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Short Communication

Possible Regulatory Roles of Cytokinins[1]

NADH OXIDATION BY PEROXIDASE AND A COPPER INTERACTION

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ABSTRACT

Apparently free-base cytokinins can interact with cupric ions in a specific manner. Oxidation of NADH by a horseradish peroxidase system was strongly promoted by such cytokinins provided cupric ions were present. Oxidation was promoted by 5 micromolar kinetin, zeatin, 6-benzylaminopurine (BA), or 6-(Δ^2-isopentenylamino)purine (2iP) but not by adenine, 6-methylaminopurine or 6,6-dimethylaminopurine. The 6-methylaminopurine promoted oxidation at 500 micromolar but adenine and 6,6-dimethylaminopurine did not. Activity of the free-base purines correlated well with their activity in cell-division assays. However, addition of methoxymethyl-, cyclohexyl-, or tetrahydropyranyl- at N-9 of BA or of ribosyl- at N-9 of BA, 2iP, kinetin, or zeatin eliminated activity in the peroxidase system. In a nonenzymic system containing cupric ions, all of the bases, including adenine, inhibited the Cu^{2+}-stimulated oxidation of ascorbic acid. As in the peroxidase system, the N-9 derivatives were inactive. The cytokinin promotion of NADH oxidation by peroxidase may result from an interaction of the hormones with copper, with peroxidase conferring a specificity similar to the cytokinin specificity observed in growth and development.

In studies of biochemical functions of cytokinins, we have stressed effects of the hormones on oxidative metabolism. The effects include both promotions and inhibitions of apparent peroxidations of several compounds by whole cells (8), promotions and/or inhibitions of O_2 consumption by cells and mitochondria (9, 10, 13), inhibition of NADH oxidation by submitochondrial particles (11), and both promotion and inhibition of NADH metabolism by cell suspensions (12). It now appears that some effects of cytokinins on oxidative metabolism possibly result from interactions with copper. Recently, we extracted from soybean tissue a soluble peroxidase-containing system that oxidizes NADH, the oxidation being promoted by cytokinins (C. O. Miller, unpublished data). Horseradish peroxidase completely substituted for the soybean proteins. The promotions by the cytokinins were especially notable in the presence of cupric ions which are known to inhibit NADH oxidation (1). Frieden and Alles (4) reported that adenine is a potent inhibitor of the nonenzymic copper-promoted oxidation of ascorbic acid and we have found that the same is true of purine cytokinins when they are in a free-base form.

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MATERIALS AND METHODS

Chemicals. HRP[2] (grade I) and NADH (grade II) were purchased from Boehringer Mannheim and other compounds were from Sigma Chemical Company. The CPPU was kindly supplied by Nelson J. Leonard. The N-9 derivatives, other than those with ribose, were synthesized as previously described (8).

Enzyme Experiments. In all peroxidase experiments, the basal reaction solution was 2.2 mM KH_2PO_4 adjusted to pH 6.3. In tests of various cytokinins or related compounds for activity, 10 μM *p*-coumaric acid, 3 μM $MnSO_4$, 0.1 μM $CuSO_4$ and 0.4 μg/ml HRP also were included. Cytokinins were added in water or DMSO and controls received the same amounts of the solvents (30 μl). NADH was used at 200 nmol/ml and was added just prior to the placing of the cuvettes in the spectrophotometer. The final reaction volume was 3.15 ml. Tests were performed at 24°C. Three replicates with and three without the tested compound were employed simultaneously in each assay. There were slight differences in control oxidation rates among the various tests, but all trends have been very repeatable.

The amount of NADH remaining in each reaction solution was measured at 340 nm. Readings were made 2 and 15 min after the addition of the NADH. Molar concentrations were calculated by dividing the absorbances by 6220 and then were converted to nmol/ml. The increase of NADH oxidation given for each concentration of a compound was calculated by subtracting the average (n = 3) of the test values from the average for controls without added compound.

Oxygen Involvement. The need for O_2 was checked by employing the procedures of others (1). Basal reaction mixture was placed in Thunberg tubes and NADH (to give 200 nmol/ml) in the arms of the tubes. After evacuation with a water aspirator, all ingredients were mixed. Fifteen min later, the solutions were gently removed so as to avoid stirring and absorbances were read. In other experiments, actual O_2 consumption was measured at 25°C with a model 53 O_2 monitor (Yellow Springs Instrument) and a Clark electrode inserted into a cell of 1.2 ml volume. After 12 min, solutions were removed from the cell and changes in absorbance determined.

NAD^+ Detection. At the end of some experiments, 0.5 ml Tris buffer (0.2 M, pH 9.5) was mixed with 3.15 ml of the reaction solutions or filtrates. The adjusted pH was 8.7. Upon addition of yeast alcohol dehydrogenase (0.03 μg/ml) and 95% ethanol (20 μl/ml), any NAD^+ present was converted to NADH which was then measured at 340 nm. This is essentially the method used by Akazawa and Cohn (1) in very similar experiments. Percentages of NADH recovered were calculated by comparing

[2] Abbreviations: HRP, horseradish peroxidase; CPPU, N-4-(2-chloropyridyl)-N′-phenylurea; BA, 6-benzylaminopurine; 2iP, 6-(Δ^2-isopentenylamino)purine.

the measurements to starting absorbances, absorbances of solutions to which no enzyme had been added, and to solutions supplied with NADH at the end of the experiment.

Ascorbate Oxidation. Ascorbate oxidation was studied in 2.2 mM KH_2PO_4 solution adjusted to pH 6.3 and containing 0.2 mM potassium ascorbate. Oxidation, as indicated by O_2 consumption, was measured with the O_2 electrode as described above. Figure 1 is an inverted and reversed copy of the recorder tracing.

RESULTS

Horseradish Peroxidase. In the presence of HRP, manganous ions, and coumarate, NADH was quickly oxidized but only minor effects of added cytokinins were observed. Oxidation was substantially slowed by adding cupric sulfate (Table I) as others have found (1). In the presence of the cupric ions, however, cytokinins strikingly promoted NADH oxidation. Mg^{2+}, Fe^{3+}, and CA^{2+} did not substitute for Cu^{2+}; Co^{2+}, however did so at 100 to 500 μM (not shown). In the mixture described in "Materials and Methods," NADH oxidation was strongly accelerated by both BA and 2iP at 5 μM (Table I). The effects were evident by 2 min but were accentuated at 15 min. Kinetin and zeatin did not promote as much as the first two cytokinins, but were always promotive at this concentration in other experiments (not shown). Adenine, 6-methylaminopurine, and 6,6-dimethylaminopurine were not effective at 5 μM. At 50 μM, both kinetin and zeatin caused highly significant increases in oxidation as did BA and 2iP; the other three purines still give no promotion. The substituted urea CPPU, which is known to elicit cytokinin-type growth responses, was without effect. At 500 μM, 2iP greatly promoted oxidation. (BA and kinetin were not included because they tended to precipitate at this concentration.) Adenine and dimethylaminopurine did not promote even at this high concentration, but methylaminopurine caused a slight promotion. In other experiments (not shown), methylaminopurine produced a greater and highly significant effect at this concentration. As others (1) have reported, EDTA (5 μM) also promoted oxidation in the mixture containing Cu^{2+}.

Table I. *Modification by Cytokinins and Related Compounds of NADH Oxidation by Horseradish Peroxidase*

NADH (200 nmol/ml) was added to a mixture of 3 μM $MnSO_4$, 0.1 μM $CuSO_4$, 10 μM *p*-coumarate, and 0.4 μg/ml HRP with/without the tested compound in 2.2 mM phosphate buffer (pH 6.3). NADH concentrations were determined 2 and 15 min later from absorbances (340 nm). Three replicates of controls without and three of assays with the tested substance were made for each concentration of a compound. Each increase of NADH oxidation was calculated by subtracting the average NADH concentration obtained with the tested compound from the average concentration of the controls.[a]

Compound Tested	Increase of NADH Oxidation	
	2 min	15 min
	nmol/ml	
At 5 μM		
BA	14.5**	109***
2iP	24.1***	101***
Kinetin	4.8	27.3*
Zeatin	1.6	16.1*
Adenine	0.0	−1.6
6-Methylaminopurine	0.0	0.0
6,6-Dimethylaminopurine	−1.6	−1.6
At 50 μM		
BA	45.0***	119***
2iP	27.3**	124***
Kinetin	25.7*	103***
Zeatin	9.6**	93.2***
Adenine	−1.6	−3.2
6-Methylaminopurine	1.6	−3.2
6,6-Dimethylaminopurine	3.2	3.2
CPPU	−1.6	−1.6
At 500 μM		
2iP	27.3**	114***
Adenine	0.0	−3.2
6-Methylaminopurine	−1.6	−3.2*
6,6-Dimethylaminopurine	1.6	0.0

[a] In a typical test in the absence of both Cu^{2+} and cytokinin, NADH concentration was reduced to 90 nmol/ml in 15 min; with 0.1 μM Cu^{2+} added, the value was 192 nmol/ml. *, **, *** The probabilities (*t* test) that control concentration averages are the same as treatment averages ≦0.1, 0.05, or 0.01, respectively. SE of the 76 NADH average concentrations (including the controls) were less than 6.4 nmol/ml, with all but 14 being below 1.6.

All tests for the presence of NAD^+ as a reaction product after 15 min indicated that at least 95% of the metabolized NADH had been oxidized to NAD^+. This was true for both the controls and the cytokinin-treated reaction solutions. This high degree of recovery of NADH occurred even though the peroxidase still was rather active at the high pH.

All tested N-9 derivatives were inactive in the HRP assay. Methoxymethyl-, cyclohexyl-, and tetrahydropyranyl- derivatives of BA and the ribosyl- derivatives of BA, 2iP, zeatin, and kinetin (not shown) were tested at 5 and 50 μM.

Ascorbate Oxidation. Confirming other reports (3, 4), we found that Cu^{2+} strongly promoted O_2 consumption by a solution containing ascorbic acid and that adenine dramatically inhibited such consumption. Furthermore, BA also strongly inhibited the oxidation (Fig. 1). One mol of adenine neutralized the influence of about 2 mol of Cu^{2+} whereas BA was slightly less effective. All of the purine bases mentioned above inhibited the ascorbate oxidation, but CPPU and the N-9 derivatives were without effect (not shown). EDTA at 5 μM completely inhibited the reaction (6) with 1 mol negating the effect of 1 mol of Cu^{2+}. When tested at 5 μM, Mg^{2+}, Ca^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Ag^+, and Hg^{2+} failed to promote ascorbate oxidation and Fe^{3+} was only slightly effective. Furthermore, addition of these cations did not lessen the effectiveness of adenine or of BA in eliminating the promotion by Cu^{2+}.

DISCUSSION

In terms of the concentrations required and of how quickly the effect could be detected, 2iP and BA were the most effective in promoting NADH oxidation although kinetin and zeatin also were quite active. Methylaminopurine was slightly effective but adenine and dimethylaminopurine were not. Similarly, in cytokinin bioassays, 2iP and BA often are very effective, kinetin somewhat less so, and methylaminopurine only slightly effective; adenine and dimethylaminopurine may be very slightly active at high concentrations (5, 7). Most of these compounds clearly have similar relative activities in cytokinin bioassays and the peroxidase test. Zeatin is somewhat of an exception. In cell-division bioassays such as that employing soybean tissue, zeatin is more effective than the other compounds. Nevertheless, in view of the great differences between short-term enzyme and long-term growth or development assays, the correspondence between relative activities in the peroxidase and growth tests is remarkable. Therefore, an interaction of the hormones with some component of the peroxidase system possibly is dependent upon a cytokinin molecular property crucial to cytokinin action in growth and

development.

Much information indicates an interaction with Cu^{2+}. The unmodified cytokinins in some way reverse the inhibition of peroxidase by Cu^{2+}. The fact that the chelator EDTA does the same is suggestive of a combination of some sort between the Cu^{2+} and the cytokinins. The ascorbate experiments are quite supportive of a combination. Adenine, which is known to combine strongly with Cu^{2+} (3, 6, 15), very effectively inhibits ascorbate oxidation by copper (4); BA (Fig. 1) and the other free cytokinin bases do likewise, and so does EDTA. Adenosine (3, 4) and N-9 derivatives of the cytokinins do not inhibit ascorbate oxidation, and the N-9 cytokinin derivatives are inactive in the peroxidase system. Apparently, adenine is effective in the ascorbate system because it combines with copper at N-3 and N-9 (15). Since adenine and BA and their N-9 derivatives gave essentially identical results in the ascorbate system (Fig. 1), we presume that BA and other cytokinins also combine with Cu^{2+} at N-3 and N-9.

Substitution onto N-9—even of a methyl group—apparently shields N-3 from combining with copper (6), so the complete inactivity of N-9 derivatives in our experiments is compatible with copper interactions at both N-9 and N-3. On the basis of crystallographic studies, Sletten (15) proposed a model in which four adenines combined with two cupric ions, whereas our results indicate that one adenine or cytokinin combines with two Cu^{2+}. Sletten also stated tha the N-3 to N-9 distance is ideal for a copper-copper bridging between the two nitrogens but he did not detect the bridge in crystals. Such a bridging would give the ratio we observed. A combination between a cytokinin and copper quite clearly might occur in the peroxidase system and perhaps in various other metabolic situations. Further examination of roles of Cu^{2+} in the peroxidative and respiratory effects we have studied (8–13) may be productive.

Specificities of responses to the purine bases differ for the peroxidase and ascorbate systems. This perhaps is because of the presence or absence of protein. Possibly the cytokinin-like specificity of the peroxidase system occurs because the purine must get into a hydrophobic region of peroxidase where Cu^{2+} is held. Adenine, not being sufficiently lipophilic, presumably does not enter this region and therefore is not active. Another possibility is that the cytokinin and copper form at such a site of complex which has a positive role in NADH oxidation. If cytokinins do affect the peroxidase system because they so interact with copper, they might similarly affect other copper-influenced biochemical systems having similar sites and perhaps of greater importance than peroxidase in growth and development.

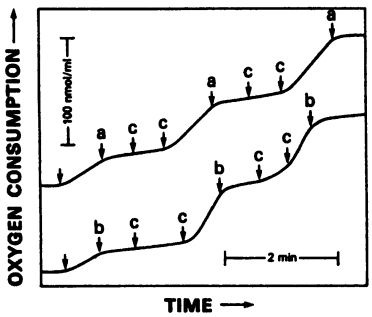

FIG. 1. Stimulation by cupric sulfate of O_2 consumption in a 0.2 mM ascorbate solution and inhibition of the stimulation by purines. Ascorbate added at first arrow on each curve. Each addition of cupric sulfate or of a purine increased its concentration by 5 μM. Arrows indicate time of additions. Additions: a, adenine; b, 6-benzylaminopurine; and c, copper sulfate.

EDTA apparently can get into the postulated hydrophobic site of peroxidase. Its failure to substitute for cytokinins in growth and development perhaps results from its not getting into the cells and to its being complexed with many other ions. Cytokinins enter cells readily and apparently do not combine very strongly with major cations at the Cu^{2+}-binding sites ("Results"). The cytokinins may have the needed lipid solubility (as in growth [5, 7]) as well as a very high specificity for binding of copper and therefore little interference from other ions. EDTA does not share these properties.

A speculative extrapolation of our results is that N-9 must be free for optimal cytokinin action in growth and development although any derivative which binds copper conceivably could be active. If N-9 indeed must be free, a meaningful assessment of current cytokinin activity in a plant requires measurement of the free bases actually present in the tissues before extraction. A tissue loaded with nucleosides or nucleotides of zeatin, for example, might contain no active cytokinin. Furthermore, any substitutent on N-9—and perhaps on N-3—would need to be metabolically removed from compounds supplied to a plant before activity could occur. A caution to these views is that adenine nucleotides bind Cu^{2+} to other nitrogens in the base (3, 4).

The two most studied types of cytokinins in growth and development are the substituted adenines and ureas. In the HRP (Table I) and soybean (C. O. Miller unpublished data) enzyme systems, CPPU was without effect. If the peroxidase system or Cu^{2+} is crucially involved in cytokinin effects on growth and development, the immediate biochemical action of the substituted ureas must differ from that of the substituted adenines.

Our findings may be of interest in reexamining related systems involved in auxin metabolism (14) or in animal peroxide-producing systems (2).

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