PEROXIDASE-H_2O_2 CATALYZED INCORPORATION OF AUXIN DERIVATIVES INTO sRNA

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SUMMARY

Derivatives from 3-indolyl(2-^{14}C) acetic acid and 2,4-dichlorophenoxy (ring UL-^{14}C) acetic acid were bound to sRNA upon reaction with a H_2O_2- horseradish peroxidase system. No radioactivity was incorporated from 3-indolyl-(1-^{14}C) acetic acid or from 2,4-dichlorophenoxy(2-^{14}C) acetic acid indicating decarboxylation of the indole acetic acid and loss of the acetate moiety by the phenoxyacetic acid upon activation or binding. These results demonstrate that H_2O_2- oxidized peroxidase can produce reactive auxin derivatives that bind covalently to cellular nucleophiles. This activation may be required to produce the ultimate phytohormone *in vivo*.

INTRODUCTION

It is presently believed that oncogenic aromatic amines including N-OH-acetylaminofluorene require metabolic activation to express their biological potential *in vivo* (1,2). Investigation of N-OH-AAF activating systems with purified horseradish peroxidase and tobacco tissue preparations has indicated that H_2O_2- oxidized peroxidase was particularly efficient in generating reactive compounds that combined with nucleic acid (3,4,5). Subsequent experiments revealed that indolic compounds such as 3-indolyl acetic acid could significantly inhibit this reaction (6). Because both indolic and phenolic compounds are known to

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Abbreviations: N-OH-AAF, N-OH-acetylaminofluorene; IAA, 3-indolyl acetic acid; 2,4-D, 2,4-dichlorophenoxyacetic acid; HRP, horseradish peroxidase, E.C.1.11.1.7; sRNA, soluble ribonucleic acid.

react with peroxidases (7,8) the natural auxin IAA and the synthetic auxin 2,4-D were examined if they could be activated by oxidized peroxidase. It is axiomatic that phytohormones have to interact with specific cellular components in order to express their biological activity in vivo. The exact nature of this interaction is not known, but is generally believed to be non-covalent (9). This report presents evidence for an oxidized-peroxidase activation of auxins and their covalent binding to sRNA.

MATERIALS AND METHODS

The auxins, 3-indolyl(2-^{14}C) acetic acid ammonium salt (56μCi/μmole), 3-indolyl(1-^{14}C) acetic acid ammonium salt (52μCi/μmole) and 2,4-dichlorophenoxy (2-^{14}C) acetic acid (28μCi/μmole) were obtained from Amersham-Searle; 2,4-dichlorophenoxy(ring UL-^{14}C) acetic acid (9.2μCi/μmole) from California Bionuclear. The binding assays were adapted from those described for N-OH-AAF (4,5). The reaction mixtures contained, in a final volume of 2.0 ml, 100 umoles sodium pyrophosphate at pH 7, 1 mg purified yeast soluble RNA grade A (Calbiochem). 50 nmoles H_2O_2 diluted daily from Merck Superoxol 30% and, where added, 1.19 nmoles IAA-2-^{14}C, 1.00 nmole IAA-1-^{14}C in 5 μl H_2O and 51.6 nmoles 2,4-D ring UL-^{14}C or 44.6 nmoles 2,4-D-2-^{14}C in 5 μl methoxyethanol. The reactions were initiated by the addition of 40 or 80 μg purified HRP (RZ 3.0-3.1, Worthington, Miles) and incubated at 37° C for times given. The reactions were terminated with 2 ml buffer saturated phenol, centrifuged and an aliquot of the deproteinized aqueous fraction was added to a 7-fold excess of 95% ethanol containing 2% potassium acetate on a fiberglass filter apparatus. The precipitated sRNA was sequentially washed with 5 ml aliquots each of 70% and 95% ethanol, acetone and ethyl ether. The filters were placed in scintillation vials, moistened with 0.15 ml H_2O, then 1 ml NCS solubilizer (Amersham-Searle) and 10 ml toluene scintillation cocktail were added. The amount of isotope bound was determined with a Searle Analytic Isocap 300 counter employing external standardization techniques.

RESULTS AND DISCUSSION

As seen in Table 1, the HRP-H_2O_2 system containing 50 nmoles H_2O_2, 40 μg HRP and 1 mg sRNA caused binding of 13.6% of the isotope from IAA and 0.4% from the 2,4-D in twenty-minute assays. When the sRNA trapping agent was added just at the termination of the experiment, with H_2O_2 and HRP present, a 12-fold increase in bound radioactivity over the control was observed for IAA indicating

Table 1. HRP-H_2O_2 catalyzed binding of ^{14}C derivatives from 3-indolyl (2-^{14}C) acetic acid and 2,4-dichlorophenoxy (ring UL-^{14}C) acetic acid to sRNA.

Reaction Components			pmoles Derivative Recovered	
			IAA	2,4-D
sRNA	H_2O_2	HRP	162 ± 32 (4)	185 ± 15 (4)
sRNA*	H_2O_2	HRP	25 ± 1 (2)	29 ± 1 (2)
sRNA	H_2O_2	---	2 ± 0 (2)	24 ± 3 (2)
sRNA	----	HRP	2 ± 0 (2)	12 ± 4 (4)
----	H_2O_2	HRP	2 ± 0 (2)	15 ± 3 (2)

*sRNA added at termination of experiment.

The experimental procedures are described in Materials and Methods. Data expressed as mean and SEM, numbers in parenthesis indicate number of determinations.

that some of the reactive species previously generated rapidly combined with the added macromolecule. 2,4-D showed about a two-fold increase over the control in identical experiments. Omitting either HRP or H_2O_2 from the reaction mixture resulted in no adduct formation with either auxin. In the absence of sRNA little radioactivity was retained in the filters.

A time course of ^{14}C incorporation is shown in Figure 1. By employing both methylene-^{14}C-IAA and carboxyl-^{14}C-IAA, it can be seen that the compound reacting with the nucleic acid retains the methylene carbon while losing the carboxyl group. Radioactivity from uniformly ring-labeled 2,4-D was incorporated, but radioactivity from 2,4-D-2^{14}C was not, demonstrating that the acetate group was removed upon activation or adduct formation.

Detailed studies from several laboratories (7,10,11,12) have provided schemes suggesting that the HRP-oxidation of IAA proceeds by the abstraction of

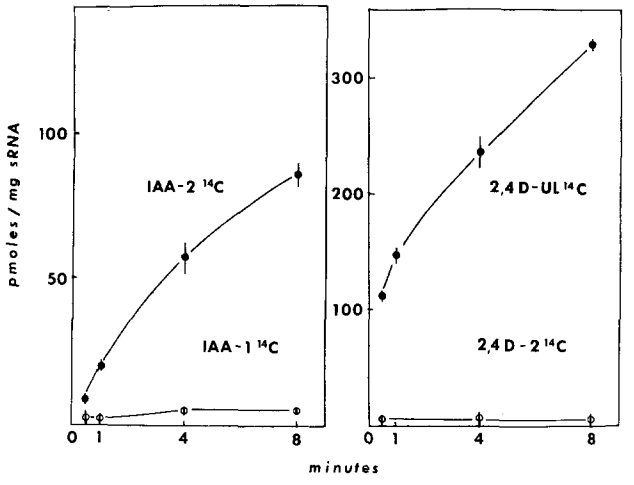

Figure 1. Time course of HRP catalyzed formation of sRNA adducts from: (left) 40 µg HRP with IAA-2^{14}C, upper curve, and IAA-1^{14}C, lower curve; (right) 80 µg HRP with 2,4-D ring UL-^{14}C, upper curve, and 2,4-D-2-^{14}C, lower curve. See methods and materials for experimental details. The vertical lines through the points indicate the range of duplicate determinations.

a single electron from the 3-position of the indole ring generating a free radical. The IAA radical, in the presence of O_2, is peroxidated and rapidly decarboxylates forming an epoxide. The epoxide subsequently can give rise to 3-indolyl aldehyde or, upon hydration, to 3-carbinol oxindole and 3- methylene oxindole (11). Because these models place decarboxylation at the time of IAA-peroxide radical formation, the binding to sRNA observed in this study could occur then. The epoxide or subsequent derivatives may also be logical candidates for the bound adduct. 3-methylene oxindole is a reactive species readily forming dimers or higher polymers and is able to react directly with sulfhydryl compounds (13).

3-methylene oxindole is of interest because of its auxin activity in three plant bioassays (14). It may be reduced in vivo to 3-methyloxindole which is devoid of auxin activity (14,15). Methyloxindole is also formed, possibly via methylene oxindole (14), by oxidation of IAA with chelated manganese at pH 6

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CHARACTERIZATION OF A MACROMOLECULAR INHIBITOR OF POLYPEPTIDE CHAIN INITIATION FROM EHRLICH ASCITES TUMOR CELLS

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SUMMARY

A non-dialyzable inhibitor of protein synthesis has been partially purified from Ehrlich ascites tumor cells. Preparations of this inhibitor have no effect on the initial rate of protein synthesis in rabbit reticulocyte lysates but cause a partial shut-off of amino acid incorporation after a lag period. The inhibitor has no appreciable endonuclease activity towards reticulocyte polysomes but under conditions of protein synthesis it causes disaggregation of polysomes. The effect of the inhibitor can be overcome by the initiation factor which forms a ternary complex with Met-tRNA$_f$ and GTP and which binds to 40S ribosomal subunits. These results suggest that the Ehrlich ascites inhibitor is very similar to an inhibitor previously identified in reticulocytes.

INTRODUCTION

The rate of polypeptide chain initiation in rabbit reticulocytes and lysates prepared from them is regulated by the availability of hemin (1-4). There is now a considerable body of evidence suggesting that this regulation is mediated by the action of a soluble translational inhibitor protein which is activated under conditions of hemin deficiency and which interferes with the formation of 40S ribosomal subunit-Met-tRNA$_f$ initiation complexes (5-13). Two pieces of evidence have led us to investigate whether this regulatory mechanism is an example of a more general phenomenon related to the control of initiation in other cell types. Firstly, hemin itself has been reported to have stimulatory effects on protein synthesis in cell-free systems from non-erythroid cells (4, 14, 15) and is capable of enhancing amino acid incorporation into protein in intact Krebs II ascites

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(16). It should be noted that a sensitive auxin bioassay requires manganese in the assay medium (17).

This peroxidase activation of auxins may be the molecular mechanism that is involved in the positive correlations observed between peroxidase activity and growth in both the crozier portion of fern fronds (18) and aspen callus cells cultured in vitro (19).

In a recent binding study (20) less ^{3}H-IAA incubated with a cellular homogenate fraction could be exchanged upon subsequent treatment with unlabeled IAA in preparations from actively growing pea buds as compared to dormant bud preparations. These incubations done under physiological conditions would include peroxidase catalyzed binding. The data are consistent with the concept of increased covalent auxin binding in actively growing tissues.

Covalent binding of IAA-2-^{14}C to sRNA has been found in mung bean hypocotyl sections (21). The radioactivity was isolated with several nucleic acid fractions, but only the sRNA still possessed auxin activity, after KOH hydrolysis and ether extraction, in the Avena curvature bioassay (22). An activation mechanism may be inferred in that the authors found no binding upon mixing isolated sRNA with IAA-2-^{14}C.

Conjugates such as 2-indoleacetyl-N-aspartate (23) and inositol esters of IAA (24) have been isolated from plant tissues. In both these complexes the carboxyl group is retained, indicating that the mechanism of their formation is different from that catalyzed by oxidized HRP.

The interaction of 2,4-D with peroxidase has not been as extensively examined as has that of IAA. In the assay described here the ^{14}C from ring labeled 2,4-D was less efficiently bound to sRNA than that of IAA-2-^{14}C. The identity of the reactive species remains to be determined, however, the loss of the acetate group could result in the formation of 2,4-dichlorophenol which is also known to react with peroxidase (8). Peroxidases have been shown to be involved in the cyclical oxidation and reduction of manganous ions and phenols (25,26).

Upon reaction with oxidized peroxidase the phenol undergoes a one-electron