Short Communication

Possible Regulatory Roles of Cytokininś²

NADH OXIDATION BY PEROXIDASE AND A COPPER INTERACTION

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CARLOS O. MILLER
Department of Biology, Indiana University, Bloomington, Indiana 47405

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-(Δ²-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 tetrahydropranyl- 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 Cu²⁺-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 O₂ consumption by cells and mitochondria (9, 10, 13), inhibition of NADH oxidation by mitochondrial 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a soluble peroxidase-containing system that oxidizes NADH, the oxidation being promoted by cytokinins (C. O. Miller, unpublished data).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.

MATERIALS AND METHODS

Chemicals. HRP² (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 KH₂PO₄ adjusted to pH 6.3. In tests of various cytokinins or related compounds for activity, 10 μM p-coumaric acid, 3 μM MnSO₄, 0.1 μM CuSO₄ 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 O₂ 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 O₂ consumption was measured at 25°C with a model 53 O₂ 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 μ, 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

²Abbreviations: HRP, horseradish peroxidase; CPPU, N-4-(2-chloropyridyl)-N⁺-phenylurea; BA, 6-benzylaminopurine; 2iP, 6-(Δ²-isopentenylamino)purine.

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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 KH₂PO₄ solution adjusted to pH 6.3 and containing 0.2 mM potassium ascorbate. Oxidation, as indicated by O₂ consumption, was measured with the O₂ electrode as described above. Figure 1 is an inverted and reversed copy of the recorder tracing.

**RESULTS**

**Horseradish Peroxidase.** In the presence of HRP, mannosanous 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. Mg²⁺, Fe³⁺, and Ca²⁺ did not substitute for Cu²⁺; Co²⁺, 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 Cu²⁺.

In agreement with the findings by Akazawa and Cohn (1), O₂ was both required for, and consumed during, NADH oxidation. 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. Methoxyethyl-, cyclohexyl-, and tetrahydropropyl 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 Cu²⁺ strongly promoted O₂ 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 Cu²⁺ 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 Cu²⁺. When tested at 5 µM, Mg²⁺, Ca²⁺, Mn²⁺, Co²⁺, Ni²⁺, Ag⁺, and Hg²⁺ failed to promote ascorbate oxidation and Fe³⁺ was only slightly effective. Furthermore, addition of these cations did not lessen the effectiveness of adenine or BA in eliminating the promotion by Cu²⁺.

**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...
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 Cu²⁺-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 substituent 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 Cu²⁺ to other nitrogens in the base (3, 4).

The two most studied types of cytokinins in growth and development are the substituted adenines and uracils. In the HRP (Table I) and soybean (C. O. Miller unpublished data) enzyme systems, CPPU was without effect. If the peroxidase system or Cu²⁺ is crucially involved in cytokinin effects on growth and development, the immediate biochemical action of the substituted uracils 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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LITERATURE CITED


FIG. 1. Stimulation by cupric sulfate of O₂ 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.