Plant Physiol. (1987) 84, 726–731
0032-0889/87/84/0726/06/$01.00/0

Cytokinin Oxidase from *Phaseolus vulgaris* Callus Tissues[1]

ENHANCED *IN VITRO* ACTIVITY OF THE ENZYME IN THE PRESENCE OF COPPER-IMIDAZOLE COMPLEXES

Received for publication December 18, 1986

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ABSTRACT

The effects of metal ions on cytokinin oxidase activity extracted from callus tissues of *Phaseolus vulgaris* L. cv Great Northern have been examined using an assay based on the oxidation of N^6-(Δ^2-isopentenyl)-adenine-2,8-^{3}H (i^{6}Ade) to adenine (Ade). The addition of cupric ions to reaction mixtures containing imidazole buffer markedly enhanced cytokinin oxidase activity. In the presence of optimal concentrations of copper and imidazole, cytokinin oxidase activity was stimulated more than 20-fold. The effect was enzyme dependent, specific for copper, and observed only in the presence of imidazole. The substrate specificity of the copper-imidazole enhanced reaction, as judged by substrate competition tests, was the same as that observed in the absence of copper and imidazole. Similarly, in tests involving DEAE-cellulose chromatography, elution profiles of cytokinin oxidase activity determined using a copper-imidazole enhanced assay were identical to those obtained using an assay without copper and imidazole. On the basis of these results, the addition of copper and imidazole to reaction mixtures used to assay for cytokinin oxidase activity is judged to provide a reliable and specific assay of greatly enhanced sensitivity for the enzyme. The mechanism by which copper and imidazole enhance cytokinin oxidase activity is not certain, but the reaction catalyzed by the enzyme was not inhibited by anaerobic conditions when these reagents were present. This observation suggests that copper-imidazole complexes are substituting for oxygen in the reaction mechanism by which cytokinin oxidase effects cleavage of the N^6-side chain of i^{6}Ade.

Cytokinin degradation in plant tissues appears to be due in large measure to the presence of a specific cytokinin oxidase (4, 10, 11, 16, 17, 19, 22). This enzyme catalyzes the oxidative cleavage of the N^6-side chains of zeatin (io^{6}Ade),[3] i^{6}Ade, and their ribonucleosides (io^{6}Ado and i^{6}Ado) to form Ade or Ado and the corresponding side chain fragment (2, 10). Details of the reaction mechanism and the nature of the prosthetic group of the enzyme have yet to be established. However, molecular oxygen is required for side chain cleavage (22), and an imino-purine intermediate postulated by Whitty and Hall (22) has recently been isolated from reaction mixtures containing 2-mercaptoethanol (6).

We have previously described the substrate specificity and regulatory properties of a cytokinin oxidase activity isolated from callus tissues of *Phaseolus vulgaris* cv Great Northern (4). Transient increases in the supply of exogenous cytokinin were observed to induce elevated levels of this enzyme activity in the Great Northern callus tissue. The increase in cytokinin oxidase activity occurred relatively rapidly (within a few hours following cytokinin treatment) and was sensitive to inhibitors of RNA and protein synthesis.

The effects of metal ions on the *in vitro* activity of the cytokinin oxidase extracted from callus tissues of *P. vulgaris* cv Great Northern have been examined in the present investigation. We report here that the activity of the enzyme is greatly enhanced in the presence of copper-imidazole complexes and present evidence that such complexes substitute for oxygen in the reactions catalyzed by cytokinin oxidase.

MATERIALS AND METHODS

Chemicals. Picloram (4-amino-3,5,6-trichloropicolinic acid) was purchased from Aldrich. Thidiazuron (*N*-phenyl-*N'*1,2,3-thidiazol-5-ylurea, DROPP, SN 49537) was a gift from NOR-AM Chemical Co. N^6-Isopentyladenine (hi^{6}Ade) was synthesized as described by Leonard *et al.* (8). All other cytokinin-active compounds used in this study were purchased from Sigma.

Polymin P (polyethyleneimine, 50% w/v) was obtained from Miles Laboratories, Inc. Ammonium sulfate (enzyme grade) was purchased from Schwarz/Mann. PVPP was purchased from Sigma and prepared for use as previously described (4). DE52-cellulose was obtained from Whatman. Sephadex LH-20 was purchased from Sigma. The Si-C_{18} TLC plates (without fluorescent indicator) used in this study are products of J. T. Baker Co.

Adenosine-2,8-^{3}H (40 Ci/mmol) was obtained from ICN Radiochemicals. Ready-Solv HP/b is a Beckman product.

Plant Materials. Seeds of *Phaseolus vulgaris* L. cv Great Northern were obtained from Atlee Burpee Co. The seeds were surface sterilized for 5 min in 50% Chlorox containing 0.1% (v/v) Tween 20, rinsed three times in sterile water, and germinated under aseptic conditions. Tissue cultures of *P. vulgaris* cv Great Northern were established from the hypocotyl tissue of 5-d-old seedlings as described previously (13). The callus tissues were subcultured on fresh medium at approximately 24 d intervals.

Tissue Culture Medium. The medium used to culture *Phaseolus* callus tissues consisted of the mineral nutrients defined by Murashige and Skoog (15) with the following organic substances added: sucrose (30 g/L), *myo*-inositol (100 mg/L), thiamine·HCl (1 mg/L), pyridoxine·HCl (0.5 mg/L), picloram (2.5 μM), and kinetin (5 μM). The pH of the medium was adjusted to 5.7 and Difco Bacto-agar (10 g/L) was added. The medium was

[1] Supported by the Science and Education Administration of the United States Department of Agriculture under grant 86-CRCR-1-1988 from the Competitive Research Grants Office.

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[3] Abbreviations: io^{6}Ade, zeatin; Ade, adenine; Ado, adenosine; b^{6}Ade, N^6-benzyladenine; hi^{6}Ade, N^6-isopentyladenine; i^{6}Ade, N^6-(Δ^2-isopentenyl)adenine; i^{6}Ado, N^6-(Δ^2-isopentenyl)adenosine; PVPP, polyvinylpolypyrrolidone; TDZ, thidiazuron (*N*-phenyl-N'-1,2,3-thidiazol-5-ylurea).

dispensed into 125 ml Erlenmeyer flasks (50 ml/flask) and autoclaved at 120°C for 15 min.

Preparation of i^{6}Ade-2,8-^{3}H. Ado-2,8-^{3}H was adjusted to a specific activity of 250 mCi/mmol by the addition of unlabeled Ado and used in the synthesis of i^{6}Ado-2,8-^{3}H according to methods previously described for the synthesis of i^{6}Ado-8-^{14}C (14). The i^{6}Ado-2,8-^{3}H was purified by chromatography on a Sephadex LH-20 column in 33% (v/v) ethanol (1) and deribosylated to i^{6}Ade as described by Laloue *et al.* (7). The i^{6}Ade-2,8-^{3}H was isolated from the reaction mixture by chromatography on a Sephadex LH-20 column in 20% (v/v) ethanol and stored at −20°C in 50% (v/v) ethanol. The yield from Ado-2,8-^{3}H was about 20%. Prior to use in enzyme assays, the i^{6}Ade-2,8-^{3}H was adjusted to a specific activity of 100 μCi/μmol with unlabeled i^{6}Ade, and the ethanol was removed by evaporation.

Extraction and Assay of Cytokinin Oxidase Activity. Callus tissues of *P. vulgaris* cv Great Northern (5–7.5 g/flask, 19–22 d old) were homogenized (1 min, Sorvall Omnimixer, full speed) with an equal volume of cold 100 mM bis-Tris-HCl (pH 6.5). All subsequent operations were performed at 4°C unless otherwise indicated. Homogenate aliquots equivalent to 5 g of tissue were each mixed with 1.5 g (dry weight) of PVPP hydrated with 50 mM bis-Tris-HCl (pH 6.5). The resulting suspensions were filtered under pressure (2 lb/in^2 N_2) through two layers of Miracloth. For each homogenate aliquot, the solids retained by Miracloth were washed with two 5 ml aliquots of the 50 mM buffer, and the filtrates were combined and centrifuged (10,000*g*, 10 min). Polymin P (1% v/v, pH 6.5) was added dropwise with stirring to the supernatant (40 μl Polymin P/ml supernatant). After 10 min, the precipitated nucleic acids and associated proteins (3) were removed by centrifugation (10,000*g*, 10 min). Solid ammonium sulfate was added to the supernatant from the Polymin P step to give 80% saturation. (The pH was maintained at 6.5.) The resulting suspension was allowed to stand without stirring for 30 min prior to centrifugation (20,000*g*, 20 min). If necessary, the pellets of precipitated protein were stored at −20°C. At this temperature, pellets sealed under nitrogen could be stored for at least 10 weeks with no detectable loss in cytokinin oxidase activity. The yield of protein was approximately 1.2 mg/g of tissue. The yield of cytokinin oxidase activity (assayed in 100 mM imidazole buffer, pH 6.5, without the addition of copper) was equivalent to approximately 1.5 nmol/h·g of tissue.

The protein pellets from above were dissolved in bis-Tris or imidazole buffer (pH 6.5), centrifuged (10,000*g*, 10 min) to remove any traces of particulate material, and assayed for cytokinin oxidase activity using i^{6}Ade-2,8-^{3}H as a substrate. The assay mixtures contained bis-Tris-HCl or imidazole-HCl buffer (both at pH 6.5) at the concentrations indicated (50, 100, or 200 mM), 0.01 mM i^{6}Ade-2,8-^{3}H (0.05 μCi, 100 μCi/μmol), metal ions and other additives as indicated, and enzyme in a total volume of 50 μl. The assays were incubated for 30 min at 37°C. The reactions were terminated by the addition of 0.1 volumes of 200 mM Na_4EDTA and 2 volumes of cold 95% (v/v) ethanol containing unlabeled Ade and i^{6}Ade (0.75 mM each). After 10 min on ice, precipitated protein was removed by centrifugation. Aliquots (100 μl) of the supernatants were streaked on 5 cm wide Si-C_{18} TLC plates. The chromatograms were developed to a height of 5 cm in 38% (v/v) ethanol containing 100 mM Na_4EDTA. (The addition of Na_4EDTA to the developing solvent was necessary to prevent copper and other metals added to the reaction mixtures from interfering with the chromatography.) The locations of the Ade and i^{6}Ade standards were determined by inspection under UV light, and bands (1.3 cm wide) centered on the standards were removed and counted in 5 ml Ready-Solv HP/b in a Beckman model 1801 scintillation counter.

Preparation of the DEAE-Cellulose Column. DE52-cellulose was precycled in HCl and NaOH (each at 0.5 N), degassed, and fines removed as recommended by Whatman (21). The exchanger was titrated to pH 7.5 with Tris base, washed with 10 bed volumes of 20 mM Tris-HCl (pH 7.5) containing 1 M KCl and packed into a 2 × 32 cm column (100 ml bed volume) in the same buffer-salt solution at a flow rate of 300 ml/h. The packed column was washed with 20 mM Tris-HCl (pH 7.5) until the pH and conductivity of the eluate were the same as those of the wash buffer.

Assay of Cytokinin Oxidase Activity by a Modified Procedure using Solvent Extraction. The cytokinin oxidase activity in fractions resulting from chromatography on DEAE-cellulose was determined using a modified assay procedure in which undegraded i^{6}Ade was removed from the reaction mixtures by solvent extraction. For the copper-enhanced assay, the reaction mixtures contained 100 mM imidazole-HCl (pH 6.5), 0.01 mM i^{6}Ade-2,8-^{3}H (0.05 μCi, 100 μCi/μmol), 10 mM $CuCl_2$, and 40 μl aliquots of the column fractions in a final volume of 50 μl. For the unenhanced enzyme assays, 100 mM bis-Tris-HCl (pH 6.5) was substituted for imidazole and $CuCl_2$ was omitted from the reaction mixtures. All assays were incubated at 37°C for 30 min and terminated by the addition of 50 μl of 20 mM Na_4EDTA containing i^{6}Ado and Ade (each at 1 mM) followed by the addition of 300 μl of butyl acetate saturated with a 1 mM Ade solution. The assay tubes were vortexed for 15 s, and the two phases separated by centrifugation. The organic phase, containing undegraded i^{6}Ade, was removed, and the extraction with butyl acetate (saturated with 1 mM Ade) was repeated two times. Aliquots (80 μl) of the aqueous phase (containing the adenine produced in the enzyme reaction) were mixed with 5 ml of Ready-Solv HP/b and counted in a Beckman LS 1801 scintillation counter.

Protein Assays. Protein solutions were mixed with equal volumes of cold 20% (w/v) TCA and allowed to stand on ice for 30 min. The precipitated protein was collected by centrifugation (20,000*g*, 10 min) and dissolved in 0.1 N NaOH for protein determination using the Folin phenol method of Peterson (18). BSA was used as a standard.

RESULTS

Effects of Metal Ions on Cytokinin Oxidase Activity. The effects of metal ions on the activity of cytokinin oxidase preparations from callus tissues of *P. vulgaris* cv Great Northern were compared using two alternative buffers (100 mM bis-Tris, pH 6.5, and 100 mM imidazole, pH 6.5). The metals were tested at 1 mM concentrations. As shown in Table I, copper was the only one of the eight metals tested that had a significant effect on cytokinin oxidase activity. Copper slightly stimulated the activity of the enzyme assayed in bis-Tris buffer and markedly enhanced the activity when imidazole was used as the assay buffer. Compared to controls assayed without copper, cytokinin oxidase activity was stimulated approximately 4-fold by the addition of 1 mM $CuCl_2$ to assay mixtures containing imidazole buffer. As noted previously (4), imidazole itself somewhat stimulated cytokinin oxidase activity relative to controls assayed in bis-Tris.

Enhanced Activity of Cytokinin Oxidase in the Presence of Copper-Imidazole Complexes. The effects of copper concentration on cytokinin oxidase activity were tested at two imidazole concentrations (100 and 200 mM) (Fig. 1). At both imidazole concentrations, cytokinin oxidase activity increased as a function of copper concentration until the molar ratio of copper to imidazole reached a value of 1 to 10. At higher copper concentrations, where imidazole was present in less than 10-fold excess, cytokinin oxidase activity decreased sharply. The cytokinin oxidase activities observed at optimal copper concentrations were similar for both imidazole concentrations and corresponded to approximately 30-fold increases in enzyme activity.

The enhanced degradation of i^{6}Ade observed in the presence

Table I. *Effects of Metals on Cytokinin Oxidase Activity*

Metal (1 mM)	Cytokinin Oxidase Activity[a] with Buffer:	
	Bis-Tris	Imidazole
	% of control[b]	
$CuCl_2$	113	410
$CaCl_2$	102	99
$NiCl_2$	98	95
$CoCl_2$	100	97
$ZnCl_2$	91	105
$MgSO_4$	96	104
$MnSO_4$	102	106
$FeSO_4$	98	91

[a] Cytokinin oxidase activity was determined in 50 μl reaction volumes containing 100 mM buffer (pH 6.5), 0.01 mM i⁶Ade-2,8-³H (0.05 μCi, 100 μCi/μmol), enzyme equivalent to 250 mg (fresh weight) of *P. vulgaris* cv Great Northern callus tissue, and the indicated concentration of metal salts. The enzyme preparation used here had been partially purified by chromatography. [b] The cytokinin oxidase activities of controls incubated without metal additions were equal to 0.97 and 1.51 nmol/h·mg protein for the bis-Tris and imidazole assays, respectively. These values were taken as 100% activity. All values are the average of three assays. Standard errors averaged ±1% of the means and did not exceed ±2% of the means.

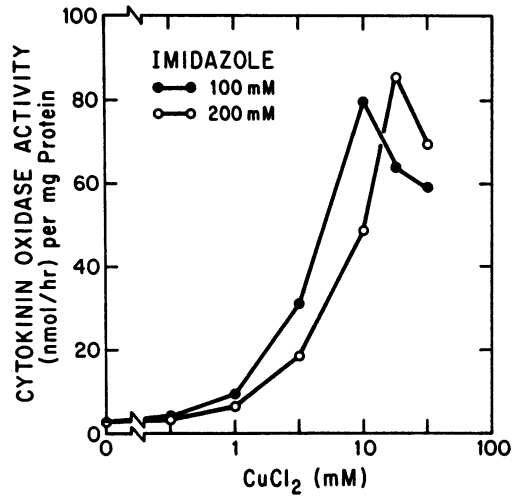

FIG. 1. Effects of varying concentrations of $CuCl_2$ and imidazole on the *in vitro* activity of cytokinin oxidase extracted from callus tissues of *P. vulgaris* cv Great Northern. Cytokinin oxidase activity was determined in reaction mixtures containing 100 or 200 mM imidazole-HCl (pH 6.5), 0.01 mM i⁶Ade-2,8-³H (0.05 μCi, 100 μCi/μmol), the indicated concentrations of $CuCl_2$, and partially purified enzyme equivalent to 21 or 125 mg (fresh weight) of *P. vulgaris* cv Great Northern callus tissue. All values are the average of three assays. SE values averaged ±1% of the means and did not exceed ±2% of the means.

of copper-imidazole complexes was enzyme dependent and inhibited by the chelating agent EDTA (Table II). The degradation of i⁶Ade to Ade did not occur in either the presence or absence of copper if the enzyme preparations were denatured or omitted from the reaction mixture. The addition of EDTA to reaction mixtures containing copper and imidazole reduced the observed activity of cytokinin oxidase to that of controls without copper.

Substrate Specificity of the Copper-Imidazole Enhanced Reaction. The specificity of the enhanced reaction catalyzed by cytokinin oxidase in the presence of copper and imidazole was examined by substrate competition tests in which unlabeled cytokinins were added to cytokinin oxidase assay mixtures at

Table II. *Copper-Imidazole Stimulation of Cytokinin Oxidase Activity from P. vulgaris cv Great Northern Callus Tissues*

Assay Conditions	Cytokinin Oxidase Activity[a]
	pmol/h ± SE
Standard assay[b]	6 ± 1
Standard assay + 10 mM $CuCl_2$	124 ± 1
Standard assay + 10 mM $CuCl_2$ + 20 mM EDTA	6 ± 1
Standard assay + 10 mM $CuCl_2$ − enzyme	0
Standard assay + 10 mM $CuCl_2$ + boiled enzyme[c]	0

[a] Cytokinin oxidase activity was assayed as described in "Materials and Methods." Each value is the average of three assays. [b] Standard assay mixtures contained 200 mM imidazole-HCl (pH 6.5), 0.01 mM i⁶Ade-2,8-³H (0.05 μCi, 100 μCi/μmol), and 8 μg of protein in a volume of 50 μl. The enzyme preparation was obtained by the standard extraction procedure described in "Materials and Methods." [c] Prior to assay, the enzyme solution was incubated in boiling water for 5 min.

Table III. *Effects of Unlabeled Cytokinins on the Degradation of N^6-(Δ²-Isopentenyl)adenine-2,8-³H by Cytokinin Oxidase in Bis-Tris, Imidazole, and Copper-Imidazole Buffers*

Buffer[a] (pH 6.5)	Unlabeled Cytokinin Concentration	Cytokinin Oxidase Activity[b] with Unlabeled Cytokinin Tested[c]				
		i⁶Ade	hi⁶Ade	TDZ	io⁶Ade	b⁶Ade
	μM	*% of control*				
Bis-Tris	10	61	98	54	82	98
	100	13	91	17	65	90
Imidazole	10	62	98	56	81	98
	100	15	92	18	65	95
Imidazole + $CuCl_2$	10	60	92	52	82	95
	100	16	87	17	45	92

[a] Assay mixtures contained 100 mM buffer, 0.01 mM i⁶Ade-2,8-³H (0.05 μCi, 100 μCi/μmol), 120 μg protein (bis-Tris and imidazole assays) or 5 μg protein (copper-imidazole assays), and the indicated concentrations of unlabeled cytokinin. The copper-imidazole assay mixtures contained (in addition) $CuCl_2$ at 10 mM. The enzyme preparation used here had been partially purified by chromatography. All other conditions were as described in "Materials and Methods." [b] The cytokinin oxidase activities of bis-Tris, imidazole, and copper-imidazole controls incubated without the addition of unlabeled cytokinins were equal to 1.02, 1.24, and 29.8 nmol/h·mg protein, respectively. These values were taken as 100% activity. All values are the average of three assays. SE averaged ± 1% of the means and did not exceed ±2% of the means. [c] Abbreviations are as follows: i⁶Ade, N^6-(Δ²-isopentenyl)adenine; hi⁶Ade, N^6-isopentyladenine; TDZ, Thidiazuron (*N*-phenyl-*N'*-1,2,3-thiadiazol-5-ylurea); io⁶Ade, zeatin; b⁶Ade, N^6-benzyladenine.

concentrations equal to that of the ³H-labeled i⁶Ade substrate (10 μM) or in 10-fold excess (100 μM). In this manner, the specificity of the copper-enhanced assay was compared with the specificity of unenhanced assays performed in the absence of copper and using either imidazole or bis-Tris as the assay buffer (Table III). Similar tests of the substrate specificity of cytokinin oxidase in imidazole buffer (without copper) have been reported previously (4). The results obtained here were essentially the same for all three assay systems tested. Unlabeled i⁶Ade and the cytokinin-active urea derivative, Thidiazuron, were equally effective in inhibiting the degradation of the labeled substrate. Zeatin rather weakly inhibited the reaction, and the addition of b⁶Ade and hi⁶Ade to the reaction mixtures had no significant effect on the rate of the reaction.

Enzyme Specificity of the Copper-Imidazole Enhanced Reaction. The specificity and reliability of the copper-imidazole enhanced assay for cytokinin oxidase activity were further tested

by chromatographic fractionation of enzyme preparations extracted from Great Northern callus tissues. To accommodate the large number of samples generated by column chromatography, the standard assay procedure for cytokinin oxidase activity was modified and simplified. Solvent extractions were used to remove undegraded i^{6}Ade from the reaction mixtures and thus eliminate the necessity of separating the reaction products by TLC. To establish the validity of the assay procedure based on solvent extraction, assays in which i^{6}Ade was removed by extraction with either ethyl acetate or butyl acetate were compared with assays using the standard TLC procedure. The results of these tests are shown in Table IV. The i^{6}Ade in the cytokinin oxidase reaction mixtures was quantitatively extracted into both solvents. However, Ade recovery in the aqueous phase from the ethyl acetate extraction was 52% of that measured by the standard assay, compared to 77% when butyl acetate was used. Therefore, butyl acetate was selected as the extraction solvent in subsequent assays for cytokinin oxidase activity in fractions generated by column chromatography.

The cytokinin oxidase activity extracted from callus tissues of *P. vulgaris* cv Great Northern was fractionated by DEAE-cellulose chromatography. The column fractions were assayed for cytokinin oxidase activity using the copper-imidazole enhanced assay and an unenhanced assay in which copper was omitted from the reaction mixtures and bis-Tris was used as the assay buffer. In both cases, the butyl acetate extraction procedure described above was employed for quantitation. The elution profiles for cytokinin oxidase activity assayed in the presence and absence of copper-imidazole complexes are compared in Figure 2. As expected, the cytokinin oxidase activity observed with the copper-imidazole assay was markedly higher than the activity determined in bis-Tris, but the profiles were otherwise identical.

Effects of Anaerobic Conditions on the Copper-Imidazole Enhanced Reaction. The relatively high copper concentrations required for enhancement of cytokinin oxidase activity suggested that the metal was acting in some manner other than as a cofactor for the enzyme. The possibility that copper-imidazole complexes might be functioning as alternative oxidants (replacing oxygen) in the assay was tested by examining the effect of copper-imidazole additions on enzyme activity measured under anaerobic conditions (Table V). Reducing oxygen concentration in assay mixtures from which copper was omitted and bis-Tris was used as the assay buffer resulted in a reduction in cytokinin oxidase activity to 16% of that observed in the same reaction mixtures in the presence of oxygen. In contrast to this result, the reduction of oxygen concentration in reaction mixtures containing copper and imidazole had little effect on the enhanced rate of the cytokinin oxidase reaction.

DISCUSSION

The effects of a number of metal ions on the *in vitro* activity of cytokinin oxidase extracted from callus tissues of *Phaseolus vulgaris* cv Great Northern have been examined here using an assay based on the degradation of i^{6}Ade-2,3-^{3}H to Ade. The *in vitro* activity of the enzyme was markedly enhanced in reaction mixtures containing copper and imidazole. As judged by substrate competition tests, the substrate specificity of the copper-imidazole enhanced reaction was identical to that observed in assays of cytokinin oxidase activity performed without copper and using either imidazole or bis-Tris as the reaction buffer. Elution profiles for cytokinin oxidase preparations fractionated by DEAE-cellulose chromatography were qualitatively identical using either bis-Tris or copper-imidazole as the assay buffers. On the basis of these results, the copper-imidazole enhanced reaction appears to be specific for cytokinin oxidase.

The ability of cytokinin-active urea derivatives to inhibit the degradation of i^{6}Ade in standard assays of cytokinin oxidase activity was reported previously by Chatfield and Armstrong (4) and by Laloue and Fox (6). It has yet to be determined whether these urea derivatives inhibit the degradation of i^{6}Ade by serving as competitive substrates for cytokinin oxidase or whether another mechanism is involved. However, it is of interest that the cytokinin-active urea derivative Thidiazuron inhibited the degradation of i^{6}Ade by cytokinin oxidase under all assay conditions tested here, including the enhanced reaction conditions provided by the presence of copper-imidazole complexes.

The degradation of i^{6}Ade to Ade catalyzed by cytokinin oxidase was observed to proceed under anaerobic conditions in the reaction mixtures containing copper-imidazole complexes. This result suggests that complexes of copper and imidazole enhance cytokinin oxidase activity by substituting for oxygen in the reaction mechanism. If oxygen-dependent cleavage of an iminopurine intermediate normally limits the rate of the cytokinin oxidase reaction, copper-imidazole complexes may enhance the reaction rate by increasing the effective concentration and/or

Table IV. *Partitioning of N^6-(Δ^2-Isopentenyl)adenine and Adenine from Cytokinin Oxidase Reaction Mixtures into Ethyl Acetate and Butyl Acetate*

Reaction Mixture[b]	Labeled Compounds Recovered	Cytokinin Oxidase Assay Method[a]				
		Standard assay (single phase)	Ethyl acetate extraction		Butyl acetate extraction	
			Aqueous phase	Organic phase	Aqueous phase	Organic phase
		% of total radioactivity recovered per assay[c]				
Control (− enzyme)	Ade	2	1	1	1	1
	i^{6}Ade	98	1	97	1	97
Assay (+ enzyme)	Ade	37	18	16	29	9
	i^{6}Ade	63	1	65	1	61

[a] The standard assay procedure and the modified assay procedure based on solvent extraction are described in "Materials and Methods." [b] Reaction mixtures contained 100 mM imidazole-HCl (pH 6.5), 0.01 mM i^{6}Ade-2,8-^{3}H (0.05 μCi, 100 μCi/μmol), and 10 mM $CuCl_2$ in a total volume of 50 μl. Protein (8 μg) from a standard cytokinin oxidase preparation was added to the assay (+ enzyme) reaction mixtures. [c] Aliquots of the phases recovered after termination of the reactions as described in "Materials and Methods" were chromatographed on SiC_{18} TLC plates in 38% (v/v) ethanol containing 100 mM Na_4EDTA and radioactivity corresponding to i^{6}Ade and Ade standards was determined as usual. Each value is the average of three assays. SE values averaged less than ±1% of the means.

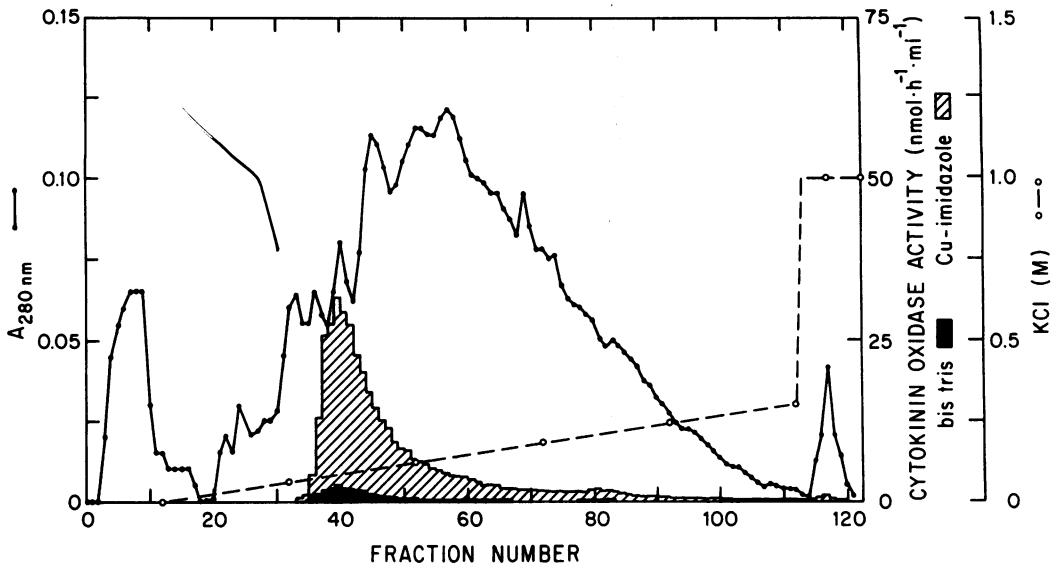

FIG. 2. DEAE-cellulose chromatography of cytokinin oxidase activity from callus tissues of *P. vulgaris* cv Great Northern. Cytokinin oxidase preparations from callus tissues of *P. vulgaris* cv Great Northern were obtained as described in "Materials and Methods." The protein recovered following ammonium sulfate precipitation was dissolved in 20 mM Tris-HCl (pH 7.5) and desalted on a Sephadex G-25 column equilibrated in the same buffer. An aliquot of desalted protein (300 mg in 140 ml buffer) was applied to a DE52-cellulose column (2 × 32 cm, 100 ml bed volume) equilibrated in 20 mM Tris-HCl (pH 7.5). The column was rinsed with 100 ml of the sample buffer and then eluted with a linear gradient of KCl (from 0–0.3 M over a volume of 2 L) dissolved in the same buffer. Fractions of 20 ml were collected at a flow rate of 50 ml/h. Cytokinin oxidase activity was assayed in bis-Tris and imidazole-copper buffers as described in "Materials and Methods."

Table V. *Effect of Anaerobic Conditions on Cytokinin Oxidase Activity*

Reaction Buffer[a]	Cytokinin Oxidase Activity[b]	
	+ O_2	− O_2
	pmol/h ± SE[c]	
Bis-Tris	65 ± 1	10 ± 1
Copper-Imidazole	186 ± 6	188 ± 5

[a] Bis-Tris reaction mixtures contained 100 mM bis-Tris-HCl (pH 6.5) and 160 μg protein from a standard enzyme preparation prepared as described in "Materials and Methods." Copper-imidazole assays contained 100 mM imidazole-HCl (pH 6.5), 10 mM $CuCl_2$, and 16 μg protein from a standard enzyme preparation. The reaction mixtures (50 μl total volumes) were contained in 0.5 ml centrifuge tubes. [b] The centrifuge tubes containing the reaction mixtures were sealed with serum stoppers. To produce anaerobic conditions, the appropriate tubes were connected to a manifold, evacuated to 0.05 atm, and then flushed with purified N_2. This process was repeated nine times. The reactions were then initiated in all tubes by adding the i^6Ade-2,8-^{3}H substrate (0.05 μCi, 100 μCi/μmol) to the enzyme mixtures with a Hamilton syringe. All other assay conditions were as described in "Materials and Methods." [c] All values are the average of three assays.

reactivity of the oxidant required for the breakdown of the intermediate. It is possible that the formation of complexes involving cytokinins and copper may also play a role in enhancing the reaction. Adenine is known to combine strongly with cupric ions (5, 9, 20), and Miller (12) has described an interaction of copper with cytokinin bases that stimulated the oxidation of NADH by horseradish peroxidase.

The extent to which the results obtained with cytokinin oxidase may be extended to other enzyme catalyzed reactions involving molecular oxygen is not certain. In preliminary experiments with galactose oxidase (data not shown), we have found that the addition of copper and imidazole to reaction mixtures used to assay for this enzyme resulted in enhanced rates of oxidation of the substrate 3-methoxybenzyl alcohol to the corresponding aldehyde. However, we cannot be certain that the mechanism of enhancement in this reaction is the same as that in the cytokinin oxidase reaction.

Whatever the mechanism of enhancement, the evidence presented here indicates that the reaction occurring in the presence of copper and imidazole provides a reliable and specific assay of cytokinin oxidase activity. The increase in assay sensitivity observed in the presence of copper and imidazole should facilitate work with the enzyme, particularly in studies involving chromatographic or electrophoretic separations. The ability of copper-imidazole complexes to drastically alter the rates of cytokinin oxidase catalyzed reactions also raises the interesting possibility that manipulation of reaction rates could be important in the *in vivo* regulation of cytokinin degradation by the enzyme.

Acknowledgment—We would like to thank Carolyn Paynter for technical assistance.

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