Experimental section. This analysis suggests that only two important spectroscopic components are required to describe the experimental data (**S** values for the first four components were 91.6, 14.0, 2.4 and 1.6). The first two **V** columns were fitted to the titration of two independent ionizable groups to obtain their p*K* values (p*K*₁ 5.7; p*K*₂ 7.9; Figure 3A) and the values that the **V** matrix elements would assume if each species were pure (**V**_{fit}), which were used to reconstruct the spectra. In this analysis three species of the reduced enzyme were

Figure 2 Absorption spectra of putrescine-reduced LSAO

To 1 ml of 100 mM potassium phosphate buffer containing 22 μ M enzyme active sites, 2 mM putrescine was added in the absence of oxygen at pH 6 (trace a), pH 7 (trace b), pH 8 (trace c), and pH 9 (trace d); at pH 5.5 the spectrum was identical with that at pH 9.

Table 2 Molar absorption coefficients of oxidized LSAO and its substrate-reduced radical species at different pH values

Numbers in parentheses are wavelengths.

pH	ϵ ($M^{-1} \cdot cm^{-1}$)		
	Oxidized enzyme	Radical species	
5	3500 (478)		
6	3600 (504)	6700 (464)	4000 (434)
7	4100 (498)	7100 (464)	4600 (434)
8	4200 (478)	3500 (464)	2500 (434)
9	4400 (483)	1600 (464)	1200 (434)

considered: doubly protonated, singly protonated and non-protonated. Clearly the doubly protonated and non-protonated species (present maximally at pH 5 and 10 respectively) correspond to the same spectroscopic state of the cofactor, i.e. the bleached Cu(II)-aminoresorcinol [d], whose reconstructed spectrum is shown in Figure 3(B). This attribution is confirmed by the observation that the absorptions at the wavelengths corresponding to the absorption peaks of the Cu(I)-semiquinolamine radical were close to zero at extremes of pH, in good agreement with previous kinetic attributions [10,11].

For singly protonated species, whose maximal population (83 %) was attained at pH 6.8, the correlation between protonation and electronic structure of the TPQ cofactor was less certain and our results do not allow us to calculate whether this species represented the pure Cu(I)-semiquinolamine; there is indeed EPR evidence that the radical never occurs at more than 40 % (see [6] and the Discussion section). The spectrum recon-

Figure 3 pH titration of the visible spectrum of substrate-reduced LSAO

(A) Fitting of the first two columns of the **V** matrix, as obtained from the application of SVD to the spectra reported in Figure 2, to two ionizable groups. (B) Spectra of pure singly protonated species (trace a) and doubly protonated and non-protonated species (trace b), as reconstructed from the fitted values of the first two columns of the **V** matrix. Trace b corresponds to the pure Cu(II)-aminoresorcinol state of the TPQ cofactor, whereas trace a is assigned to a mixture of this species and Cu(I)-semiquinolamine.

structed for the pure singly protonated species is shown in Figure 3(B); the molar absorption coefficients of the fully oxidized and fully reduced species are summarized in Table 2.

Because the reduced enzyme is a pH-dependent equilibrium mixture of two different electronic states of the TPQ cofactor, it seemed to be of interest to measure the pH dependence of its reactivity on oxygen. To obtain a solution of fully reduced LSAO we used benzylamine, a slowly reacting reducing substrate whose second-order combination constant is much smaller than that of oxidation (see [10] and below).

The reoxidation rate constant of substrate-reduced LSAO, in the presence of 2.5 mM benzylamine, was measured by mixing the enzyme with oxygen-containing buffer in a stopped-flow apparatus. The second-order rate constant was $1.56 \times 10^7 M^{-1} \cdot s^{-1}$ at pH 7.2 and 25 °C, in good agreement with the published value [10], and $8.5 \times 10^5 M^{-1} \cdot s^{-1}$ at pH 9.7 (Figure 4). This result confirms the expectation that the Cu(I)-semiquinolamine radical reacts faster with O_2 than the Cu(II)-aminoresorcinol. Owing to the contribution of the 0.8–1.5 % Cu(I)-semiquinolamine still present at pH 9.7, whose rate of interconversion to Cu(II)-aminoresorcinol is unknown, we estimate that the rate constant for oxidation of the latter species is approx. $5 \times 10^5 M^{-1} \cdot s^{-1}$.

Figure 4 Time course of the reoxidation of substrate-reduced LSAO

Symbols: ●, pH 7 and $15 \mu\text{M}$ O_2 ; ○, pH 9.7 and $135 \mu\text{M}$ O_2 . Other experimental conditions: buffer, 100 mM potassium phosphate or 100 mM glycine/NaOH; temperature 25°C . Abbreviation: d absorbance, change in absorbance.

Figure 5 Time-dependent spectral changes during the reaction of LSAO with GABA

To 1 ml of 100 mM potassium phosphate buffer containing $22 \mu\text{M}$ enzyme active sites, 2 mM GABA was added under anaerobic conditions. Spectra were recorded from 4 min (trace a) to 28 min (trace g) at intervals of 4 min. In the first 3 min the spectrum was identical with the spectrum in trace c of Figure 1 (oxidized enzyme).

Catalytic activity towards GABA

GABA is a very poor substrate for LSAO (Table 1). When GABA was added to LSAO in the absence of air there was a lag period (approx. 4 min; Figure 5, trace a) before the disappearance of the 498 nm band, indicating that the formation of the Cu(II)-quinolaldimine was slow. The species that accumulates during

Figure 6 Reduction of LSAO in the presence of putrescine and variable amounts of sodium dithionite

(A) Time courses of the putrescine-driven reduction of LSAO in the presence of dithionite. Experimental conditions: LSAO, $28 \mu\text{M}$ monomer; putrescine, $50 \mu\text{M}$ in 100 mM potassium phosphate buffer, pH 6; temperature 25°C ; $\lambda = 464 \text{ nm}$; dithionite concentrations were $0 \mu\text{M}$ (□), $25 \mu\text{M}$ (■), $275 \mu\text{M}$ (○), 2.75 mM (●) and 15 mM (△) (all concentrations after mixing). (B) Dependence of the rate constant (k) of the bleaching on dithionite concentration at pH 7 (●) and pH 6 (○).

the lag phase is assumed to be the Cu(II)-quinone ketimine [b], to which the same spectrum as that of the Cu(II)-quinone [a] is assigned. The appearance of the spectral features of the Cu(I)-semiquinolamine radical [e] is synchronous with the bleaching of the 498 nm band, more clearly at pH 7 than at pH 6 (results not shown). No further change was seen in the visible spectrum. In this spectral change, isosbestic points at 380, 414 and 474 nm were observed, indicating that the bleached Cu(II)-amino-resorcinol [d] did not accumulate. This is obviously consistent with the fact that once the aldehyde product has been released, the rate of the reaction becomes independent of the amine substrate and hence relatively rapid with respect to the reaction steps involving GABA.

Transient spectroscopy experiments

On mixing oxidized LSAO with putrescine in the absence of oxygen in a photodiode array-stopped flow apparatus, the peak centred on 498 nm disappeared rapidly, synchronously with the appearance of the optical features of the reduced enzyme. Owing to the rapid oxidation of the bound substrate, the intermediate bleached species observed with *p*-[(dimethylamino)methyl]-benzylamine [10,11] was not present and the reaction time course conformed to a single exponential process, completed within 1 s after mixing (Figure 6A). Evidence for the bleached Michaelis complex was, however, obtained in steady-state stopped-flow experiments; there is no reason to suggest that the catalytic cycle of this substrate has any peculiarity aside from the large rate constants for the first-order processes [11]. Interestingly, in the presence of sodium dithionite the spectrum of the reduced enzyme was not stable but decayed with a time course consistent with a pseudo-first-order reaction (Figure 6A). The same results were obtained in the absence and in the presence of 2 mM sodium cyanide, which is known to trap the semiquinone form by stabilizing the Cu(I) [22]. The apparent rate constant of this process over a large range of dithionite concentration is linearly dependent on the square root of the sodium dithionite concentration, as expected for the bimolecular reduction of the Cu(I)-semiquinolamine radical by SO_3^- [23] (Figure 6B). Because, in the absence of oxygen, ammonia remained bound to the reduced enzyme, further reduction of the fully reduced enzyme is tentatively assigned to the formation of Cu(I)-aminoresorcinol (Scheme 1 [f]). Consistent with this assignment and with the fact that the visible spectrum was dominated by TPQ (rather than Cu), the spectrum of the species present at the end of the reaction was fully bleached (results not shown) and indistinguishable from that of the Cu(II)-aminoresorcinol. The spectral features of the Cu(I)-aminoresorcinol derivative of LSAO were independent of pH but its rate of formation was higher at pH 6 than at pH 7 (results not shown).

Reaction with benzylamine

The time course of the optical changes recorded on mixing LSAO with benzylamine under anaerobic conditions was well described by a single exponential at each significant wavelength (250, 464 and 498 nm) with a measured rate constant, k , of 0.87 s^{-1} at 0.38 mM benzylamine, $10 \mu\text{M}$ enzyme and 25°C . This rate constant was much smaller than those already measured for the reoxidation under similar conditions (see above), suggesting that the catalytic cycle is rate-limited by the bimolecular formation of the Michaelis complex. Extrapolation from data at different benzylamine concentrations suggests the following values for the rate constants of benzylamine association and dissociation: $k_1 = 620 \text{ M}^{-1} \cdot \text{s}^{-1}$; $k_{-1} = 0.64 \text{ s}^{-1}$. Experiments performed in the presence of tiny amounts of dithionite (where the formation of the aldehyde could not be followed) confirmed the above results.

DISCUSSION

After the discovery that TPQ is the cofactor of copper-containing amine oxidases [24], various models of the catalytic mechanism have been suggested [7,8,11,24–26]. Plant amine oxidases provided a wealth of experimental evidence because they contain the yellow Cu(I)-semiquinolamine radical and because of the availability of so many substrates with different catalytic properties. The results obtained in this study extend the characterization of the reaction intermediates of the TPQ cofactor and suggest some modification to the previously proposed catalytic scheme [11].

The single most relevant result reported in this study is that the enzyme, as reduced by the amine substrates, is not a pure species but an equilibrium mixture of the Cu(II)-aminoresorcinol [d] and the Cu(I)-semiquinolamine radical [e]. The pH dependence of the relative proportions of the two species allowed us to assign to one of them [the Cu(II)-aminoresorcinol] a spectrum and a rate constant for the reaction with oxygen. Fitting of the spectra of the reduced enzyme on the basis of two ionizable groups confirms that the spectrum of the Cu(II)-aminoresorcinol is bleached (Figure 3), consistent with that of similar enzymes from mammals [which do not contain the Cu(I)-semiquinolamine radical].

The Cu(II)-aminoresorcinol reacts with oxygen in a second-order process with a rate constant of approx. $5 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$, still slightly higher than the comparable value for most mammalian copper-containing amine oxidases. This value is consistent with the pH dependence of the enzyme activity; the observation that on increasing the pH from 7.2 to 9.7 the reaction with O_2 slows down to 1/30 but the V_{max} decreases only to one-fifth demonstrates that the rate-limiting step changes with pH.

The actual spectrum and reactivity of the Cu(I)-semiquinolamine radical cannot be determined with certainty from our results because it cannot be postulated that the singly protonated species of reduced LSAO is totally in the form of the radical. In this respect we note that greatest population of the singly protonated species of reduced LSAO is twice (or more) that of the radical, as calculated from EPR spectra (20–40%) [6,9]. It is tempting to speculate that the higher figure obtained by EPR might correspond to the prevalent population of a species of the enzyme containing only one cofactor in the radical form, a possibility suggested by the half-sites reactivity occasionally observed in some mammalian amine oxidases [27]. The reoxidation of the enzyme at pH 7 proceeds mainly through the reaction of the Cu(I)-semiquinolamine radical with O_2 and the conversion of the Cu(II)-aminoresorcinol to the former species; because the reaction is completed in a single second-order process, the interconversion of the two reduced species must be relatively rapid. If the dimeric enzyme is heterogeneous because the radical state of one cofactor favours the aminoresorcinol state of the other, our results suggest that such a constraint is released by oxidation of the radical. It is important to stress that we did not attempt to calculate the intrinsic oxidation rate constant of the Cu(I)-semiquinolamine radical, in view of the uncertainties in the mechanism of the overall reaction; thus the oxidation rate constant of $1.56 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ reported above is an apparent value.

The first enzyme–substrate complex, obtained with GABA, proceeds through the catalytic cycle so slowly that the initial steps can be resolved with manual techniques. This substrate is unique in displaying the formation of the Cu(II)-quinolaldimine [c] as the rate-limiting step of the catalytic cycle and allows us to infer the formation of the Cu(II)-quinone ketimine intermediate [b] from the lag preceding the bleaching of TPQ.

Finally, the species present when the Cu(I)-semiquinolamine radical [e] reacts with dithionite does not present absorption bands in the visible region and is thus suggested to have the aromatic TPQ ring characteristic of the quinol derivatives, as depicted in Scheme 1.

In conclusion, our experiments assign a spectrum to three previously elusive intermediates, namely the Cu(II)-quinone ketimine, the Cu(I)- and Cu(II)-aminoresorcinol and the Cu(I)-semiquinolamine, and provide evidence for the reversible chemical equilibrium between the Cu(I)-semiquinolamine and the Cu(II)-aminoresorcinol, thus explaining at least in part the difference in the catalytic-centre activity ('turnover number') between copper-containing amine oxidases from plants and

mammals. A definitive explanation for the absence of the radical species in mammalian copper-containing amine oxidase is, however, still elusive and will probably be established by site-directed mutagenesis and X-ray diffraction studies in the near future.

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