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CHORISMATE MUTASE OF *CHLAMYDOMONAS REINHARDI*

PARTIAL PURIFICATION AND SOME PROPERTIES

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Summary

Chorismate mutase (chorismate pyruvatemutase, EC 5.4.99.5) was extracted from *Chlamydomonas reinhardi* by sonication. Fractionation of crude sonic extracts with $(NH_4)_2SO_4$ and by DEAE-cellulose and Sephadex gel chromatography indicated a single peak of chorismate mutase activity with molecular weight 61 000. The Michaelis constant for 20-fold purified enzyme was 0.46 mM. Prephenate dehydrogenase (EC 1.3.1.9) and prephenate dehydratase (EC 4.2.1.40) activities were not detected in our crude or partially purified preparations of chorismate mutase.

Tyrosine (1.25 mM) inhibited chorismate mutase activity by approx. 85% in crude and partially purified preparations. Phenylalanine (1.25 mM) inhibited 20%. Tryptophan (1.25 mM) by itself had no detectable effect on chorismate mutase activity but it completely reversed inhibition by tyrosine and phenylalanine. No repression of chorismate mutase was observed when the minimal growth medium was supplemented with aromatic end products.

Introduction

Little is known of the regulation of algal biosynthetic enzymes. This paper reports the first investigation on the control of biosynthesis of the aromatic amino acids and vitamins in the unicellular chlorophycete *Chlamydomonas reinhardi*, a species chosen for its ease of culture and genetic manipulation. Chorismate mutase was selected as the starting point for this work as it is the first enzyme specific to phenylalanine and tyrosine biosynthesis in a number of organisms [1,2] (Fig. 1). It was therefore expected to be subject to negative feedback control by these amino acids. It is also a relatively easy enzyme to assay and appeared stable and present in fairly high concentration in *C. reinhardi*, thus facilitating its extraction and purification.

A brief abstract of this work has appeared earlier [3].

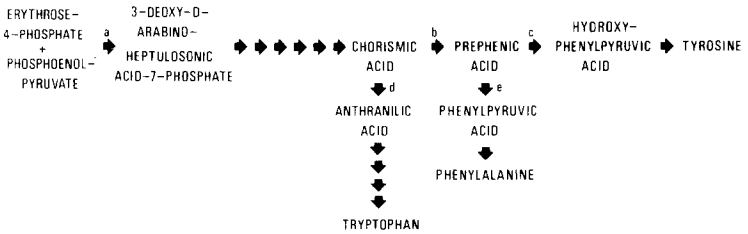

Fig. 1. An outline of aromatic amino acid biosynthesis. Enzymes: a, 3-deoxy-D-arabinoheptulosonic acid 7-phosphate synthetase; b, chorismate mutase; c, prephenate dehydrogenase; d, anthranilate synthetase; e, prephenate dehydratase.

Materials and Methods

Organisms

The organism used in this work was *C. reinhardi* C. 137 (+) provided by P.M. Gresshof.

Chemicals

Chorismic acid was prepared by the method of Gibson [4]. Amino acids were the L-isomers of the highest grade commercially obtainable. $(NH_4)_2SO_4$ (A.R. grade, Ajax Chemical Co., Australia) DEAE-cellulose (Sigma, St. Louis, Mo., U.S.A.) and Sephadex G-100 and G-200 (Pharmacia) were used for enzyme fractionation. Protein standards used to calibrate gel filtration columns for molecular-weight determinations were cytochrome *c* type III (horseheart), ribonuclease A (bovine pancreas), eggwhite lysozyme Grade 1 (all from Sigma), pepsin (swine stomach; Worthington Biochem. Corp., U.S.A.) and bovine plasma albumin (Calbiochem). All other chemicals used were of analytical reagent grade.

Buffers

Buffer I was 0.01 M KH_2PO_4/NaOH, pH 7.4. Buffer II was 0.1 M KH_2PO_4/NaOH, pH 7.4.

Growth of cells

Basal growth medium was the Tris/acetate/phosphate medium of Gorman and Levine [5]. This was supplemented as indicated. Cells were grown in 250-ml Erlenmeyer flasks containing 100 ml of medium. Flasks were incubated at 25° C in a Gallenkamp reciprocating shaker (4 cm stroke length; shaking rate 110 strokes per min) and illuminated by four 20-W "Biolux" fluorescent tubes at a distance of approx. 67 cm above the surface of the medium. The mean generation time under these conditions was 8.4 h.

Preparation of crude extracts

Cells in exponential phase were harvested by centrifugation at 1000 × *g* for 10 min at 4° C then washed once in cold Buffer II. The washed cells were resuspended in Buffer I, held in an ice/alcohol bath and sonicated for 5 min (Biosonik II (Bronwill) sonicator). Cell debris was removed by centrifugation at

48 000 × *g* for 30 min and the supernatant dialysed against 100 vol. of Buffer I for 1 h at 0°C then changed with the same buffer and dialysed for a further 1 h.

Enzyme assays

Chorismate mutase and prephenate dehydratase were assayed by the method of Cotton and Gibson [6]. Prephenate dehydrogenase was assayed by the method of Dayan and Sprinson [7]. The temperature of enzyme reaction mixtures was 25°C. All enzyme activities are reported in international units (μmol product formed/min).

Protein assay

Proteins were determined by the method of Lowry et al. [8] with bovine plasma albumin (Calbiochem) as standard.

Concentration of enzyme solutions

Solutions containing enzyme were concentrated by vacuum dialysis in colloidin tubes (Sartorius membrane filter SM13200) against Buffer II.

$(NH_4)_2SO_4$ fractionation of crude extracts

Dialysed crude extracts were treated with protamine sulphate (final concentration, 0.75%) in an ice/alcohol bath and the precipitate removed by centrifugation at 48 000 × *g* for 20 min. An appropriate amount of crystalline $(NH_4)_2SO_4$ was slowly added to the supernatant with stirring. Each fraction was collected by centrifugation at 48 000 × *g* for 20 min, dissolved in 1 ml Buffer II and dialysed against 200 vol. of Buffer II for 1 h.

Gel-filtration on Sephadex G-100

The protein which precipitated between 0.3—0.4 fractional saturation $(NH_4)_2SO_4$ (2 ml) was layered onto a Sephadex G-100 column (1.5 cm × 40 cm) and eluted with Buffer I. 2-ml fractions were collected. Void volume was 22 ml. A single peak of chorismate mutase activity was eluted with a maximum at Fraction 17. Fractions 15—21 were pooled and concentrated to 1.5 ml.

Gel filtration on Sephadex G-200

The fractions pooled after G-100 chromatography were layered onto a Sephadex G-200 column (1.5 cm × 40 cm) and eluted with Buffer I. 2-ml fractions were collected. Void volume was 26 ml. A single peak of chorismate mutase activity was eluted with a maximum at Fraction 21. Fractions 19—23 were pooled for subsequent analysis.

Results

Chorismate mutase activity in crude extracts of C. reinhardi

Chorismate mutase activity was found in crude extracts. The enzyme activities reported were measured under conditions of linearity with respect to time and protein concentration. In general the duration of the assay was 20 min. Activity was unaffected by addition of EDTA (1 mM), dithiothreitol (1

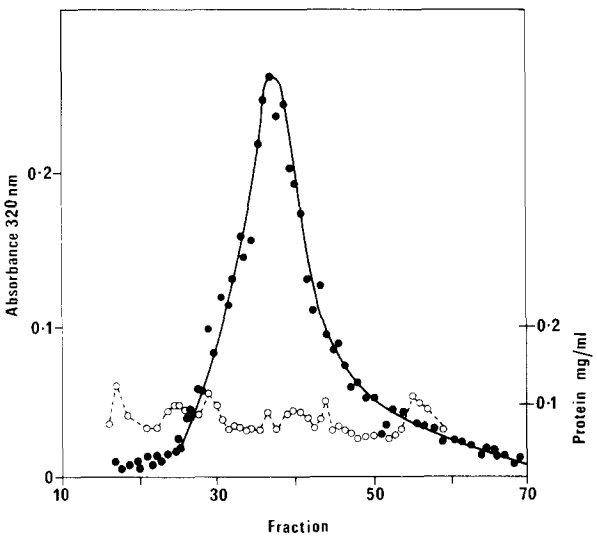

Fig. 2. DEAE-cellulose chromatography of chorismate mutase in crude extract of *C. reinhardi*. The column (1.4 cm × 40 cm) was equilibrated in Buffer II, 2 ml of crude extract (53 mg protein) was applied and a gradient of NaCl used to elute (at any volume k, NaCl concentration (c) was given by $c = 0.5 - 0.5e^{-k/1000}$). Fraction volume was 6.5 ml. •, chorismate mutase activity (0.25 ml of column fraction, 60 min. incubation time); ○, protein, mg/ml.

mM) or Cl^- (0.5 M) to the reaction mixture and was unchanged after storage for 50 days at −19° C.

DEAE-cellulose chromatography of chorismate mutase

When crude sonic extracts of cells grown in Tris/acetate/phosphate medium were fractionated on DEAE-cellulose only a single peak of chorismate mutase activity could be detected (Fig. 2). This peak showed inhibition by phenylalanine and tyrosine similar to that of the crude extract (see below). Neither prephenate dehydratase nor prephenate dehydrogenase activity (the enzymes catalyzing step 2 of phenylalanine and tyrosine biosynthesis, respectively) could be detected in crude extracts or in these fractions. Total recovery of chorismate mutase activity in the eluate was 57%. Total recovery of protein at 0.24 M NaCl was 63%.

Partial purification of chorismate mutase

The purification procedure is outlined in Table I. A single peak of activity was recovered following $(NH_4)_2SO_4$ fractionation and two gel filtration steps (Materials and Methods). Final recovery after Sephadex G-200 chromatography was 7% and purification was approx. 20-fold over the enzyme concentration in crude extract. The Sephadex G-200 column was calibrated with proteins of known molecular weight and the apparent molecular weight of chorismate mutase was calculated, using the procedure of Andrews [9] to be 61 000.

Michaelis constant

The Michaelis constant (K_m) for chorismate mutase was determined using the 20-fold purified enzyme preparation. The K_m was calculated to be 0.46

TABLE I

PURIFICATION OF CHORISMATE MUTASE

Stage	Total protein (mg)	Total activity (units)	Recovery (percent)	Specific activity (units/mg protein)	Purification
Crude extract	260	2.6	100	0.01	1
$(NH_4)_2SO_4$ ppt. (0.3—0.4 fractional saturation)	14	0.77	30	0.055	5.5
Sephadex G-100*	8.3	0.92	36	0.111	11.1
Sephadex G-200*	0.7	0.14	7	0.198	19.8

* Values shown are for the pooled fractions indicated in Materials and Methods.

TABLE II

EFFECT OF AROMATIC AMINO ACIDS AND THEIR ANALOGUES ON CHORISMATE MUTASE ACTIVITY IN *C. REINHARDI*

Enzyme activities were measured as described in Materials and Methods. Reaction time was 20 min. The peak enzyme from Sephadex (G-200 gel filtration) was contained in the batched fractions (Materials and Methods). The DEAE-cellulose fraction numbers refer to fractions taken from the fractionation shown in Fig. 2. The specific activities of the uninhibited enzymes were: crude extract, 0.018; Sephadex G-200 pool, 0.111; DEAE-cellulose Fraction 31, 0.033; DEAE-cellulose Fraction 37, 0.123; DEAE-cellulose Fraction 43, 0.046.

Source of enzyme	Additions to reaction mixture	Inhibition (percent)
Crude extract		
	None	0
	Tyr (1.25 mM)	85
	Tyr (0.3 mM)	30
	Phe (1.25 mM)	20
	Trp (1.25 mM	1
	Tyr + Phe (1.25 mM each)	52
	Tyr + Trp (1.25 mM each)	5
	Phe + Trp (1.25 mM each)	2
	Tyr + Phe + Trp (1.25 mM each)	4
	D,L-3,4-Dihydroxyphenylalanine (0.1 mM)	31
	D,L-3,4-Dihydroxyphenylalanine (0.25 mM)	60
	D,L-3,4-Dihydroxyphenylalanine (1 mM)	95
	D,L-*p*-Fluorophenylalanine (1 mM)	7
	β-2-Thienylalanine (1 mM)	3
	D,L-*p*-aminophenylalanine (1 mM)	49
	D,L-*p*-aminophenylalanine (5 mM)	61
Peak enzyme (Sephadex G-200)		
	Tyr (1.25 mM)	85
	Phe (1.25 mM)	21
DEAE-cellulose, Fraction 31		
	Tyr (1.25 mM)	69
	Phe (1.25 mM)	23
DEAE-cellulose, Fraction 37		
	Tyr (1.25 mM)	83
	Phe (1.25 mM)	16
DEAE-cellulose, Fraction 43		
	Tyr (1.25 mM)	69
	Phe (1.25 mM)	23

mM chorismate (S.E. ±0.07 mM). Substrate concentrations used were 1.25—0.25 mM chorismate.

Regulation of chorismate mutase activity by the aromatic amino acids

The effects of tyrosine, phenylalanine and tryptophan on chorismate mutase activity in crude extracts are summarised in Table II. At a concentration of 1.25 mM tyrosine inhibited 85%, phenylalanine inhibited 20% while tryptophan did not inhibit at all. Tyrosine inhibition was relieved, partially by phenylalanine, and almost completely by tryptophan. A mixture of all three amino acids gave no inhibition. Tyrosine and phenylalanine had similar effects upon chorismate mutase activity in crude extract, in 20-fold purified preparations and in DEAE-cellulose fractions. Table II also shows the effects of aromatic amino acid analogues on chorismate mutase activity in crude extracts. 3,4-Dihydroxyphenylalanine and 4-aminophenylalanine were the most effective inhibitors of those tested.

Effect of exogenous aromatic end products on synthesis of chorismate mutase

Growth in basal medium containing tyrosine (1 mM) or a complete aromatic supplement consisting of tyrosine, phenylalanine, tryptophan (1 mM each) plus 4-aminobenzoic acid, 4-hydroxybenzoic acid, 2,3-dihydroxybenzoic acid (1 mM each) plus shikimic acid (1 μ g/ml) failed to repress chorismate mutase below the specific activity seen in cells grown in basal medium. This result is not conclusive, however, and could mean either that chorismate mutase is constitutive (i.e. is not repressible) or that it is already repressed by endogenously synthesized compounds in basal-grown cells and is not further repressed by addition of exogenous aromatic end products. A further possible explanation is that aromatic amino acids are not permeable to *C. reinhardi* and hence cannot repress. This possibility is rendered unlikely by the finding that exogenous aromatic amino acid analogues such as 3,4-dihydroxyphenylalanine, 5-methyltryptophan and β-2-thienylalanine are able to inhibit growth and hence may be assumed to be permeable to the cell (Table III).

TABLE III

EFFECT OF AROMATIC AMINO ACID ANALOGUES ON GROWTH OF *C. REINHARDI*

Analogues were incorporated in Tris/acetate/phosphate agar. Plates were incubated 7 days at room temperature and illuminated with a 40-W white fluorescent light. Growth was scored as follows: +++, heavy growth; ++, moderate growth; +, slight growth; —, negative.

Analogue	Concentration (mM)	Growth
None	—	+++
D,L-3,4-Dihydroxyphenylalanine	0.25	++
D,L-3,4-Dihydroxyphenylalanine	0.5	+
D,L-3,4-Dihydroxyphenylalanine	1.0	—
D,L-5-Fluorotryptophan	5	—
D,L-5-Methyltryptophan	2.5	—
D,L-*p*-Fluorophenylalanine	2.0	+++
β-2-Thienylalanine	5.0	+

Discussion

Our fractionation of *C. reinhardi* extracts provides evidence for only a single molecular species of chorismate mutase. Since we could not detect prephenate dehydratase and prephenate dehydrogenase activities in crude extracts we are unable to say whether one or more of these activities is associated with chorismate mutase. The finding of a single chorismate mutase resembles the situation in *Saccharomyces cerevisiae* [10,11], *Neurospora crassa* [12], *Claviceps paspali*, SD58 and Pb156-E16) [13—15] and *Euglena gracilis* [16]. It contrasts with *Bacillus subtilis* [17—19], *Claviceps paspali* Pb156 [14,15] and mung beans (*Phaseolus aureus*) [20] where two or more molecular species of chorismate mutase have been reported and with *Escherichia coli* [21—23], *Aerobacter aerogenes* [6] and *Salmonella typhimurium* [24,25,7] where there are two proteins, one with chorismate mutase (P) and prephenate dehydratase activity and another with chorismate mutase (T) and prephenate dehydrogenase activity. The chorismate mutase of *C. reinhardi* is compared with that of other species in Table IV.

We found that the chorismate mutase of *C. reinhardi* is feedback-inhibited by tyrosine. Of the other aromatic amino acids, phenylalanine inhibited only weakly and partially reversed the inhibition of tyrosine; tryptophan did not inhibit and completely reversed tyrosine and phenylalanine inhibition. This pattern of chorismate mutase regulation has some resemblance to that of several other eukaryotes, viz. *S. cerevisiae*, *N. crassa*, *C. paspali*, *E. gracilis*, *Ph. aureus* and *Pisum sativum* (Table IV). Whilst most of these species differ from *C. reinhardi* in that phenylalanine is an effective inhibitor and does not reverse tyrosine inhibition they all show a reversal of tyrosine and phenylalanine inhibition by tryptophan (and in most cases show activation by tryptophan when it is present alone). The regulation of the chorismate mutases of these eukaryotes is thus accomplished by interaction between the three aromatic amino acids. This contrasts with the situation in the procaryotes (Table IV) where the chorismate mutases are regulated either by product inhibition by prephenate or by specific inhibition of one molecular species by phenylalanine. The characteristic occurrence of reversal of tyrosine and phenylalanine inhibition by tryptophan among such widely divergent eukaryotes indicates evolutionary conservation of this regulatory circuit and suggests that it confers a selective advantage. Before any such selective advantage can be elucidated in *C. reinhardi*, however, other possible sites of feedback inhibition must be tested. Possible sites include the first enzyme of aromatic biosynthesis, 3-deoxy-D-arabinoheptulosonic acid 7-phosphate synthetase (EC 4.1.2.15), prephenate dehydratase, prephenate dehydrogenase and anthranilate synthetase.

Our failure to observe repression of chorismate mutase by exogenous aromatic amino acids in wild-type *C. reinhardi* does not exclude the possibility that such regulation exists. It is possible that the endogenous concentration of aromatic amino acids in wild-type cells grown in basal medium is repressing and that addition of exogenous amino acids does not cause further repression. This is the case for chorismate mutase in *E. coli* where it has been necessary to starve aromatic auxotrophs of phenylalanine or tyrosine in order to observe significant derepression [1,2,28,29]. To rigorously test for repression of choris-

TABLE IV

COMPARISON OF CHORISMATE MUTASES OF VARIOUS SPECIES

Organism	Molecular species	M_r (daltons)	Apparent K_m (mM chorismate)	Regulatory properties
C. reinhardi	1	61 000	0.46	Tyr strongly inhibits Phe weakly inhibits. Trp reverses both inhibitions. Phe partially reverses Tyr inhibition
S. cerevisiae [10,11]	1	35 000	*	Tyr inhibits, Phe does not inhibit. Trp activates.
N. crassa [12]	1	*	*	Tyr, Phe inhibit. Trp reverses both inhibitions and activates.
C. paspali SD58 [14,15]	1	60 000	*	Tyr, Phe inhibit. Trp activates.
C. paspali Pbl56-E16 [13, 14]	1	*	*	Tyr, Phe inhibit. Trp reverses both inhibitions and activates.
C. paspali Pbl 56 [14,15]	2	55 000 59 000	*	Major species: Tyr, Phe inhibit. Trp activates. Minor species: Not inhibited. Trp activates
E. gracilis [16]	1	160 000	1.0	Tyr, Phe inhibit; Trp reverses both inhibitions and activates.
Mung beans *(P. aureus)* [20]	2	CM-1 50 000 CM-2 36 000	* 0.33	CM-1: Tyr, Phe inhibit. Trp reverses both inhibitions and activates CM-2: not affected by aromatic amino acids.
Peas *(P. sativum)* [27]	1	*	*	Tyr, Phe inhibit. Trp reverses both inhibitions and activates.
B. subtilis 168 [17—19]	1	(CM_3) 140 000	2.6	Prephenate inhibits. Unaffected by Phe, Tyr, Trp.
B. subtilis 23 [17—19]	3	(CM_1) 78 000 (CM_2) 135 000 (CM_3) 140 000	* 1.0 2.6	All molecular species inhibited by prephenate; unaffected by Phe, Tyr, Trp.
E. coli K12 [21—23]	2	(T) 82 000 (P) 85 000	0.39 0.045	Tyr inhibits associated prephenate dehydrogenase. Phe weakly inhibits. Phe inhibits associated prephenate dehydratase.
A. aerogenes [6]	2	(T) 75 000 (P) *	1.3 *	as for *E. coli* K12. as for *E. coli* K12.
S. typhimurium [24,25,7]	2	(T) *	*	as for *E. coli* K12. as for *E. coli* K12.

* Not known.

mate mutase in *C. reinhardi* it may be necessary to carry out analogous starvation experiments with aromatic auxotrophs. Such experiments are not feasible at present since these auxotrophs of *C. reinhardi* are not available. Other approaches might include testing for derepression by examining cells grown in the presence of partially inhibitory concentrations of aromatic amino acid analogues.

Acknowledgements

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