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Distinctive Enzymes of Aromatic Amino Acid Biosynthesis That Are Highly Conserved in Land Plants Are Also Present in the Chlorophyte Alga *Chlorella sorokiniana*

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Considerable enzymological diversity underlies the capacity for biosynthesis of aromatic amino acids in nature. For this biochemical pathway, higher plants as a group exhibit a uniform pattern of pathway steps, compartmentation, and catalytic, physical and allosteric properties of enzymes. This biochemical pattern of higher plants contains a collection of features which are completely different from photosynthetic prokaryotes such as the cyanobacteria. A unicellular representative of the chlorophyte algae, *Chlorella sorokiniana*, was found to be strikingly similar to higher-plant plastids in possessing the following distinctive enzymes: a Mn^{2+}-stimulated, dithiothreitol-activated isoenzyme of 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) synthase, a probable bifunctional protein competent as both dehydroquinase and shikimate dehydrogenase, an allosterically controlled isoenzyme of chorismate mutase, a highly thermotolerant species of prephenate aminotransferase, an $NADP^+$-dependent, tyrosine-inhibited arogenate dehydrogenase, and an arogenate dehydratase. In addition an isoenzyme of DAHP synthase shown in higher plants to be cytosolic, absolutely dependent upon the presence of divalent metals, and able to substitute other sugars for erythrose-4-phosphate, was also demonstrated in this alga. A broad-specificity 3-deoxy-D-*manno*-octulosonate 8-phosphate synthase, recently discovered in higher plants, is also present in this *Chlorella* species.

Keys words: L-Arogenate — Aromatic amino acid biosynthesis — *Chlorella sorokiniana* — Chlorophyte algae — Shikimate pathway.

In higher plants an entire complement of enzymes which specify aromatic amino acid biosynthesis is located within the plastid compartment (Jensen 1986). The common portion of the biosynthetic pathway consists of seven steps which thus far appear to be universal. These steps are performed by 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) synthase which catalyzes the condensation of erythrose-4-phosphate and phosphoenolpyruvate (PEP), dehydroquinate (DHQ) synthase, dehydroquinase, an NADPH-utilizing shikimate (SHK) dehydrogenase,

Abbreviations: ADH, arogenate dehydrogenase; ADT, arogenate dehydratase; AGN, L-arogenate; AroD·E, bifunctional protein exhibiting catalytic domains for dehydroquinase and shikimate dehydrogenase; AS, anthranilate synthase; CHA, chorismate; CM-1, plastid-localized isoenzyme of chorismate mutase in higher plants; CM-2, cytosolic isoenzyme of chorismate mutase; CM-G, species of chorismate mutase from *Chlorella* recovered in the gradient fractions following HA chromatography; CM-R, general designation for the class of chorismate mutase which includes CM-1, CM-W and CM-G and is monofunctional and subject to both allosteric activation by Trp and feedback inhibition by Tyr and Phe; CM-W, species of chorismate mutase from *Chlorella* recovered in the wash fractions following HA chromatography; DAHP, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate; DHQ, dehydroquinate; DS-Co, cytosolic isoenzyme of DAHP synthase; DS-Mn, plastid-localized isoenzyme of DAHP synthase; DTT, dithiothreitol; EPPS, *N*-(2-hydroxyethyl)-piperazine-*N'*-(3-propanesulfonic acid); HA, hydroxylapatite; KDOP, 3-deoxy-D-*manno*-octulosonate-8-phosphate; OPA, *ortho*-pthalaldehyde; PDT, prephenate dehydratase; PEP, phosphoenolpyruvate; PIPES, 1,4-piperazinediethanesulfonic acid; PLP, pyridoxal-5'-phosphate; PMSF, phenylmethylsulfonyl fluoride; PPA, prephenate; PPY, phenylpyruvate; SHK, shikimate.

SHK kinase, 3-enolpyruvyl shikimate 3-phosphate synthase, and chorismate (CHA) synthase. CHA, located at the major branchpoint position of the pathway, is utilized in one direction by anthranilate synthase (AS) to yield ultimately Trp and in another direction by chorismate mutase to produce prephenate (PPA). Subsequent steps to Phe and Tyr are not universal in nature. In higher plant plastids PPA is transaminated in the presence of an amino-donor substrate and pyridoxal 5'-phosphate (PLP) via PPA aminotransferase to produce L-arogenate (AGN). AGN is a precursor of both Phe and Tyr. Although some prokaryotes synthesize either Phe or Tyr from AGN (Byng et al. 1982), none (including cyanobacteria) have yet been found which possess AGN as the branchpoint of divergence to both endproducts. AGN dehydratase (ADT) eliminates water and CO_2 from AGN to produce Phe, while a NADP-dependent AGN dehydrogenase (ADH) eliminates CO_2 but retains the 4-hydroxy substituent to produce Tyr.

In addition to the variant pathway steps, other enzyme features have been found which are both unique and conserved in higher plants. Thus, a plastid-localized isoenzyme of DAHP synthase (DS-Mn) is stimulated by Mn^{2+} and displays hysteretic activation by dithiothreitol (DTT) (Ganson et al. 1986). DS-Mn has been shown to be feedback inhibited by AGN (Doong et al. 1993), a variation of a pattern of multi-circuit allosteric control called sequential feedback inhibition. Two catalytic domains present on AroD·E specify dehydroquinase and SHK dehydrogenase. This bifunctional protein is thus far unique to higher plants (Bonner and Jensen 1994). The plastid-localized isoenzyme of chorismate mutase (CM-1) is subject to a complex pattern of allosteric control, being feedback inhibited by Tyr and by Phe and activated by Trp. This three-effector type of chorismate mutase is not commonly found in prokaryotes, but exists in fungi such as *Neurospora* and *Saccharomyces*. Xia and Jensen (1992) have proposed CM-R as a general designation for this class of chorismate mutase. PPA aminotransferase in higher plants is highly distinctive because of its specificity for PPA and because it possesses an unusually high temperature optimum for catalytic activity of about 70°C. ADH is fairly widespread in prokaryotes, although it may sometimes utilize NAD^+ as an alternative or exclusive co-substrate (in contrast to the plant enzyme which always is $NADP^+$-specific). ADT is rarely found in prokaryotes, although the more common cyclohexadienyl dehydratase (able to use AGN and PPA as alternative substrates) can catalyze the same reaction (Byng et al. 1982).

Higher plant cells possess an isoenzyme of DAHP synthase denoted DS-Co which is located in the cytosol, requires a divalent metal for activity, and is insensitive to allosteric control. DS-Co exhibits a striking breadth of substrate recognition for sugars that can be substituted for erythrose-4-phosphate as a co-substrate with PEP (Doong et al. 1992). A cytosolic isoenzyme of chorismate mutase (CM-2), in contrast to CM-1, is unaffected by Phe, Tyr or Trp (Jensen 1986). It is known that both *Euglena* (Byng et al. 1981) and cyanobacteria (Hall et al. 1982) differ from higher plants and from one another in some features of enzymatic organization and regulation of aromatic amino acid biosynthesis (see Discussion). Thus, in an evolutionary context of consideration of the endosymbiotic hypothesis of plastid origin, it was of interest to ascertain the distribution of these pathway characteristics in a representative of the chlorophyte algae.

Materials and Methods

Organisms—When extracts were prepared from cultures using nitrate as the sole source of nitrogen, *C. sorokiniana* cells were cultured asynchronously in continuous light under conditions described by Prunkard et al. (1986). When the culture turbidity reached an A_{640} value of 6.4 (as determined in an 1 cm cuvette using a Bausch and Lomb Spectronic 70), the cells were harvested by centrifugation at $10,000 \times g$, washed twice with 250 ml of 0.01 M Tris-HCl at pH 7.5, and stored at −20°C as 2 g (fresh weight) pellets. When extracts were prepared from cultures using ammonium as the sole source of nitrogen, 29 mM ammonium chloride was used under conditions described by Bascomb et al. (1987).

Preparation of C. sorokiniana extracts—Extract preparation A. Frozen pellets of *C. sorokiniana* cultures were suspended 1 : 1 (w/v) in the extraction buffer (50 mM 1,4-piperazinediethane sulfonic acid (PIPES) buffer (pH 7.3) containing 1 mM DTT, 1 mM phenylmethyslulfonyl fluoride (PMSF) and 35 mM KCl), and were broken by 3 or 4 passes through a French pressure cell at 124.2 MPa. The slurry was first centrifuged at $35,000 \times g$ at 4°C for 15 min, and the supernatant was then centrifuged at $150,000 \times g$ for 1 h at 4°C. The supernatant thus obtained was either desalted on a PD10 Sephadex column (Pharmacia, Piscataway, NJ, U.S.A.) or dialyzed against the same PIPES buffer specified above. To assay activities of chorismate mutase, DAHP synthase, and 3-deoxy-D-*manno*-octulosonate 8-phosphate (KDOP) synthase in crude, unfractionated extracts, gel filtration on PD10 or dialysis was performed using 50 mM *N*-(2-hydroxyethyl) piperazine-*N'*-(3-propanesulfonic acid) (EPPS)-KOH buffer (pH 8.0) containing 1 mM DTT, 1 mM PMSF and 35 mM KCl. To assay activities of PPA aminotransferase, ADH, ADT, SHK dehydrogenase, and dehydroquinase in crude, unfractionated preparations, extracts were applied to PD10-Sephadex columns equilibrated with 50 mM EPPS-KOH at pH 8.6 containing 20% (v/v) glycerol and 0.1 mM PLP.

Extract preparation B. Chlorella cell pellets were suspended into 50 mM K-phosphate buffer (pH 7.25) containing 0.5% β-mercaptoethanol. Further preparation of ex-

tracts was the same as the method described above.

Extracts from the cells grown in nitrate-based medium contained 1.6 to 4.0 mg protein ml^{-1}, while extracts from the cells grown in ammonium-based medium contained 10–11 mg protein ml^{-1}. Protein was estimated by the method of Bradford (1976) using bovine serum albumin as a standard.

Column chromatography of C. sorokiniana extracts—DEAE-cellulose chromatography. Two DEAE-cellulose (DE-52 from Fisher Scientific, Orlando, FL, U.S.A.) columns were prepared to locate aromatic amino acid enzymes. Proteins were applied to columns equilibrated at 4°C with the extraction buffers. Column I (1.5 × 11 cm) was prepared and equilibrated in PIPES buffer before applying about 29 mg of protein (18 ml) from nitrate-grown *Chlorella* cells. To each fraction tube 0.1 mM PLP and 20% (v/v) glycerol were added to protect aminotransferases and dehydrogenases, respectively. The column was washed with 40 ml of the same buffer before 200 ml of a 35-to-500 mM KCl/PIPES gradient was applied. This wash was followed by addition of 1.0 M KCl/PIPES buffer. These fractions were assayed for all enzymes included in this study, except for the DAHP synthase isoenzymes. Eluate fractions having dehydroquinase, SHK dehydrogenase and chorismate mutase activities in the gradient were pooled and dialyzed against 5 mM K-phosphate buffer, 20% (v/v) glycerol, and 1 mM DTT at pH 7.2.

Column II (1.5 × 30 cm) was equilibrated with 50 mM K-phosphate (pH 7.25) and loaded with 175 mg of extract protein from the ammonium-grown *Chlorella* cultures. Following a 110-ml wash of the same buffer, a 200-ml gradient (0 to 1 M KCl in 50 mM K-phosphate) was applied.

Hydroxylapatite (HA) chromatography. An HA (Biogel HTP from BioRad, Rockville Center, NY, U.S.A.) column (0.6 × 10 cm) was equilibrated with 5 mM K-phosphate (pH 7.25) at 4°C. The 15-ml dialyzed sample from DEAE-cellulose column I was applied to the HA column. Following a 60 ml wash with buffer, a 125 ml gradient from 5-to-400 mM K-phosphate (pH 7.25) was applied.

Enzyme assays—Aminotransferases were assayed as described by Bonner and Jensen (1985). PPA aminotransferase was incubated with 20 mM L-glutamate and 0.8 mM PPA unless otherwise stated, and then the products were assayed by use of HPLC. Peaks of Phe were detected (as an ortho-pthalaldehyde (OPA) derivative) in methanol : 20 mM K-phosphate [6 : 4 (v/v)] before and after acid conversion (1 M HCl for 10 min at 37°C) of the product of the reaction (AGN) to Phe. Other keto-acid or amino-donor substrates tested in assays were included at 10 mM unless otherwise stated.

Chorismate mutase activity was assayed spectrophotometrically at 320 nm as described by Goers and Jensen (1984a). An extinction coefficient of 17,500 for phenylpyruvate (PPY) was used to calculate activity.

Dehydroquinase was assayed using a coupled assay with excess SHK dehydrogenase (Bonner and Jensen 1994). The reaction mixture contained 1.0 mM DHQ and 0.2 mM NADPH. The oxidation of NADPH was followed fluorometrically (340 nm excitation and 460 nm emission) and expressed as fluorometric units (Fu) min^{-1}. ADT was assayed by HPLC detection of OPA derivatives of the acid-converted product (Jung et al. 1986). PPA dehydratase (PDT) was assayed by measurement of PPY at alkaline pH (Fischer and Jensen 1987). An extinction coefficient of 17,500 for PPY was used for calculations.

For the assay of dehydrogenases, the formation of NADPH or NADH was followed fluorometrically and expressed as Fu min^{-1}. ADH (Bonner and Jensen 1987), PPA dehydrogenase and quinate dehydrogenase activities were assayed in the forward direction with 0.5 mM $NADP^+$ plus 0.5 mM AGN, 0.5 mM PPA or 1.0 mM quinate, respectively. SHK dehydrogenase activity was assayed in the reverse-of-physiological direction with 0.5 mM $NADP^+$ or NAD^+ and 4 mM SHK.

The selective assays which discriminate between DS-Mn, DS-Co, and KDOP synthase (Doong and Jensen 1992) were used.

Biochemicals—AGN (90% pure) and PPA (85% pure) were isolated from culture supernatants of a Tyr auxotroph of *Salmonella typhimurium* (Dayan and Sprinson 1970, Bonner et al. 1990). PPA was prepared as the barium salt and was converted to the potassium salt with excess K_2SO_4 prior to use. Lyophilized AGN was dissolved into 20 mM K-phosphate (pH 7.0). DHQ was chemically synthesized (Haslam et al. 1963). All other biochemicals were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.

Results

Detection of aromatic-pathway enzymes in crude extracts from Chlorella cells—Key enzymes at early-pathway, mid-pathway and late-pathway positions from cultures grown with nitrate as the source of nitrogen were assayed (Table 1). For comparison, the corresponding assays of exponential-phase cell cultures of *Nicotiana silvestris* are shown. Specific activities of enzymes from *Chlorella* were uniformly lower than those from *Nicotiana*. Selective assays have been developed to discriminate between isoenzymic species of DAHP synthase, DS-Mn and DS-Co, in higher plants (Doong and Jensen 1992). Both activities were detected, although DS-Mn activities were very low and varied non-linearly as a function of protein concentration. The activity ratio of SHK dehydrogenase and dehydroquinase (present as a bifunctional protein in higher-plant chloroplasts; Bonner and Jensen 1994) was 5-fold lower in *Chlorella* than in *Nicotiana*. Chorismate mutase activity was readily detected in *Chlorella* and potentially could represent the combined activities of the two isoen-

Table 1 Comparison of specific activities from unfractionated extracts[a] of *N. silvestris* and *C. sorokiniana*

Enzyme	Specific activity (nmol min^{-1} (mg protein)$^{-1}$)	
	N. silvestris	*C. sorokiniana*
DAHP synthase isoenzymes:		
DS-Mn (E4P/PEP)	29.9	16.2
DS-Co (glycolaldehyde/PEP)	341.7	40.0
Dehydroquinase	12.0	6.2
SHK dehydrogenase/NADP	60.4	6.0
Chorismate mutase	55.7	11.4
PPA aminotransferase	16.2	3.8
AGN dehydrogenase/NADP	1.1	ND[b]
AGN dehydrogenase/NAD	ND	ND
PPA dehydrogenase/NADP	ND	ND
PPA dehydrogenase/NAD	ND	ND
AGN dehydratase	0.6	ND
PPA dehydratase	ND	ND

[a] Crude extracts obtained from suspension cultures of *N. silvestris* were grown as detailed by Bonner et al. (1988). *Chlorella* cultures were grown as specified under Methods using nitrate as the source of nitrogen.
[b] ND, not detected.

zymes characteristic of higher plants (Goers and Jensen 1984a, b). At the post-PPA level of pathway function, PPA aminotransferase was detected, but not ADH or ADT. The

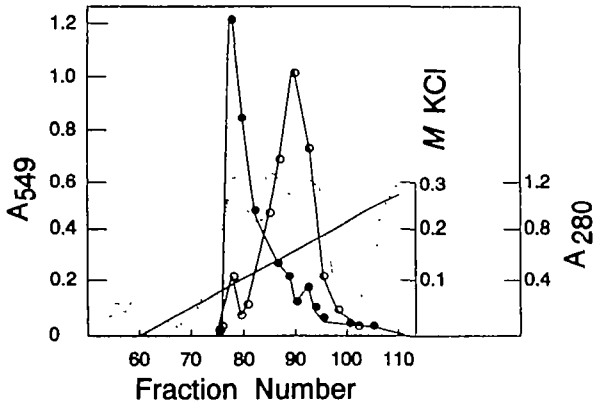

Fig. 1 Separation of DS-Mn and DS-Co by DEAE-cellulose chromatography. A column was prepared as described in Methods (column II) for elution of DAHP synthase isoenzymes from ammonium-grown *Chlorella* cells. DS-Co (●) and DS-Mn (○) were partially separated on this column, and fractions 78 (DS-Co) and 90 (DS-Mn) were used further to characterize the isoenzymes. The leading peak detected with the DS-Mn assay is KDOP synthase. The protein profile (···) was measured at A_{280} and the gradient is indicated by the straight line.

possible alternative steps of Phe (PDT) and Tyr (PPA dehydrogenase) biosynthesis (see Byng et al. 1982) were not detected in *Chlorella*.

When ammonium was used as the source of nitrogen, much higher yields of soluble protein per cell weight in initial extracts were obtained. Specific activity calculated for each enzyme shown in Table 1 was similar for both nitrogen sources, except that DS-Co, assayed with 4 mM glycolaldehyde, 3 mM PEP, and 20 mM Mg^{2+}, exhibited a specific activity of only 6.8 nmol min^{-1}, and DS-Mn was barely detectable (<0.3 nmol min^{-1}) when ammonium was the nitrogen source. The activities of DS-Mn in the latter extracts must have been largely masked by protease sensitivity or some inhibitor since substantial activities were recovered after DEAE-cellulose chromatography (Fig. 1).

The DS-Co/DS-Mn pair of isoenzymes—When discriminated by the selective assay procedures, DS-Co and DS-Mn could be seen to elute as partially separated peaks (Fig. 1). KDOP synthase, recognized by its ability to use erythrose-4-P in the absence of divalent metals (unlike DS-Co) and in the absence of DTT (unlike DS-Mn), was also located within the elution profile where its position overlapped that of DS-Co. The identity of KDOP synthase was confirmed by its 9-fold greater activity when arabinose-5-P was substituted for erythrose-4-P, by its low pH optimum, and by its insensitivity to EDTA (data not shown).

Peak fractions obtained from the DEAE-cellulose col-

Table 2 Effects of metals on DS-Mn and DS-Co activities in *Chlorella*

Metal	Concentration (mM)	Specific activity (nmol min^{-1} (mg protein)$^{-1}$)	
		Fraction 90[a]	Fraction 78[a]
None	—	24.6	—
$MgCl_2$	1.0	23.4	11.1
	5.0	23.9	34.2
	10.0	26.1	36.5
	20.0	25.4	41.8
$MnCl_2$	0.125	56.5	
	0.25	59.2	4.3
	0.50	62.0	3.1
	1.0	60.0	2.7
$CoCl_2$	0.125	4.0	
	0.25	2.4	10.5
	0.50	0.9	8.8
	1.0	0.2	8.3

[a] The source of DS-Mn was fraction 90 and the source of DS-Co was fraction 78 eluted from the DEAE-cellulose column shown in Fig. 1.

umn were used to characterize the differential properties of DS-Mn and DS-Co. DS-Mn was active in the absence of divalent metals and was unaffected by Mg^{2+} (Table 2). A greater than 2-fold stimulation was obtained by Mn^{2+}, while Co^{2+} was highly inhibitory (>99% inhibition at 1.0 mM Co^{2+}). On the other hand, DS-Co exhibited an absolute requirement for a divalent metal and was able to accept Mg^{2+}, Mn^{2+} or Co^{2+} as alternative activating metals. Mn^{2+} was the least effective metal. Whereas Co^{2+} was the most effective metal on a molar basis, the range of 10–20 mM Mg^{2+} is much more consistent with physiological conditions than is 0.25 mM Co^{2+}.

DS-Mn exists in an inactive form in the absence of DTT, and activation by DTT is slow relative to catalysis, i.e., hysteretic activation (Ganson et al. 1986). Figure 2 shows that hysteretic activation by DTT takes roughly 25 min at 37°C before DS-Mn achieves the catalytic rate of fully activated enzyme. Thus, in order to obtain conditions of proportionality with respect to time in various determinations of the characteristics of DS-Mn, it was important to use the activated species of enzyme.

Apparent K_m values were calculated for DS-Mn and DS-Co isoenzymes separated in the peak fractions of Fig. 1. DS-Mn exhibited K_m values of 0.67 mM and 0.83 mM for erythrose-4-P and PEP, respectively. The corresponding values for DS-Co were 3.20 mM and 1.43 mM. The K_m value of DS-Co for glycolaldehyde was 3.08. Similar to the higher-plant isoenzymes (Ganson et al. 1986), it was observed that the *Chorella* DS-Mn was sensitive

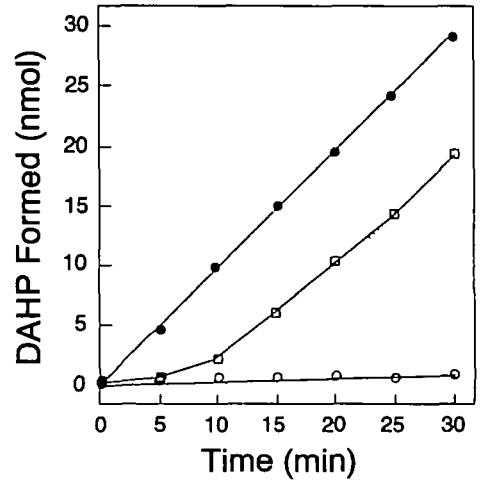

Fig. 2 DS-Mn and hysteretic activation by DTT. DS-Mn was assayed without addition of DTT (○), with 1.0 mM DTT added at zero time (□) and after pre-assay incubation of enzyme with 1.0 mM DTT for 30 min (●). Activation was complete after 25 min.

to substrate inhibition by erythrose-4-P, whereas DS-Co was not. Thus, all of the novel properties of DS-Mn, DS-Co and KDOP synthase noted in higher plants (Doong and Jensen 1992) are virtually identical in *Chlorella*.

The mid-pathway enzymes—Figure 3 shows that SHK dehydrogenase and dehydroquinase co-eluted from a DEAE-cellulose column. When the peak fractions were applied to an HA column (Fig. 4), co-eluting activities were again observed. This result is consistent with their possible co-existence as domains on a single bifunctional protein. Such a bifunctional protein occurs within the plastid com-

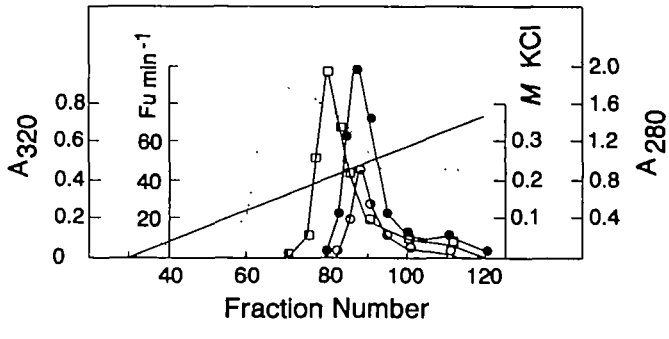

Fig. 3 DEAE-cellulose chromatography of aromatic pathway enzymes in *C. sorokiniana*. *Chlorella* extract was applied to a DEAE-cellulose column as described in Materials and Methods (column I). The first thirty wash fractions (no enzyme activities found) are not shown in the column profile. Enzyme activities for SDH (●), dehydroquinase (○) and chorismate mutase (□) are shown.

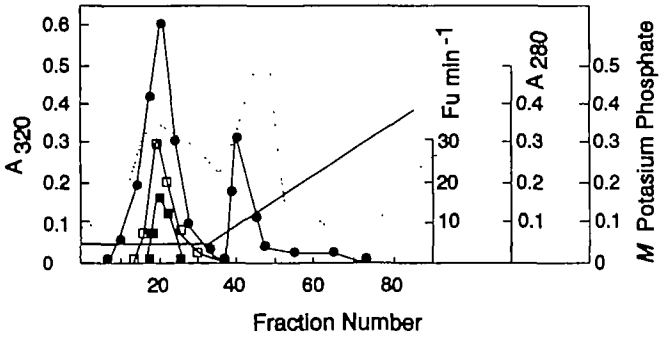

Fig. 4 HA chromatography. Fractions 75–95 from the DE-52 column were pooled and dialyzed for application onto an HA column as described in Methods. Chorismate mutase (●) eluted in the wash (CM-W) and in the gradient fractions (CM-G) at about 75 mM KPO_4. SDH (□) and DHQase (■) eluted in the wash fractions. Protein was monitored at A_{280} (····) and the gradient is denoted by the solid line.

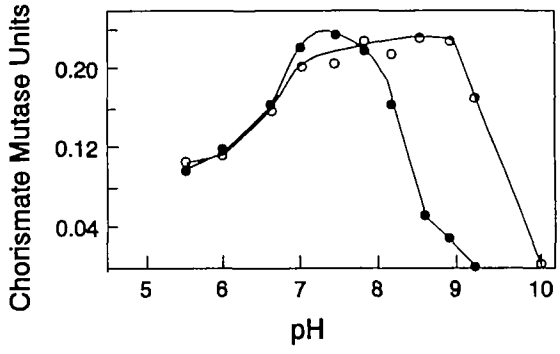

Fig. 5 Effect of Trp upon the pH profile of chorismate mutase. CM-W (Fig. 4) was assayed from pH 5.5 to pH 10 under standard chorismate mutase assay conditions (●) and with addition of 0.5 mM Trp (○).

partment of higher plants (Bonner and Jensen 1994).

A single peak of chorismate mutase was obtained (Fig. 3). Plastid-localized isoenzyme CM-1 of higher plants is highly sensitive to allosteric control (Goers and Jensen 1984a, b). Assays performed on the peak fractions in the presence of all possible combinations of Phe, Tyr and Trp (data not shown) suggested that most or all of this peak was CM-1. In order to examine the expected presence of at least a small amount of CM-2, the cytosol-localized and allosterically insensitive species of chorismate mutase, fractions 75–95 from the DE-52 column eluate were pooled and dialyzed prior to application to an HA column as described under Methods. Two species of chorismate mutase were indeed separated (Fig. 4), one of which migrated unretarded in the wash eluate (CM-W) and the other which eluted in the gradient (CM-G) at about 0.085 M KCl. However, both species exhibited similar properties of allosteric regulation (Table 3). Either Phe or Tyr alone was capable of causing complete inhibition. Although Trp alone had no effect on activity at the substrate concentrations used, it was effective in reversing inhibition caused by Phe and Tyr. Whether CM-W and CM-G might exhibit quantitatively different allosteric sensitivities was not examined. It seemed possible that the *Chlorella* counterpart of CM-2 in higher plants might be present in crude extracts, but labile to fractionation. In an attempt to detect a residual chorismate mutase activity that might be unaffected by endproducts, crude extracts were assessed for allosteric sensitivity to endproducts (i.e., CM-2). The results (Table 3) are consistent with the presence of a minor CM-2 species which did not survive fractionation, and which might constitute up to 2% of the total activity.

Trp is known to broaden the pH curve for CM-1 in plants such as *N. silvestris* (Goers and Jensen 1984b). Figure 5 shows that CM-W behaves the same way. At neu-

Table 3 Allosteric sensitivity of chorismate mutase

Effector[a]	Relative activities of chorismate mutase from:		
	CM-W	CM-G	Crude extract
None	100	100	100
Phe	0	0	22
Tyr	0	0	9
Trp	100	100	100
Phe+Tyr	0	0	2
Phe+Tyr+Trp	57	75	98

[a] Phe, Tyr, and Trp were present where specified at 10 mM, 5 mM, and 0.5 mM concentrations, respectively.

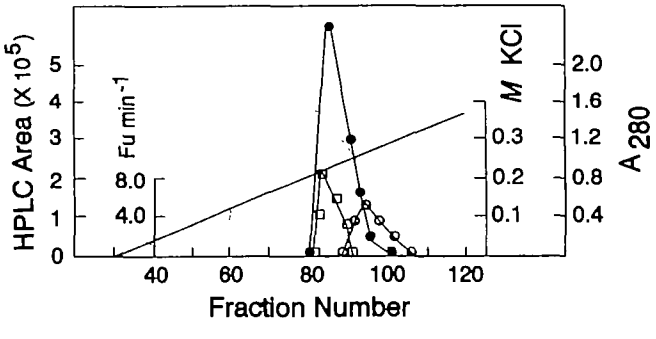

Fig. 6 Partial purification of post-PPA enzymes from *C. sorokiniana*. *Chlorella* extract was applied to a DEAE-cellulose column as described in Materials and Methods. The first thirty wash fractions (no enzyme activities found) are not shown in the column profile. Enzyme activities for PPA aminotransferase (●), ADH (□), and ADT (○) are shown.

Table 4 PPA aminotransferase activity after thermal treatment in *C. sorokiniana*[a]

Aminotransferase couple	No treatment	65°C 10 min	70°C 10 min	70°C 20 min
PPA/Glu	0.61	0.60	0.61	0.25
PPY/Glu	0.29	0	0	0

[a] Assays were incubated at 37°C for 10 min following thermal treatment. Concentrations of 1 mM PPA, 10 mM PPY, and 10 mM Glu were used in these assays.

tral pH and saturating substrates, Trp alone had no effect upon activity; however, it had a potent ability to reverse inhibition caused by Phe and/or Tyr. At pH values greater than 8.0, Trp was an increasingly potent activator. The effect at pH 9.0, for example, is very strong. Similar data were obtained for CM-G (not shown).

The post-PPA enzymes—Figure 6 shows the elution profiles of ADH, PPA aminotransferase, and ADT from a DEAE cellulose column. ADH required $NADP^+$ and did not accept NAD^+ (data not shown). It was feedback inhibited by Tyr (60% inhibition by 0.125 mM Tyr at 1.0 mM AGN). ADT was specific for AGN and did not use PPA as an alternative substrate. It was clearly sensitive to feedback inhibition by Phe, but the technically difficult quantitative analyses (Jung et al. 1986) were not carried out.

PPA aminotransferase utilized L-glutamate (10 mM) as the preferred amino donor in combination with 1 mM PPA, and peak fractions from the experiment of Fig. 6 yielded an activity of 0.8 nmol min^{-1}, compared to 0.4 nmol min^{-1} with 10 mM L-aspartate and 0.1 nmol min^{-1} with 10 mM Tyr. Other amino acids were ineffective amino donor substrates.

Table 4 shows that crude, unfractionated extracts exhibit activity with either PPA or PPY in combination with L-glutamate. The enzyme(s) responsible for the PPY/glutamate activity were completely abolished by thermal treatment at 65°C. In contrast, PPA aminotransferase activity resisted denaturation when treated at 70°C for 10 min. The profile obtained when the activity of PPA aminotransferase (obtained as shown in Fig. 6) was plotted as a function of temperature revealed a catalytic optimum of 70°C, with a profile nearly identical to that of *N. silvestris* (Bonner and Jensen 1985).

Discussion

All of the key enzymes sought in *Chlorella* as counterparts of established plastid-localized enzymes of aromatic amino acid biosynthesis in higher plants were found. The results indicate that *Chlorella* and higher plants utilize the same assemblage of enzymatic steps, probably possess a common bifunctional protein AroD·E, and share a common overall pattern of allosteric regulation. Of the key enzymes assayed, only the counterpart of higher-plant cytosolic CM-2 was not found. However, this isoenzyme was not always found when a number of higher plant species were surveyed (Singh et al. 1986). In one case (*Solanum tuberosum*) where CM-2 appeared to be absent, a more comprehensive reappraisal revealed its presence (Kuroki and Conn 1989). Indeed some evidence for a low level of CM-2 in crude extracts of *Chlorella* was obtained.

Higher plants were recently shown to possess a KDOP synthase which can be mistaken for DAHP synthase because of its ability to use erythrose-4-phosphate in place of arabinose-5-phosphate (Doong et al. 1991). The presence of KDOP synthase in *Chlorella* as well indicates an important role related to cell wall composition.

Table 5 shows a comparison among data obtained from *Chlorella* with that previously obtained from higher plants (Jensen 1986), *Euglena gracilis* (Byng et al. 1981) and cyanobacteria (Hall et al. 1982). *Euglena* possesses the same AGN-mediated routes to Phe and Tyr as higher plants and *Chlorella*, including the same pattern of allosteric regulation at the mid-pathway and terminal-pathway branchpoints. However, *Euglena* differs from higher plants and *Chlorella* in having a Tyr-regulated type of DAHP synthase instead of DS-Mn, and *Euglena* also differs in having a pentafunctional (steps 2–6) protein in the common pathway and a quadrifunctional (steps 2–5) protein in the Trp pathway. Cyanobacteria do not resemble the other phylogenetic groups in any of the distinctive character states analyzed. Although cyanobacteria do possess a $NADP^+$-dependent AGN dehydrogenase, it is insensitive to feedback inhibition by Tyr. Tyr exercises an indirect but potent form of feedback regulation by acting as a potent activator of PDT, a Phe-inhibited enzyme of Phe biosynthesis. DAHP synthase exhibits varied patterns of allosteric control in different phylogenetic subgroupings of cyanobacteria, but none of these resemble DS-Mn of higher plants and *Chlorella*.

Distinctive features of aromatic-pathway construction and regulation separate the cyanobacteria from all presently characterized representatives of green algae, higher-plant chloroplasts, and *Euglena*. The way that these individual features integrate to yield two completely different overall patterns of pathway regulation is shown in Fig. 7. Additional features which separate *Euglena* from green algae and higher-plant chloroplasts involve the regulation of DAHP synthase and a number of multifunctional proteins in the early-pathway and Trp-pathway segments (Table 5). These observations are consistent with evidence summarized by Bonen et al. (1979) that red algae chloroplasts arose via endosymbiosis of cyanobacteria, whereas chloroplasts of chlorophyte algae and higher

Table 5 Comparison of key character states of aromatic amino acid biosynthesis in photosynthetic organisms

Feature	Higher plants	*Chlorella*	*Euglena*	Cyanobacteria
Multifunctional proteins	AroD·E	AroD·E[a]	AroB·D·E·L·A TrpD·C·A·B	None
Branchpoint to Phe+Tyr	AGN	AGN	AGN	PPA
Precursor of Tyr	AGN	AGN	AGN	AGN
Precursor of Phe	AGN	AGN	AGN	PPY
Allosteric control points				
Early-pathway	DS-Mn	DS-Mn	DS-Tyr	Many[b]
Mid-pathway	CM-1[c]	CM-W/CM-G[d]	CM-R	Unregulated
Tyr branch	ADH	ADH	ADH	Unregulated
Phe branch	ADT	ADT	ADT	PDT
Trp branch	AS	?	AS	AS
PPA aminotransferase				
Present?	Yes	Yes	Yes	Yes
Thermotolerant?	Yes	Yes	?	No[e]
DS-Co present?	Yes	Yes	No	No
KDOP synthase present?	Yes	Yes	?	?

See text for higher plant, *Euglena* and cyanobacteria references. AroD·E refers to a bifunctional protein which catalyzes steps 3–4. The pentafunctional AROM protein of *Euglena* catalyzes steps 2–6 and is designated according to the monocistronic units of *E. coli* (i.e., AroB·D·E·L·A). The same convention is used to designate the quadrifunctional Trp-pathway protein (TrpD·C·A·B) which catalyzes the last four steps. Question marks indicate character states which have not been examined.

[a] Suggestive but not absolute evidence exists.

[b] Multiple patterns of DAHP synthase control exist (Hall et al. 1982).

[c] Synonymous with CM-R as defined by Xia and Jensen (1992).

[d] Both are synonymous with CM-R as defined by Xia and Jensen (1992).

[e] Unpublished data, Hall and Jensen.

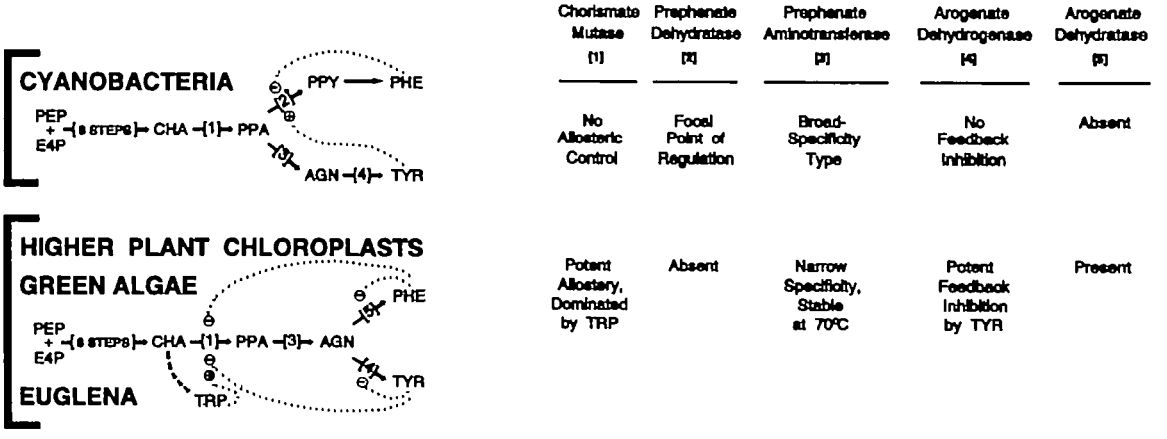

Fig. 7 Conserved character states of aromatic amino acid biosynthesis which distinguish cyanobacteria (top) from chlorophyte algae, higher-plant chloroplasts and *Euglena* (bottom). Enzymes identified by bracketed numbers in the table on the right are shown as they function in pathway operation at the left. The pathway is shown beginning with PEP and erythrose-4-phosphate (E4P) which initiates the common pathway leading to CHA, the mid-pathway point of branching to Trp and Phe/Tyr. In cyanobacteria PDT is the focal point of a competition between Phe and Tyr as inhibitor (⊖) and activator (⊕) agents, respectively. In the other organisms (bottom) enzymes [1], [4] and [5] are controlled by positive or negative allosteric effects as indicated.

plants arose from endosymbionts which might have been similar to present-day non-cyanobacterial oxygenic photosynthesizers such as *Prochlorothrix* (Bullerjahn et al. 1987). *Euglena* chloroplasts originated as an offshoot from a common ancestor of the higher plant/chlorophyte algae lineage. In this context it would be most intriguing to know the character states of aromatic biosynthesis in red algae plastids and in *Prochlorothrix*.

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