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Reactions of plant copper/topaquinone amine oxidases with N^6-aminoalkyl derivatives of adenine

ZBYNĚK LAMPLOT[1], MAREK ŠEBELA[1], PETR FRYČÁK[2], SILVIA LONGU[3], ALESSANDRA PADIGLIA[3], ROSARIA MEDDA[3], GIOVANNI FLORIS[3] & PAVEL PEČ[1]

[1]*Department of Biochemistry, Faculty of Science, Palacký University, Šlechtitelů 11, CZ-783 71 Olomouc, Czech Republic,* [2]*Department of Analytical Chemistry, Faculty of Science, Palacký University, Třída Svobody 8, CZ-771 46 Olomouc, Czech Republic, and* [3]*Department of Applied Sciences in Biosystems, University of Cagliari, I-09042 Monserrato (CA), Italy*

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Abstract

Plant copper/topaquinone-containing amine oxidases (CAOs, EC 1.4.3.6) are enzymes oxidising various amines. Here we report a study on the reactions of CAOs from grass pea (*Lathyrus sativus*), lentil (*Lens esculenta*) and *Euphorbia characias*, a Mediterranean shrub, with N^6-aminoalkyl adenines representing combined analogues of cytokinins and polyamines. The following compounds were synthesised: N^6-(3-aminopropyl)adenine, N^6-(4-aminobutyl)adenine, N^6-(4-amino-*trans*-but-2-enyl)adenine, N^6-(4-amino-*cis*-but-2-enyl)adenine and N^6-(4-aminobut-2-ynyl)adenine. From these, N^6-(4-aminobutyl) adenine and N^6-(4-amino-*trans*-but-2-enyl)adenine were found to be substrates for all three enzymes ($K_m \sim 10^{-4}$ M). Absorption spectroscopy demonstrated such an interaction with the cofactor topaquinone, which is typical for common diamine substrates. However, only the former compound provided a regular reaction stoichiometry. Anaerobic absorption spectra of N^6-(3-aminopropyl)adenine, N^6-(4-amino-*cis*-but-2-enyl)adenine and N^6-(4-aminobut-2-ynyl)adenine reactions revealed a similar kind of initial interaction, although the compounds finally inhibited the enzymes. Kinetic measurements allowed the determination of both inhibition type and strength; N^6-(3-aminopropyl)adenine and N^6-(4-amino-*cis*-but-2-enyl)adenine produced reversible inhibition ($K_i \sim 10^{-5}$–10^{-4} M) whereas, N^6-(4-aminobut-2-ynyl)adenine could be considered a powerful inactivator.

Keywords: *Adenine, amine oxidase, biogenic amine, cytokinin, substrate, inhibition*

Abbreviations: *ABAD, N^6-(4-aminobutyl)adenine, ABYAD, N^6-(4-aminobut-2-ynyl)adenine, ACBAD, N^6-(4-amino-cis-but-2-enyl)adenine, APAD, N^6-(3-aminopropyl)adenine, ATBAD, N^6-(4-amino-trans-but-2-enyl)adenine, CAO, copper-contaning amine oxidase, GPAO, grass pea seedling amine oxidase, ELAO, Euphorbia characias latex amine oxidase, ESI, electrospray ionisation, LSAO, lentil seedling amine oxidase, MS, mass spectrometry, PSAO, pea seedling amine oxidase*

Introduction

Copper/topaquinone-containing amine oxidases (CAOs, EC 1.4.3.6) play a crucial role in the metabolism of primary amines. These enzymes are widely distributed in nature [1]. In microorganisms, CAOs have a nutritional role. In mammals and plants CAOs appear to be tissue specific and they are implicated in wound healing, detoxification, cell growth, signalling and apoptosis [1]. The oxidative deamination of amine substrates catalysed by CAOs yields the corresponding aldehydes with the concomitant production of hydrogen peroxide and ammonia [2].

The reaction proceeds through a transamination mechanism and it is mediated by an active site cofactor topaquinone (TPQ). The cofactor is derived from the post-translational self-processing of a specific tyrosine residue that requires both active site copper and molecular oxygen. [2] The key step

Correspondence: M.Šebela, Department of Biochemistry, Faculty of Science, Palacký University, Šlechtitelů 11, CZ-783 71 Olomouc, Czech Republic. Tel: 420585634927. Fax: 420585634933. E-mail: sebela@prfholnt.upol.cz

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in the oxidative deamination is the conversion of the initial substrate Schiff base (quinoimine) to a product Schiff base (quinoaldimine) facilitated by Cα-proton abstraction through a conserved aspartate residue acting as a general base at the active site. [3] This step is followed by hydrolytic release of the aldehyde product and the reduced cofactor is finally reoxidised by molecular oxygen with the release of H_2O_2 and NH_4^+. The reduced TPQ occurs in two molecular forms: the first form is a Cu(II)-aminoresorcinol derivative which is in equilibrium with the second form, the Cu(I)-semiquinolamine radical. [3] The role of active site copper in the reoxidation step still has not been sufficiently elucidated. Recently published evidence for CAO from *Hansenula polymorpha*, however, suggest it might be involved in electron transfer from substrate-reduced TPQ to oxygen that is bound at a site separate from copper. [2]

Plant CAOs are often classified as diamine oxidases, since they preferentially act on aliphatic diamines like putrescine or cadaverine. [4] From this group of enzymes, amine oxidases from garden pea (*Pisum sativum*, PSAO), lentil (*Lens esculenta*, LSAO) and grass pea (*Lathyrus sativus*, GPAO) seedlings have been subjected to an extensive investigation. [1] Plant CAOs have been exploited as biorecognition parts of biosensors for the detection of various amine compounds. [5,6] The enzymes are also utilised in new approaches to controlling the cellular physiopathology. [7] Substrates and inhibitors of plant CAOs have been reviewed in detail. [8,9] A special place is reserved for the mechanism-based inhibitors but-2-yne-1,4-diamine, [10,11] 2-bromoethylamine and 2-chloroethylamine [12] resembling the normal amine substrates but, however, causing irreversible inactivation. An initial hypothesis was that the enzymes could oxidise cytokinins (plant hormones derived from adenine [13]) however, detailed experiments did not prove this. Conversely, it has been shown that both natural and artificial cytokinins function as CAO inhibitors. [14]

Here we describe reactions of three plant CAOs with five N^6-aminoalkyl derivatives of adenine, which were synthesised by an established method based on the reaction of 6-chloropurine with primary amines in the presence of triethylamine. [15] The synthesised compounds were tested with CAOs in kinetic experiments, including activity and stoichiometry measurements, and by absorption spectroscopy under both aerobic and anaerobic conditions. Only N^6-(4-aminobutyl)adenine, a derivative of putrescine, was shown to be a normal substrate of plant CAOs. Nevertheless, some substrate properties were demonstrated also in the case of N^6-(4-amino-*trans*-but-2-enyl)adenine. The other compounds showed inhibitory properties with differing potency and mode of inhibition.

Materials and methods

Chemicals

6-Chloropurine, hydrazine hydrate, *trans*-1,4-dibromo-2-butene, *cis*-1,4-dichloro-2-butene, 1,4-dichloro-2-butyne, propane-1,3-diamine and putrescine (butane-1,4-diamine) were purchased from Sigma-Aldrich Chemie (Steinheim, Germany). Potassium phthalimide was from Acros Organics (Geel, Belgium). *N*,*N*-Dimethylformamide and triethylamine were from ICN (Aurora, OH, USA). n-Butanol and phenylhydrazine hydrochloride were from Fluka (Buchs, Switzerland). All other chemicals were of analytical purity grade.

Organic syntheses

trans-But-2-ene-1,4-diamine, *cis*-but-2-ene-1,4-diamine and but-2-yne-1,4-diamine were synthesised by the Gabriel synthesis according to a published protocol, [16] however, with a substantial modification. The respective intermediate products (*bis*-phthalimido derivatives) were hydrolysed using hydrazine in absolute ethanol. [17] During the hydrazinolyses performed with heating under reflux (110°C, 3 h), white spongy precipitates appeared. These precipitates were recovered using a rotary vacuum evaporator and dissolved in 30% (w/v) KOH. Free diamines (bases) were then each triple-extracted by 50 ml portions of diethylether, the extracts combined and dried using anhydrous sodium sulphate. The extracts were then concentrated on a rotary vacuum evaporator. Except for but-2-yne-1,4-diamine (a yellow liquid), the above mentioned diamines were obtained as yellowish solids by spontaneous evaporation of the solvent at 23°C.

N^6-aminoalkyl derivatives of adenine were synthesised following a method for the preparation of cytokinin-like compounds. [15] This was based on the reactions of 6-chloropurine with propane-1,3-diamine, putrescine, *trans*-but-2-ene-1,4-diamine, *cis*-but-2-ene-1,4-diamine or but-2-yne-1,4-diamine (in a ratio 6-chloropurine to diamine of 1:1.5) in the presence of triethylamine using *n*-butanol as a solvent. The reaction mixtures were heated under reflux for 3 h. The following compounds, with the indicated temperature of heating during reflux, were prepared: N^6-(3-aminopropyl)adenine, APAD (from propane-1,3-diamine, 110°C); N^6-(4-aminobutyl)adenine, ABAD (from putrescine, 110°C); N^6-(4-amino-*trans*-but-2-enyl)adenine, ATBAD (from *trans*-but-2-ene-1,4-diamine, 110°C); N^6-(4-amino-*cis*-but-2-enyl)adenine, ACBAD (from *cis*-but-2-ene-1,4-diamine, 110°C) and N^6-(4-aminobut-2-ynyl)adenine, ABYAD (from but-2-yne-1,4-diamine, 90°C). After refluxing was complete, crude products were crystallised from *n*-butanol overnight at 4°C. The crystalline preparations were dissolved in a minimal amount of

diluted hydrochloric acid and recrystallised by adding absolute ethanol.

Analyses of synthetic compounds

Crystalline synthetic preparations of the above N^6-aminoalkyl adenines were analysed using various methods. Absorption spectra (measured in water) showed a maximum at 260 nm revealing the presence of the purine moiety. Melting points were measured using an SMP 3 apparatus for glass sample capillaries (Stuart Scientific, Redhill, Surrey, UK). All the synthesised N^6-aminoalkyl adenines did not provide clear melting points, since they started to decompose at particular temperatures with the crystals turning brown–black. Elemental analyses were carried out using a CHN-O analyzer Finnigan Flash EA1112 (Thermo Electron Corporation, San Jose, CA, USA). Mass spectra were obtained using an ion trap mass spectrometer Finnigan MAT LCQ (Thermo Electron Corporation, San Jose, CA, USA) equipped with an electrospray ionisation interface. All samples were directly introduced to the electrospray interface of the mass spectrometer by a syringe (flow rate of 5 $\mu l\,min^{-1}$). Parameters of the electrospray were as follows: source voltage 5.6 kV, sheath gas flow 20 units, cone voltage 33.43 V, capillary temperature 250°C. The used ionisation mode produced positively charged quasimolecular ions (molecular mass +1). ^{1}H-NMR measurements were performed at 25°C on a Bruker AVANCE 300 NMR spectrometer (Bruker Biospin, Rheinstetten, Germany), operating at a magnetic field strength of 7.05 T. NMR spectra were measured only for ABAD and ATBAD which showed substrate properties in the present study. Deuterium oxide (D_2O, 99.96%) was from Sigma-Aldrich Chemie.

The following characteristics were obtained: APAD (**I**, **Figure** 1); Calculated for $C_8H_{12}N_6{\cdot}3HCl$: C, 31.86; H, 5.01; N 27.86. Found: C, 31.14; H, 5.28; N, 27.49%; decomposition – 214°C; ESI MS: *m/z* 193.2, MS/MS: *m/z* 176.1 and 118.9. ABAD (**II**, **Figure** 1); Calculated for $C_9H_{14}N_6{\cdot}3HCl$: C, 34.25; H, 5.43; N, 26.63. Found: C, 34.67; H, 6.18; N, 26.56%; decomposition – 235°C; ESI MS: *m/z* 207.1, MS/MS: *m/z* 190.1 and 118.7; ^{1}H-NMR (D_2O): δ 1.77 (m, 4H), 3.05 (m, 2H), 3.58 (t, 2H, $\mathcal{J} = 6$ Hz), 8.07 (s, 1H), 8.16 (s, 1H). ATBAD (**III**, **Figure** 1); Calculated for $C_9H_{12}N_6{\cdot}3HCl$: C, 34.47; H, 4.82; N, 26.80. Found: C, 34.76; H, 5.28; N, 26.40%; decomposition – 227°C; ESI MS: *m/z* 205.2, MS/MS: *m/z* 188.1 and 118.9; ^{1}H-NMR (D_2O): δ 3.60 (d, $\mathcal{J} = 6$ Hz, 2H), 4.32 (d, $\mathcal{J} = 4$ Hz, 2H), 5.82 (m, 1H), 5.95, (m, 1H), 8.10 (s, 1H), 8.19 (s, 1H). ACBAD (**IV**, **Figure** 1); Calculated for $C_9H_{12}N_6{\cdot}3HCl$: C, 34.47; H, 4.82; N, 26.80. Found: C, 34.91; H, 4.84; N, 26.50%; decomposition – 221°C; ESI MS: *m/z* 205.2, MS/MS: *m/z* 188.1 and 118.9. ABYAD (**V**, **Figure** 1); Calculated for $C_9H_{10}N_6{\cdot}3HCl$: C, 34.69; H, 4.21; N, 26.97. Found: C, 33.78; H, 4.20; N, 27.16%; decomposition – 228°C; ESI MS: *m/z* 203.1, MS/MS: *m/z* 186.1 and 118.7.

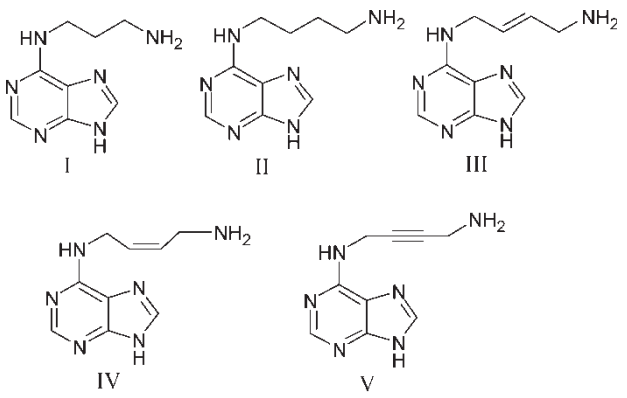

Figure 1. Structure of N^6-aminoalkyl adenines tested as substrates and inhibitors of plant amine oxidases: **I** – N^6-(3-aminopropyl) adenine, APAD; **II** – N^6-(4-aminobutyl)adenine, ABAD; **III** – N^6-(4-amino-*trans*-but-2-enyl)adenine, ATBAD; **IV** – N^6-(4-amino-*cis*-but-2-enyl)adenine, ACBAD; **V** – N^6-(4-aminobut-2-ynyl) adenine, ABYAD.

Enzymes

GPAO was isolated according to a published protocol. [18] The enzyme was additionally purified by ion-exchange chromatography on a Mono S HR 5/5 column (Amersham Biosciences, Uppsala, Sweden) connected to a BioLogic Duo Flow liquid chromatograph (Bio-Rad, Hercules, CA, USA). The following buffers were used: A, 20 mM potassium phosphate buffer, pH 5.8; B, 20 mM potassium phosphate buffer, pH 5.8, containing 2 M NaCl. Enzyme samples were loaded onto the column equilibrated with buffer A. Then retained proteins were eluted at a flow rate of 1 $ml\,min^{-1}$ by a linear gradient from 0 to 100% of the buffer B in the time interval 1.5–17.0 min. Fractions showing activity were pooled, dialysed against 5 mM potassium phosphate buffer, pH 7.0, and concentrated in an ultrafiltration cell (Amicon, Danvers, CA, USA) equipped with an XM10 filter. LSAO and *Euphorbia characias* latex amine oxidase (ELAO) were purified according to previously published protocols. [19,20] The purified enzymes were stored frozen at −80°C. Horseradish (*Armoracia rusticana*) peroxidase was purchased from Fluka. Rapid removal of low-molecular-weight compounds from enzyme samples was carried out using a HiTrap Desalting chromatographic column (Amersham Biosciences).

Activity and protein assay

Amine oxidase activity was measured either on a spectrophotometer using the guaiacol method [21] or

by the determination of oxygen consumption. The latter measurements were performed using a DW1 oxygen electrode connected to an OXYG1 computer controlled oxygen control unit (Hansatech Instruments, King's Lynn, Norfolk, UK) thermostated at 30°C. An enzyme sample (0.05–0.2 nmol) was added to 1 ml of 100 mM potassium phosphate buffer, pH 7.0, in the electrode chamber and then the reaction was started by the addition of 70 mM substrate – putrescine or N^6-aminoalkyl adenine (20 µl). The rate of oxygen consumption (nmol min^{-1}) was obtained from the slope of a computer plot. For K_m, V and K_i measurements, substrate concentrations in the electrode chamber or spectrophotometric cuvette varied between 0.1 and 2 mM. Kinetic constants were calculated from initial rates using the computer program GraFit 4 (Erithacus Software, Horley, Surrey, UK). For stoichiometry measurements based on oxygen consumption, 200 nmol of a substrate was added to amine oxidase (2 nmol) in 1 ml of 100 mM potassium phosphate buffer, pH 7.0. The amount of oxygen consumed for the total conversion of all substrates was obtained from the respective computer plot. The protein content in enzyme samples was estimated by a modified Lowry method. [22]

Spectroscopic methods

Rapid scanning experiments under admission of air at 25°C were carried out using a DU 7500 photodiode array spectrophotometer (Beckman, Fullerton, CA, USA) essentially according to a previously published protocol. [11] Anaerobic spectra were measured on a Ultrospec 2100 pro-spectrophotometer (Biochrom, Cambridge, UK). Anaerobiosis was achieved by several cycles of vacuum followed by flushing with oxygen-free argon at 25°C in a Thunberg-type spectrophotometer cuvette (Vetroscientifica, Rome, Italy) in which anaerobic additions of various reagents could be made through a rubber cap.

Results

Syntheses of N^6-aminoalkyl adenines

We describe a biochemical study performed with five N^6-aminoalkyl adenines (Figure 1). Crude synthetic preparations of these compounds were purified by repeated crystallisation and analysed by several methods including elemental analysis, mass spectrometry and, in the case of ABAD and ATBAD, also ^{1}H-NMR spectroscopy. Details are summarised in Materials and Methods. Elemental analysis and NMR confirmed the structure of the synthesised compounds. ESI MS showed single peaks corresponding to quasimolecular ions of the N^6-aminoalkyl adenines. MS/MS experiments provided fragmentation patterns showing a loss of the primary aminogroup in the form of ammonia. Additionally, there was always observed a fragment peak with m/z 118.9, probably reflecting a release of the respective diamine moiety.

Table I. Substrate preference of plant CAOs to N^6-aminoalkyl adenines.

	Relative reaction rate (%)	
Enzyme	ABAD	ATBAD
GPAO	93.7	20.6
LSAO	88.2	20.1
ELAO	94.7	20.5

Activity was determined by oxygen consumption in 100 mM potassium phosphate buffer, pH 7.0, at 30°C as decribed in Materials and Methods. The final concentration of substrates in the reaction mixture was 1.5 mM. The rate of putrescine oxidation was arbitrarily taken as 100%.

Kinetic measurements

Two of the synthesised adenine derivatives, ABAD and ATBAD, were oxidised by GPAO, LSAO and ELAO. No activity was detected with APAD, ACBAD and ABYAD. Table I shows relative reaction rates measured for ABAD and ATBAD by oxygen consumption. ABAD oxidation was comparable with that of putrescine, whereas ATBAD was oxidised to a lesser extent. K_m values for ABAD and ATBAD (Table II) were of the same order of magnitude as those for putrescine. Table II also provides an overview of the relative V values (those for putrescine were taken as 100%). For GPAO and putrescine, $V = 800$ nmol s^{-1} per mg of protein; for LSAO and putrescine, $V = 2070$ nmol s^{-1} per mg; for ELAO and putrescine, $V = 570$ nmol s^{-1} per mg. The results for PSAO given in Table II were measured previously [23] with a sample of the enzyme ($V = 980$ nmol s^{-1} per mg) purified as described for GPAO (see Materials and Methods). As can be seen, ABAD was generally oxidised at a much higher rate than ATBAD.

Table II. K_m and relative V values measured for substrates of plant CAOs.

	Relative V (%)		K_m (mM)		
Enzyme	ABAD	ATBAD	Putrescine	ABAD	ATBAD
GPAO	107	18	0.30	0.48	0.33
LSAO	114	18	0.20	0.77	0.15
ELAO	96	27	0.20	0.25	0.20
PSAO*	99	16	0.27	0.95	0.69

Activity was determined by oxygen consumption in 100 mM potassium phosphate buffer, pH 7.0, at 30°C as described in Materials and Methods. An asterisk indicates results previously obtained with pea seedling enzyme (PSAO).[23] Activity measurements were in this case performed using the guaiacol spectrophotometric method.[21] The presented V values were subtracted from the corresponding double reciprocal plots, those for putrescine were taken as 100%.

Table III. Determination of stoichiometry of ABAD and ATBAD oxidation by plant CAOs.

	O_2 consumed (nmol)		
Enzyme	Putrescine	ABAD	ATBAD
GPAO	184	187	59
LSAO	182	172	77
ELAO	194	182	62

Samples (200 nmol) of the compounds were individually incubated with amine oxidase (2 nmol). The oxygen consumed in the reaction was determined polarographically.

Stoichiometry measurements were performed by incubating 200 nmol of ABAD or ATBAD with a relatively high amount of amine oxidase. Our results, presented in Table III, suggest that ABAD could be regarded as a normal substrate of plant CAOs giving a regular stoichiometry of oxidation (1:1). However, only 0.3 mol of oxygen was consumed in the oxidation of 1 mol of ATBAD.

Some additional experiments were carried out to further evaluate substrate properties of ABAD and ATBAD. GPAO, LSAO and ELAO (2 μM) were each incubated with ABAD (1 mM) in the presence of catalase and, after 10 minutes of incubation, aliquots were used for an activity assay with putrescine. The enzymes were not inhibited at all. Instead, complete inhibition was obtained with ATBAD in the presence of catalase. When GPAO, LSAO and ELAO were incubated with 0.1 mM ATBAD for 2 h, their residual activity was negligible, so that they became fully inactivated. After prolonged dialysis (72 h) or passing through a Sephadex G-25 desalting column of the inhibited GPAO, LSAO and ELAO samples, activity was partially recovered (40–60%).

Since neither oxygen was consumed, nor hydrogen peroxide formation was registered by the guaiacol assay method in the reaction of APAD, ACBAD and ABYAD, these three compounds were tested as inhibitors towards putrescine oxidation after preincubation for 10 minutes. Figure 2 shows the Lineweaver-Burk plot for the GPAO reaction with putrescine in the presence of APAD. As can be seen, APAD functioned as a competitive inhibitor of the enzyme ($K_i = 0.02$ mM). For ACBAD, the same inhibition type and a higher K_i value of 0.20 mM were found. LSAO was inhibited competitively by both APAD and ACBAD, the following inhibition constants being obtained: APAD, 0.02 mM; ACBAD, 0.21 mM. Surprisingly, ELAO showed a non-competitive inhibition with the following K_i values: APAD (0.62 mM) and ACBAD (0.48 mM). ABYAD was found to inhibit GPAO, LSAO and ELAO in a time-dependent manner. The enzymes were incubated with various concentrations of ABYAD (2–20 μM) for 5–30 min and residual activity values were measured. Then IC_{50} plots were constructed from the residual activities obtained after the incubation for 30 min (not shown). From these plots, the following IC_{50} values were determined: GPAO, 4.4 μM; LSAO, 7.1 μM; ELAO, 4.0 μM. It is worth recalled that but-2-yne-1,4-diamine, [10,11] a structurally related compound, provided approximately half values of 2.5, 2.5 and 2.8 μM, respectively.

APAD and ACBAD inhibited the investigated enzymes reversibly. GPAO, LSAO and ELAO activities recovered to original values after dialysis of 2 μM solutions of the enzymes, which had previously been incubated with 1 mM APAD or ACBAD for 1 h. When GPAO (2 μM in 20 mM potassium phosphate buffer, pH 7.0) was incubated with 2.5 mM APAD or ACBAD for 2 h and then dialysed against the working

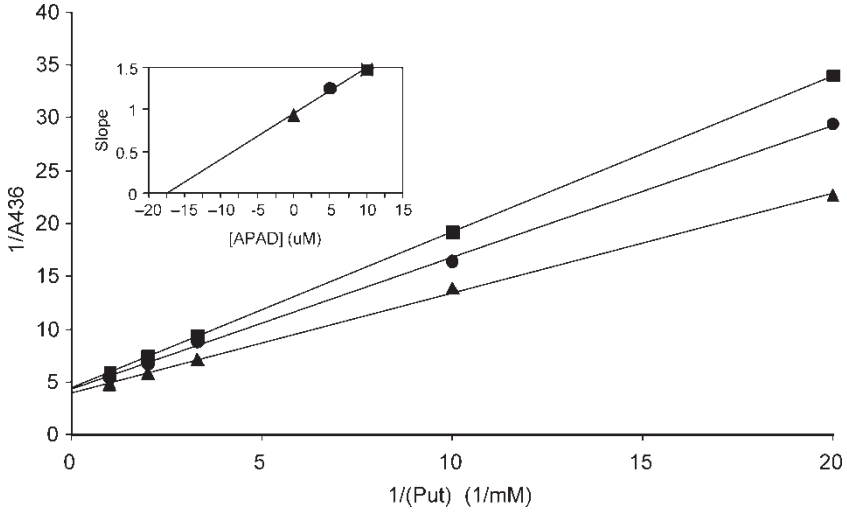

Figure 2. Lineweaver-Burk plot of competitive inhibition of GPAO by APAD. Final concentrations of the inhibitor were as follows: ▲, 0 μM; •, 5 μM; ■, 10 μM, preincubation 10 min. The inset shows a secondary plot of slopes against the inhibitor concentration ($K_i = 17.5$ μM). Measured with putresine as a substrate using the guaiacol spectrophotometric method.[21]

buffer, in both cases the enzyme recovered about 80% of its activity after the dialysis for 96 h (triple changed buffer). Instead, ABYAD caused irreversible inhibition that could not be reversed by dialysis or gel chromatography.

Absorption spectroscopy

Resting native CAOs have a characteristic pink colour with a broad absorption band centred at 498 nm reflecting the presence of the oxidized TPQ cofactor. [1–4] The anaerobic addition of a substrate to CAOs causes immediate disappearance of this band. New absorption maxima at 364, 434 and 464 nm arise in the spectrum. These are diagnostic of the free radical intermediate which accumulates in the absence of oxygen. [3] Subsequent oxygenation restores the original enzyme colour. [1–4]

Aerobic spectra of GPAO reactions with APAD, ABAD and ATBAD were measured using a rapid scanning spectrophotometry [11] approach. The reaction was achieved through the addition of these compounds to amine oxidase in an excess of 100:1. After correcting the baseline to that of the enzyme solution, the respective adenine derivative was rapidly added by a syringe and full scans in the range 300–700 nm were recorded each 100 ms (99 spectra in total). The obtained results are shown in Figures 3 and 4. Mixing of GPAO with ABAD resulted in a decrease in absorbance at 500 nm followed by the appearence of three new maxima at 364, 434 and 464 nm that are characteristic for substrate-reduced CAOs (Figure 3). A similar spectrum showing the same maxima was recorded during the reaction with ATBAD. However, the corresponding maxima were lower (not shown). Instead, the reaction of GPAO with APAD brought about a decrease in absorbance at 500 nm and a new maximum at 350 nm appeared (Figure 4).

All the investigated enzymes provided the characteristic spectrum of substrate-reduced CAO when reacted with ABAD, ATBAD and ACBAD in anaerobiosis. The absorption spectrum of GPAO recorded after mixing the enzyme with APAD under anaerobic conditions showed stable maxima at 364, 434 and 464 nm. After admission of air, the enzyme did not recover its original spectrum. There was a clear absorption peak preserved at 350 nm that finally disappeared after dialysis of the enzyme sample (not shown). During the dialysis, the enzyme gradually recovered its original activity (see kinetic measurements). Similar results were also obtained for the other enzymes.

When GPAO, LSAO and ELAO were individually incubated with an excess of ABYAD in anaerobiosis, the spectrum of substrate-reduced CAOs was observed (not shown). Subsequent oxygenation of the mixtures restored the characteristic colour of the resting enzyme. In the case of ELAO and GPAO, the formation of the pink spectral species was rapid with an absorption maximum at 500 nm, whereas LSAO became pink slowly with a red-shift absorption at 525 nm. Surprisingly after 120 min, the enzymes completely lost their original spectra without the possibility of recovery by dialysis and gained a yellow–brown colour (λ_{max} around 300 nm). The enzymes fully inactivated by ABYAD did not react anymore with phenylhydrazine, a typical carbonyl reagent. This result suggested that the TPQ cofactor was no longer available for such a reaction. [24] Conversely, APAD- and ACBAD-reacted and exhaustively dialysed

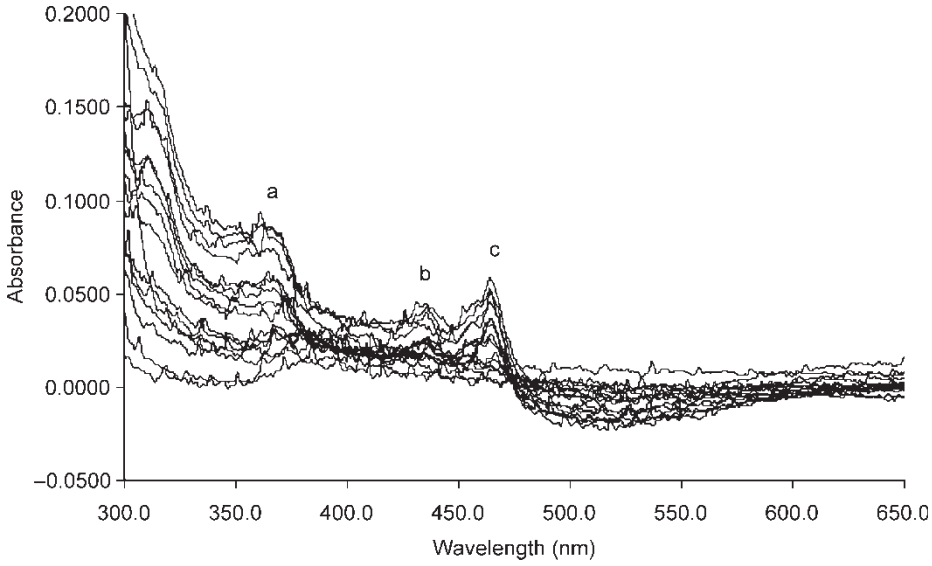

Figure 3. Rapid scanning difference absorption spectra of GPAO (20 μM) in early phase of the reaction with ABAD (1 mM) in 20 mM potassium phosphate buffer, pH 7.0. The spectra were recorded in 0.1 s intervals for 9.9 s at 25°C. Absorption maxima at 364 (a), 434 (b) and 464 nm (c) represent a spectrum of the substrate-reduced cofactor. Several measured spectra were removed for clarity.

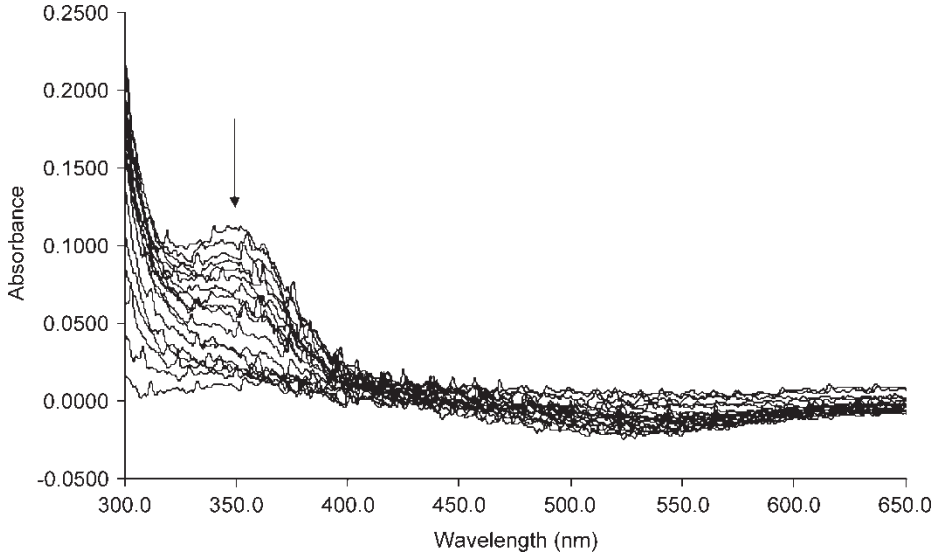

Figure 4. Rapid scanning difference absorption spectra of GPAO (20 μM) in early phase of the reaction with APAD (1 mM) in 20 mM potassium phosphate buffer, pH 7.0 The spectra were recorded in 0.1 s intervals for 9.9 s at 25°C. Several measured spectra were removed for clarity. An arrow indicates the discussed absorption peak at 350 nm.

enzymes provided the reaction giving stoichiometry ratios of 1:1.5 and 1:1.7, respectively. Finally, the dialysed ATBAD-treated enzyme reacted with phenylhydrazine in the same way.

Discussion

Cytokinins were once discovered as factors promoting cell division in tobacco tissue cultures. [25] Since then, they have been shown to regulate a wide range of additional developmental events in plants, such as *de novo* bud formation, release of buds from apical dominance, leaf expansion, delay of senescence, promotion of seed germination and chloroplast formation. [26] Naturally occurring cytokinins are N^6-derivatives of adenine and cytokinins with a hydroxylated side-chain at that position, such as zeatin, are major constituents in plants. Synthetic cytokinins include adenine derivatives, such as kinetin, as well as compounds structurally unrelated to natural cytokinins, such as certain phenylureas. [26] Several processes have been identified as playing a role in cytokinin metabolism in plants, among them also the oxidation of the the N^6-side-chain of adenine. [26] Formerly there was a hypothesis that the mechanism of enzymatic oxidation is the same as that of degradation of biogenic amines by CAOs. [13] However, as has been shown quite recently, cytokinin oxidase/dehydrogenase is a member of the group of FAD-containing enzymes [27,28] and it follows a different reaction mechanism. [29]

N^6-aminoalkyl adenines might represent combined analogues of cytokinins and biogenic amines. Hence studying their effect on amine oxidising enzymes seems to be of interest. To our knowledge, such compounds have not yet been subjected to a biochemical study. Some N^6-aminoalkyl adenines (including ABAD and APAD) have already been synthesised but, however, only partially characterised. [30] In the present study, a nucleophilic substitution reaction was utilised to synthesise five N^6-aminoalkyl adenines from 6-chloropurine and the structure and purity of the synthesised compounds carefully checked. Contrary to a previous report describing crystalline monohydrochlorides, [30] the compounds used in this study were crystallised as trihydrochlorides. This was achieved due to using hydrochloric acid for recrystallisation. There are reports regarding crystals of trihydrochlorides of some N^6-derivatives of adenine in the literature. [31] Without using HCl, we obtained ABAD from ethanol as a monohydrochloride which however, was not so pure.

Our results show that the presence of an adenine moiety does not exclude the interaction of diamines with plant CAOs. Based on the described kinetic measurements, both ABAD and ATBAD can be regarded as substrates. These compounds bind to the topaquinone cofactor engaged in the enzyme turnover and they are converted to the respective products. This binding and the subsequent oxidation were witnessed using absorption spectroscopy, where the characteristic turnover intermediates were clearly recorded. The measured K_m values for ABAD and ATBAD resemble those for putrescine as determined with the individual enzymes. In general, ATBAD provided slightly lower K_m values than ABAD (especially in the case of LSAO), hence it seems to bind better at the active site. On the other hand, ABAD is oxidised faster than ATBAD. The production of H_2O_2, which was shown by the guaiacol assay method, confirms that both ABAD and ATBAD

pass through the whole CAO turnover. However, a clear difference between these two compounds was recognised during the reaction stoichiometry measurements. ABAD oxidation is in accordance with the reaction of a typical substrate of plant CAOs. On the contrary, the ratio substrate/oxygen (Table 3) measured for ATBAD indicates that the reaction is slowed down in this case. Plant CAOs in the reaction with ATBAD are probably inhibited by the product aminoaldehyde when initial oxidation proceeds normally. The presence of catalase has no effect on the reaction and therefore H_2O_2 may be excluded as an inhibitor. Since the activity could be recovered by dialysis, the inhibition seems to be caused by reversible blocking of the active site. A similar inhibition by the reaction product was reported for the substrate cysteamine. [32]

APAD and ACBAD represent reversible inhibitors of the studied plant CAOs. The aerobic reaction of APAD is in accordance with that of the "parent compound" propane-1,3-diamine, where the 350-nm spectral intermediate was shown to be the corresponding substrate Schiff base. [33] It is interesting, that ATBAD and ACBAD as geometric isomers show different kinetic properties. Probably it comes from the length of the aliphatic chain, which is shorter for the *cis*-isomer and hence ACBAD does not bind in a correct orientation. Similarly, the related diaminobutenes show different inhibitory properties in the reaction with bovine serum amine oxidase. [16] Absorption spectra revealed that ACBAD reacted with the cofactor to form the semiquinolamine radical. It seems likely that the enzyme-ACBAD complex is reoxidised only very slowly. The inhibition is diminished by dialysis as is the case for diaminoketones as CAO substrate analogues. [34] The difference in the inhibition modes of ACBAD and APAD measured with GPAO and LSAO (competitive) and ELAO (non-competitive) could come from the substrate specificity of the *Euphorbia* enzyme [8,20] or from a different binding of the adenine moiety. Differences among plant CAOs with respect to particular inhibitors have recently been reviewed. [9]

ABYAD inhibits irreversibly and might be suggested as a mechanism-based inhibitor of plant CAOs. Surprisingly its oxidation is too slow and cannot be registered by kinetic activity assay method. However, absorption spectra clearly showed ABYAD interaction with the cofactor and passage through the whole enzyme turnover under the admission of air. The formation of a 300-nm absorbing intermediate after prolonged incubation resembles the GPAO reaction with but-2-yne-1,4-diamine as a typical diamine-derived mechanism-based inhibitor of plant CAOs. [11] Thus we could anticipate a similar inhibition mechanism involving a nucleophilic attack on the product aldehyde. The most likely explanation for the lack of observed phenylhydrazine derivatization is that access of phenylhydrazine to the quinone cofactor is sterically blocked by a covalent adduct of an inhibitor-derived species to the active site channel. [24]

The present study deals with several N^6-aminoalkyl derivatives of adenine that might prospectively show interesting biological effects. Due to the presence of a short aliphatic diamine moiety, they could interact not only with CAOs, but also with the other enzymes involved in the metabolism of biogenic amines, [35] for example with aminotransferases or polyamine synthases. In addition, their structure resembles that of natural purine-derived cytokinins (zeatin, isopentenyl adenine) as plant hormones. Thus they may also provide interesting results when tested with cytokinin oxidase/dehydrogenase. Purine-derived compounds are currently being studied in biomedicinal research as potential anti-cancer drugs. [36–38] Various substituted derivatives of N^6-(benzylamino)purine have been subjected to a systematic screening in order to find selective and potent inhibitors of cyclin-dependent kinase, an enzyme showing a crucial role in the processes of cell division and proliferation. [36–38] Evaluations of the described adenine derivatives for their possible biological activity using various *in vitro* tests are in progress in our laboratory.

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