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Subcellular localization of chorismate-mutase isoenzymes in protoplasts from mesophyll and suspension-cultured cells of *Nicotiana silvestris*

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Abstract. The subcellular locations of two readily discriminated chorismate-mutase (EC 5.4.99.5) isoenzymes from *Nicotiana silvestris* Speg. et Comes were determined in protoplasts prepared from both leaf tissue and isogenic suspension-cultured cells. Differential centrifugation was used to obtain fractions containing plastids, a mixture of mitochondria and microbodies, and soluble cytosolic proteins. Isoenzyme CM-1 is sensitive to feedback inhibition by L-tyrosine and comprises the major fraction of total chorismate mutase in suspension-cultured cells. Isoenzyme CM-2 is not inhibited by L-tyrosine and its expression is maximal in organismal (leaf) tissue. Isoenzyme CM-1 is located in the plastid compartment since (i) proplastids contained more CM-1 activity than chloroplasts, (ii) both chloroplast and proplastid fractions possessed the tyrosine-sensitive isoenzyme, and (iii) latency determinations on washed chloroplast preparations confirmed the internal location of a tyrosine-sensitive isoenzyme. Isoenzyme CM-2 is located in the cytosol since (i) the supernatant fractions were heavily enriched for the tyrosine-insensitive activity, and (ii) a relatively greater amount of tyrosine-insensitive enzyme was present in the supernatant fraction derived from organismal tissue.

Key words: Chorismate mutase – Isoenzyme – *Nicotiana* (chorismate mutase).

Introduction

The metabolic fates of aromatic amino acids synthesized by higher-plant cells extend well beyond their crucial roles as substrates for protein synthesis. It is not unexpected that such divergencies of biochemical function would prove to equate with some mechanism of compartmentation (for a recent review, see Stafford 1981). It has been shown that isolated chloroplasts are capable of assimilating labelled CO_2 and shikimate into aromatic amino acids in spinach (Bickel et al. 1978). Thus, the entire biosynthetic pathway appears to be located within the chloroplast. However, the incorporation was quite low, and chloroplast-localized biosynthesis does not account for 100% of aromatic amino-acid biosynthesis in spinach (Buchholz et al. 1979). Thus, at least in this plant, the existence of separate biochemical pathways for aromatic biosynthesis localized in plastid and extra-plastidial compartments seems to be a distinct possibility.

In *Nicotiana silvestris* the recent characterization of chorismate-mutase (EC 5.4.99.5) isoenzymes has indicated very marked advantages for initiating experiments to elucidate the extent to which aromatic biosynthesis may be partially or entirely compartmentalized in different subcellular locations. Two isoenzymes denoted CM-1 and CM-2 were separated (Goers and Jensen 1984a), and these possessed properties of regulation (Goers and Jensen 1984b) that can be conveniently exploited to discriminate one isoenzyme from the other. Isoenzyme CM-1 is subject to feedback inhibition by L-tyrosine and by L-phenylalanine and is activated by L-tryptophan. Isoenzyme CM-2 is inhibited only by caffeic acid. For compartmentation studies, we have used L-tyrosine to discriminate between isoenzymes CM-1 and CM-2.

A second advantage of employing chorismate-mutase isoenzymes for compartmentation studies derives from the finding that the ratio of isoenzyme CM-1 to isoenzyme CM-2 is much higher in suspension-cultured cells than in organismal (leaf) tissue, varying by well over an order of magnitude. Thus, if, for example, one of the two isoenzymes were localized within plastids, one would expect to find the plastid fraction to be heavily enriched for that isoenzyme, which then could be identified as either CM-1 or CM-2 by its regulatory properties. For example, if the plastid-localized isoenzyme proved to be the CM-1 species, then one would expect to find much greater levels of isoen-

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zyme CM-1 in proplastids of cultured cells than in chloroplasts of leaf mesophyll cells. On the other hand, one would expect to find greater levels of isoenzyme CM-2 in an extra-plastidial location of leaf mesophyll cells than of cultured cells. The converse expectations would apply if isoenzyme CM-2 were the plastid-localized enzyme.

By fractionating protoplasts derived from both suspension-cultured cells and mesophyll cells, we were able to compare the subcellular distribution of the CM-1 and CM-2 isoenzymes in preparations where the starting ratios of the two isoenzymes differed. Use of tyrosine inhibition to discriminate between the isoenzymes, together with comparison of preparations with different ratios of isoenzyme levels, has provided conclusive information about the different subcellular locations of isoenzymes CM-1 and CM-2.

Material and methods

Plant material. Seeds of *Nicotiana silvestris* Speg. et Comes were originally obtained from Dr. L.G. Burk (U.S. Department of Agriculture Tobacco Research Laboratory, Oxford, N.C., USA). The plants were grown as described in Gaines et al. (1982).

Cell-culture procedures and characteristics of cell line. The initiation of callus cultures from leaf discs and the establishment of the isogenic experimental cell line designated ANS-1 have been described in Gaines et al. (1982). This line was obtained from haploid ($1n=1x=12$) green leaf material and has since become aneuploid in liquid culture. The average chromosome number of 42 has remained stable for at least 24 months. Procedures used for chromosone counts in cells of suspension cultures have also been described in Gaines et al. (1982).

Preparation and purification of protoplasts. Fifteen to twenty g of young leaves were excised, two to four still-expanding leaves per plant, from plants eight to ten weeks old (from seeding) that had been dark adapted for 4 d. The leaves were washed in glass-distilled water and plasmolyzed for 1 h at 25° C in K_3 medium supplemented with 0.4 M sucrose (Kao 1974), hereafter referred to as K_3-sucrose. The medium was removed, and the leaves were sliced into 1-mm strips. Fresh K_3-sucrose medium containing 1.5% (w/v) cellulysin (Calbiochem, La Jolla, Cal., USA) and 1.0% (w/v) macerase (Calbiochem) was added. Digestion of the cell walls was completed with 5–6 h at room temperature. Cell-wall debris was removed by filtration through a 43-μm stainless-steel sieve, and protoplasts were collected following centrifugation at 100 *g* for 4 min. Under these conditions, cellular debris sedimented and protoplasts floated to the surface. The protoplasts were removed by aspiration, diluted tenfold with a wash solution (Maliga 1982) consisting of 154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl and 5 mM glucose, pH 5.6. Protoplasts were collected by centrifugation as above. Protoplasts from ANS-1 cells were prepared by similar methods. Cultures were harvested in mid-exponential phase by centrifugation at 300 *g* for 5 min and washed once with K_3-sucrose medium prior to plasmolysis and enzymatic digestion. Protoplasts were usually released within 6–8 h.

Protoplast lysates and cell fractions. All procedures were conducted at 4° C. Protoplasts were resuspended in a fractionation buffer originally described by Frankel et al. (1979) and modified to consist of 0.5 M sorbitol, 50 mM Pipes (1,4-piperazinediethanesulfonic acid) buffer adjusted to pH 7.2 with NaOH, 2 mM ethylenediaminetetraacetic acid (EDTA) disodium salt, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 0.8 mM K_2HPO_4, 10 mM KCl, 5 mM 2-mercaptoethanol, 2 mM isoascorbate and 0.1% (w/v) fraction-V bovine serum albumin (BSA). Protoplast disruption was accomplished by three or four passes through a 20-μm mesh (Spectrum Medical Industries, Los Angeles, Cal., USA) fitted to the end of a syringe, and monitored by light microscopy. To an aliquot of this lysate, Triton X-100 was added to a final concentration of 0.05% (v/v). This preparation was designated as total homogenate and served as the reference point for calculation of recovery of enzyme activities in the various derivative fractions. The remainder of the lysate was centrifuged for 60 s in a HB-4 rotor (Sorvall, DuPont Instruments, Wilmington, Del., USA) at 4000 *g*. The supernatant was centrifuged for 30 min at 15000 *g* in a SS-34 rotor (Sorvall). Both the 4000-*g* and the 15000-*g* pellets were resuspended in 3.0 ml of 10 mM Pipes buffer (pH 7.2), 1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.05% (v/v) Triton X-100. Triton X-100 was added to the final supernatant at a concentration of 0.05% (v/v). Aliquots were removed from both of the resuspended pellets, the supernatant, and the total homogenate for chlorophyll determinations. All four fractions were clarified by centrifugation at 38000 *g* for 30 min and then desalted on Sephadex G-25 columns (PD-10; Pharmacia, Piscataway, N.J., USA) previously equilibrated with 10 mM Pipes buffer (pH 7.2), 1 mM DTT and 0.1 mM PMSF.

Enzyme assays. All assays were carried out under conditions of proportionality with respect to enzyme concentration and reaction time. Chorismate mutase was assayed according to Cotton and Gibson (1968) with modifications (Goers and Jensen 1984a). For latency analysis, for which a protein-precipitation step was required to remove chloroplast debris, the method of Nishioka and Woodin (1972) was used. Nitrite-reductase (EC 1.7.7.1) activity was measured by following the disappearance of nitrite (Dalling et al. 1972). Catalase (EC 1.11.1.6) activity was measured with a continuous spectrophotometric assay (Lück 1965). Fumarase (EC 4.2.1.2) was assayed according to the method of Racker (1950).

Washed chloroplast preparations for latency analysis. Isolated, washed chloroplasts were prepared essentially as described by Rhodes and Kung (1981). However, the fractionation buffer used for extraction and resuspension of the chloroplasts was as described above for protoplast lysis and fractionation, except that the sorbitol concentration was decreased to 0.33 M. Briefly, 50 g of leaf material (coarsely ground in liquid nitrogen) was warmed to −5° C, and ten volumes of the ice-cold fractionation buffer was added. Chloroplasts were filtered through one layer of Miracloth (Calbiochem), and the resulting filtrate was centrifuged for 15 min at 1500 *g* in a GSA rotor (Sorvall) at 4° C. The chloroplasts were washed by resuspending the pellet in five volumes of buffer, and sedimented as before. This procedure was repeated to yield a twice-washed preparation of chloroplasts. The final pellet was resuspended in two volumes of ice-cold 10 mM Pipes buffer (pH 7.2) containing 0.33 M sorbitol, 1 mM DTT and 0.1 mM PMSF, and was then used directly for latency analysis.

Latency analysis. Assays performed on washed chloroplast preparations were done under conditions where a final concentration of 0.33 M sorbitol was maintained as an osmoticum. Identical assays were done under conditions where sorbitol was diluted fourfold, thereby causing osmotic rupture of the plastids and release of their stromal contents into the reaction mixture.

Chlorophyll and protein. Chlorophyll content was determined by the method of Arnon (1949), protein content by the method

of Bradford (1976) using BSA as a standard reference, and corrected for interference by Triton X-100.

Biochemicals. Chorismate (98% pure) was prepared by the method of Gibson (1964) from culture fluids of the multiply-auxotrophic *Klebsiella pneumoniae* 62-1. Protoplast-releasing enzymes were obtained from Calbiochem. Prior to their use, both cellulysin and macerase were dissolved separately in distilled water (5 g in 25 ml) at 4° C, centrifuged for 30 min at 38000 *g* in a SS-34 rotor (Sorvall) to remove precipitates, desalted on a 2.5-cm-diameter, 90-cm-long P-6 column (Bio-Rad, Richmond, Cal., USA) previously equilibrated with glass-distilled water, and lyophilized. All other biochemicals were obtained from Sigma Chemical Co., St. Louis, Mo., USA.

Results and discussion

Protoplast fractionation. The subcellular locations of chorismate-mutase isoenzymes were in part determined by means of fractionation of protoplast lysates by differential centrifugation. Protoplasts are very good starting materials because the mechanical stress imposed upon organelles during cell disruption is minimized. Protoplast lysates yielded sufficient material to locate chorismate-mutase and marker-enzyme activities in a single experiment. The dilution of constituents of disrupted protoplasts upon fractionation was small compared with that of intact tissue, facilitating the quantitation of chorismate mutase and organellar marker enzymes in subcellular fractions in relation to the total homogenate. Appropriate enzymes were used as markers to locate fractions enriched in particular subcellular components (see review by Quail 1979).

The distribution of total chorismate-mutase activity in protoplast lysates from both mesophyll cells and suspension-cultured cells is shown in Table 1 and is compared with the distribution of the marker enzymes: nitrite reductase (chloroplasts or proplastids), fumarase (mitochondria), and catalase (microbodies). Most of the chlorophyll (Table 1A) was found in the 4000-*g* pellet. This agreed well with the distribution of nitrite reductase, which has been established as a specific marker enzyme for chloroplasts (Wallsgrove et al. 1979; Dalling et al. 1972) and proplastids (Quail 1979). As expected, the majority of mitochondria and microbodies sedimented with the 15000-*g* pellet in fractionated mesophyll protoplasts. However, in fractionated cultured cells (Table 1B) approximately one-fifth of the catalase activity was found in the 4000-*g* pellet, and the greatest proportion of fumarase was detected in the supernatant. Nitrite reductase was detected primarily in the 4000-*g* pellet of fractionated cultured cells, although nearly 40% of the recovered activity was found in the other fractions. We have observed that proplastids do not sediment as efficiently as chloroplasts when centrifuged at 4000 *g* for 1 min, and it was necessary to double the centrifugation time in order to pellet more proplastids. This may account for the high activity observed for catalase in the 4000-*g* fraction. Mills and Joy (1980) have suggested that even though microbodies have a greater density, chloroplasts sediment more rapidly because of their greater size. It is possible that a portion of the microbody population may be sufficiently large to sediment with the proplastids, the latter having less mass than chloroplasts. The activity could also be attributed to an adsorption of catalase onto the surface of the proplastid as was noted for chloroplasts by Allen (1977), who was able to remove this non-plastidial activity by washing isolated type-A chloroplasts. Even though the disruption of protoplasts is a relatively gentle procedure, the fragility of the single membrane of

Table 1. Distribution of chorismate-mutase activity in protoplast lysate fractions prepared from (A) leaf mesophyll cells or from (B) suspension-cultured cells of *Nicotiana silvestris*. Numbers are %; the sum of the three numbers in each column equals 100% of the total chorismate-mutase activity or total marker-enzyme activity recovered from the three fractions. Each number given in parentheses is the ratio of the latter sum to the activity determined for total homogenate (×100%)

A. From mesophyll protoplasts

Fraction[a]	Chorismate mutase	Chlorophyll	Nitrite reductase	Fumarase	Catalase
4000-*g* pellet	19	84	89	18	8
15000-*g* pellet	6	4	5	66	80
Supernatant	75	12	6	16	12
	(109)	(111)	(109)	(106)	(107)
Total homogenate activity[c]	76	[d]	102	28	155

B. From cultured-cell protoplasts

Fraction[b]	Chorismate mutase	Nitrite reductase	Fumarase	Catalase
4000-*g* pellet	64	61	8	23
15000-*g* pellet	19	22	34	61
Supernatant	17	17	58	16
	(100)	(101)	(126)	(121)
Total homogenate activity[c]	390	65	246	160

[a] A total of 6.6 mg of protein was fractionated
[b] A total of 39 mg of protein was fractionated
[c] Activity is expressed as nmol min^{-1}
[d] A total of 2.4 mg of chlorophyll was fractionated

Table 2. Allosteric sensitivity of chorismate mutase to L-tyrosine inhibition in fractions prepared from protoplast lysates of leaf mesophyll cells and cultured cells of *N. silvestris*. The cell fractions used were those described in Table 1

Fraction	Leaf protoplasts			Cultured-cell protoplasts		
	Total activity[a]	Inhibition (%)[b]	CM-1 isoenzyme (%)[c]	Total activity[a]	Inhibition (%)[b]	CM-1 isoenzyme (%)[c]
4000-*g* Pellet	14	62	76	250	68	83
15000-*g* Pellet	5	13	16	75	68	83
Supernatant	57	8	10	68	36	44
Total homogenate	70	42	51	390	70	86

[a] Total activity is expressed as nmol prephenate formed min^{-1}
[b] Inhibition in the presence of 0.5 mM L-tyrosine in 10 mM Pipes buffer, pH 7.2
[c] The percent of CM-1 isoenzyme contributing to the chorismate-mutase activity of any given fraction was calculated by using a maximal sensitivity of 82% inhibition by 0.5 mM L-tyrosine at pH 7.2 for isoenzyme CM-1 compared with a value of 0% for CM-2 (Goers and Jensen 1984b)

microbodies may result in the loss of some constituents (Vigil 1983). Moreover, the interconnectedness of different components can markedly contribute to contamination during isolation and purification (Vigil et al. 1979).

In leaf protoplasts, most of the chorismate mutase was recovered in the supernatant except for approx. 20% which fractionated with the 4000-*g* pellet. The fumarase marker indicated mitochondrial contamination of the crude chloroplast pellet such that the percentage of recovered activity in the 4000-*g* pellet was similar to that of chorismate mutase. However, the possibility that the chorismate mutase recovered at 4000-*g* might be a contaminant from the mitochondrial constituent can be ruled out since this possibility would require more than ten times the amount of chorismate mutase that was in fact found in the 15000-*g* fraction. The proportion of total chorismate-mutase activity which was found in the 4000-*g* pellet agreed well with the proportion of total chorismate mutase existing as isoenzyme CM-1 in green leaf tissue (Goers and Jensen 1984a).

In protoplasts from cultured cells where CM-1 is the major isoenzyme the proplastid fraction contained the greatest fraction of total chorismate-mutase activity. Presumably this fraction would have been even greater if the recovery of proplastids had been as efficient as the recovery of chloroplasts (compare the recoveries of the nitrite-reductase marker in parts A and B of Table 1). As was the case with protoplasts from leaf material, the distribution patterns of microbody and mitochondrial markers did not parallel the distribution of chorismate mutase that would be expected if either isoenzyme CM-1 or isoenzyme CM-2 were located in these organelles.

Use of L-tyrosine for isoenzyme discrimination. The identification of chorismate-mutase isoenzymes in fractionated protoplast lysates was approached by comparing the L-tyrosine sensitivities of activities in each fraction (Table 2). Leaf tissue contains at least as much isoenzyme CM-2 (an isoenzyme whose activity is unaffected by aromatic amino acids) as isoenzyme CM-1 (which is feedback-inhibited by L-tyrosine). Cultured cells, on the other hand, possess much more CM-1 than CM-2. Comparison of the L-tyrosine sensitivities of the chorismate-mutase activities in protolast lysates from both mesophyll and suspension-cultured cells clearly indicated that the activity which fractionated with the 4000-*g* pellet was isoenzyme CM-1, while the activity in the supernatant was isoenzyme CM-2. The percent inhibition obtained for chorismate mutase in the 4000-*g* pellet corresponded favorably to that found for partially purified isoenzyme CM-1 (Goers and Jensen 1984b). Most of the chorismate-mutase activity from mesophyll cells was found in the supernatant (Table 1 A) and this activity exhibited very low sensitivity to inhibition by L-tyrosine (Table 2), thus identifying it as isoenzyme CM-2. In contrast, the major portion of total chorismate mutase activity was detected in the 4000-*g* pellet fraction from tissue-culture protoplasts (Table 1 B), and this activity was strongly inhibited by L-tyrosine (Table 2). We conclude that isoenzyme CM-1 is a constituent of both the chloroplasts and the proplastids while isoenzyme CM-2 is cytosolic.

Latency analysis. To determine if the chorismate-mutase activity associated with the 4000-*g* fraction was an internal constituent of the chloroplast, latency determinations were made on washed chloroplast preparations which were assayed before and after organelle rupture (Table 3). Latency analysis is a rigorous criterion of plastid intactness, and a dramatic enhancement of stroma-localized activities following osmotic rupture of intact chloro-

Table 3. Affirmation of localization of isoenzyme CM-1 by use of latency analysis in isolated chloroplasts of *N. silvestris*. Chloroplasts were prepared as described in Material and methods. Twice-washed chloroplasts were used in the enzyme assays. The units of enzyme activity given are expressed as nmol min^{-1}. Each reaction mixture contained 37 μg of chlorophyll

Enzyme	Intact chloroplasts[a]	Disrupted chloroplasts[b]	Latency factor[c]
Nitrite reductase	42	675	16.1
Chorismate mutase	0.04	0.89	22.3

[a] Maintained in 0.33 M sorbitol as an osmoticum
[b] Disrupted by osmotic shock as specified under Material and methods
[c] Defined as the ratio of the activity of broken plastids divided by the activity measured in intact plastids

plasts is expected. Activities for chorismate mutase and nitrite reductase were indeed increased substantially following chloroplast disruption. The latency value for nitrite reductase was 25% less than for chorismate mutase. This is probably because a portion of the exogenous nitrite can enter the chloroplast compartment where it is assimilated (Miflin and Lea 1976, 1977; Givan 1979), while chorismate is probably not transported. The identification of the chorismate-mutase activity in washed chloroplast preparations as isoenzyme CM-1 was confirmed by its sensitivity to inhibition (75%) by L-tyrosine.

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