Formation of *N*-Arylacylhydroxamic Acids from Nitroso Aromatic Compounds in Isolated Spinach Leaf Cells

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The formation of *N*-arylacetohydroxamic acids from nitroso aromatic compounds in the presence of pyruvate was investigated using isolated spinach leaf cells. The activity was enhanced by the addition of TPP, $MgSO_4$, and pyruvate, requirements for pyruvate dehydrogenase complex (PDHC). Measurement of the kinetic parameters revealed that the K_m values of nitroso aromatic compounds tested were identical and that electron-donating ring substituents decreased the catalytic efficiency. The activation energy of the formation of *N*-phenylacetohydroxamic acid was lower than that reported for porcine heart PDHC. With α-oxo acids tested, α-oxobutyrate served as a substrate to give the corresponding *N*-phenylpropionylhydroxamic acid. The activity of spinach leaf cells in *N*-phenylacetohydroxamic acid formation was found in both mitochondria and chloroplasts. The contribution of chloroplast PDHC to total activity in the formation of *N*-phenylacetohydroxamic acid was estimated to be 50% under the conditions used.

Keywords: *N-Arylacylhydroxamic acid; nitroso aromatic compound; PDHC; spinach leaf cells*

INTRODUCTION

There have been many studies on the metabolic pathways and metabolites of xenobiotics in plants, such as fungicides, herbicides, and insecticides, mainly to reveal their mode of action and basis of selectivity and to characterize the residues found in plant crops. The transport and metabolism of xenobiotics in plants and soils (Goodman et al., 1992) and xenobiotic conjugation in plants (Lamoureux and Rusness, 1986; Cole, 1994; Neuefeind et al., 1997) have been reviewed with respect to their detoxification and activation. In spite of the increasing public concern regarding the environmental use of xenobiotics, there is little information on the xenobiotic metabolism in plants from the viewpoint of toxicological effects on animals and humans.

In our previous studies, the formation of proximate carcinogenic *N*-arylacetohydroxamic acids from pyruvate and nitroso aromatic compounds (Chart 1) was found in mammalian isolated cells and perfused organs (Yoshioka et al., 1989, 1992, 1996). This biotransformation has been shown to be mediated by pyruvate dehydrogenase complex (PDHC) (Yoshioka and Uematsu, 1993), a common enzyme for aerobes including plants. Nitroso derivatives have been reported to be intermediates of arylamine oxidation or nitroarene reduction in the environment (Shimotori and Kuwatsuka, 1978; Aizawa, 1982; Draper and Casida, 1983). Photoreduction of nitro aromatic compounds by spinach chloroplasts has been also reported (Wessels, 1965; Suzuki and Uchiyama, 1975). Therefore, the formation of *N*-arylacetohydroxamic acids from the nitroso aromatic intermediates could potentially proceed in plants. For studying plant cell biochemistry, isolated cells and protoplasts of plants have been a useful system. The objective of the present study was to determine whether PDHC-catalyzed formation of *N*-arylacetohydroxamic acids from pyruvate and nitroso aromatic compounds proceeds in isolated leaf cells of spinach, which can be cultivated almost anywhere during most of year.

Chart 1. Structures of Nitroso Aromatic Compounds and *N*-Arylacylhydroxamic Acids

MATERIALS AND METHODS

Materials. Macerozyme R-10 (major enzymes: pectinase and hemicellulase) and cellulase onozuka R-10 (major enzyme: β-D-glucan-4-glucanohydrolase) were obtained from Yakult Honsha Co. (Tokyo, Japan). Potassium dextran sulfate was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). TPP and sodium salts of α-oxobutyrate, α-oxovalerate, α-oxoisovalerate, and α-oxoisohexanoate were obtained from Sigma Chemical Co. (St. Louis, MO). Sodium pyruvate was obtained from Merck (Darmstadt, Germany). Nitrosobenzene, obtained from Tokyo Kasei Kogyo Co. (Tokyo, Japan), was recrystallized from ethanol before use. Other nitroso aromatic compounds and *N*-arylacylhydroxamic acids used were synthesized as described previously (Sakamoto et al., 1989; Yoshioka and Uematsu, 1993). All other chemicals used were of reagent grade.

Preparation of Isolated Cells. Leaves of fresh spinach (*Spinacia oleracea* L.) were obtained from a local market. Isolated cells were prepared from spinach leaves by using macerozyme R-10, according to the first step of the enzymatic procedure for the preparation of protoplasts, as described by Nishimura and Akazawa (1975). The number of isolated cells was counted by a Thoma ruling hemocytometer.

Preparation of Spinach Leaf Mitochondria and Chloroplasts. Intact mitochondria and chloroplasts were isolated from spinach leaf protoplasts by differential centrifugation followed by Percoll discontinuous gradient centrifugation, according to the procedures described by Nishimura et al. (1982) and Joyard et al. (1987), respectively.

Chlorophyll and Protein Measurements and Cytochrome *c* Oxidase Assay. Chlorophyll concentrations were

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Table 1. Gradient Elutions for the Analysis of *N*-Arylacetohydroxamic Acids

ring substituent	solvent system	gradient elution
H	A [MeOH−H_2O (3:7)]	0−12 min linear gradient to 24% B
	B [MeOH−H_2O (4:1)]	12−17 min linear gradient to 100% B, 17−22 min 100% B, 22−27 min linear gradient to 100% A
4-OEt	A [MeOH−H_2O (9:11)]	0−7 min 100% A
	B [MeOH−H_2O (4:1)]	7−12 min linear gradient to 100% B, 12−15 min 100% B, 15−18 min linear gradient to 100% A
4-Me	A [MeOH−H_2O (2:3)]	0−7 min 100% A
	B [MeOH−H_2O (4:1)]	7−12 min linear gradient to 100% B, 12−15 min 100% B, 15−18 min linear gradient to 100% A
4-Cl	A [MeOH−H_2O (1:1)]	0−7 min 100% A
	B [MeOH−H_2O (4:1)]	7−17 min linear gradient to 100 % B, 17−22 min 100% B, 22−25 min linear gradient to 100% A

determined spectrophotometrically at 652 nm as described by Walker et al. (1987). Cytochrome *c* oxidase activity was measured as described by Briskin et al. (1987). Protein was determined by a modification of the Lowry procedure, as described by Markwell et al. (1981), with BSA as the standard.

Assay of *N*-Arylacylhydroxamic Acid Formation Activity. Isolated spinach leaf cells were suspended at about 10^6 cells in 1 mL of an assay medium comprising 0.32 M mannitol, 10 mM 3-(*N*-morpholino)propanesulfonic acid−NaOH buffer (pH 6.9), 5 mM $MgSO_4$, 0.5 mM TPP, 10 mM sodium salt of α-oxo acid, and 2 mM nitroso aromatic compound [added as 25 μL of a bis(2-methoxyethyl) ether solution]. Each incubation was initiated by the addition of the nitroso aromatic compound and carried out at 25 °C with shaking (110 strokes/min) in a 2-mL screw-cap vial equipped with a Teflon-faced seal to prevent nitroso aromatic compound volatilization. At the required time, 700 mg of ammonium sulfate was added, and then the mixture was extracted with 1 mL of isopropyl ether (purified by passage through a basic aluminum oxide column) saturated with the buffer, by shaking for 10 min. After brief centrifugation, an aliquot (50 μL) was analyzed by HPLC. For the 4-chloro derivative, the isopropyl ether layer (500 μL) was washed with 0.1 M sulfuric acid (500 μL) by shaking for 10 min, and then the organic layer (50 μL) was injected into the column.

HPLC Assay. HPLC analyses were carried out with a column of LiChrosorb RP-8 (Merck, 4 × 250 mm) with detection at 260 nm. Methanol−water containing 0.01% desferal mesylate (Corbett and Chipko, 1979) was used as the eluting solvent, at a flow rate of 1 mL/min and at 40 °C. Gradient elutions for the analysis of *N*-arylacetohydroxamic acids are summarized in Table 1.

Identification of *N*-Phenylacetohydroxamic Acid. For the isolation of *N*-phenylacetohydroxamic acid as a metabolite, the incubation mixture was extracted with isopropyl ether, and the extract was evaporated under reduced pressure. *N*-Phenylacetohydroxamic acid was separated by silica gel TLC (Merck, article 5554) for high-resolution mass spectrometry (JEOL DX303): calcd for $C_8H_9O_2N$, 151.0633; found, 151.0640 (error 0.7 millimass unit).

Calculation of Kinetic Constants. Data obtained from the initial velocity studies on the formation of *N*-arylacetohydroxamic acids in isolated spinach leaf cells were plotted in a double-reciprocal form to check the fitting with the Michaelis−Menten equation. The best-fit values of K_m and V_{max} were obtained by the method of least-squares with the Taylor expansion (Sakoda and Hiromi, 1976). Data without SD are derived from a single experiment.

RESULTS

***N*-Phenylacetohydroxamic Acid Formation by Isolated Spinach Leaf Cells.** When nitrosobenzene and pyruvate were incubated in the presence of isolated spinach leaf cells, *N*-phenylacetohydroxamic acid, *N*-phenylhydroxylamine, aniline, and azoxybenzene, as well as an unknown metabolite with a retention time of 4.8 min, were formed. The same metabolites were found in spinach leaf protoplasts under the conditions used (data not shown). A typical HPLC chromatogram, of isolated spinach leaf cells, is shown in Figure 1. As

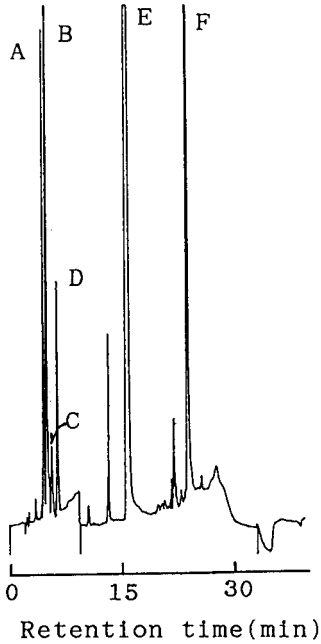

Figure 1. Typical HPLC chromatogram: A, *N*-phenylhydroxylamine (4.4 min); B, unknown metabolite (4.8 min); C, aniline (5.5 min); D, *N*-phenylacetohydroxamic acid (6.2 min); E, nitrosobenzene (15.5 min); and F, azoxybenzene (23.2 min).

Table 2. Requirements for the Formation of *N*-Phenylacetohydroxamic Acid by Isolated Spinach Leaf Cells[a]

incubation mixture	activity (nmol/30 min/10^8 cells)
complete mixture	14.0 (100)
− pyruvate	8.4 (60)
− TPP	11.8 (84)
− Mg^{2+}	12.6 (90)
− nitrosobenzene	nd[c]
− cells	nd
heated cells[b]	nd

[a] The concentrations used were as follows: pyruvate, 10 mM; TPP, 0.5 mM; Mg^{2+}, 5 mM; nitrosobenzene, 2 mM. Each incubation was carried out at 25 °C with shaking (110 strokes/min) for 30 min. Values in parentheses are activities expressed relative to the complete incubation mixture, arbitrarily taken as 100. [b] Isolated spinach leaf cells were heated in boiling water saturated with ammonium sulfate for 20 s. Incubation was performed in the complete mixture with the heated cells. [c] nd, not detected.

shown in Table 2, the activity in the formation of *N*-phenylacetohydroxamic acid was enhanced by the addition of $MgSO_4$, TPP, and pyruvate, requirements for PDHC. Since a detectable amount of *N*-phenylacetohydroxamic acid was not formed with heated cells, the formation of the compound seems to be an enzymatic reaction. *N*-Phenylhydroxylamine, aniline, and azoxybenzene were formed with heated cells (data not shown), indicating that the formation of these compounds seems to be a nonenzymatic reaction. Nitroso aromatic compounds are known to be reduced nonenzymatically and

Table 3. Kinetic Constants of Substituted Nitrosobenzenes in the Formation of *N*-Arylacetohydroxamic Acids by Isolated Spinach Leaf Cells

ring substituent[a]	K_m (mM)	V_{max} (nmol/min/10^8 cells)
H	0.230 ± 0.080	16.6 ± 1.4
Cl	0.298 ± 0.063	19.8 ± 1.3
Me	0.240 ± 0.057	10.5 ± 0.6
OEt	0.282 ± 0.081	7.3 ± 1.0

[a] Ring substituents at the 4-position of nitrosobenze. H, nitrosobenzene; Cl, 4-chloronitrosobenzene; Me, 4-methylnitrosobenzene; OEt, 4-ethoxynitrosobenzene.

to react with thiol compounds such as GSH (Corbett and Corbett, 1994; Zuman, 1994).

The activity in the formation of *N*-phenylacetohydroxamic acid was linear to the number of the isolated spinach leaf cells and was 14.3 ± 0.6 nmol/min/10^8 cells at the concentration of 5 mM nitrosobenzene (data not shown). The activity of isolated spinach leaf cells was stable for at least 3 h, when stored in an ice bath in the dark (data not shown).

The activation energy of the formation of *N*-phenylacetohydroxamic acid was 61.8 ± 2.9 kJ/mol (data not shown). This value is lower than that obtained with porcine heart PDHC (Yoshioka and Uematsu, 1993).

Since *N*-arylacetohydroxamic acids have been reported to be deacetylated by hepatocytes (Yoshioka et al., 1996), further metabolism of *N*-phenylacetohydroxamic acid in isolated spinach leaf cells was studied. A decrease in the compound, at a final concentration of 12.5 nmol/mL, was not found during incubation for 30 min (data not shown).

Effect of Substituents of Nitrosobenzenes on the Activity. To estimate the kinetic constants for the formation of *N*-phenylacetohydroxamic acid, the activities were measured in the presence of 0.5 mM TPP and 10 mM pyruvate, which are sufficiently high concentrations to saturate PDHC in plants (Randall et al., 1989). To elucidate the effect of the ring substituents on the enzymatic reaction, K_m and V_{max} values were measured with 4-chloro-, 4-methyl-, and 4-ethoxynitrosobenzenes. The kinetic parameters obtained are summarized in Table 3. The K_m values were identical within the experimental error. The V_{max} values, however, were affected by ring substituents. The order of V_{max}/K_m values, parameters indicating the catalytic efficiency of formation of *N*-arylacetohydroxamic acids, was 4-chloro- > unsubstituted > 4-methyl- > 4-ethoxynitrosobenzene. By the application of the Hammett equation (Hammett, 1970), a good correlation was obtained, as shown in Figure 2. The equation ($n = 4$, $r = 0.982$) is as follows:

$$\log(V_{max}/K_m) = (0.503 \pm 0.068)\sigma^+ + (1.807 \pm 0.029)$$

The positive ρ value in the equation suggests that nitroso aromatic compounds serve as electrophiles. This result is comparable with that obtained for porcine heart PDHC (Yoshioka and Uematsu, 1993).

When nitrobenzene, instead of nitrosobenzene, was incubated under the same conditions for 60 min, the formation of *N*-phenylacetohydroxamic acid was not found (data not shown).

***N*-Phenylacylhydroxamic Acids from α-Oxo Acids.** To investigate the specificity of α-oxo acids as substrates in the formation of corresponding *N*-phenylacylhydroxamic acids, nitrosobenzene was incubated with five kinds of α-oxo acids (pyruvate, α-oxobutyrate, α-oxovalerate, α-oxoisovalerate, and α-oxoisohexanoate) in the presence of isolated spinach leaf cells. Of the α-oxo acids tested, pyruvate and α-oxobutyrate were found to be substrates, though the catalytic rate with α-oxobutyrate was 17% of that with pyruvate (data not shown). This result is comparable with the report that α-oxobutyrate is the substrate utilized by plant PDHC (Randall et al., 1989).

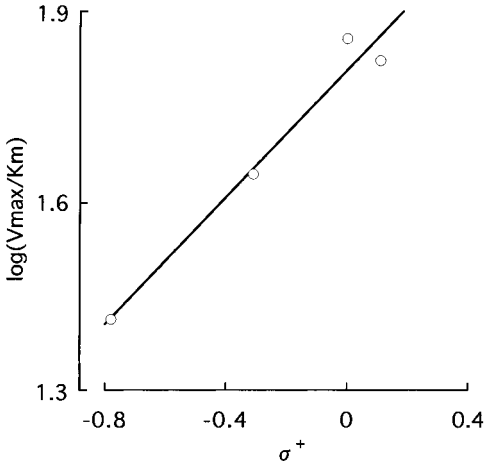

Figure 2. Correlation of $\log(V_{max}/K_m)$ for nitroso aromatic compounds in the formation of *N*-arylacetohydroxamic acids versus Hammett's σ^+ constant.

Table 4. Activities of Mitochondria and Chloroplasts in the Formation of *N*-Phenylacetohydroxamic Acid

	activity
homogenate[a]	53 nmol/30 min/mg of chlorophyll
chloroplasts	27 nmol/30 min/mg of chlorophyll
mitochondria	28 nmol/30 min/mg of protein

[a] Spinach protoplasts were homogenized with a Teflon−glass homogenizer, and the activity is shown as per mg of chlorophyll contained in the homogenate to compare with the activity of the purified chloroplasts.

Activities in Mitochondria and Chloroplasts. Plants are known to have two distinct types of PDHC localized within different subcellular compartments: mitochondria and chloroplasts (Randall et al., 1989). Therefore, activities in the formation of *N*-phenylacetohydroxamic acid from nitrosobenzene and pyruvate were determined with mitochondria and chloroplasts obtained from spinach. As shown in Table 4, both mitochondria and chloroplasts showed activity. The contamination of organelles in each fraction was estimated by cytochrome *c* oxidase (a marker enzyme for mitochondria) activity and chlorophyll concentration. No significant cross-contamination was observed (data not shown). From the chlorophyll contents of the homogenate of spinach leaf protoplasts and the purified intact chloroplasts, the activity of the chloroplasts in the formation of *N*-phenylacetohydroxamic acid was estimated to be 50% of that obtained with the homogenate, under the assay conditions used.

DISCUSSION

The present results indicate that the formation of *N*-arylacetohydroxamic acids from nitroso aromatic compounds proceeds in isolated spinach leaf cells and protoplasts. The enzymatic properties of this biotransformation are comparable with those obtained for mammalian cells (Yoshioka et al., 1989, 1996) and for porcine heart mitochondria (Yoshioka and Uematsu, 1993). Several lines of information on the PDHC-catalyzed

biotransformation in isolated spinach leaf cells were obtained as follows: (1) The enzymatic activity was enhanced by TPP and Mg^{2+}, cofactors of PDHC (Table 2). This result indicates that TPP is taken up by isolated spinach leaf cells as well as by mammalian cells (Yoshioka et al., 1992, 1996). (2) K_m values of the nitroso aromatic compounds tested (Table 3) and the ρ value obtained in the Hammett plot (Figure 2) are comparable with those obtained with porcine heart PDHC (Yoshioka and Uematsu, 1993). These results indicate that the catalytic characteristics of PDHC in isolated spinach leaf cells are similar to those of mammalian PDHC. (3) The substrate specificity of α-oxo acids in this biotransformation agreed with that reported in plant PDHC (Randall et al., 1989). This substrate specificity is comparable with that obtained for porcine heart PDHC-catalyzed formation of *N*-arylacetohydroxamic acids (Yoshioka and Uematsu, 1993), though the effectiveness of α-oxobutyrate was lower than that obtained with mammalian PDHC. (4) Activity of isolated spinach leaf cells was found in both mitochondria and chloroplasts, which have been reported to possess PDHC (Lernmark and Gardenstrom, 1994). Therefore, the enzymatic activities of isolated spinach leaf cells in this biotransformation are thought to be derived from PDHCs in both organelles. While these plant PDHCs are structurally different from each other, the in vitro catalytic characteristics of chloroplast PDHC are generally similar to those of mitochondrial PDHC (Randall et al., 1989).

As shown in Table 2, the activity of prepared spinach leaf cells, without the addition of pyruvate, showed 60% of the activity of that under the complete incubation conditions. Although thiamin content of spinach has been reported to decrease during storage both at room temperature and under refrigeration (Hebrero et al., 1988), the prepared spinach leaf cells, without the addition of TPP, showed 84% of the activity of that obtained under the complete incubation conditions. The lower activiation energy than that of porcine heart PDHC (Yoshioka and Uematsu, 1993) suggests that the formation of *N*-arylacetohydroxamic acids from nitroso aromatic compounds and pyruvate proceeds more smoothly and is less affected by temperature in spinach than in homoisothermal animals.

PDHC plays central roles particularly in catabolism and anabolism of cells; mitochondrial PDHC is a key enzyme participating in a metabolic link between glycolysis and the TCA cycle, and chloroplast PDHC is reported to provide acetyl-CoA for the biosyntheses of fatty acids and isoprenoids (Camp and Randall, 1985). Therefore, PDHC-catalyzed formation of *N*-arylacetohydroxamic acids from nitroso aromatic compounds and pyruvate may exert toxic effects on plant cells by diminishing the intracellular concentration of acetyl-CoA and NADH. From studies on the regulation of these PDHCs in plants (Randall et al., 1989; Gemel and Randall, 1992; Moore et al., 1993; Luethy et al., 1996), interdependence of photosynthesis and respiration in plant cells is thought to participate in the regulation mechanisms. Although mitochondrial PDHC, but not chloroplast PDHC, was shown to be regulated by reversible phosphorylation with ATP (Randall et al., 1989), the activity of mitochondrial PDHC in the formation of *N*-arylacetohydroxamic acids might be freed from the regulation because of restriction of the TCA cycle followed by a decrease in ATP production.

Furthermore, this biotransformation in plants may provide a possible route for entry of *N*-arylacetohydroxamic acids into humans as well as livestock through the food chain. Since *N*-phenylacetohydroxamic acid was not further metabolized, the *N*-arylacetohydroxamic acids formed are thought to be retained within plants. This needs to be studied further in vivo. Nitroso derivatives were reported to be intermediates of arylamine oxidation or nitroarene reduction in the environment (Shimotori and Kuwatsuka, 1978; Aizawa, 1982; Draper and Casida, 1983). Photoreduction of nitro aromatic compounds was also reported to proceed by spinach chloroplasts (Wessels, 1965; Suzuki and Uchiyama, 1975). Therefore, the formation of *N*-arylacetohydroxamic acids may proceed in plants that take up nitro and/or amino aromatic pesticides used both directly and indirectly.

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