

## Catalytic reaction of cytokinin dehydrogenase: preference for quinones as electron acceptors

Jitka FRÉBORTOVÁ\*, Marco W. FRAAIJE†, Petr GALUSZKA‡, Marek ŠEBELA§, Pavel PEČ§, Jan HRBÁČ||, Ondřej NOVÁK\*, Kristin D. BILYEU¶, James T. ENGLISH\*\* and Ivo FRÉBORT‡<sup>1</sup>

\*Laboratory of Growth Regulators, Faculty of Science, Palacký University/Institute of Experimental Botany of the Academy of Science, Šlechtitelů 11, 783 71 Olomouc, Czech Republic, †Laboratory of Biochemistry, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands, ‡Division of Molecular Biology, Faculty of Science, Palacký University, Šlechtitelů 11, 783 71 Olomouc, Czech Republic, §Department of Biochemistry, Faculty of Science, Palacký University, Šlechtitelů 11, 783 71 Olomouc, Czech Republic, ||Department of Physical Chemistry, Faculty of Science, Palacký University, tř. Svobody 26, 771 46 Olomouc, Czech Republic, ¶USDA/ARS, 210 Waters Hall, Columbia, MO 65211, U.S.A., and \*\*Department of Plant Microbiology and Pathology, University of Missouri, Columbia, MO 65211, U.S.A.

The catalytic reaction of cytokinin oxidase/dehydrogenase (EC 1.5.99.12) was studied in detail using the recombinant flavoenzyme from maize. Determination of the redox potential of the covalently linked flavin cofactor revealed a relatively high potential dictating the type of electron acceptor that can be used by the enzyme. Using 2,6-dichlorophenol indophenol, 2,3-dimethoxy-5-methyl-1,4-benzoquinone or 1,4-naphthoquinone as electron acceptor, turnover rates with *N*<sup>6</sup>-(2-isopentenyl)adenine of approx. 150 s<sup>-1</sup> could be obtained. This suggests that the natural electron acceptor of the enzyme is quite probably a *p*-quinone or similar compound. By using the stopped-flow technique, it was found that the enzyme is rapidly reduced by *N*<sup>6</sup>-(2-isopentenyl)adenine ( $k_{\text{red}} = 950 \text{ s}^{-1}$ ). Re-oxidation of the reduced enzyme by molecular

oxygen is too slow to be of physiological relevance, confirming its classification as a dehydrogenase. Furthermore, it was established for the first time that the enzyme is capable of degrading aromatic cytokinins, although at low reaction rates. As a result, the enzyme displays a dual catalytic mode for oxidative degradation of cytokinins: a low-rate and low-substrate specificity reaction with oxygen as the electron acceptor, and high activity and strict specificity for isopentenyladenine and analogous cytokinins with some specific electron acceptors.

**Key words:** cytokinin dehydrogenase (cytokinin oxidase), cytokinin, flavoprotein, plant hormone metabolism, quinone.

### INTRODUCTION

Both prokaryotes and eukaryotes have been shown to utilize flavoprotein enzymes for a wide variety of oxidoreductase reactions. A family of flavin-containing oxidoreductases was previously identified on the basis of the presence of a shared FAD-binding domain [1]. Members of this group include enzymes with covalently and non-covalently bound FAD capable of performing oxidation as well as dehydrogenation reactions, but it is not clear how the FAD-binding domain provides access to the electron acceptor. Electron transport resulting in re-oxidation of FAD appears to be a divergent feature of this group of enzymes. To understand the evolution of different mechanisms of enzyme flavin utilization, it is important to compare the reaction mechanisms and physical properties of different enzymes containing the FAD-binding domain.

Cytokinins are important plant hormones [2], which are selectively inactivated by oxidative cleavage of their side chain. The enzyme catalysing this degradation has been historically classified as a cytokinin oxidase [3], but recent findings indicate that the enzyme uses an electron acceptor other than oxygen [4–6]; re-classification of the enzyme as cytokinin dehydrogenase (CKX; EC 1.5.99.12) was approved by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology in 2001. The reaction catalysed by the enzyme is shown schem-

atically in Figure 1 [4]. CKX was revealed to be a member of the oxidoreductase family after the enzyme was cloned from maize and shown to contain the FAD-binding domain and a covalent association with flavin [5,9]. To avoid confusion with the use of the traditional and the new names, only the abbreviation CKX will be used throughout the paper.

CKX removes the isoprenoid side chain of iP [*N*<sup>6</sup>-(2-isopentenyl)adenine] and zeatin, converting them into adenine and the corresponding aldehyde [3,7,8]. Genes encoding the enzyme were cloned from maize [9,10], *A. thaliana* [5,11,12], *Dendrobium* orchid [13,14] and recently also from barley (P. Galuszka, unpublished work; GenBank<sup>®</sup> accession no. AF491590). To date, CKX was not known to act on aromatic side-chain cytokinins such as kinetin and benzyladenine derivatives [7].

Recently, it has been shown that the addition of DCPIP (2,6-dichlorophenol indophenol) to *in vitro* reaction increases the reaction rate of degradation of *trans*-zeatin by the ZmCKX1 [the CKX enzyme from maize, encoded by the gene *ZmCKX1* (GenBank<sup>®</sup> accession no. AF044603)] nearly 4000-fold faster than the oxygen-dependent rate [6]. In the present study, we present further characterizations of the reaction mechanism of CKX from maize, ZmCKX1, which focus on explaining the kinetic and redox properties of the enzyme and the role of the electron acceptor. These studies form the basis for broader comparisons among FAD oxidoreductases and within the plant CKX gene family.

Abbreviations used: CAN, citraconic anhydride; CKX, cytokinin dehydrogenase (EC 1.5.99.12); CPPU, *N*-(2-chloro-4-pyridyl)-*N*<sup>8</sup>-phenylurea; DCPIP, 2,6-dichlorophenol indophenol; DEPC, diethyl pyrocarbonate; EDC, *N*-(3-dimethyl-aminopropyl)-*N*-ethylcarbodi-imide; iP, *N*<sup>6</sup>-(2-isopentenyl)adenine; iPR, *N*<sup>6</sup>-(2-isopentenyl)adenosine; NAI, *N*-acetylimidazole; NBS, *N*-bromosuccinimide; NHE, normal hydrogen electrode; NEM, *N*-ethylmaleimide; PHG, phenylglyoxal; Q<sub>0</sub>, 2,3-dimethoxy-5-methyl-1,4-benzoquinone; Q<sub>1</sub>, 2,3-dimethoxy-5-methyl-6-(3-methyl-2-butenyl)-1,4-benzoquinone; SCE, saturated calomel electrode.

<sup>1</sup> To whom correspondence should be addressed (e-mail frebort@prfholt.upol.cz).

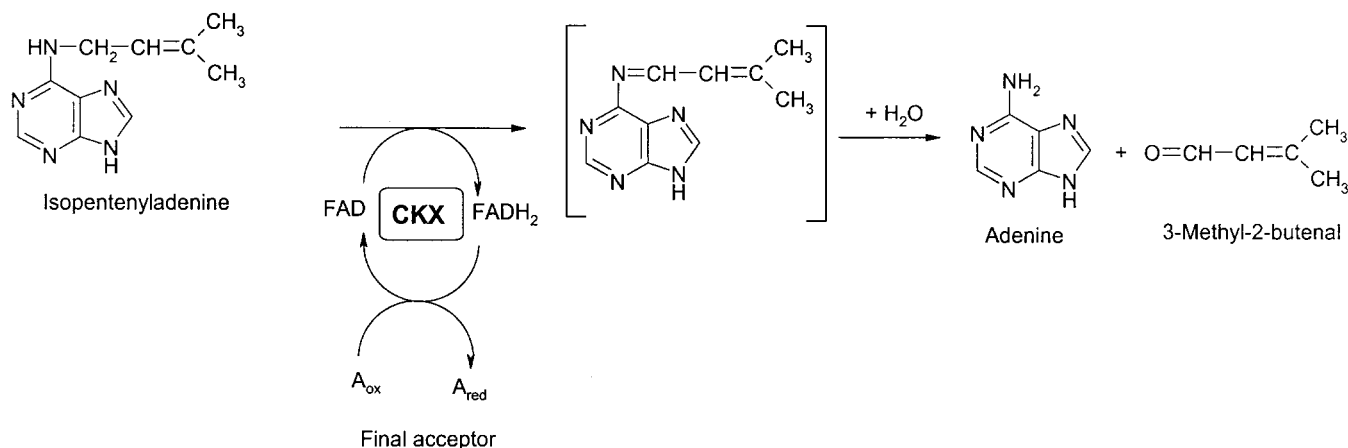


Figure 1 Reaction scheme of CKX (EC 1.5.99.12)

## EXPERIMENTAL

### Enzymes and chemicals

The enzyme, recombinant ZmCKX1 produced by *Pichia pastoris*, was purified as described previously [5] and stored as a concentrated stock solution (87.7  $\mu\text{M}$ ) in TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0), containing 1 M KCl at 4 °C. Partially purified plantacyanin from spinach leaves was prepared by the method described in [15]. Horse heart cytochrome *c* was obtained from Biomed (Cracow, Poland); bovine milk xanthine oxidase (EC 1.1.3.22) was from Sigma (St. Louis, MO, U.S.A.).

Rosmaric acid, scopoletin, caffeic acid, daphnoretin, *o*- and *p*-coumaric acid, luteolin, hydrocaffeic acid and umbelliferone were donated by Dr Lubomír Opletal (Charles University, Hradec Králové, Czech Republic). Coenzyme Q<sub>1</sub> [2,3-dimethoxy-5-methyl-6-(3-methyl-2-butenyl)-1,4-benzoquinone] was a gift from Professor Kazunobu Matsushita (Yamaguchi University, Japan) and atrazine was donated by Dr Pavel Adamovský (Department of Analytical Chemistry, Palacký University, Olomouc, Czech Republic). 1,4-Naphthoquinone was obtained from BDH Chemicals (Poole, Dorset, U.K.) and DCPIP and indole-3-acetic acid were from LOBA Feinchemie (Fischamend, Austria). Acetosyringone (3,5-dimethoxy-4-hydroxyacetophenone) was from Aldrich (Milwaukee, WI, U.S.A.), ferulic acid was from Theodor Schuchardt (Munich, Germany). *N*<sup>6</sup>-(2-isopentenyl)-adenosine-5'-monophosphate, *N*<sup>6</sup>-benzyladenosine-5'-monophosphate, (*R*/*S*)-dihydrozeatin riboside-5'-monophosphate, dihydrozeatin and zeatin riboside-5'-monophosphate were purchased from Apex Organics (Honiton, U.K.). *N*<sup>6</sup>-(2-hydroxybenzyl)adenine, *N*<sup>6</sup>-(3-hydroxybenzyl)adenine, *N*<sup>6</sup>-(4-hydroxybenzyl)adenine and CPPU [*N*-(2-chloro-4-pyridyl)-*N*<sup>9</sup>-phenylurea] were from OlChemIm (Olomouc, Czech Republic). *N*<sup>6</sup>-(2-Methoxybenzyl)adenine, *N*<sup>6</sup>-(3-methoxybenzyl)adenine and *N*<sup>6</sup>-(4-methoxybenzyl)adenine were provided by the Laboratory of Growth Regulators (Palacký University/Institute of Experimental Botany ASCR, Olomouc, Czech Republic). *N*-Methyl-isopentenyladenine [*N*<sup>6</sup>-methyl-*N*<sup>6</sup>-(3-methyl-2-butenyl)adenine] was synthesized as in [16]. DEPC (diethyl pyrocarbonate), EDC [*N*-(3-dimethyl-aminopropyl)-*N*'-ethylcarbodiimide], CAN (citraconic anhydride), PHG (phenylglyoxal) monohydrate and NBS (*N*-bromosuccinimide) were from Fluka (Zwijndrecht, The Netherlands). iP, *trans*-zeatin ((*E*)-2-methyl-4-(1*H*-purin-6-ylamino)-2-buten-1-ol), *N*<sup>6</sup>-benzyladenine, kinetin [*N*<sup>6</sup>-(2-furfuryl)adenine], 4-aminophenol, vitamin K<sub>1</sub> (phylloquinone), 2-methyl-3-phytyl-1,4-naphthoquinone, vitamin K<sub>3</sub> (menadione,

2-methyl-1,4-naphthoquinone), coenzyme Q<sub>0</sub> (2,3-dimethoxy-5-methyl-1,4-benzoquinone), NAI (*N*-acetylimidazole), NEM (*N*-ethylmaleimide), kaempferol [3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one], 4-methylcatechol, Diuron [3-(3,4-dichlorophenyl)-1,1-dimethylurea], guaiacol (2-methoxyphenol), tyrosine, xanthine, Methyl Viologen, thionin, Methylene Blue, resorufin and other chemicals were from Sigma.

### Differential pulse polarography

Differential pulse polarography measurements were performed on ZmCKX1 in a PRG 4 apparatus (Tacussel, Villeurbanne, France) as described previously [17], using a mercury dropping electrode and SCE (saturated calomel electrode) as the reference. The reaction mixture in a glass polarographic cell (2 ml) contained ZmCKX1 (1.5  $\mu\text{M}$ ) in 43 mM Mops/NaOH (pH 7.2) and 0.24 mM EDTA. The reaction mixture was de-aerated by bubbling with argon gas for 10 min and polarographed over the potential range between -1.4 and 0.0 V.

### Cyclic voltammetry

Cyclic voltammetry measurements were performed on ZmCKX1 using EG&G M-273 apparatus (Princeton Applied Research, Oak Ridge, TN, U.S.A.). For enzyme reduction assessments in the absence of substrate, a reaction mixture of 2.5 ml of ZmCKX1 (87.7  $\mu\text{M}$ ) in TE buffer (pH 8.0), containing 1 M KCl was scanned from 0.0 to -1.4 V at scan rates 100–500 mV/s using mercury stationary drop as working electrode, SCE as the reference and a Pt wire as the auxiliary electrode. The scan was also performed with addition of the substrate, iP, at final concentrations of 40–120  $\mu\text{M}$ . For enzyme re-oxidation experiments, the enzyme was first reduced with 120  $\mu\text{M}$  iP and then the reaction mixture was scanned from 0.0 to 0.8 V at the scan rate of 250 mV/s using an MF-2012 glassy carbon working electrode (Bioanalytical Systems, West Lafayette, IN, U.S.A.), SCE as the reference and a Pt wire as the auxiliary electrode. A control experiment was performed with the enzyme without adding iP.

The redox potential of ZmCKX1 was also determined by the method of Massey [18]. The enzyme (5  $\mu\text{M}$ ) in the presence of 2  $\mu\text{M}$  Methyl Viologen, 200  $\mu\text{M}$  xanthine and a reference dye in 50 mM potassium phosphate (pH 7.5) was made anaerobic in a spectrophotometric cell by flushing with nitrogen. Thionin (+0.056 V), Methylene Blue (+0.011 V) and resorufin (-0.051 V) were used as reference dyes. Reduction was initiated

by adding 0.5  $\mu\text{g/ml}$  xanthine oxidase and subsequently monitored using an 8453A diode array spectrophotometer (Hewlett-Packard, Foster City, CA, U.S.A.). Spectra (300–700 nm) were recorded every 1 min until reductions of both the dye and the enzyme were complete (approx. 1.5 h).

### Spectral studies on CKX

Spectrophotometric titrations were performed using methods described in studies of other flavoproteins [19,20]. Titrations were performed in a stirred quartz cell on a DU 7500 photodiode array spectrophotometer (Beckman, Fullerton, CA, U.S.A.) at 30 °C. The enzyme solution (20–25  $\mu\text{M}$ ), of total volume 1.5 ml, in 75 mM imidazole/HCl (pH 6.5) was titrated with 2  $\mu\text{l}$  aliquots of a reagent ( $Q_0$ , iP and sodium dithionite) at a concentration, in the same buffer, that allowed approximately ten additions for complete reduction/oxidation. The aliquots were added at 1 min intervals.

Rapid-scanning experiments that monitored time course of the reduction of 5.85  $\mu\text{M}$  ZmCKX1 with iP, *N*-methyl-isopentenyladenine, 2-hydroxybenzyladenine, 4-hydroxybenzyladenine and 0.15 mM dihydrozeatin were performed in 75 mM imidazole/HCl (pH 6.5) at 30 °C, using the multi-wavelength kinetics mode that allows recording of spectra of photodiode readings in 0.1 s interval and can store up to 99 recorded spectra at chosen time intervals. The experiment was also performed with iP in the presence of 1.25  $\mu\text{M}$   $\text{CuCl}_2$ , which, in the imidazole buffer used, acts as an electron acceptor in the CKX reaction.

Stopped-flow kinetics was performed with an SX17MV stopped-flow instrument apparatus equipped with a diode-array detector (Applied Photophysics, Leatherhead, U.K.). Spectral scans were collected at 2.56 ms intervals. Spectral deconvolution was performed using the Pro-K software (Applied Photophysics, Leatherhead, U.K.). For anaerobic experiments, the solutions were flushed with nitrogen and simultaneously 10 mM glucose and 0.1  $\mu\text{M}$  glucose oxidase were included. For a more accurate estimation of reduction rates, the single-wavelength detection mode was used. Single-wavelength kinetic traces were recorded at 445 nm after mixing 2.0  $\mu\text{M}$  ZmCKX1 with different concentrations of iP [75 mM imidazole/HCl (pH 6.5) at 30 °C].

### Quantification of CKX-mediated aldehyde formation

The assay was performed using previously described methods [21]. An enzyme sample was incubated in a reaction mixture (total volume of 0.6 ml in an Eppendorf tube) composed of buffer [75 mM Tris/HCl (pH 8.0) or 75 mM imidazole/HCl, pH 6.5], DCPIP (or other electron acceptor) in appropriate concentration (0.5 mM taken as reference/'saturation') and 0.15 mM iP (or appropriate concentration of a substrate) at 37 °C. The incubation period varied from 0.5 to 2 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 then centrifuged at 12000 g for 5 min. After the addition of 0.2 ml of 4-aminophenol (2% solution in 6% trichloroacetic acid) to the supernatant, the whole spectrum in the range 300–700 nm was scanned and the absorbance was measured at a specific wavelength (352 nm for iP,  $\epsilon_{352}$  15.2  $\text{mM}^{-1} \cdot \text{cm}^{-1}$ ), against a blank without the substrate.

For enzyme inhibition studies, inhibitors were dissolved in a minimal amount of DMSO (2–10  $\mu\text{l}$ ) and added to the assay mixtures at various concentrations. The same aliquot of DMSO was always added to the blank without substrate. After incubation, 4-aminophenol was added to the reaction mixture and absorption was measured as before.

All kinetic data were analysed using the program GraFit 4.0.12 (Erithacus Software, Horley, Surrey, U.K.).

### Monitoring of CKX reaction by LC-MS

Reaction mixtures consisting of 0.44  $\mu\text{M}$  ZmCKX1, 0.15 mM substrate (iP, *N*-methyl-isopentenyladenine and dihydrozeatin) in 75 mM imidazole/HCl (pH 6.5), alternatively containing 0.5 mM  $Q_0$ , were incubated for 30 min at 37 °C and then cooled on ice. Aliquots (0.01 ml) were diluted 100-fold with 15 mM formic acid adjusted to pH 4.0 by ammonium hydroxide. The samples (10  $\mu\text{l}$ ) were separated on a Symmetry C18 column (2.1 mm inner diameter  $\times$  150 mm, 5  $\mu\text{m}$ ), using an Alliance 2690 Separations Module equipped with a 996 Photodiode Array Detector (Waters, Milford, MA, U.S.A.) with a linear gradient from 2 to 70% (v/v) methanol and 98–30% (v/v) 15 mM ammonium formate (pH 4.0) in 15 min, followed by an increase to 100% methanol from 20 to 21 min, at a flow rate of 0.25 ml/min, the column being thermostatically maintained at 30 °C. Mobile-phase flow was split (1:1) in between the photodiode array detector (scanning range 200–400 nm, resolution 1.2 nm) and a ZMD 2000 single quadrupole mass spectrometer equipped with an electrospray interface (Micromass, Manchester, U.K.). The ionization mode used produced positively charged ions corresponding to the pseudo-molecular one (molecular mass + 1 Da). In the LC-MS experiments, full scans were performed with the following electrospray parameters: source temperature 100 °C; capillary voltage, + 3.0 kV; cone voltage, + 30 V; ion energy, 0.3 V and desolvation temperature, 250 °C; nitrogen was used both as desolvation gas (450 l/h) and as cone gas (50 l/h). Data were evaluated with the software Masslynx (Micromass).

### Chemical modifications of active-site amino acid residues

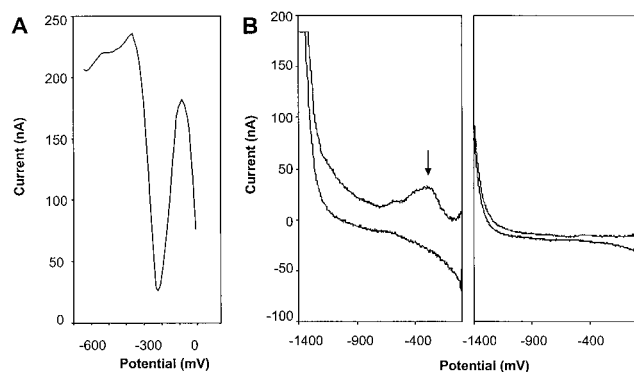
Chemical modifications of specific amino acids were performed to determine residues that are essential for the enzyme activity [22]. NEM (10 mM) was used to modify cysteine residue. DEPC (5 mM) and EDC (10 mM) were used to modify histidine and carboxylate residues (Glu and Asp) respectively. PHG (10 mM) and CAN (10 mM) were used to modify arginine and lysine residues respectively. NAI (10 mM) was chosen for the reaction with tyrosine and NBS (1 mM) was used to modify tryptophan. The following buffers were used: 0.08 M Tris/HCl, pH 8.5 (CAN, NEM, PHG), 0.08 M Tris/HCl, pH 8.0 (EDC, NAI, NBS) and 0.05 M potassium phosphate, pH 7.0 (DEPC). ZmCKX1 samples (1.8  $\mu\text{M}$ ) were incubated with the reagents at room temperature (24 °C) and aliquots were taken out for the activity assay with iP and DCPIP as above.

## RESULTS

### Electrochemical characterization of the CKX reaction

Flavoproteins show distinct electrochemical signals that can be used for their characterization. When native ZmCKX1 was studied by differential pulse polarography, a signal corresponding to FAD reduction was found at  $-0.27$  V (Figure 2A), which corresponded to  $E'_0 = -0.014$  V [peak at  $-0.27$  V, correction to SCE versus NHE (normal hydrogen electrode) (+0.244 V) and pH 7.2 versus pH 7.0 (+0.012 V)].

Using cyclic voltammetry, the effect of substrate on the reduction of the FAD cofactor of ZmCKX1 was studied. In the reductive half-reaction without substrate (Figure 2B), the peak for cofactor FAD occurred at  $E'_0 = +0.013$  V [peak at  $-0.290$  V, correction to SCE versus NHE (+0.244 V) and pH 8.0 versus



**Figure 2** Electrochemical properties of ZmCKX1

(A) Differential pulse polarography. ZmCKX1 (1.5  $\mu\text{M}$ ) in 0.043 M Mops (pH 7.2), containing 0.24 mM EDTA (peak at  $-0.27$  V). The current was measured using a mercury-dropping electrode with SCE as the reference. (B) Cyclic voltammetry of reductive half-reaction: ZmCKX1 (87.7  $\mu\text{M}$ ) in TE buffer (pH 8.0), containing 1 M KCl and scan rate 250 mV/s; without substrate (left-hand panel) and with 0.12 mM iP (right-hand panel); the arrow shows the FAD peak at  $-0.29$  V (which disappeared after adding the substrate). The current versus voltage curve was measured using a mercury stationary drop electrode with SCE as the reference.

pH 7.0 (+0.059 V)]. In the presence of substrate, this characteristic peak disappeared confirming the substrate-mediated reduction of the cofactor. In the oxidative half-reaction with substrate-reduced enzyme, a low-amplitude wave appeared at approx.  $E'_0 = +0.75$  V (not shown). In the process of assaying for production of hydrogen peroxide in the CKX reaction using a coupled assay with peroxidase/guaiacol [23], it was discovered that the product of guaiacol oxidation, 3,3'-dimethoxy-4,4'-biphenylquinone [24], can actually act as an electron acceptor in the dehydrogenase reaction of ZmCKX1 (results not shown). Therefore hydrogen peroxide was assayed and detected for the CKX with iP (without any other electron acceptor) reaction using a discontinuous assay (results not shown). The formation of hydrogen peroxide in the reaction can explain the wave observed by cyclic voltammetry at approx.  $E'_0 = +0.75$  V, which agrees well with the standard redox potential for conversion of hydrogen peroxide.

The redox properties of ZmCKX1 were also studied spectrophotometrically. For this, the enzyme was anaerobically reduced by the xanthine oxidase/xanthine system [18]. Reduction of ZmCKX1 revealed that a red anionic flavin semiquinone is significantly stabilized during the redox titration as evidenced by the typical absorbance of approx 360 nm. Using several reference dyes (resorufin, Methylene Blue and thionin), redox potentials of the two couples (oxidized/semiquinone and semiquinone/quinol) could be estimated. Titration with thionin indicated that the potential of the first electron transfer is approx. +0.022 V (Nernst plot gave 29.7, the theoretical value being 29.5 mV). Titration with resorufin revealed that the potential for reduction of the red anionic radical is  $-0.007$  V (Nernst plot gave 30.1 mV as slope, the theoretical value being 29.5 mV). Using Methylene Blue, the redox potential for reduction of the radical could be determined at  $-0.004$  V. The difference in redox potential between the two redox couples agrees well with the observed amount of semiquinone formed during the redox titration. Taken together, the midpoint potential for a two-electron redox potential of ZmCKX1 is estimated to be  $+0.008 \pm 0.005$  V, which is similar to the values determined by differential pulse polarography and cyclic voltammetry. This relatively high redox potential is typical for a covalently bound flavin cofactor [25], compared with the free FAD that has a standard redox potential of  $-0.219$  V. The high redox potential also limits the usage of physiologically available

**Table 1** Kinetic constants of ZmCKX1 with various electron acceptors

3-Methyl-2-butanal produced by oxidative cleavage of the side chain of iP was detected by the 4-aminophenol assay [21]. Final concentrations of the reagents were: electron acceptors, 0.5 mM, except for cytochrome *c* (0.05 %) and plantacyanin (approx. 0.2 mg of partially purified preparation); iP, 0.15 mM; ZmCKX1, 4.5 nM to 0.73  $\mu\text{M}$  (higher with lower turnover rate).

Compound	$E'$ (V) (if known)	Turnover rate ( $\text{s}^{-1}$ )	
		pH 6.5*	pH 8.0†
O <sub>2</sub> (no compound added)	+0.82	0.6	< 0.1
DCPIP	+0.22	178.4	278.0
Quinones			
Q <sub>0</sub>	+0.08	143.6	n.a.‡
Q <sub>1</sub>	+0.08	123.1	n.a.‡
Vitamin K <sub>1</sub>	+0.13	2.3	1.6
Vitamin K <sub>3</sub>	+0.14	85.4	n.a.‡
1,4-Naphthoquinone	+0.06	169.6	n.a.‡
Phenolics			
Rosmaric acid	–	0.4	0.0
Scopoletin	–	1.4	1.1
Caffeic acid	–	0.3	< 0.1
Daphnoretin	–	1.0	4.2
<i>o</i> -Coumaric acid	–	0.3	< 0.1
Luteolin	–	< 0.1	< 0.1
Hydrocaffeic acid	–	0.2	< 0.1
Umbelliferone	–	< 0.1	< 0.1
<i>p</i> -Coumaric acid	–	< 0.1	< 0.1
Guaiacol	–	< 0.1	< 0.1
Acetosyringone	–	< 0.1	< 0.1
Protein electron carriers			
Cytochrome <i>c</i>	+0.24	0.8	–
Plantacyanin from spinach	+0.34	0.9	–

\* 75 mM imidazole/HCl (pH 6.5).  
† 75 mM Tris/HCl (pH 8.0).  
‡ Quinones are not applicable as electron acceptors in alkaline pH due to their polymerization.

electron acceptors. Although molecular oxygen, according to its redox potential, could be a potential electron acceptor, it hardly reacts with reduced ZmCKX1 [4]. The reluctance to use oxygen as electron acceptor may suggest shielding of the flavin cofactor. It was recently shown for a sequence-related flavoenzyme, cholesterol oxidase, that oxygen can only enter the active site via a strictly defined narrow tunnel [26]. Also the active-site architecture can prevent molecular oxygen from reacting with a reduced cofactor. Apparently, ZmCKX1 activity is strictly dependent on a specific type of electron acceptor.

### Search for electron acceptors of CKX

Since the natural electron acceptor of the enzyme is unknown and oxygen has very limited reactivity [4,6], a variety of redox-active compounds was tested, including caffeic acid and acetosyringone, which were shown previously to enhance CKX activity [16,27]. Although some of the compounds, for which an activating effect has been described, decreased the activity, several compounds markedly increased CKX activity (Table 1). Particularly, *p*-quinones, including analogues of ubiquinone, Q<sub>0</sub> and Q<sub>1</sub>, 1,4-naphthoquinone and the artificial electron acceptor DCPIP enhanced ZmCKX1 activity. Using 0.15 mM iP, the  $K'_m$  values for the most effective electron acceptors were determined: DCPIP, 0.036 mM; Q<sub>0</sub>, 0.21 mM; and 1,4-naphthoquinone, 0.011 mM (all at pH 6.5). The redox potentials of all effective electron acceptors vary from +0.06 to +0.22 V. As found previously for CKX from wheat, the  $E'_0$  of the sought natural electron acceptor should indeed lie in this range [4]. The electron acceptor DCPIP was effective at both alkaline and neutral pH, whereas

**Table 2** Activity of ZmCKX1 with various cytokinins

Aldehydes produced by oxidative cleavage of the side chain of studied cytokinins were detected by the 4-aminophenol assay [21]. Reaction mixtures included 4.5 nM to 0.73  $\mu$ M ZmCKX1 (higher with lower turnover rate) and 0.15 mM substrates for saturation in 75 mM imidazole/HCl (pH 6.5).

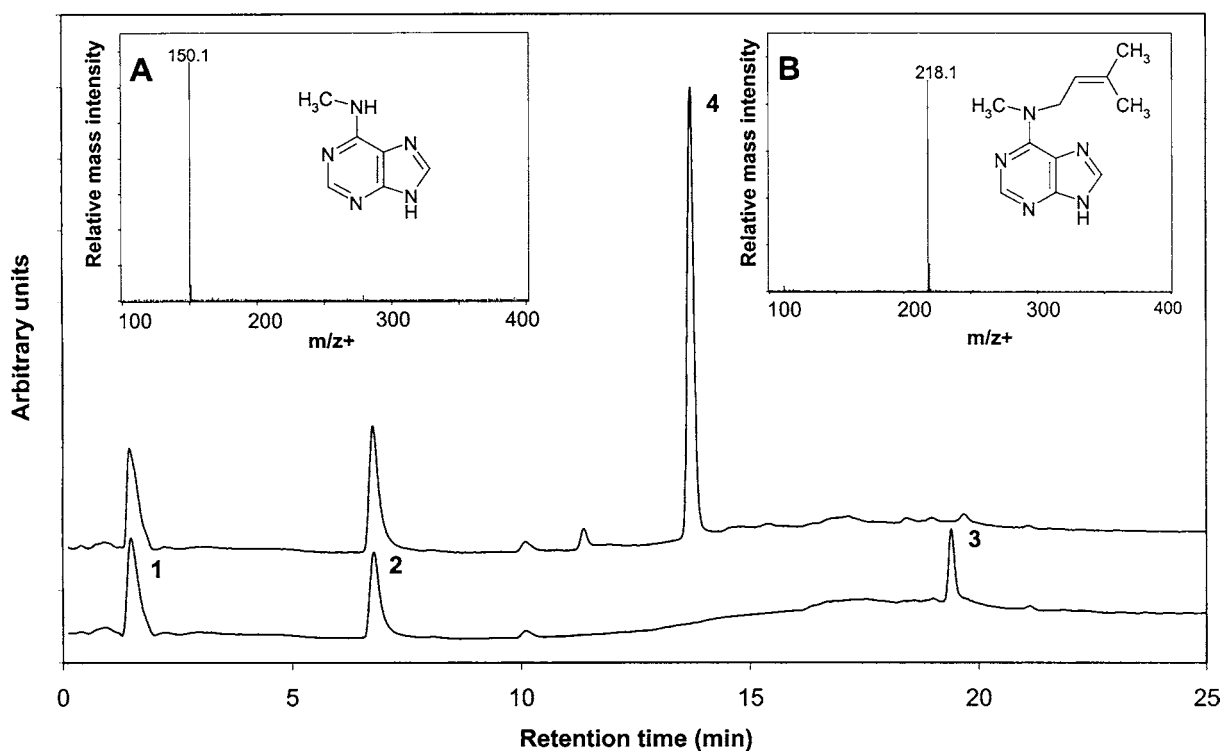
Substrate	Turnover rate ( $s^{-1}$ )		
	Q <sub>0</sub> (0.5 mM)	Aerobic	Anaerobic
Isopentenyladenine	143.6	0.6	< 0.1
Isopentenyladenine riboside	129.8	0.7	< 0.1
Benzyladenine	0.2	–	–
Kinetin	1.2	0.7	< 0.1
2-Hydroxybenzyladenine	0.1	0.1	< 0.1
3-Hydroxybenzyladenine	0.1	–	–
4-Hydroxybenzyladenine	0.8	0.8	< 0.1
2-Methoxybenzyladenine	0.7	0.4	< 0.1
3-Methoxybenzyladenine	0.2	–	–
4-Methoxybenzyladenine	1.0	0.5	< 0.1
Benzyladenine riboside monophosphate	0.1	–	–
Isopentenyladenine riboside monophosphate	0.5	–	–
<i>N</i> -Methyl-isopentenyladenine	0.6	0.4	–

at the alkaline pH, the quinones gave non-reproducible results due to their spontaneous oxidative side-reactions. It is notable that under aerobic conditions, the enzyme displayed low but measurable activity (turnover rate approx.  $0.5 s^{-1}$ ), even without any electron acceptor added. This reaction was not inhibited by either KCN or sodium azide, indicating no contamination of the enzyme with electron acceptors from the respiratory chain of yeast used to produce the recombinant enzyme. Therefore this activity has been further referred to as an oxidase activity. For subsequent

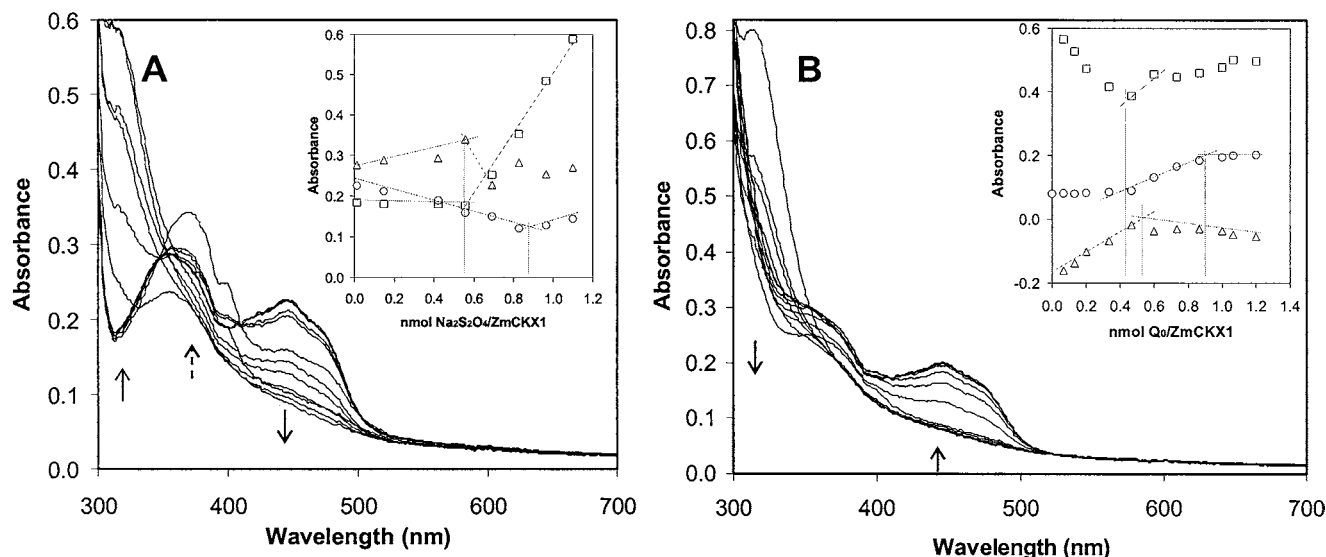
studies, 0.5 mM concentration of the electron acceptor Q<sub>0</sub> was chosen as a reference, since at higher concentrations Q<sub>0</sub> was found to interfere with the *p*-aminophenol assay used.

### Other cytokinins as potential substrates of CKX

Until recently, aromatic cytokinins such as hydroxy-derivatives of *N*<sup>6</sup>-benzyladenine found in plants were considered not to be substrates of CKX [7]. In a previous report, we designed a method for quantification of aldehydes that might be produced by oxidative cleavage of the side chain of aromatic cytokinins [21]. Using this method, it was found that ZmCKX1 is in fact capable of cleaving aromatic cytokinins, although at low turnover rates compared with iP (Table 2). The rates obtained were of the same magnitude as those for iP-type cytokinins without the addition of a functional electron acceptor. Surprisingly, the reaction of ZmCKX1 with *N*-methyl-isopentenyladenine also proceeded with a turnover rate similar to those of aromatic cytokinins (Table 2) and resulted in the production of 3-methyl-2-butenal that was identified by the 4-aminophenol method [21]. Evidently, the replacement of H with the methyl group at N-6 does not prevent catalysis. Owing to the low turnover rate compared with iP, this compound can act as a competitive inhibitor of iP degradation as reported earlier for CKX from tobacco [16] and maize [5]. Reaction mixtures of ZmCKX1 with iP, *N*-methyl-isopentenyladenine and dihydrozeatin were analysed by LC-MS to observe the formation of products. The control reaction with iP (retention time, 17.1 min;  $m/z^+$  204.1) showed a production of adenine (retention time, 3.3 min;  $m/z^+$  136.1). Analysis of the reaction mixture of *N*-methyl-isopentenyladenine (retention time, 19.4 min;  $m/z^+$  218.1) showed its degradation to *N*-methyladenine (retention time, 6.8 min;  $m/z^+$  150.1) as presented in Figure 3.

**Figure 3** LC-MS analysis of ZmCKX1 reaction with *N*-methyl-isopentenyladenine

Samples were incubated for 30 min at 37 °C, and analysed by LC-MS (see the Experimental section for details). Lower trace: 0.44  $\mu$ M ZmCKX1, 0.15 mM *N*-methyl-isopentenyladenine in 75 mM imidazole/HCl, pH 6.5; upper trace: the same reaction mixture containing 0.5 mM Q<sub>0</sub>. Peak identification (268 nm): 1, imidazole ( $m/z^+$  68.9); 2, *N*-methyladenine ( $m/z^+$  150.1, mass spectrum inset A); 3, *N*-methyl-isopentenyladenine ( $m/z^+$  218.1, mass spectrum inset B); 4, Q<sub>0</sub> ( $m/z^+$  183.2).



**Figure 4** Spectrophotometric redox titration of the FAD cofactor of ZmCKX1

(A) Stepwise reduction of the enzyme (20  $\mu$ M) in 75 mM imidazole/HCl (pH 6.5) with sodium dithionite and (B) re-oxidation with  $Q_0$ . Insets: profiles at 315 nm, FADH<sub>2</sub> (□), difference 370–330 nm, FADH ( $\Delta$ ) and 445 nm, FAD (○).

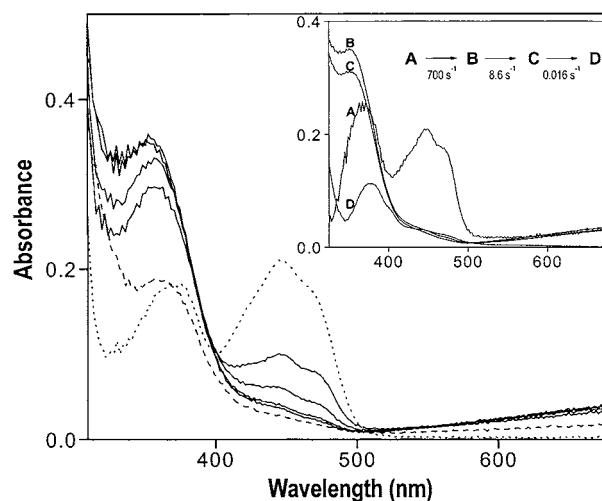
Dihydrozeatin (retention time, 11.6 min;  $m/z^+$  222.3) was not degraded at all.

Using 0.5 mM  $Q_0$ , the concentration-dependent rate of the cytokinin degradation resembled Michaelis–Menten kinetics with apparent  $K'_m$  values of 0.040 and 0.044 mM for iPR and iPR [ $N^6$ -(2-isopentenyl)adenosine] respectively. Apparently, the ribose moiety in iPR does not significantly affect the catalytic efficiency since the turnover rates for both iPR and iP are similar (Table 2). When only molecular oxygen is present, however, an apparent  $K'_m$  of 0.44 mM for iP was measured, whereas for kinetin and 2-hydroxybenzyladenine (two aromatic cytokinins) again relatively low  $K'_m$  values were found (respectively 0.070 and 0.040).

Quite surprising was the fact that the rates for cleavage of aromatic cytokinin side chains in the presence of  $Q_0$  did not differ significantly from rates observed in the presence of molecular oxygen. The low rates for both iP type and aromatic cytokinins decreased when the reaction mixtures were purged with nitrogen gas, suggesting that indeed molecular oxygen is needed for the low-rate reaction. The enzyme thus displays a dual catalytic activity mode of a high dehydrogenase activity with a suitable electron acceptor that is specific for iP-type cytokinins and a low oxidase activity that is non-specific towards other cytokinins. A similar activity with oxygen/ $Q_0$  for the aromatic cytokinins may indicate that flavin reduction by the substrate has become rate-limiting.

### Spectral detection of reaction intermediates

The characteristic absorption spectrum of flavoproteins shifts in accordance with the redox status of the flavin cofactor and typically three peaks were observed at approx. 445 nm for FAD, 370 nm for FADH and 315 nm for FADH<sub>2</sub> [19,20,28–30]. When the catalytic centre of ZmCKX1 was stepwise reduced with sodium dithionite, the resulting spectra showed a clear two-step reaction with stoichiometric formation of the flavin semiquinone as observed during the redox titration (Figure 4A). When the enzyme was then re-oxidized with  $Q_0$ , the formation of semiquinone was less obvious, but still noticeable (Figure 4B). However, when the enzyme was aerobically mixed with an excess

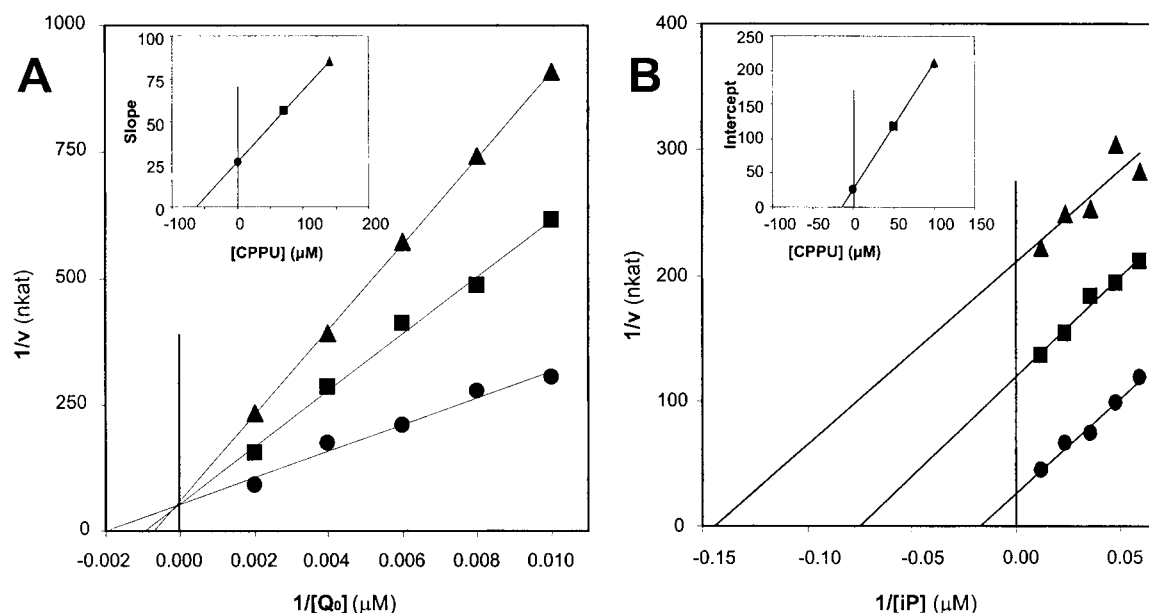


**Figure 5** Spectrophotometric monitoring of anaerobic ZmCKX1 reduction by iP

Using the stopped-flow apparatus, 20  $\mu$ M ZmCKX1 was mixed with 30  $\mu$ M iP (final concentrations) after which spectra were recorded every 2.56 ms. A total of 500 spectra were collected in logarithmic distribution during 49 s. The spectra collected at 1.3 ms, 2.86 ms, 6.42 ms, and 8.98 ms (solid lines), and 49 s (---) are shown. The spectrum of ZmCKX1 obtained after mixing with buffer is also shown (· · ·). Spectral deconvolution revealed four spectral species (A, B, C, D) and three corresponding exponential decay rates (inset).

of iP, no formation of semiquinone was observed, even though yielding a fully reduced enzyme. Partial reduction of the flavin cofactor was observed during the aerobic reaction with low turnover rate substrates *N*-methyl-isopentenyladenine, 2-hydroxybenzyladenine and 4-hydroxybenzyladenine, indicating that flavin re-oxidation by molecular oxygen is a relatively slow event. With dihydrozeatin, no flavin reduction could be detected.

ZmCKX1 could be rapidly reduced by iP as evidenced by stopped-flow analysis (Figure 5). Spectral analysis of the diode array data revealed that flavin is rapidly and fully reduced in the first step. Complete reduction of flavin indicates that this reaction



**Figure 6** Double-reciprocal plots of inhibition of ZmCKX1 with CPPU

(A)  $Q_0$  varied;  $iP$ , 150  $\mu M$ ; CPPU: 0 ( $\bullet$ ), 70 ( $\blacksquare$ ) and 140  $\mu M$  ( $\blacktriangle$ ); competitive inhibition,  $K_i = 63 \mu M$ ; (B)  $iP$  varied;  $Q_0$ , 500  $\mu M$ ; CPPU: 0 ( $\bullet$ ), 50 ( $\blacksquare$ ) and 100  $\mu M$  ( $\blacktriangle$ ); uncompetitive inhibition,  $K_i = 14 \mu M$ . All experiments were performed in 75 mM imidazole/HCl (pH 6.5), using the assay with 4-aminophenol [21] with 30 min incubation at 37 °C. The results are averages of two independent measurements; lines were fitted with linear regression. Insets: secondary plots of slopes/intercepts versus inhibitor concentration.

step is essentially irreversible. Substrate-concentration-dependent analysis of the rate of reduction using single-wavelength-absorption detection mode (445 nm) of the stopped flow revealed a  $K_d < 2 \mu M$  and a maximal reduction rate of 950  $s^{-1}$ . Spectral analysis revealed that, after reduction, two other kinetic events can be observed. During these relatively slow processes, the formed absorbance of approx. 352 nm disappears, resulting in a final spectral species that resembles a reduced flavin species. Formation of an imine product would correspond to the spectral properties of this intermediate as it represents a highly conjugated compound. The observed increased absorbance at higher wavelength (> 550 nm), after the enzyme was mixed with  $iP$ , suggests the formation of a charge-transfer complex. This indicates that the observed spectral intermediate represents a binary complex of reduced enzyme and a bound oxidation product, probably the imine. From these results, it can be deduced that release of the product or imine from the reduced enzyme is a relatively slow process that might be triggered by re-oxidation of the cofactor. Such a ternary kinetic mechanism resembles the mechanism of vanillyl-alcohol oxidase [31], another member of the oxidoreductase family.

### Elucidation of the enzyme reaction mechanism

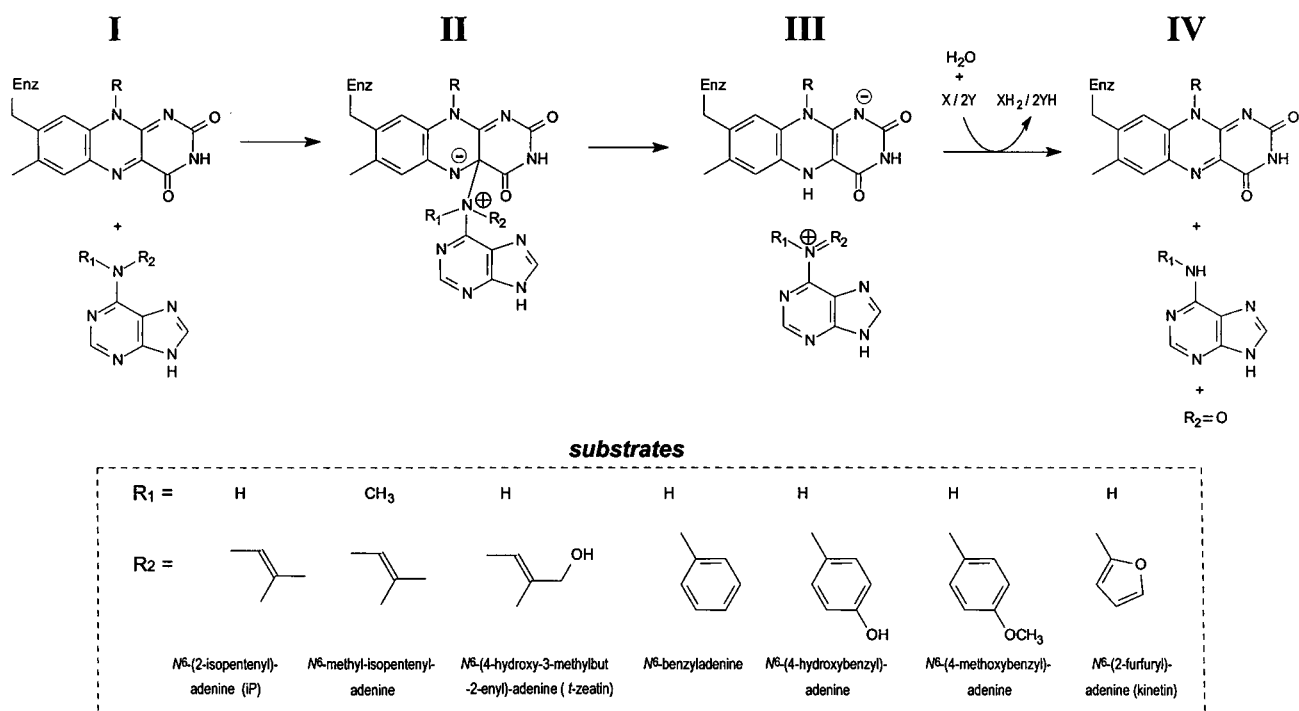
The kinetics of ZmCKX1, in the presence of  $Q_0$  or DCPIP as electron acceptors, were characterized to elucidate the mechanism of the dehydrogenase reaction. Saturation curves of ZmCKX1 with  $iP$  as substrate showed a decrease in specific activity after reaching a maximum rate, previously observed also for the recombinant enzyme AtCKX2 from *A. thaliana* [21] and for the ZmCKX1 with *trans*-zeatin [6]. This characteristic is typical for two-substrate kinetics, in which a ternary complex forms among the enzyme and both substrates [32]. Thus the ZmCKX1 dehydrogenase reaction is unlikely to follow the Ping-Pong mechanism, where only binary complexes are formed that

has been described for some FAD-dependent oxidases such as cholesterol oxidase [26].

In general, the order in which the substrates bind in the ordered bireactant system can be determined by use of a specific inhibitor [32,33]. The inhibitor CPPU, which is known to inhibit ZmCKX1 [5], was chosen for the studies. Analysis of the double-reciprocal plot showed that with the two-electron carrier  $Q_0$  as the electron acceptor, the inhibition by CPPU was competitive towards  $Q_0$  ( $K_i = 0.063$  mM) and uncompetitive towards  $iP$  ( $K_i = 0.014$  mM); see Figures 6(A) and 6(B). This characteristic is typical for ordered bireactant systems as described by Segel [33], with  $iP$  binding first and  $Q_0$  next. However, when the one-electron carrier DCPIP was used, the CKX reaction became a ter-reactant system, because two molecules of the one-electron carrier DCPIP are needed to oxidize fully the cytokinin substrate. With DCPIP as the electron acceptor, the inhibition by CPPU appeared to be competitive towards  $iP$  ( $K_i = 0.004$  mM), which agrees with the results obtained by measuring DCPIP bleaching [5] and uncompetitive towards DCPIP ( $K_i = 0.075$  mM).

When measuring the enzyme activities by the 4-aminophenol assay [21], the amount of CKX had to be carefully set to avoid substrate exhaustion or product inhibition in the given incubation period. In general, the experiments with  $iP < 0.01$  mM were difficult to perform; the amount of enzyme had to be kept low and the absorbances measured were low ( $\epsilon_{352}$  15.2  $mM^{-1} \cdot cm^{-1}$ ). On the other hand, the 4-aminophenol method has an advantage over measuring the DCPIP bleaching [5], since, unlike the DCPIP bleaching method, it allows measurement under saturating concentrations of the electron acceptor.

ZmCKX1 readily reduces analogues of ubiquinone,  $Q_0$  and  $Q_1$ ; however, there seems to be no similarity of its quinone-binding site to the  $Q_b$  site of respiratory enzymes, since the  $Q_b$  site inhibitors diuron and atrazin [34] showed only little effect on the enzyme activity. Atrazin was totally ineffective, whereas diuron showed weak inhibition with behaviour similar to CPPU



**Figure 7** Putative reaction mechanism of ZmCKX1

The proposed catalytic reaction of ZmCKX1 follows a ternary complex mechanism. In this mechanism, the oxidized enzyme (**I**) is reduced by a cytokinin via a covalent intermediate (**II**) forming a binary complex of reduced enzyme and a product intermediate (**III**). After the reaction of the binary complex with a suitable electron acceptor, the imine product is released and hydrolysed to form oxidized enzyme, adenine and the respective side-chain aldehyde (**IV**).

(competitive towards Q<sub>0</sub>, K<sub>i</sub> = 0.27 mM; uncompetitive towards iP, K<sub>i</sub> > 0.5 mM).

The substrate analogues *N*-methyl-isopentenyladenine and dihydrozeatin showed rather low inhibition effects when tested as inhibitors towards iP in the reaction mediated by Q<sub>0</sub>. *N*-Methyl-isopentenyladenine showed competitive inhibition for K<sub>i</sub> > 0.2 mM, whereas dihydrozeatin inhibited only approx. 30% of the CKX activity at 0.5 mM concentration.

#### Identification of critical amino acid residues

Specific amino acid modifiers were used to elucidate residues that are important for the enzymic activity of ZmCKX1 (see the Experimental section for details). Strong time-dependent inhibition was observed on Trp modification by NBS (30 and 10% of control after 5 and 15 min respectively) and His modification by DEPC (70 and 3% of control after 30 and 60 min respectively). Activity of ZmCKX1 modified on other amino acid residues was within 80–95% of the control. On the basis of amino acid sequence alignments with other oxidoreductases, H105 and H394 are candidates for covalent flavination. Indeed, an isolated lysyl endopeptidase cleavage fragment of ZmCKX1-containing FAD was found to contain H105 (K. D. Bilyeu, unpublished work). Further understanding of important amino acid residues will involve structure determination and site-directed mutagenesis.

#### DISCUSSION

A conserved domain search [35] on the protein sequence of ZmCKX1 revealed the presence of a FAD-binding domain (pfam01565) represented by residues 69–231. This domain has

been found in a superfamily of FAD-dependent oxidoreductases [1,36]. Members of this flavoprotein oxidoreductase superfamily have been shown to use a variety of electron acceptors: e.g. vanillyl-alcohol oxidase (covalent FAD) efficiently uses molecular oxygen [25], D-lactate dehydrogenase (non-covalent FAD) uses membrane quinones [37], whereas UDP-*N*-acetylmuramate dehydrogenase is an NADP<sup>+</sup>-dependent enzyme [38]. The results of our experiments demonstrate the formation of a ternary complex for CKX during a dehydrogenase reaction utilizing a suitable electron acceptor other than oxygen. CKX (covalent FAD) is therefore an important member of the oxidoreductase family because it follows an analogous reaction mechanism although differing in re-activation of the flavin.

By the identification of several highly effective electron acceptors, the classification of the cytokinin degrading enzyme as a dehydrogenase has been affirmed. As can be expected, the redox potentials of these electron acceptors are somewhat higher than the redox potential of the covalent flavoprotein. On the basis of the kinetic data presented in this study, we propose a ternary kinetic mechanism of the CKX-mediated oxidation reaction (Figure 7), which includes flavin reduction via a 'concerted covalent catalysis mechanism' proposed for monoamine oxidase [39]. In this mechanism, the cytokinin substrate reacts with the oxidized flavin to form a transient flavin covalent adduct to the C4a atom. Such a reduction reaction is in line with the reluctance of *N*-methyl-isopentenyladenine to reduce the flavin cofactor as the tertiary amine is sterically hindered to approach the cofactor. The formed covalent intermediate breaks down to the oxidized flavin cofactor and an aldimine form of the cytokinin. Spectral analysis of the reductive half-reaction confirms the formation of such a product intermediate. The rapid reaction and inhibition results suggest that, for re-oxidation of the reduced flavin, the electron acceptor



reacts with a binary complex. After re-oxidation, the imine dissociates from the active site and subsequently hydrolyses to adenine and a side-chain aldehyde.

Our first pre-steady-state kinetic experiments with iP indicate that reduction of the flavin by this cytokinin is extremely fast ( $950\text{ s}^{-1}$ ), whereas the enzyme displays a high affinity for the substrate ( $K_d < 2\ \mu\text{M}$ ). As the measured turnover rate with iP is significantly lower for the identified electron acceptors (approx.  $150\text{ s}^{-1}$ ), it is probable that, for this substrate, the rate of conversion is limited by the rate of re-oxidation or product release. As the turnover rate with the electron acceptors is still several orders of magnitude faster when compared with using molecular oxygen as electron acceptor ( $0.5\text{ s}^{-1}$ ), it is clear that the low rate of conversion with molecular oxygen is limited by the re-oxidation rate. With the aromatic cytokinins, no increase in the turnover rate was found when using a range of potential electron acceptors. This suggests that, in this case, the cytokinin-mediated reduction of the cofactor is relatively slow. Apparently, the enzyme is highly efficient only with iP-type cytokinins when appropriate electron acceptors are available.

As described here, the reaction rate of cytokinin degradation strictly depends on the electron acceptor used. Therefore the cytokinin turnover rates shown here are much higher than those available in the literature, where the type or concentration of the electron acceptor was inadequate. For example, with  $0.05\text{ mM}$  DCPIP, the observed conversion rate of ZmCKX1 with iP was only  $67\text{ s}^{-1}$  [5]. The values  $36$  and  $54\text{ s}^{-1}$  obtained for *trans*-zeatin, using  $0.05$  and  $0.075\text{ mM}$  DCPIP respectively [5,6], cannot be compared directly because the *p*-aminophenol method shows poor molar absorption coefficient with *trans*-zeatin product, 4-hydroxy-3-methyl-2-butenal [21], and the reaction is affected by a side reaction with DCPIP [6]. It is expected, however, that the rates observed would increase significantly with a proper type and concentration of the electron acceptor. In retrospect, all currently applied methods for CKX activity measurements suffer from undersaturation of the system with the (artificial) electron acceptor used. It is probable that the native electron acceptor allows the enzyme to achieve relatively high turnover rates. Since the reduction of the flavin cofactor with iP is very fast and the enzyme displays a relatively low  $K_d$  for iP ( $< 2\ \mu\text{M}$ ), theoretically, the enzyme is able to degrade cytokinins very efficiently even at low concentrations. Our results correspond to the observation that the apparent  $K_m$  value for iP conversion by ZmCKX1 was only  $1.5\ \mu\text{M}$  when measured with DCPIP bleaching [5]. Fast turnover, however, is achieved only if a suitable electron acceptor is available. If a highly effective electron acceptor is present, the enzyme should be theoretically capable of degrading cytokinins in turnover rates approaching  $1000\text{ s}^{-1}$ . When the reduced form of flavin is re-oxidized with molecular oxygen, the reaction proceeds with all cytokinins studied in the present study with turnover rate of approx.  $0.5\text{ s}^{-1}$ , which is doubtful to have any physiological significance. Since the aerobic low turnover rates may be greatly affected in the presence of different redox compounds such as membrane quinones, activity assay methods that do not use any electron acceptor in the reaction mixture can produce ambiguous results when applied to plant homogenates [3,11,12,40,41].

Out of the seven CKX genes in *Arabidopsis*, four encode N-terminal signal peptides that probably direct the proteins to the secretory pathway, two signal peptides predict transport to mitochondria and one protein has a presumably cytosolic location [2]. The total number of CKX genes in maize is not known at present, but at least four different CKX genes are expressed in developing maize kernels [42]. Differences in local environments of CKX activity therefore dictate differences in modes of biochemical action on cytokinin substrates, electron

acceptors, or both. In addition to the broad comparisons with other oxidoreductase family members, corresponding similarities and differences among CKX enzymes could be evaluated, which relate to electron acceptor use *in vivo*.

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## REFERENCES

- 1 Fraaije, M. W., van Berkel, W. J. H., Benen, J. A. E., Visser, J. and Mattevi, A. (1998) A novel oxidoreductase family sharing a conserved FAD-binding domain. *Trends Biochem. Sci.* **23**, 206–207
- 2 Schmülling, T. (2002) New insights into the functions of cytokinins in plant development. *J. Plant Growth Regul.* **21**, 40–49
- 3 Hare, P. D. and van Staden, J. (1994) Cytokinin oxidase: biochemical features and physiological significance. *Physiol. Plant.* **91**, 128–136
- 4 Galuszka, P., Frébort, I., Šebela, M., Sauer, P., Jacobsen, S. and Peč, P. (2001) Cytokinin oxidase or dehydrogenase? Mechanism of the cytokinin degradation in plants. *Eur. J. Biochem.* **268**, 450–461
- 5 Bilyeu, K. D., Cole, J. L., Laskey, J. G., Riekhof, W. R., Esparza, T. J., Kramer, M. D. and Morris, R. O. (2001) Molecular and biochemical characterization of a cytokinin oxidase from maize. *Plant Physiol.* **125**, 378–386
- 6 Laskey, J. G., Patterson, P., Bilyeu, K. D. and Morris, R. O. (2003) Rate enhancement of cytokinin oxidase/dehydrogenase using 2,6-dichloroindophenol as an electron acceptor. *Plant Growth Regul.* **40**, 189–196
- 7 Mok, D. W. and Mok, M. C. (2001) Cytokinin metabolism and action. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **52**, 89–118
- 8 Galuszka, P., Frébort, I., Šebela, M. and Peč, P. (2000) Degradation of cytokinins by cytokinin oxidases in plants. *Plant Growth Reg.* **32**, 315–327
- 9 Morris, R. O., Bilyeu, K. D., Laskey, J. G. and Cheikh, N. N. (1999) Isolation of a gene encoding a glycosylated cytokinin oxidase from maize. *Biochem. Biophys. Res. Commun.* **255**, 328–333
- 10 Houba-Herlin, N., Pethe, C., d'Alayer, J. and Laloue, M. (1999) Cytokinin oxidase from *Zea mays*: purification, cDNA cloning and expression in moss protoplasts. *Plant J.* **17**, 615–626
- 11 Werner, T., Motyka, V., Strnad, M. and Schmülling, T. (2001) Regulation of plant growth by cytokinin. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 10487–10492
- 12 Werner, T., Motyka, V., Laucou, V., Smets, R., van Onckelen, H. and Schmülling, T. (2003) Cytokinin-deficient transgenic *Arabidopsis* plants show multiple developmental alterations indicating opposite functions of cytokinins in the regulation of shoot and root meristem activity. *Plant Cell* **15**, 1–20
- 13 Yang, S. H., Yu, H. and Goh, C. J. (2002) Isolation and characterization of the orchid cytokinin oxidase DSCCKX1 promoter. *J. Exp. Bot.* **53**, 1899–1907
- 14 Yang, S. H., Yu, H. and Goh, C. J. (2003) Functional characterisation of a cytokinin oxidase gene DSCCKX1 in *Dendrobium* orchid. *Plant. Mol. Biol.* **51**, 237–248
- 15 Aikazyan, V. T. and Nalbandyan, R. M. (1975) Plantacyanin from spinach. *FEBS Lett.* **55**, 272–274
- 16 Wang, J. and Letham, D. S. (1995) Cytokinin oxidase – purification by affinity chromatography and activation by caffeic acid. *Plant Sci.* **112**, 161–166
- 17 Šebela, M., Studničková, M. and Wimmerová, M. (1996) Differential pulse polarographic study of the redox centres in pea amine oxidase. *Bioelectrochem. Bioenerget.* **41**, 173–179
- 18 Massey, V. (1991) A simple method for the determination of redox potentials. In *Flavins and Flavoproteins 1990* (Curti, B., Ronchi, S. and Zanetti, G., eds.), pp. 59–66, de Gruyter, Berlin
- 19 Pace, C. P. and Stankovich, M. T. (1987) Redox properties of electron-transferring flavoprotein from *Megasphaera elsdenii*. *Biochim. Biophys. Acta* **911**, 267–276
- 20 Gutierrez, A., Lian, L.-Y., Wolf, C. R., Scrutton, N. S. and Roberts, C. K. G. (2001) Stopped-flow kinetic studies of flavin reduction in human cytochrome P450 reductase and its component domains. *Biochemistry* **40**, 1964–1975
- 21 Frébort, I., Šebela, M., Galuszka, P., Werner, T., Schmülling, T. and Peč, P. (2002) Cytokinin oxidase/dehydrogenase assay: optimized procedures and applications. *Anal. Biochem.* **306**, 1–7
- 22 Lundblad, R. L. and Noyes, C. M. (1984) *Chemical Reagents for Protein Modification*, CRC Press, Boca Raton, FL

- 23 Haviger, A., Peč, P. and Frébort, I. (1991) Simultaneous determination of peroxidase and catalase activities using modified guaiacol method. *Biológia (Bratislava)* **46**, 337–343
- 24 Doerge, D. R., Divi, R. L. and Churchwell, M. I. (1997) Identification of the colored guaiacol oxidation product produced by peroxidases. *Anal. Biochem.* **250**, 10–17
- 25 Fraaije, M. W., van den Heuvel, R. H. H., van Berkel, W. J. H. and Mattevi, A. (1999) Covalent flavinylation is essential for efficient redox catalysis in vanillyl-alcohol oxidase. *J. Biol. Chem.* **274**, 35514–35520
- 26 Coulombe, R., Yue, K. Q., Ghisla, S. and Vrieland, A. (2001) Oxygen access to the active site of cholesterol oxidase through a narrow channel is gated by an Arg–Glu pair. *J. Biol. Chem.* **276**, 30435–30441
- 27 Armstrong, D. J. (1994) Cytokinin oxidase and the regulation of cytokinin degradation. In *Cytokinins: Chemistry, Activity and Function* (Mok, D. W. S. and Mok, M. C., eds.), pp. 139–154, CRC Press, Boca Raton, FL
- 28 Massey, V. and Palmer, G. (1966) On the existence of spectrally distinct classes of flavoprotein semiquinones. A new method for the quantitative production of flavoprotein semiquinones. *Biochemistry* **5**, 3181–3189
- 29 Dwyer, T. M., Mortl, S., Kemter, K., Bacher, A., Fauq, A. and Frerman, F. E. (1999) The intraflavin hydrogen bond in human electron transfer flavoprotein modulates redox potentials and may participate in electron transfer. *Biochemistry* **38**, 9735–9745
- 30 Yin, Y., Sampson, N. S., Vrieland, A. and Lario, P. I. (2001) The presence of a hydrogen bond between asparagine 485 and the  $\pi$  system of FAD modulates the redox potential in the reaction catalyzed by cholesterol oxidase. *Biochemistry* **40**, 13779–13787
- 31 Fraaije, M. W. and van Berkel, W. J. H. (1997) Catalytic mechanism of the oxidative demethylation of 4-(methoxymethyl)phenol by vanillyl-alcohol oxidase. *J. Biol. Chem.* **272**, 18111–18116
- 32 Copeland, R. A. (1996) *Enzymes. A Practical Introduction to Structure, Mechanism, and Data Analysis*, pp. 263–278, VCH Publishers, Cambridge, U.K.
- 33 Segel, I. H. (1993) *Enzyme Kinetics. Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems*, pp. 320–329, J. Wiley and Sons, New York
- 34 Lancaster, C. R. and Michel, H. (1999) Refined crystal structures of reaction centres from *Rhodospseudomonas viridis* in complexes with the herbicide atrazine and two chiral atrazine derivatives also lead to a new model of the bound carotenoid. *J. Mol. Biol.* **286**, 883–898
- 35 Marchler-Bauer, A., Panchenko, A. R., Shoemaker, B. A., Thiessen, P. A., Geer, L. Y. and Bryant, S. H. (2002) CDD: a database of conserved domain alignments with links to domain three-dimensional structure. *Nucleic Acids Res.* **30**, 281–283
- 36 Fraaije, M. W. and Mattevi, A. (2000) Flavoenzymes: diverse catalysts with recurrent features. *Trends Biochem. Sci.* **25**, 126–132
- 37 Dym, O., Pratt, E. A., Ho, C. and Eisenberg, D. (2000) The crystal structure of *D*-lactate dehydrogenase, a peripheral membrane respiratory enzyme. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 9413–9418
- 38 Benson, T. E., Fillman, D. J., Walsh, C. T. and Hogle, J. M. (1995) An enzyme–substrate complex involved in bacterial cell wall biosynthesis. *Nat. Struct. Biol.* **2**, 644–653
- 39 Binda, C., Mattevi, A. and Edmondson, D. E. (2003) Structure–function relationships in flavoenzyme-dependent amine oxidations: a comparison of polyamine oxidase and monoamine oxidase. *J. Biol. Chem.* **277**, 23973–23976
- 40 Motyka, V., Faiss, M., Strnad, M., Kamínek, M. and Schmölling, T. (1996) Changes in cytokinin content and cytokinin oxidase activity in response to de-repression of *ipt* gene transcription in transgenic tobacco calli and plants. *Plant Physiol.* **112**, 1035–1043
- 41 Motyka, V., Vaňková, R., Čapková, R., Petrášek, J., Kamínek, M. and Schmölling, T. (2003) Cytokinin-induced upregulation of cytokinin oxidase activity in tobacco includes changes in enzyme glycosylation and secretion. *Physiol. Plant.* **117**, 11–21
- 42 Bilyeu, K. D., Laskey, J. G. and Morris, R. O. (2003) Dynamics of expression and distribution of cytokinin oxidase/dehydrogenase in developing maize kernels. *Plant Growth Regul.* **39**, 195–203

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