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Biosynthesis of Phytosiderophores[1]

In Vitro Biosynthesis of 2′-Deoxymugineic Acid from L-Methionine and Nicotianamine

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ABSTRACT

2′-Deoxymugineic acid (DMA), one of mugineic acid-family phytosiderophores (MAs), was synthesized *in vitro* both from L-methionine and from nicotianamine (NA) with a cell-free system derived from root tips of iron-deficient barley (*Hordeum vulgare* L.). The reactions producing DMA from NA needed an amino group acceptor (*i.e.* 2-oxoglutarate, pyruvate, or oxalacetic acid) and a reductant (*i.e.* NADH or NADPH). The activity of the enzymes to produce NA from L-methionine was the highest at about pH 9. This biosynthetic activity was markedly induced by iron-deficiency stress. The synthesis of NA from *S*-adenosyl-L-methionine was more efficient than from L-methionine. From the results with the cell-free system reported here, we propose a revised biosynthetic pathway of MAs.

Plants with 'Strategy 2' for iron acquisition (16) excrete iron-chelators, called phytosiderophores, from the roots to solubilize the external insoluble Fe^{3+}, and the amount of the secreted phytosiderophores increases under iron deficiency stress. MAs[2] are the only examples of the phytosiderophores known so far (21). Their chemical properties (9, 14, 20, 23) and their physiological significance (4, 7, 8, 11, 12, 15, 22) have already been established.

The biosynthetic pathway of MAs is presently considered to be that three molecules of L-methionine are combined to form NA, which is then converted to DMA by deamination and hydroxylation at the 3″-carbon, and subsequent series of hydroxylation of DMA lead to other members of MAs. This pathway has been supported by the incorporation of L-[^{14}C]methionine or L-[^{13}C]methionine into MAs *in vivo* (5, 10) and the inhibition of deamination *in vivo* (6, 18). To characterize the biosynthesis furthermore, we recently developed a cell-free system for the biosynthesis of NA from L-methionine, using crude protein fraction from the roots of iron-deficient barley (18). For a larger scale preparation of the sample, we also reported the cell-free system from suspension culture of *Nicotiana megalosiphon* (19).

On the above-mentioned hypothetical pathway of the biosynthesis of MAs, NA is a key substance, not only because its structure is much similar to MAs but also because the biosynthetic steps from NA to MAs can be thought to be evolutionally acquired by the 'strategy 2' plants, or lost by the 'strategy 1' plants: NA is widely distributed among the plants of both strategy 1 and 2, but MAs can be found only in some graminaceous plants (1, 3, 17). Nevertheless, there has been no direct evidence that NA is converted to DMA. The principal reason for this lack of data may be that there have been no systems to produce DMA *in vitro*.

We thus improved our previous cell-free system in the following order. First, the cell-free system was optimized so that a large quantity of [^{14}C]NA could be produced and supplied to the enzymes synthesizing DMA by the *in vitro* system itself. We also tested to see if SAM could be the precursor to NA. Second, effects of several cofactors on the cell-free reactions were examined to find conditions where DMA could be synthesized from L-methionine. By this improvement, we could obtain a cell-free system producing DMA from L-methionine. Finally, we incubated purified [^{14}C]NA in that cell-free system.

In the present paper, we report the biosynthesis of DMA *in vitro* both from L-methionine and from NA, and we propose a revised pathway for the biosynthesis of MAs.

MATERIALS AND METHODS

Plant Material

Seeds of barley (*Hordeum vulgare* L. cv Ehimehadakamugi No. 1) were germinated on wet filter paper and transferred into the standard culture solution (10) in a growth cabinet at 20°C/15°C (day/night) under natural light. The pH of the culture solution was adjusted at 5.5 every day. Sixty plants were cultured in a container of 20 L. When the third leaves developed, about 14 d after germination, the plants were transferred into the culture solution which contained all the other nutrients except iron and was prepared with deionized water. The pH was maintained at 7.0. The culture solution was renewed every 5th d. After about another 2 weeks, when severe iron-deficient chlorosis appeared on the fourth and the

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[2] Abbreviations: MAs, mugineic acid-family phytosiderophores; DMA, 2′-deoxymugineic acid; NA, nicotianamine; SAM, S-adenosyl-L-methionine; Taps, *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid

fifth leaves, the plants were sampled and crude proteins were extracted.

Chemicals

L-[1-^{14}C]Methionine (2.0 GBq/mmol, American Radiolabeled Chemicals Inc.) and S-adenosyl-L-[1-^{14}C]methionine (2.1 GBq/mmol, Du Pont Company) were used. Enzyme cofactors were purchased from Sigma Chemical Co. Most of the other chemicals were obtained from Wako Pure Chemical Industries, Tokyo, Japan. Authentic nicotianamine was synthesized (2). Authentic DMA was prepared from the root washing of iron deficient wheat (*Triticum aestivum* L. cv Haruhikari) by the method of Kawai *et al.* (4).

Cell-Free System

Root tips (about 3 g fresh mass) were collected by cutting the primary roots of 60 barley plants at a point about 1 cm from the apex and homogenized with 5 mL of the buffer (pH 8.5 adjusted with KOH) that contained 100 mM Hepes, 5 mM $MgCl_2$, 5 mM KCl, 1 mM EDTA, 30 mM DTT, 1.4 μM leupeptin, 1% (w/v) PVP360, and 5% (v/v) glycerol. The homogenate was passed through three layers of nylon cloth with pore size of 70 μm and centrifuged at 160*g* for 5 min. The supernatant (8 mL) was applied onto a column (2.7 × 50 cm) of Sephadex G-25 (medium). The column was previously washed with the buffer (pH 8.5) that contained 50 mM Hepes, 5 mM $MgCl_2$, 5 mM KCl, 1 mM EDTA, and 10 mM DTT. The sample was eluted with the same buffer and absorbance was detected at 280 nm. The first peak was collected and concentrated to about 10 mL by ultrafiltration with N_2 gas pressure. The molecular mass cutoff was 20 kD. This concentrated sample with the protein concentration ranging from 1.0 to 1.5 mg/mL was referred to as the crude protein fraction and used for *in vitro* reactions. Conditions such as the incubation period, the amount of the radioactive substrate added, or the pH of the reaction mixture were slightly different in each experiment of this study from our original ones (18), and the modifications are described in the following sections. The reactions were stopped by addition of ethanol at the final concentration of 70% (v/v).

The procedure for the analysis of the reaction products by HPLC (a conventional system to analyze amino acids with a Li-type cation exchanger column, Japan Spectroscopic Co., Ltd., Tokyo, Japan) was previously reported (18). Protein concentration was determined with a Bio-Rad protein assay kit with bovine plasma gamma globulin as a standard.

Effect of Iron-Deficiency Stress

For the comparison of the *in vitro* activity to produce NA from L-methionine between iron-deficient and iron-sufficient barley, two containers of the barley plants were cultured. While one group of the plants was grown without iron, the other group was kept in the standard culture solution. Both groups were grown during the same period from germination and sampled on the same day. The iron-sufficient barley showed no chlorosis on the day of sampling. L-[1-^{14}C]Methionine of 925 kBq and 10 mM ATP was reacted in the crude protein fractions (8 mL) from the iron-deficient and iron-sufficient plants at pH 8.8 for 3 h at 25°C. The experiment was conducted twice with different plants.

Effect of pH

The root tips of the iron-deficient plants were homogenized and centrifuged as above. The supernatant (8 mL) was divided into eight aliquots of 1 mL, and they were applied onto eight different columns of Sephadex G-25 (prepacked columns, PD10 from Pharmacia LKB, Sweden) for buffer exchange. The columns were previously washed with 30 mL of buffers adjusted at different pH: for pH 6 with 50 mM Mes (pK_a 6.15), for pH 7 with 50 mM Mes or 50 mM Hepes (pK_a 7.55), for pH 8 with 50 mM Hepes or 50 mM Taps (pK_a 8.4), for pH 9 with 50 mM Taps or 50 mM monoethanol amine (pK_a 9.5), for pH 10 with 50 mM monoethanol amine. All the buffers also contained 5 mM $MgCl_2$, 5 mM KCl, 1 mM EDTA, and 10 mM DTT. The samples were eluted with the respective buffers. High-mol wt fraction (3.5 mL) was collected and the pH was checked. ATP (10 mM at final) was added into this fraction, and the pH was checked again. L-[1-^{14}C]Methionine

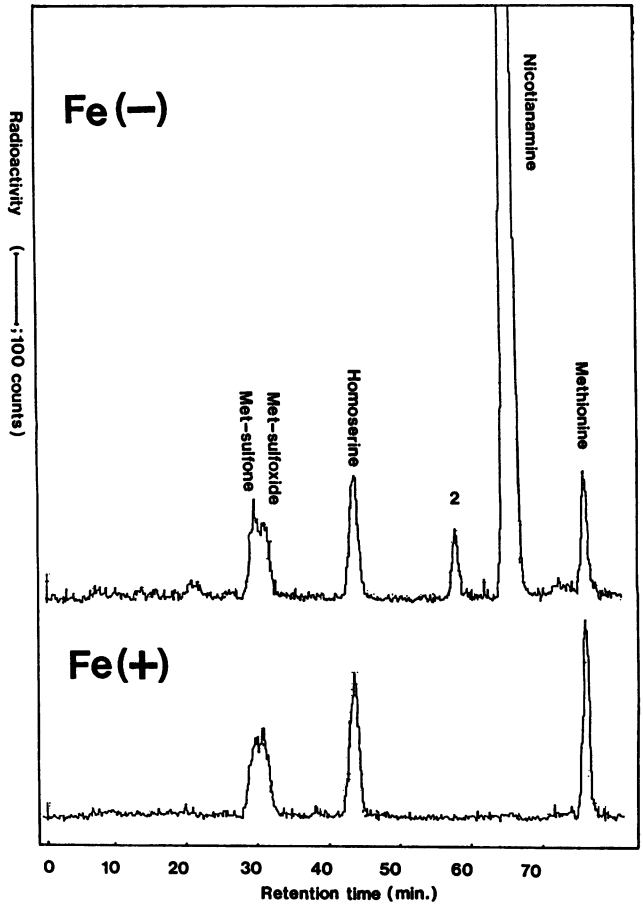

Figure 1. Effect of iron-deficiency stress on the synthesis of nicotianamine from L-methionine *in vitro*, analyzed by HPLC. Radioactivity was counted at time constant of 10 s. Fe(−), *in vitro* products by iron-deficient barley; Fe(+), *in vitro* products by iron-sufficient barley. Unidentified peak 2 corresponds to those in Figures 4 and 6.

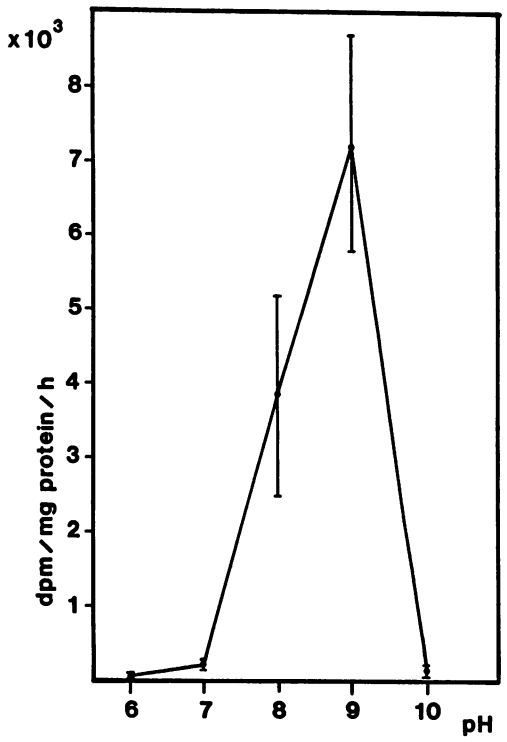

Figure 2. Effect of the pH of the reaction mixture *in vitro* on the synthesis of nicotianamine from L-methionine. Radioactivity incorporated into nicotianamine (dpm/mg protein/h) was compared at pH 6 (n = 2), pH 7 (n = 4), pH 8 (n = 4), pH 9 (n = 4), and pH 10 (n = 2). Means ± SD (vertical lines) are shown.

of 74 KBq was reacted in 1.5 mL of this mixture in duplicate at 25°C for 1 h. The pH was checked once again just before the reactions were stopped. Because the concentration by the ultrafiltration was omitted, the protein concentration ranged from 0.7 to 0.8 mg/mL.

Comparison between L-Methionine and SAM

The crude protein fraction (16 mL [pH 8.5]) was divided into 16 fractions of equal volume (1 mL). ATP (10 mM at final) was added into the fractions for the reaction of L-methionine, but not for SAM. L-[1-^{14}C]Methionine of 37 kBq or S-adenosyl-L-[1-^{14}C]methionine of 37 kBq was reacted at 25°C for 15, 30, 45, or 60 min, in duplicate. Just before the reactions were stopped, the pH was checked.

In Vitro Biosynthesis of DMA

2-Oxoglutarate and/or NADH was added into the crude protein fractions of 4 mL at the final concentration of 5 mM. L-[1-^{14}C]Methionine of 555 kBq was reacted at pH 8.2 for 2 h at 25°C. In the experiments for the synthesis of DMA, we omitted passage of the reaction products through a cation exchanger column of Amberlite IR 120 (H^+) before the analysis by HPLC (18) to avoid any loss of acidic amino acids such as MAs during this process. The effects of pyruvate, oxalacetic acid, or NADPH was compared with those of 2-oxoglutarate or NADH in other experiments on the same conditions as above.

In addition to the conventional HPLC system to analyze amino acids, the reaction products were also analyzed by a high-resolution system of HPLC, where more acidic elution buffer (pH 2.75 instead of pH 2.97) was run for 50 min from the sample injection so that each member of MAs could be separated well from one another (10).

The compound eluted at the retention time of DMA from the HPLC was collected, desalted (18), and analyzed by TLC. The sample and authentic DMA were spotted on cellulose plates (Avicel SF, purchased from Asahi Chem. Industry Co., Tokyo), and the plates were developed with three different solvent systems: (a) phenol:*n*-butanol:formate:water (12:3:2:3, v/v); (b) phenol:28% ammonia:water (15:2:3, v/v); (c) chloroform:ethanol:methanol:1 M ammonium carbonate (3:1:8:3, v/v). Radioactivity was detected by autoradiography. After the exposure, DMA was identified on the same plate by iron solubilizing activity; that is, the TLC plates were sprayed with 1 mM $FeCl_3$ in 60% (v/v) acetone and exposed to ammonia vapor, then iron-DMA complex was washed away by soaking the plates in methanol, and the remaining iron was detected with *o*-phenanthroline. The spot where DMA had been present could be identified as a white spot against reddish background. Details of the above mentioned methods of TLC and

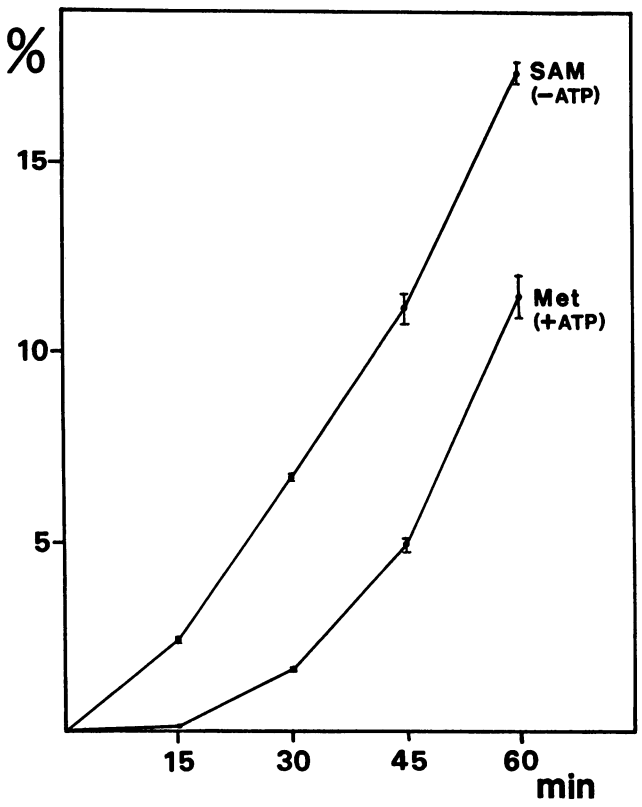

Figure 3. *In vitro* synthesis of NA from [^{14}C]SAM (without 10 mM ATP) or L-[^{14}C]methionine (with 10 mM ATP). The substrates were reacted for 15, 30, 45, or 60 min. Percentage of radioactivity incorporated into nicotianamine to the total radioactivity added was compared and means ± SD (vertical lines, n = 2) are shown.

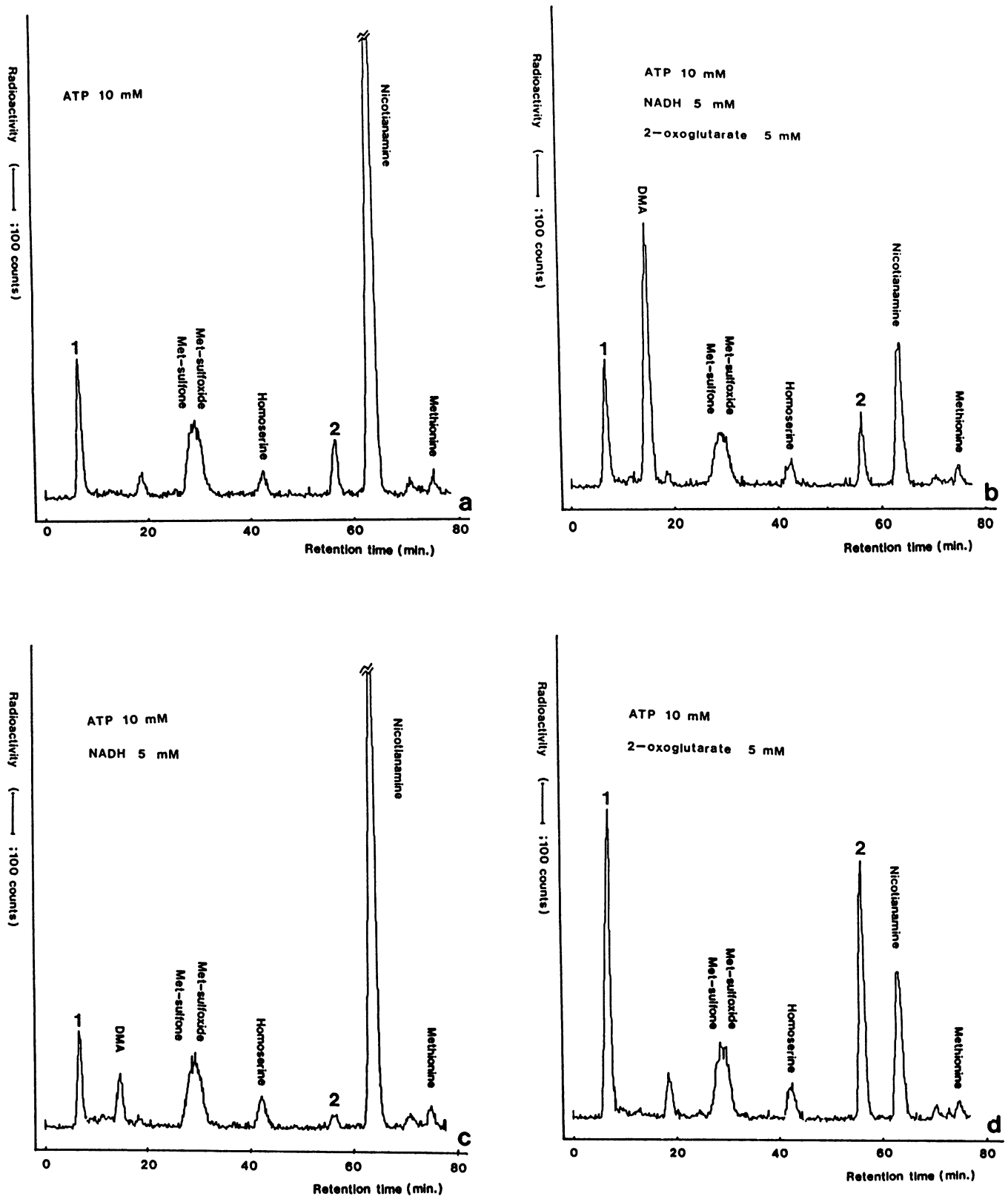

Figure 4. *In vitro* synthesis of DMA from L-[1-^{14}C]methionine, analyzed by HPLC. Effect of 2-oxoglutarate and NADH was examined. Radioactivity was counted at time constant of 10 s. Compounds at peaks 1 and 2 are not identified.

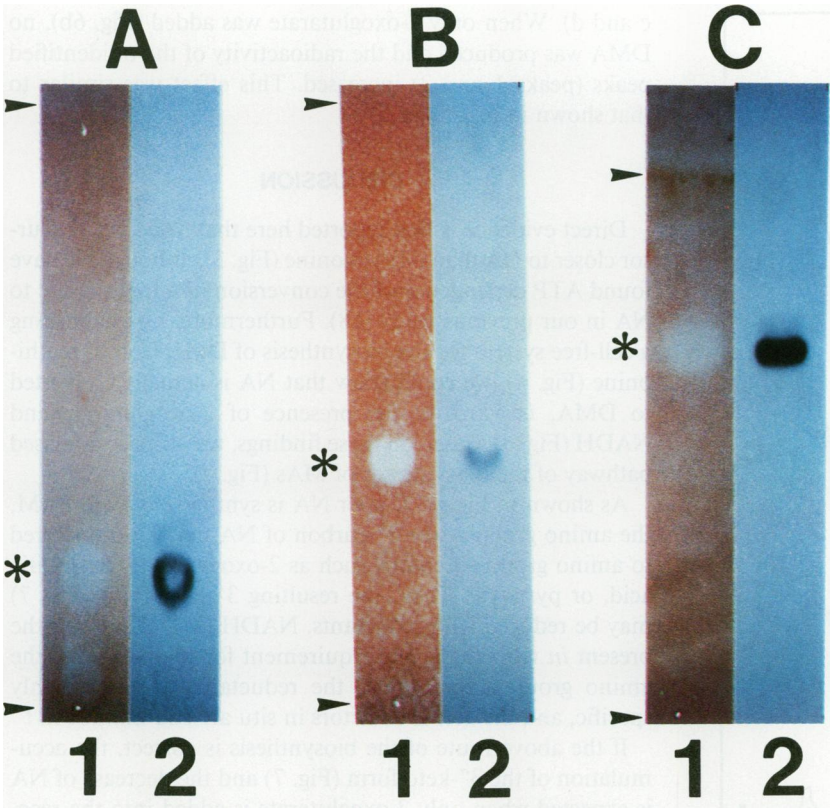

Figure 5. TLC analysis of the eluate from the HPLC at the retention time of DMA. The sample itself could not be detected by iron solubilizing activity because of its low amount. R_F of the sample detected by autoradiography was compared with that of the authentic DMA on the same plate. A, Chloroform:ethanol:methanol:1 M ammonium carbonate (3:1:8:3, v/v); B, phenol:28% ammonia:water (15:2:3, v/v); C, phenol:*n*-butanol:formate:water (12:3:2:3, v/v); lane 1, authentic DMA detected by iron solubilizing activity; lane 2, the sample detected by autoradiography; arrow head, start (bottom) and solvent front (top); asterisk, DMA.

the detection of MAs by the iron-solubilizing activity were previously reported by Kawai *et al.* (4).

Incorporation of [^{14}C]NA

[^{14}C]NA was synthesized from L-[^{14}C]methionine by the cell-free system, collected from the HPLC, and desalted. The specific radioactivity of [^{14}C]NA was 4.8 GBq/mmol. [^{14}C]NA of 6.3 kBq was reacted in the crude protein fraction of 2.5 mL at pH 8.1 for 1.5 h at 25°C. Effects of ATP (10 mM), 2-oxoglutarate (5 mM), or NADH (5 mM) were examined.

RESULT

Induction of NA Synthesis by Iron-Deficiency Stress

Figure 1 shows that the activity to produce NA from L-methionine was induced in barley plants by iron-deficiency stress. Radioactivity was incorporated into NA at the rate of 1.36 kBq/mg protein/h by the crude protein fraction from the iron-deficient barley plants. The formation rate of NA was estimated to be more than 220 pmol/mg protein/h based on the assumption that the highest possible specific radioactivity of NA is three times as high as that of the substrate L-methionine. The fraction from the iron-sufficient barley roots, however, did not show any detectable activity to produce NA from L-methionine. The protein concentration of the reaction mixture from the iron-deficient and the iron-sufficient barley plants used in the experiment shown in Figure 1 was 1.1 and 1.4 mg/mL, respectively.

Effect of pH on the Synthesis of NA from L-Methionine

The optimum pH for the synthesis of NA from L-methionine was about pH 9.0 (Fig. 2). The activity was hardly detectable at pH 10.0. However, in the range of pH lower than pH 9.0, the activity decreased more gradually and was undetectable at pH 6.0. The initial pH of all the buffers remained unchanged after 1 h incubation.

Incorporation of SAM into NA

NA was synthesized from SAM more rapidly than from L-methionine as shown in Figure 3. If compared at time 60 min, the rate of the incorporation of radioactivity from SAM into NA was about 1.5 times higher than that from L-methionine. In addition, the synthesis of NA from L-methionine showed a time lag for at least 15 min. The conversion of SAM to NA did not need ATP. The pH did not change during the incubation.

In Vitro Synthesis of DMA from L-Methionine

High radioactivity was detected at the retention time of DMA (14.8 min) when both 2-oxoglutarate and NADH, together with ATP, were present in the reaction mixture (Fig. 4b). The same sample was also analyzed by the high-resolution system of HPLC, and we found high radioactivity at the retention time of DMA (27.0 min, not shown). Figure 5 shows that the R_F of the radioactivity of the eluate collected at 14.8 min in Figure 4b was identical with that of authentic DMA in all the three different solvent systems of TLC. We concluded, therefore, that DMA was synthesized *in vitro* from L-

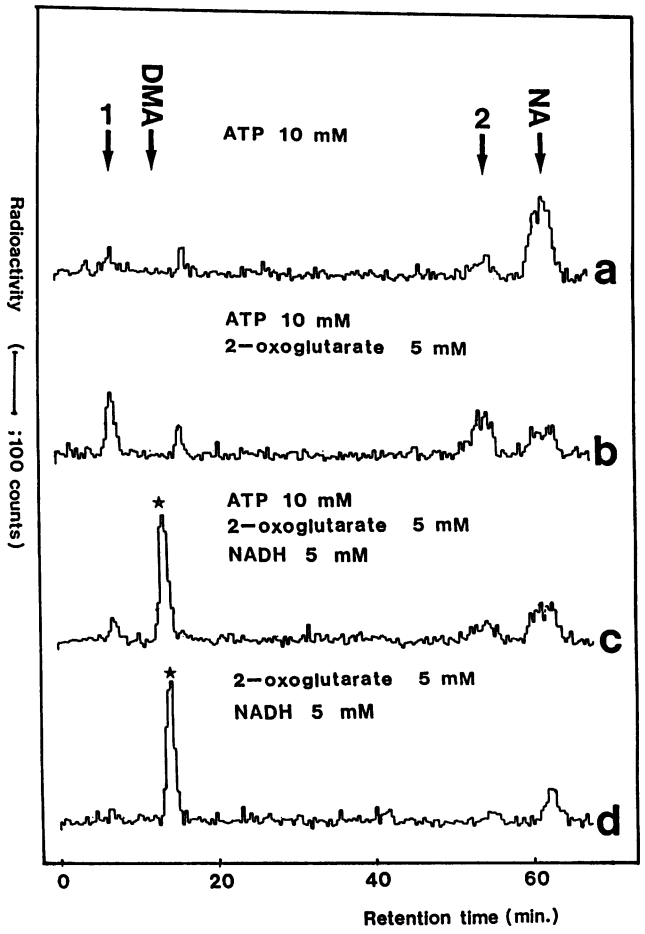

Figure 6. *In vitro* synthesis of DMA from [^{14}C]NA, analyzed by HPLC. Radioactivity was counted at time constant of 20 s. Effects of ATP, 2-oxoglutarate and NADH were examined. Peaks 1 and 2 correspond to those in Figure 4. The peak of DMA is also marked with a star.

methionine. The specific radioactivity of DMA in Figure 4b was about 5.8 GBq/mmol.

When ATP and NADH were added, but no 2-oxoglutarate (Fig. 4c), most of NA remained and a small amount of DMA was synthesized. When ATP and 2-oxoglutarate were added, but no NADH (Fig. 4d), the radioactivity of NA was as small as in Figure 4b and the radioactivity of unidentified peaks (peaks 1 and 2 in Fig. 4) markedly increased, but DMA was hardly produced. In all the cases shown in Fig. 4, most of L-methionine was consumed and there was no difference in the radioactivity of methionine-sulfone, methionine-sulfoxide, and homoserine among the treatments.

Pyruvate or oxalacetic acid was as effective as 2-oxoglutarate in the synthesis of DMA. NADPH could also replace NADH without any difference in the formation of DMA (not shown). No radioactivity was detected at the retention time of methyl methionine sulfonium (172.8 min, not shown), which is known as a common product in feeding experiments with labeled methionine.

In Vitro Synthesis of DMA from NA

NA was converted to DMA when there was 2-oxoglutarate and NADH, and this conversion did not require ATP (Fig. 6, c and d). When only 2-oxoglutarate was added (Fig. 6b), no DMA was produced and the radioactivity of the unidentified peaks (peaks 1 and 2) increased. This effect was similar to that shown in Figure 4d.

DISCUSSION

Direct evidence is first reported here that SAM is a precursor closer to NA than L-methionine (Fig. 3), although we have found ATP dependence of the conversion of L-methionine to NA in our previous study (18). Furthermore, by establishing a cell-free system for the biosynthesis of DMA from L-methionine (Fig. 4), we could show that NA is actually converted to DMA, *in vitro*, in the presence of 2-oxoglutarate and NADH (Fig. 6). Based on these findings, we propose a revised pathway of the biosynthesis of MAs (Fig. 7).

As shown in Figure 7, after NA is synthesized from SAM, the amino group at the 3″-carbon of NA may be transferred to amino group acceptors, such as 2-oxoglutarate, oxalacetic acid, or pyruvate. Then, the resulting 3″-keto form (Fig. 7) may be reduced with reductants, NADH or NADPH. In the present *in vitro* study, the requirement for the cofactors (the amino group acceptor and the reductant) was not highly specific, and the actual cofactors in situ are still unknown.

If the above route of the biosynthesis is correct, the accumulation of the 3″-keto form (Fig. 7) and the decrease of NA is expected when only 2-oxoglutarate is added into the reaction mixture. Figures 4d and 6b show the accumulation of compounds at peaks 1 and 2 on such a condition. In addition, the results in Figure 6 suggest that the compounds at the peaks 1 and 2 are the products from NA. Therefore, it is possible that the 3″-keto form is in the compounds at the peak 1 or 2, although it should be noted that the peak 1 is the front peak which may contain several kinds of anionic or weakly basic materials and that it was removed from the sample for HPLC by pretreatment with Amberlite IR 120, a cation exchanger resin (Fig. 1). The peak 2 was designated as peak 4 in our previous study (18), and it disappeared by addition of aminooxyacetic acid, a specific inhibitor of transamination mediated by pyridoxal phosphate. Therefore, the

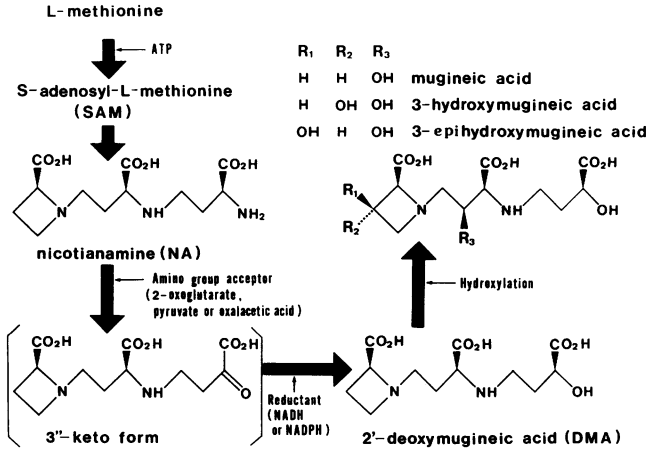

Figure 7. A revised pathway for the biosynthesis of MAs.

previous data indicated that the compound at the peak 2 in this report might be the 3″-keto form.

Assuming that the peak 1 or 2 contains the 3″-keto intermediate and a trace of endogenous amino group acceptors is still present in the crude protein fraction after gel filtration, it can be expected that some DMA will be produced and the height of the peak 1 and/or 2 will decrease when NADH is the only cofactor added into the reaction mixture. This idea can explain the difference between Figure 4, a and c.

To establish the cell-free system synthesizing DMA from L-methionine, it was necessary to have NA produced to such a degree that the concentration of NA is not limiting to the subsequent enzymatic steps. Therefore, according to the results shown in Figures 1 and 2, we extracted the crude protein fraction from severely chlorotic plants and adjusted the pH of the reaction mixture between pH 8.1 and 8.8.

Although the buffering capacity of Hepes (pKa 7.55) is weak at higher pH than pH 8.2, the pH of the crude protein fraction adjusted at pH 8.5 with 50 mM Hepes and KOH did not change after 1 h incubation. In addition, Hepes did not interfere with the analysis of the products by HPLC with a cation exchanger column. Accordingly, 50 mM Hepes was used as the buffer in this study. The pH was adjusted at pH 8.8 or 8.5 for the synthesis of NA (Figs. 1 and 3) because the optimum pH was pH 9.0 and the activity decreased steeply beyond pH 9.0 (Fig. 2). The pH of the crude protein fraction for the synthesis of DMA was adjusted at pH 8.1 or 8.2 (Figs. 4 and 6) because the optimum pH for the steps from NA to DMA was unknown and we thought that pH higher than pH 8.5 might be too basic for this conversion. Consequently, high enzyme activity was achieved in all the cases.

The optimum pH, pH 9.0, for the synthesis of NA from L-methionine is higher than physiological pH in the cytoplasm. Those enzymes may be localized in a certain compartment, where high pH is maintained; for example, vesicles appearing in the root epidermal cells of iron-deficient barley (13).

The specific radioactivity of [^{14}C]DMA (5.8 GBq/mmol) synthesized *in vitro* in this study was much higher than that synthesized by feeding L-[^{14}C]methionine *in vivo, e.g.* 7.8 MBq/mmol, because most of the endogenous materials of low mol wt such as L-methionine, SAM, or DMA itself had been removed by gel filtration before the cell-free reactions. *In vitro* synthesized NA or DMA of high specific radioactivity will be useful as a radiolabeled tracer for future experiments.

The cell-free system will also provide a powerful tool to determine the accurate mechanism for the biosynthesis of NA from SAM, to identify the intermediate between NA and DMA, to achieve the *in vitro* synthesis of other members of MAs from DMA, and to purify the biosynthetic enzymes of MAs from L-methionine.

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