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NADH- and NAD(P)H-Nitrate Reductases in Rice Seedlings

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ABSTRACT

By use of affinity chromatography on blue dextran-Sepharose, two nitrate reductases from rice (*Oryza sativa* L.) seedlings, specifically, NADH:nitrate oxidoreductase (EC 1.6.6.1) and NAD(P)H:nitrate oxidoreductase (EC 1.6.6.2), have been partially separated. Nitrate-induced seedlings contained more NADH-nitrate reductase than NAD(P)H-nitrate reductase, whereas chloramphenicol-induced seedlings contained primarily NAD(P)H-nitrate reductase. NAD(P)H-nitrate reductase was shown to utilize NADPH directly as reductant. This enzyme has a preference for NADPH, but reacts about half as well with NADH.

When rice seedlings are induced with nitrate or nitrite, the nitrate reductase formed reacts with NADH and NADPH, with a preference for NADH; but when chloramphenicol or a related nitrocompound is the inducer, the enzyme formed has a preference for NADPH (5). This study presents evidence that the difference in reactivity with the two pyridine nucleotides is not due to secondary reactions occurring in the extracts, but that there are in fact two different enzymes formed in response to the different inducers.

MATERIALS AND METHODS

Plant Materials. Rice (*Oryza sativa* L. cv. IR-8) seedlings were cultured aseptically in nutrient solutions as described previously (4). Forty seedlings were cultured from excised embryos in 160 ml of nutrient solution/500-ml Erlenmeyer flask. Flasks were reciprocally shaken at 27 C and illuminated with tungsten lamps (6000 lux).

Induction and Extraction of Nitrate Reductase. Nitrate-induced nitrate reductase was extracted from rice seedlings grown for 5 days in nutrient solution containing 4 mM KNO_3 as the sole nitrogen source. Chloramphenicol-induced nitrate reductase was extracted from rice seedlings grown for 5 days in nutrient solution containing 2 mM $(NH_4)_2SO_4$ as the nitrogen source. Four hrs prior to harvest, the nutrient solution was supplemented with chloramphenicol (2 mg/ml). Seedlings were disrupted by grinding in a glass homogenizer with 4 ml of 0.02 M K-phosphate, pH 7.6/g fresh weight. The homogenate was cleared by centrifugation at 40,000*g* for 40 min.

Assay of Nitrate Reductase Activity. NADH-nitrate reductase activity was assayed by measuring nitrite production under standard conditions as described by Wray and Filner (11). When appropriate, NADPH was substituted for NADH in the assay. One unit of enzyme is defined as that amount which catalyzes the reduction of 1 nmol nitrate/hr.

Blue Dextran-Sepharose Chromatography. Blue dextran-Sepharose was prepared as described by Ryan and Vestling (3). Enzyme extract in 0.02 M K-phosphate, pH 7.6, was applied to a column (0.9 × 6 cm) of blue dextran-Sepharose equilibrated with 0.01 M phosphate, pH 7.6. After application of the sample, the column was washed with 10 ml of equilibration buffer before specific elution. When tightly bound nitrate reductase was eluted with NADH, the NADH present in the eluates was removed by passing the corresponding fractions through a Sephadex G-25 column (0.9 × 10 cm) before assaying for NADPH-nitrate reductase activity.

Separation and Measurement of Pyridine Nucleotides and Nitrite. Pyridine nucleotides and nitrite were adsorbed on a DEAE-cellulose column (Whatman DE-52; 0.9 × 6 cm) equilibrated with 0.05 M tris-HCl, pH 8, and were eluted by washing with buffers of increasing ionic strength. Nitrite and NAD^+ (if present) were removed first by 0.05 M tris-HCl, pH 8; NADH (if present) and $NADP^+$ by 0.1 M tris-HCl, pH 8, containing 0.05 M NaCl; and finally NADPH by 0.1 M tris-HCl, pH 8, containing 0.2 M NaCl. Oxidized pyridine nucleotides were estimated by measuring the extinction at 260 nm, and reduced pyridine nucleotides were estimated at 340 nm with a Zeiss PM QIII spectrophotometer. Nitrite was measured as described by Snell and Snell (8).

RESULTS AND DISCUSSION

Solomonson (9) has described the successful use of affinity chromatography on blue dextran-Sepharose to obtain highly purified preparations of nitrate reductase from *Chlorella*. The present procedures were derived from his method, but were aimed at separating different molecular species of nitrate reductase suggested to be present in rice seedlings (5). Purification in itself was not an object of the present study, because the quantities of enzyme employed were so small.

In the present experiments, nitrate reductase was bound by blue dextran-Sepharose regardless of which induction method was used. When the column was washed with phosphate buffer, most of the activity induced by chloramphenicol was removed, but most of the activity induced by nitrate remained bound to the column and was removed later by micromolar concentrations of NADH. Representative results are shown in Figure 1.

Most of the nitrate reductase induced in rice seedlings by nitrate is NADH-specific and behaves on the column like the NADH-specific nitrate reductase of *Chlorella* (9). In contrast, the enzyme induced by chloramphenicol is active with both NADH and NADPH. Most of this NADH activity is removed by phosphate. That is, it remains associated with the NADPH activity, and does not behave like the nitrate-induced enzyme on the column. This suggests that chloramphenicol may induce the formation of an enzyme which reacts with both NADPH and NADH, with relative reaction velocities of about 2 to 1. Accordingly, the enzyme has been called NAD(P)H-nitrate reductase.

Neither type of induction resulted exclusively in one type of

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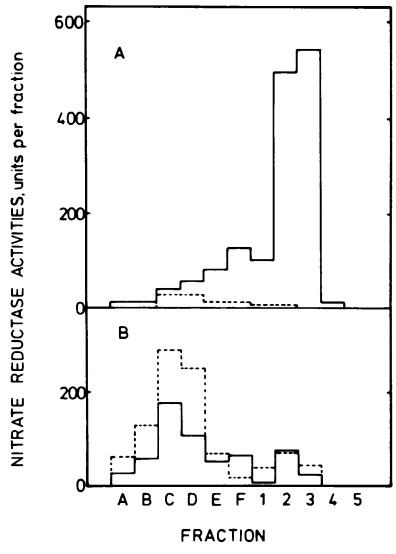

FIG. 1. Elution of nitrate reductase from blue dextran-Sepharose. A: nitrate-induced enzyme; B: chloramphenicol-induced enzyme. Total activities applied: A: NADH-nitrate reductase, 800 units; NADPH-nitrate reductase, 280 units. B: NADH-nitrate reductase, 460 units; NADPH-nitrate reductase, 2500 units. Weakly bound nitrate reductase was eluted with a step gradient of phosphate buffer, pH 7.6. Six 4-ml fractions were collected, A through F, with 0.04, 0.08, 0.10, 0.12, 0.16, and 0.20 M phosphate, respectively. Tightly bound nitrate reductase was eluted with 200 μM NADH. Five 2-ml fractions (1 to 5) were collected. —: NADH-nitrate reductase activity; - - -: NADPH-nitrate reductase activity.

enzyme. Thus, the nitrate reductase from nitrate-induced rice seedlings includes a small amount of NAD(P)H-nitrate reductase which can be eluted from the column by 0.08 or 0.10 M phosphate buffer (Fig. 1A). On the other hand, there is also a small amount of NADH-nitrate reductase in chloramphenicol-induced seedlings. Its presence is indicated by the increased NADH-nitrate reductase to NADPH-nitrate reductase activity ratio in those fractions eluted by 0.16 and 0.20 M phosphate buffer and NADH (Fig. 1B). Variations in this ratio observed at low enzyme activities are the result of limitations of the assay. The amount of nitrite produced was too small to be measured accurately.

The differential behavior of the two enzymes observed in Figure 1 might be explained by secondary differences in the two extracts which would alter the elution profiles. To eliminate this possibility, extracts of nitrate- and chloramphenicol-induced seedlings were mixed and processed together on the same column. NAD(P)H-nitrate reductase was first removed by washing with 0.1 M phosphate buffer and NADH-nitrate reductase was eluted later by NADH (Fig. 2). However, the NADH-nitrate reductase to NADPH-nitrate reductase activity ratio in the fractions eluted out by 0.1 M phosphate buffer was higher than the values observed with samples of chloramphenicol-induced tissues. A small quantity of NADH-nitrate reductase is apparently eluted with 0.1 M phosphate. Separate experiments showed that the peak was in the fifth fraction, separated somewhat from the peak of the NAD(P)H activity.

Besides physical separation, additional evidence has been reported suggesting that different types of nitrate reductases are present in rice seedlings. The *Km* for nitrate of the NADH-nitrate reductase induced by nitrate or nitrite is about 10-fold higher than that of NADH-nitrate reductase activity induced by nitrocompounds. On the other hand, the *Km* for nitrate of the NADPH-nitrate reductase activity is the same regardless of the type of induction (6).

The recovery of the NADPH-nitrate reductase activity from the column was generally around 40% while the recovery of NADH-nitrate reductase activity was usually higher than 100% regardless of the source. A possible explanation for this fact could be the presence in these extracts of an inhibitor of NADH-nitrate reductase activity. Such an inhibitor has been reported by Kadam *et al.* (2). The NADPH activity is apparently less susceptible to inhibition.

In order to demonstrate that NADPH is a direct electron donor for the NAD(P)H-nitrate reductase of rice seedlings, the reaction products formed when NADPH was used as reductant were separated and measured. A column of DEAE-cellulose was used. Of the 100 nmol of NADPH added to the reaction mixture, 78 nmol were oxidized to $NADP^+$ with the concomitant formation of 78 nmol of nitrite (Fig. 3). The remaining nucleo-

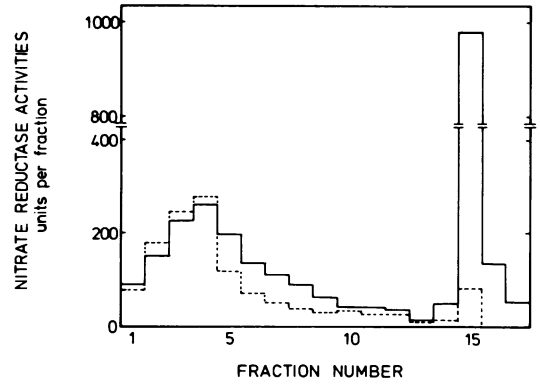

FIG. 2. Separation of mixed induction products. A mixture of nitrate- and chloramphenicol-induced nitrate reductase containing 2090 units of NADH-nitrate reductase and 3212 units of NADPH-nitrate reductase was applied to a blue dextran-Sepharose column. The column was washed first with 36 ml of 0.1 M phosphate buffer, pH 7.6 (fractions 1 to 12), followed by 10 ml of 200 μM NADH (fractions 13 to 17). —: NADH-nitrate reductase activity; - - -: NADPH-nitrate reductase activity.

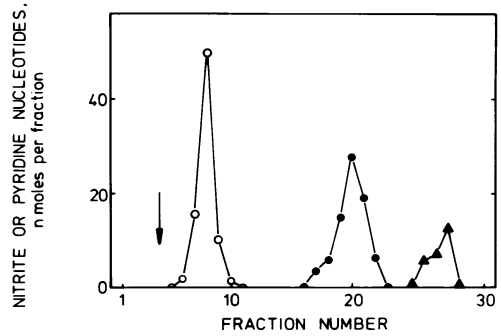

FIG. 3. Separation of the products of the reaction catalyzed by NAD(P)H-nitrate reductase. Enzyme from chloramphenicol-induced rice seedlings was used after elution from blue dextran-Sepharose with 0.1 M phosphate. The reaction mixture which contained enzyme (107 units in 0.1 M phosphate, pH 7.6), KNO_3 (10 μmol), and NADPH (100 μmol) in a final volume of 1.2 ml was placed in a Warburg vessel. The vessel was flushed with argon for 10 min, and the reaction was started by the addition of NADPH from a side-arm. Incubation was for 60 min at 30 C, after which the reaction mixture was diluted with water to 10 ml. The mixture was passed through a column of DEAE-cellulose (Whatman DE-52; 0.9 × 6 cm) equilibrated with 0.05 M tris-HCl, pH 8. The column was washed consecutively with 20 ml of 0.05 M tris-HCl, pH 8; 26 ml of 0.1 M tris-HCl, pH 8, containing 0.05 M NaCl; and finally, 14 ml of 0.1 M tris-HCl, pH 8, containing 0.2 M NaCl. Two-ml fractions were collected. ○: nitrite; ●: $NADP^+$; ▲: NADPH. The arrow indicates the position where NAD^+ would appear if present.

tide was quantitatively recovered as NADPH. Neither NADH nor NAD^+ was found in the reaction mixture. These results demonstrate that the NAD(P)H-nitrate reductase of rice seedlings can utilize NADPH directly as electron donor.

These results exclude the possibility that the reactivity of the enzyme with NADPH is due to a phosphatase converting NADPH to NADH, as has been suggested for the nitrate reductase from maize and soybean leaves (10). A preliminary report has appeared, however, that two isoenzymes of nitrate reductase have been separated from soybean leaves by column chromatography on DEAE-cellulose (1). One of these enzymes was designated NADH-nitrate reductase. The second nitrate reductase had greater activity with NADPH as electron donor than with NADH and was designated NADPH-nitrate reductase. Perhaps the NADH- and NAD(P)H-nitrate reductases suggested by the present work are commonly present in higher plant tissues rather than a NADH-specific and a NADPH-specific nitrate reductase as originally suggested by Sims *et al.* (7).

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LITERATURE CITED

1. Jolly, S. O., W. H. Campbell, and N. E. Tolbert. 1975. NADH- and NADPH-nitrate reductase isoenzymes isolated from soybean leaves. Plant Physiol. 56: S-73.
2. Kadam, S. S., A. P. Gandhi, S. K. Sawhney, and M. S. Naik. 1974. Inhibitor of nitrate reductase in the roots of rice seedlings and its effect on the enzyme activity in the presence of NADH. Biochim. Biophys. Acta 350: 162-170.
3. Ryan, L. D. and C. S. Vestling. 1974. Rapid purification of lactate dehydrogenase from rat liver and hepatoma: A new approach. Arch. Biochem. Biophys. 160: 279-284.
4. Shen, T. C. 1969. The induction of nitrate reductase and the preferential assimilation of ammonium in germinating rice seedlings. Plant Physiol. 44: 1650-1655.
5. Shen, T. C. 1972. Nitrate reductase of rice seedlings and its induction by organic nitro compounds. Plant Physiol. 49: 546-549.
6. Shen, T. C. and W. J. Broughton. 1975. Substrate affinities of differently induced nitrate reductase in rice seedlings. Plant Physiol. 56: S-36.
7. Sims, A. P., B. F. Folkes, and A. H. Bussey. 1968. Mechanisms involved in the regulation of nitrogen assimilation in microorganisms and plants. *In*: E. J. Hewitt and C. V. Cutting, eds., Recent Aspects of Nitrogen Metabolism in Plants. Academic Press, New York. pp. 91-114.
8. Snell, F. D. and S. T. Snell. 1949. Colorimetric Methods of Analysis. Van Nostrand, New York. p. 804.
9. Solomonson, L. P. 1975. Purification of NADH-nitrate reductase by affinity chromatography. Plant Physiol. 56: 853-855.
10. Wells, G. N. and R. H. Hageman. 1974. Specificity for nicotinamide adenine dinucleotide by nitrate reductase from leaves. Plant Physiol. 54: 136-141.
11. Wray, J. L. and P. Filner. 1970. Structural and functional relationships of enzyme activities induced by nitrate in barley. Biochem. J. 119: 715-725.