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Separation and characterization of two chorismate-mutase isoenzymes from *Nicotiana silvestris*

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Abstract. Two isoenzymes of chorismate mutase (EC 5.4.99.5) were isolated and partially purified from leaves of diploid ($2n=24$) *Nicotiana silvestris* Speg. et Comes and from isogenic cells in a suspension culture originally established from haploid tissue. An isoenzyme denoted CM-1 ($M_r=52{,}000$) accounted for the major fraction of total activity recovered from suspension-cultured cells, while isoenzyme CM-2 ($M_r=65{,}000$) represented the major fraction of activity recovered from green leaf tissue. The ratio of isoenzyme levels from these two sources differed more than 20-fold. The subcellular location of isoenzyme CM-1 is known to be in the chloroplasts of green leaves or in proplastids of cultured cells, while isoenzyme CM-2 is located in the cytosol. Both isoenzymes were stable during partial purification, possessed broad pH optima for catalysis between 6.0 and 8.0, and were active without denaturation at temperatures at least as high as 45° C. Thiol reagents were unnecessary for either stability or activity of both isoenzymes. The affinity of isoenzyme CM-2 for substrate ($K_m=0.24$ mM) was almost an order of magnitude better than that of CM-1. The kinetic behavior of isoenzyme CM-1 was influenced by pH, while that of isoenzyme CM-2 was not. At pH 7.2, hyperbolic substrate-saturation curves ($K_m=1.7$ mM) were obtained for isoenzyme CM-1. At pH 6.1, however, isoenzyme CM-1 displayed relatively weak positive cooperativity, Hill plots yielding an n value of 1.2. At pH 6.1 the half-saturation ($[S]_{0.5}$) value was 2.5 mM.

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

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Abbreviations: DEAE = diethylaminoethyl; M_r = molecular weight

Introduction

Diverse control patterns for chorismate mutase in nature. Chorismate mutase (EC 5.4.99.5) generates prephenate molecules fated for two-step transformations to either L-phenylalanine or L-tyrosine. In microorganisms this is a major metabolic pathway to supply these amino acids for protein synthesis. In higher plants L-tyrosine, and especially L-phenylalanine, are in addition important as initial precursors of quantitatively large amounts of phenylpropanoid compounds and other secondary metabolites. It is hardly surprising that chorismate mutase stands as one of the most-studied branchpoint enzymes, contributing to a growing appreciation of the control patterns which act to partition the flow of carbon to divergent endproducts.

Microorganisms illustrate the variety of regulatory strategies for chorismate mutase which exist in nature (for review, see Byng and Jensen 1983). A mechanism for balanced allosteric regulation of a branchpoint enzyme activity by multiple endproduct molecules which is presently receiving considerable attention is the elaboration of differentially controlled isoenzymes. In *Escherichia coli* two isoenzymic chorismate mutases are bifunctional, one possessing prephenate-dehydratase activity (P-protein) and the other possessing prephenate-dehydrogenase activity (T-protein). The P-protein and T-protein compete to channel chorismate toward L-phenylalanine or to L-tyrosine, re-

spectively. The P-protein is widely distributed in nature (Byng et al. 1983). In *Pseudomonas aeruginosa* (Fiske et al. 1983) a P-protein co-exists with a monofunctional, unregulated chorismate mutase. The latter is a critical element in a second flow route which can overproduce L-phenylalanine under conditions of increased carbon input into the aromatic pathway. The role of this unregulated chorismate mutase in metabolite hyperproduction under particular conditions of substrate availability is perhaps reminiscent of the role of isoenzyme CM-2 (see below) in secondary metabolism in higher plants.

Isoenzymic chorismate mutases of higher plants. Where higher plants have been studied, two or more isoenzymes of chorismate mutase are typically found. One species (denoted isoenzyme CM-1) is feedback-inhibited by L-tyrosine or L-phenylalanine and is activated by L-tryptophan. A second species (denoted isoenzyme CM-2) is not affected by aromatic amino acids. Organisms having this general arrangement include *Vigna radiata* (Gilchrist and Kosuge 1974, 1975), *Quercus pedunculata* (Gadal and Bouyssou 1973), *Medicago sativa* (Woodin et al. 1978) and *Nicotiana silvestris* (Goers and Jensen 1984). The finding (d'Amato et al. 1984) that isoenzyme CM-1 isolated from leaves of *N. silvestris* occupies the chloroplast compartment, while isoenzyme CM-2 is extrachloroplastic, indicated to us that aromatic biosynthesis within the chloroplasts is tightly regulated by aromatic amino acids. In contrast, L-tyrosine and L-phenylalanine formed in the cytosol may overflow without restraint into connecting pathways of secondary metabolism.

Material and methods

Plant material. Seeds of *Nicotiana silvestris* Speg. et Comes ($2n=24$) were obtained from Dr. L.G. Burk (U.S. Department of Agriculture Tobacco Research Laboratory, Oxford, N.C., USA). Plants were grown to flowering in a growth chamber with 18 h of illumination (2500 lx). Details of the growth conditions were described earlier (Gaines et al. 1982).

Cell-culture procedures. Callus cultures were obtained from leaf discs, surface sterilized for 30 s with 95% ethanol followed by a 5-min treatment with 0.5% (w/v) sodium hypochlorite. The discs were inoculated on medium (Murashige and Skoog 1962) containing 1 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 1 µg L^{-1} kinetin (N^6-furfurylaminopurine), and 1% (w/v) agar. Suspension cultures from callus were initiated and maintained as described in Gaines et al. (1982).

Cell lines employed in tissue culture. Two cell lines were studied in the preliminary characterization of chorismate-mutase isoenzymes. Line ANS-1 was originally established from haploid tissue, thereby excluding isoenzyme complications resulting from heterozygosity (Gaines et al. 1982). Line DNS-1 was obtained from a callus culture derived from a normal, diploid green leaf. Both ANS-1 and DNS-1 cultures produced similar levels of CM-1 and CM-2 isoenzyme activities. Other chorismate-mutase isoenzymes were not expressed in line DNS-1, and the detailed enzymological work reported here was done with line DNS-1. The doubling time of line DNS-1 during exponential growth was 72 h (dry weight). Line DNS-1 has been in suspension culture for over two years and has become a stable aneuploid, exhibiting an average chromosome number of 44 throughout 20 months of culture as judged by periodic chromosome spreads (Gaines et al. 1982).

Preparation of crude extracts. Exponentially growing cell cultures, subcultured 4 d earlier, were harvested on Miracloth filters (Calbiochem, La Jolla, Cal., USA) and washed with 3% mannitol. The cell pack was immediately frozen in liquid nitrogen and ground to a fine powder in a Waring blender. For experiments with leaf material, the midribs were excised and the leaves washed with distilled water. Leaves were cut into pieces of approx. 2 cm^2, frozen in liquid nitrogen, and ground to a fine powder in a Waring blender. When powder from cell culture or leaf material was not used immediately for enzyme extraction, chorismate-mutase activities proved to be stable for at least one month at −40° C.

All extraction procedures were carried out at 4° C and as quickly as possible. The frozen cell or leaf powder was combined (1:1:1:1, w/w/w/v) with hydrated polyvinylpolypyrrolidone, acid-washed Amberlite adsorbent XAD-4 (Loomis et al. 1979) and cold (4° C) extraction buffer containing: 10 mM Pipes (1,4-piperazine diethanesulfonic acid) buffer (pH 7.2), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 35 mM KCl, and 50 mM Na-ascorbate. These were mixed until thawed. The slurry was filtered through two layers of cheesecloth and one layer of Miracloth, and centrifuged for 10 min at 55000 *g* at 4° C in a Beckman ultracentrifuge (Beckman Instruments, Palo Alto, Cal., USA). The resulting supernatant was passed over a column of Sephadex G-25 (2.5 cm diameter, 48.5 cm long) equilibrated with 10 mM Pipes buffer (pH 7.2), 0.1 mM PMSF, and 35 mM KCl to remove small molecules. The resultant fractions containing protein were pooled and designated as crude extracts. Protein concentration was determined by the method of Bradford (1976) using Fraction-V bovine serum albumin as a reference protein for standard curves.

Diethylaminoethyl(DEAE)-cellulose chromatography. Crude extract (100 mg protein) was loaded onto a DEAE-cellulose (DE-52; Whatman, Clifton, N.J., USA) column (1.5 cm diameter, 20 cm long) equilibrated with 10 mM Pipes buffer (pH 7.2), 0.1 mM PMSF and 35 mM KCl. The column was washed with 100 ml of equilibration buffer prior to the application of a 300-ml linear salt gradient (35 mM–0.4 M KCl) in the same buffer. Fractions of 2 ml were collected at a flow rate of 30 ml h^{-1}. Those fractions containing isoenzyme CM-1 and isoenzyme CM-2 activities were pooled separately and concentrated by ultrafiltration on an Amicon PM10 membrane with $2{,}8 \cdot 10^5$ Pa (40 psi) N_2.

Hydroxylapatite chromatography of isoenzyme CM-1. The concentrated CM-1 isoenzyme was loaded onto the bed of a Sephadex G-25 column (1.5 cm diameter, 22 cm long) equilibrated with 50 mM K-phosphate buffer (pH 7.2) and 0.1 mM PMSF. The desalted enzyme was adsorbed onto a hydroxylapatite (Bio-Gel HTP) column (2.0 cm diameter, 5 cm long) equilibrated with the same buffer used for the Sephadex G-25 column, and the column was washed with 100 ml of buffer. A 300-ml linear

gradient between 50 mM and 200 mM K-phosphate buffer (pH 7.2) containing 0.1 mM PMSF was applied, and fractions of 2 ml were collected at a flow rate of 30 ml h^{-1}. Isoenzyme CM-1 eluted off hydroxylapatite in the gradient as a single peak of activity and was used for all assays described in this study.

Separation of protein interfering with assay of CM-2 activity. Fractions containing CM-2 activity after DEAE-cellulose chromatography co-eluted with a protein whose presence interfered with the testing of the substituted hydroxycinnamic acids as possible effector molecules. The contaminating protein was apparently responsible for a reaction which converted caffeic acid to a purple chromophore. Examination of the purple reaction mixture by high-performance liquid chromatography (HPLC) showed that little or no caffeic acid was present after 20 min at 37° C. Samples (25 µl each) were fractionated by HPLC using an Altex (Altex Scientific, Berkeley, Cal., USA) Ultrasphere ODS (5 µm particle size) column (4.6 mm diameter, 150 mm long) where the mobile phase was 5% methanol in 5 mM K-phosphate buffer (pH 7.3) pumped with an Altex Model 110A metering pump at a flow rate of 1 ml min^{-1} at room temperature (23° C). Solutes were detected by ultraviolet (UV) absorption at 215 nm on a model 250 HM UV-VIS Holochrome Dual Beam spectrophotometer (Gilford Instrument Laboratories, Oberlin, O., USA). Retention times of caffeic acid, phenylpyruvate and chorismate at 215 nm were determined separately prior to application of the reaction-mixture samples.

The CM-2 activity was separated from the contaminating protein by hydroxylapatite chromatography. Concentrated CM-2 isoenzyme was desalted as described for the CM-1 isoenzyme and applied to the same column used in the purification of isoenzyme CM-1. The column was washed with 100 ml of equilibration buffer prior to application of a 300-ml linear gradient between 50 mM and 200 mM K-phosphate buffer (pH 7.2) with PMSF present at 0.1 mM. Fractions of 2 ml were collected with a flow rate of 30 ml h^{-1}. Under these conditions, isoenzyme CM-2 eluted without retardation in the wash volume. The contaminating protein was strongly adsorbed onto the hydroxylapatite, concentrations in excess of 0.5 M K-phosphate (pH 7.2) being required to elute the protein. In all subsequent CM-2 isoenzyme preparations the use of the linear gradient was therefore omitted. Fractions containing CM-2 activity were pooled, concentrated by ultrafiltration on an Amicon PM-10 membrane ($2{,}8 \cdot 10^5$ Pa N_2) and used for all assays described in this paper unless otherwise noted.

Gel filtration. Preparations of CM-1 and CM-2 obtained following the DEAE-cellulose purification step were desalted on a Sephadex G-25 column (1.5 cm diameter, 22 cm long) equilibrated with 50 mM K-phosphate (pH 7.2) and 0.1 mM PMSF and then applied separately to an Ultrogel AcA34 gel filtration column (2.5 cm, 44 cm) equilibrated with the aforementioned buffer. Fractions of 1.5 ml were collected at a flow rate of 13.0 ml h^{-1}. The column was calibrated with the following molecular-weight standards: γ-globulin (205000), albumin (67000), ovalbumin (43000), α-chymotrypsinogen A (25000) and ribonuclease A (13700). The void volume was determined by blue dextran ($M_r = 2000000$).

Enzyme assays. Published procedures were used to assay for the possible presence of activities for arogenate dehydrogenase (Gaines et al. 1982), prephenate dehydrogenase (Champney and Jensen 1970), or prephenate dehydratase (Patel et al. 1977).

The assays of chorismate-mutase activities were carried out under conditions of proportionality with respect to protein concentration and reaction time. Chorismate mutase was usually assayed as described by Cotton and Gibson (1968) with the following modifications. Reaction mixtures for assaying isoenzyme CM-1 contained (200 µl total volume) 2 mM chorismate, 50 mM K-phosphate buffer (pH 7.2) and an appropriate aliquot of enzyme. Reaction mixtures for assaying isoenzyme CM-2 contained (200 µl total volume) 1 mM chorismate, 50 mM K-phosphate buffer (pH 7.2) and an appropriate aliquot of enzyme. When assaying at other pH values, 250 mM K-phosphate buffers were used. Buffer concentration had no effect on enzyme activity. Reaction mixtures were incubated 20 min at 37° C unless otherwise indicated. Addition of 0.1 ml 1 N HCl stopped the reaction, and a second incubation for 20 min at 37° C converted the prephenate formed to phenylpyruvate. After the addition of 0.7 ml 2.5 N NaOH, the absorbance of phenylpyruvate was measured at 320 nm. An extinction coefficient of 17500 was used for calculating specific activity (Cotton and Gibson 1965).

Interference of caffeic acid with the assay for phenylpyruvate in base. When assaying for sensitivity of chorismate mutase to caffeic acid using the 2.5 N NaOH method, the absorbance of phenylpyruvate was quenched by the presence of caffeic acid. The absorbances of phenylpyruvate and caffeic acid were not additive, and 85–100% of apparent inhibition by caffeic acid could be attributed to the quenching effect. This problem was circumvented by assay of the enol tautomer of phenylpyruvate in borate buffer as described by Nishioka and Woodin (1972). Reaction mixtures (200 µl) were incubated for 20 min at 37° C at pH 7.2 unless otherwise noted. The reaction was stopped by the addition of 50 µl 20% trichloroacetic acid. This precipitated the protein as well as converting the prephenate formed to phenylpyruvate. Reaction mixtures were centrifuged in a table-top centrifuge for 5 min to pellet the protein, and 200 µl of the supernatant were removed for mixture with 800 µl of 1 M borate – 2 M K-phosphate buffer (pH 6.5). After 30 min at room temperature, the absorbance of phenylpyruvate was read at 300 nm. An extinction coefficient of 9000 was used for calculating specific activity. Using these assay conditions, the absorbances of phenylpyruvate and caffeic acid were additive.

Biochemicals. Chorismate (92% pure) was prepared from culture fluids of the multiply-auxotrophic *Klebsiella pneumoniae* 62-1 (ATCC 23506) as described by Gibson (1964). L-Arogenate was prepared from a multiple auxotroph of *Neurospora crassa* ATCC 36373 (Jensen et al. 1977; Zamir et al. 1980). Barium prephenate was prepared from culture supernatants of a tyrosine auxotroph of *Salmonella typhimurium* (Dayan and Sprinson 1970). All other biochemicals were obtained from Sigma Chemical Company (St. Louis, Mo., USA).

Results

Resolution of two isoenzymes of chorismate mutase. When crude extracts prepared either from suspension-cultured cells or green leaf tissue were fractionated by DEAE-cellulose chromatography, two distinct peaks of chorismate-mutase activity were discerned. Clean separation of these isoenzymes was facilitated by use of 35 mM KCl in the starting buffer, a condition which prevented binding of isoenzyme CM-2 to the resin. Thus, the CM-2 isoenzyme washed through the column without retardation while the CM-1 isoenzyme eluted at 0.1 M KCl in the salt gradient. Table 1 shows that

Table 1. Partial purification of chorismate-mutase isoenzymes, CM-1 and CM-2, from suspension-cultured cells and from green leaves of *N. silvestris*

Source of enzyme	Fractionation step	Total activity[a]	Specific activity[b]	Purification (×)
Cultured cells	Unfractionated	29.52	0.29	
	CM-1	[25.97][c]	[0.26][c]	1.0
	CM-2	[3.55][c]	[0.03][c]	1.0
	DEAE-cellulose			
	CM-1	15.47	1.07	4.1
	CM-2	1.17	0.17	4.8
	Hydroxylapatite			
	CM-1	3.37	7.67	29.5
	CM-2	0.63	0.17	4.8
Green leaves	Unfractionated	30.29	0.30	
	CM-1	[7.57]	[0.07]	1.0
	CM-2	[22.67]	[0.23]	1.0
	DEAE-cellulose			
	CM-1	5.93	0.23	3.0
	CM-2	12.37	0.81	3.6

[a] Expressed as SI units (nkat) at pH 7.2. Since assays were done at 2 mM concentrations of chorismate, CM-1 activities were determined at non-saturating levels of substrate. At saturating substrate concentrations of 5 mM chorismate, activities of CM-1 would have been very close to double the values shown. A 2-mM concentration of chorismate was saturating for isoenzyme CM-2

[b] Expressed as nkat mg^{-1} protein

[c] Numbers in brackets refer to an estimate calculated as described in the text

fivefold greater specific activities for isoenzyme CM-1 were recovered after DEAE-cellulose chromatography of extracts made from suspension-cultured cells than from green leaves. In contrast, the specific activity of CM-2 from green leaves was fivefold greater than from suspension-cultured cells after DEAE-cellulose chromatography. The fractional portions of isoenzymes CM-1 and CM-2 in the starting crude extracts were calculated by comparing the ability of 0.5 mM L-tyrosine to inhibit total chorismate-mutase activity present in unfractionated, crude extracts with the ability of 0.5 mM L-tyrosine to inhibit partially purified isoenzyme CM-1 (Goers and Jensen 1984).

Both chorismate-mutase isoenzymes recovered from cultured cells appeared to be identical with the isoenzymes obtained from green leaves. Most of our detailed characterizations were carried out with chorismate-mutase isoenzymes from suspension-cultured cells, using preparations carried through the second fractionation step of hydroxylapatite chromatography (Table 1). Figure 1 shows the elution profiles. The CM-1 preparation thus obtained was free of any detectable prephenate-aminotransferase activity, which can complicate chorismate-mutase assays carried out in the presence of L-tyrosine or L-phenylalanine (the latter being potential amino-donor reactants).

Application of isoenzyme CM-2 to a hydroxylapatite column, as shown in Fig. 1B, resulted in unretarded passage of isoenzyme CM-2 through the column, while a protein that reacts with caffeic acid (thereby interfering with inhibition assays) was retained. The latter protein could be eluted with 0.5 M K-phosphate buffer. The contaminating protein also reacted with chlorogenic acid, and reactions with either caffeic acid or chlorogenic acid were qualitatively obvious by virtue of formation of a purple chromophore. When reactions with caffeic acid were allowed to go to completion, high-performance liquid chromatography demonstrated loss of most caffeic acid originally present. Two absorbance bands characteristic of caffeic acid in Pipes buffer (pH 7.2) at λ_{max} 284 and λ_{max} 310 were substantially reduced. Two new bands appeared: a broad band in the visible range (λ_{max} 520) and a second band in the ultraviolet range (λ_{max} 278). When reaction mixtures were freed of small molecules by passage over Sephadex G-25, subsequent incubation with added caffeic acid again produced a purple chromophore. See Material and methods for additional details.

Characterization of isoenzyme CM-1. Partially purified (DEAE-cellulose) isoenzyme CM-1 from suspension-cell cultures was applied to a gel filtration column as specified under Material and methods. An estimated molecular weight of 52000 was obtained. The following characterizations were made with 30-fold purified isoenzyme CM-1 (line 5 of Table 1). Isoenzyme CM-1 was stable for at least one month at either 0° C or −20° C, both catalytic and regulatory properties remaining intact. Reducing agents such as 1 mM dithiothreitol affected neither stability nor activity.

Substrate-saturation curves were carried out with isoenzyme CM-1 at pH 7.2 and pH 6.1. Michaelis-Menten kinetics were observed at pH 7.2 (Fig. 2), and a relatively high concentration of chorismate (at least 4 mM) was required to approach saturation. A double-reciprocal plot (inset) yielded a K_m value of 1.7 mM for chorismate. At pH 6.1 the substrate-saturation curve (Fig. 3) was sigmoidal in shape, indicating positive cooperativity of chorismate binding to CM-1. The possibility was considered that velocity was not proportional to reaction time (20 min) at low concentrations of chorismate, thus causing a cooperative re-

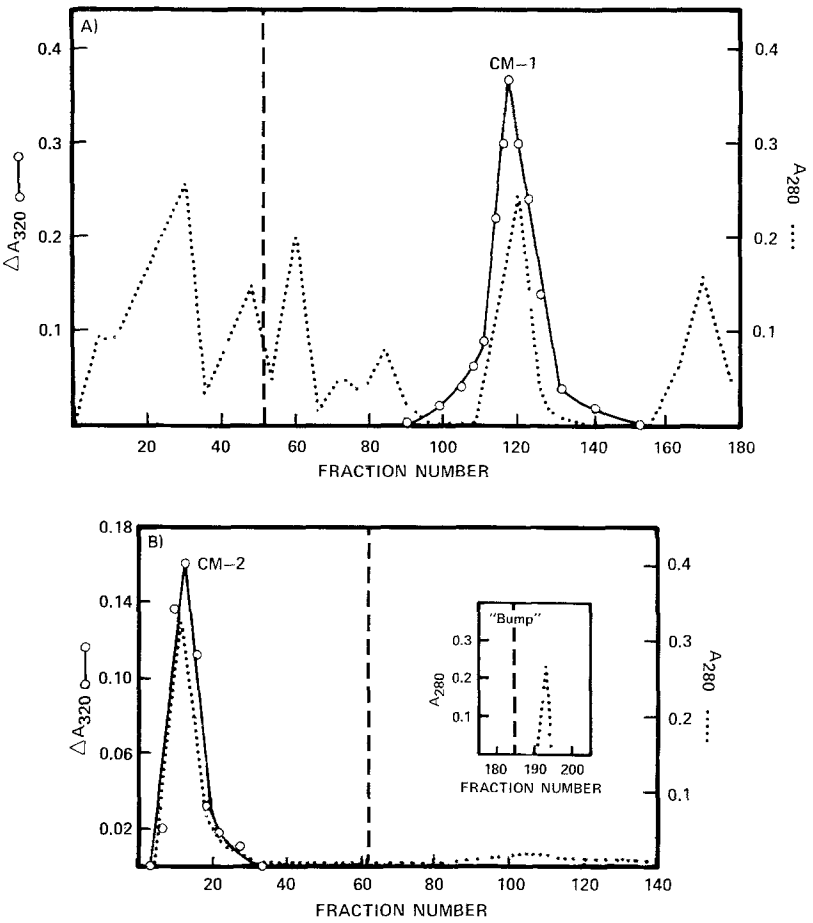

Fig. 1A, B. Isolation of chorismate-mutase isoenzymes of *N. silvestris* by ion-exchange chromatography. Preparations of isoenzyme CM-1 (**A**) and isoenzyme CM-2 (**B**) obtained after DEAE-cellulose chromatography of extract made from suspension-cultured cells were then applied to hydroxylapatite as specified under Material and methods. The *vertical dashed line* indicates the start point of gradient elution. A value on the ordinate scale of 0.10 at 320 nm corresponds to a velocity of 0.004 nkat. Elution of protein was monitored at 280 nm. The *inset* shows the elution position of protein (which proved to possess a caffeic-acid-reactive activity) displaced ("Bump") from the resin with 0.5 M K-phosphate buffer

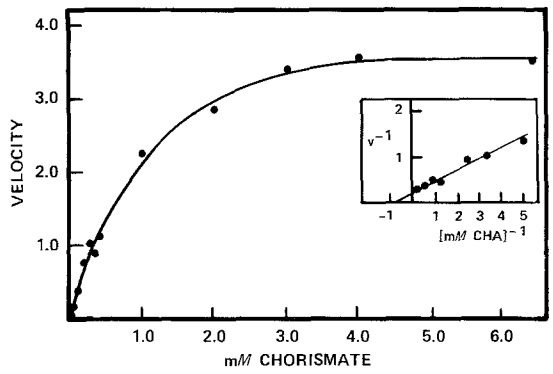

Fig. 2. Substrate-saturation curve at pH 7.2 for isoenzyme CM-1 from suspension-cultured cells of *N. silvestris*. The CM-1 preparation used was partially purified by DEAE-cellulose and hydroxylapatite chromatography steps (see Table 1). Reaction mixtures contained 9 µg of protein. A value of 1.0 on the ordinate scale corresponds to a specific activity of 1.85 nkat mg^{-1}. *Inset*: replot of these data using double-reciprocal coordinates

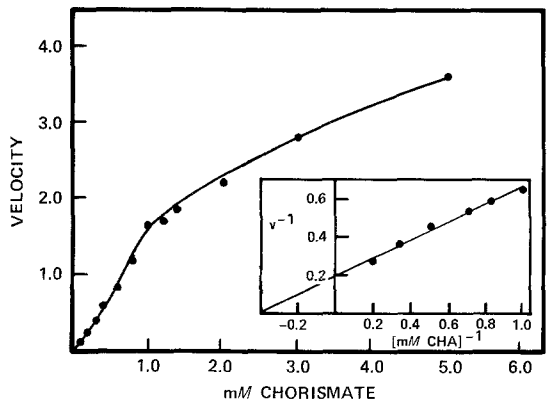

Fig. 3. Substrate-saturation curve at pH 6.1 for isoenzyme CM-1 from suspension-cultured cells of *N. silvestris*. A CM-1 preparation isolated as specified under Fig. 2 was used, 7 µg of protein being present in reaction mixtures. A value of 1.0 on the ordinate scale corresponds to a specific activity of 2.38 nkat mg^{-1}. *Inset*: replot of these data using double-reciprocal coordinates

lationship that was only apparent. However, proportionality was demonstrated, thus eliminating this trivial explanation. Statistical analysis of multiple data points yielded a correlation coefficient (r) of 0.989 for the best-fit line drawn through the points by means of linear regression. This was significant beyond the 0.001 level. Thus, the observed A_{320} values within the sigmoidal section of the saturation curve are significant and accurately reflect the velocity of the reaction. The inset of Fig. 3

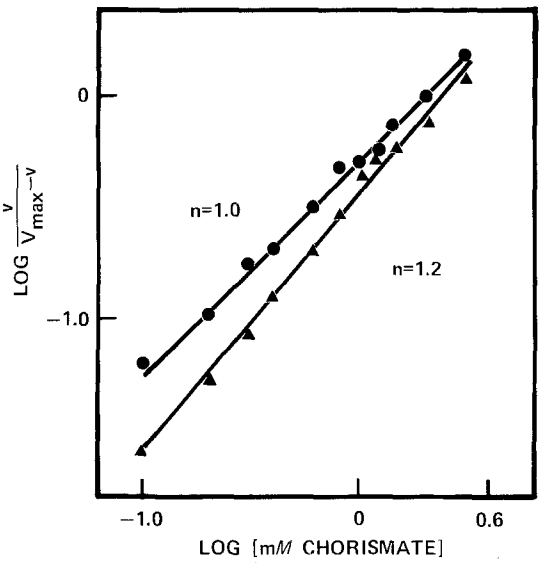

Fig. 4. Hill plots of data shown in Fig. 2 (pH 7.2) and Fig. 3 (pH 6.1). Lines were fitted to data points by linear regression analysis. Values shown for n are equal to the slopes obtained. At pH 7.2, $n=1.0$, while at pH 6.1, $n=1.2$. The values taken for $[S]_{0.5}$ were the chorismate concentrations on the abscissa scale corresponding to zero on the ordinate scale

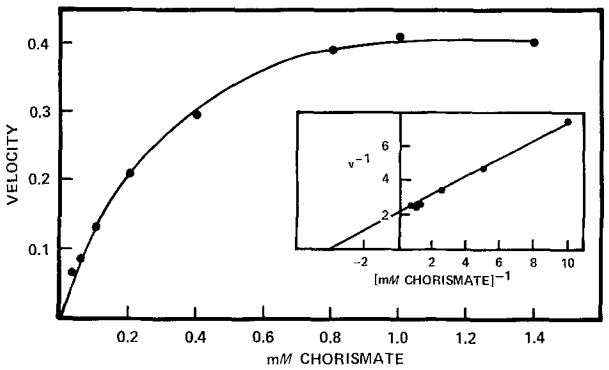

Fig. 5. Substrate-saturation curve of isoenzyme CM-2 of *N. silvestris* at pH 7.2. Isoenzyme CM-2 prepared from suspension-cultured cells and partially purified from DEAE-cellulose and hydroxylapatite resins was assayed as described under Material and methods. Reaction mixtures contained 39 µg of protein, and a value of 0.1 on the ordinate scale corresponds to a specific activity of 0.04 nkat mg^{-1}. A replot of these data points on double-reciprocal coordinates is shown in the *inset*

shows a double-reciprocal plot containing only data points obtained at concentrations above 1 mM chorismate, from which an apparent K_m of 2.5 mM was determined. Since the half-saturation ($[S]_{0.5}$) value is more aptly descriptive of affinity for substrate when sigmoidal substrate-saturation curves are encountered, Hill plots were constructed (Fig. 4). Hill plots based upon data obtained at pH 6.1 were used to calculate an $[S]_{0.5}$ value of 2.4 mM, which compared favorably with the aforementioned apparent K_m value.

Isoenzyme CM-1 exhibited a broad pH optimum for catalysis within a range of 6.0–8.0. Relative to activity measured at pH 6.0, activity increased gradually with increasing pH (25% greater activity at pH 8.0). Isoenzyme CM-1 was active at relatively high assay temperatures. High catalytic rates were maintained constant at 45° C with no evidence of denaturation for at least 20 min of reaction time. Over 50% of CM-1 activity was lost after incubation at 52° C for 20 min.

Characterization of isoenzyme CM-2. Following the separation of isoenzymes CM-2 and CM-1 with the initial DEAE-cellulose fractionation step (Table 1), isoenzyme CM-2 was applied to a gel filtration column as specified under Material and methods. A molecular weight of 65000, somewhat larger than that of isoenzyme CM-1 was obtained. The following characterizations were made with fivefold purified isoenzyme CM-2 (line 6 of Table 1). Isoenzyme CM-2 was stable for at least two weeks at either 0° C or −20° C, both catalytic and regulatory properties remaining intact. Reducing agents such as dithiothreitol did not influence stability or activity. The pH profile of isoenzyme CM-2 was unusually broad, being nearly flat between pH 6.0 and 8.0. Like isoenzyme CM-1, CM-2 catalysis was operative at 45° C, no denaturation occurring over a reaction time of 20 min. However, at 52° C, CM-2 activity was reduced to less than 50% after an incubation time of 5 min. In contrast, isoenzyme CM-1 required 20 min duration at 52° C for comparable inactivation.

Substrate-saturation curves obeyed conventional Michaelis-Menten kinetics (Fig. 5) at pH 7.2. Unlike isoenzyme CM-1, assay at lower pH did not alter the kinetic behavior of isoenzyme CM-2. A K_m of 0.24 mM chorismate was obtained from a double-reciprocal plot (inset of Fig. 5).

Discussion

Isoenzymic chorismate mutases in higher plants. The presence of two isoenzymic forms of chorismate mutase having the general properties of CM-1 and CM-2 appears to be characteristic of higher plants (see Introduction). The regulatory properties of isoenzymes CM-1 and CM-2 (Goers and Jensen 1984) implicate a physiological linkage of their functions to primary biosynthesis of aromatic amino acids and to secondary metabolism (biosynthesis of phenylpropanoid compounds), respectively. Although a third isoenzyme has been reported in some cases, e.g. with *N. tabacum* (Woodin et al.

1978), no physiological role of a third isoenzyme is apparent, and trivial explanations could account for an extra isoenzyme. For example, *N. tabacum* is an amphidiploid and presents the possibility of heterozygosity at four allelic positions. Isoenzyme CM-3 of *N. tabacum* was very similar to CM-1 except that it was activated by 3,4-dimethoxycinnamic acid, the latter a property of no apparent physiological utility. Isoenzyme CM-3 may prove to be an electrophoretic variant of one of the four allelic CM-1 cistrons of *N. tabacum*, perhaps being a neutral mutation in which the activation is merely an anomalous in-vitro reflection of the alteration. A similar explanation could apply to the third isoenzyme of chorismate mutase which was detected in alfalfa (Woodin and Nishioka 1973). Our enzyme preparations from *N. silvestris* were from isogenic cell populations which had originated from haploid material, thus largely eliminating isoenzyme complications that may occur with heterozygosity.

Differential expression of isoenzymes CM-1 and CM-2. In rapidly growing cells in tissue culture, isoenzyme CM-1 was the major fraction of chorismate-mutase activity present. In contrast, young green leaves contained isoenzyme CM-2 as greater than 50% of the total chorismate mutase expressed. The ratio of isoenzymes CM-1/CM-2 varied by a factor of more than 20 when organismal tissue and cultured cells were compared, and this was a repeatable result. During exponential growth of cultured cells, secondary metabolism is minimal while demand for biosynthesis of aromatic amino acids for protein synthesis would be high. This seems consistent with the relatively high level of isoenzyme CM-1 and the diminished level of isoenzyme CM-2 observed. On the other hand, in the adult plant considerable metabolic output to secondary metabolism occurs. Again, this seems consistent with the elevated level of isoenzyme CM-2 and the decreased level of isoenzyme CM-1 found in organismal tissue.

Isoenzyme CM-2 has a much greater affinity for substrate than does isoenzyme CM-1, almost an order of magnitude. (Since this kinetic parameter is influenced by pH with isoenzyme CM-1, the difference increases as pH of catalysis is lowered.) It may be that isoenzyme CM-2 (in the cytosol) receives a low-concentration flow of chorismate that is reflective of low-flux carbon flow to secondary metabolites while isoenzyme CM-1 (in the chloroplast) intercepts a high-flux flow of carbon, the latter being consistent with the sensitive and delicately balanced pattern of end-product control observed for isoenzyme CM-1 (Goers and Jensen 1984).

Positive cooperativity of substrate binding at low pH. Isoenzyme CM-1 exhibits positive cooperativity with respect to chorismate binding only at low pH. At pH 6.1, the cooperativity of substrate binding is weak, as indicated by sigmoid-shaped substrate-saturation curves ($n=1.2$). In contrast, at pH 7.2, no positive cooperativity could be detected. Since pH also strongly influences the sensitivity of isoenzyme CM-1 to allosteric effectors (Goers and Jensen 1984), pH variation within the intracellular microenvironment may prove to be of physiological importance.

Positive cooperativity of substrate binding has commonly been observed for chorismate mutase in eukaryotic organisms, e.g., *Hansenula henricii* (Bode and Birnbaum 1978), *Claviceps paspeli* (Sprössler et al. 1970), *Euglena gracilis* (Weber and Böck 1970) and *Vigna radiata* (Gilchrist and Kosuge 1974). It seems likely that some organisms whose chorismate-mutase activity has been studied in detail only at a given pH might prove to exhibit positive cooperativity at another pH. For example, chorismate mutase (CM-1) of *Quercus pedunculata* exhibited Michaelis-Menten kinetics at pH 7.5, but the potential for positive cooperativity is indicated by a sigmoidal substrate-saturation curve that was observed in the presence of L-phenylalanine or L-tyrosine at pH 7.5 (Gadal and Bouyssou 1973). The exact influence of pH upon cooperativity of substrate binding differs in the three systems where adequate information exists, namely *E. gracilis*, *V. radiata* and *N. silvestris*. In *V. radiata* cooperativity decreased with pH (with $n=2.4$ at pH 7.5 and $n=1.8$ at pH 6.5). In *E. gracilis* cooperativity was distinct at pH 8.4 ($n=2.0$) but was completely lost at pH 7.0 ($n=1$). Isoenzyme CM-1 of *N. silvestris* exhibits low-pH enhancement of positive cooperativity in contrast to the high-pH enhancement of positive cooperativity seen in *E. gracilis* and *V. radiata*.

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