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Enzymatic and Mechanistic Studies on the Formation of *N*-Phenylglycolohydroxamic Acid from Nitrosobenzene and Pyruvate in Spinach Leaf Homogenate

RYOSUKE TATSUNAMI AND TADAO YOSHIOKA*

Department of Pharmacy, Hokkaido Pharmaceutical University School of Pharmacy, 7-1 Katsuraoka-cho, Otaru, Hokkaido 047-0264, Japan

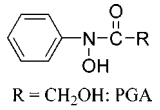
The biotransformation mechanism of an unknown metabolite formed enzymatically from nitrosobenzene (NOB) and pyruvate in spinach (*Spinacea oleracea* L.) was investigated using spinach leaf homogenate. The unknown metabolite was identified as *N*-phenylglycolohydroxamic acid (PGA). The activity of PGA formation was decreased by L-alanine, increased by L-serine, and completely inhibited by aminooxyacetic acid, an inhibitor of transaminases. These results indicate that the transaminase participates in PGA formation. Indeed, hydroxypyruvate and alanine were produced in the transamination between pyruvate and serine. Hydroxypyruvate served as a direct-acting glycoloyl donor for PGA formation. A good correlation between the activities of the 200*g* supernatant of spinach homogenate and commercial yeast transketolase for PGA formation from several glycoloyl donors was obtained. These results suggest the following mechanism for PGA formation from NOB and pyruvate: transamination of L-serine into hydroxypyruvate, which serves as a glycoloyl donor to NOB.

KEYWORDS: ***N*-Phenylglycolohydroxamicacid (PGA); transaminase; transketolase; hydroxypyruvate; *Spinacea oleracea* L.**

INTRODUCTION

Artificial organic compounds (so-called xenobiotics), including agricultural chemicals, pharmaceuticals, and other chemically engineered products, through their widespread use, make their way into the environment, where they can be found in the form of countless metabolites. These xenobiotics, as well as their metabolites, accumulate in plants, which lack effective excretion systems (*1*, *2*); as a result, the ingestion of plants that have been exposed to xenobiotics is believed to represent a toxicological risk. Therefore, information on the biotransformation of xenobiotics in plants is needed from the viewpoint of toxicological effects on animals and humans. There are many reports on the metabolic pathways and metabolites of xenobiotics in plants (*3*): aromatic compounds taken up by green leaves (*4*); factors affecting metabolism and absorption of xenobiotics by green leaves and roots (*5*); metabolic pathways including hydroxylation activity of aromatic compounds; dealkylation (*6*); and acetyl (*7*), glucose (*8*), and GSH (*9*) conjugations. The metabolites and metabolic pathways for agricultural chemicals such as herbicides have also been investigated (*10*−*12*).

N-Substituted aromatic compounds, including nitro and/or amino aromatics, are organic substances that are widely used in the environment, and some of these are known to be carcinogenic or mutagenic. We have studied the biotransformation and toxicology of these compounds, because their bioactivation has been considered as a critical step for their carcinogenicity and mutagenicity (*13*, *14*). In our previous study, a nonoxidative pathway for the formation of proximate carcinogenic *N*-arylacetohydroxamic acids from nitroso aromatic compounds and pyruvate, catalyzed by pyruvate dehydrogenase complex, was found in animals and also in isolated spinach leaf cells (*15*). In the spinach experiment with nitrosobenzene (NOB) and pyruvate, an unknown metabolite was formed in addition to *N*-phenylacetohydroxamic acid (PAA) (**Figure 1**). The aim of this study, therefore, was to identify the unknown metabolite and to reveal the mechanism of its formation from NOB and pyruvate.



R = CH_2OH: PGA
CH_3: PAA

Figure 1. Structures of PGA and PAA.

MATERIALS AND METHODS

Apparatus. HPLC was performed with a LC-6A (Shimadzu) equipped with a SPD-6A spectrophotometric detector (Shimadzu). ^{1}H NMR spectra were recorded on a JNM-GX270 spectrometer (JEOL, Japan). Mass spectra were recorded on a M-2000 (HITACHI). Spectrophotometry was carried out on a UV-200S (Shimadzu).

Materials. NOB, obtained from Tokyo Kasei Kogyo Co. (Tokyo, Japan), was recrystallized from EtOH before use. Sodium pyruvate and thiamin diphosphate (TPP) were purchased from Merck (Darmstadt,

* To whom correspondence should be addressed. Tel: +81-134-62-1894. Fax: +81-134-62-5161. E-mail: yoshioka@hokuyakudai.ac.jp.

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Germany). L-Serine, magnesium sulfate, D-fructose 6-phosphate disodium salt, and amino acids standard solution type H were purchased from Wako Chemical Industries (Osaka, Japan). L-Alanine and aminooxyacetic acid were purchased from Kanto Kagaku (Tokyo, Japan) and Tokyo Kasei Kogyo Co. (Tokyo, Japan), respectively. Sodium D-xylulose 5-phosphate, glyoxylic acid, lithium β-hydroxypyruvate, spinach glyoxylate reductase, and yeast transketolase were purchased from Sigma-Aldrich. PGA was synthesized according to a reported procedure (*16*). All other chemicals used were of reagent grade.

Preparation of Spinach Homogenate. Spinach (*Spinacea oleracea* L.) was obtained from a local market. Leaves were washed and homogenized for 20 s in a Waring blender with a medium containing 0.32 M mannitol and 50 mM morpholinopropanesulfonic acid (MOPS)−KOH (pH 6.9). The homogenates were filtered through two layers of gauze. After centrifugation of the filtrate at 200*g* for 5min, an aliquot of the supernatant was centrifuged at 105 000*g* for 60 min. A portion of the 105 000*g* supernatant was dialyzed using a Spectra/Por (MWCO 25000, SPECTRUM) against 200 volumes of homogenization buffer to remove low molecular weight material.

Partial Purification of the Spinach Transketolase. The partial purification of transketolase from spinach leaf homogenates was performed according to the reported procedure (*17*). Fractions obtained by ion exchange column chromatography were assayed by the standard transketolase test, and the pooled active fractions were used for the assay of PGA formation activity.

Assay of PGA Formation Activity. The activity of PGA formation was essentially assayed according to the previous report (*15*). The assay medium (final volume, 1 mL) consisted of 50 mM MOPS−KOH (pH 6.9), 0.32 M mannitol, 0.5 mM TPP, 5 mM $MgSO_4$, 10 mM pyruvate, and 2 mM NOB [added as 25 μL of a bis(2-methoxyethyl) ether solution]. Each incubation was initiated by the addition of supernatants of spinach homogenate (200*g* and 105 000*g*), dialyzed 105 000*g* supernatant, or partially purified transketolase. Incubation was carried out at 25 °C with shaking (70 strokes/min) in a 2.5 mL screw-cap vial equipped with a Teflon-faced seal to prevent NOB volatilization. The reaction was stopped by heating for 15 s in boiling water, and then the reaction mixture was cooled immediately and kept on ice until HPLC analysis. For the HPLC analysis, 800 mg of ammonium sulfate was added to the heat-treated sample and then the mixture was extracted with 1 mL of diisopropyl 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.

HPLC Analysis. HPLC analysis was carried out using a LiChrosorb RP-8 column (Merck, 4 × 250 mm) with detection at 260 nm. CH_3CN:CH_3OH:H_2O containing 0.01% desferal mesylate (*16*) was used as the mobile phase, at a flow rate of 1 mL/min and at 40 °C. The mobile phase was a gradient of solvent A [CH_3CN:CH_3OH:H_2O (1.5:1:18, v/v/v)] and solvent B [CH_3OH:H_2O (4:1, v/v)] with 0−12 min 100% A, 12−15 min linear gradient to 100% B, 15−20 min 100% B, and 20−23 min linear gradient to 100% A.

Identification of the Metabolite as PGA. For the isolation of PGA from a preparative scale of incubation mixture (100 mL), 70 g of ammonium sulfate was added after 21 h of incubation, and then the resultant mixture was extracted with 200 mL of diethyl ether. From the ether solution, PGA was then extracted with 0.1 mol/L NaOH (50 mL) and the aqueous solution was washed with diethyl ether (2 × 10 mL). After the pH of the solution was adjusted to ca. 3 with 5 mol/L phosphoric acid, the resultant solution was extracted with diethyl ether (5 × 20 mL). The extract was evaporated under reduced pressure. Final purification of PGA (R_f = 0.38) was carried out by a preparative silica gel TLC (Merck, article 1.13895) with ethyl acetate:benzene (3:1, v/v) as a developing solvent. Spectral data of the metabolically formed PGA were as follows. ^{1}H NMR (DMSO-d_6): δ 4.30 (2H, d, J = 6.1 Hz), 4.75 (1H, t, J = 6.2 Hz, exchangeable with D_2O), 7.14 (1H, t, J = 7.4 Hz), 7.38 (2H, t, J = 8.1 Hz), 7.65 (2H, d, J = 7.6 Hz), 10.48 (1H, s, exchangeable with D_2O). MS (EI): m/z 167 (M^+), 151, 149, 119, and 109 (base peak). High-resolution MS: calcd for $C_8H_9NO_3$, 167.0581; found, 167.0564 (error −1.7 millimass unit).

The spectral data of PGA, synthesized chemically, were as follows. ^{1}H NMR (DMSO-d_6): δ 4.30 (2H, d, J = 5.9 Hz), 4.76 (1H, t, J = 6.2 Hz, exchangeable with D_2O), 7.14 (1H, t, J = 7.4 Hz), 7.38 (2H, t, J = 7.9 Hz), 7.65 (2H, d, J = 7.8 Hz), 10.49 (1H, s, exchangeable with D_2O). MS (EI): m/z 167 (M^+), 151, 149, 119, and 109 (base peak).

Assay of Hydroxypyruvate and Alanine in the Transamination. The incubation mixture (final volume, 7.5 mL) consisted of 50 mM MOPS−KOH (pH 6.9), 10 mM pyruvate, and 10 mM L-serine. The incubation was initiated by the addition of the dialyzed 105 000*g* supernatant of leaf homogenate (1.5 mL) and carried out at 25 °C. Samples were removed at various times to determine the amounts of hydroxypyruvate and alanine. Since the transamination was not affected by TPP and $MgSO_4$, they were omitted from the incubation mixture used for the assay of PGA formation activity.

(1) Determination of Hydroxypyruvate. To an aliquot of the incubation mixture (300 μL) was added 5.5 mM aminooxyacetic acid (30 μL) to stop the reaction. Hydroxypyruvate was quantified with hydroxypyruvate reductase according to the literature (*18*) with some modifications. Briefly, the activity was measured by monitoring the oxidation of NADH spectrophotometrically at 340 nm in 1 mL reaction mixture consisting of 50 mM MOPS−KOH (pH 6.9), 0.125 mM NADH, sample solution (50 μL), and 20 μL of hydroxypyruvate reductase (Sigma-Aldrich as glyoxylate reductase) at 25 °C. Pyruvate is not a substrate for this enzyme. Additionally, L-serine and aminooxyacetic acid did not affect the reductase activity.

(2) Determination of Alanine. To the second aliquot of the incubation mixture (300 μL), 25 mg of 5-sulfosalicylic acid was added to precipitate proteins and stop the reaction. After brief centrifugation, the supernatant (60 μL) was removed and then 20 μL of 1 mol/L NaOH (20 μL) was added to it for neutralization. An aliquot (50 μL) was analyzed by an amino acid analysis system (Shimadzu), according to the operating instructions. After separation on an ion exchange column (Shim-pack ISC-07/S1504Na) with a sodium hydroxide gradient in citrate buffer at pH 3.2−10, amino acids were derivatized with *o*-phthalaldehyde and then detected at 450 nm by fluorescence excited at 348 nm. A standard solution containing 17 amino acids including L-serine and L-alanine was used for the check of the retention times and of good separation.

PGA Formation Activity Catalyzed by Transketolase. The activities of PGA formation were assayed with commercial yeast transketolase and the 200*g* supernatant of spinach leaf homogenate. The incubations were carried out according to the assay of PGA formation activity using known as glycoloyl donors instead of pyruvate, hydroxypyruvate, D-fructose 6-phosphate, D-xylulose 5-phosphate, and fructose at concentrations of 4 times each K_m except for D-fructose at 10 mM.

Calculation of Kinetic Constants. Data obtained from the initial velocity studies on PGA formation 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 (*19*).

RESULTS

Structure Confirmation of the Unknown Metabolite. As in the isolated cell experiment (*15*), the unknown metabolite was also formed in an incubation mixture consisting of NOB, pyruvate, and the 200*g* supernatant of spinach leaf homogenate. MS spectral analysis of the isolated metabolite revealed M^+ at m/z 167 and a fragment ion $(M - 18)^+$ at m/z 149. Another ion equivalent to $(M - 16)^+$ at m/z 151 seems to be the characteristic one observed for hydroxamic acid compounds (*20*). On the basis of the result of high-resolution mass spectrometry, the molecular formula of the metabolite was presumed to be $C_8H_9NO_3$. ^{1}H NMR spectrum of the metabolite showed signals of two protons (δ 4.76 and 10.49, each 1H) exchangeable with D_2O, as well as aromatic protons (5H). The spectrum also showed a doublet signal of methylene protons (δ 4.30, 2H), which collapsed to a singlet signal by the addition of D_2O. These results indicate the metabolite to be PGA (**Figure 1**).

Therefore, PGA was chemically synthesized according to the reported procedure (*16*), and comparison of authentic PGA with

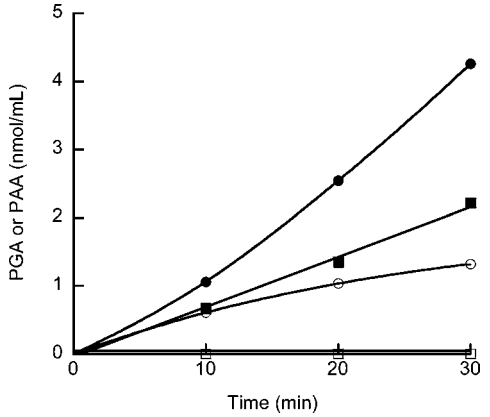


Figure 2. Time courses of PGA and PAA formations in 200*g* supernatant. PGA formation in the presence of pyruvate (●) and in the absence of pyruvate (○); PAA formation in the presence of pyruvate (■) and in the absence of pyruvate (□). Pyruvate was added at the final concentration of 10 mM.

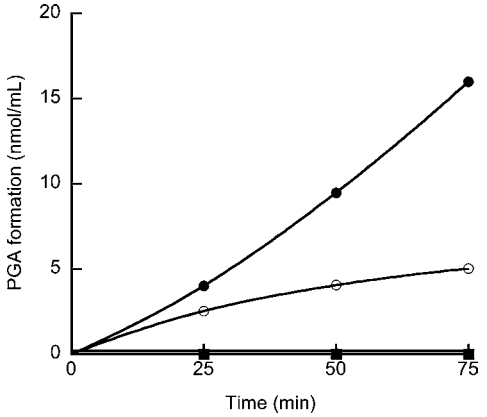


Figure 3. Time courses of PGA formation in the 105 000*g* supernatant. PGA formation with the 105 000*g* supernatant in the presence of pyruvate (●) and in the absence of pyruvate (○); PGA formation with dialyzed 105 000*g* supernatant in the presence or absence of pyruvate (■). Pyruvate was added at the final concentration of 10 mM.

the metabolite proved its identity by producing consistent ^{1}H NMR and MS spectra. In addition, the retention times in HPLC and the R_f values in TLC for these compounds were also consistent, and both compounds reacted positively to ferric chloride (hydroxamic acid−iron reaction). On the basis of all these findings, the unknown metabolite was identified as PGA.

Pyruvate-Dependent Formation of PGA from Spinach Leaf Homogenate. In comparison with the complete incubation mixture, PGA formation activity was decreased by approximately 70% in the absence of pyruvate. PGA formation was not observed when the 200*g* supernatant was heat-treated for 15 s in a boiling water bath (data not shown). The time courses of the formation of PGA and PAA were investigated under conditions with and without the addition of pyruvate using the 200*g* supernatant. The formation of PAA was almost completely dependent on pyruvate, while PGA formation was observed in the absence of pyruvate, as mentioned above. Furthermore, although PAA formation increased in proportion to the incubation time, PGA formation increased at an accelerating rate (**Figure 2**). A proportional relationship was observed between the amount of supernatant added and PAA formation; PGA formation was not linear but parabolic (data not shown).

The activity of PGA formation in the 200*g* supernatant was further fractionated by a differential centrifugation. Approximately 80% of the activity of the 200*g* supernatant remained in the 105 000*g* supernatant (data not shown). **Figure 3** shows the time courses of PGA formation catalyzed by the 105 000*g* supernatant both in the presence and absence of pyruvate. PGA formation was shown to be pyruvate-dependent, similar to the results obtained with the 200*g* supernatant (**Figure 2**). With the dialyzed 105 000*g* supernatant, however, no PGA formation was observed, even when pyruvate was added.

Participation of Transamination in PGA Formation. On the basis of the results obtained and the chemical structure of PGA, it is difficult to believe that pyruvate serves as the direct-acting substrate for PGA formation. An intrinsic dialyzable component, which can react with pyruvate, might participate in PGA formation. Since pyruvate was proven to serve as the direct substrate for PAA formation (*21*), hydroxypyruvate, an α-oxo acid having a glycoloyl group (−$COCH_2OH$), is considered to be highly likely to serve as the direct substrate for PGA formation. Therefore, further experiments were conducted using the 105 000*g* supernatant, and a transamination between pyruvate and L-serine was examined in order to confirm whether hydroxypyruvate is formed. As shown in **Table 1**, the results revealed a 4-fold increase in the activity when L-serine was added and a 60% decrease for the addition of L-alanine.

Table 1. Effects of L-Serine and L-Alanine on PGA Formation

additive	relative amount (%) of PGA formation[a]
no additive (control)	100
L-serine[b]	390
L-alanine[b]	60

[a] Values are expressed as the percentage of the control value for the amount of PGA formed with pyruvate during the 30-min incubation. [b] Each amino acid was added at the final concentration of 10 mM to the incubation mixture containing 10 mM pyruvate and 105000*g* supernatant.

Various amino acids, including L-serine, were detected in the 105 000g supernatant of spinach leaf homogenate by amino acid analysis (data not shown), as reported by others (*22*). Since these results strongly suggest transamination between pyruvate and L-serine, the formation of hydroxypyruvate and L-alanine was measured by using the dialyzed 105 000*g* supernatant. As shown in **Figure 4**, formation of hydroxypyruvate with concomitant formation of alanine was observed. When aminooxyacetic acid (AOA), an inhibitor of transaminase (*23*, *24*), was added to the incubation mixture after 20 min, the formation of both hydroxypyruvate and alanine was stopped.

Effects of Amino Acids and α-Oxo Acids in Transamination. Using the dialyzed 105 000*g* supernatant, PGA formation activity was measured with some amino acids and α-oxo acids. The formation of PGA in the presence of pyruvate and L-serine was inhibited by the addition of D-serine, an inhibitor of serine:glyoxylate transaminase (*25*), but no decrease in the activity of PGA formation was observed with the addition of L-glutamate or L-aspartate (data not shown). **Table 2** shows the highest PGA formation activity (13.5-fold increase) with glyoxylate in the presence of L-serine. The amount of PGA formed by the addition of other α-oxo acids was less than 30% of that observed with pyruvate. These results indicate that the spinach transaminase(s) participating in hydroxypyruvate formation has high specificity to glyoxylate as the amino acceptor.

Formation of PGA from Hydroxypyruvate in the 200*g* Supernatant. The results obtained suggested that hydroxypyruvate formed by transamination between pyruvate and L-serine

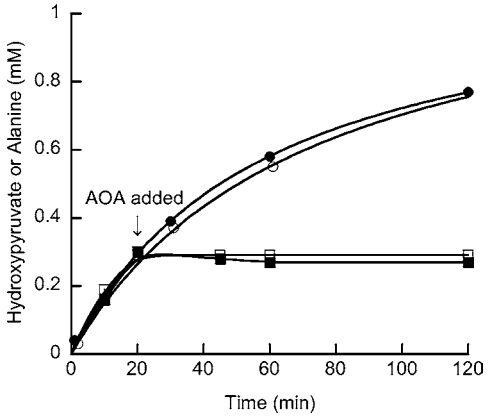


Figure 4. Hydroxypyruvate and alanine formation in transamination between pyruvate and L-serine and inhibitory effect of aminooxyacetic acid. Hydroxypyruvate formation (●) and in the addition of aminooxyacetic acid (■); Alanine formation (○) and in the addition of aminooxyacetic acid (□). The incubation was started with 10 mM pyruvate and 10 mM L-serine in the presence of dialyzed 105 000g supernatant. Aminooxyacetic acid was added at the final concentration of 0.1 mM.

Table 2. Relative Amount of PGA Formation from L-Serine and Various α-Oxo Acids

α-oxo acid[a]	relative amount (%) of PGA formation[b]
pyruvate (control)	100
glyoxylate	1350
oxalacetate	29
mercaptopyruvate	19
2-ketoglutarate	17
ketoisovalerate	7
2-keto-3-methylvalerate	5

[a] Each α-oxo acid was added at the final concentration of 1 mM to the incubation mixture containing 10 mM L-serine and dialyzed 105 000*g* supernatant. [b] Values are expressed as the percentage of the control value for the amount of PGA formed with pyruvate during the 20-min incubation.

is directly involved in PGA formation. Accordingly, PGA formation from hydroxypyruvate and NOB was examined using the 200*g* supernatant. PGA was shown to be formed with no time lag (data not shown). Moreover, as shown in **Table 3**, the activity level was markedly higher than that observed with pyruvate. The activity of PGA formation observed with hydroxypyruvate was not affected by the addition of AOA (data not shown).

Kinetic Analysis of PGA Formation in the 200*g* Supernatant. Kinetic analyses of PGA formation with the 200*g* supernatant in hydroxypyruvate were performed. PGA formation with the 200*g* supernatant exhibits Michaelis−Menten kinetics. The kinetic parameters of this reaction are as follows: $K_m = 2.40 \pm 0.01$ mM and $V_{max} = 186 \pm 1$ nmol/min/g of spinach leaf.

Participation of Transketolase in the Formation of PGA. Hydroxypyruvate is known to be a substrate for transketolase (*26*), which catalyzes the transfer of glycoloyl groups of ketoses into aldoses. Nitroso aromatic compounds have been reported to serve as glycoloyl acceptors in transketolase-catalyzed *N*-arylglycolohydroxamic acids (*27*). Therefore, a correlation for PGA formation activities of the 200*g* supernatant of spinach leaf homogenate, which is known to present transketolase activity (*28*, *29*), and commercial yeast transketolase was examined with various types of glycoloyl donors. As shown in

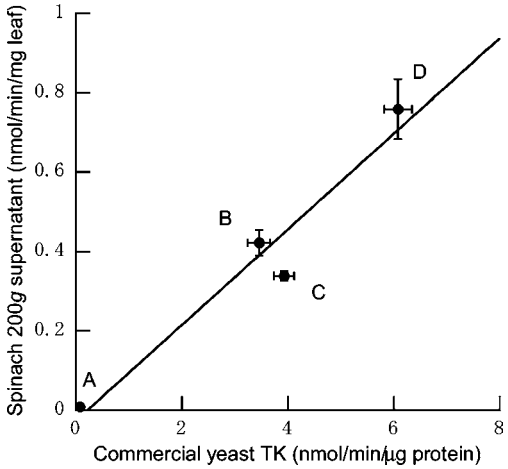


Figure 5. Correlation of activities of PGA formation in the spinach 200*g* supernatant and commercial yeast TK: (**A**) D-fructose, (**B**) D-fructose 6-phosphate, (**C**) D-xylulose 5-phosphate, and (**D**) hydroxypyruvate. Initial velocities of PGA formation were measured under the condition described in the Materials and Methods. Values are means ±SD ($n = 3$).

Table 3. Initial Velocities of PGA Formation

substrate[a]	V_0 (nmol/min)[b]
hydroxypyruvate	9.4 ± 0.8
pyruvate	0.089 ± 0.004

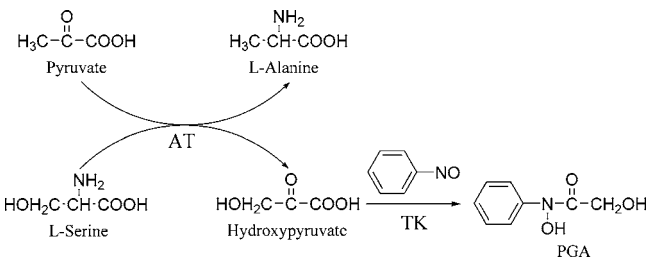
[a] Each substrate was added at the final concentration of 0.5 mM (hydroxypyruvate) and 10 mM (pyruvate) to the incubation mixture containing the 200*g* supernatant. [b] Values are obtained by the least-squares method.

Figure 5, there was a positive correlation in the activities of PGA formation ($r = 0.971$, $n = 4$, $p < 0.05$). In experiments using the partially purified spinach transketolase, PGA formation with hydroxypyruvate increased in proportion to the incubation time, and a proportional relationship was observed between the amount of the transketolase added and PGA formation activity (data not shown).

DISCUSSION

Mechanism of PGA Formation in Spinach Leaf Homogenate. In the pyruvate-dependent PGA formation using the 200*g* supernatant of a spinach leaf homogenate, PGA was formed at an accelerating rate (**Figure 2**) and no proportional relationship between the amount of supernatant added and PGA formation was observed (data not shown). From the structure of PGA, pyruvate was considered not to be the direct-acting substrate for PGA formation. These results, therefore, suggest a mechanism by which pyruvate is converted into the actual substrate that reacts with NOB to produce PGA. This hypothesis is supported by the observation of pyruvate-dependent PGA formation with 105 000*g* supernatant but not with the dialyzed supernatant (**Figure 3**). An intrinsic low molecular weight material in the homogenate, which is removed by dialysis, is thought to play an important role in PGA formation. Furthermore, the activity of PGA formation was enhanced by the addition of L-serine together with pyruvate (**Table 1**). On the other hand, the activity was reduced by the addition of L-alanine (**Table 1**). These results suggest that a transaminase catalyzed the transamination between pyruvate and L-serine participates in PGA formation. This was also confirmed by the production of alanine and hydroxypyruvate from pyruvate and L-serine in the presence of the dialyzed 105 000*g* supernatant (**Figure 4**).

Scheme 1. Two Stages of the Mechanism for Pyruvate-Dependent Formation of PGA from NOB[a]



[a] AT, transaminase; TK, transketolase.

This transamination reaction was also completely inhibited by the addition of AOA (**Figure 4**). As shown in **Table 3**, hydroxypyruvate was shown to serve as the direct-acting substrate for PGA formation from NOB, in which PGA formation was shown to exhibit Michaelis−Menten kinetics. On the basis of these results, the enzyme that catalyzed PGA formation from hydroxypyruvate and NOB is thought to be transketolase.

It is thus assumed that pyruvate-dependent PGA formation from NOB proceeds in spinach leaves by way of the two-stage mechanism shown in **Scheme 1**. Stage 1 of this mechanism is a transaminase-catalyzed reaction between pyruvate and L-serine to give alanine and hydroxypyruvate, and stage 2 is a transketolase-catalyzed glycoloyl ($-COCH_2OH$) transfer reaction between hydroxypyruvate and NOB. L-Serine is the abovementioned intrinsic low molecular weight material in the spinach homogenate. Stages 1 and 2 of the PGA formation are discussed separately below.

Stage 1: Transamination. In limited studies (*30*−*32*) relating to spinach leaves, three types of transaminase have been reported: serine:glyoxylate transaminase (EC 2.6.1.45; SGT), glutamate:glyoxylate transaminase (EC 2.6.1.4), and aspartate:2-oxoglutarate transaminase (EC 2.6.1.14). SGT and glutamate:glyoxylate transaminase are localized in leaf peroxisomes, but aspartate:2-oxo-glutarate transaminase isozymes are found in the chloroplasts, mitochondria, and peroxisomes. As for SGT, glyoxylate is reportedly more effective than pyruvate as an amino acceptor (*30*), and both L-serine and L-alanine have particularly high capacity to donate amino groups among amino acids, with the capacity being higher in L-serine than L-alanine (*33*). The transamination activity of SGT is also reported to be inhibited by D-serine and ammonium sulfate (*25*).

In this study, as shown in **Table 1**, the activity of PGA formation was inhibited by the addition of L-alanine. The activity of L-serine-dependent PGA formation was markedly enhanced by the addition of glyoxylate compared with that in the case of addition of pyruvate (**Table 2**). On the basis of these results and the inhibition of PGA formation by D-serine and ammonium sulfate, SGT is thought to be the major enzyme involved in the transamination reaction between pyruvate and L-serine.

No increase in PGA formation was observed even when PLP was added to the spinach leaf homogenate. Related findings showing that the activity of partially purified SGT from spinach leaves (*30*) and the activity of asparagine transaminase from pea plant leaves in the initial stage of purification (*34*) are not amplified by the addition of PLP have also been reported. Moreover, in general, binding to PLP is reportedly stronger with transaminase derived from plant tissue than with those derived from animal tissue or microorganisms (*35*). Consequently, this is believed to be the reason that the addition of PLP resulted in no further increase in PGA formation in spinach leaves.

Stage 2: Glycoloyl Group Transfer Reaction. Hydroxypyruvate formed by transamination between pyruvate and L-serine is likely to serve as the direct-acting substrate for transketolase, which catalyzes the glycoloyl group transfer from hydroxypyruvate into NOB. With regard to PGA formation in nonplant systems, PGA has been reported to be formed from NOB and D-xylulose 5-phosphate using yeast transketolase (*27*). We have also reported PGA formation from NOB and hydroxypyruvate in experiments using rat liver homogenate (*36*). Furthermore, it has been reported that hydroxypyruvate serves as a glycoloyl donor in transketolase reactions (*26*). As shown in **Figure 5**, PGA formation was observed even when D-fructose, D-fructose 6-phosphate, and D-xylulose 5-phosphate, known glycoloyl donors in transketolase reactions (*26*), were used. Moreover, a positive correlation was found between yeast transketolase and spinach leaf homogenate in the activities for PGA formation when various types of glycoloyl donors were used. Stereochemistry of glycoloyl donors in transketolase has shown a high specificity for ketoses with C3-L, C4-D configuration (*37*). Structurally, hydroxypyruvate is very different from these ketoses and has been reported to be an irreversible glycoloyl donor (*26*). Furthermore, the formation of PGA from hydroxypyruvate was also observed in the presence of partially purified transketolase. Taken together, these findings strongly suggest that transketolase is involved in the formation of PGA in spinach leaves.

Even without the addition of pyruvate, PGA formation was observed (**Figure 2**). This pyruvate-independent PGA formation might be accounted for by the presence of intrinsic glycoloyl donors, such as D-fructose 6-phosphate and hydroxypyruvate.

Transketolase is a TPP-dependent enzyme; however, no enhanced PGA formation activity was observed when TPP was added (data not shown). This result is believed to be due to the fact that TPP is strongly embedded in the transketolase active site (*38*) and the fact that spinach leaves contain TPP (calculated as thiamin, approximately 7 μg/g of dried spinach) (*39*).

Intracellular Localization of Enzymes Involved in PGA Formation. It was strongly suggested that SGT and transketolase contribute to the formation of PGA. SGT in spinach leaves has been reported to localize in the peroxisomes (*32*), while transketolase has been reported to localize in the chloroplasts in spinach leaves (*28*, *29*). Consequently, given this intracellular localization of SGT and transketolase, activity of PGA formation could not be observed in the 105 000*g* supernatant. However, in this study, approximately 80% of the activity of PGA formation observed with the 200*g* supernatant of spinach leaf homogenate remained in the 105 000*g* supernatant. These results suggest that during the homogenization process used in this experiment, destruction of chloroplasts and peroxisomes causes the elution of transketolase and SGT, respectively.

Toxicological Aspects. The formation of PGA in spinach using the model compound NOB illustrates possible toxicological problems.

First, nitro and/or amino aromatic compounds that are widely used in the environment are taken up by plants and then converted into nitroso compounds and subsequently into the corresponding *N*-acylhydroxamic acids such as *N*-acetyl and *N*-glycoloyl derivatives through pathways described in this report and a previous report (*15*). In plants, which have no effective systems for excretion (*1*, *2*), these metabolites may accumulate in structures such as vacuoles or cell walls (*1*, *2*, *40*) and be potentially harmful for animals and humans that consume them. 2-*N*-(2-Fluorenyl)glycolohydroxamic acid has been reported to show the same level of mutagenicity as that

of the corresponding *N*-acetohydroxamic acid derivative (*41*). Furthermore, both the hydroxamic acid derivatives have the ability to form a covalent bond to the DNA of isolated rat cells (*42*).

Second, because SGT and transketolase, enzymes for intermediate metabolism, are involved in PGA formation, the formation of *N*-arylglycolohydroxamic acids from the corresponding aromatic nitroso compounds in plants could disrupt both amino acid and sugar metabolisms, which may affect plant growth and nutritional values.

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