

**Kinetic and chemical analyses of the cytokinin oxidase/dehydrogenase catalysed reaction:  
Correlations with the crystal structure**

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**Abbreviations used:** CKX, cytokinin dehydrogenase (EC 1.5.99.12); ZmCKX1, the CKX enzyme from maize, encoded by the gene *Zmckx1* (GenBank AF044603).

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## Synopsis

Cytokinin dehydrogenase (CKX) is a flavoprotein that cleaves cytokinins to adenine and the corresponding side-chain aldehyde using a quinone-type electron acceptor. Reactions of maize CKX with five different substrates (*N*<sup>6</sup>-isopentenyladenine, *trans*-zeatin, kinetin, *p*-topolin and *N*-methyl-isopentenyladenine) were studied. By stopped-flow analysis of the reductive half-reaction, spectral intermediates were observed indicative of the transient formation of a binary enzyme-product complex between the cytokinin imine and the reduced enzyme. The reduction rate was high for isoprenoid cytokinins that showed formation of a charge transfer complex of reduced enzyme with bound cytokinin imine. For the other cytokinins, flavin reduction was slow and no charge transfer intermediates were observed. The binary complex of reduced enzyme and imine product intermediate decays relatively slowly to form an unbound product, cytokinin imine, which accumulates in the reaction mixture. The imine product only very slowly hydrolyses to adenine and an aldehyde derived from the cytokinin *N*<sup>6</sup>-side chain. Mixing of the substrate-reduced enzyme with Cu<sup>2+</sup>/imidazole as an electron acceptor to monitor the oxidative half-reaction revealed a high rate of electron transfer for this type of electron acceptor when using *N*<sup>6</sup>-isopentenyladenine. The stability of the cytokinin imine products allowed their fragmentation analysis and structure assessment by Q-TOF MS/MS. Correlations of the kinetic data with the known crystal structure are discussed for reactions with different cytokinins.

**Key words:** cytokinin, cytokinin oxidase/dehydrogenase, flavoprotein, maize, mass spectrometry, stopped-flow spectrophotometry

## INTRODUCTION

Flavoproteins fulfill a wide variety of biological functions. While being recognised for catalysing a range of redox reactions, flavoproteins have also evolved to mediate non-redox processes, e.g. light emission and sensing, transcriptional regulation and DNA repair. They typically contain flavin adenine dinucleotide (FAD) or flavin mononucleotide (FMN) as a cofactor covalently or non-covalently bound to the apoprotein. Flavoproteins often also harbour other cofactors, such as heme, 4Fe-4S cluster or molybdenum [1].

Flavin-containing oxidoreductases mediate the cleavage of a C-H bond with concomitant transfer of two electrons from a donor molecule to oxygen (oxidases) or another electron acceptor (dehydrogenases) [1]. Cytokinin oxidase/dehydrogenase (CKX; EC 1.5.99.12) is a flavoprotein in which the FAD cofactor is covalently linked to a histidine residue. The enzyme catabolises plant hormones (cytokinins) by irreversible cleavage of their side chain (Fig. 1a). This function makes it an important regulator of cytokinin level in plants [2]. Cytokinin substrate, enzyme and an electron acceptor form a ternary complex during catalysis that leads to conversion of the cytokinin to adenine and the corresponding aldehyde [3]. CKX was originally thought to be an oxidase with molecular oxygen as the primary electron acceptor. Kinetic data on wheat and maize CKXs have shown, however, that oxygen is a poor electron acceptor [4,5] (despite a high redox potential +0.82 V) that favourably accepts electrons from many other covalent flavoproteins [6]. Quinone-type electron acceptors were found to function more efficiently for CKX indicating that it is a much more effective dehydrogenase than oxidase [4,5,7,8]. A crystal structure of the CKX from *Zea mays* (ZmCKX1) has been solved recently and confirmed that ZmCKX1 is a covalent flavoprotein in which FAD is covalently tethered to His105. This monomeric protein of 57 kDa is most active with the isoprenoid cytokinins, *trans*-zeatin and isopentenyladenine. X-ray analyses revealed that, upon binding, these substrates seal the pore that leads to the catalytic site leaving no space for electron acceptor entry [9]. So far, the crystallographic data has not revealed potential electron acceptor binding sites.

This work presents steady-state and pre-steady-state kinetic analyses of the ZmCKX1 reaction with five different substrates (Fig. 1b), the typical isoprenoid cytokinins (isopentenyladenine and *trans*-zeatin), aromatic cytokinins (kinetin and *p*-topolin [4-hydroxybenzyladenine]), and the cytokinin analogue *N*-methyl-isopentenyladenine that was used earlier for mechanistic studies [5]. We also present the first structural identification of a CKX reaction intermediate that was postulated as a cytokinin-derived imine by Laloue and Fox in 1985 [10]. The structure of the intermediate was determined for all five studied cytokinins using MS fragmentation analysis and accurate mass determination by MS/MS spectroscopy.

## MATERIALS AND METHODS

### Enzyme and chemicals

The recombinant ZmCKX1 enzyme used in this study was the same as that described earlier [5,9]. The molar extinction coefficient of the ZmCKX1 enzyme was determined spectrophotometrically by comparing the absorption spectrum of the native enzyme and of the enzyme mixed with SDS. It was found that incubation with 0.1 % SDS for 5 minutes ensured its complete unfolding as the obtained flavin spectrum was similar to free FAD while higher SDS concentrations or longer incubation with SDS did not change the final spectrum. By using  $\epsilon_{\text{FAD}} = 11.3 \text{ mM}^{-1} \text{ cm}^{-1}$  at 450 nm [11], the molar extinction coefficient of ZmCKX1 at 450 nm was found to be  $11.5 \text{ mM}^{-1} \text{ cm}^{-1}$ .

*N*<sup>6</sup>-(2-isopentenyl)adenine (isopentenyladenine), *trans*-zeatin [6-((*E*)-4-hydroxy-3-methylbut-2-enylamino)purine], and kinetin [*N*<sup>6</sup>-(2-furfuryl)adenine] were obtained from Sigma (St. Louis, MO, U.S.A.). *p*-Topolin [*N*<sup>6</sup>-(4-hydroxybenzyl)adenine] was from OlChemIm (Olomouc, Czech Republic). *N*-Methyl-isopentenyladenine [*N*<sup>6</sup>-methyl-*N*<sup>6</sup>-(3-methyl-2-butenyl)adenine] was synthesised as in Wang and Letham [12]. Absorption spectra of used cytokinins and corresponding product aldehydes (3-methyl-2-butenal, furfural and 4-hydroxybenzaldehyde; all from Sigma) were measured in 75 mM imidazole/HCl buffer, pH 6.5, and 50 mM Tris/HCl buffer, pH 8.0, respectively.

### Stopped-flow kinetics of FAD reduction

Stopped-flow kinetics was performed using a SX17MV stopped-flow instrument equipped with a diode-array detector (Applied Photophysics, Leatherhead, U.K.). Spectral scans were collected either in 2.56 ms or 7.68 ms intervals. To determine the rates for specific processes accurately, the single-wavelength detection mode was used. (451 nm for flavin reduction, 360 nm for intermediate decay and 650 nm for monitoring the charge-transfer complex). Anaerobic conditions were accomplished by flushing solutions with nitrogen and by subsequent inclusion of 20 mM glucose and glucose oxidase (20 µg/ml). The ZmCKX1 enzyme (23 µM) was mixed with different concentrations of cytokinin substrates in 75 mM imidazole/HCl, pH 6.5, at 25 °C. The experiment with *p*-topolin was also done in 75 mM Tris/HCl buffer series, pH range from 7.5 to 8.5.

### Monitoring the redox state of the FAD cofactor during catalysis

The experiment was carried out using the stopped-flow apparatus in the single-wavelength detection mode at 451 nm as above, in 75 mM imidazole/HCl, pH 6.5, at 25 °C. The enzyme (5 µM, final concentration) was first anaerobically reduced with 0.1 mM isopentenyladenine and then mixed with an equal volume of oxygen saturated buffer (75 mM imidazole/HCl, pH 6.5) or with anaerobically prepared buffer of the same composition containing 0.5 mM CuCl<sub>2</sub>.

### **Spectrophotometric measurements of the reaction intermediate decay**

Spectrophotometric measurements were performed using a DU 7500 photodiode array spectrophotometer (Beckman, Fullerton, CA, U.S.A.) in the range of 250-700 nm, in aerobic mode. The enzyme (5  $\mu$ M) was incubated at 25 °C with various concentrations of cytokinin substrate in imidazole/HCl (75 mM, pH 6.5) and Tris/HCl (75 mM, pH 8.0) buffers.

### **Determination of kinetic parameters for the turnover reaction**

The assay was performed using previously described methods (Libreros Minota and Tipton, 1995; Frébort et al., 2002). An enzyme sample (0.44 - 120 nM) was incubated in a reaction mixture (total volume of 0.6 ml in an Eppendorf tube) composed of 75 mM imidazole/HCl, pH 6.5, containing 1.25 mM CuCl<sub>2</sub> and appropriate concentration of the substrate, at temperatures of 25 °C and 37 °C. The incubation period varied from 0.5 to 1.5 h, depending on the activity. The reaction was stopped by adding 0.3 ml of 40% (w/v) trichloroacetic acid to the tube, which was followed by an addition of 0.2 ml of 4-aminophenol (2% solution in 6% trichloroacetic acid). The absorption of the sample was then scanned using a UV-2401PC double-beam spectrophotometer (Shimadzu, Tokyo, Japan) in the range 300–700 nm against a blank without the substrate. Concentrations of the reaction products of different cytokinins were then determined from absorptions at specific wavelengths according to Frébort et al. (2002). All kinetic data were analysed using the program GraFit 4.0.12 (Erithacus Software, Horley, Surrey, U.K.).

### **Intermediate identification by MS/MS**

The high resolution MS measurements of cytokinin dehydrogenase reaction intermediates were done on a hybrid mass analyser Q-TOF *micro* with MassLynx data system software (Micromass, Manchester, U.K.). Cytokinins were diluted from a stock solution of 1 mM in DMSO to 0.05 mM concentration in 30 mM (NH<sub>4</sub>)<sub>2</sub>CO<sub>3</sub>, pH 8.0. ZmCKX1 (stock solution 87.7  $\mu$ M in TE buffer, pH 8.0, containing 1 M KCl) was desalted using Microcon YM-30 filter (Millipore, Bedford, MA, U.S.A.). For the reaction intermediate analysis, a reaction mixture that contained 1.5  $\mu$ M ZmCKX1 and 0.05 mM cytokinin in 30 mM (NH<sub>4</sub>)<sub>2</sub>CO<sub>3</sub>, pH 8.0 was prepared and incubated at 25 °C for 2 min before the measurement. The samples were applied in the form of an in-line spray at the injection rate of 10  $\mu$ l/min. In the full scan mode, the data acquisition was done in the range 50-1000 Da with the cycle time 47  $\mu$ s, scan time 0.5 s, collision energy 5 V. The electrospray ionization in the positive mode was done using the following parameters: source block/desolvation temperature 80/150 °C, capillary/cone voltage 2500/30 V, spray/cone gas flow (N<sub>2</sub>) 50/250 l/h. For the exact mass determination experiments, the external calibration was done using a lock spray technology with a mixture of 0.1 M NaOH, 10% formic acid and acetonitrile (1:1:8) as a reference. For the MS/MS experiments, the fragmentation was done in an argon gas-filled collision cell with the collision energy 15, 20, 25, 30

and 35 V. Other parameters were the same as in the simple MS experiments. The masses of the parent ions and their fragments were calculated and used for the determination of the elementary composition and structure with accuracy within 5 ppm for full scan and within 15 ppm for MS/MS experiments.

## RESULTS

### Reductive half-reaction

To study the kinetic properties of ZmCKX1 in more detail, a pre-steady state kinetic study was performed. The five different cytokinins chosen for the kinetic study were two naturally occurring isoprenoid cytokinins (isopentenyladenine and *trans*-zeatin), two aromatic cytokinins (kinetin and *p*-topolin), and a cytokinin analogue (*N*-methyl-isopentenyladenine), that was earlier shown to be a substrate of ZmCKX1 (see Fig. 1b for structural formulas) [5]. As a reference, the absorption spectra of the cytokinins and available product aldehydes (3-methyl-2-butenal, furfural and 4-hydroxybenzaldehyde) were measured (data shown in Supplement 1). None of the compounds showed significant absorption above 300 nm, except for 4-hydroxybenzaldehyde (product of cleavage of *p*-topolin) that has a typical absorption spectrum with a peak at 330 nm at pH 8.0 ( $\epsilon_{330}$  of 17 900 M<sup>-1</sup>cm<sup>-1</sup>).

When ZmCKX1 was anaerobically mixed with individual cytokinins using a stopped-flow apparatus equipped with a diode array detector, the time-resolved spectral analysis (300-670 nm) revealed the presence of at least two distinct kinetic events (Fig. 2). During the first process, the FAD cofactor was fully reduced as evidenced by an absorption decrease around 451 nm. Concomitantly with FAD reduction, the formation of a species characterised by a shoulder at 360 nm was observed with most substrates. This kinetic event was extremely fast for the isoprenoid cytokinins where the rates were 200-1000 s<sup>-1</sup> (Fig. 2a,b), but slow for the aromatic cytokinins and *N*-methyl-isopentenyladenine (Fig. 2c-e) with the rates  $\leq 1$  s<sup>-1</sup>. For isopentenyladenine and to some extent also for *trans*-zeatin, a broad absorbance band above 550 nm was also observed concomitantly with the FAD reduction (Fig. 2a,b), and this feature was previously ascribed to a charge-transfer complex [5].

While the flavin was fully reduced in this first kinetic process by all natural cytokinins, the cofactor was only partially reduced by *N*-methyl-isopentenyladenine. Only in a second slow phase was the flavin fully reduced. With most substrates, kinetic processes other than the relatively fast flavin reduction were observed. For the isoprenoid cytokinins, isopentenyladenine and *trans*-zeatin, the reduction was followed by two relatively slow phases in which the formed intermediates, absorbing around 360 nm, slowly disappeared. In the case of kinetin, the species formed upon flavin reduction also showed a relatively high absorbance around 360 nm and disappeared in a single and slow subsequent step. Unique for *p*-topolin, the anaerobic reduction of ZmCKX1 revealed a single kinetic event in which the flavin was fully reduced while a species was formed that absorbs at ~390 nm. The

pH dependence of this band suggests that it is indeed the phenolic imine of *p*-topolin, since the aldehyde product 4-hydroxybenzaldehyde would give a peak at 330 nm (see Supplement 1). The  $pK_a$  of the imine is apparently similar to that of the aldehyde, as would be expected.

To further elucidate the kinetic behaviour of the individual spectral species as described above, single wavelength detection modes at 451 nm, 360 nm, and 650 nm were applied for higher accuracy. The FAD reduction rates were measured as absorbance decay at 451 nm and their dependencies on different concentrations of individual cytokinins were used to determine the reduction rate constants and dissociation constants of the enzyme-substrate complex. The rates of the enzyme-product binary complex decay were measured at 360 nm and the charge transfer complex was monitored at 650 nm. The resulting data are summarised in Table 1.

Data in this table demonstrate that isopentenyladenine provided very fast and efficient reduction of the enzyme cofactor with an extremely high rate constant of  $1150\text{ s}^{-1}$  and a dissociation constant of  $15\text{ }\mu\text{M}$ . In contrast to previous measurements that showed  $K_d < 2\text{ }\mu\text{M}$  [5], the present analysis allowed better fitting for the fast reduction process. Previously, only one fast phase could be discerned, while now two phases were observed. Along with the fast cofactor reduction, a strong charge-transfer complex absorption band appeared that was relatively stable and decayed in a biphasic process that was synchronised with the binary complex decay. It is supposed that the strong charge-transfer complex is formed due to the tight binding of the intermediate product (imine), positioned next to the isoalloxazine ring system of the flavin cofactor. Repositioning of this product in the binary complex makes the charge-transfer complex weaker, and dissociation of the binary complex leads to its complete loss and to a formation of imine-product (Fig. 2a and the inset graph).

*trans*-Zeatin was also effective in reducing the FAD cofactor, although with a lower rate constant ( $244\text{ s}^{-1}$ ) while with a similar dissociation constant ( $13\text{ }\mu\text{M}$ ) when compared to isopentenyladenine (Table 1). A charge transfer complex was also observed and decayed in a biphasic process. It seems that product binding in the first binary complex results in less extensive orbital overlap as compared to isopentenyladenine and evidenced by a less intense charge-transfer band. This may also explain why the spectral species showing the strong absorption at 360 nm that was observed for isopentenyladenine (Fig. 2a) is less abundant in the *trans*-zeatin spectral data (Fig. 2b).

*N*-Methyl-isopentenyladenine showed much slower FAD reduction than isopentenyladenine and *trans*-zeatin, and showed no evidence for the charge-transfer complex formation. The measurements at 451 nm provided a reduction rate fitted by two exponentials, a faster process characterised by  $k_{\text{red}} = 1.0\text{ s}^{-1}$  and  $K_d = 22\text{ }\mu\text{M}$ , and a second reductive event with an apparent rate of  $0.2\text{ s}^{-1}$  and a similar apparent affinity ( $K_d = 12\text{ }\mu\text{M}$ ). In the first phase, again a relative increase at around 360 nm was observed that disappeared during the second phase. However, the intensity at 360 nm of this intermediate was much lower when compared to the above mentioned cytokinin substrates.

For the aromatic cytokinins, the reduction rate constants were very low (0.1-0.8 s<sup>-1</sup>). The single wavelength stopped-flow kinetic analyses showed that kinetin affinity to the enzyme is somewhat low ( $K_d = 66 \mu\text{M}$ ), while *p*-topolin showed relatively stronger binding ( $K_d = 24 \mu\text{M}$ ). The rate constants of the binary complex decay were about 0.01 s<sup>-1</sup>, so the binary complex was quite stable, but no charge transfer was observed.

### Oxidative half-reaction

To determine the reactivity of reduced ZmCKX1 with molecular oxygen, the oxidative half-reaction was also studied. For pre-steady-state reoxidation experiments, 20  $\mu\text{M}$  ZmCKX1 was reduced anaerobically by adding 25  $\mu\text{M}$  dithionite. By mixing the dithionite-reduced enzyme with air-saturated buffer, ZmCKX1 was reoxidised in a slow monophasic process yielding fully oxidised enzyme. The reoxidation rate was 1.83 s<sup>-1</sup>. Using a buffer which was saturated with molecular oxygen (0.62 mM oxygen upon mixing) a higher reoxidation rate was observed yielding a reoxidation rate constant of  $1.5 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ . Compared to other flavoprotein oxidases, this reoxidation rate constant is relatively low indicating that *in vivo* the enzyme will not act as an efficient oxidase.

The turnover rate of ZmCKX1, which was previously measured by the formation of the corresponding cytokinin-derived aldehyde product, was found to be much faster when using alternative electron acceptors such as 2,3-dimethoxy-5-methyl-1,4-benzoquinone and 2,6-dichlorophenol indophenol [4,5]. Although highly effective, these compounds cannot be used for spectrophotometric analysis of FAD reoxidation due to strong absorption at the studied wavelength range. To overcome this disadvantage, a Cu(II)/imidazole complex was used, since it also functions as an electron acceptor of CKX [4], but does not interfere with the FAD absorption. The enzyme was first anaerobically reduced with an excess of isopentenyladenine and then mixed, using the stopped/flow apparatus, with either an oxygen-saturated buffer or an anaerobically prepared CuCl<sub>2</sub>/imidazole solution. With oxygen, the enzyme needed about 30 s to consume the substrate before starting to revert to the oxidised form in an exponential process (rate of  $\sim 0.6 \text{ s}^{-1}$ ). Using CuCl<sub>2</sub>/imidazole, the substrate was consumed within 0.7 s and the oxidised form restored with a rate of 28 s<sup>-1</sup>. These results clearly indicate that the Cu(II)/imidazole complex is about fifty-fold more effective as an electron acceptor than molecular oxygen. For the remainder of this kinetic study, the steady-state parameters of ZmCKX1 with the studied cytokinins were determined using CuCl<sub>2</sub>/imidazole.

The apparent  $K_m$ ' values and turnover rates measured by cytokinin-derived aldehyde production [13] with Cu(II)/imidazole at pH 6.5 at 25 °C (the temperature used for spectrophotometric measurements and for corresponding MS/MS experiments) are shown in Table 1. The turnover rate was 40 s<sup>-1</sup> for isopentenyladenine, but much less for kinetin, *N*-methyl-isopentenyladenine and *p*-topolin. The  $K_m$ ' was as low as 1  $\mu\text{M}$  for isopentenyladenine, while with the other cytokinins it ranged from 14-85  $\mu\text{M}$ . At 37 °C, the turnover rates for all studied cytokinins increased about 3-fold and the apparent Michaelis constants by approximately 50% (data not shown).



Anaerobic substrate-mediated ZmCKX1 reduction experiments (Fig. 2) showed that the binary complex, consisting of reduced enzyme and cytokinin-derived imines, is converted to a species absorbing at 310 nm. This species was proposed previously to be the free imine product [10]. Under aerobic conditions, the enzyme undergoes slow turnover that results in accumulation of the 310 nm-species in the reaction mixture [5], before it is hydrolysed to adenine and the corresponding cytokinin-derived aldehyde. In this work, the accumulation and decay of this spectral species were monitored using diode-array spectrophotometry. The data in Fig. 3 show that the 310 nm species derived from isopentenyladenine, *trans*-zeatin, and *N*-methyl-isopentenyladenine, respectively, accumulate in the reaction mixture until all corresponding substrate is degraded. For isopentenyladenine, this species showed exponential decay, being relatively stable at pH 6.5, but rapidly disappearing at pH 8.0. In the case of *N*-methyl-isopentenyladenine, this species was also stable, not being significantly affected by pH. In contrast, the 310 nm species derived from *trans*-zeatin was rapidly decaying at both pH values as documented by a rather sharp decreases in absorption (Fig. 3c). The time needed for the substrate consumption and enzyme re-oxidation (detected as absorbance change at 451 nm) was the same in acidic and alkaline conditions when the enzyme was reduced by isopentenyladenine or *N*-methyl-isopentenyladenine. For *trans*-zeatin, however, alkaline conditions led to a prolonged re-oxidation phase (three-fold) compared to that at pH 6.5 (Fig. 3c) that could be caused by further cyclisation of the *trans*-zeatin-derived aldehyde product, 4-hydroxy-3-methyl-2-butenal.

*p*-Topolin showed different behaviour than the other cytokinins. The anaerobic reduction of CKX by this cytokinin already revealed that the enzyme-bound imine product has different spectral properties with a pH-sensitive absorbance peak at around 390 nm (Fig. 2e). This imine product has similar stability under acidic and alkaline conditions (Fig. 4). Upon prolonged incubation, the imine is hydrolysed to 4-hydroxybenzaldehyde that has a typical absorption spectrum with a peak at 330 nm at pH 8.0 ( $\epsilon_{330}$  of 17 900 M<sup>-1</sup>cm<sup>-1</sup>), while the absorption at pH 6.5 is negligible ( $\epsilon_{330}$  of 2750 M<sup>-1</sup>cm<sup>-1</sup>). The striking observation was made that re-oxidation of *p*-topolin-reduced enzyme (451 nm trace in Fig. 4) does not occur even after prolonged incubation (12 h). Only an equimolar amount of the aldehyde is formed before the enzyme is reduced as can be seen from the 330 nm progress curve at pH 8.0 (Fig. 4b – right panel). This observation suggests that the reaction product of *p*-topolin acts as an inhibitor.

### Mass spectrometry identification of cytokinin-derived imines

Q-TOF mass spectrometry is a powerful tool for analysing structures of small molecules that allows identification of a structural formula from accurate measurements of masses of the parent ion and its fragments in the MS/MS mode [14]. Cytokinins were analysed in the direct injection mode and gave parent ions with *m/z* values that are presented in Table 2. For the analysis of cytokinin intermediates and products in the ZmCKX1 catalytic reaction, a reaction mixture composed of the enzyme and cytokinin was injected into the mass spectrometer shortly after component mixing. For all

studied cytokinins, formation of a compound having the  $m/z$  of 2 Da lower than the original cytokinin substrate was found (Table 2). The elementary compositions were calculated from accurate masses of the parent ions and the results showed a loss of two hydrogens. This observation is in general agreement with the early proposal of the cytokinin imine formation, a cytokinin[-2H] intermediate of isopentenyladenine [10].

Parent ions of cytokinins were further fragmented to observe and analyse the fragmentation patterns in correlation with known structures. Fragmentation analysis was then applied on cytokinin[-2H] intermediates to analyse their structures. The results of the study are summarised in Fig. 5 and Supplements 2-4.

Fragmentation of the isopentenyladenine parent ion with a calculated molecular formula  $C_{10}H_{14}N_5^+$  (Fig. 5a) yielded the typical ions of  $C_6H_6N_5^+$  ( $m/z$  148.0623),  $C_5H_6N_5^+$  ( $m/z$  136.0623) and  $C_5H_3N_4^+$  ( $m/z$  119.0359), respectively, and the  $N^6$ -side chain ion  $C_5H_9^+$  ( $m/z$  69.0704). The isopentenyladenine[-2H] intermediate ion  $C_{10}H_{12}N_5^+$  (Fig. 5b) showed a much more complex fragmentation pattern that was rich in purine ring fragments ( $m/z$  146.0458, 136.0610 and 119.0356) with ions of the side chain structure,  $m/z$  84.8014 ( $C_{10}H_4N^+$ ) and  $m/z$  67.0548 ( $C_5H_7^+$ ). Besides other peerless fragments ( $m/z$  175.0980, 173.0826, 159.0663), a fragment  $C_{10}H_9N_4^+$  ( $m/z$  185.0818) showed the maximal relative intensity. The same ion was also present in the MS/MS spectrum of *trans*-zeatin, although with lower intensity.

The fragmentation pattern of *N*-methyl-isopentenyladenine ion ( $C_{11}H_{16}N_5^+$ ), see Fig. 5c, showed two ions  $C_7H_8N_5^+$  ( $m/z$  162.0767) and  $C_6H_8N_5^+$  ( $m/z$  150.0770) that contained the *N*-methyladenine ring and fragments  $C_6H_8N^+$  ( $m/z$  94.0662) and  $C_5H_9^+$  ( $m/z$  69.0711) that originated from a side chain. Other ions with lower intensity (e. g.  $C_5H_7N_4^+$ , 123.0661) were observed. The *N*-methyl-isopentenyladenine[-2H] intermediate of the  $C_{11}H_{14}N_5^+$  ion (Fig. 5d) showed a more complex MS/MS spectrum than that of *N*-methyl-isopentenyladenine. The presence of fragment pair  $C_6H_8N_5^+$  ( $m/z$  150.0756) and  $C_5H_7^+$  ( $m/z$  67.0553) corresponded with the fragment spectrum of *N*-methyl-isopentenyladenine. Also, the pattern of purine ring fragments and associates ( $m/z$  185.0838, 175.0962, 159.0670, 148.0640) indicated similarity with the fragmentation of the isopentenyladenine[-2H] intermediate (compare Fig. 5b). However, unique ions of  $m/z$  199.0954 ( $C_{11}H_{11}N_4^+$ ),  $m/z$  134.0591 ( $C_6H_6N_4^+$ ) and  $m/z$  121.0515 ( $C_5H_5N_4^+$ ), as well as  $m/z$  96.0804 ( $C_6H_{10}N^+$ ) and  $m/z$  82.0647 ( $C_5H_8N^+$ ) that originated from the side chain, were also observed.

The fragmentation pattern of the *trans*-zeatin  $C_{10}H_{14}N_5O^+$  ion (Supplement 2a) showed, besides various purine ring fragments and associates ( $m/z$  185.0815, 148.0614, 136.0618 and 119.0356), a loss of water and a rising of ion  $C_{10}H_{12}N_5^+$  ( $m/z$  202.1088). Fragments of the *trans*-zeatin side chain were not found. The *trans*-zeatin[-2H] intermediate ( $C_{10}H_{12}N_5O^+$ ) fragmentation (Supplement 2b) showed a similar loss of water ( $C_{10}H_{10}N_5^+$ ,  $m/z$  200.0909) as the *trans*-zeatin MS/MS spectrum, one unique ( $C_7H_6N_5^+$ ,  $m/z$  160.0631) and three corresponding purine ring ions  $C_6H_6N_5^+$  (148.0604),  $C_5H_6N_5^+$

(136.0622) and  $C_5H_3N_4^+$  (119.0368). A side chain fragment  $C_5H_7O^+$  ( $m/z$  83.0494) was identified as a pair fragment of  $C_5H_6N_5^+$ .

The fragmentation of the kinetin parent ion with calculated molecular formula  $C_{10}H_{10}N_5O^+$  (Supplement 3a) showed the fragments  $m/z$  188.0929 ( $C_9H_{10}N_5^+$ ),  $m/z$  148.0608 ( $C_6H_6N_5^+$ ),  $m/z$  119.0357 ( $C_5H_3N_4^+$ ) and the most intense ion  $m/z$  81.0338 ( $C_5H_5O^+$ ). Only  $m/z$  81.0338 was produced from the  $N^6$ -side chain, the other fragments contained the purine ring. The kinetin[-2H] intermediate ion with elementary composition  $C_{10}H_8N_5O^+$  (Supplement 3b) underwent the loss of water under a rising of ion  $C_{10}H_6N_5^+$  ( $m/z$  196.0617) and made the most intensive fragment  $m/z$  119.0345 ( $C_5H_3N_4^+$ ) with a pair fragment of the side chain  $m/z$  96.0453 ( $C_5H_6NO^+$ ). Both a three-fold higher intensity of ions  $m/z$  186.0758 ( $C_9H_8N_5^+$ ) and  $m/z$  146.0453 ( $C_6H_4N_5^+$ ) compared to the corresponding kinetin fragments  $m/z$  188.0929 ( $C_9H_{10}N_5^+$ ) and  $m/z$  148.0608 ( $C_6H_6N_5^+$ ) and an intensity loss of ion  $m/z$  81.0347 ( $C_5H_5O^+$ ) were observed.

The fragmentation of the *p*-topolin ion of  $C_{12}H_{12}N_5O^+$  (Supplement 4a) showed a major occurrence of ion  $m/z$  136.0608 ( $C_5H_6N_5^+$ ) and also an ion of  $m/z$  107.0499 ( $C_7H_7O^+$ ) derived from the cytokinin side chain. Fragments  $C_6H_6N_5^+$  ( $m/z$  148.0616) and  $C_5H_3N_4^+$  ( $m/z$  119.0346) were also observed. On the other hand, the *p*-topolin[-2H] intermediate of  $C_{12}H_{10}N_5O^+$  (Supplement 4b) produced the most intensive fragment pair of  $m/z$  119.0354 ( $C_5H_3N_4^+$ ) and  $m/z$  122.0598 ( $C_7H_8NO^+$ ) originating from the side chain. Ions of  $m/z$  146.0460 ( $C_6H_4N_5^+$ ) and  $m/z$  95.0495 ( $C_6H_7O^+$ ) were the second pair products of fragmentation. The relative intensity of the ion fragment  $C_5H_6N_5^+$  decreased from 100 % in the *p*-topolin MS/MS spectrum to 4.3 % in the MS/MS spectrum of the *p*-topolin[-2H] intermediate.

## DISCUSSION

Cytokinin oxidase/dehydrogenase is a covalent flavoprotein that plays a crucial role in tuning the level of plant cytokinins. It is capable of catalysing the oxidative side chain cleavage of a number of cytokinins. A homology model [15] and later the crystal structure [9] revealed a two-domain folding topology similar to members of the vanillyl-alcohol oxidase flavoprotein family [16]. Similar to vanillyl-alcohol oxidase, ZmCKX1 can pass electrons from the covalently bound flavin to molecular oxygen. However, this process is not very efficient, as the reaction turnover of ZmCKX1 can proceed much faster with quinone-type electron acceptors [5]. The active site of ZmCKX1, where cytokinin catabolism takes place, was found to be unique among three-dimensional structures of proteins. A narrow pore, leading to the active site, allows entry only to an aliphatic or aromatic side-chain of the cytokinin, the substrate moiety that is oxidatively cleaved. Adenine, the second entity of the cytokinin substrate, protrudes on the protein surface and seals the hydrophobic active site. Such a conformation

leaves no space for an electron acceptor to bind in the active site [9] indicating that electron transfer to the protein surface may take place.

Although all cytokinin substrates bind, in principle, in the same structural way to the enzyme, some cytokinins, such as *trans*-zeatin or isopentenyladenine, react very quickly with the enzyme while others, such as *N*-methyl-isopentenyladenine, *p*-topolin or kinetin, react slowly [5]. In fact, until recently, aromatic cytokinins were considered not to be substrates of CKX [17]. However, data presented in the work of Frébortová et al. (2004) and in this study show that ZmCKX1 is capable of cleaving aromatic cytokinins, albeit at very low rates. All substrates tested in this study show a similar affinity for the enzyme as binding constants only varied to a small extent with  $K_d$  ranging from 13 to 66  $\mu\text{M}$ . This indicates that the enzyme mainly recognises the cytokinins by interacting with the adenine moiety while it can easily accommodate varying types of side chains. This is in agreement with the crystallographic data which show that there are several specific H-bond interactions between the protein and the adenine part of the substrate or product. The  $K_d$  values also nicely correspond with the measured  $K_m$ ' values. Only the  $K_m$ ' value observed for isopentenyladenine is relatively low which can be explained by the fact that the rate of flavin reduction is extremely fast ( $1150 \text{ s}^{-1}$ ).

To elucidate the mechanism of CKX-mediated catalysis, including intermediate formation, a pre-steady state kinetic analysis of CKX from *Zea mays* (ZmCKX1) has been performed in this study. The advantage of stopped-flow kinetic analysis, employed here, is the possibility to measure the reductive and oxidative half-reactions of the flavin-containing oxidoreductase separately. The time-resolved analysis also allows detection of possible spectral intermediates. It is known that isopentenyladenine degradation by CKX is connected with formation of an intermediate reported to be the cytokinin imine product [10] that absorbs at 310 nm. Chemical hydrolysis of this imine would produce adenine and a corresponding aldehyde. The measurements presented here show that catalytic cleavage of all presented cytokinins proceeds first through a formation of a reaction intermediate detectable around 360 nm (Fig. 2). This intermediate is not very stable and dissociates within seconds or even tenths of seconds. It can be ascribed to a formation of the binary complex between the corresponding imine and the reduced enzyme. A similar binary complex was previously observed in the reaction of a flavoprotein homolog, vanillyl-alcohol oxidase, with 4-(methoxymethyl) phenol [18]. In that case, the absorbance increase at 364 nm was ascribed to the formation of a binary complex between the enzyme and the quinone-methide of 4-(methoxymethyl)phenol.

For the cytokinins that rapidly reduce the flavin cofactor, isopentenyladenine and *trans*-zeatin, the interaction with ZmCKX1 also showed another unique feature that was disclosed by stopped-flow kinetic analysis: a charge transfer complex is formed resulting in an absorbance band above 550 nm. This complex consists of reduced ZmCKX1 and a tightly bound cytokinin-derived imine positioned near the flavin cofactor. Binding of the imine product in close proximity of the flavin cofactor was also confirmed by X-ray analysis [9]. The crystal structures of ZmCKX1 in complex with the imines of isopentenyladenine and *trans*-zeatin showed that the imines are bound on top of the isoalloxazine

ring of the cofactor. The structure of ZmCKX1 with the imine of *trans*-zeatin disclosed two possible conformations in which it is bound next to the flavin. In one conformation, charge-transfer is not feasible which might explain the lower intensity observed for the *trans*-zeatin induced charge-transfer absorbance when compared to isopentenyladenine. However, the two conformations of binding might also reflect the transition between the first formed binary complex of reduced ZmCKX1 with the imine and the subsequent conformation (spectra B and C in Fig. 2a and 2b). The reductive half-reaction of ZmCKX1 with isopentenyladenine showed that it is undoubtedly the best substrate of the enzyme, being able to reduce the enzyme with a rate of about  $1150\text{ s}^{-1}$ . An additional hydroxyl group present in *trans*-zeatin (Fig. 1) apparently has a negative effect on the flavin reduction reaction, decreasing the reduction rate by  $\sim 75\%$  (Table 1).

*N*-Methylation of isopentenyladenine (Fig. 1) has a profound effect on the reactivity of this substrate with the enzyme. While the  $K_m$ ' value is in the same range as other substrates, the  $k_{\text{cat}}$  is low (Table 1). This can be explained by the poor reactivity of the carbon atom that should be oxidised by the flavin. Reduction of ZmCKX1 by *N*-methyl-isopentenyladenine revealed two reductive phases. Both reductive phases are slow, indicating that flavin reduction is limiting the turnover rate for this substrate. The observed reduction rates for both phases are a thousand-fold lower than that with isopentenyladenine. The rates for both reductive phases were strongly dependent on substrate concentration with similar apparent binding constants ( $25.1$  and  $13.3\ \mu\text{M}$ ) and relatively slow reduction rates ( $1.08$  and  $0.24\ \text{s}^{-1}$ , respectively). The extent by which the flavin was reduced in both phases, indicated by the absorbance decrease at around  $450\ \text{nm}$  as analysed by global diode array data analysis, did not change depending on the substrate concentration. The observed rates obeyed Michaelis-Menten type kinetic behaviour in which the reduction rate approaches zero when decreasing the substrate concentration. This is indicative for a reduction step that is virtually irreversible. Our data are consistent with a mechanism in which two species of ZmCKX1 exist, one reflecting a conformation that is slightly more efficient in oxidising *N*-methyl-isopentenyladenine when compared with the other population. No charge transfer complex was observed during reduction of the enzyme with *N*-methyl-isopentenyladenine, indicating that the formed oxidised cytokinin is positioned in a different way when compared with the non-methylated cytokinin, isopentenyladenine.

Relatively slow reduction rates were also observed for kinetin and *p*-topolin. In both cases the reduction of the flavin cofactor involved a one step process. In this first relatively fast phase of flavin reduction, the absorbance around  $450\ \text{nm}$  decreased significantly suggesting full reduction. This indicates that the reduction is an irreversible process. This was confirmed when analysing the observed substrate concentration-dependent reduction rates: the data could be fitted using a Michaelis-Menten equation. The rate of oxidation for these two cytokinins appears to be limited to the rate of flavin reduction.

Once the binary complex between the reduced enzyme and the cytokinin-derived imine is dissociated, a free cytokinin imine-product is formed. It is noteworthy for all substrates tested in this

study that the binary complex decay is by one or two orders of magnitude slower than flavin reduction (Table 1). This indicates that the binary complex accumulates during the reductive half-reaction. Despite this fact, however, binary complexes are not very stable, and are lost within a few seconds or tenths of seconds.

It is known that hydrolysis converts the imine-products to adenine and the corresponding aldehydes that are routinely detected as final products of the enzymatic reaction. As the non-enzymatic hydrolysis is a relatively slow process, the imine accumulates in the reaction mixture as long as a supply of the substrate is available (Fig. 3). Cytokinin imines generally accumulate more in slightly acidic rather than in slightly alkaline pH, whereas extreme pH values facilitate instant hydrolysis. For *trans*-zeatin, an additional reaction occurs in slightly alkaline pH that slows down hydrolytic cleavage and delays enzyme reoxidation (Fig. 3c). The aldehydes derived from isopentenyladenine, *N*-Methyl-isopentenyladenine, *trans*-zeatin, and kinetin have absorption maxima around or below 300 nm (Supplement 1), and thus were not directly detectable in the presented measurements. On the other hand, the *p*-topolin-derived aldehyde, 4-hydroxybenzaldehyde, has an absorption maximum at 330 nm and is easily detectable in alkaline pH. When the enzyme was reduced by *p*-topolin, only the amount of the aldehyde equimolar to the enzyme was formed, and FAD reoxidation was observed. This indicates that the active site of the enzyme becomes blocked by the *p*-topolin imine.

Each cytokinin studied gave specific fragmentation in the MS/MS experiments, but a neutral loss of the side chain amine resulting in formation of the cation  $C_5H_3N_4^+$  that had low relative intensity (2-13%) was conserved. Cytokinins, when converted by ZmCKX1, produced cytokinin[-2H] intermediates that fragmented differently. Fragmentation patterns of these cytokinin[-2H] intermediates clearly supported the structure of cytokinin imine products. Based on our results, the likeliest molecular structures of cytokinin[-2H] intermediates are shown in Figure 5 and Supplements 2-4. The [-2H] intermediates of kinetin, *p*-topolin and isopentenyladenine produced the ion  $C_5H_3N_4^+$  and the corresponding ions formed from the side chain ( $C_5H_6NO^+$ ,  $C_7H_8NO^+$  and  $C_5H_{10}N^+$ , respectively) that became one of the major ions in the spectrum. The presence of the ion  $C_6H_4N_5^+$  in the MS/MS spectrum of the kinetin[-2H] intermediate (Supplement 3b) and the *p*-topolin[-2H] intermediate (Supplement 4b) was indicative of a double bond on the N6 nitrogen of adenine. In contrast, the  $C_6H_4N_5^+$  ion and the intensive pair ions ( $C_5H_6N_5^+$  and  $C_5H_7^+$ ) were observed in the isopentenyladenine[-2H] intermediate fragmentation spectrum, indicating two possible locations of the double bond on the  $N^6$ -side chain (Fig. 5b).

Cleavage of *trans*-zeatin to the ion  $C_5H_3N_4^+$  led to a neutral loss of the side chain, but a side chain fragment  $C_5H_7O^+$  was observed as a pair fragment to the ion  $C_5H_6N_5^+$ . The presence of ions  $C_7H_6N_5^+$  and  $C_6H_6N_5^+$  in the same MS/MS spectrum (Supplement 2b) pointed out two possible double bond locations on the  $N^6$ -side chain, but neither double bond was observed on the N6.

For *N*-methyl-isopentenyladenine, the fragmentation of the [-2H] intermediate did not clearly show the structure of an imine. The pair fragments ( $C_6H_8N_5^+$ ,  $C_5H_7^+$ ) and fragments of the side chain ( $C_6H_{10}N^+$ ,  $C_5H_8N^+$ ) were observed and indicated double bond location on the  $N^6$ -side chain but not its exact position. On the basis of the results we propose that the cleavage of *N*-methyl-isopentenyladenine proceeds via a different mechanism than for isopentenyladenine. Fig. 6a shows the reductive half-reaction of ZmCKX1 with isopentenyladenine [9], where a hydride ion is transferred from the  $\alpha$ -carbon of the cytokinin to the N5 atom of FAD, which is followed by proton abstraction from N6 of the cytokinin by a catalytic base Asp169 to form the imine product. In case of the *N*-methyl derivative, we propose that again a hydride is transferred to the flavin N5 atom (Fig. 6b). However, the proton abstraction from N6 of the cytokinin substrate is impossible due to the methylation of the amine group. Therefore, in this case the  $\alpha$ -methylene group of the isoprenoid side chain is deprotonated resulting in the formation and migration of a double bond. The identity of the base in this mechanism is as yet unknown. Asp169 or the reduced flavin cofactor could fulfill this function. Alternatively, they could both be able to act as a base which would be in line with the observation that reduction of the enzyme with *N*-methyl-isopentenyladenine appears to follow two kinetic routes (see above). An analysis of an Asp169 mutant enzyme might reveal more details concerning this reaction step.

The results of this paper show that cytokinin cleavage via ZmCKX1 undergoes cascade catalysis and the resulting products adenine and a side chain aldehyde are not directly formed by the enzyme, but result from a hydrolytic cleavage of cytokinin imine products. These products are rather stable and might have some physiological role during the cytokinin degradation in plants. In the case of *trans*-zeatin, the resulting aldehyde 4-hydroxy-3-methyl-2-butenal has been recognised as an oxidation product of isoprene emitted from vegetation [19]. This aldehyde is likely to undergo a cyclization reaction to form 2-methylfuran as the product. This reaction is facilitated in alkaline pH and slows down the catalytic reaction of ZmCKX1 (see Fig. 3c). A similar compound, 4-hydroxy-2-butenal is known as a precursor of furan during decomposition of polyunsaturated fatty acids [20]. The possible physiological role of cytokinin imines, resulting aldehydes and their metabolites remains to be investigated.

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**Table 1 Kinetic parameters of the catalytic reaction of ZmCKX1 with various cytokinins**

Rate constants of the processes of flavin reduction, FADH<sub>2</sub>-imine complex decay, and charge transfer formation and decay were monitored under anaerobic conditions at 451, 360 and 650 nm, respectively, using single-wavelength stopped flow spectrophotometry for 0.25 mM cytokinin substrates with 10 μM ZmCKX1, in 75 mM imidazole/HCl, pH 6.5, at 25 °C. The enzyme-substrate complex dissociation constants ( $K_d$ ) were determined at 451 nm with 3 μM ZmCKX1 enzyme reduced with cytokinin substrates in concentration range 15-250 μM, under the same conditions. Turnover rates and the respective apparent  $K_m'$  were determined using the 4-aminophenol method (Frébort et al., 2002) with 0.44 - 120 nM ZmCKX1 and 1.25 mM CuCl<sub>2</sub> as the electron acceptor in 75 mM imidazole/HCl, pH 6.5, at 25 °C. For determining the rate of reduction and the  $K_d$  value, the observed reduction rates were measured using at least 7 different substrate concentrations. Both the steady-state and the reduction kinetic data could be analysed using a Michaelis-Menten equation, yielding steady state kinetic parameters ( $k_{cat}$ ,  $K_m'$ ) and kinetic parameters for the enzyme reduction ( $k_{red}$ ,  $K_d$ ). The standard deviations obtained upon fitting the data are indicated. For the rates of imine decay and charge transfer decay, the kinetic data were derived from curve fitting using an exponential decay model. The derived values exhibited standard deviations of < 5%.

Cytokinin substrate	FAD reduction		FADH <sub>2</sub> -imine complex decay	Charge transfer decay		Aldehyde product formation	
	$k_{red}$ (s <sup>-1</sup> )	$K_d$ (μM)	Rate constant (s <sup>-1</sup> )	Amplitude (A <sub>650</sub> )	Observed rate (s <sup>-1</sup> )	$k_{cat}$ (s <sup>-1</sup> )	$K_m'$ (μM)
Isopentenyladenine	1150 ± 50	15.1 ± 2.5	62 7.0 <sup>1)</sup>	0.0115 0.0028 <sup>1)</sup>	60 8.0 <sup>1)</sup>	40.0 ± 2.8	1.0 ± 0.6
<i>trans</i> -Zeatin	244 ± 5	13.1 ± 1.1	179 3.1 <sup>1)</sup>	0.0140 0.0028 <sup>1)</sup>	222 2.6 <sup>1)</sup>	- <sup>2)</sup>	14 <sup>2)</sup>
<i>N</i> -Methyl-isopentenyladenine	1.08 ± 0.02 0.24 ± 0.01 <sup>1)</sup>	25.1 ± 1.4 13.3 ± 3.2 <sup>1)</sup>	0.16 0.024 <sup>1)</sup>	0	-	0.13 ± 0.01	23.9 ± 1.6
Kinetin	0.82 ± 0.02	66 ± 3	0.013	0	-	1.90 ± 0.04	84.9 ± 6.1
<i>p</i> -Topolin	0.11 ± 0.01	24 ± 3	0.009	0	-	0.23 ± 0.01	38.4 ± 3.3

<sup>1</sup> The event was biphasic producing a second rate constant/amplitude shown in parentheses.

<sup>2</sup> The method used for aldehyde detection is not reliable enough to determine kinetic parameters (Frébort et al., 2002). The  $K_m'$  value shown here was obtained by measuring reduction rate of 2,6-dichlorophenol indophenol (Bilyeu et al., 2001) and is given only for comparison.

**Table 2 MS/MS analysis of cytokinins and cytokinin reaction products**

Accurate masses of the parent ions of studied cytokinins (0.05 mM) in 30 mM (NH<sub>4</sub>)<sub>2</sub>CO<sub>3</sub>, pH 8.0, and their imine products (cytokinin [-2H]) after conversion by 1.5 μM ZmCKX1 (2 min incubation at 25 °C), were obtained by Q-TOF hybrid mass analyser. For experimental details see Materials and Methods. Experimental values (m/z Exp.) are compared with those calculated for respective ion formulas (m/z Calc.).

<b>Cytokinin substrate</b> <b>Cytokinin imine product</b>	<b>m/z Exp.</b>	<b>Formula</b>	<b>m/z Calc.</b>	<b>Difference</b> (ppm)
Isopentenyl adenine	204.1247	C <sub>10</sub> H <sub>14</sub> N <sub>5</sub> <sup>+</sup>	204.12492	-1.1
Isopentenyl adenine [-2H]	202.1090	C <sub>10</sub> H <sub>12</sub> N <sub>5</sub> <sup>+</sup>	202.10927	-1.3
<i>trans</i> -Zeatin	220.1204	C <sub>10</sub> H <sub>14</sub> N <sub>5</sub> O <sup>+</sup>	220.11984	2.6
<i>trans</i> -Zeatin [-2H]	218.1050	C <sub>10</sub> H <sub>12</sub> N <sub>5</sub> O <sup>+</sup>	218.10419	3.7
<i>N</i> -Methyl-isopentenyladenine	218.1400	C <sub>11</sub> H <sub>16</sub> N <sub>5</sub> <sup>+</sup>	218.14057	-2.5
<i>N</i> -Methyl-isopentenyladenine [-2H]	216.1254	C <sub>11</sub> H <sub>14</sub> N <sub>5</sub> <sup>+</sup>	216.12492	2.2
Kinetin	216.0880	C <sub>10</sub> H <sub>10</sub> N <sub>5</sub> O <sup>+</sup>	216.08854	-2.5
Kinetin [-2H]	214.0720	C <sub>10</sub> H <sub>8</sub> N <sub>5</sub> O <sup>+</sup>	214.07289	-4.1
<i>p</i> -Topolin	242.1030	C <sub>12</sub> H <sub>12</sub> N <sub>5</sub> O <sup>+</sup>	242.10419	-4.9
<i>p</i> -Topolin [-2H]	240.0881	C <sub>12</sub> H <sub>10</sub> N <sub>5</sub> O <sup>+</sup>	240.08854	-1.8

## Figure legends

### Figure 1 Cytokinin dehydrogenase and cytokinins.

(a) Reaction mechanisms of cytokinin dehydrogenase (EC 1.5.99.12) and (b) structures of cytokinins used in this study.

### Figure 2 Anaerobic reduction of ZmCKX1 with cytokinins

Using a stopped-flow equipped with a photodiode array detector, spectral data were obtained upon anaerobic mixing of ZmCKX1 with cytokinin substrates. The spectral species shown were derived from global analysis of the data obtained. The data could be fitted using a model in which species A decays in an exponential fashion to form B ( $A \rightarrow B$ ). If needed, more exponential decay steps were included in the model. Typically, 23  $\mu\text{M}$  ZmCKX1 was mixed with 0.5 mM of a cytokinin substrate (except for *N*-methyl-isopentenyladenine and kinetin, 0.25 mM was used) under anaerobic conditions, in 75 mM imidazole/HCl, pH 6.5, at 25 °C. The deconvoluted spectra are shown for: (a) isopentenyladenine, (b) *trans*-zeatin, (c) *N*-methyl-isopentenyladenine, (d) kinetin, and (e) *p*-topolin. For *p*-topolin (e), spectra of species B obtained with 75 mM Tris/HCl, pH 7.5 (dashed) and pH 8.5 (dotted) are shown. Time courses obtained by global analyses for the respective species are shown in the insets. For experimental details see the Materials and Methods section.

### Figure 3 Cytokinin intermediate formation and decay

Time courses of the reaction intermediate formation and decay (310 nm, circles) and oxidation changes of the FAD cofactor (451 nm, triangles) observed for ZmCKX1 (5  $\mu\text{M}$ ) that was mixed aerobically with 125  $\mu\text{M}$  cytokinins: (a) isopentenyladenine, (b) *N*-methyl-isopentenyladenine, and (c) *trans*-zeatin in 75 mM imidazole/HCl, pH 6.5 (left panels) and 75 mM Tris/HCl, pH 8.0 (right panels). The data could be fitted using a model in which species A is converted in a hyperbolic fashion to species B that then decays exponentially to form C.

### Figure 4 Interaction of ZmCKX1 with *p*-topolin

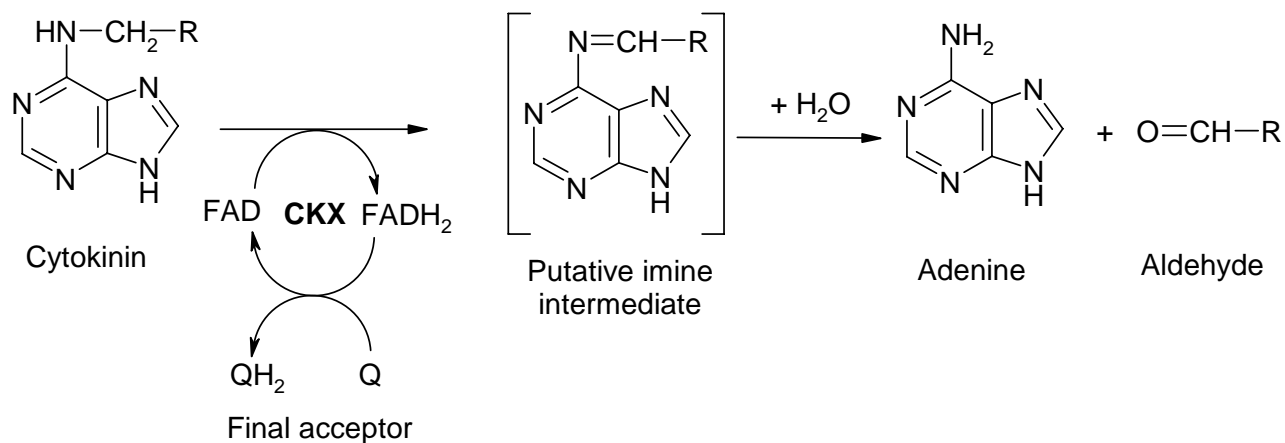
(a) Spectral changes in the range of 250-700 nm and (b) time courses of the reaction intermediate decay (360 nm, open circles), reduction of the FAD cofactor (451 nm, triangles), and formation of the product 4-hydroxybenzaldehyde (330 nm at pH 8.0, filled circles) observed for ZmCKX1 (5  $\mu\text{M}$ ) that was mixed aerobically with 125  $\mu\text{M}$  *p*-topolin in 75 mM imidazole/HCl, pH 6.5 (left panels) and with 13  $\mu\text{M}$  *p*-topolin in 75 mM Tris/HCl, pH 8.0 (right panels). Arrows in the panel (a) indicate direction

of spectral changes as observed from 1.3 min to 118.7 min of the reaction course. The data in panel (b) could be fitted using a model in which species A decays in an exponential fashion to form B. To differentiate the kinetics of the formation of 4-hydroxybenzaldehyde (330 nm) at pH 8, the species are marked as A' and B'.

**Figure 5 MS/MS analysis of the ZmCKX1 reaction** with cytokinins: (a) isopentenyladenine (50  $\mu\text{M}$ ), and (b) isopentenyladenine[-2H] intermediate, (c) *N*-methyl-isopentenyladenine (50  $\mu\text{M}$ ) and (d) *N*-methyl-isopentenyladenine[-2H] intermediate. Cytokinins were dissolved in 30 mM  $(\text{NH}_4)_2\text{CO}_3$ , pH 8.0. For the reaction intermediate analysis, a reaction mixture that contained 1.5  $\mu\text{M}$  ZmCKX1 and 50  $\mu\text{M}$  cytokinin in 30 mM  $(\text{NH}_4)_2\text{CO}_3$ , pH 8.0, was prepared and incubated at 25 °C for 2 min before the measurement. The high resolution MS/MS experiments were done on a hybrid mass analyser Q-TOF *micro* with MassLynx data system software. The samples were applied in the form of an in-line spray at the injection rate of 10  $\mu\text{l}/\text{min}$ . The data acquisition was done in the range 50-1000 Da with the cycle time 47  $\mu\text{s}$ , scan time 0.5 s, and collision energy 15, 20, 25, 30 and 35 V. The accurate masses of the parent ions and its fragments were calculated and used for the determination of the elementary composition and structure with fidelity within 15 ppm.

**Figure 6 Reaction mechanisms of cytokinin dehydrogenase** for (a) isopentenyladenine described by Malito et al. [9] and (b) *N*-methyl-isopentenyladenine proposed in this work. Note that Asp169 could also act as a base, instead of the N5 of the reduced flavin (see Discussion).

a)



b)

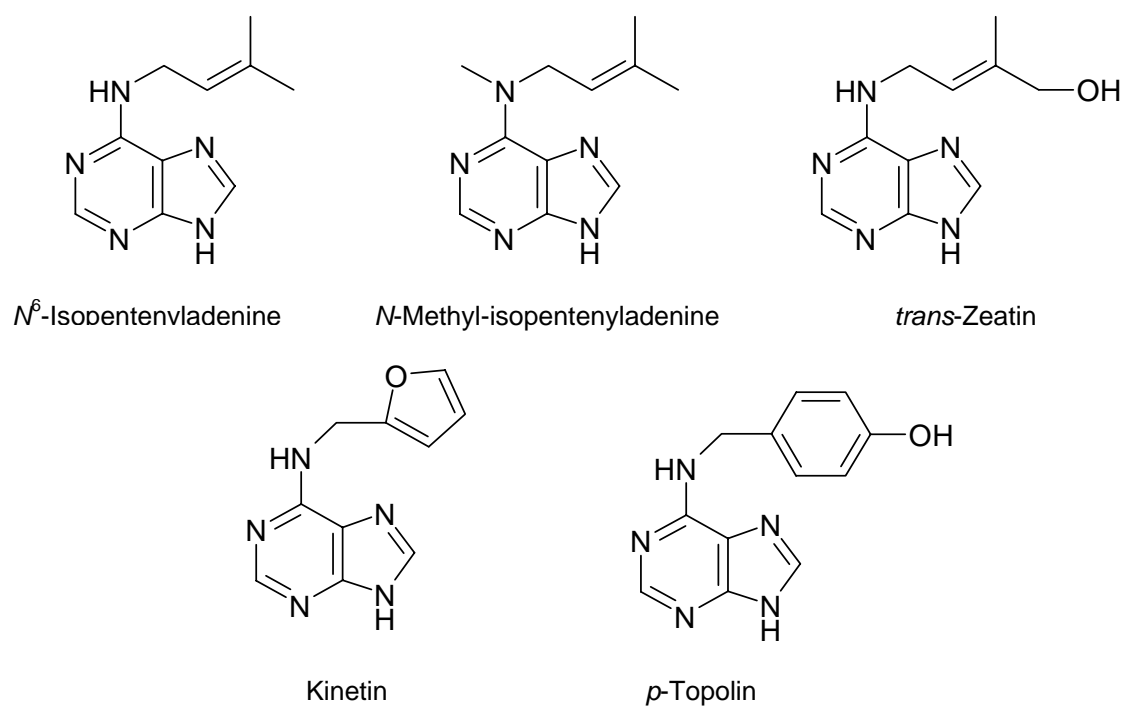


Fig. 1

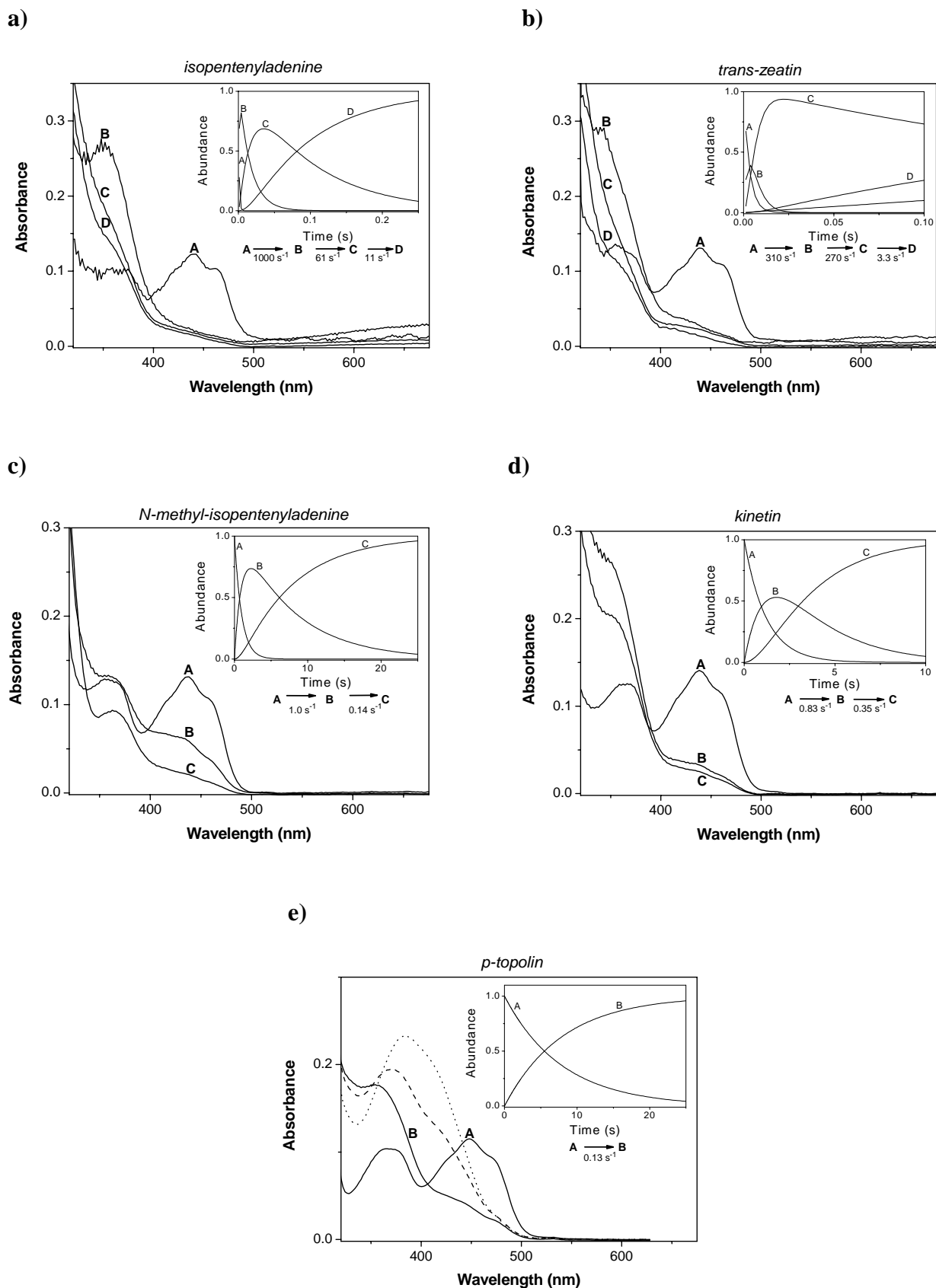


Fig. 2

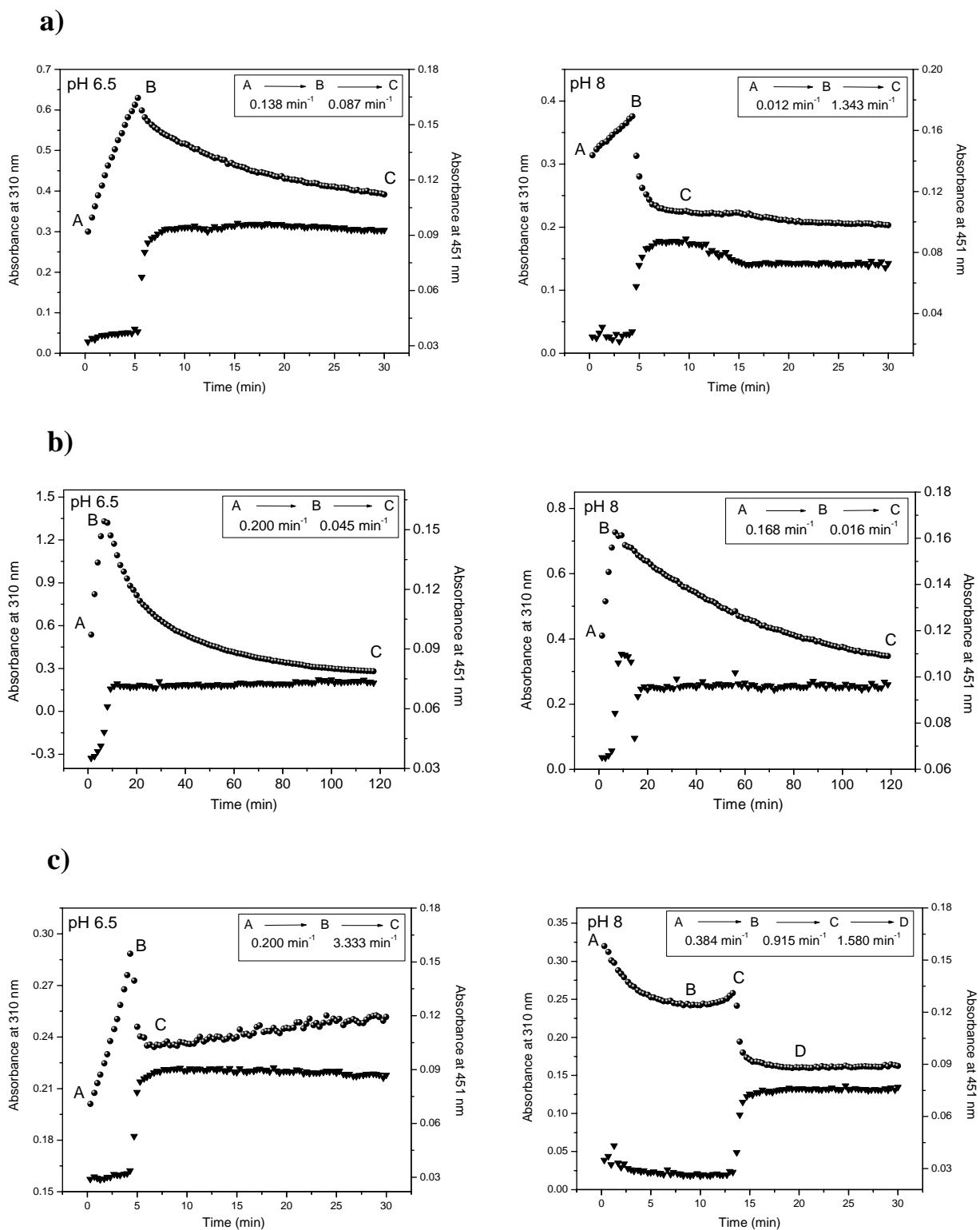
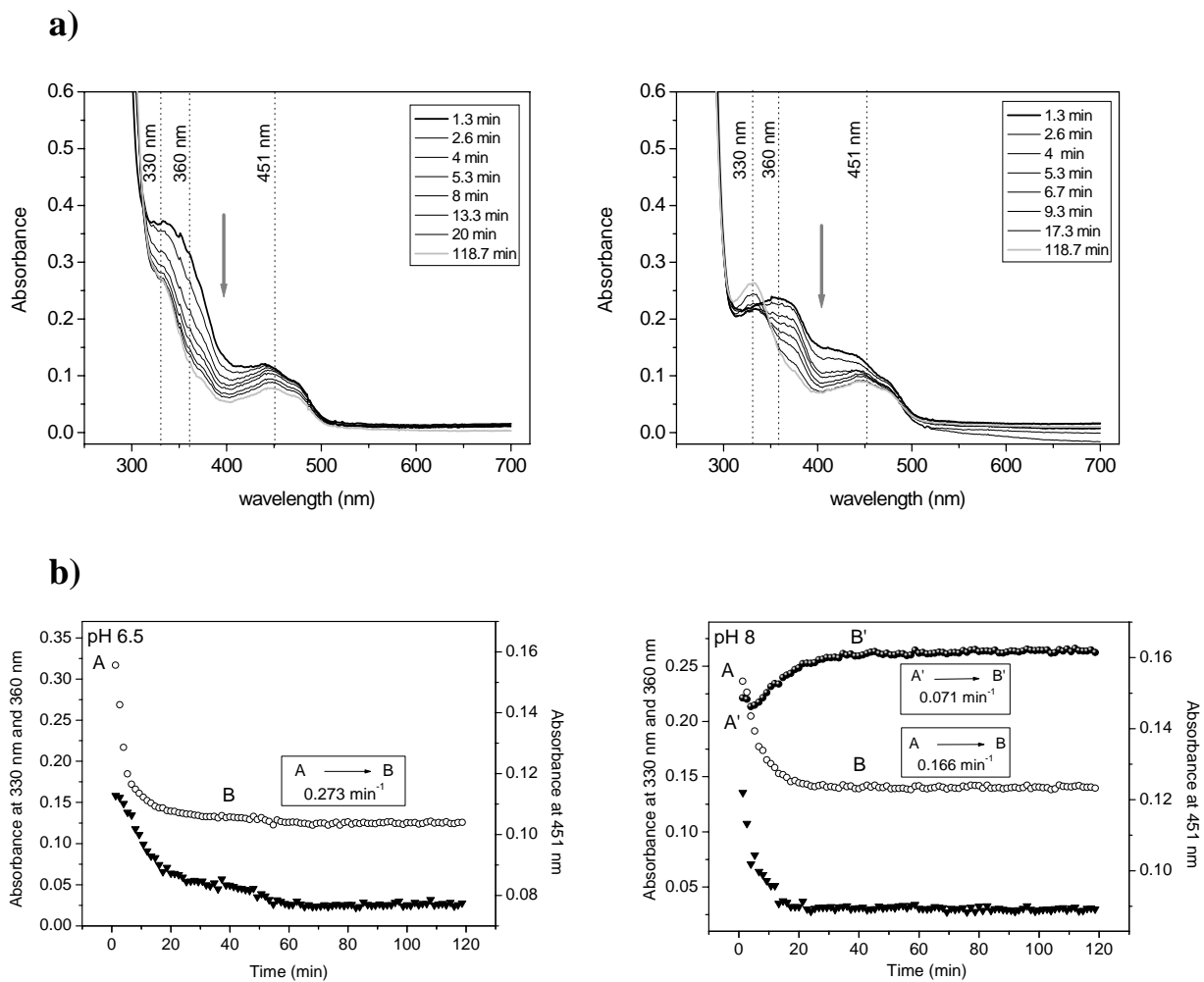


Fig. 3





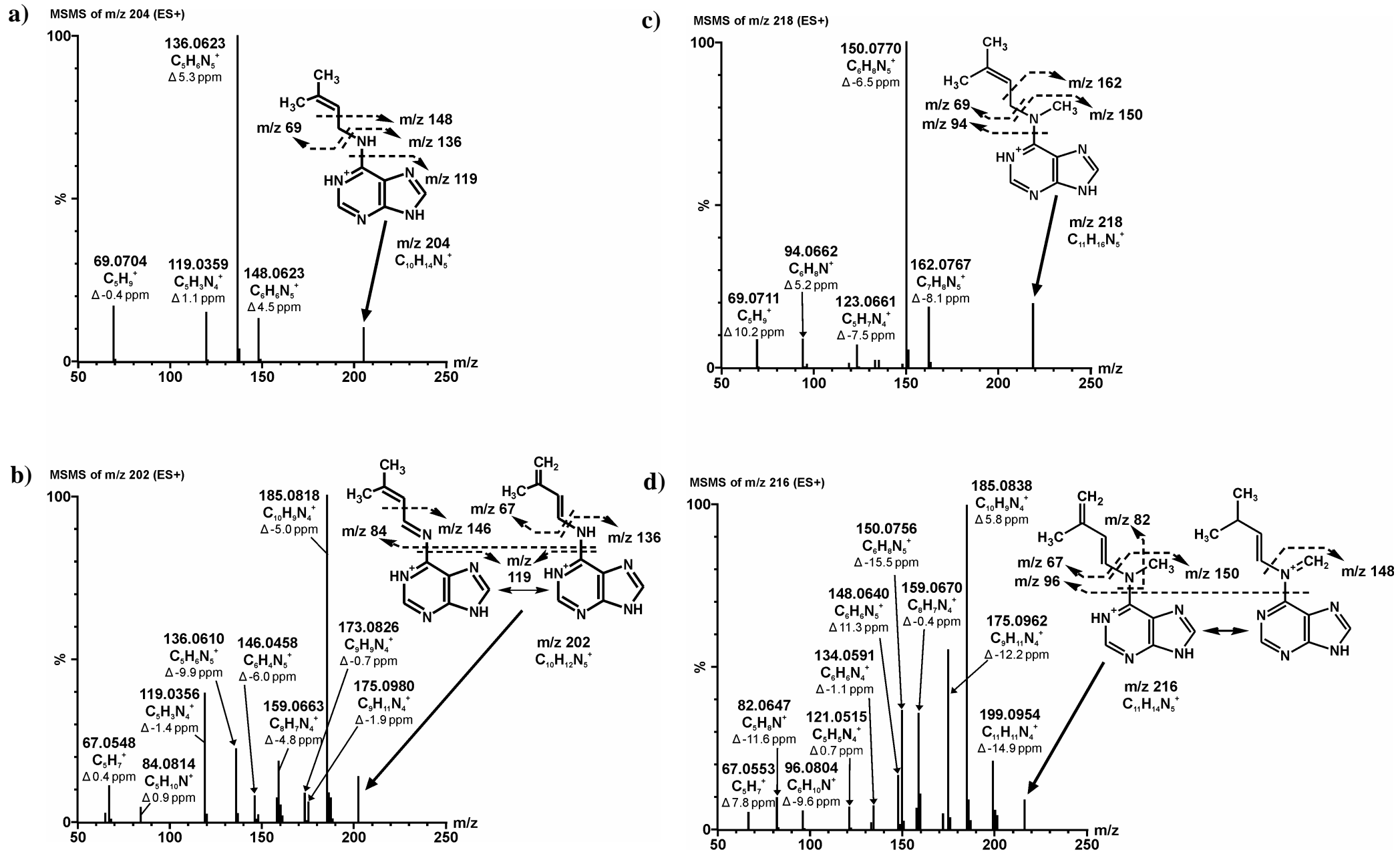


Fig. 5

