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INTRACELLULAR LOCATION OF NITRATE REDUCTASE AND NITRITE REDUCTASE

II. WHEAT ROOTS*

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SUMMARY

Approximately 15% of the total nitrite reductase of crude homogenates of wheat roots applied to sucrose gradients was separated with an organelle whose isopycnic density was about 1.22 $g \cdot cm^{-3}$. The activity recovered in the supernatant was thought to be particulate in origin, because similar ratios of activity of isoenzyme 1 and 2 of nitrite reductase were found in both particulate and supernatant fractions. The particle with nitrite reductase activity also contained glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, triose phosphate isomerase and NADPH diaphorase. This root particle and whole chloroplasts from leaves had a similar isopycnic density as well as these enzymes, and thus the data suggest that the root particle may be a proplastid.

Nitrate reductase was found only in the supernatant and it was not associated with any of the root organelles.

Mitochondria from wheat roots had an equilibrium density of 1.18 $g \cdot cm^{-3}$ and contained both NAD and NADP glutamate dehydrogenase, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, triosephosphate isomerase and NADPH diaphorase but not nitrite reductase. Microbodies of wheat roots had an equilibrium density of about 1.20 $g \cdot cm^{-3}$ on the sucrose gradient and contained catalase and glycollate oxidase.

INTRODUCTION

Most studies of nitrate and nitrite reductase have been made with leaf tissue, however, the enzymes are also present in non-green tissues[1]. Current evidence as to the intracellular location of the two enzymes in leaves indicates that nitrate reductase is a soluble (cytoplasmic) enzyme, while nitrite reductase is localized in the stroma of the chloroplast (for review see accompanying paper[2]). The intracellular location of these reductases in non-green tissues has not been widely studied. Miflin[3] reported that a

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small proportion of the total nitrate and nitrite reductase activity of the barley roots was associated with a particulate fraction. More recently he has shown that this fraction with both reductases could be separated from the mitochondria by sucrose density gradient centrifugation[4]. The data presented in this paper also show that nitrite reductase from wheat roots was in a particle fraction that was neither mitochondria nor microbodies, but probably proplastids. In contrast to Miflin's findings[4], nitrate reductase was not localized with any particle and is considered to be a soluble enzyme.

MATERIALS AND METHODS

Plant material

Wheat seeds (*Triticum aestivum* L., variety Avon) were surface sterilized with 10 % H_2O_2 for 20 min and washed with distilled water. The seeds were germinated and grown under sterile conditions suspended on cheesecloth over a continuously aerated Hoagland's solution. The plants were illuminated with 100 ft candles light on a 12-h photoperiod with 24 °C day and 16 °C night temperatures. The roots were harvested after 6–7 days and washed thoroughly before use.

Preparation of cellular organelles

The roots were cut into very small pieces with scissors and ground with a chilled mortar and pestle in an ice-cold medium, which contained 50 mM potassium phosphate at pH 7.5, 2 mM EDTA, 10 mM KCl, 10 mM $MgCl_2$, 10 mM dithiothreitol and 30 % sucrose (w/w). The ratio of grinding medium to fresh root weight was 1:1 (v/w). The homogenate was filtered through one layer of Miracloth (Chicopee Mills, Inc., New York) and 20 ml layered onto the sucrose gradient. With the exception that the sucrose solutions were made with 0.025 M glycylglycine, pH 8.0, the gradients were prepared, centrifuged and fractionated into 2.5-ml aliquots as previously described[2]. Larger quantities of particles were prepared in a B-30 zonal rotor with multi-step sucrose gradients consisting of 200 ml of 56 % and 25 ml each of 54, 52, 50, 48, 46, 44, 42, 40, 38, 36, 34, 32, 30, 27.5 and 25 % sucrose solutions (w/w). These sucrose solutions were prepared in 50 mM potassium phosphate at pH 7.5, mM EDTA, 10 mM KCl, and 10 mM $MgCl_2$. About 170 ml of root extract was added at the core of the rotor and particles were moved into the sucrose gradient by centrifuging for 1 h at 25000 rev./min. If larger preparations were used, 56 % sucrose was added at the rim to displace the homogenate from the core, and then more homogenate was loaded onto the gradient. Isopycnic sedimentation of the particles was achieved by centrifuging for 3 h at 30000 rev./min. The gradient was unloaded from the edge (densest sucrose) in 10-ml fractions.

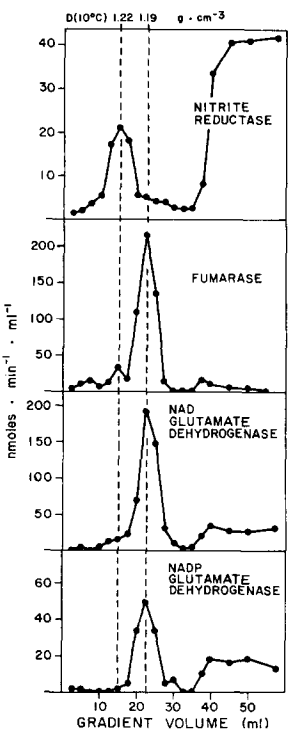
Enzyme assays

Nitrate reductase was assayed with NADH[5] or with succinate[4] as electron donor. Nitrite reductase was assayed with dithionite reduced methyl viologen as reductant, as described by Hucklesby *et al.*[6]. Their method No. 1 for 3 ml final volume was used for the sucrose density fractions and method No. 3 for 0.6 ml final volume for fractions from DEAE-cellulose columns. Assays for catalase, cytochrome *c* oxidase, and triose phosphate isomerase are mentioned in the accompanying paper[2]. Fumarase was

measured by the change in absorbance at 240 nm[7], and glycollate oxidase by the anaerobic reduction of 2,6-dichlorophenolindophenol at 600 nm[8]. For glutamate dehydrogenase the initial rate of NADH oxidation was measured in a total volume of 1 ml with 20 μmoles $(NH_4)_2SO_4$, 0.2 μmole NADH or NADPH, 0.5 μmole KCN, 100 μmoles potassium phosphate buffer at pH 7.6, 5 μmoles α-keto-glutarate, 0.025% Triton X-100 and enzyme. NADPH diaphorase was measured by the oxidation of ferricyanide as a rate of decrease of absorption at 420 nm. The reaction mixture in 1 ml contained 5 μmoles Tris–HCl at pH 8.0, 50 nmoles potassium ferricyanide, 0.2 μmole NADPH and enzyme. Glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were assayed in 1-ml cuvettes according to Waygood and Rohringer[9]. Protein was measured by the method of Lowry *et al.*[10].

RESULTS

After organelle separation by isopycnic sucrose density gradient centrifugation, mitochondria were located by determining the distribution of the marker enzyme, fumarase (Fig. 1), as well as cytochrome *c* oxidase, which data are not shown. The mitochondrial band, located at a density of 1.18 to 1.19 g·cm^{-3} corresponded to the



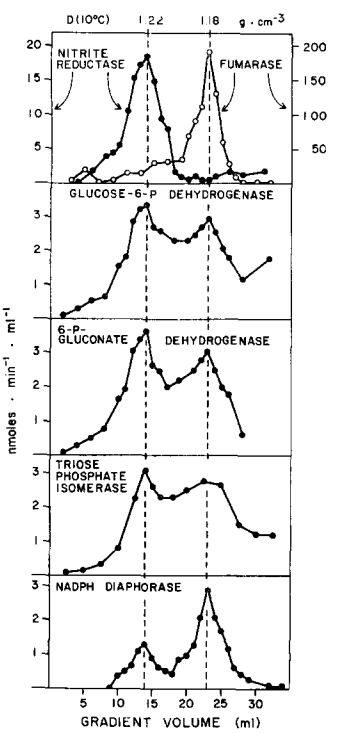


Fig. 1. Location of mitochondria and the organelle with nitrite reductase from wheat roots on isopycnic sucrose density gradients.

Fig. 2. Association of nitrite reductase with plastid enzymes on a sucrose gradient of wheat root homogenate. Four enzymes, considered to be marker enzymes for whole chloroplasts from leaf tissue, were associated with the particle containing nitrite reductase, as well as the mitochondria.

major protein band. Both NAD and NADP glutamate dehydrogenases in the wheat root exhibited a similar distribution pattern as fumarase and cytochrome *c* oxidase, and were therefore considered to be associated with the mitochondria, an observation consistent with the results of Miflin[4].

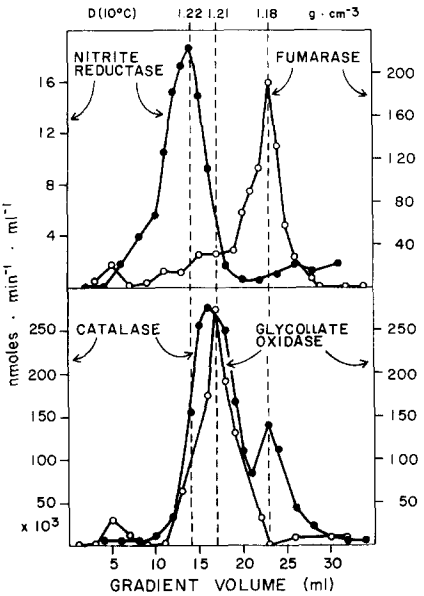


Fig. 3. Distribution on sucrose gradients of microbodies, mitochondria, and the organelle with nitrite reductase from wheat roots.

Nitrite reductase from the wheat roots was located in a band corresponding to a density of about 1.22 g·cm^{-3} which was a significantly higher density than the mitochondrial band (Figs 1, 2 and 3). The nitrite reductase in the particle represented about 15 % of the total activity, the rest being in the soluble fractions. Nitrate reductase, whether assayed with NADH or succinate as the electron donor, was not associated with any specific particle in wheat roots, for the bulk (93–95 %) of the activity was in the soluble fraction (data not shown).

The nature of the particle containing nitrite reductase at a density of 1.22 g·cm^{-3} from the wheat roots was compared with known organelles in this part of the sucrose gradient. Whole chloroplasts from leaf tissue are localized at equilibrium densities of about 1.22 g·cm^{-3} (refs 2 and 11) and microbodies at about 1.23 to 1.25 from various leaf tissue[2,11–13]. Distribution of marker enzymes for both these types of particles from wheat roots was compared with the nitrite reductase location (Figs 2 and 3). Marker enzymes for whole chloroplasts from green leaves[11] such as NADPH diaphorase, triosephosphate isomerase, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, were associated with the particle from wheat roots containing nitrite reductase. This association of enzymes suggests that nitrite reductase in wheat roots is located in some form of a proplastid, analogous to the whole chloroplast from leaves. Appreciable levels of glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, triose phosphate isomerase and NADPH

diaphorase were found throughout the sucrose density gradient between 1.24 and 1.17 $g \cdot cm^{-3}$, with maximum activity at 1.22 and 1.18 $g \cdot cm^{-3}$, the location centers of proplastids and mitochondria, respectively. This distribution pattern was different from that observed with leaf preparations in which these enzymes were found in the whole plastids and cytosol but not in the mitochondria[2,14]. Triose phosphate dehydrogenase (NAD or NADP) activity was not detected in either the wheat root mitochondria or proplastid fractions.

Catalase and glycolate oxidase, which are marker enzymes for peroxisomes[8,11–13], were localized at a density of 1.19 to 1.21 $g \cdot cm^{-3}$ on these sucrose gradients of organelles from wheat roots (Fig. 3). The mean density of this isolated microbody in the sucrose gradient from five experiments was 1.20 $g \cdot cm^{-3}$. This equilibrium density of the root microbody was somewhat less than that for other plant tissues[2,8,11–13,15,16], as Huang and Beevers[17] have also noted. Although there was overlap of enzyme acitivities, the peaks of catalase and glycollate oxidase were distinctly separate from those of nitrite reductase and those associated with plastids (Fig 2 and 3), and provide evidence for the existence of root microbodies. Isocitrate lyase, a marker enzyme for glyoxysomes[15] was not found in any fraction. Thus the root microbodies have an enzyme complement more characteristic of leaf peroxisomes than of cotyledonary glyoxysomes.

Intracellular location of nitrite reductase isoenzymes

Corn scutella[6] and corn roots[18,19] contain isoenzymes of nitrite reductase and in the scutella both isoenzymes are associated with an undefined particle. Two isoenzymes of nitrite reductase were also found in wheat roots, and a study was made of their intracellular location. To obtain adequate amounts of enzymes for assay, homogenates from large quantities of wheat roots were fractioned on sucrose gradients in a B-30 zonal rotor and the supernatant and "proplastid" fractions pooled separately. Each fraction was dialyzed for 6 h at 3 °C against 20 mM potassium phosphate at pH 7.6 containing 0.1 mM dithiothreitol, and then applied to a DEAE-cellulose column (1.4 cm × 17 cm) equilibrated with the same buffer. The column was eluted by a linear gradient of NaCl, 0 to 0.2 M in 150 ml. Nitrite reductase in both the supernatant

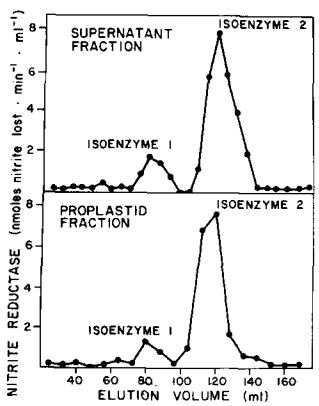


Fig. 4. DEAE-cellulose column elution patterns for nitrite reductase isoenzymes 1 and 2 in the supernatant fraction and the proplastid organelle.

and particulate fractions eluted in two peaks, previously shown to correspond to isoenzyme 1 and 2 (Fig. 4). The ratio of total activity of isoenzyme 1 to isoenzyme 2 was 0.19 and 0.14 for the supernatant and particulate fractions, respectively.

DISCUSSION

In leaves nitrite reductase is associated with whole chloroplasts[2, 20], and similarly in wheat roots part of it can be isolated in an organelle which would appear to be a proplastid. Both the chloroplast and the root plastid have a similar density and contain in addition to nitrite reductase, glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and triose phosphate isomerase. Simms *et al.*[16] introduced the term nitrosome to describe a coordinately induced and repressed relationship for nitrite reductase in yeast. Miflin[4] also applied this term to describe a particle, isolated from barley roots, which contained both nitrate and nitrite reductase activity. Since the proplastid fraction from wheat roots did not contain nitrate reductase, the term nitrosome was not used in this paper. Nitrate reductase in wheat roots was not associated with a particle, an observation consistent with its location in the cytosol of leaves[2].

Of the total nitrite reductase of the wheat root about 15 % was in the proplastid and the rest in the soluble fraction. Similarly most of the nitrite reductase of leaves after homogenization is found in the soluble fraction and none in the bulk of broken chloroplasts[2]. Only the small fraction of isolated chloroplasts contain their stroma enzymes, such as nitrite reductase and ribulose diphosphate carboxylase. By analogy we propose that for roots *in situ* all of the nitrite reductase is located in the "proplastid" particle. During the harsh grinding procedure required to break up the root tissue, most of the proplastids would be ruptured and their stroma enzymes lost or solubilized. This hypothesis was supported by the observations that the ratio of activity for isoenzyme 1 and 2 of nitrite reductase is the same for both the proplastid and soluble fraction of homogenized wheat roots. A different ratio in the two fractions would have implicated a different subcellular location for each isoenzyme.

The presence of some of the enzymes for the pentose phosphate pathway in the proplastid can be related to the formation of reductant for nitrite reduction in non-green tissue and for green tissue in the dark. Butt and Beevers[21] observed a marked stimulation of this pathway when corn roots were incubated with nitrite and concluded that the NADPH formed by the pentose phosphate pathway was coupled to nitrite reduction. Similarly Losada *et al.*[22] used this scheme to account for the generation of reductant in leaves for nitrite reduction in the dark.

The study of isoenzymes in wheat roots contributes one more example of an emerging concept regarding tissue distribution of the two isoenzymes of nitrite reductase. Green leaves contain only isoenzyme 2 (ref. 19), roots contain isoenzyme 1 and 2 in a ratio of 0.14 to 0.19 so that isoenzyme 2 also dominates, but in corn scutella with both isoenzymes there was about 1.5 times more isoenzyme 1 than 2 (ref. 6). In roots both isoenzymes are in a proplastid type of organelle.

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