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Lathyrus cicera copper amine oxidase reactions with tryptamine

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ABSTRACT

Lathyrus cicera copper amine oxidase (LCAO) rapidly formed the typical Cu(I)-TPQ semiquinone UV–visible spectrum, identical to that formed by other substrates, upon O_2 exhaustion by turnover with excess tryptamine. A new band at 630 nm formed more slowly, with intensity dependent on aldehyde and H_2O_2 concentrations. On prolonged incubation, all bands decayed in parallel, together with loss of enzymatic activity. The blue color disappeared on addition of KCN, a Cu(I) stabilizing agent, while the intensity of the radical visible bands increased. This shows that the 630 nm absorbing species is a Cu(II) derivative, as confirmed by the unchanged intensity of the EPR spectrum of the frozen blue solution from that of the native protein. Rapid kinetics experiments showed that this species derives from a reduced form of the protein, plus aldehyde and H_2O_2 and that it is not in dynamic equilibrium with the radical. Given the similar population of the semiquinone radical with all substrates, it is possible that the reaction with aldehyde and H_2O_2 occurs in all cases although substrates lacking the indole group only produce the Cu(I)-semiquinone band. The radical participation to the catalytic activity is demonstrated by the observation that its relative population (controlled by the pH) parallels changes in the reoxidation rate constant, while the 630 nm absorbing species is implied in the inactivation process, which depends on H_2O_2 and aldehyde concentration. The results of the paper are consistent with half-of-the-site reactivity, i.e. the two subunits of LCAO are kinetically and spectroscopically distinct from each other.

1. Introduction

Copper amine oxidases (CuAOs) share with other enzymes the property of having a Cu-bound organic cofactor derived from the post-translational modification of a conserved polypeptidic chain residue [1]. The modified residue of CuAOs is a tyrosine which is oxidized to 2,4,5-trihydroxyphenylalanine quinone (TPQ). In the copper protein lysyl oxidase, an enzyme required for cross-linking of collagen and elastin, the cofactor lysine tyrosylquinone (LTQ) is derived from an oxidized tyrosine linked to a conserved lysine. In alkylamine dehydrogenases from gram-negative soil bacteria the tryptophan tryptophylquinone (TTQ) is derived from two oxidized and condensed tryptophan residues. In fungal galactose oxidase the cofactor is a tyrosyl-cysteine radical. Amine oxidases are found in nearly all forms of life, where they play important biological roles, namely nutrient catabolism in microorganisms [2], for which amines are the sole source of carbon and nitrogen, wound healing in plants [3], biogenic amines catabolism and signaling in mammals [2].

The X-ray structures, available for CuAOs of different sources [4–10], show that the enzymes are dimeric, with extensively interacting subunits of 70–90 kDa. They display considerable structural homology, although the overall primary sequence identity is usually not high, <25%. Notable exceptions are CuAOs purified from plants, such as lentil and pea seedling, which share over 90% sequence identity [11], and the BSAO/VAP1 couple which share 85% sequence identity [9]. The structural homology and thirty-three residues fully conserved in the catalytic site region [12], probably account for the similar, but not identical, reactivity. They catalyze the oxidative deamination of primary amines by the two electron reduction of O_2. Aldehyde, ammonia, and H_2O_2 are produced in the reaction which occurs with ping-pong mechanism [2,13] (Scheme 1). The reductive phase of the ping-pong reaction consists [14,15] of the initial formation of the substrate Schiff-base, a iminoquinone, the subsequent conversion to the product Schiff-base, a quinolaldimine, and the formation, upon release of the aldehyde, of the reduced species Cu(II)-aminoresorcinol in equilibrium with Cu(I)-semiquinone. The conversion is assisted by a conserved aspartate, which helps proton abstraction from the α-carbon of the substrate with formation of the carbanion. The mechanism of the reoxidation reaction is not as well established as the reduction phase [15]. X-ray structural data support the binding of O_2, probably in the form of H_2O_2, between Cu(II) and the iminoquinone [16]. From this intermediate H_2O_2 is released and ammonia is hydrolyzed to restore TPQ. A debated point remains, concerning the first electron transfer to O_2. Two different mechanisms were proposed to overcome the energetically unfavorable change of O_2 spin multiplicity. According to mechanism 1, O_2 binds Cu(I)-semiquinone with transfer of one electron and formation of the

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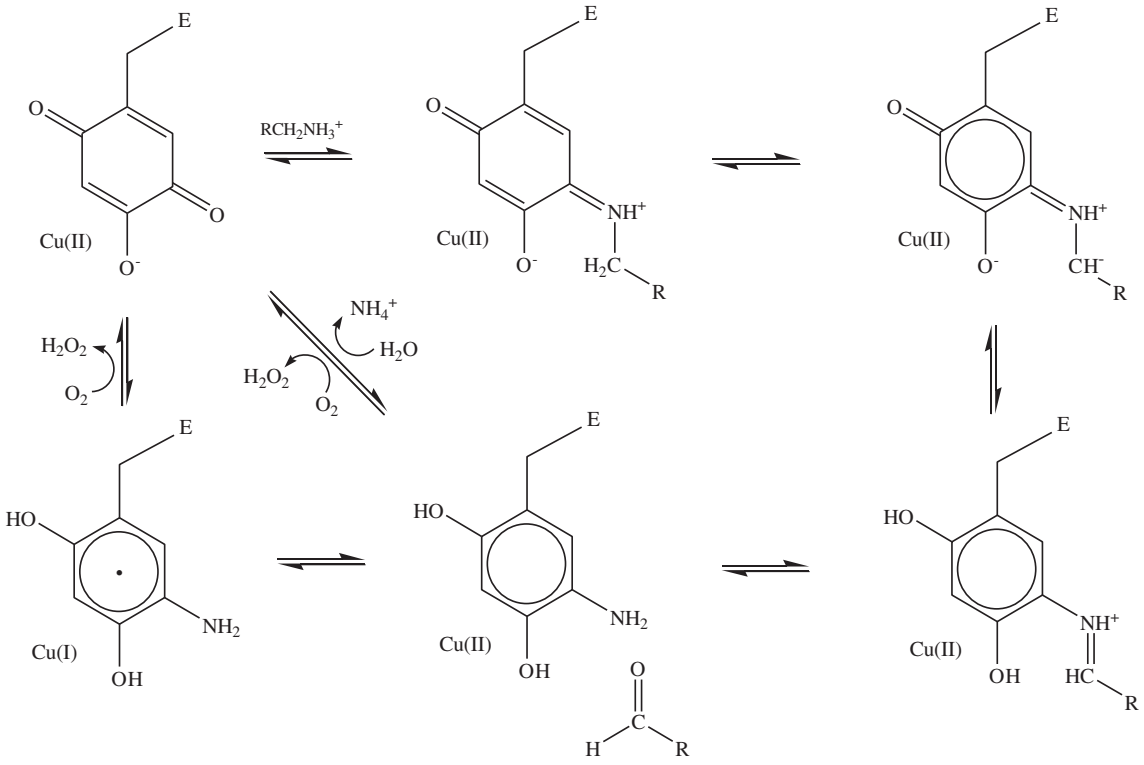

Scheme 1. Scheme of the reaction of LCAO with amine substrate.

Cu(II)-superoxide adduct, while according to mechanism 2, the binding of O_2 occurs at a hydrophobic pocket adjacent to Cu(II). Then one electron is directly transferred to O_2 from reduced TPQ (aminoresorcinol intermediate) to form the same Cu(II)-O_2^--semiquinone adduct as in the previous case. Mechanism 1 was proposed for enzymes, such as plant amine oxidases, which are able to form up to 40% of Cu(I)-semiquinone on reduction by substrate under anaerobic conditions, and is supported by the fast, single second order reaction with O_2. Mechanism 2 was proposed for enzymes such as BSAO, which do not form appreciable amounts of the semiquinone on reduction by substrate under anaerobic conditions [17], and is supported by the catalytic activity of the Co(II)-substituted derivative [18]. Furthermore, reduced BSAO reacts with O_2 with Michaelis–Menten mechanism, at a slower rate than the semiquinone forming enzymes. In conclusion, these properties were taken to suggest that the mechanism might be different in the two types of enzymes [19].

CuAOs from animal and plant sources also differ because of the specificity of the amine substrate. The natural mutation of a BSAO tyrosyl residue near the catalytic site, the so called "gate" residue, into a phenylalanine residue in PSAO might be at least in part responsible for the differences by increasing the hydrophobic nature of the site [20]. However, it was shown that in *Hansenula polymorpha* amine oxidase a single-point mutation at a second sphere ligand to copper increases the cofactor semiquinone level, on reduction by substrate under anaerobic conditions, without altering the mechanism of O_2 reduction [21]. The two types of enzymes also differ in the reactivity with inhibitors which bind the TPQ carbonyl group. Plant AOs bind two molecules of phenylhydrazine per dimer, while BSAO only binds one molecule. Half-of-the-sites reactivity was suggested to occur in the latter enzyme [22].

The present paper addresses these debated points by the study of the reactions of LCAO with tryptamine and serotonin. Previous work using various amine substrates had shown that this enzyme, like other AOs [23,24], is slowly inactivated by H_2O_2 produced on turnover, when left to incubate in the reduced state after O_2 exhaustion. The study of tryptamine-treated LCAO allows to follow two independent reactions, respectively implicated in the catalytic and in the inactivation process.

2. Experimental

2.1. Protein preparation

All chemicals were reagent grade and were used without further purification. Substrates, catalase, aldehyde dehydrogenase (ADH), superoxide dismutase (SOD), and horseradish peroxidase (HRP), were purchased from Sigma–Aldrich S.r.l. (Milan, Italy). LCAO was purified from *Lathyrus cicera* seedling by the method previously reported [24]. The purified proteins moved as single bands on SDS-PAGE. The concentration was measured using the molar extinction coefficient for phenylhydrazine, $\varepsilon_{445nm} = 64\ mM^{-1}\ cm^{-1}$, or benzylhydrazine, $\varepsilon_{380\ nm} = 58\ mM^{-1}\ cm^{-1}$, assuming a content of two TPQ per mole enzyme. As previously discussed [20], the values obtained in this way were in good agreement with those obtained by employing for the protein the molar extinction coefficients reported for *Pisum sativum* AO [24,25], namely $\varepsilon_{280\ nm} = 300\ mM^{-1}\ cm^{-1}$ and $\varepsilon_{500\ nm} = 4.9\ mM^{-1}\ cm^{-1}$. The copper content was assayed by atomic absorption spectrometry with a Perkin Elmer 3030 apparatus equipped with a graphite furnace and by the biquinoline spectrophotometric method [26]. The AO activity was assayed at 25 °C with 1.0 mM putrescine in 0.1 M potassium phosphate buffer, pH 7.2, by measuring the formation of H_2O_2 from the absorbance of the pink adduct ($\varepsilon_{515\ nm} = 26\ mM^{-1}\ cm^{-1}$) produced by the horseradish peroxidase (HRP) catalyzed oxidation of aminoantipyrine, followed by condensation with 3,5-dichloro-2-hydroxybenzensulfonic acid [27]. The HRP system was also used for the quantitation of H_2O_2 produced at the end of inactivation experiments (see below). Samples with specific activity >70 IU/mg (micromoles of substrate oxidized/min) were employed.

Potassium phosphate buffer (0.05 M) was used in the pH range 6.0–8.0 and 0.05 M sodium borate buffer in the pH range 8.4–9.2.

The ionic strength of the solutions was kept constant at 120 mM by proper addition of NaCl.

2.2. Inactivation experiments

Inactivation was achieved in two different ways i) by incubating 4.0 μM LCAO with 2.0 mM substrate, in 0.1 M potassium phosphate buffer pH 7.2 in a 1.0 mL test tube, open to air, using a 37 C° thermostatted water bath. At given time intervals, aliquots of the solutions were tested for activity with 1.0 m M putrescine at 25 °C, after dilution to approximately 2.0 nM LCAO; ii) by incubating LCAO (4.0–6.0 μM) with 1.0 mM substrate, at 37 °C, in an optical cuvette provided with a Teflon stopper, thus limiting the amount of available O_2 and allowing to monitor the UV–vis spectrum of reacting species. The amount of H_2O_2 produced in the inactivation experiments was measured as described in the previous section by the HRP method, after proper sample dilution.

2.3. Steady state kinetics

Kinetic parameters were obtained by measuring the velocity of H_2O_2 formation as described above for the enzymatic activity determination. K_m and k_{cat} values were obtained by fitting the kinetic data to the Michaelis–Menten equation $v = V_{max}\,[S]/(K_m + [S])$ by non-linear regression analysis using Microcal Origin 3.5 software. The data were the average of three experiments, carried out at 25 °C, using at least eight amine concentrations. The standard deviation was ≤12%. k_{cat} and k_{cat}/K_m values in the range pH 5.5–pH 9.1, and the dissociation constants of protonated groups that control the dependence of kinetic parameters on the pH, K_{a1} and K_{a2} were measured as previously described [20].

2.4. Rapid kinetics

Rapid kinetic experiments were carried out using an Applied Photophysics MV17 stopped flow apparatus (Applied Photophysics, Leatherhead, UK) equipped with either a monochromator and a photomultiplier tube (for single wavelength time courses) or with a photodiode array detector (for recording time resolved absorbance spectra). When the photodiode array detector was used the intensity of the 150 W lamp of the instrument was reduced to approximately 1/4 by neutral filters to prevent possible photochemical damage of the sample; this required increasing the exposure of the sensor to 5 ms/spectrum. The data collected by the instrument were transferred to a pentium IV based personal computer working under the Linux operating system and analyzed using routines written for the software package Octave.

2.5. EPR measurements

EPR spectra were recorded with a Brucker ElexSys spectrometer, operating in X band with a SHQ-type cavity. The sample was inserted in a 3 mm I.D. Suprasil tube (Wilmad), 300 μl volume, for the measurements at 150 K. EPR spectra were recorded at 1 mW microwave power, at 2 mT modulation amplitude, 0.4 s/mT sweep time to sweep field ratio, 0.3 ms time constant. A Mn^{2+} standard sample, fixed at the bottom of the cavity, was used for field reference. A HP 53150A frequency counter was used for frequency measurements.

3. Results

3.1. LCAO inactivation by tryptamine

4.0–6.0 μM LCAO was incubated with a large excess of tryptamine (2.0 mM) over O_2 (0.27 mM) in a test tube open to air, as described in a previous work carried out with several other aliphatic and aromatic amines [24]. Complete inactivation of the enzyme and loss of reactivity with phenylhydrazine were eventually produced. The presence in solution of either catalase or ADH protected in part the enzyme from inactivation, since some residual activity and reactivity with phenylhydrazine was found after a similar incubation time. After 15 min incubation with tryptamine, the loss of catalytic activity was about 95%, but did not exceed 40% in presence of either catalase or ADH. The inactivation was completely prevented by the simultaneous presence of both enzymes. This shows the involvement of both H_2O_2 and indoleacetaldehyde in the inactivation process.

In another series of experiments, the incubation with an excess of tryptamine (1.0 mM) over O_2 was carried out in a spectroscopic cuvette, closed by a teflon stopper affording protection from oxygen leaks. This allowed the observation of a spectrum with sharp absorption peaks at 464, 434, and 350 nm, typical of the Cu(I)-semiquinone radical. Since this radical only forms under strictly anaerobic conditions [28], all O_2 initially available in solution (0.27 mM at 25 °C and atmospheric pressure) was consumed by turnover at this stage. An equivalent amount of H_2O_2 was detected in solution with the HRP method described in Section 2.1. At difference from amine substrates previously examined [24], an additional intense band around 630 nm (Fig. 1) was formed by tryptamine. The maximum absorbance was reached after 6 min (Fig. 2A, curve a), when the Cu(I)-semiquinone, formed within mixing time, was already in the decreasing phase (Fig. 2B, curve a). The presence of SOD had no effect on the formation and decay of either the 630 nm band or the radical spectrum (Fig. 2A, curve b). When LCAO was treated with tryptamine in presence of ADH a much less intense 630 nm band was initially formed, which then rapidly disappeared (Fig. 2A, curve c). A similar effect was produced by catalase (Fig. 2A, curve d). The seemingly slower decay of the 630 nm band and the slower formation rate of 465 nm band in presence of catalase (Fig. 2A, curve d and B, curve d, respectively) is imputable to the catalase reaction with H_2O_2, which releases O_2 and H_2O. Catalase addition also produced the rapid bleaching of a fully developed 630 nm band. A presumably similar band (not shown), whose absorption is shifted to 650 nm and with slightly lower absorbance, was produced by 5-hydroxytryptamine (serotonin).

The turnover reaction with 0.2 mM tryptamine, less than O_2 concentration (0.27 mM), left LCAO in the oxidized state. Neither inactivation nor optical effects occurred, although 0.2 mM H_2O_2 was detected in solution by the method described in Sections 2.1 and 2.2. A first important implication of this result is that the oxidized protein does not form the Cu(I)-semiquinone spectrum neither the

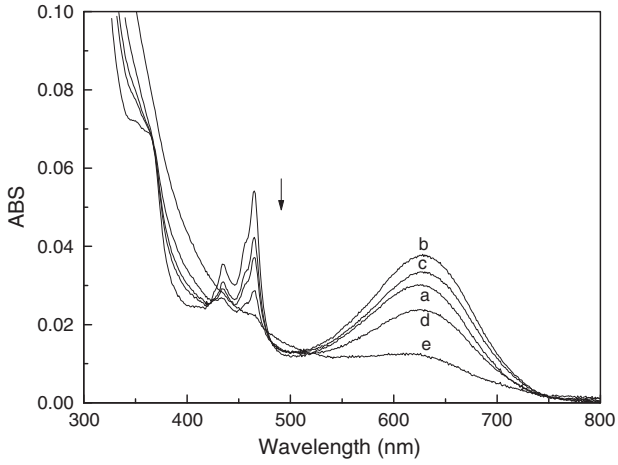

Fig. 1. Optical spectra of tryptamine reacted LCAO in a closed spectrophotometer cuvette. 4.9 μM enzyme in 0.1 M K-phosphate buffer pH 7.2 plus excess tryptamine, 1.0 mM, over O_2, recorded at 25 °C immediately after mixing (a), and after 12 (b), 20 (c), 40 (d), and 90 (e) min. The arrow indicates the decrease of the 465 nm band in the order a > b > c > d > e.

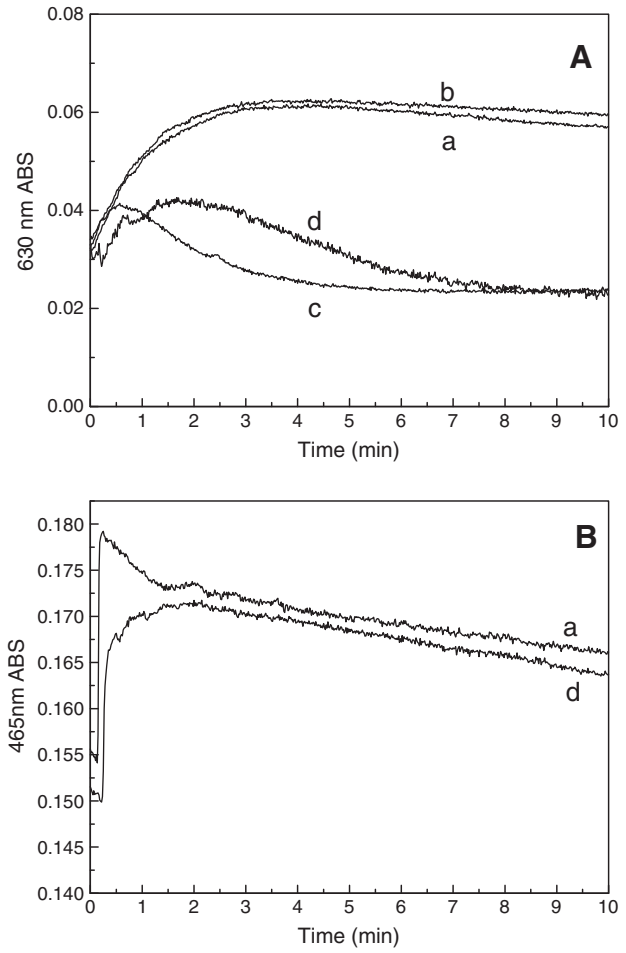

Fig. 2. Time course of LCAO–tryptamine reaction in a closed spectrophotometer cuvette. 5.6 μM enzyme in 0.1 M K-phosphate buffer pH 7.2, at 25 °C, plus 1.0 mM tryptamine. Panel A: recorded at 630 nm (a), in presence of 2.3 μM SOD (b), or 60 units ADH (c), or 100 units catalase (d). Panel B: recorded at 465 nm in the absence (a) or in the presence of catalase (d).

630 nm band with catalytic products. Re-oxygenation by stirring the solution opened to air and subsequent addition of excess tryptamine (1.0 mM) produced a Cu(I)-semiquinone spectrum of the usual intensity and a band at 630 nm, of approximately double intensity that produced with a single 1.0 mM tryptamine treatment. The increased intensity of the 630 nm band is apparently related to the accumulation of catalytic products, H_2O_2 and indoleacetaldehyde as confirmed by the value close to 0.47 mM H_2O_2 measured after the second tryptamine addition. The 0.47 mM H_2O_2 value is the sum of 0.2 mM, produced by the first tryptamine addition and 0.27 mM produced with excess substrate, corresponding to the amount of O_2 reintroduced by stirring the solution in air. H_2O_2 concentrations measured at any stage of the tryptamine reaction were in good agreement with expected values. The acetaldehyde concentration is assumed to be identical to that of H_2O_2. In order to evaluate the effect of increasing products concentrations on the intensity of the 630 nm band, several 0.2 mM tryptamine aliquots were added stepwise, stirring the solution in air. As in the case of a single 0.2 mM tryptamine addition, no optical effects occurred. The final addition of excess tryptamine, in a closed cuvette, required to obtain the Cu(I)-semiquinone cofactor in the reduced state, showed a progressive increase of the intensity of the 630 nm band, up to a limit value of approximately 22 mM^{-1} cm^{-1} (Fig. 3). The optical absorption of the Cu(I)-semiquinone was unaffected throughout. In order to test the separate effect of each catalytic product, the 1.0 mM tryptamine excess was used in presence of exogenous H_2O_2. A high H_2O_2 concentration (2.0 mM) afforded a 20% increase of the

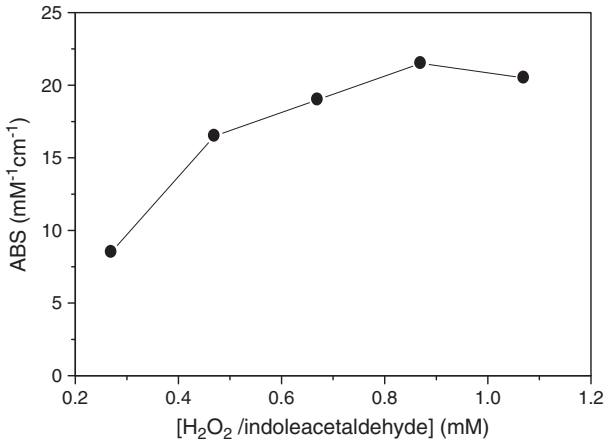

Fig. 3. Dependence of the 630 nm band intensity on products (indoleacetaldehyde and H_2O_2) concentration. 4.8 μM LCAO in 0.1 M K-phosphate buffer pH 7.2, at 25 °C, was treated with 1.0 mM tryptamine in a closed cuvette after the addition to solutions stirred in air of 0, 1, 2, 3, 4 aliquots of 0.2 mM tryptamine, respectively. The points correspond to 0.27, 0.47, 0.67, 0.87, 1.07 mM products.

630 nm band intensity (Fig. 4) showing that the large increase of Fig. 3 is mainly related to indoleacetaldehyde concentration. Fig. 4 also shows that the initial intensity of the 465 nm radical band is not affected by the presence of exogenous H_2O_2 and that the loss of intensity with time is faster, parallel to the 630 nm band decay. A different behavior of the two sets of bands was also observed as a function of pH on a single addition of 1.0 mM tryptamine. The intensity of the Cu(I)-semiquinone spectrum changed with pH in the order pH 8.5 < pH 7.2 ≈ pH 6.5, while the intensity of the 630 nm band was almost independent of pH (Fig. 5). The latter band was instead substantially temperature dependent. The rate of formation at 15 °C was lower than at 25 °C, but the band reached a higher intensity and the decay time was longer. If the limiting absorbance of 22 mM^{-1} cm^{-1} is taken as the extinction coefficient of the derivative, the absorbance recorded in these experiments accounted for about 30% and 50% of reacted molecules at 25 °C and 15 °C, respectively. An even higher ratio of 75% was measured immediately after thawing a frozen solution. The Cu(I)-TPQ semiquinone spectrum was unchanged in the 25–15 °C temperature range, but the intensity was greatly reduced in the spectrum measured after thawing.

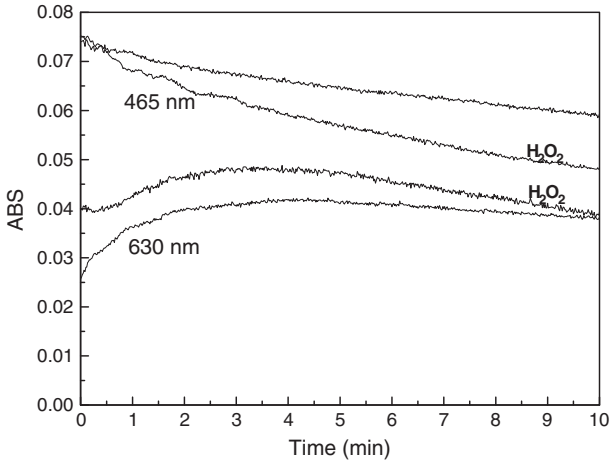

Fig. 4. Effect of exogenous H_2O_2 on the time course of the LCAO–tryptamine reaction in a closed spectrophotometer cuvette. 4.8 μM enzyme in 0.1 M K-phosphate buffer pH 7.2, at 25 °C, plus 1.0 mM tryptamine recorded at 465 nm (upper curves) or 630 nm (lower curves) without and with 2.0 mM H_2O_2.

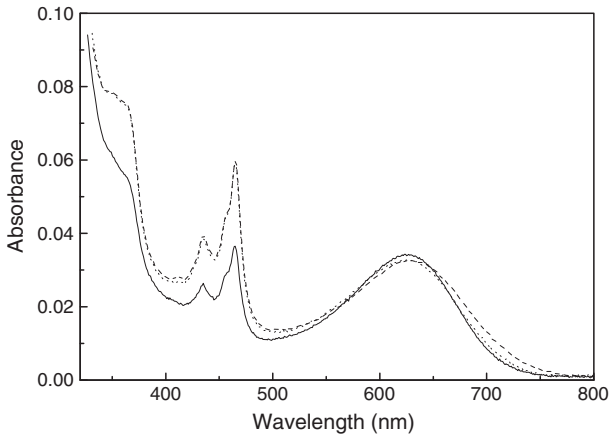

Fig. 5. Dependence on pH of the optical spectra of tryptamine reacted LCAO in a closed spectrophotometer cuvette.5.3 μM LCAO in 0.1 M K-phosphate buffer, at 25 °C, plus 1.0 mM tryptamine, at pH 6.5 (dashed line), at pH 7.2 (dotted line), and in 0.1 M borate buffer pH 8.5 (solid line). The spectra were recorded 2 min after tryptamine addition.

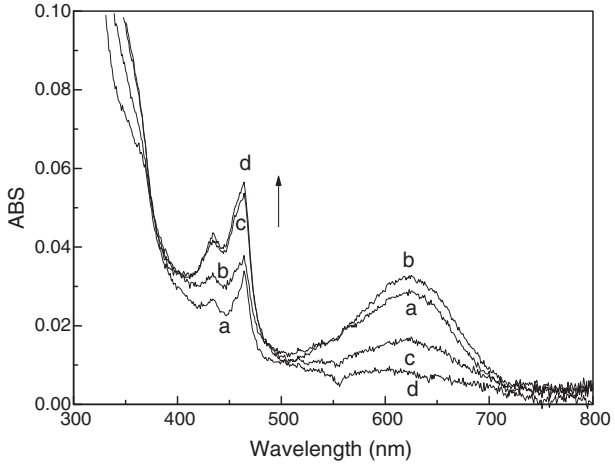

Fig. 7. Effect of KCN on the reaction of LCAO with tryptamine in a closed spectrophotometer cuvette. 6.3 μM enzyme plus 1.0 mM tryptamine, at 25 °C, in Na-borate buffer pH 8.5 (a), plus 1.0 mM KCN recorded immediately (b), after 9 min (c) and 18 min (d).

3.2. EPR measurements

EPR spectra were recorded at 100 K. The spectrum of 110 μM LCAO in 0.1 M phosphate buffer pH 7.2 was recorded before and after the addition of 2.0 mM tryptamine. The intensity of the solution blue color increased on freezing, while the EPR copper signal was almost unaffected by the presence of tryptamine (Fig. 6). This may be taken as an indication that the blue species is a Cu(II) derivative. It is well known that the radical disappears at low temperature and that it does not exceed 40% of total Cu at room temperature [28]. The EPR spectra of Fig. 6 are very similar to those reported by Medda et al. at low temperature [29]. This is not surprising as they were obtained before and after addition of excess tryptamine to lentil seedling amine oxidase (LSAO) under comparable conditions.

3.3. Reactions in the presence of Cu binding inhibitors

In phosphate buffer pH 8.5, addition of 1.0 mM KCN, a reagent known to stabilize the Cu(I) species [28], made the spectrum of the radical substantially more intense while the 630 nm band of tryptamine treated LCAO disappeared (Fig. 7). When NaN_3 was used in the place of KCN both bands disappeared, while the addition of tryptamine to NaN_3 treated LCAO only produced the reduction of the 500 nm band of the native protein.

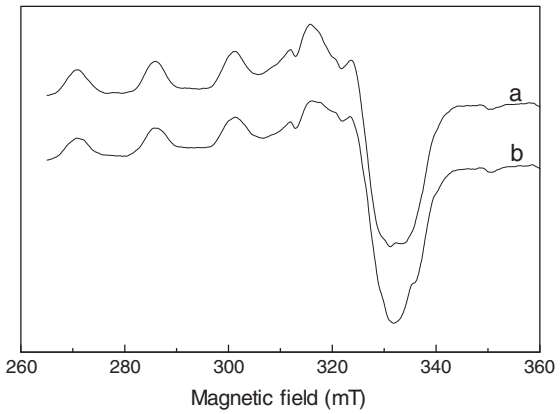

Fig. 6. EPR spectra of native and tryptamine reacted LCAO at 150 K.130 μM LCAO in 0.1 M potassium phosphate buffer, pH 7.2 before (a); after 2.0 mM tryptamine addition (b). Acquisition parameters: X-band, 1 mW microwave power, 1 mT modulation amplitude, 150 K temperature. A ElexSys spectrometer operating in X-band at 150 K with a SHQ-type cavity was used.

3.4. Steady state kinetics

Steady state kinetics were measured for tryptamine in the range pH 6.5–pH 9.3. A bell shaped curve was obtained by plotting k_{cat} against hydrogen ion concentration. The curve showed a maximum at pH 7.5 and apparent $pK_{a1} = 7.12 \pm 0.03$ and $pK_{a2} = 8.14 \pm 0.02$ values were calculated as reported in a previous paper [20]. Serotonin kinetics were only measured at a single pH because of the very low k_{cat} value of 0.09 s^{-1} at pH 7.2. The k_{cat} value for tryptamine, in the same conditions, was instead of 10.7 s^{-1}, two orders of magnitude higher. Serotonin and tryptamine K_m values, measured in 0.1 M K-phosphate buffer pH 7.2, were 0.095 mM and 1.12 mM, respectively.

3.5. Rapid kinetics

Two types of rapid kinetic experiments were carried out, i.e. (i) the characterization of the full catalytic cycle and (ii) the rapid mixing of reduced LCAO with indoleacetaldehyde and H_2O_2. The steady state process followed by stopped flow reveals three clearly distinguishable intermediates, namely the oxidized and reduced derivatives of the enzyme and the putative enzyme-product complex which absorbs at 630 nm. The absorbance spectrum of the steady state mixture cannot be described as a linear combination of the spectra of the three species described above, indicating that at least a fourth spectroscopic component is present, tentatively identified as the quinol-aldimine enzyme substrate complex [30]. The fully reduced LCAO, which is described as a mixture of the aminoresorcinol and Cu(I)-semiquinone radical derivatives of the TPQ cofactor, is never obtained in these experiments since the enzyme-product complex usually starts accumulating before complete reduction of the enzyme (Fig. 8A, B). The time courses recorded in this type of experiments can be satisfactorily described using the reaction scheme already described for other plant CuAOs (Scheme 1). In order to test whether or not the latter reaction requires cycling, the following experiment was carried out: a 20 to 25 μM solution of LCAO reduced with 1.0 mM putrescine was rapidly mixed in the stopped flow apparatus with a solution of the indoleacetaldehyde, produced using a minute (2.0 to 2.5 μM) concentration of the same enzyme (Fig. 8C, D). Under these experimental conditions, both solutions are strictly anaerobic and the enzyme is fully reduced; no cycling is expected to occur. The enzyme product complex in these experiments formed in an exponential process, governed by a rate constant of 0.015 s^{-1} irrespective of the aldehyde or the H_2O_2 concentrations. This result indicates unequivocally that the enzyme derivative capable of reacting with the aldehyde of tryptamine is reduced and that cycling is not required. We were puzzled by the

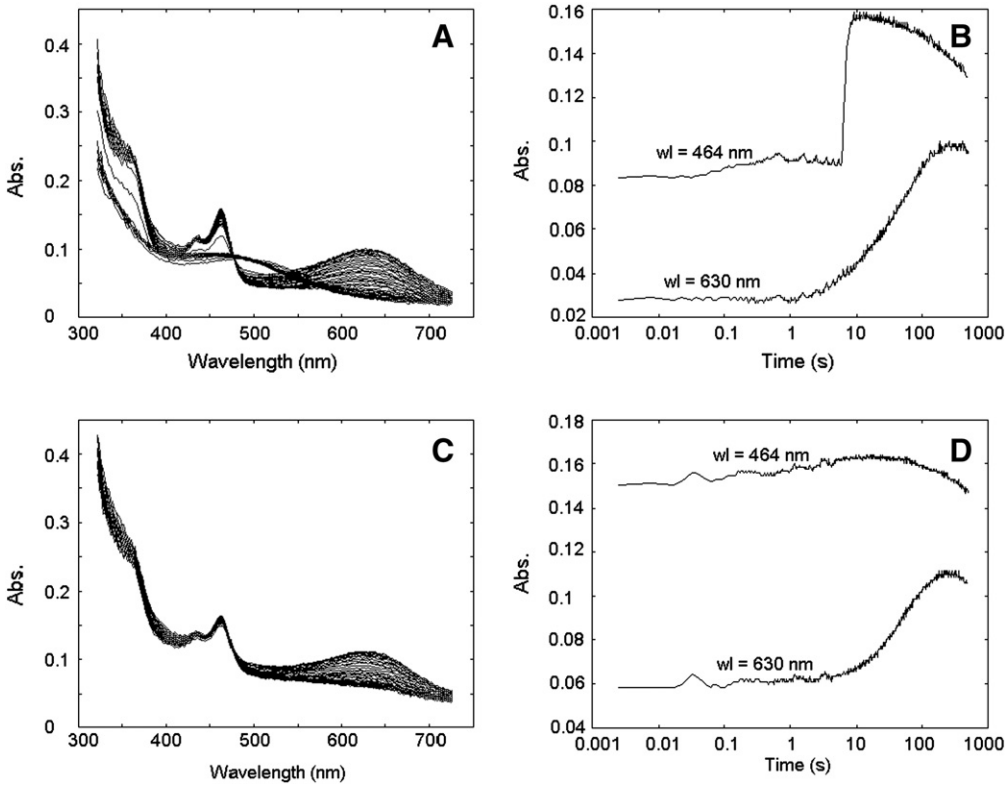

Fig. 8. Time courses of the appearance and decay of the inhibited intermediates.Experimental conditions: 0.1 M K-phosphate buffer pH 7.2, 25 °C.Panels A and B: oxidized LCAO (33 μM) was mixed with 1 mM tryptamine, 270 μM O_2 (all concentrations after mixing). Only 50 of the original 500 spectra are shown in Panel A.Panels C and D: 21 μM LCAO reduced with 1.0 mM putrescine was mixed with 120 μM indoleacetaldehyde (produced by 2.1 μM LCAO in the presence of 120 μM tryptamine, 150 μM putrescine, 270 μM O_2 and 1.0 mM H_2O_2). The concentrations of the relevant reagents after mixing were: 11.6 μM LCAO, 60 μM indoleacetaldehyde, 770 μM H_2O_2.

reaction rate constant being insensitive to the reagents' concentrations. This probably indicates that a semi-stable reaction intermediate is formed and that its monomolecular decay to the inactive enzyme-product complex is associated to the spectroscopic change.

4. Discussion

The oxidation of tryptamine by pea seedling amine oxidase to 3-indoleacetaldehyde and the subsequent enzyme inactivation by H_2O_2 was reported by Clarke and Mann [31]. The inactivation of LSAO by stoichiometric tryptamine was reported by Medda et al. [29]. The latter reaction was carried out under anaerobic conditions and assigned to the slow formation of an irreversible covalent derivative of the reduced protein with the two initially formed moles indoleacetaldehyde per mole of enzyme. The full inactivation of the enzyme and the loss of reactivity with phenylhydrazine required more than 4 h. In the present work, LCAO was rapidly reduced by an excess tryptamine over O_2 in a closed spectrophotometer cuvette, as shown by the appearance of the typical Cu(I)-semiquinone spectrum with peaks at 464 nm, 434 mm, and 360 nm. A high concentration of indoleacetaldehyde, equivalent to that of O_2 consumed during turnover (0.27 mM) was produced together with 0.27 mM H_2O_2. Subsequently, substrate reduced LCAO was inactivated by indoleacetaldehyde, more rapidly than in the conditions used by Medda et al. [29]. H_2O_2 also contributed to the inactivation since both ADH and catalase had a protective action. The inactivation of reduced LCAO by H_2O_2 was reported in a previous work carried out with other primary amines [24].

Another important new result induced by the high products concentration was the formation of an intense band at 630 nm (Fig. 1). A similar band was produced by serotonin at 650 nm. The intensity of the Cu(I)-semiquinone spectrum was similar to that produced by all other examined substrates [24], independent of the presence of the 630 nm transition. This was only produced by tryptamine and serotonin and was dependent on both indoleacetaldehyde and H_2O_2 concentration. It was probably not observed by Medda et al. with LSAO [29] because the products concentration, stoichiometric with the protein, was too low in their experiments. The 630 nm band was formed at slower rate than the radical band and was suppressed by ADH or by catalase, without effect on the radical spectrum (Fig. 2). This implies the absence of a dynamic equilibrium between the species responsible for the two sets of bands. This is supported by the pH-dependence of the radical spectrum at difference from the 630 nm band, which is pH insensitive (Fig. 5). An increase of the radical species was only observed in presence of KCN, which stabilizes Cu(I) and bleaches the 630 nm band (Fig. 7). On the contrary, freezing the solution stabilizes the Cu(II) state, as shown by the EPR spectrum (Fig. 6) in the presence of tryptamine and all other substrates [28]. By considering that the amount of Cu(I)-semiquinone was reported to never exceeded 40% of the copper sites [28] and that it was not in equilibrium with the 630 nm absorbing species, it seems possible to suggest that each of the two species involves a single protein subunit. This conclusion might apply to all substrates, since the intensity of the radical vis–UV spectrum is independent of the substrate nature [24].

Medda et al. [29] proposed the formation of an irreversible enamine derivative between reduced LSAO and indoleacetaldehyde because the two aldehyde molecules, formed by the reductive half-reaction under anaerobic conditions, were progressively lost upon incubation, in parallel with protein inactivation. In the present case an additional effect is induced by H_2O_2 of producing the 630 nm band and supporting the assignment to a back-reaction of the reduced protein which binds both indoleacetaldehyde and H_2O_2. It is doubtful whether the binding site of indoleacetaldehyde is the aminoresorcinol or some other residue of the active site, e.g. Lys [32]. The bleaching effect of copper binding inhibitors such as NaN_3 on the 630 nm band together with the reported high

affinity of reduced TPQ-Cu(II) for H_2O_2 [33] suggest that a charge-transfer complex involving copper, H_2O_2, and indoleacetaldehyde is possibly formed by the reduced cofactor. The implication of the indole group is supported by the longer maximum wavelength, 650 nm, of the 5-hydroxytryptamine (serotonin) derivative. It may be noted that low energy transitions imputable to the indole group (420 and 650 nm) are found in the TTQ spectrum [34]. With substrates lacking the indole group the back reaction with aldehyde and H_2O_2 may produce colorless adducts.

The above results were confirmed by stopped flow experiments. They were aimed to determine two points in particular, namely (i) the mechanism and rate of the reaction and (ii) which enzyme derivative was responsible for the formation of the enzyme-product complex. As far as the first point is concerned, we can confidently state that it involves at least one spectroscopically silent intermediate since the reaction is first order at all reagents' concentrations explored, with a rate constant of 0.015 s^{-1}. Identification of the reduced enzyme derivative which is most likely responsible for reacting with the aldehyde of tryptamine is more difficult. Indeed the complex formation does not require that the enzyme carries out its catalytic cycle and occurs also on the completely reduced species, in which the TPQ cofactor is approximately 40% in the state of the Cu(I)-semiquinone radical and 60% in that of the aminoresorcinol [28]. Since the spectroscopic features of the semiquinone radical do not change appreciably during the formation of the 630 nm band, we conclude that the radical is not consumed (Fig. 7D). The observation that formation of the 630 nm absorbing species is not associated with significant re-equilibration of the two forms of reduced TPQ (namely the Cu(II)-aminoresorcinol and the Cu(I)-semiquinone) may be explained by two alternative hypothesis (i) the reaction site is the Cu(II)-aminoresorcinol on one monomer of the dimer, whereas the other monomer remains in the Cu(I)-semiquinone radical state. This hypothesis implies a strong functional inequivalence of the two subunits of the same dimer. An alternative, and equally plausible hypothesis, is that (ii) the TPQ is not the reaction site and that both forms of the reduced enzymes react without affecting the equilibrium between Cu(II)-aminoresorcinol and Cu(I)-semiquinone.

5. Conclusions

In the reaction of LCAO with inhibitors, such as phenyl- or benzylhydrazine, adducts with identical optical properties are formed by both LCAO subunits [24]. Present results may be interpreted by assuming that two different, independent reactions with tryptamine occur at the TPQ sites, namely the formation of Cu(I)-semiquinone absorbing at 464 nm, 434 nm and 360 nm and the formation of the Cu(II)-H_2O_2-aminoresorcinol-indoleacetaldehyde transient, absorbing at 630 nm. A similar conclusion can be extended to other amine substrates, based on the similar intensity of the produced Cu(I)-semiquinone optical spectrum. Enzyme inactivation was previously found to correlate with the decay of the radical [24]. The involvement of H_2O_2 and aldehyde in both enzyme inactivation and the formation of the 630 nm band suggests that the latter species is responsible for this process, while the decay of the radical might rather be a consequence. The radical is implicated in catalytic activity, which is pH dependent as is the intensity of the radical spectrum, while the intensity of the 630 nm band is not affected by pH.

6. Abbreviations

ADH	aldehyde dehydrogenase
AO	amine oxidase
CuAO	copper amine oxidase
BSAO	bovine serum amine oxidase
HRP	horseradish peroxidase
LCAO	*Lathyrus cicera* amine oxidase
LSAO	lentil seedling amine oxidase
LTQ	lysyl trihydroxyphenylalanine quinone
SOD	superoxide dismutase
TPQ	2,4,5-trihydroxyphenylalanine quinone
TTQ	tryptophyl tryptophanquinone
VAP-1	vascular adhesion protein-1

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