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The localization of nitrite reductase, glutamate synthase and malate metabolism enzymes in *Pisum arvense* L. roots

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Abstract

Centrifugation of a homogenate made from *Pisum arvense* L. roots in a sucrose density gradient enabled the separation of the plastid fraction from mitochondria and microsomes. The presence of nitrite reductase and glutamate synthase was demonstrated in the plastids. Malic enzyme activity was not linked with any organelle fraction and was found only in the cytosol. High malate dehydrogenase activity was found in the mitochondria fraction, although its activity was also determined in plastids. The results suggest that malic acid metabolism in plastids may be the source of reduced pyridine nucleotides for reactions catalysed by nitrite reductase and glutamate synthase.

Key words: roots, nitrogen metabolism enzymes, malate dehydrogenase

INTRODUCTION

Both nitrite reductase (NiR) and glutamate synthase (GOGAT) require reduced pyridine nucleotides. Studies by Kłobus (1984) on *Pisum arvense* seedlings have shown that the $NADPH_2$ used in the reduction of nitrites can come from the pentose phosphate pathway as well as from the oxidation of malic acid. The sources of reduction equivalents for plant root GOGAT are still unknown. However, studies by Matoh and Takahashi (1982), who observed a rise in the amount of ^{14}C-glutamate produced by isolated chloroplasts in the presence of exogenous malate, point to malate as an intermediate in providing electrons for glutamate synthase. Malic acid metabolism involving malate dehydrogenase (MDH) and malic enzyme (ME) can then play a key role as one of the sources of reduction equivalents for reduction processes catalysed by NiR and GOGAT in roots. Studies by Zschoche and Ting (1973) have shown that *Pisum* root malate dehydrogenase is found mainly in the mitochondria and cytosol

as well as in microsomes, whereas NiR and GOGAT have been shown in root plastids (Emes and Fowler 1979, Suzuki et al. 1981, Kłobus et al. 1983). Taking into account the fact that plastid membranes are impermeable to pyridine nucleotides (Heber 1974, Walker 1976), it must be assumed that malate dehydrogenase is found in plastids, or, that there exist specific shuttle systems. This is why we attempted to localize the NAD^+ and $NADP^+$—dependent malate dehydrogenases and malic enzyme in subcellular fractions from *Pisum arvense* roots based on the activity of NiR and GOGAT.

MATERIAL AND METHODS

All experiments were carried out on 5 day-old *Pisum arvense* L. var. "Nieznaniecka" roots. The plants were cultivated under conditions described by Kłobus et al. (1983).

Extracts from 20 g of *Pisum arvense* roots were made in 20 cm^3 phosphate buffer, pH 7.5 (linear sucrose density gradient) or Hepes-NaOH, pH 7.5 (discontinuous sucrose density gradient) containing, in addition, 0.4 M sucrose, 5 mM EDTA, 10 mM dithiothreitol (DTT), 10 mM KCl, 2 mM $MgCl_2$ and 1% bovine serum albumin. Homogenate was strained through a double layer of Miracloth and layered onto previously prepared sucrose density gradient.

The linear sucrose density gradient was composed of 18 cm^3 25-55% (w/w) sucrose overlaying on 2 cm^3 60% (w/w) sucrose cushion. The sucrose solutions contained 50 mM phosphate buffer (pH 7.5), 3 mM EDTA and 10 mM dithiothreitol. The tubes were placed in a SW 3×35 rotor and centrifuged in a Vac 602 centrifuge for 5 min at 2900×g and then for 180 min at 18 000×g.

The discontinuous sucrose density gradient was made with 4 cm^3 60%, 8 cm^3 50% and 8 cm^3 30% (w/w) sucrose in 50 mM Hepes-NaOH buffer (pH 7.5) with the addition of 3 mM EDTA and 10 mM DTT. The samples were centrifuged in a SW 3×35 rotor for 5 min at 2900×g, then for 30 min at 18 000×g. Fractions 0.87 cm^3 in volume were collected from the sucrose gradients. All of the above described steps were carried out at a temperature of 0-4 °C.

The activities of all of the studied enzymes with the exception of glucose-6-phosphate dehydrogenase were assayed after previous treatment of the fractions with Triton X-100. The activity of glucose-6-phosphate dehydrogenase was determined according to Devlin and Galloway (1968) by measuring spectrophotometrically at 340 nm the rate of NADP reduction. A measure of cytochrome c oxidase activity was the speed with which reduced cytochrome c was oxidized, followed at 550 nm (Smith 1955).

Nitrite reductase activity was assayed using methyl viologen reduced by sodium dithionite (Hucklesby et al. 1972). Malic enzyme activity was measured according to Davies et al. (1974) by determining the rate of $NADP^+$ reduction. Determination of NAD^+-malate dehydrogenase activity was based on the method of Wakiuchi et al. (1971) whereas $NADP^+$-malate dehydrogenase activity was assayed according to the method of Vidal et al. (1980) by spectrophotometrically measuring the rate of $NADH_2$ or $NADPH_2$ oxidation. The activity of glutamate synthase was analysed according to Emes and Fowler (1979) by determining the rate of $NADH_2$ oxidation. Luck's method (1965) was used to determine catalase activity and Spalding and Edwards' method (1978) to measure phosphoenolopyruvate carboxylase activity. Enzyme activity is presented in nmoles substrate or product used or produced per min in 1 cm^3 of a fraction. The sucrose concentration of each fraction was determined refractometrically. Each experiment was repeated three times.

RESULTS

Figure 1 presents the separation of subcellular fractions from *Pisum arvense* roots as obtained in a linear sucrose density gradient. Clear separation of the plastid fraction from mitochondria was obtained. This can be seen from the sharp, clearly removed from each other, activity peaks of cytochrome c oxidase (a marker of the inner mitochondrial membrane) and glucose-6-phosphate dehydrogenase (used as a plastid marker) (Fig. 1a). Only a part of the total glucose-6-phosphate activity was found in the plastid fraction. However, a clear activity peak of this enzyme in the 1.230 $g \cdot cm^{-3}$ sucrose density region, which corresponds to plastids, and the inhibition of plastid glucose-6-phosphate dehydrogenase (Table 1) by Triton X-100 indicates that in *Pisum arvense* roots there is a form of this enzyme specifically connected with plastids. Measurements of NiR and GOGAT activities run simultaneously showed that peak activities of both enzymes coincided with the maximum glucose-6-phosphate dehydrogenase activity (Fig. 1b).

Table 1

The effect of Triton X-100 (0.5%) on the activity of glucose-6-phosphate dehydrogenase in the cytosol fraction and plastids from *Pisum arvense* roots cells

Fraction	Glucose-6-phosphate dehydrogenase, nmoles $NADP^+ \times min^{-1} \times cm^{-3}$ fraction	
	−Triton X-100	+Triton X-100
Cytosol	193.0	225.0
Plastids	16.1	4.0

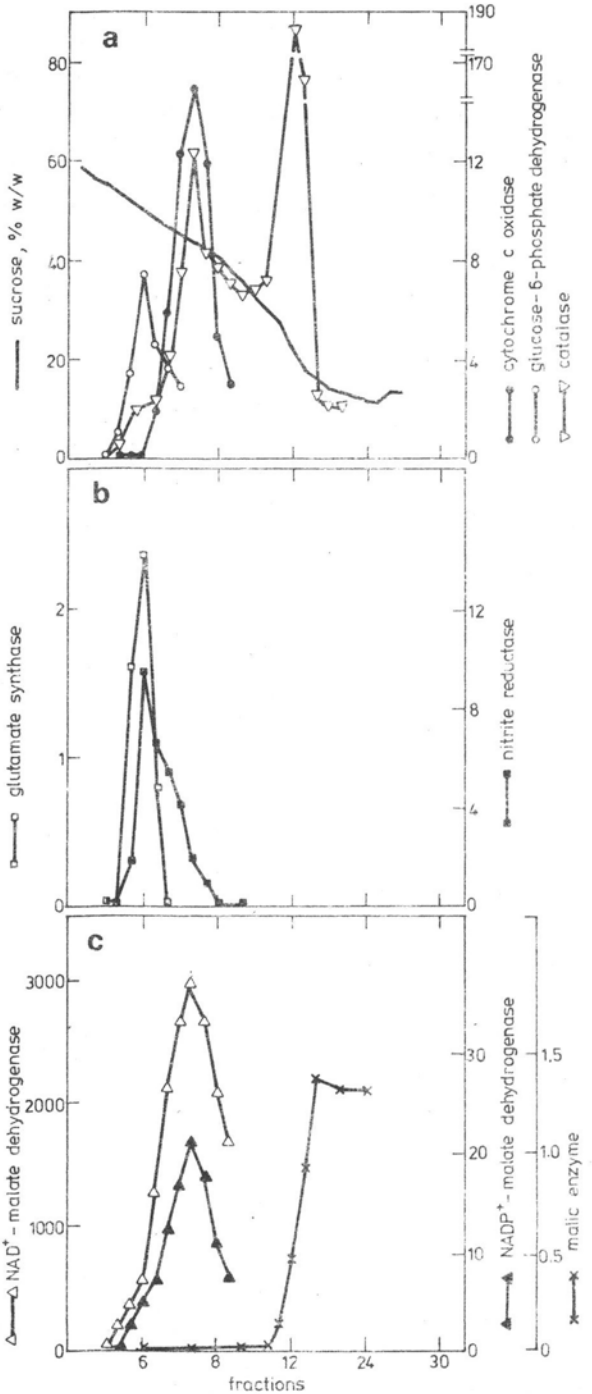

Fig. 1. The distribution of enzyme activity from homogenate of *Pisum arvense* roots in a linear sucrose density gradient. The activity of all of the enzymes is expressed in nmoles of product or substrate per minute per cm^3 fraction

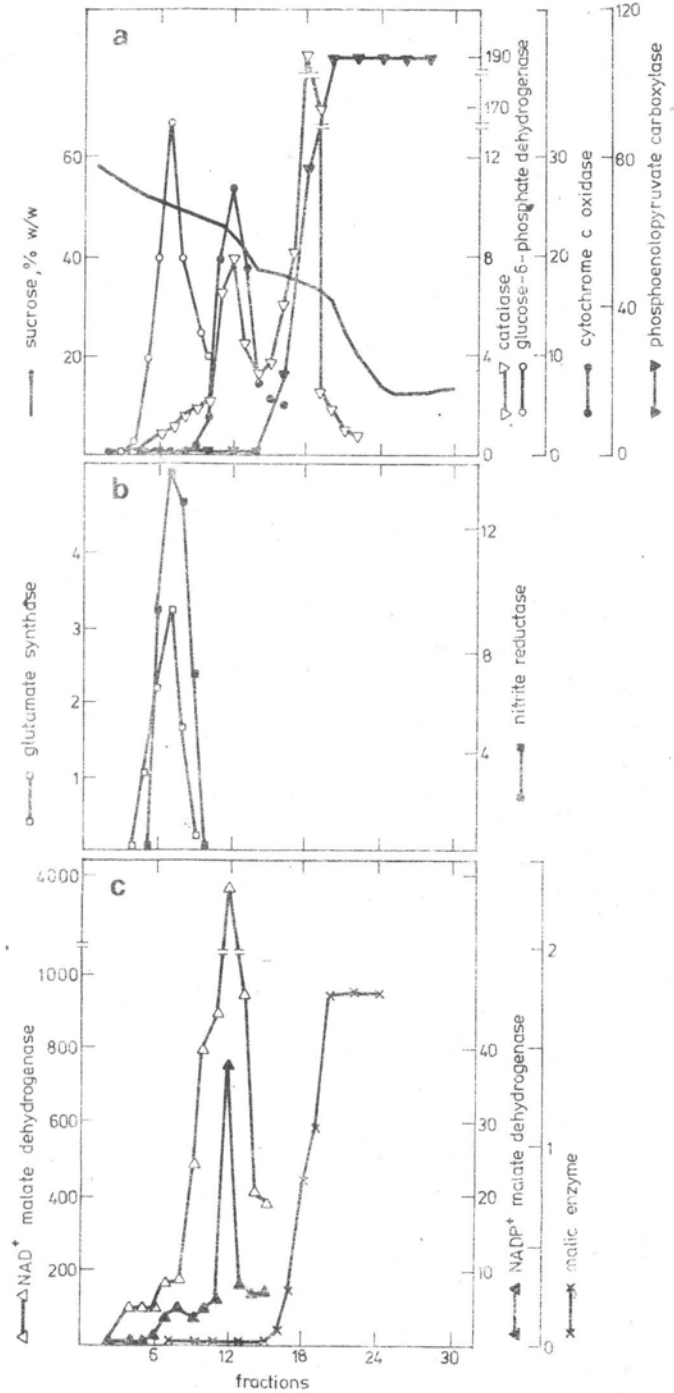

Fig. 2. The distribution of enzyme activity from homogenate of *Pisum arvense* roots in a discontinuous sucrose density gradient. Enzyme activity is expressed as in Fig. 1

Catalase activity was found in two peaks (Fig. 1a). The first coincided with the maximum activity of cytochrome c oxidase, which indicates that mitochondria and microsomes migrated together to the density of 1.197 g·cm^{-3}. Whereas, the density of the second catalase activity peak (1.072 g·cm^{-3}) suggests that the enzyme forms associates with membrane fragments most likely originating from destroyed organelles. Maximum NAD^+- or $NADP^+$-malate dehydrogenase activities coincided with the peak cytochrome c oxidase activity. Both dehydrogenases were also found in the plastid fraction, however, their activity levels were decidedly lower (Fig. 1c). Malic enzyme activity (Fig. 1c) was, however, not found in the studied organelles. The entire ME activity was found in the cytosol fraction.

Mitochondria and plastids from *Pisum arvense* roots also clearly separated in the discontinuous sucrose density gradient, which is shown by the activity peaks of marker enzymes (Fig. 2a). The maximum activity of glucose-6-phosphate dehydrogenase, corresponding to plastids, was found in the sucrose density fraction of 1.230 g·cm^{-3}. Maximum activities of NiR and GOGAT (Fig. 2b) were also found in this fraction. As in the case of the linear sucrose density gradient, maximum catalase activity coincided with the maximum cytochrome c oxidase activity, which points to joint sedimentation of mitochondria and microsomes during centrifugation. Maximum NAD^+- and $NADP^+$-MDH activities were found in the mitochondria fraction, although plastids also were not devoid of the presence of both dehydrogenases (Fig. 2c). Phosphoenolopyruvate cyrboxylase and malic enzyme were found jointly in the cytosol of cells from *Pisum arvense* roots (Fig. 2a)

The effect of $NADH_2$ and $NADPH_2$ on the level of NAD^+-MDH activity in the motochondria fraction was also tested. It was found that NAD^+-MDH showed high activity in the presence of $NADH_2$ as a cofactor and low activity in the presence of $NADPH_2$ (Table 2).

Table 2

The activity of NAD^+-malate dehydrogenase from the mitochondria fraction in the presence of different electron donors

Cofactor	NAD^+-malate dehydrogenase, nmoles $NAD/P/H_2 \times min^{-1} \times cm^{-3}$ fraction
$NADH_2$	4292.5
$NADPH_2$	19.3

DISCUSSION

By employing the technique of centrifugation in a sucrose density gradient it was possible to clearly separate plastids and mitochondria from a homogenate of *Pisum arvense* roots. Plastids sedimented at a sucrose

density of 1.230 $g \cdot cm^{-3}$, which is in agreement with the results of Schnarrenberger et al. (1972) and Suzuki et al (1981). The marker enzyme for root plastids was glucose-6-phosphate dehydrogenase, which, like triosephosphate isomerase, exhibits maximum activity in the plastid fraction (Miflin and Beevers 1974). Satoh et al. (1983) showed that the glucose-6-phosphate dehydrogenase found in plastids, in contrast with the cytosol form of this enzyme, was inactivated with Triton X-100, which has also been confirmed in our study on *Pisum arvense* root plastids. The maximum activity of glucose-6-phosphate dehydrogenase coincided with peaks of nitrite reductase and glutamate synthase activities. These results confirm our previous suppositions (Kłobus et al. 1983) based on the activity pattern of NiR and GOGAT in cell organelles obtained by differential centrifugation, that both enzymes are located in *Pisum arvense* root plastids.

The activities of malate dehydrogenase dependent on NAD^+ or $NADP^+$ were mainly connected with the mitochondria fraction, which is in agreement with the results of other authors (Ting et al. 1966). Washitani and Sato (1977) did not find NAD^+-MDH activity in the proplastids or cytosol of cells from tobacco cultures. Our studies show that the NAD^+--MDH isolated from *Pisum arvense* root mitochondria was also active in the presence of $NADPH_2$ as cofactor. It can then be supposed that the activity of the enzyme called by us $NADP^+$-MDH, in fact was the result of the catalytic activity of the NAD^+-malate dehydrogenase. It seems worthy to underscore the fact that *Pisum arvense* root cells exhibited MDH activity in plastids. As results from studies by Kubik-Dobosz (1984), the MDH enzymatic proteins from plastids and mitochondria differ in their isoelectric points, which indicates that the observed activity of MDH in the plastid fraction is connected with the presence of a specific molecular form of the studied dehydrogenase in these organelles. These reaults are analogous then to the subcellular distribution of MDH in green tissues, where in addition to mitochondria, also chloroplasts contained malate dehydrogenase (Ting and Rocha 1971, Heber and Krause 1971, Nainawatte et al. 1974, Jaquot et al. 1977, Vidal and Gadal 1981). However, we were not able to demonstrate malic enzyme activity in any of the studied *Pisum arvense* root cell organelles; as in green tissues (Slack and Hatch 1967, Mukerji and Ting 1968), this activity was found only in the cytosol.

Kubik-Dobosz (manuscript in preparation) determined enzymatically that after placing plastids isolated from *Pisum arvense* roots in a solution of glutamine, glutamate began to appear in the incubation medium. Addition of exogenous malate stimulated the transport of glutamate out of the plastids. These results can point to the participation of plastid MDH in providing reduced nucleotides for the glutamic acid synthesis

going on in these organelles. The presence of both nitrite reductase and malate dehydrogenase in root plastids supports the hypothesis put forth in a previous paper (Kłobus 1984), that malic acid oxidation in plastids coupled with the simultaneous reduction of pyridine nucleotides can be the source of reduction equivalents for the process catalyzed by NiR.

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Lokalizacja reduktazy azotynowej i syntazy glutaminianowej oraz enzymów szlaku przemian jabłczanu w korzeniach Pisum arvense L.

Streszczenie

Wirowanie homogenatu korzeni *Pisum arvense* L. w gradiencie gęstości sacharozy pozwoliło na oddzielenie frakcji plastydów od mitochondriów i mikrociał. W plastydach wykazano obecność reduktazy azotynowej i syntazy glutaminianowej. Aktywność enzymu jabłczanowego nie była związana z żadną frakcją organelli komórkowych i występowała jedynie w cytosolu. Dużą aktywność dehydrogenazy jabłczanowej stwierdzono w frakcji mitochondrialnej, jakkolwiek także w plastydach oznaczono aktywność tego enzymu. Otrzymane wyniki sugerują, że przemiany kwasu jabłkowego w plastydach mogą stanowić źródło zredukowanych nukleotydów pirydynowych dla reakcji katalizowanych przez reduktazę azotynową i syntazę glutaminianową.