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INTRACELLULAR LOCATION OF NITRATE REDUCTASE AND NITRITE REDUCTASE

I. SPINACH AND TOBACCO LEAVES*

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SUMMARY

The intracellular location of nitrate and nitrite reductase was determined by extraction and isolation of organelles from spinach and tobacco leaves using sucrose based extraction media and isopycnic sucrose density gradient centrifugation. Nitrite reductase was located in the chloroplasts and nitrate reductase in the cytosol. With certain extraction media, nitrate reductase was found to be associated with all organelles but especially with the broken chloroplasts. This scattered and variable distribution was attributed to indiscriminate adsorption of nitrate reductase by all organelles, since bovine serum albumin eliminated this phenomenon. A low activity of nitrate reductase in crude homogenates or the supernatant fraction of tobacco leaves was due to a heat-stable, small molecular weight inhibitor. Neither soluble or insoluble polyvinylpyrollidone nor sulfhydryl reagents protected nitrate reductase from the inhibitor.

INTRODUCTION

Studies of the intracellular location of both nitrate and nitrite reductase in green tissue have yielded conflicting results. Because light enhances the *in vivo* assimilation of both nitrate and nitrite, especially the latter[1–4], an association with the chloroplast was at first thought possible. Although Del Campo *et al.*[5] isolated in aqueous medium chloroplasts which were reported to contain nitrate reductase, the amounts were negligible, and in later experiments they[6] had to add exogenous nitrate reductase to isolated chloroplasts to obtain reduction of nitrate. Ritenour *et al.*[7], using non-aqueous media and marker enzymes to fractionate and identify the components, concluded that nitrate reductase was located in the cytosol while nitrite reductase was associated with the chloroplast. On the other hand, Coupé *et al.*[8], also using non-aqueous techniques and basing location on protein and chlorophyll

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distribution, concluded that nearly 60 % of the total nitrate reductase was associated with the chloroplasts. Recently Swader and Stocking[9] have also concluded that nitrate reductase was not associated with aqueously isolated chloroplasts from *Wolffia arrhiza*. Most workers seem to agree that nitrite reductase is found in the chloroplast fraction after aqueous or non-aqueous isolation[7,9–11]. However, Grant *et al.*[12], who found low levels of nitrate and nitrite reductases to be associated with the chloroplast, suggested that *in vivo* both enzymes were located in the cytosol. Adding to this confusion Lips[13] has recently reported that peroxisomes, isolated on sucrose gradients by isopycnic centrifugation from aqueous extracts of tobacco leaves, contained almost the entire complement of nitrate and nitrite reductases.

Indirect evidence for the location of nitrite reductase within the chloroplasts and nitrate reductase with the cytosol comes from two lines of evidence. Schrader *et al.*[14] found that chloramphenicol inhibited the synthesis of nitrite reductase but not nitrate reductase. Previously Anderson and Smillie[15] had shown that appropriate levels of chloramphenicol binds with chloroplast ribosomes and thus inhibits protein synthesis at that site but is without effect on cytoplasmic ribosomes. Since nitrate reductase is NADH dependent, it has been suggested that this reduction is coupled to the oxidation of phospho-3-glyceraldehyde which moves from the chloroplasts into the cytoplasm[3]. In a similar vein, the dependence of nitrite reductase on ferredoxin as reductant suggests a location within the chloroplast[16].

The objective of this work was to reexamine the intracellular location of nitrate and nitrite reductase by extraction and isolation of organelles from spinach and tobacco leaves by means of sucrose based extraction media and isopycnic sucrose density gradient centrifugation. This technique has been successful in separating the various organelles from leaves[17].

MATERIALS AND METHODS

Plant material

Spinach (*Spinacia oleracea* L., var. Long Standing Bloomsdale) was grown in soil during a 12-h photoperiod with 24 °C day and 16 °C night temperatures. Illumination of about 1700 ft candles at leaf level was provided by fluorescent and incandescent bulbs. Tobacco (*Nicotiana tabacum* L., var. Burley) plants were grown in soil in a greenhouse with 14 h of supplemental illumination. The temperature varied from 29 °C at noon to 21 °C at midnight. Both groups of plants were watered every other day with full-strength Hoagland nutrient solution.

Preparation of cellular organelles

Leaves were deribbed, washed with distilled water and blotted dry. The laminae were finely minced with a scalpel in approximately 1 ml of grinding medium for each g of tissue. The slurry was then gently ground in a mortar for 1 to 2 min and the homogenate filtered through one layer of Miracloth (Chicopee Mills Inc., New York). All preparatory work was done at 0 to 5 °C. In most experiments grinding medium A was used, and it contained 30 g sucrose in 70 g of solution with 50 mM potassium phosphate, pH 7.5, 2 mM EDTA, 10 mM KCl, 10 mM $MgCl_2$ and 10 mM dithiothreitol. Grinding medium B was 30 g of sucrose dissolved in 70 g of solution containing 50 mM (*N*-morpholino)ethanesulfonic acid buffer (pH 7.0), 2 mM iso-

ascorbate, 0.1 % bovine serum albumin, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, sucrose in 75 g of glycylglycine solution (0.025 M, pH 8.0) with or without 5 mM 20 mM NaCl, 0.5 mM KH_2PO_4 and 10 mM dithiothreitol. Medium C was 25 g of EDTA, 5 mM cysteine and 0.1 % soluble polyvinylpyrollidone.

Sucrose density centrifugation

Discontinuous sucrose density gradients were prepared at 4 °C by adding 5 ml of 60 % sucrose followed by 2.5 ml of 56.5, 51.5, 50, 47.5, 45, 42.5, 40, 37.5, 35, 32.5, 30, 27.5 and 25 % (w/w) sucrose solutions to Beckman cellulose nitrate tubes (2.2 cm × 8.6 cm). The sucrose solutions had been prepared in 50 mM potassium phosphate buffer at pH 7.5, 2 mM EDTA, 10 mM KCl and 10 mM $MgCl_2$. 20 ml of filtered leaf homogenate were then layered on top of the gradient and centrifuged for 3 h at 25000 rev./min (44700 × *g* to 106900 × *g*) in a Spinco SW 25.2 rotor. The gradients were fractionated by puncturing the bottom of the tube and collecting 2.5-ml aliquots.

Enzyme assays

Nitrate reductase was measured in a final volume of 1.0 ml containing 10 μmoles KNO_3, 20 μmoles potassium phosphate at pH 7.5 and the enzyme; the reaction was started by adding 0.1 ml of NADH solution (1.5 mg/ml). After 30 min at 30 °C the reaction was stopped by 0.1 ml of 1 M zinc acetate, the samples were centrifuged, and aliquots of the supernatant were analyzed colorimetrically for nitrite[18,19].

Nitrite reductase was driven by methyl viologen reduced by dithionite as the electron donor and measured as the disappearance of nitrite. In a total volume of 2.5 ml the reaction medium contained 480 nmoles $NaNO_2$, 284 μmoles potassium phosphate buffer at pH 6.9, 0.76 mg methyl viologen, and enzyme. The reaction was initiated by the addition of 0.5 ml sodium dithionite solution (7.2 mg/ml of 50 mM potassium phosphate buffer at pH 6.9). After 30 min of incubation at 30 °C the reaction was stopped by agitation of the reaction mixture with a Vortex mixer to oxidize the dithionite. Chlorophyll and protein were precipitated by the addition of 0.1 ml of 1 M zinc acetate, samples were centrifuged, and the remaining nitrite was measured[18].

Catalase was determined by the rate of H_2O_2 disappearance[20]. Cytochrome *c* oxidase was measured by the rate of oxidation of dithionite-reduced cytochrome *c* at 550 nm and the activity calculated according to Schnarrenberger *et al.*[21]. Triose phosphate isomerase was measured as described by Gibbs and Turner[22]. Chlorophyll was extracted with 80 % acetone and the concentration determined spectrophotometrically[23].

RESULTS AND DISCUSSION

Organelles from spinach and tobacco leaves were partially resolved by isopycnic sucrose gradient centrifugation and located on the gradient by characteristic marker enzymes (Fig. 1). The peak of mitochondria was located by cytochrome *c* oxidase at a density of 1.19 $g \cdot cm^{-3}$. Peroxisomes were located by catalase activity at a density of 1.25 $g \cdot cm^{-3}$. Glycollate oxidase and hydroxypyruvate reductase were also at this same peroxisomal location[17], however, these data are not shown. Broken and whole chloroplasts were identified by chlorophyll peaks at a density of 1.15 $g \cdot cm^{-3}$

and 1.22 g·cm^{-3}, respectively. The location of the whole chloroplasts was conclusively confirmed to be at 1.22 g·cm^{-3} by marker enzymes[24], one of which was triose phosphate isomerase (Fig. 1).

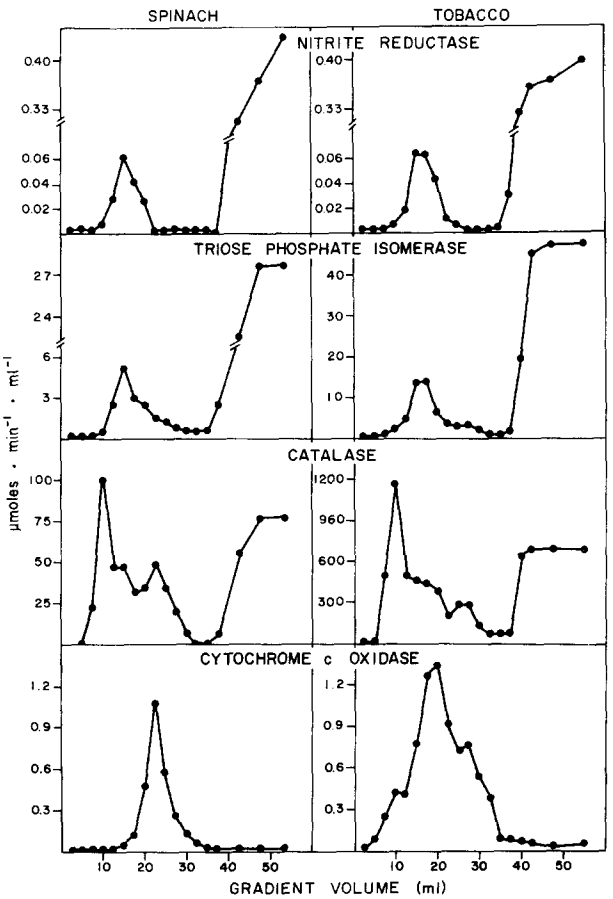

Fig. 1. Distribution of nitrite reductase on isopycnic sucrose gradients of homogenates from spinach and tobacco leaves. The leaves were ground in grinding medium A and the homogenate filtered through miracloth and placed on top of the gradient.

In preparations from both spinach and tobacco the distribution of nitrite reductase on the sucrose gradient coincided with that of the markers for the whole chloroplasts. While the percent of the total nitrite reductase associated with the whole chloroplast fraction was low, the yields were comparable with those obtained for triose phosphate isomerase and chlorophyll in this fraction. For example, from the total leaf homogenate applied to the gradient, the yields observed in the whole chloroplast fraction from tobacco leaves were 16 % of the nitrite reductase, 13 % of triose phosphate isomerase, and 13 % of the chlorophyll. There were insignificant amounts of nitrite reductase in the broken chloroplasts which contained the bulk of the chlorophyll. It is concluded that nitrite reductase is located in the stroma of whole chloroplasts, and like other stroma enzymes, is solubilized when the chloro-

plasts are ruptured by the isolation procedure. Nitrite reductase was not associated with either the peroxisomes (catalase) or mitochondria (cytochrome *c* oxidase) (Fig. 1).

Preliminary results, obtained by sucrose gradient fractionation of homogenates of leaves of spinach and wheat, showed that the bulk of the nitrate reductase was in the supernatant fraction. Only low amounts (3–5%) of the total activity were associated with the various organelles. If one considered only the distribution of nitrate reductase activity among the organelles, most of the bound activity was associated with the broken chloroplasts. Similar results were obtained with homogenates from tobacco leaves, except that there was little or no activity in the supernatant fraction. Thus it appeared, initially, that bulk of the total activity from tobacco leaves was associated with the organelles, especially the broken chloroplasts. However, the lack of activity in the supernatant was an artifact due to the presence of an inhibitor of nitrate reductase. The inhibitor did not adhere to the organelles. The inhibitor could be removed by passing the supernatant through Sephadex G-25. The addition of EDTA, cysteine and soluble polyvinylpyrrollidone, compounds known to give protection to nitrate reductase during extraction[19], to grinding medium C gave a 3-fold increase in activity in the supernatant (Table I). However, these additives did not provide sufficient protection from the inhibitor, as Sephadex G-25 filtration gave an additional 8-fold increase in nitrate reductase activity. When the inhibitor was removed from the tobacco homogenates, the bulk of the nitrate reductase was also found in the supernatant. In contrast to the results obtained with spinach and wheat, a higher proportion (about 20%) of the total nitrate reductase activity of tobacco homogenates prepared with grinding medium C was associated with the combined organelle fractions (Table I).

TABLE I

EFFECT OF GRINDING MEDIA ON EXTRACTION OF NITRATE REDUCTASE FROM TOBACCO LEAVES

Grinding media	*μmoles NO_2^- produced/h per ml homogenate* *		
	Supernatant	*Supernatant after Sephadex G-25*	*Pellet* **
25% (w/w) sucrose with 0.025 M glycylglycine, pH 8.0	0.06	1.05	0.21
Same *plus* 0.005 M EDTA, 0.005 M cysteine, and 0.1% polyvinylpyrrollidone	0.19	1.60	0.41

* All values are relative as homogenization was incomplete.

** The pellet fraction was obtained by centrifugation of the original homogenate at 27000 × g for 8 min and resuspension of it in the original grinding medium.

Typical distribution patterns of total nitrate reductase activity of filtered homogenates from tobacco leaves obtained by sucrose gradient separation are shown in Fig. 2. Activity in the supernatant fractions was determined after passage through Sephadex G-25, but this treatment was not necessary for the particle fractions. Organelle location (shown only by arrows) was determined by measurement of activity of appropriate marker enzymes. When the leaves were ground in grinding medium A there was measurable activity of nitrate reductase over the whole gradient with a

pronounced peak corresponding to the region of broken chloroplasts (density: 1.17 $g \cdot cm^{-3}$). Thus some of the nitrate reductase is in some manner associated with the broken chloroplast or protein found in this part of the gradient. It is known that the bulk of the chlorophyll and protein is located in this region of the gradient[17]. In either case the association of nitrate reductase with the broken chloroplast fraction is affected by the nature of the grinding medium. When grinding medium B, which contained bovine serum albumin, was used to prepare the homogenate, only negligible amounts of nitrate reductase were associated with the various organelles (Fig. 2).

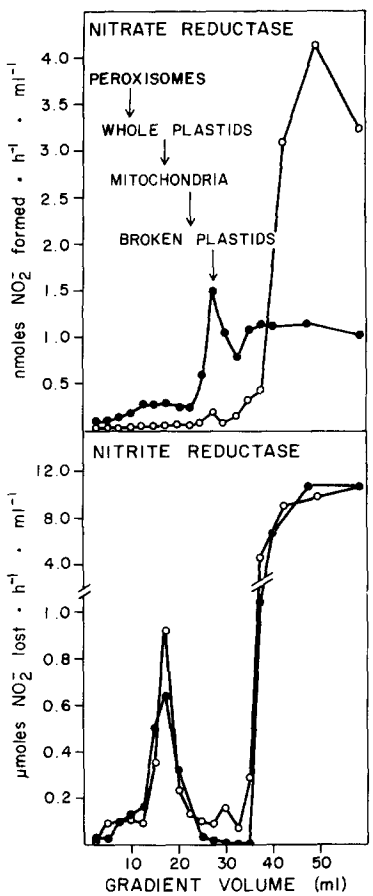

Fig. 2. Effect of different grinding media on the distribution of nitrate reductase and nitrite reductase from tobacco leaves on sucrose gradients. ●—●, grinding medium A; ○—○, grinding medium B. Both homogenates were prepared from a common composite leaf sample and centrifuged at the same time.

In other experiments it was noted that the distribution of nitrate reductase among the various fractions (organelles) varied from preparation to preparation when grinding medium A was used, although the bulk of the bound activity was always associated with the broken chloroplasts. This suggests that during homogenization part of the enzyme may become indiscriminately bound to membrane (protein)

surfaces and appear to be associated with various organelles. Such binding could account for the conflicting reports as to the intracellular location of nitrate reductase. It is not known whether nitrate reductase *in situ* is associated with the external membrane of the chloroplast. Such a cellular organization would promote nitrate reduction because of its intimate interrelationship with photosynthesis and glycolysis[3] and because it would provide directly the nitrite for nitrite reductase located within the chloroplasts[10].

It is concluded that all of the nitrate reductase activity was associated with the supernatant fraction in homogenates of tobacco and spinach leaves. In contrast to the variable distribution pattern of nitrate reductase, the amount of nitrite reductase activity in the whole chloroplast fraction was not affected by the change in the grinding medium (Fig. 2B) or from preparation to preparation. This specific distribution of nitrite reductase was consistent with its location in the intact plastid.

The nature or *in vivo* function of the inhibitor of nitrate reductase is not known. However, its discovery and removal from the supernatant is most important for the valid calculation of the percentage of total enzymatic activity associated with the various organelles. Some properties of the inhibitor of nitrate reductase were noted in a preliminary investigation. (a) The inhibitor did not bind tightly to nitrate reductase, as it could be removed by Sephadex filtration. (b) The action of the inhibitor was reversible, as the material in tobacco supernatant inhibited nitrate reductase found in the various particulate fractions and Sephadex G-25 treated supernatant, as well as nitrate reductase from spinach leaves. (c) When increments of untreated supernatant were added to active enzyme preparations, the inhibitory effects were linear over a certain range and then saturated. (d) The inhibitor was of such small molecular weight that it was removed by Sephadex G-25. (e) It was heat stable. (f) The inhibitor was not removed or bound by soluble or insoluble polyvinylpyrrollidone.

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