

# Purification and Characterization of Chorismate Synthase from *Euglena gracilis*<sup>1</sup>

## Comparison with Chorismate Synthases of Plant and Microbial Origin

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### ABSTRACT

Chorismate synthase was purified 1200-fold from *Euglena gracilis*. The molecular mass of the native enzyme is in the range of 110 to 138 kilodaltons as judged by gel filtration. The molecular mass of the subunit was determined to be 41.7 kilodaltons by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Purified chorismate synthase is associated with an NADPH-dependent flavin mononucleotide reductase that provides *in vivo* the reduced flavin necessary for catalytic activity. *In vitro*, flavin reduction can be mediated by either dithionite or light. The enzyme obtained from *E. gracilis* was compared with chorismate synthases purified from a higher plant (*Corydalis sempervirens*), a bacterium (*Escherichia coli*), and a fungus (*Neurospora crassa*). These four chorismate synthases were found to be very similar in terms of cofactor specificity, kinetic properties, isoelectric points, and pH optima. All four enzymes react with polyclonal antisera directed against chorismate synthases from *C. sempervirens* and *E. coli*. The closely associated flavin mononucleotide reductase that is present in chorismate synthase preparations from *E. gracilis* and *N. crassa* is the main difference between those synthases and the monofunctional enzymes from *C. sempervirens* and *E. coli*.

The three aromatic amino acids are synthesized via the shikimate pathway in microorganisms and plants (Fig. 1). Although the reaction sequence in the prechorismate pathway is identical in all organisms investigated so far, there are considerable differences in enzyme organization, as well as regulation, between organisms of different taxonomic groups. In *Escherichia coli*, the seven enzymatic activities of the prechorismate pathway are associated with monofunctional polypeptides. In contrast, in fungi the five enzyme activities

converting DAHP<sup>5</sup> to EPSP reside in a single pentafunctional polypeptide, the *arom* complex. In higher plants, two activities (3-dehydroquinase and shikimate dehydrogenase) form a bifunctional polypeptide, although the other five enzymes are monofunctional (reviewed in ref. 4). In *Euglena gracilis*, the organization of the prechorismate pathway appears to resemble that found in fungi. The first and the last steps are catalyzed by single enzymes (DAHP-synthase and chorismate synthase, respectively), whereas activities 2 to 6 form a large complex resembling the fungal *arom* complex (27). *Euglena* and fungi are strikingly similar in other biochemical pathways as well. Thus, both groups of organisms use the L- $\alpha$ -amino adipate pathway for lysine biosynthesis, whereas bacteria, algae, and plants follow the diaminopimelate route (30). On the basis of such findings, a close evolutionary relationship was proposed for euglenoids and fungi. But aromatic biosynthesis shows several features that, among all organisms studied so far, are unique for *E. gracilis*. Anthranilate synthase, the first enzyme in the tryptophan branch (Fig. 1), is monomeric in *Euglena*, whereas in all other anthranilate synthases characterized so far the chorismate and glutamine binding sites reside on distinct polypeptide chains (16). The synthesis of tryptophan from anthranilate is catalyzed by a single protein in *Euglena* (17), but in all other known examples tryptophan synthase is separable from the other activities of the tryptophan branch (17). Unlike other organisms, *E. gracilis* possesses only a single chorismate mutase isozyme, which is unusually large (31). In contrast to microorganisms, L-arogenate is the last common precursor of both phenylalanine and tyrosine in *Euglena* (7), which also appears to be the case for higher plants.

Chorismate synthase is the only shikimate pathway enzyme previously not detected in *E. gracilis*, except indirectly (6), which remained to be characterized. A comparison of the *Euglena* enzyme with chorismate synthases of other species is particularly interesting considering the data mentioned above.

Chorismate synthase (EC 4.6.1.4) catalyzes the formation

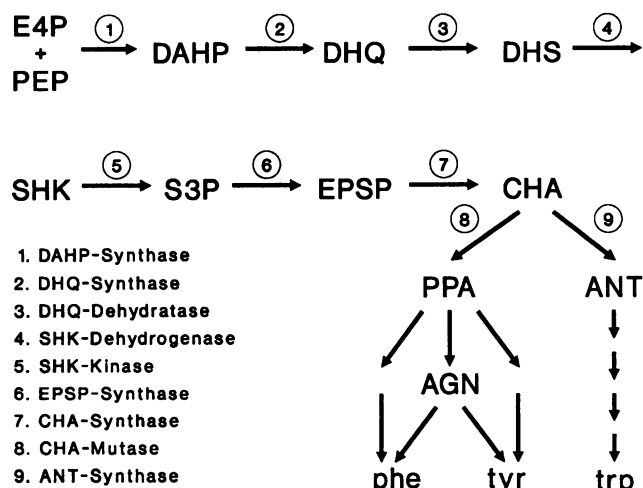
<sup>1</sup> Supported at Ruhr-Universität Bochum by a grant from the Deutsche Forschungsgemeinschaft and at the Swiss Federal Institute of Technology by ETHZ grant 07592/41–1030.5.

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<sup>5</sup> Abbreviations: DAHP, 3-deoxy-D-arabino-heptulosonate 7-phosphate; EPSP, 5-enolpyruvylshikimate 3-phosphate; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide.



**Figure 1.** Overview of the shikimate pathway. E4P, D-erythrose 4-phosphate; PEP, phosphoenolpyruvate; DHQ, 3-dehydroquinate; DHS, 3-dehydroshikimate; SHK, shikimate; S3P, shikimate 3-phosphate; CHA, chorismate; PPA, prephenate; AGN, arogenate; ANT, anthranilate.

of chorismate—the last common precursor for all three aromatic amino acids—by 1,4-elimination of orthophosphate from EPSP. The enzyme strictly requires a reduced flavin as cofactor, although no net change in redox state of the substrate is observed. Chorismate synthases have been purified and characterized from *E. coli* (25, 33), *Neurospora crassa* (32, 33), and *Bacillus subtilis* (18). Although chorismate synthase activity was only recently discovered in an extract from a higher plant (26), the enzyme has been isolated and characterized from a cell suspension culture of *Corydalis sempervirens* in our laboratory (28). Furthermore, a cDNA coding for *C. sempervirens* chorismate synthase has been cloned and analyzed (A. Schaller, J. Schmid, U. Leibinger, N. Amrhein, submitted for publication). Here we report the purification and characterization of chorismate synthase from *E. gracilis* as a representative of lower photoautotrophic eukaryotes, together with a more detailed analysis of the properties of the *C. sempervirens* chorismate synthase. The results are discussed in the context of data obtained for chorismate synthases isolated from *E. coli* and *N. crassa*. We find that chorismate synthase, unlike other shikimate pathway enzymes, is highly similar in different organisms. The main difference appears to be the presence of a flavin reductase activity that is associated with chorismate synthase in some fungi and *E. gracilis*.

## MATERIALS AND METHODS

### Strains and Cultures

*Euglena gracilis* strain 1224-5/25 was obtained from the German Collection of Microorganisms (Göttingen, FRG). It was grown in NL medium (5) either on plates or in liquid cultures of 25, 100, or 750 mL in Erlenmeyer flasks on a rotary shaker at 25°C under constant illumination with incandescent white light ( $100 \mu\text{E m}^{-2}\text{s}^{-1}$ ). The 750 mL cultures were used to inoculate a 20 L culture in an airlift fermenter.

Cells were harvested after 7 d (late logarithmic phase of growth) at a cell density of  $2 \times 10^6/\text{mL}$ . A cell suspension culture of *C. sempervirens* was grown as described previously (28). The *E. coli* strain AB2849 carrying the plasmid pGM602 (33) was a gift of Dr. J.R. Coggins (University of Glasgow, UK). It was grown in 2YT medium in the presence of ampicillin.

### Materials

All chemicals purchased from commercial sources were of the highest purity available. Chorismate was isolated and purified according to the method of Gibson (15). Shikimate 3-phosphate was prepared as in ref. 21 and EPSP was synthesized from shikimate 3-phosphate as described in ref. 22. Anthranilate synthase from *Aerobacter aerogenes* was purified following the procedure of Egan and Gibson (11) with the modifications described in ref. 28. Chorismate synthase was purified from *C. sempervirens* as described previously (28). Chorismate synthases were purified from *E. coli* about 400-fold and from *N. crassa* 260-fold essentially as described in ref. 33 with omission of the last chromatographic step. Specific enzyme activities were 48 and 6 nkat/mg, respectively. An antiserum was raised in rabbits against *C. sempervirens* chorismate synthase (28) and the immunoglobulin G fraction was purified on protein A beads (Pharmacia). The rabbit anti-*E. coli* chorismate synthase serum was a gift of Dr. J.R. Coggins.

### Assay of Chorismate Synthase Activity

All enzyme reactions were at 30°C unless otherwise stated. Chorismate synthase activity can be assayed either by detection of the reaction products (chorismate or phosphate) or by forward coupling to the reaction of anthranilate synthase and monitoring the anthranilate production. Throughout the enzyme purification, the discontinuous coupled assay was used, according to ref. 32 for *E. gracilis* and *N. crassa*, and according to ref. 28 for *C. sempervirens* and *E. coli*. The coupled assay was not used for the characterization of chorismate synthases, so as to exclude the influence of anthranilate synthase on the results. For characterization of the *E. gracilis* chorismate synthase, the formation of chorismate was followed at 274 nm using the assay system described in ref. 33. The assay mixture contained 50 mM triethanolamine/HCl, pH 8.0, 50 mM KCl, 2.5 mM  $\text{MgCl}_2$ , 83  $\mu\text{M}$  NADPH, 10  $\mu\text{M}$  FMN, and 80  $\mu\text{M}$  EPSP. This assay is not compatible with chorismate synthases that lack an intrinsic flavin reductase activity. When measuring the *E. coli* and *C. sempervirens* chorismate synthase activities, the flavin had to be reduced either chemically (5 mM dithionite) or by illumination (white incandescent light,  $750 \mu\text{E m}^{-2}\text{s}^{-1}$ ). Both treatments interfered, however, with the spectrophotometric assay of chorismate formation. Therefore, the rate of phosphate release was determined for the characterization of the plant enzyme as well as for the comparative studies with all four chorismate synthases. The reaction mixture (total volume 250  $\mu\text{L}$ ) contained 50 mM triethanolamine/HCl, pH 8.0, 10  $\mu\text{M}$  FMN, 2 mM DTT, 25 mM KCl, 320  $\mu\text{M}$  EPSP. The reaction was initiated after 10 min illumination (to reduce the FMN) by addition of EPSP. After 15 or 30 min under continuous white

light ( $750 \mu\text{E m}^{-2}\text{s}^{-1}$ ), free Pi was determined according to Lanzetta *et al.* (24).

### Purification of Chorismate Synthase from *Euglena gracilis*

All steps were carried out at 4°C unless otherwise stated.

#### 1. Protein Extraction and Precipitation

*E. gracilis* cells were harvested by centrifugation and resuspended in an equal volume of 100 mM Tris/HCl, pH 7.5, 5 mM EDTA, 1.2 mM PMSF, 1 mM DTT, and disintegrated by sonication. Cell debris was removed by centrifugation (30 min, 27,000 g). The resulting supernatant, designated crude extract, was subjected to fractionated ammonium sulfate precipitation from 35 to 65% saturation. The precipitate was collected by centrifugation (as above) and resuspended in a small volume of 40 mM Tris/HCl, pH 7.5, 5 mM EDTA, 1.2 mM PMSF, 1 mM DTT (buffer A). Then the solution was desalted by chromatography on Sephadex G-25 (Pharmacia, equilibrated in buffer A).

#### 2. DEAE-Sephacel Chromatography

The desalted protein solution was loaded onto a DEAE-Sephacel (Pharmacia) column (100 mL bed volume) pre-equilibrated in buffer A. The column was first washed with buffer A, then protein was eluted with a linear gradient (500 mL) of 10 to 270 mM KCl in buffer A (flow rate 1 mL/min, 10 mL fractions). Fractions containing chorismate synthase activity were pooled.

#### 3. Phenyl-Sepharose Chromatography

The combined fractions from the previous step were loaded directly onto a Phenyl-Sepharose column (Pharmacia, 40 mL bed volume), equilibrated in buffer A. Protein was eluted with a gradient (400 mL) of 0 to 30% (v/v) ethylene glycol (flow rate 0.8 mL/min, 10 mL fractions). Fractions containing chorismate synthase activity were pooled and concentrated by ultrafiltration (Amicon PM-30). Glycerol and DTT were added to a final concentration of 50% (v/v) and 1 mM, respectively. This preparation could then be stored for several months without any loss of chorismate synthase activity.

#### 4. MonoQ Chromatography

Chromatography on a MonoQ HR 5/5 Fast Protein Liquid Chromatography column (Pharmacia) was used as final step in chorismate synthase purification when the purified enzyme was to be used for biochemical characterization because of the high yield and speed of the method. The protein sample containing 50% (v/v) glycerol was applied to the column that had been pre-equilibrated in 20 mM Tris/HCl, pH 7.5 (buffer B). Protein was eluted using first a gradient (14 mL) of 0 to 230 mM KCl in buffer B, then a second gradient (8 mL) of 230 to 275 mM KCl in buffer B (flow rate 1 mL/min, 1 mL fractions). Combined fractions with chorismate synthase activity were desalted on Sephadex G-25 equilibrated in buffer B containing 1 mM DTT.

#### 5. Cellulose Phosphate Chromatography

In some cases, chromatography on cellulose phosphate (P11, Whatman) was used as the last purification step, replacing MonoQ chromatography. This step was more time consuming and gave a lower yield but resulted in a much higher purification and, therefore, was used when chorismate synthase was intended to be analyzed by PAGE. Ammonium sulfate was added to the combined fractions of step 3 up to 80% saturation. Precipitated protein was collected by centrifugation and resuspended in a small volume of 10 mM potassium phosphate buffer, pH 7.5, 5 mM EDTA, 1 mM DTT (buffer C), and desalted on Sephadex G-25 equilibrated in the same buffer. The sample was then loaded onto a cellulose phosphate column (2 mL bed volume) that had been pre-equilibrated in buffer C. The column was washed with 100 mM phosphate in buffer C; then chorismate synthase was eluted with 200 mM phosphate in buffer C. The eluate was dialyzed against 40 mM Tris/HCl, pH 7.5, 5 mM EDTA, 1 mM DTT, 50% glycerol, and subsequently stored at -20°C.

#### Determination of Molecular Mass and of Isoelectric Point

The molecular mass of native *Euglena* chorismate synthase was determined by gel filtration on an Ultropac TSK G3000SW HPLC column (LKB) pre-equilibrated with 20 mM Tris/HCl, pH 7.5, 100 mM KCl, at a flow rate of 0.5 mL/min. The molecular mass of chorismate synthase was determined in comparison with those of standard proteins (alcohol dehydrogenase, 150 kD; ovalbumin, 45 kD; carboanhydrase, 29 kD; Cyt c, 12.4 kD). The isoelectric point was determined by chromatofocusing on PBE 94 (Pharmacia, 11 mL bed volume), equilibrated in 25 mM imidazol/HCl, pH 7.4 or pH 6.8, 1 mM DTT, for chorismate synthases of *E. gracilis* and *N. crassa*, respectively. The protein sample was dialyzed against equilibration buffer prior to application. Protein was eluted by a linear pH gradient created by Polybuffer 74 (Pharmacia)/HCl, pH 4.0, 1 mM DTT, with a flow rate of 1 mL/min. Polybuffer 74 was used in eightfold dilution.

#### PAGE and Western Blot

SDS-PAGE was performed on 12.5% polyacrylamide slab gels using the buffer system described by Laemmli (23). Gels were silver stained after electrophoresis according to Ansoerg (1). Alternatively, proteins were transferred to nitrocellulose membranes using a semidry blotting apparatus (Millipore). Immunodetection of proteins on the blots was done at room temperature with 3% BSA in Tris-buffered saline as blocking agent. Goat anti-rabbit immunoglobulin G alkaline phosphatase conjugate obtained from Bio-Rad was used as secondary antibody.

#### Protein Concentration

Protein concentration was determined using the Bio-Rad protein assay following the manufacturer's instructions with BSA as a standard.

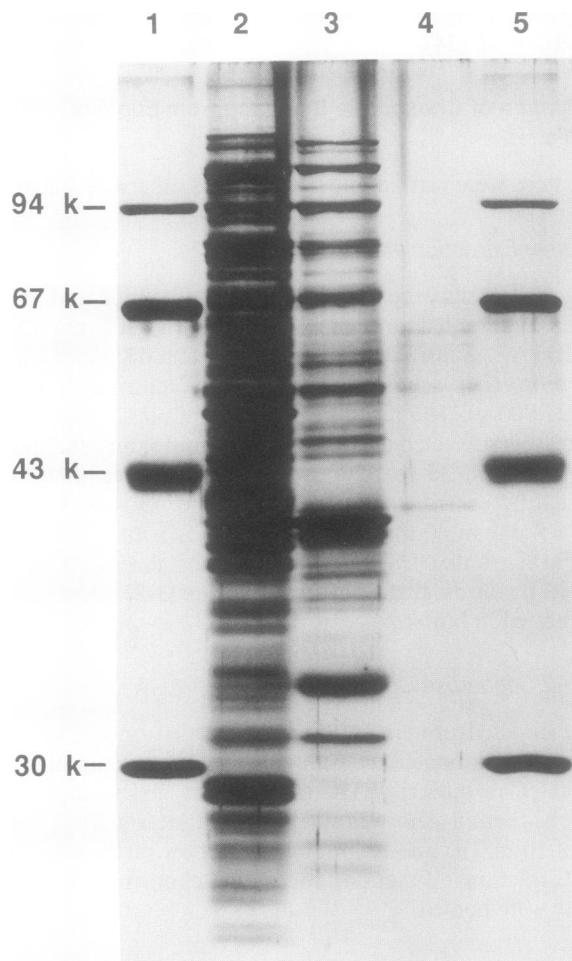
## RESULTS AND DISCUSSION

Purification of Chorismate Synthase from *E. gracilis*

The purification of chorismate synthase starting from 50 g (wet weight) of *E. gracilis* cells is summarized in Table I. Chorismate synthase activity was not readily detectable in crude extracts of *E. gracilis* due to the presence of low molecular weight inhibitory material. To determine chorismate synthase activity in the crude extract, this material could be removed by gel filtration and it was lost during ammonium sulfate precipitation. Chorismate synthase activity eluted at a conductivity of 9.7 msiemens/cm from DEAE-Sepharose, well separated from the bulk protein. Fractions containing chorismate synthase activity could then be applied directly to Phenyl-Sepharose. The major portion of proteins did not bind to the column. Chorismate synthase activity eluted at 5% (v/v) ethyleneglycol. Chromatography on MonoQ was the final step during routine enzyme preparation, resulting in a 269-fold enrichment of chorismate synthase with a yield of 54%. In this preparation, enzyme activity was linear both with time and with protein concentration in the enzyme assay, and therefore it was considered suitable for enzyme characterization. A better, about 1200-fold purification could be achieved at expense of the yield when chromatography on cellulose phosphate was performed as the final step in the purification procedure. But even this highly purified chorismate synthase preparation was not homogeneous as judged by SDS-PAGE (Fig. 2). There are still at least four different protein bands visible. One of them, with a  $M_r$  of 41,700, reacts with antisera directed against chorismate synthases of both *E. coli* and *C. sempervirens* (Fig. 3) and could thus be identified as *E. gracilis* chorismate synthase. The apparent molecular mass of the native enzyme was determined to be in the range of 110 to 138 kD by gel filtration (data not shown). The 41.7 kD polypeptide, therefore, is a subunit of native chorismate synthase. Chorismate synthases of other organisms are also composed of multiple subunits. White *et al.* (33) reported that chorismate synthases of *E. coli* and *N. crassa* are both homotetramers. Gaertner (13) observed at least two active forms of the enzyme in *N. crassa*, the homodimer and the homotetramer. In *C. sempervirens*, native chorismate synthase consists of at least two identical subunits (28). Native chorismate synthase in *Bacillus subtilis* is composed of three polypeptides carrying chorismate synthase, 3-dehydroquinate synthase, and a flavin reductase activity, respectively (18). The apparent molecular mass of native *E. gracilis* chorismate synthase

**Table I.** Chorismate Synthase Purification from *E. gracilis* Cells (50 g)

Purification Stage	Total Activity	Specific Activity	Recovery	Purification
	nkat	nkat/mg	%	-fold
Crude extract	27.4	0.013	100	1
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> precipitate	26.4	0.023	96	1.8
DEAE-Sepharose	30.5	0.182	111	14.1
Phenyl-Sepharose	23.3	0.899	85	69.7
MonoQ	15.0	3.474	54	269
Cellulose-phosphate	5.2	15.250	19	1173

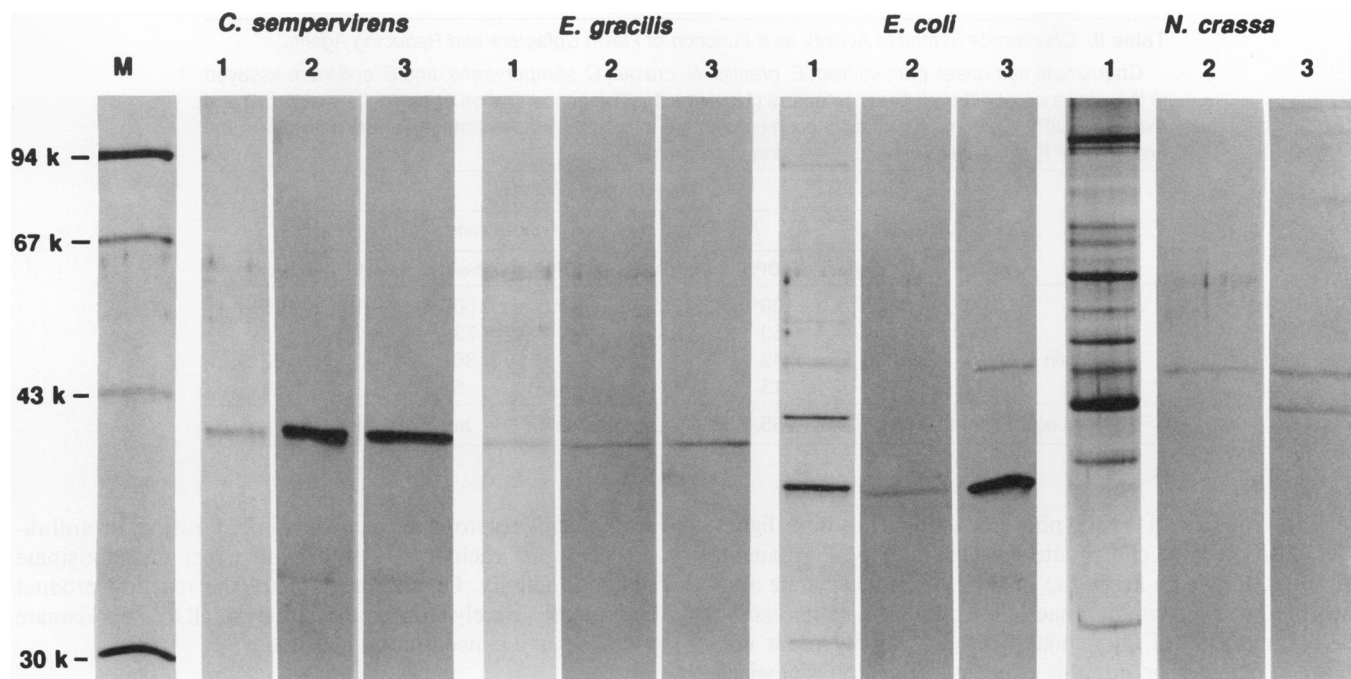


**Figure 2.** Silver-stained SDS-PAGE documenting the progress of chorismate synthase purification from *E. gracilis*. Lanes 1 and 5, protein standards (Low Molecular Weight Calibration Kit, Pharmacia). Lanes 2 to 4, combined fractions containing chorismate synthase activity after chromatography on DEAE-Sepharose (12  $\mu$ g of protein), Phenyl-Sepharose (6  $\mu$ g), and cellulose phosphate (0.8  $\mu$ g), respectively.

suggests either a homotrimeric structure of three 41.7 kD polypeptides or an oligomeric structure of different subunits. The latter possibility is supported by the observation that the  $M_r$  of 41,700 is similar to that of monofunctional chorismate synthases (41,900 and 39,138 in *C. sempervirens* [28] and *E. coli* [8], respectively). The flavin reductase activity, which is still present in the highly purified *Euglena* enzyme, may therefore be located on a different subunit.

## Cofactor Requirement of Chorismate Synthases

Chorismate synthase strictly requires a reduced flavin cofactor for enzymatic activity. For *in vitro* analysis, the reduced flavin has either to be supplied exogenously, as in *E. coli* (33) and *C. sempervirens* (28), or it can be generated by a flavin reductase activity that may be associated either covalently or noncovalently with chorismate synthase as described for *N. crassa* (32, 33) and *B. subtilis* (18), respectively. In *E. gracilis*,



**Figure 3.** Immunological cross-reactivity of chorismate synthases from different organisms. Lanes 1: silver-stained SDS-PAGEs of chorismate synthase purified from *C. sempervirens* (20 pkat/0.5  $\mu$ g), *E. gracilis* (15 pkat/1  $\mu$ g), *E. coli* (25 pkat/0.5  $\mu$ g), and *N. crassa* (12 pkat/5  $\mu$ g). Lanes 2 and 3: proteins detected on Western blots by polyclonal antisera directed against chorismate synthase from *C. sempervirens* and *E. coli*, respectively. Compared with lane 1, four times as much protein was loaded in lanes 2 and 3. Only in *C. sempervirens* (lane 2) and *E. coli* (lane 3), where homologous antisera were used, the protein amount was the same as in lanes 1. Lane M: protein standards (Low Molecular Weight Calibration Kit, Pharmacia).

there is a flavin reductase activity associated even with the highly purified chorismate synthase, which strictly depends on NADPH as a source of electrons for FMN reduction (data not shown). The four chorismate synthases that are the subject of the present investigation all show a very similar behavior when supplied with flavin derivatives exogenously reduced by 5 mM dithionite. Chorismate synthase activity is stimulated most effectively by FMN, which can be partially replaced by FAD and, to a lesser extent, also by riboflavin (Table II). When *E. gracilis* chorismate synthase is assayed with NADPH instead of dithionite as a reducing agent, *i.e.* by using the intrinsic flavin reductase activity, the specificity for FMN is much more pronounced. Under these conditions, FAD and riboflavin do not stimulate chorismate synthase to more than background activity (without addition of any flavin). We conclude from these data that the FMN specificity of chorismate synthase from *E. gracilis* reflects in fact the specificity of the flavin reductase. This was also reported for the bifunctional *N. crassa* enzyme by White *et al.* (33). In contrast with their observation, we could also detect activity of *N. crassa* chorismate synthase in the presence of only NADPH and FAD. This result may well be explained by the fact that our enzyme preparation was less pure than that used by White *et al.* (33), and that, thus, a second contaminating flavin reductase may have been present in our preparation that reduced FAD at the expense of NADPH. The flavin reductase that is noncovalently associated with chorismate synthase in *B. subtilis* accepts FMN and FAD as equivalent electron donors

(18). *B. subtilis* chorismate synthase has so far not been assayed independently of its flavin reductase.

#### Light Stimulation of Chorismate Synthase Activity

The reduction of a site in chorismate synthase, which has been suggested to be either Fe(III) (25) or a disulfide bond (32), appears to be a prerequisite for maintaining the active state of the enzyme. FMN appears to be involved in this process in conjunction with a flavin reductase activity. The flavin reductase is associated with chorismate synthase in *N. crassa*, *B. subtilis*, and *E. gracilis*. In *E. coli*, FMN is presumably reduced by an endogenous diaphorase activity (33). The *in vivo* source of reduced flavin for plant chorismate synthases is unknown. Because plant chorismate synthase activity is associated with the chloroplast compartment, a possible involvement of a photosynthetic electron transport chain component has been discussed (26). This would allow for the regulation of the shikimate pathway by light. A light stimulation of the overall activity of the shikimate pathway, *in vivo* or in isolated chloroplasts, has been reported earlier (19, 20), but light-regulated enzymes have not been identified yet (29). We found that light (white incandescent light, 750  $\mu$ E m<sup>-2</sup>s<sup>-1</sup>), in the presence of 1 mM DTT, can provoke chorismate synthase activity in the absence of a flavin reductant (Table III). This light stimulation of enzyme activity is most likely not a direct effect of light on the enzyme itself, but is rather due to photoreduction of FMN. We observed that only light

**Table II.** Chorismate Synthase Activity as a Function of Flavin Cofactors and Reducing Agents

Chorismate synthases purified from *E. gracilis*, *N. crassa*, *C. sempervirens*, and *E. coli* were assayed in the presence of different flavin cofactors (10  $\mu\text{M}$  each). The concentration of dithionite was 5 mM and that of NAD(P)H 200  $\mu\text{M}$ . Activities in each column are expressed as percentages of the activities in the presence of FMN. All experiments were done in triplicate.

Flavin	Chorismate Synthase Activity							
	<i>E. gracilis</i>		<i>N. crassa</i>		<i>C. sempervirens</i>		<i>E. coli</i>	
	NADPH	Dithionite	NADPH	Dithionite	NADPH	Dithionite	NADH	Dithionite
FMN	100 <sup>a</sup>	100 <sup>b</sup>	100 <sup>c</sup>	100 <sup>d</sup>	— <sup>e</sup>	100 <sup>f</sup>	— <sup>e</sup>	100 <sup>g</sup>
FAD	29	84	63	84	—	73	—	73
Riboflavin	26	39	49	14	—	36	—	22
None	25	33	13	15	—	5	—	9

<sup>a-g</sup> 100% is equivalent to <sup>a</sup> 103, <sup>b</sup> 270, <sup>c</sup> 255, <sup>d</sup> 381, <sup>e</sup> not detectable, <sup>f</sup> 364, and <sup>g</sup> 584 pkat/mL.

qualities capable of FMN photoreduction (*i.e.* blue light) could also promote chorismate synthase activity. Permanent illumination, which keeps the FMN in its reduced state and thus produces essentially anaerobic conditions, is necessary for maintenance of chorismate synthase activity (data not shown). Whether or not this effect is of any physiological relevance is not clear. The fact that *E. coli* chorismate synthase also is stimulated by light to some extent may argue against a direct physiological role of light in the activation of chorismate synthase. On the other hand, free flavins have been proposed to act as primary photoreceptors in the photoreactivation of *N. crassa* nitrate reductase (12). On the basis of our data, we cannot clearly assign to chorismate synthase a function in the regulation of the shikimate pathway.

Using light as reducing power, it was now possible to assay the activity of the monofunctional chorismate synthases without forward coupling of the reaction to anthranilate synthase (see "Materials and Methods"). This assay system, which dispenses with the highly tryptophan-sensitive anthranilate synthase, allowed us to test a possible influence of aromatic amino acids on chorismate synthase activity. In isolated spinach chloroplasts, each of the aromatic amino acids was shown to exert strict feedback inhibition of its own synthesis (2). Chorismate synthase activity, however, was not affected by end products of the pathway (Table IV). None of the aromatic

amino acids tryptophan, phenylalanine, tyrosine, or anthranilate (1 mM each) had a significant effect on chorismate synthase activity. On the other hand, the reaction product chorismate severely reduced the activity of all four chorismate synthases at a concentration of 1 mM.

### Kinetic Properties

Apparent  $K_m$  values were deduced from steady-state kinetic analyses. In all cases, triethanolamine/HCl buffer was used at a pH of 8.0, which is close to the pH optima of the known chorismate synthases (8.0, 8.2, and 8.1 for *C. sempervirens*, *E. gracilis*, and *N. crassa*, respectively). Figure 4 shows the Michaelis-Menten and linearized plots from which the apparent  $K_m$  values were calculated for the *C. sempervirens* and *E. gracilis* enzymes. The apparent  $K_m$  values for EPSP were 53 and 27  $\mu\text{M}$ , and those for FMN were 37 and 76 nM for the *C. sempervirens* and *E. gracilis* chorismate synthases, respectively. The *N. crassa* enzyme has a comparably high affinity for FMN (data not shown), whereas the affinity of the *B. subtilis* chorismate synthase (18) is much lower (66 nM and 12.5  $\mu\text{M}$ , respectively). An apparent  $K_m$  for EPSP of 2.7  $\mu\text{M}$  was reported earlier (3) for *N. crassa* chorismate synthase, although we calculated a value of 7  $\mu\text{M}$ . With the exception

**Table III.** Stimulation of Chorismate Synthase Activity by Light

Chorismate synthases purified from *E. gracilis*, *N. crassa*, *C. sempervirens*, and *E. coli* were assayed under continuous illumination (750  $\mu\text{E m}^{-2} \text{s}^{-1}$  white incandescent light), which could partially replace the reducing agents dithionite (5 mM) or NADPH (200  $\mu\text{M}$ ). The activity is given as percentages of the value obtained in the presence of 5 mM dithionite. All experiments were done in triplicate.

Reducing Agent	Chorismate Synthase Activity			
	<i>E. gracilis</i>	<i>N. crassa</i>	<i>C. sempervirens</i>	<i>E. coli</i>
Dithionite, dark	100 <sup>a</sup>	100 <sup>b</sup>	100 <sup>c</sup>	100 <sup>d</sup>
NADPH, dark	69	71	0	0
None, dark	0	0	0	0
None, light	50	42	54	27

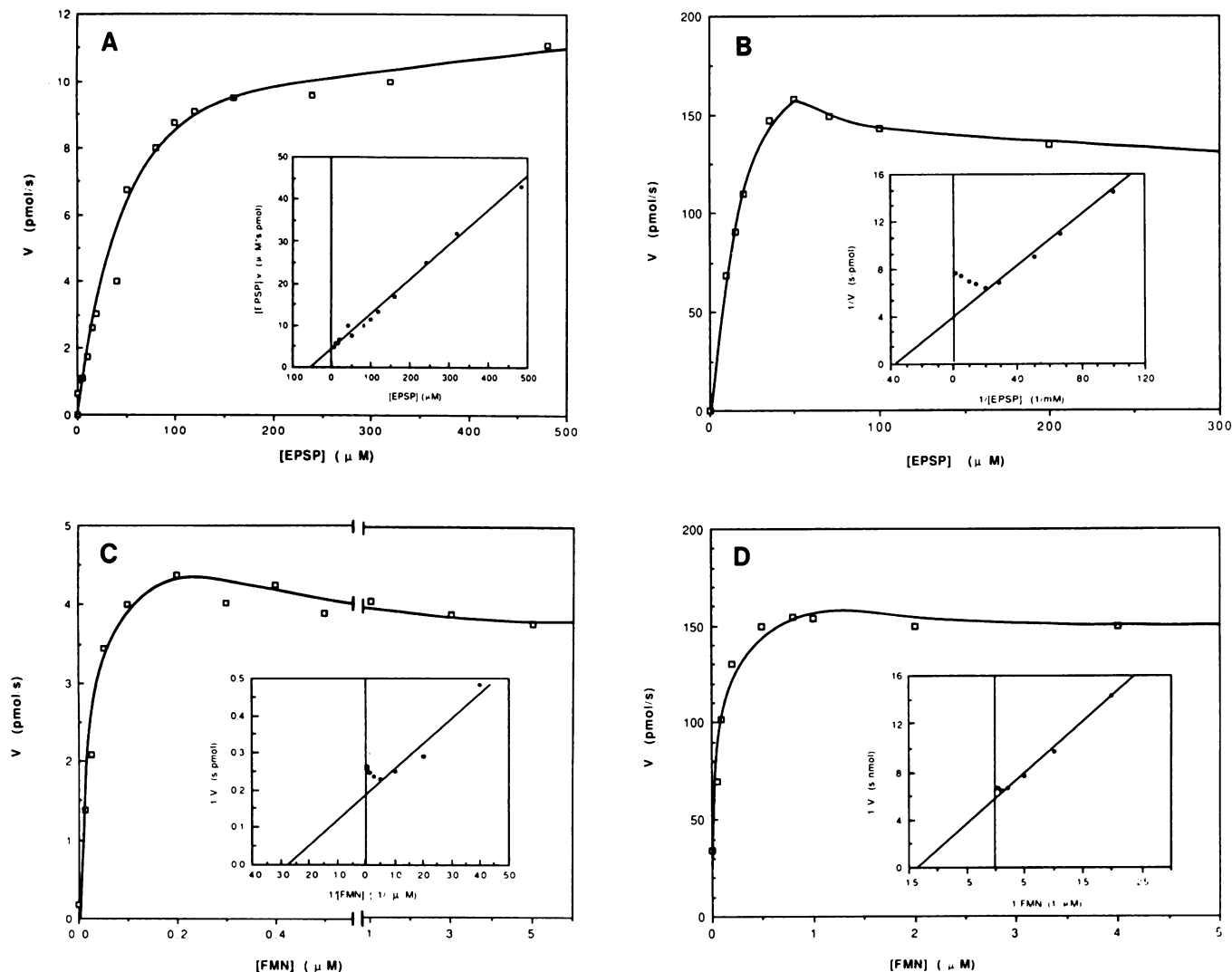
<sup>a-d</sup> 100% is equivalent to <sup>a</sup> 272, <sup>b</sup> 387, <sup>c</sup> 328, <sup>d</sup> 445 pkat/mL.

**Table IV.** Effect of Aromatic Amino Acids on Chorismate Synthase Activity

Chorismate synthases purified from *E. gracilis*, *N. crassa*, *C. sempervirens*, and *E. coli* were assayed in the presence of aromatic amino acids and chorismate (1 mM each). The activity is given as percentages of the value obtained in the photochemical assay without addition of any effector. All experiments were done in triplicate.

Amino Acid	Chorismate Synthase Activity			
	<i>E. gracilis</i>	<i>N. crassa</i>	<i>C. sempervirens</i>	<i>E. coli</i>
Tryptophan	105 <sup>a</sup>	108 <sup>b</sup>	108 <sup>c</sup>	108 <sup>d</sup>
Phenylalanine	102	108	109	98
Tyrosine	102	105	91	96
Anthranilate	112	93	99	105
Chorismate	35	16	36	31

<sup>a-d</sup> 100% is equivalent to <sup>a</sup> 871, <sup>b</sup> 826, <sup>c</sup> 1067, <sup>d</sup> 972 pkat/mL.



**Figure 4.** Determination of apparent  $K_m$  values for EPSP and FMN. Enzyme activity is plotted versus EPSP and FMN concentrations for *C. sempervirens* (A, C) and *E. gracilis* (B, D) chorismate synthases, respectively. The inserts show the corresponding linearized plots, which were used for determination of the apparent  $K_m$  values.

of the *B. subtilis* enzyme, chorismate synthases show similar affinities for EPSP and FMN in all organisms from which data are available. High concentrations of FMN were inhibitory for all chorismate synthases investigated here. An inhibitory effect of high EPSP concentrations was observed for *E. gracilis* and *N. crassa* chorismate synthases.

#### Immunological Relationships between Chorismate Synthases

Polyclonal antisera directed against the chorismate synthases of *C. sempervirens* and *E. coli* were used to detect chorismate synthases of different species on Western blots (Fig. 3). Both antisera react with chorismate synthase of *E. gracilis*, as mentioned above, but they also recognize the other chorismate synthases under investigation. Chorismate synthases, or rather the domains of the polypeptides carrying

chorismate synthase activity (in the case of bifunctional enzymes), therefore seem to be highly similar in organisms that are widely apart in evolutionary terms. Whereas the interpretation in quantitative terms of differing intensities of stained protein bands may be deceiving, we wish to point out that the antiserum directed against plant chorismate synthase gives bands of equal intensity with the three other chorismate synthases, although the amount of chorismate synthase protein present on the gels seems to be by far the smallest in case of *E. gracilis* as judged by the silver-stained lanes. This may indicate a closer relationship between the chorismate synthases of *Corydalis* and *Euglena* on the one hand than between the *Corydalis* enzyme and the bacterial and fungal chorismate synthases on the other hand. It is at present not possible to specify the immunological relationship more precisely because we cannot be sure of the amount of antigen present in each reaction. Similar amounts were loaded onto



**Table V.** Synopsis of Chorismate Synthase Properties

The data presented in the "Results and Discussion" section are summarized. Numbers in parentheses indicate the reference for data published elsewhere.

	<i>E. gracilis</i>	<i>N. crassa</i>	<i>C. sempervirens</i>	<i>E. coli</i>
Apparent $K_m$ (EPSP)	27 $\mu\text{M}$	7 (2.7) $\mu\text{M}$ (3)	53 $\mu\text{M}$	n.d. <sup>a</sup>
Apparent $K_m$ (FMN)	76 nM	66 nM	37 nM	n.d.
Cofactor specificity	FMN > FAD	FMN > FAD	FMN > FAD	FMN > FAD
Flavin reductase	Present	Present (32)	Absent (28)	Absent (33)
Apparent $K_m$ (NADPH)	7.4 $\mu\text{M}$	21.4 $\mu\text{M}$		
Mol. mass (native)	110–138 kD	198 kD (33)	80.1 kD (28)	144 kD (33)
Mol. mass (denatured)	41.7 kD	50 kD (33)	41.9 kD (28)	39.1 kD (8)
No. of subunits	n.d.	2 or 4 (13, 33)	2 (28)	4 (33)
Isoelectric point	5.5	4.9	5.0 (28)	n.d.
pH optimum	8.2	8.1	8.0	8.0–8.5 (25)

<sup>a</sup> Not determined.

the gel in terms of enzyme activity, but the specific activities of the preparations differed. Also, the intensity of the silver stain may not accurately reflect the actual amount of protein present, because not all proteins stain equally in this procedure.

The immunological cross-reactivity of all four chorismate synthases indicates that antigenic determinants of this enzyme were highly conserved during evolution. Also, the primary structures of chorismate synthases turn out to be highly similar throughout species. There is 48% sequence identity between the enzymes from *C. sempervirens* and *E. coli* (A. Schaller, J. Schmid, U. Leibinger, N. Amrhein, submitted for publication) and 53% between *C. sempervirens* and *Saccharomyces cerevisiae* (D. Jones, G. Braus, personal communication). EPSP synthase appears to be the only other enzyme in the prechorismate pathway that shows a similar degree of conservation (54% identity between EPSP-synthases of petunia and *E. coli* [14]). In contrast, the individual domains of the *arom* complex from *S. cerevisiae* are only between 21 and 38% identical to the respective corresponding monofunctional *E. coli* enzymes (9), and potato DAHP-synthase shows only as little as 22% identity with the three *E. coli* DAHP-synthases (10). Clearly, until sequences of other enzymes of the pathway become available for comparison, it is too early for a generalization.

## CONCLUSIONS

We have shown here that chorismate synthases from *E. gracilis*, *C. sempervirens*, *N. crassa*, and *E. coli*, i.e. of species representing four distinct taxonomic phyla, must be considered very similar proteins (see synopsis in Table V). The enzyme from *B. subtilis*, which was not included in the present investigation, seems to be somewhat different, however (18). The four chorismate synthases listed in Table V resemble each other very much in their cofactor specificities, kinetic properties, isoelectric points, and pH optima. Also, the structures of the enzymes must be similar as judged from the possession of common antigenic determinants as well as from sequence comparisons. In the prechorismate pathway, conservation of the amino acid sequences, although to a much lesser extent, has also been observed between the individual

monofunctional *E. coli* enzymes and the corresponding domains of the fungal *arom* complex (9). These enzymes, therefore, appear to have originated from common ancestors, but presumably at some later stage during evolution the organizational forms of the pathway diverged. Enzymes were clustered to different degrees in bacteria, plants, fungi, and euglenoids. Enzyme organization also appears to be the major difference between the chorismate synthases of these organisms. Chorismate synthase can either be monofunctional, as in plants (26, 28) and in many bacteria (33), or it can be associated with a flavin reductase activity, as in *N. crassa* (13, 33) and *B. subtilis* (18). Chorismate synthase from *E. gracilis* was also found to be bifunctional. Whether or not the flavin reductase in *E. gracilis* is associated covalently with the chorismate synthase remains to be elucidated. As outlined in the introduction, a common feature of *Euglena* and higher fungi, in contrast with plants and bacteria, is the high degree to which different enzyme activities are aggregated. Chorismate synthase appears to fit into this scheme.

## ACKNOWLEDGMENT

We wish to thank Professor J.R. Coggins (University of Glasgow, UK) for the kind gift of the strain *E. coli* AB2849/pGM602 and of a polyclonal rabbit antiserum directed against *E. coli* chorismate synthase.

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