Phosphate Starvation and a Glycolytic Bypass Catalyzed by Phosphoenolpyruvate Carboxylase in Suspension-Cultured *Catharanthus roseus* Cells

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Pathways involved in the conversion of phosphoenolpyruvate (PEP) to pyruvate, the final step in glycolysis, were investigated after transfer of stationary-phase cells from suspension cultures of *Catharanthus roseus* to fresh complete or phosphate (Pi)-deficient Linsmaier and Skoog medium. In addition to pyruvate kinase (PK), enzymes that can function in an alternative pathway, namely, PEP carboxylase (PEPC), NAD-malate dehydrogenase and NAD-malic enzyme, were present in significant amounts in *C. roseus* cells. The activity of PEPC in Pi-starved cells was more than twice that in cells in the complete medium (Pi-fed cells), while that of PK in Pi-starved cells was lower than that in Pi-fed cells. No significant differences were observed in the levels of NAD-malate dehydrogenase and NAD-malic enzyme between these two types of cell. At cytosolic pH, the K_m value of PEP (45 μM) for PEPC was lower than that for PK (100 μM). The activity of PEPC was inhibited by malate, citrate, aspartate and ATP. The activity of PK was also inhibited by ATP, but to a lesser extent. The cellular levels of PEP, adenylates and malate, which are substrates and effectors of PK and PEPC, in Pi-fed and Pi-starved cells suggest that the contribution of PEPC to the metabolism of PEP increased in Pi-starved cells *in vivo*. Evidence for operation of a bypass from malate to pyruvate *in vivo* was supported by the rapid release of $^{14}CO_2$ from organic compounds derived from fixed $NaH^{14}CO_3$ and from [4-^{14}C]malate.

Introduction

The metabolic adaptation of respiratory pathways to phosphate (Pi) deficiency have recently been investigated in cultured cells of *Brassica nigra* and *Catharanthus roseus* (Duff *et al.,* 1989; Li and Ashihara, 1990; Theodorou *et al.,* 1992; Theodorou and Plaxton, 1993; Nagano and Ashihara, 1993). Duff *et al.* (1989) indicated that adenylate-independent, alternative enzymes of glycolysis, inducible by Pi starvation, such as PPi: fructose-6-phosphate phosphotransferase (PFP, EC 2.7.1.90) and phosphoenolpyruvate phosphatase (PEPase, EC 3.1.3.60), bypass nucleotide phosphate-dependent glycolytic reactions in Pi-starved cells. Although little change in the maximum activity of PFP is observed during Pi starvation of *C. roseus* cells (Li and Ashihara, 1990; Nagano and Ashihara, 1993), PFP bypass the reaction catalyzed by phosphofructokinase (PFK, EC 2.7.1.11) as a result of the fine control of the activities of PFK and PFP (Nagano and Ashihara, 1993). Thus, mechanisms of metabolic adaptation to Pi deficiency seem to differ among plant species (Duff *et al.,* 1989; Li and Ashihara, 1990; Theodorou and Plaxton, 1993; Nagano and Ashihara, 1993).

Compared with information available about *B. nigra* cells (Theodorou and Plaxton, 1993), little is known about the conversion of PEP to pyruvate in *C. roseus* cells, although it has been suggested that this step is also important for regulation of glycolysis (Copeland and Turner, 1987; Kubota and Ashihara, 1990). We found previously that the activity of phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) is higher in an extract of Pi-starved *Catharanthus roseus* cells than in an ex-

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tract of cells supplied with 1.25 mM Pi (Nagano and Ashihara, 1993). An increase in the level of PEPC during Pi deficiency has also been found in suspension-cultured *B. nigra* cells (Duff *et al.*, 1989), rape roots (Huffland *et al.*, 1992) and a green alga (Schuller *et al.*, 1990a). In the present study, we examined whether PEPC can function as an alternative enzyme in glycolysis and can bypass the reaction catalyzed by pyruvate kinase (PK, EC 2.7.1.40) in Pi-starved *C. roseus* cells. From the results obtained, we discuss the physiological significance of the PEPC bypass.

Materials and Methods

Cell cultures

Suspension cultures of cells of *Catharanthus roseus* (L.) G. Don were maintained heterotrophically and subcultured at 7 day intervals in complete LS medium (Linsmaier and Skoog, 1968) that contained 2.2 μM 2,4-dichlorophenoxyacetic acid and 3% sucrose (Ashihara *et al.*, 1988a). For preparation of Pi-deficient cultures, portion (7 ml) of the suspension of 7-day-old cultures were transferred to 43 ml aliquots of the fresh LS culture medium excluding Pi in 300 ml Erlenmeyer flasks. The flasks were incubated on a horizontal rotary shaker (90 strokes min^{-1}, 80 mm amplitude) at 27 °C in the dark.

Preparation of enzymes for determinations of maximum activity

C. roseus cells (1.5–2.0 g fresh weight) were collected and washed with distilled water by vacuum filtration through a layer of filter-paper on a Buchner funnel. Washed cells were homogenized with 5–10 vol. of extraction medium as follows. The medium for assays of PEPC contained 50 mM imidazole-HCl (pH 7.2), 0.1% (v/v) 2-mercaptoethanol, 2 mM $MgCl_2$ and EDTA. The media for assays of NAD-malate dehydrogenase (NAD-MDH, EC 1.1.1.37) and NAD-malic enzyme (NAD-ME, EC 1.1.1.39) were the same as that for assays of PEPC but they contained 2 mM $MnCl_2$ as an additional inorganic salt and 5 mM dithiothreitol (DTT) instead of 0.1% 2-mercaptoethanol. Furthermore, 0.5% (w/v) Triton X-100 was added to the extraction medium for assays of NAD-ME. The medium for extraction of PK consisted of 50 mM potassium phosphate buffer (pH 7.6), 2.5 mM $MgCl_2$, 2 mM EDTA, 2 mM DTT, 50 mM NaF, 0.1 mM PEP, 1 mM phenylmethylsulfonylfluoride (PMSF), 20% glycerol and 2.5% (w/v) polyvinylpolypyrolydone (PVP). Each homogenate was centrifuged at 30,000×*g* for 20 min at 2 °C. A portion of the supernatant (2.5 ml) was desalted on a column of Sephadex G-25 (PD-10 column; bed volume, 9.0 ml; Pharmacia, Uppsala, Sweden) that had been equilibrated with the appropriate extraction buffer, except in the case of PK, when the column was equilibrated with 50 mM potassium phosphate buffer (pH 7.1). The fraction containing eluted protein (3.5 ml) was used immediately for assays of enzymatic activities.

Purification of PEPC

PEPC was partially purified from *C. roseus* cells by the fractionation with ammonium sulphate and chromatography on Q-Sepharose as described elsewhere (Nagano *et al.*, 1994). The enzyme was purified about 8-fold, as compared to the level in the supernatant of the homogenate with 40% recovery of total activity. No PEP phosphatase activity, which may interfere with accurate kinetic studies of PEPC, was present in the preparation of PEPC. The specific activity of the final preparation was approximately 95 nkat mg^{-1} protein.

Purification of cytosolic PK

The cytosolic form of PK (PKc) was partially purified by the method of Plaxton (1988) with modifications. Freshly harvested cells (25 g fresh weight) were homogenized with 2 volumes of extraction buffer, which consisted of 50 mM potassium phosphate buffer (pH 7.6), 2 mM EDTA, 2.5 mM $MgCl_2$, 2 mM DTT, 50 mM NaF, 0.1 mM PEP, 1 mM PMSF, 20% (v/v) glycerol and 2.5% (w/v) PVP. The homogenate was centrifuged at 17,000×*g* for 20 min, and the resultant supernatant was filtered through Miracloth (Calbiochem, La Jolla, CA, U.S.A.). To remove plastid-associated form PK, the supernatant obtained was divided equally between two 500 ml flasks and heated at 60 °C for 5 min. The extract was then cooled on crushed ice to 4 °C and centrifuged as above. Polyethylene glycol (PEG; molecular mass 8000; Sigma Chem. Co., St. Louis, MO, U.S.A.) was added to

the resultant supernatant fraction to bring the final concentration of PEG to 3% (w/v) and the mixture was stirred for 20 min. After centrifugation as above, the pellets were discarded, and the supernatant was adjusted to 8.5% (w/v) with PEG, and stirred for 60 min, and then it was centrifuged as described above. The surface of pellets that contained PKc was briefly washed with the extraction buffer to remove PEG, and then pellets were dissolved in 4 ml of elution buffer, which consisted of 10 mM potassium phosphate buffer (pH 7.1), 5 mM $MgCl_2$, 1 mM EDTA, 2 mM DTT and 20% (v/v) glycerol. The enzyme fraction was centrifuged at 27,000×*g* for 20 min, and the resultant supernatant was applied to a column of Q-Sepharose (15 mm i.d. × 200 mm) which had been equilibrated with the elution buffer. The column was washed with the same buffer, at 2.5 ml min^{-1}, until the absorbance at 280 nm (A_{280}) decreased to 0.05. PK was eluted with a 300 ml linear gradient of KCl (0–700 mM) and fractions (3.5 ml each) were collected. The active fraction which was eluted by approximately 200 mM KCl, was used as the preparation of enzyme. The enzyme was purified about 65-fold and the overall recovery was about 10%. The specific activity of the final preparation was approximately 100 nkat mg^{-1} protein.

Assays of enzymatic activities

The assays of activities of individual enzymes were based on the published methods, as described in the references cited below. Concentrations of reagents and pH values for the various assays were optimized for assays of the activities of enzymes from *C. roseus*. The total volume of each reaction mixture was 1 ml. Reactions were performed at 30 °C.

Activities of PEPC, PK, NAD-MDH and NAD-ME were determined spectrophotometrically. The composition of reaction mixtures was as follows, with references cited in parentheses:

PEPC (Zipfel *et al.*, 1990): 25 mM Tris(hydroxymethyl)aminomethane (Tris)-HCl buffer (pH 8.0), 2 mM PEP, 1 mM $KHCO_3$, 5 mM $MgCl_2$, 2 mM DTT, 0.2 mM NADH and 33 nkat MDH.

PK (Plaxton, 1988): 50 mM Tris-HCl buffer (pH 7.5), 1 mM PEP, 2 mM ADP, 75 mM KCl, 10 mM $MgCl_2$, 2 mM DTT, 0.2 mM NADH, 33 nkat lactate dehydrogenase.

NAD–MDH (Hatch *et al.*, 1982): 50 mM (N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]) (Hepes)-NaOH buffer (pH 7.2), 0.2 mM NADH, 1 mM oxaloacetate.

NAD-ME (Fathi and Schnarrenberger, 1990): 50 mM Hepes-NaOH buffer (pH 7.2), 5 mM malate, 2 mM NAD^+, 5 mM DTT, 0.1 mM sodium EDTA, 0.05 mM CoA and 2 mM $MnCl_2$.

Native electrophoresis of PEPC

Non-denaturing gels containing 7.5% polyacrylamide (Ishida and Ashihara, 1993) were used. Gels were stained for PEPC activity by the method of Queiroz-Claret and Queiroz (1992).

Quantitation of metabolites

Metabolites were extracted with 6% perchloric acid (PCA) and quantitated enzymatically (Kubota and Ashihara, 1990, 1993). Experiments to assess recovery were performed in parallel with assays, as described elsewhere (Kubota and Ashihara, 1993). Recoveries were above 85% in all cases.

Metabolism of $NaH^{14}CO_3$

Cells (100 mg fresh weight) were suspended in 2 ml of 25 mM Hepes-NaOH buffer (pH 7.2) that contained 0.5 mM $NaH^{14}CO_3$ (specific activity 74 kBq μmol^{-1}, ICN Biomedicals, Inc., Irvine, CA, U.S.A.) in the main compartment of a 30 ml Erlenmeyer flask with a centre well. Filter-paper wetted with 0.1 ml of 20% KOH was inserted in the centre well to trap $^{14}CO_2$. After incubation at 27 °C for 30 min, cells were collected on a layer on Miracloth by filtration under vacuum and washed briefly with distilled water. Washed cells were left in a flask so that radioactivity could be "chased". Harvested cells were homogenized with 80% ethanol and treated with 6% PCA to remove any remaining $^{14}CO_2$. The radioactivity of the ethanol-soluble and ethanol-insoluble fractions was measured separately. The amount of radioactivity in each fraction was summed and the total was regarded as the radioactivity that had been fixed by the cells.

Metabolism of [4-^{14}C]malate

[4-^{14}C]Malate was synthesized enzymatically from commercially purchased $NaH^{14}CO_3$ (specific activity, 2.1 GBq mmol^{-1}) by the method described by Amino (1992). Methods for the administration of radiochemicals, extraction of metabolites and analysis of labelled compounds were the same as those described in a previous paper (Nagano and Ashihara, 1993) except that [U-^{14}C]-glutamine was replaced by [4-^{14}C]malate.

Results and Discussion

In the conventional glycolic pathway, conversion of PEP to pyruvate is catalyzed by PK. In addition, this conversion can also be performed by sequential reactions that are catalyzed by PEPC, NAD-dependent malate dehydrogenase (NAD-MDH) and NAD-dependent malic enzyme (ME), respectively, in plant cells (Wiskich and Dry, 1985; Bryce and ap Rees, 1985). Oxaloacetate, a product of the reaction catalyzed by PEPC, is converted to malate by cytosolic NAD-MDH. In cells of *C. roseus,* some malate, after transport into mitochondria, was metabolized directly by the TCA cycle, but the rest was converted to pyruvate by mitochondrial NAD-ME, with the release of CO_2 (Fig. 1). In the present study, we examined the coarse and fine control of the key enzymes in these two pathways. From our results, we examined whether the alternative pathway, catalyzed by PEPC, is the predominant pathway in Pi-starved cells.

Coarse control of the activities of various enzymes

Fig. 2 shows the maximum catalytic activities of PK, PEPC, MDH and ME in Pi-fed (+Pi) and Pi-starved (−Pi) cells. The level of the extractable PEPC activity increased 2.3- to 2.6-fold when cells that had been cultured in complete LS medium for 7 days were transferred to the fresh Pi-deficient LS medium. Since the increase in the activity of PEPC in Pi-starved cells was suppressed completely by cycloheximide (25 μg ml^{-1}), it seems that PEPC was synthesized *de novo* during the 24 h after transfer of the cells to the Pi-deficient medium. By contrast, the level of PK activity was higher in Pi-fed cells than in Pi-starved cells. Similar results were also obtained in an earlier study (Li and Ashihara, 1990). However, the activity of PK reported in our previous paper was significantly lower than that obtained in this study. The difference is mainly due to a difference between the extraction media used. In the present study, we found that Plaxton's extraction medium (Plaxton, 1988), which contains 50 mM phosphate buffer and 20% glycerol, is very suitable for stabilization of the activity of PK. The activities of MDH and ME in the Pi-starved cells were slightly lower than or similar to those in the Pi-fed cells. Our previous analysis indicated that the flux of glycolysis in

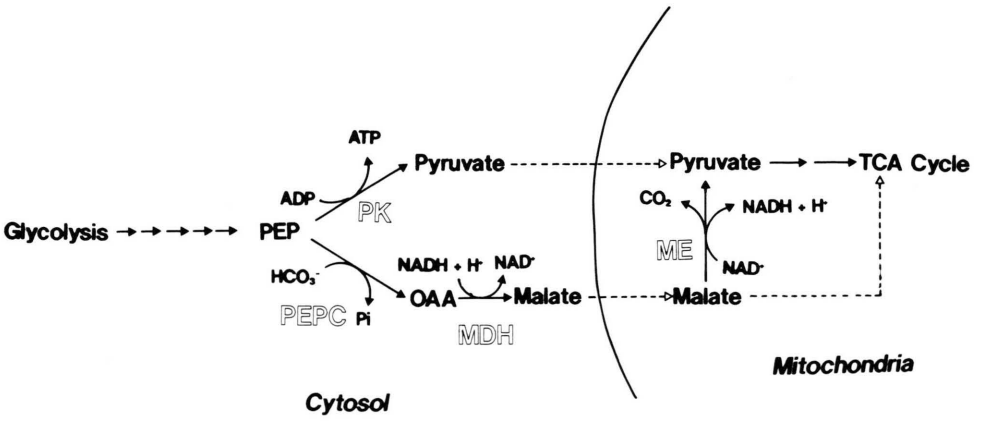

Fig. 1. Reactions catalyzed by pyruvate kinase and phosphoenolpyruvate carboxylase and possible pathways for conversion of phosphoenolpyruvate to pyruvate in *Catharanthus roseus* cells. MDH, NAD-malate dehydrogenase; ME, NAD-malic enzyme; PEPC, phosphoenolpyruvate carboxylase; PK, pyruvate kinase; PEP, phosphoenolpyruvate; OAA, oxaloacetate.

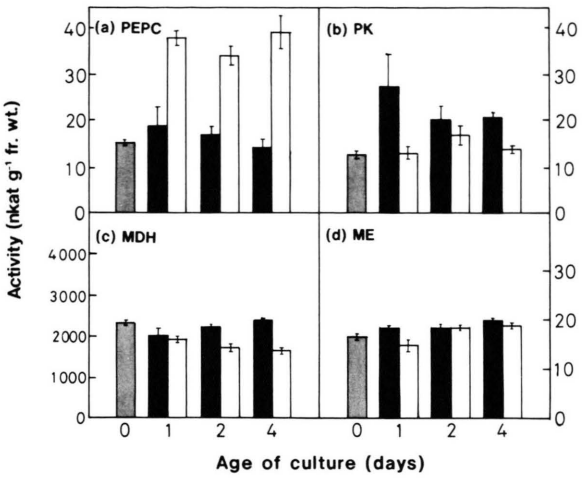

Fig. 2. Maximum activities of phosphoenolpyruvate carboxylase (a), pyruvate kinase (b), NAD-malate dehydrogenase (c) and NAD-malic enzyme (d) in suspension-cultured *Catharanthus roseus* cells grown in complete (heavily shaded columns) or in Pi-depleted (open columns) LS medium. Values are means ± s.d. of results from four separate experiments. Initial enzymatic activities in cells in the inoculum are shown by lightly shaded columns.

C. roseus cells is equivalent to less than 1.7 nmol PEP consumed per sec per fr. wt. (Kubota and Ashihara, 1990). Thus, maximum catalytic activities of PEPC and PK seemed to be at least 6 times higher than that expected from the flux of glycolysis *in vivo*. It is also noteworthy that the maximum activity of ME was almost the same as that of PK, but the activity of MDH was 50–100 times higher than those of the other three enzymes, PEPC, PK and NAD-ME. Therefore, it is difficult to conclude that the increased level of PEPC activity (protein), *i.e.*, coarse control of PEPC, leads directly to the increase in the contribution of the alternative pathway to glycolysis in Pi-starved cells. These results simply suggest that both PK and PEPC are functional as the final enzymes of glycolysis in Pi-starved cells. The activities of both enzymes *in vivo* are controlled by the concentrations of the substrates and effectors for these enzymes, which may change greatly in Pi-fed and Pi-starved cells. We can expect that the fine control of the activities of PK and PEPC is important in an analysis of the role of the bypass in Pi-starved cells.

Fine control of the activities of PK and PEPC

In order to examine the fine control of the activities of PK and PEPC, cytosolic PK and PEPC were partially purified for kinetic studies. Although PEPC seemed to be synthesized *de novo* in Pi-starved cells, activities of PEPC from both Pi-fed and Pi-starved cells were eluted as a single peak from a column of Q-Sepharose by approximately 0.3 M KCl (Fig. 3). The PEPC in each fraction was then analyzed by PAGE on native gels. A clear stained band of activity that corresponded to PEPC was detected at the same position in each case (insets in Fig. 3). Furthermore, the kinetic properties of PEPC from Pi-starved cells were very similar to those from Pi-fed cells.

It has been argued that the affinity for PEP of PEPC is at least one order of magnitude lower than that of PK in higher plants (Copeland and Turner, 1987; Davies, 1979; O'Leary, 1982). However, the K_m of PEP for PEPC from *C. roseus* was smaller than that for PKc at physiological pH (Table I). Therefore, in *C. roseus* cells, the parti-

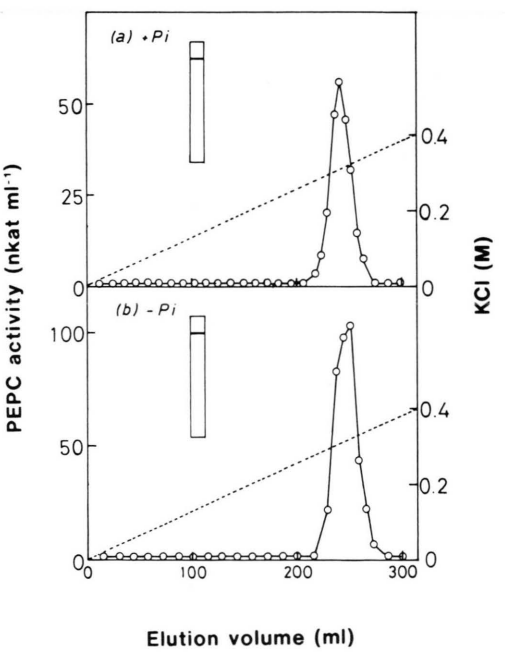

Fig. 3. Profiles of elution of phosphoenolpyruvate carboxylase (PEPC) after column chromatography on Q-Sepharose. Insets show the distribution of activity of PEPC on 7.5% polyacrylamide gels. (a) PEPC from 1-day-old Pi-fed cells, (b) PEPC from 1-day-old Pi-starved cells. (Age refers to days after transfer to fresh medium in this and other legends.)

Table I. Comparison of K_m values of substrates for PKc and PEPC that had been partially purified from *Catharanthus roseus* cells. The enzymatic activities were measured at physiological pH (7.5). K_m values were determined from double-reciprocal plots. Kinetic data were subjected to linear regression analysis and the correlation coefficient in each case was greater than 0.98.

Substrate	PKc [μM]	PEPC [μM]
PEP	100	45
ADP	90	–

tioning of PEP between PEPC and PKc is likely to favor PEPC if inhibitors and/or activators of these two enzymes are absent. A similar low K_m (50 μM) for PEPC was also reported recently in the case of PEPC from soybean nodule and it was determined in the presence of 15% glycerol at pH 7 (Schuller *et al.*, 1990b). The K_m of ADP for PKc from *C. roseus* cells was 90 μM. This result also suggests that PEPC has an advantage over PK when the availability of ADP is limited.

The effects of various effectors at different concentrations of on the activities of PEPC and PKc, purified from Pi-fed cells, were examined (Fig. 4). Malate inhibited the activity of PEPC but did not greatly influence the activity of PK within the range of concentrations shown in Fig. 4. Citrate and aspartate also inhibited PEPC but to a lesser extent. ATP inhibited both enzymatic activities. The concentration of ATP for 50% inhibitions were 1.9 mM (PEPC) and 3.1 mM (PKc), respectively. ADP and AMP were weak inhibitors of PEPC.

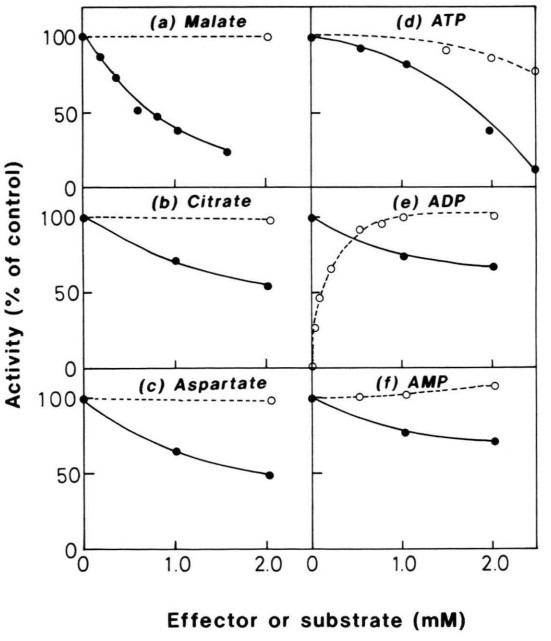

Fig. 4. Effects of malate (a), citrate (b), aspartate (c), ATP (d). ADP (e) and AMP (f) on the activities of phosphoenolpyruvate carboxylase (—●—) and pyruvate kinase (– –○– –) from 1-day-old Pi-fed *Catharanthus roseus* cells.

Levels of metabolites in Pi-fed and Pi-starved cells

In order to estimate the activity of PEPC and PKc *in vivo*, cellular levels of the substrates and effectors of these enzymes were measured. Until two days after the transfer of cells to complete and Pi-depleted medium, the level of PEP in the Pi-fed and Pi-starved cells was similar. However, after 4 days, the level in the Pi-fed cells become lower than in the Pi-starved cells. These results are consistent with the observation that the level of PEP was low when the rate of respiration was high (Kubota and Ashihara, 1993).

Estimated cytoplasmic concentrations of PEP varied from 90 to 210 μM, if we assume that the cytoplasm represents 10% of the total volume of the cell and that PEP is present only in the cytoplasm. These values were similar to or slightly higher than the K_m values of PEP for PEPC and PKc.

Levels of malate and ATP, potent inhibitors of PEPC, were significantly reduced in Pi-starved cells (Table II and Li and Ashihara, 1990). If malate and ATP are exclusively located in the cytosol, the activity of PEPC would seem to be

Table II. Levels of PEP, malate and pyruvate in suspension-cultured *Catharanthus roseus* cells grown in complete (+Pi) and Pi-deficient (– Pi) medium. The levels are expressed as nmol g fr. wt.$^{-1}$.

Metabolite	Age of culture [days]			
	0		1	2	4
PEP	12 ± 1	+Pi	20 ± 6	21 ± 5	9 ± 1
		– Pi	20 ± 4	18 ± 4	18 ± 2
Malate	1391 ± 17	+Pi	513 ± 22	2215 ± 128	1265 ± 79
		– Pi	212 ± 34	176 ± 19	119 ± 16
Pyruvate	65 ± 21	+Pi	55 ± 7	80 ± 16	124 ± 4
			165 ± 12	75 ± 7	69 ± 10

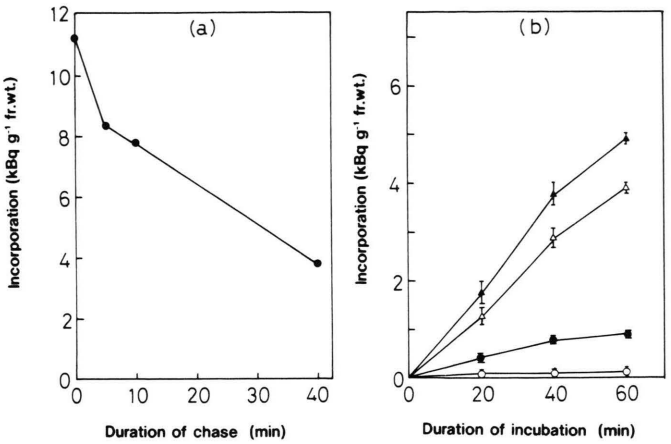

Fig. 5. (a) Loss of ^{14}C from components fixed by the 1-day-old Pi-starved *Catharanthus roseus* cells after administration of $NaH^{14}CO_3$ for 30 min. (b) Metabolism of [4-^{14}C]malate by 1-day-old Pi-starved *Catharanthus roseus* cells. Total uptake of ^{14}C by the cells (▲); incorporation into CO_2 (△), perchloric acid-soluble fraction (●) and perchloric acid-insoluble fraction (○).

inhibited completely in Pi-fed cells. However, significant amounts of malate may be present in vacuoles. Nevertheless, it is obvious that inhibition of PEPC by these effectors is relieved to a greater or lesser extent in Pi-starved cells.

Metabolism of $NaH^{14}CO_3$ and [4-^{14}C]malate in Pi-starved cells

The enzymatic studies mentioned above only provided and indication that the bypass by PEPC, NAD-MDH and NAD-ME could occur in Pi-starved *C. roseus* cells. Bryce and ap Rees (1985) concluded that the bypass operates in roots of *Pisum* and *Plantago* from pulse-chase experiments with $^{14}CO_2$. Similar experiments were performed using $NaH^{14}CO_3$ and Pi-starved *C. roseus* cells (Fig. 5a). Consistent with the results of Bryce and ap Rees (1985), there was appreciable fixation of ^{14}C at the end of the pulse, and subsequently substantial loss of ^{14}C from the cells during the chase. This rapid decarboxylation of the products of dark fixation of HCO_3^- in Pi-starved cells suggests that PEP from glycolysis was converted to pyruvate with the release of $^{14}CO_2$. We also observed that 5 mM 2-*n*-butylmalonate, an inhibitor of the transport of malate into mitochondria (Wiskish, 1975) caused 40% inhibition of the fixation of HCO_3^-. This result is compatible with the results from pea roots, in which 36% inhibition of fixation was observed. Bryce and ap Rees (1985) suggested that 2-*n*-butylmalonate inhibits the transport of malate into mitochondria and that the resultant accumulation of malate in the cytosol would be expected to inhibit PEPC.

To examine the metabolism of malate in further detail, the fate of radioactivity from [4-^{14}C]malate in Pi-starved cells was investigated (Fig. 5b). Rapid release of large amounts of $^{14}CO_2$ from [4-^{14}C]malate was observed. In addition, approximately 20% of the radioactivity that was taken up was distributed in the soluble fraction and the rest was incorporated into the PCA-insoluble fraction (possibly as protein). After a 60 min incubation, 79.5%, 7.8%, 10.3% and 2.5% of the total radioactivity taken up by the cells was recovered as CO_2, organic acids, free amino acids and protein, respectively. These results indicate that exogenously supplied malate was almost exclusively metabolized and most of it seemed to be converted to pyruvate with the release of CO_2. These results support the hypothesis that the bypass for conversion of PEP to pyruvate is functional in *C. roseus* cells. Similar results have been obtained in suspension-cultured photoautotrophic cells of *Chenopodium rubrum* (Amino, 1992). In darkness, 96% of radioactivity from [4-^{14}C]malate that had been taken up by *Chenopodium* cells was metabolized, and more than 66% of the radioactivity was released as $^{14}CO_2$.

The rapid release of $^{14}CO_2$ from recently fixed HCO_3^- and from [4-^{14}C]malate was also observed in Pi-fed cells (data not shown). Thus, the bypass is probably functional as a relief pathway in both

Pi-fed and Pi-starved cells, although the extent of its contribution may be controlled by several factors, as mentioned above.

Role of the PEPC bypass in Pi-starved cells

Results of the present study suggest that the activity of PEPC relative to that of PK increased in Pi-starved *C. roseus* cells *in vivo* since the maximum catalytic activity of PEPC increased and the concentrations of negative effectors of PEPC decreased in Pi-starved cells. When the cellular concentration of Pi is extremely low, the production of PEPC protein is increased, as is that of other Pi-recycling enzymes, such as several phosphatases and RNases (Ueki and Sato, 1971; Duff *et al.*, 1991; Nurnberger *et al.*, 1990). Thus, one of the functions of PEPC in Pi-starved cells seems to be the production of Pi from PEP. A second function of PEPC is the conversion of PEP to pyruvate without adenine nucleotides. The levels of adenine nucleotides are greatly reduced in Pi-starved cells (Ukaji and Ashihara, 1986; 1987; Ashihara *et al.*, 1988b; Kubota *et al.*, 1989). Thus, PEPC appears to be a member of a group of glycolytic bypass enzymes whose activities are stimulated by Pi starvation and which include PFP and non-phosphorylating NADP-glyceraldehyde 3-phosphate dehydrogenase (Duff *et al.*, 1989; Theodorou and Plaxton, 1993). The anaplerotic role of PEPC is also considerable, but it does seem to be limited since most of the carbon fixed by PEPC in Pi-starved cells is immediately released as CO_2. However, compared with the reaction catalyzed by PK, the reaction catalyzed by PEPC is energetically wasteful because ATP is not generated during the conversion of PEP to pyruvate. Therefore, in normally growing cells, the activity of PEPC seems to be suppressed by effectors, such as ATP and malate, and PK functions predominantly as the enzyme that catalyzes the final step in glycolysis.

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