Intracellular Localization of Nitrate Reductase, Nitrite Reductase, and Glutamic Acid Dehydrogenase in Green Leaf Tissue

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Summary. Greenhouse grown seedlings of corn (Zea mays L.) and foxtail (Setaria faberii Herrm.) were used as source material in determining the intracellular localization of nitrate reductase, nitrite reductase, and glutamic acid dehydrogenase. Nonaqueous and aqueous isolation techniques were used to establish that nitrate reductase is localized within the chloroplasts, but that nitrite reductase and glutamic acid dehydrogenase are not. Nonaqueous isolation gives distribution patterns of nitrite reductase which are the same as those observed for NADP-dependent 3-phosphoglyceraldehyde dehydrogenase but which differ drastically from the patterns observed for pyruvic acid kinase. The distribution patterns for nitrate reductase are the same as those of pyruvic acid kinase. The techniques used do not eliminate the possibility that nitrate reductase and pyruvic acid kinase are localized on the external chloroplast membrane.

The data obtained establish that glutamic acid dehydrogenase of green leaves is localized within the mitochondria.

Most of the nitrogen absorbed by plants growing under field conditions is in the form of nitrate (30) which must be reduced prior to elaboration into amino acids. Evidence presented to date suggests that these steps are completed by 3 enzymes, nitrate reductase (NR), nitrite reductase (a complex), and glutamic acid dehydrogenase (13). These enzymes then must be functioning in a coordinated manner to provide the aminated organic compounds needed by the plant for growth and development.

Since in the past, light has been implicated as having a major, albeit not well defined role in nitrate metabolism (7), it appeared logical to assume that NR and nitrite reductase were localized in the chloroplast. The failure to detect NR in albinio corn seedlings (unpublished work of R. H. Hageman) also tends to support this view. Losada et al. (20) concluded that isolated spinach chloroplasts contain all of the enzymes necessary for reduction of nitrate to ammonia. This conclusion was based upon the association of NR with aqueously isolated chloroplasts (11), the reduction of nitrate in chloroplasts (23), and the demonstration that grana supplemented with ferredoxin and FMN could couple light to nitrate and nitrite reductase. Losada and co-workers (20) also noted that FMN and NADP-reductase were required to couple NADPH to NR and concluded that reduced flavins (FADH₂ or FMNH₂) were the natural electron donors for NR (21). A major factor in the assignment of this role for flavins was the failure of NADPH to serve directly as an electron donor to NR. This is not surprising because other work (2) has shown that 15 of 16 plant species (including spinach) tested, NADH is the preferred if not exclusive electron donor for NR. Exogenous or free flavins are not required when NADH is used. The function of ferredoxin as an electron donor for nitrite reductase reported by Losada et al. (20) is supported by other investigations (3, 18).

In contrast, other workers have found that NR is readily solubilized and appears primarily in the soluble fraction after aqueous extraction of algae (8) and higher plant tissue (14). With respect to localization of NR, this must be accepted with caution as Heber (16) has reported that extensive loss of chloroplastic proteins occurs during aqueous extraction. Stocking (28) warns not only against loss of, but adsorption of, soluble enzymes by the chloroplasts. Thus small amounts of NR associated with chloroplasts may not be a valid indication

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of localization. Since NR occurs in root tissue (14, 25) and excised roots have been successfully cultured on a nitrate medium (25), it is apparent that chloroplasts are not an absolute requirement for functional NR. Therefore, the evidence for or against the association of NR with the chloroplasts is not conclusive.

Although Bulen (6) extracted and purified glutamic acid dehydrogenase from corn leaves, no report was found concerning the intracellular localization of this enzyme in green leaves. Several investigations (4, 9, 10, 24) with etiolated seedling tissue have indicated that glutamic acid dehydrogenase is localized in the mitochondrion. Little work has been done with green leaf mitochondria, although aqueous extraction methods have been developed (22).

The objective of this work was to utilize nonaqueous and aqueous fractionation techniques in an attempt to establish the intracellular localization of nitrate reductase, nitrite reductase, and glutamic acid dehydrogenase in green leaves.

Materials and Methods

Plant Material. Corn (Zea mays L.), variety Hy2 × Oh7, and foxtail (Setaria faberii Herrm.) plants were grown in the greenhouse on a 1:1 mixture of soil and peat moss that was watered daily with full strength Hoagland’s nutrient solution. Natural sunlight was supplemented with fluorescent lights that provided an additional 2.5 × 10^4 lux for 16 hours each day. The condition and amount of leaf tissue was adequate for experimentation when corn and foxtail plants were 10 and 28 days old, respectively. The plants were given a 24-hour dark treatment just prior to harvest to deplete starch from the chloroplasts. Leaf material was removed from the plants and quickly frozen between layers of dry ice. The frozen leaves, mixed with additional dry ice, were pulverized with a mortar and pestle. This material was then lyophilized without thawing and the dry powder stored under vacuum at −10°C until used for the nonaqueous experiments. Identical procedures, except for freezing and lyophilization, were used to produce the leaf material for the aqueous extractions.

Nonaqueous Fractionation. The general procedure followed is illustrated by the scheme presented by Stocking (28): however, specific details varied and will be listed. One-half gram of lyophilized material was ground (homogenizer, Virtis Co., Gardiner, New York) with 30 ml of a mixture (density 1.30) of hexane and carbon tetrachloride and 10 g glass homogenizing beads (Virtis Co.) for 45 seconds at 70% of line voltage. The slurry was filtered through cheesecloth, then centrifuged for 15 minutes at 15,000 × g. This centrifugation gave a sediment and a supernatant solution capped with a green (pellet) layer. The supernate and green pellet were decanted, measured and diluted with an equal volume of hexane, thus reducing the density to less than 1.30. This diluted mixture was centrifuged for 10 minutes at 5000 × g to collect the suspended chloroplasts. The sedimented material designated Fraction I was composed almost exclusively of chloroplasts of which 80% appeared to be intact by microscopic inspection. The supernatant liquid was discarded.

The material sedimented by the initial centrifugation was suspended in a mixture (density 1.35) of hexane and carbon tetrachloride and centrifuged for 15 minutes at 13,000 × g to give a third sediment and supernatant liquid. Again the supernatant liquid was capped with a green pellet. This second pellet was handled exactly as the first pellet. The resulting chlorophyllous material had fewer intact (25%) chloroplasts, and was contaminated to a greater extent with other organelles, i.e., mitochondria, and pieces of cell wall, and was designated Fraction II (density 1.30 to 1.35). The third sediment (density greater than 1.35) contained some chloroplasts, pieces of cell wall and broken cells, and was designated Fraction III.

The Fraction I, II and III precipitates were dried under vacuum, then suspended in 0.025 M phosphate, pH 7.5, which was 0.01 M in cysteine. An aliquot was removed for chlorophyll determinations and the rest of the suspension was centrifuged 10 minutes at 10,000 × g. Each supernate was assayed for the various enzymes. All steps from tissue homogenization through centrifugation of the enzyme were carried out at 3°C or lower.

Results of all enzyme assays were computed as μmol of substrate consumed or product formed (or equivalent), μg chlorophyll, M, min⁻¹. However, for convenient comparison most of the results have been converted to ratio values with Fraction I values being established at unity. Ratio values that remain at or near unity for all 3 fractions indicate that the enzyme is localized with or within the chloroplast. Conversely, ratio values that increase above unity for Fractions II and III (amount of chlorophyll decreases with each successive fraction) indicate that the enzyme is not associated with the chloroplast.

In addition to the 3 enzymes concerned with nitrogen metabolism, 2 other enzymes, NADP-dependent 3-phosphoglycerate dehydrogenase and pyruvic acid kinase, were included as criteria or reference enzymes. Heber and Heber et al. (16, 17) and Smillie and Fuller (26) have shown that NADP-dependent 3-phosphoglycerate dehydrogenase is associated with the chloroplasts while pyruvic acid kinase is not (16, 17).

Aqueous Fractionation. After macerating the tissue as described by Leech (19), the centrifugal fractionation procedure described by Pierpoint (22) was used to obtain 3 fractions designated chloro-
Plants, mitochondria, and soluble.

Ten grams of leaves, cut into pieces 1 mm$^2$, and 50 ml of extracting medium (0.15 m phosphate, 0.3 m sucrose, and 0.01 m cysteine with pH adjusted to 7.5 and chilled to 2°C) was macerated for 5 minutes using a plastic spatula and an ice-cold mortar. The slurry was filtered through cheesecloth and the filtrate centrifuged for 2 minutes at 200 × g. The sediment contained cells and cell debris and was discarded. The 200 × g supernate was recenterfuged for 7 minutes at 1000 × g. The sediment formed was resuspended in 20 ml of the extracting medium and designated as chloroplasts. The 1000 × g supernatant was recenterfuged for 15 minutes at 13,000 × g. This sediment was resuspended in 20 ml of extracting medium and designated mitochondria. The 13,000 × g supernate was designated the soluble fraction. All operations were carried out to 2 to 3°C. The 3 fractions were assayed for nitrate reductase, nitrite reductase, and glutamic acid dehydrogenase activity only. The results for each enzyme are expressed as the mmoles of substrate consumed or product produced, min$^{-1}$ for each fraction as well as the percentage of the total activity (sum of activities of the 3 fractions) found in each fraction.

**Assay.** Nitrate reductase was assayed with NADH as the electron donor by a modification (14) of the original method described by Evans and Nason (12). A modification of the method of Paneque et al. (21) was used when FMNH$\_2$ was the electron donor. The other enzymes were assayed as follows: nitrite reductase (18), glutamic acid dehydrogenase (6), pyruvic acid kinase (5), and NADP-dependent 3-phosphoglyceraldehyde dehydrogenase (15). Chlorophyll was determined as described by Arnon (1).

### Results and Discussion

Data presented in table I show that nitrate reductase is associated with the chloroplasts while NR and glutamic acid dehydrogenase are not. Similar conclusions as to the localization of NR were reached with both electron donors, NADH and FMNH$\_2$. The finding that NR is not associated with the chloroplasts isolated by aqueous or nonaqueous techniques is divergent from the reports of Del Campo et al., (11) and Losada et al., (20). The data (table II) show that only a small portion (5% of total) of NR activity is associated with aqueously isolated chloroplasts. The origin of this small amount of activity is not known because aqueously (0.35 m NaCl) isolated chloroplasts are considered to be largely devoid of external membranes and stroma (19). This small amount of activity could be due to occlusion, or adsorption of a small amount of soluble NR, or to

| Table I. The Intracellular Localization of NADP-Dependent 3-Phosphoglyceraldehyde Dehydrogenase, Nitrate Reductase Assayed by NADH and FMNH$\_2$, Procedures, Pyruvic Acid Kinase, Nitrite Reductase, and Glutamic Acid Dehydrogenase of Green Leaves as Determined by Nonaqueous Isolation of Chloroplasts |
|---|---|---|---|---|---|---|---|---|
| | Pyruvic acid kinase | Nitrate reductase | NADPH 3-phosphoglyceraldehyde dehydrogenase | | | | |
| Frac* | Expt. | | | | | |
| I | 0.6 | 1.0 | 0.4 | 1.0 | 0.3 | 1.0 | 7.9 | 1.0 | 0.07 | 1.0 | 0.4 | 1.0 |
| b | 0.7 | 1.0 | 0.3 | 1.0 | 0.2 | 1.0 | 6.5 | 1.0 | 0.08 | 1.0 | 0.5 | 1.0 |
| | a | 1.2 | 2.0 | 0.8 | 2.0 | 0.8 | 2.7 | 7.6 | 1.0 | 0.07 | 1.0 | 0.9 | 2.3 |
| II*** | b | 1.5 | 2.1 | 0.7 | 2.4 | 0.7 | 3.3 | 6.5 | 1.0 | 0.10 | 1.2 | 1.6 | 3.1 |
| III | a | 1.9 | 3.2 | 1.3 | 3.3 | 1.2 | 1.9 | 7.1 | 0.9 | 0.08 | 1.1 | 1.4 | 1.0 |
| b | 2.5 | 3.6 | 0.9 | 2.9 | 0.8 | 3.9 | 5.9 | 0.9 | 0.07 | 1.0 | 0.8 | 1.5 |
| | | | | | | | | | | | | |
| I | a | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| b | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| II*** | a | 3.6 | 1.5 | 1.2 | 1.2 | 0.9 | 2.1 | 2.1 | 1.5 | 1.5 | 3.0 | 3.0 |
| b | 3.3 | 1.7 | 1.2 | 1.2 | 1.1 | 2.1 | 2.1 | 1.5 | 1.5 | 3.0 | 3.0 |
| III | a | 5.5 | 2.2 | 3.5 | 0.9 | 1.2 | 1.2 | 1.2 | 1.2 | 1.2 | 1.2 | 1.2 |
| b | 4.1 | 3.7 | 4.5 | 0.9 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |

* Chlorophyll content of Fractions I, II and III were 37.4, 13.1 and 7.8 μg ml$^{-1}$.

** Ratio values that remain at or near unity for all 3 fractions indicate that the enzyme is localized with or within the chloroplast (27). Conversely, ratio values that exceed unity for Fraction II and III indicate that the enzyme is not associated with the chloroplast.

*** Fraction II contains a relatively large amount of mitochondria.
retention of a small amount of external membranes holding NR.

Although Stocking and Ongun (29) showed by electron microscopy that chloroplasts isolated by nonaqueous techniques are apparently devoid of membranes, later improvements in technique indicated that only the outer membrane was lost (personal communication from C. R. Stocking). The retention of the inner membrane explains the much higher level of minerals and protein found in chloroplasts isolated by nonaqueous techniques than in chloroplasts isolated by aqueous (0.5 M sucrose) techniques (29). Thus it is conceivable that NR could be bound or associated with the external chloroplast membrane. Since this membrane is apparently stripped away during nonaqueous as well as aqueous extraction, and if NR is localized on the membrane neither technique would reveal the true in vivo localization of NR. It is also obvious from the nonaqueous data (table 1) that NR is not held within the bounding membrane that retains mineral elements (29), nitrite reductase, or enzymes associated with the chloroplasts like NADP-dependent 3-phosphoglyceraldehyde dehydrogenase (the reference enzyme). It is on this basis that NR is stated to be an exo-chloroplastic enzyme.

In many ways the association of NR with the plastid membrane would make it easier to visualize a coordinated nitrate-nitrite reducing system. Since nitrite reductase appears to be closely associated with the chloroplasts, a soluble NR requires a shuttling of NO₂⁻. This does not preclude exo-chloroplast localization for NR because it is obvious that NH₄ must somehow move from the chloroplast to the mitochondrion for the reductive amination step. Localization of NR on the plastid membrane might facilitate utilization of electron donors from either chloroplast or cytoplasm.

It should be emphasized that the low level of nitrite reductase associated with the aqueously isolated chloroplasts in no way conflicts with the nonaqueous data (table 1). Both Heber (16) and Leech (19) have shown that aqueously isolated chloroplasts lose much of their protein and stroma.

Additional support for the conclusion that nitrite reductase is associated with the chloroplasts (data of table 1) is provided by the association of ferredoxin with chloroplasts (27). Ferredoxin has been indicated as the natural electron donor for nitrite reductase (3, 18).

The divergence of the glutamic acid dehydrogenase patterns of activity in the 3 fractions (table I) from that of soluble (reference) enzymes, pyruvic acid kinase, and NADP-dependent 3-phosphoglyceraldehyde dehydrogenase, is attributed to the presence of mitochondria in Fraction II. Based on microscopy and related studies, Stocking (personal communication) has concluded that mitochondria are collected in Fraction II. Further evidence that glutamic acid dehydrogenase is localized with the mitochondria is provided by the aqueous isolation techniques (table II). The localization of glutamic acid dehydrogenase with mitochondrial fractions of aqueous preparations from etiolated seedlings is well established (4, 9, 10, 24). The occurrence of glutamic acid dehydrogenase activity in the soluble fraction and the trace amount noted with the chloroplast fraction is attributed to leaching from the mitochondria (10).

Initial nonaqueous experiments were made with material not subjected to the 24-hour dark treatment. Although this material gave preparations with higher levels of NR activity and a greater number of more intact chloroplasts in Fraction I than the dark treated material, there were no alterations in the distribution patterns of the enzymes tested.

Literature Cited

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