Plant Physiol. (1989) 91, 581-586
0032-0889/89/91/0581/06/$01.00/0

Received for publication February 22, 1989
and in revised form May 19, 1989

Pyrroline-5-Carboxylate Reductase Is in Pea (*Pisum sativum* L.) Leaf Chloroplasts[1]

P. John Rayapati*, Cecil R. Stewart, and Ethan Hack
Department of Botany, Iowa State University, Ames, Iowa 50011

ABSTRACT

Proline accumulation is a well-known response to water deficits in leaves. The primary cause of accumulation is proline synthesis. Δ^1-Pyrroline-5-carboxylate reductase (PCR) catalyzes the final reaction of proline synthesis. To determine the subcellular location of PCR, protoplasts were made from leaves of *Pisum sativum* L., lysed, and fractionated by differential and Percoll density gradient centrifugation. PCR activity comigrated on the gradient with the activity of the chloroplast stromal marker NADPH-dependent triose phosphate dehydrogenase. We conclude that PCR is located in chloroplasts, and therefore that chloroplasts can synthesize proline. PCR activities from chloroplasts and etiolated shoots were compared. PCR activity from both extracts is stimulated at least twofold by 100 millimolar KCl or 10 millimolar $MgCl_2$. The pH profiles of PCR activity from both extracts reveal two separate optima at pH 6.5 and 7.5. Native isoelectric focusing gels of samples from etiolated tissue reveal a single band of PCR activity with a pI of 7.8.

Induction of proline accumulation by water deficit is a well-known, but little understood, phenomenon in plant stress physiology. Proline accumulation is caused primarily by increased synthesis from glutamic acid; the biosynthetic pathway is postulated to be analogous to that which converts glutamic acid to proline in *Escherichia coli* (21). The first two enzymes of this pathway have yet to be defined in plant extracts. The third and final enzyme PCR,[2] has been measured from several plant sources (12, 13), and Krueger *et al.* (10) have purified it to apparent homogeneity from wilted barley leaves.

The subcellular location of proline biosynthesis has not been clearly established. The involvement of light in this process has been indicated. Noguchi *et al.* (17) have shown that inhibition of PSII inhibits proline synthesis in tobacco leaf discs. Rajaopal *et al.* (20) have shown that the pattern of proline accumulation in drought-stressed wheat parallels the pattern of diurnal change in light intensity. PCR activity has been reported in chloroplast-enriched fractions from tobacco leaves (16), but Kohl *et al.* (9) found that in soybean root nodules this enzyme is in the cytosol and not in plastids. To obtain an indication of the subcellular location of proline biosynthesis in leaves, we have investigated the subcellular location of PCR, the only proline biosynthetic enzyme for which a reliable assay in higher plants is available. Our results indicate that, like many other amino acid biosynthetic enzymes, PCR is located in the chloroplasts. We have also compared the properties of PCR from leaves and from etiolated shoots and find that they are similar.

MATERIALS AND METHODS

Plant Material

Peas (*Pisum sativum* L. var Argenteum) were grown in soil flats in a growth chamber under the following conditions: 16 h light, 8 h dark, 20°C, 270 μmol s^{-1} m^{-2} combined fluorescent and incandescent light. Plants were watered with Hoagland solution (7) every third day and were allowed to wilt for 1 d each week. The wilting ensured that increased proline synthesis was stimulated. The Argenteum variety was used because its leaf epidermis is easily peeled away. Protoplasts were prepared from peeled leaves. Etiolated peas (*P.* sativum L. var Progress No. 9) were grown in coarse vermiculite at 30°C in darkness for 9 to 12 d and watered with deionized water. Progress No. 9 was used for experiments with etiolated tissue because sufficient Argenteum seed was not available.

Protoplast Preparation

Pea protoplasts were prepared from 4-week-old leaves of the Argenteum variety (24). Plants were destarched by placing them in darkness for 24 h. Adaxial epidermises were peeled and 40 leaves were floated on protoplast buffer (500 mM sorbitol, 5 mM Mes-KOH, 1 mM $CaCl_2$, [pH 6.0]) containing wall digesting enzymes (2% [w/v] Onozuka cellulase, 0.5% [2/v] Macerozyme, 1% [w/v] hemicellulase) and 0.2% (w/v) BSA in 8.5 cm Petri dishes in darkness at 30°C. After 1 h, the digestion medium was aspirated and 10 mL of protoplast buffer was added to each dish. Protoplasts were released by gentle rocking and decanted into a beaker, then another 10 mL of protoplast buffer was added, and the remaining protoplasts were decanted. Protoplasts were collected by centrifugation in a swinging bucket rotor at $100g_{max}$ for 1 min at 4°C. The supernatant was aspirated and discarded. Protoplasts were resuspended in 5 mL of chloroplast buffer (300 mM sorbital, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 0.2% [w/v] BSA, 50 mM Mops-KOH [pH 7.2]).

[1] Supported by U.S. Department of Agriculture Competitive Research Grants Office grant 85-CRCR-1-1671.

[2] Abbreviations: PCR, Δ^1-pyrroline-5-carboxylate reductase; MMT, Mes, Mops, Tricine; CAPS, 3-(cyclohexylamino)propanesulfonic acid; CHAPSO, 3-([3-cholamidopropyl]-diethylammonio)-2-hydroxy-1-propanesulfonate; GK, γ-glutamyl kinase; GPR, γ-glutamyl phosphate reductase; TPDH, triose phosphate dehydrogenase.

Protoplast Fractionation

Protoplasts were ruptured by passing the suspension three times through 20-μm nylon mesh at the end of a syringe barrel. Microscopic examination revealed complete lysis of all protoplasts after this treatment. The resulting suspension was centrifuged in a Sorvall HB-4 swinging bucket rotor at $250g_{max}$ for 2 min. Pellets, which contained both damaged and intact chloroplasts, were resuspended in 3 mL chloroplast buffer and overlaid on a Percoll (Pharmacia) gradient. The gradient was generated by mixing 15 mL Percoll with 15 mL 2× chloroplast buffer in 50-mL polypropylene tubes and centrifuging at $40,000g_{max}$ in a Sorvall SS-34 fixed angle rotor for 30 min; the rotor was stopped without the brake. The overlaid gradient was centrifuged at $8,000g_{max}$ for 20 min in a Sorvall HB-4 swing-out rotor without the brake. A 12-cm-long, 20-gauge needle was inserted into the gradient to the bottom of the tube, and the gradient was fractionated into 1.6-mL aliquots using a peristaltic pump.

Extraction of PCR

Because Percoll interfered with enzyme assays, protein was precipitated from each Percoll gradient fraction by addition of 2.4 mL of a 50% (w/v) polyethylene glycol (3,500 average molecular mass) solution buffered with 50 mM Mops-KOH (pH 7.2). Samples were vortexed, incubated 10 min, and centrifuged in a Sorvall SM-24 fixed angle rotor at $20,000g_{max}$ for 10 min. Supernatants were aspirated and the pellets resuspended in 0.2 mL of 20 mM Tricine-KOH (pH 8.0), 5 mM $MgCl_2$, 10 mM β-mercaptoethanol, and 20% (v/v) glycerol.

Etiolated pea shoots were harvested and stored at −20°C. Five hundred g of shoots were homogenized in 500 mL grinding buffer (100 mM Mops-KOH, 1 mM EDTA, 20 mM $MgCl_2$, 10 mM β-mercaptoethanol, 5% [w/v] insoluble PVP) with a Polytron tissue homogenized (Brinkmann Instruments) at 4°C for 5 min. The slurry was filtered through four layers of cheesecloth. After filtration, the extract was brought to 30% saturation with $(NH_4)_2SO_4$ at 40°C, incubated 15 min, and centrifuged in a Sorvall GSA rotor at $25,000g_{max}$ for 10 min. The supernatants were pooled, brought to 60% saturation with $(NH_4)_2SO_4$, incubated 15 min, and centrifuged again. The pellets were resuspended in 5 ml 20 mM Tricine-KOH (pH 8.0) and desalted on a Sephadex G-25 column (2.5 × 28 cm) equilibrated with the same buffer. This preparation was made 20% (v/v) with glycerol and stored at −20°C.

For assays of crude chloroplast preparations, PCR was rapidly extracted from chloroplasts by the following modification of the procedure used for gradient purification of chloroplasts. The $250g$ pellet from a lysed protoplast suspension was resuspended in 6 mL of chloroplast buffer and recentrifuged at $250g$ for 2 min in a Sorvall HB-4 rotor. This pellet was resuspended in 2 mL of lysis buffer (20 mM Tricine [pH 8.0], 0.1 mM PMSF, 1 mM DTT) plus 1% (w/v) CHAPSO. After 10 min at 4°C, the sample was centrifuged in a Sorvall SS-34 rotor at $43,500g_{max}$ for 15 min at 4°C. The supernatant, which was yellow-green, was assayed.

Assays

The following assays were performed as previously described. $NADP^+$-dependent triose phosphate dehydrogenase was used as a stromal marker (11), Chl as a thylakoid marker (11), Cyt *c* oxidase as a mitrochondrial marker (1), and catalase as a peroxisome marker (2). The general PCR assay buffer contained 50 mMTricine-KOH (pH 8.0), 1 mM DTT, 200 μM NADH, and 2 mM D,L-Δ^1-pyrroline-5-carboxylate (D,L-P5C). For the subcellular fractionation experiments the buffer also contained 100 mM KCl and 0.01% (w/v) Triton X-114. D,L-P5C was prepared by the method of Williams and Frank (26). P5C-dependent NADH oxidation was measured at 340 nm. The extinction coefficient of NADH (6.2 mM^{-1} cm^{-1}) was used to calculate PCR activity. Kinetic parameters were determined by iterative fitting of the Michaelis-Menten equation (25).

Isoelectric Focusing

A modification of a previously described method (15) was used. Native isoelectric focusing was carried out in gels that were poured and run in a Mighty Small electrophoresis apparatus (Hoefer). Gels contained 4% (w/v) acrylamide, 0.0016% (w/v) methylene-*bis*-acrylamide, 1% (v/v) NP-40, 10% (v/v) glycerol, and 5% (v/v) Pharmalytes (pH 3–10). Gels were run at 200 V for 2 h followed by 400 V for 2 h. To locate PCR, gels were rocked for 2 h in 50 mL of 40 mM CAPS-HCl (pH 8.5), 100 mM L-proline, 1 mM NAD^+, 1 mM $Mg(OAc)_2$, 100 mM KOAc, 300 μg/mL nitroblue tetrazolium, 20 μg/mL phenazine methosulfate. This staining system gives purple bands by the reverse (proline dehydrogenase) reaction of PCR, which is active at pH >9. Two-mm-thick gel slices were also assayed spectrophotometrically for PCR activity in the forward reaction. Slices were incubated in PCR assay buffer for 15 h. Reverse activity of the same samples were measured spectrophotometrically as previously described (12). In these assays forward PCR activity was fivefold greater than reverse activity.

RESULTS

Subcellular Localization of PCR Activity

A crude chloroplast suspension prepared from lysed protoplasts contained 25% of the PCR activity in the protoplast suspension (Table I). The suspension contained comparable proportions of two chloroplast markers, $NADP^+$-TPDH activity (15%) and Chl (19%). This result suggests that PCR may be localized in plastids; however, the suspension also contained significant amounts of activity of a mitochondrial marker enzyme, Cyt *c* oxidase (6% of total activity), and a peroxisomal marker enzyme, catalase (13% of total activity). When this chloroplast-enriched preparation was fractionated by isopycnic Percoll density gradient centrifugation, the maximum PCR activity coincided with the maximum TPDH activity and Chl concentration (fraction 3, Fig. 1). There was almost no Cyt *c* oxidase activity in this region of the gradient, but some catalase activity was apparent. The proportion of catalase activity recovered in fraction 3 was only one-third the proportion of chloroplast markers and PCR recovered in

Table I. *Distribution of PCR and Markers in Subcellular Fractions from Pea Protoplasts*

Marker	Total Units[a]			Percent of Total Along Gradient[b]	
	3 mL protoplast suspension	3 mL chloroplast suspension	Total along Percoll gradient[c]	Fraction No. 3	Fraction No. 17
PCR	0.8	0.20	1.02	35	9
TPDH	3.3	0.50	0.54	29	6
Chl	5.0	0.95	1.44	33	21
Cyt *c* oxidase	6.3	0.35	0.003	3	0
Catalase	4.2	0.55	0.14	11	17

[a] 1 unit = 1 μmol min^{-1} for enzymes and 1 mg for Chl. [b] Numbers in the third column represent 100%. [c] Sum of activities in all fractions.

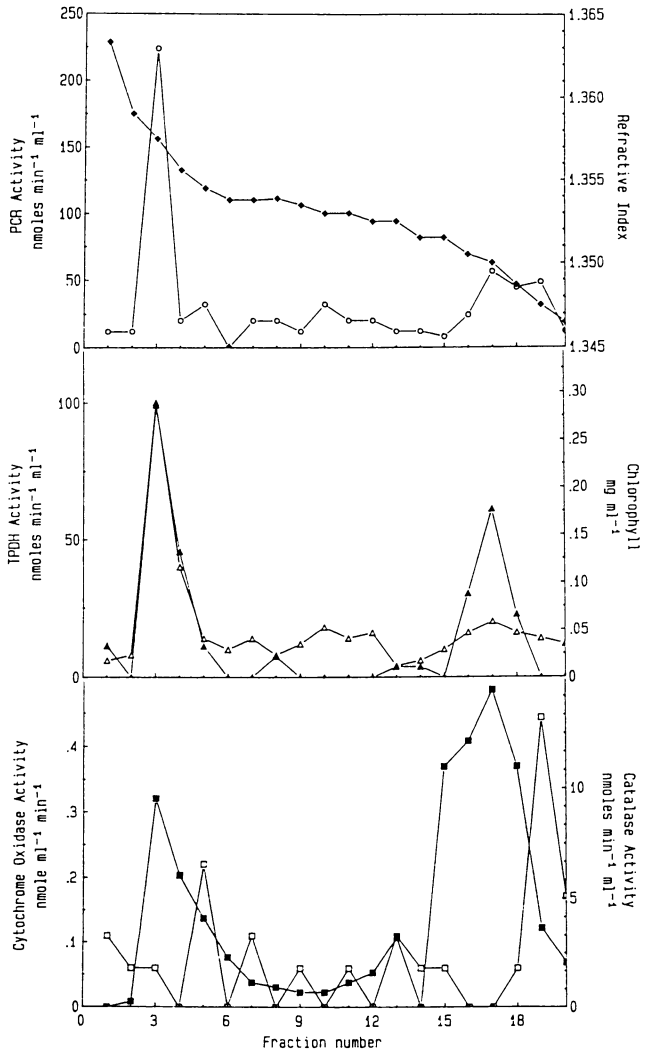

Figure 1. Distribution of PCR activity and subcellular markers along a percoll gradient: PCR (○), refractive index (♦), triose phosphate dehydrogenase (△), Chl (▲), Cyt oxidase (□), and catalase (■). The overlaid samples contained 60 mg protein. Fraction 1 is the bottom of the gradient.

that fraction (Table I). The lower recoveries of Cyt oxidase and catalase indicate that fraction 3 contained lesser proportions of mitochondria and peroxisomes than of chloroplasts. Moreover, the distribution of PCR activity in the gradient as a whole coincided more closely with the distribution of TPDH than with that of catalase or Cyt oxidase.

In three of seven replicate gradients (data not shown), PCR activity was prominent in the ruptured chloroplast region (fraction 17). This distribution suggests that PCR is associated with thylakoid membranes. Several nonionic detergents were added to the chloroplast lysis buffer to test their ability to dissociate interactions between PCR and thylakoids. The yield of PCR activity in the soluble faction from chloroplasts was increased twofold by NP-40, CHAPS, or CHAPSO (data not shown).

Whole etiolated shoots yielded 100-fold greater PCR activity than chloroplasts from green leaves. When etioplasts were purified from pea shoots on a Percoll gradient, PCR activity (42 nmol min^{-1} mg protein^{-1}) was detected, but the yield was less than 1% of the PCR activity of whole shoot extracts. The yield of etioplasts was itself very low: only 1.3% of the total NADP$^+$-TPDH activity of etiolated shoots was present in the etioplast fraction (102 nmol min^{-1} mg protein^{-1}). These results suggest that PCR is present in etioplasts, but do not provide conclusive evidence as to its subcellular distribution in etiolated shoots.

Kinetic Properties of PCR

The specificity of chloroplast PCR for pyridine nucleotide cofactors was investigated. At pH 7.5 and in the presence of 2 mM D,L-P4C, the enzyme had apparent K_ms for NADPH and NADH of 0.12 mM and 0.19, respectively, whereas V_{max} was greater with NADPH (0.19 μmol min^{-1} mg protein^{-1}) than with NADH (0.15 μmol min^{-1} mg protein^{-1}). The kinetic parameters of PCR from etiolated shoots were also examined. PCR from etiolated shoots had apparent K_ms for NADPH and NADH of 0.1 mM and 0.43 mM, respectively. The V_{max} was greater with NADH (3.2 μmol min^{-1} mg protein^{-1}) than with NADPH (0.83 μmol min^{-1}).

There were two pH optima for PCR activity in chloroplast preparations, at pH 6.5 and at pH 7.5 (Fig. 2A). These optima are also present in PCR prepared from etiolated pea shoots (Fig. 2B) and similar optima were observed with both enzymes when NADPH was the substrate (data not shown).

Salt stress causes proline to accumulate (3, 8). Light increases the Mg^{2+} concentration in the stroma *in vivo* (19). The effects of salts on PCR activity were investigated to test the hypothesis that proline biosynthesis can respond to changes in ion concentration. One hundred mM KCl (Fig. 3) or 10 mM $MgCl_2$ (Fig. 4) caused an approximately twofold increase in the PCR activity of both chloroplast and etiolated preparations. Potassium acetate, NH_4Cl, and NH_4OAc stimulated PCR activity to the same extent as KCL (Table II). Maximal stimulation was observed at concentrations between 50 and 100 mM KCl. Activity declined at concentrations above 100 mM; at 200 mM KCl, activities were only slightly higher than the control activity of the chloroplast enzyme increased to a maximum at 10 mM $MgCl_2$ and did not differ between 10 and 20 mM (Fig. 4). Activity of the enzymes from

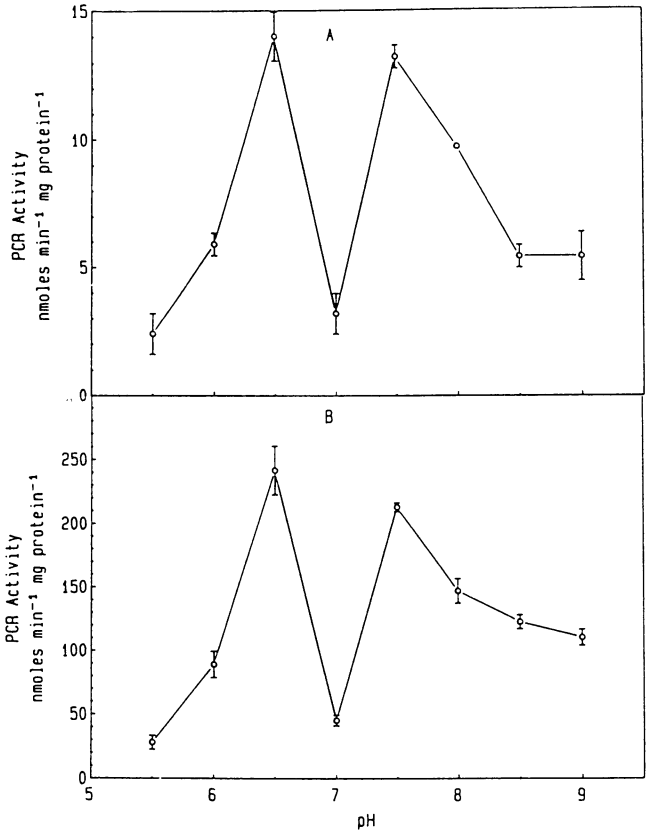

Figure 2. Effect of pH on PCR activity from pea chloroplasts (A) and etiolated pea shoots (B). MMT buffer used for the pH curve contained 50 mM Mes, 50 mM Mops, and 50 mM Tricine. HCl or KOH was used to adjust pH. Except for the change in buffer, the general PCR assay conditions were used, NADH being the substrate. Results are the means and standard errors of four assays.

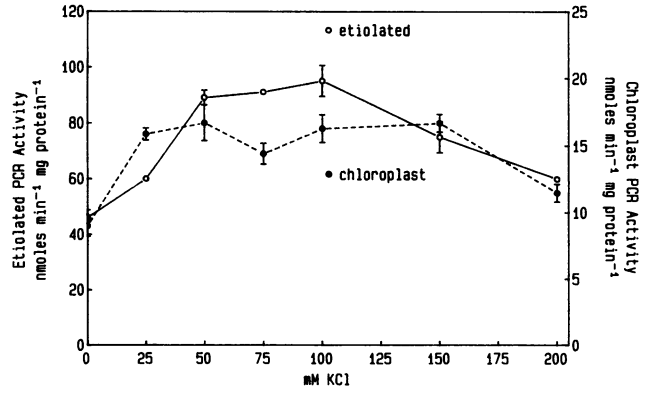

Figure 3. Effect of KCl on PCR activity from pea chloroplasts (●) and etiolated pea shoots (O). Assay was as described in "Materials and Methods," but KCl was added. Results are the means and standard errors of four assays.

etiolated tissue increased with increasing $MgCl_2$ concentration to a maximum at 5 mM, but activity at 10 and 20 mM was lower than it was at 5 mM (Fig. 4). Sucrose and sorbitol of osmolalities equal to that of 100 mM KCl did not cause any significant stimulation (Table II). Potassium acetate, ammonium chloride, and ammonium acetate stimulated PCR activity to the same extent as KCl (Table II). Manganese chloride, calcium chloride, and magnesium acetate caused the same stimulation as magnesium chloride (Table II). The effects of KCl and $MgCl_2$ were not additive at concentrations that gave maximal stimulation alone (Table II).

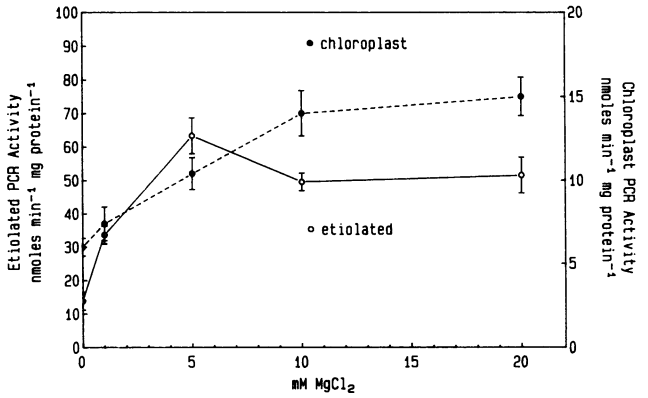

Figure 4. Effect of $MgCl_2$ on PCR activity from pea chloroplast (●) and etiolated pea shoots (O). Assay was described in "Materials and Methods," but $MgCl_2$ was added. Results are the means and standard errors of four assays.

Table II. *In Vitro Effects of Salts and Sugars on PCR Activities[a] from Pea Leaf Chloroplasts and Etiolated Pea Shoots*

Treatment	Chloroplast	Etiolated Shoot
	nmol min^{-1} mg protein^{-1}[a]	
Control[b]	4[c] (1.0)	35 (7)
Monovalent Cations		
+100 mM KCl	9 (0.2)	138 (31)
+100 mM KOAc	9 (1.1)	127 (3)
+100 mM NH_4Cl	10 (0.6)	162 (3)
+100 mM NH_4OAc	8 (1.2)	142 (39)
+200 mM Sucrose	3 (0.5)	39 (5)
+200 mM Sorbitol	3 (0.6)	39 (4)
Divalent cations		
+10 mM $MgCl_2$	13 (0.7)	132 (19)
+10 mM $MnCl_2$	14 (1.3)	170 (10)
+10 mM $CaCl_2$	13 (1.3)	164 (23)
+10 mM $Mg(OAc)_2$	14 (0.7)	117 (33)
Combined cations		
+10 mM $MgCl_2$ + 100 mM KCl	8 (1.0)	147 (8)

[a] Mean (SE). Each value represents the mean of four assays. [b] Assay described in "Materials and Methods."

Isoelectric Focusing

Separation of the pH 6.5 and pH 7.5 PCR activities from etiolated shoots was attempted by native isoelectric focusing. One zone of activity ($R_f = 0.44$, pI 7.8) contained PCR activity (Fig. 5). Activity of the reverse PCR reaction (P5C dehydrogenase activity, pH 9.5) produced bands of purple precipitate in the same region. The results were the same when $NADP^+$ was the substrate.

DISCUSSION

Noguchi *et al.* (16) described the localization of PCR activity in a chloroplast-enriched fraction from tobacco leaves.

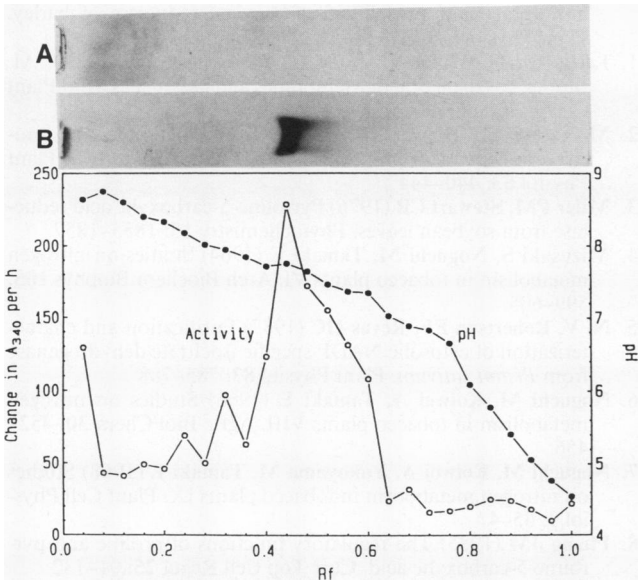

Figure 5. PCR activity (○) and pH (●) in 2 mm segments of a native isoelectric focusing gel. R_f = position of proteins in segment relative to position of a pI marker, methyl red. The general PCR assay was used with the addition of 100 mM KCl. This gel was stained for proline dehydrogenase activity (pH 9.5), (A) without proline, and (B) with proline.

Kohl *et al.* (9), however, found that in soybean nodules, PCR was localized in the cytosol and not in plastids. The results in Figure 1 and Table I demonstrate that in green pea leaves PCR is present in chloroplasts, since the distribution of PCR activity when leaf protoplasts are fractionated most closely resembles the distribution of the stomal marker $NADP^+$-triose phosphate dehydrogenase. Most significantly, PCR activity was most abundant in the same fraction of Percoll gradients as TPDH. About 25% of the PCR activity measured in protoplast suspensions (Table I, column 1) was recovered in chloroplast suspensions (Table I, column 2), while the recovery of intact chloroplasts from protoplasts was approximately 15 to 20% based upon the recovery of $NADP^+$-TPDH and Chl. Because the chloroplast-to-protoplast ratio for PCR activity is greater than the same ratios for chloroplast markers, it appears that PCR in plastids can account for all of the pea leaf PCR activity. This distribution contrasts with the localization of PCR activity in the cytosol of soybean root nodules (9). PCR from pea leaf chloroplasts uses both NADH and NADPH as electron donors, but has a lower K_m and higher V_{max} for NADPH. Thus, like PCR from barley (10) and tobacco leaves (16), it is more active with NADPH. This specifity is consistent with the chloroplast localization of this enzyme, although the enzyme from soybean root nodules is also more active with NADPH except at very high cofactor concentrations (9).

Preliminary evidence indicates that PCR is present in etioplasts from etiolated pea shoots. Although PCR from etiolated shoots has a lower K_m for NADPH than for NADH, its V_{max}/K_m ratios with the two cofactors indicates that it is more active with NADH than with NADPH. Thus, the most abundant form of the enzyme in etiolated shoots may be different from that in leaves.

The bimodal pH curve for pea PCR is analogous to that reported previously for PCR in extracts from etiolated barley (*Hordeum vulgare*) and etiolated mung bean (*Vigna radiata*), both of which give broad pH-activity curves with optima or shoulders at pH 6.4 and 8.0 (4). The bimodal pH curve was also observed when NADPH was used as the cofactor. The pH 7 minimum is not an artifact, because the pH profile was reproducible with batches of MMT buffer prepared three different times. The bimodal curve could be produced by a single enzyme or two isoforms. If there are two forms in green leaves, both are probably present in chloroplasts. The ratio of the pH 6.5 to pH 7.5 activities in chloroplast-enriched preparations was equal to the ratio in whole etiolated shoot extracts. PCR in whole protoplast extracts from green leaves produced a broad pH curve. Activities at pH 6.5 and 7.5 were similar (data not shown), as in the chloroplast-enriched fraction and in etiolated shoots. There was no minimum apparent at pH 7.0, but the enzyme was difficult to assay precisely in whole protoplast extracts because of its low activity. This investigation focused on the PCR activity with the pH 7.5 optimum.

The native isoelectric focusing gel revealed only one region of PCR activity, with a pI of 7.8; the same pattern of PCR activity was detected with NADH and NADPH. Thus, it appears that if there are to isoenzymes in etiolated shoots, they have the same pI. An alternative explanation is that one of the isoenzymes is inactivated during native isoelectric focusing. This activity does not represent the activity of mtiochondrial proline dehydrogenase: the mitochondrial proline dehydrogenase is not active at pH 9.5 (4), and it does not donate electrons to NAD^+. The genetic basis for the pH dependence of PCR activity remains uncertain.

The stimulation of PCR by salts is unclear but it appears to be a function of ionic charge. Because potassium ion concentrations in chloroplasts are normally around 100 mM, the enzyme should be fully active *in vivo*. Changes in stromal pH between light and dark occur in the range that includes the pH optima observed in this work. Stromal pH in the dark is approximately 7, corresponding to the trough in activity; stromal pH approaches 8 in the light, so that the pH 7.5 activity would function in the light. Proline synthesis occurs in leaves in both light and darkness (6, 16, 17, 23), but is stimulated in light.

A role for proline as a redox shuttle molecule has been established for some mammalian tissues (20) and has been proposed for nitrogen fixing nodules (9). The existence of such a shuttle mechanism in leaves needs to be investigated. If such a shuttle operates in leaves under nonstressed conditions, then the localization of PCR in chloroplasts would function to transport reducing potential to mitochondria in the form of proline. To test such a redox shuttle hypothesis, a mechanism that transports proline across the chloroplast envelope needs to be identified. An uncoupled redox shuttle mechanism would contribute to proline accumulation. If water deficit caused such a shuttle to become uncoupled by decreasing proline oxidation without decreasing proline synthesis, then proline accumulation could be a symptom of metabolic dysfunction. Because proline has biocompatible characteristics and proline accumulation is not toxic, metab-

olism may be temporarily shunted in this direction until homeostasis is regained.

Since the product of P5C reductase is proline, the results of this investigation provide evidence that, like many other amino acids, proline is synthesized in chloroplasts. It will be of considerable interest to determine whether the enzymes catalyzing conversion of glutamate to P5C have the same subcellular location.

ACKNOWLEDGMENT

We are grateful to Professor G. A. Marx for supplying the Argenteum pea mutant.

LITERATURE CITED

1. **Chance B** (1953) Techniques for the assay of the respiratory enzymes. Methods Enzymol **4:** 273–329
2. **Decker LA** (ed) (1977) Catalase. Worthington Enzyme Manual, Worthington Biochemical Corporation, Freehold, NJ, pp 63–65
3. **Demming B, Winter K** (1986) Sodium potassium, chloride and proline concentrations of chloroplasts isolated from a haplotype, *Mesembryanthemum crystallinum* L. Planta **168:** 421–426
4. **Elthon TE, Stewart CR** (1981) Submitochondrial location and electron transport characteristics of enzymes involved in proline oxidation. Plant Physiol **67:** 780–784
5. **Elthon TE, Stewart CR** (1984) Effects of the proline analog L-thiazolidine-4-carboxylic acid on proline metabolism. Plant Physiol **74:** 213–218
6. **Hanson AD, Tulley RE** (1979) Light stimulation of proline synthesis in water-stressed barley leaves. Planta **145:** 45–51
7. **Johnson CM, Stout PR, Broyer TC, Carlton AB** (1957) Comparative chlorine requirements of different plant species. Plant Soil **8:** 337–353
8. **Kaiser WM, Weber H, Sauer M** (1983) Photosynthetic capacity, osmotic response and solute content of leaves and chloroplasts from *Spinacia oleracea* under salt stress. Z Pflanzenphysiol **113:** 15–27
9. **Kohl DH, Schubert KR, Carter MB, Hagedorn CH, Shearer G** (1988) Proline metabolism in N_2 fixing root nodules: energy transfer and regulation of purine synthesis. Proc Natl Acad Sci USA **85:** 2036–2040
10. **Krueger R, Jager HJ, Hintz M, Pahlich E** (1986) Purification to homogeneity of pyrroline-5-carboxylate reductase of barley. Plant Physiol **80:** 142–144
11. **Leegood RC, Walker DA** (1983) Chloroplasts. *In* JL Hall, AL Moore, eds, Isolation of Membranes and Organelles from Plant Cells, Academic Press, New York, pp 185–210
12. **McNamer AD, Stewart CR** (1974) Nicotinanide adenine dinucleotide-dependent proline dehydrogenase in *Chlorella*. Plant Physiol **53:** 440–444
13. **Miler PM, Stewart CR** (1976) Pyrroline-5-carboxylic acid reductase from soybean leaves. Phytochemistry **15:** 1855–1857
14. **Mizusaki S, Noguchi M, Tamaki E** (1964) Studies on nitrogen metabolism in tobacco plants VI. Arch Biochem Biophys **105:** 599–605
15. **Ni V, Robertson EF, Reves HC** (1987) Purification and characterization of cytosolic NADP specific isocitrate dehydrogenase from *Pisum sativum*. Plant Physiol **83:** 785–788
16. **Noguchi M, Koiwai A, Tamaki E** (1966) Studies on nitrogen metabolism in tobacco plants VIII. Agric Biol Chem **30:** 452–456
17. **Noguchi M, Koiwoi A, Yokoyama M, Tamaki E** (1968) Studies on nitrogen metabolism in tobacco plants IX. Plant Cell Physiol **9:** 35–47
18. **Phang JM** (1985) The regulatory functions of proline and pyrroline-5-carboxylic acid. Curr Top Cell Regul **25:** 91–132
19. **Portis AC** (1981) Evidence of a low stromal Mg concentration in intact chloroplasts in the dark. Plant Physiol **67:** 985–989
20. **Rajagopal V, Ralasubramanian V, Sinha SK** (1977) Diurnal fluctuations in relative water content, nitrate reductase and proline content in water-stressed and non-stressed wheat. Physiol Plant **40:** 69–71
21. **Stewart CR** (1981) Proline accumulation: biochemical aspects. *In* LG Paleg, D Aspinall, eds, Physiology and Biochemistry of Drought Resistance in Plants. Academic Press, New York, pp 243–259
22. **Taylor AA, Stewart GR** (1981) Tissue and subcellular localization of enzymes of arginine metabolism in *Pisum sativum*. Biochem Biophys Res Commun **101:** 1281–1289
23. **Vansuyt G, Vallée JC, Prevost J** (1979) La pyrroline-5-carboxylate reductase et la proline deshydrogenase chez *Nicotiana tabacum* var. Xanthi n.c. en fonction de son dévelopement. Physiol Veg **17:** 95–105
24. **Weigel P, Weretilnyk EA, Hanson AD** (1986) Betaine aldehyde oxidation by spinach chloroplasts. Plant Physiol **82:** 753–759
25. **Wilkinson GN** (1961) Statistical estimations in enzyme kinetics. Biochem J **80:** 324–332
26. **Williams I, Frank L** (1975) Improved chemical synthesis and enzymatic assay of Δ^1-pyrroline-5-carboxylic acid. Anal Biochem **64:** 85–97