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CHORISMATE MUTASE ISOZYME PATTERNS IN THREE FUNGI

TERRY S. WOODIN AND LEO NISHIOKA
Division of Biochemistry, University of Nevada, Reno, Nev. 89507 (U.S.A.)

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SUMMARY

As judged by results of gel electrophoresis and effector sensitivity, the number of chorismate mutase isozymes differs in extracts of the three fungi investigated. *Neurospora crassa* contains only one form of chorismate mutase designated CM_1. It is inhibited by phenylalanine, tyrosine and caffeic acid, activated by tryptophan and unaffected by ferulic and 3,4-dimethoxycinnamic acid. *Penicillium chrysogenum* contains two mutases; a CM_1 similar in electrophoretic mobility and effector sensitivity to that of *N. crassa* and CM_3 which is inhibited by phenylalanine and tyrosine, activated by tryptophan and 3,4-dimethoxycinnamic acid but unaffected by caffeic acid. *Penicillium duponti*, a thermophilic fungus, contains three forms of the mutase; a CM_1 and a CM_3 similar in electrophilic mobility and effector sensitivity to those of *P. chrysogenum* and CM_2, which is inhibited by caffeic acid, slightly activated by tryptophan and unaffected by phenylalanine, tyrosine or ferulic acid.

INTRODUCTION

In all organisms studied so far the biosynthetic pathway for the aromatic amino acids appears to be the same, the shikimic acid pathway, Fig. 1 (ref. 1). More detailed investigations using numerous organisms reveal significant diversity in the processes controlling aromatic amino acid production, such as key points in the pathway where isozymes appear as well as the number and characteristics of such isozymes. Jensen[2] has proposed comparison of control and isozyme patterns of this pathway as a useful taxonomic tool.

Certain features of the shikimic acid pathway in fungi have been elucidated by: (a) Lingen's[3,4] detailed investigations of chorismate mutase (Enzyme 8, Fig. 1) and 3-deoxy-D-arabinoheptulosonate-7-phosphate synthetase (DAHP synthetase, Enzyme 1, Fig. 1) in *Claviceps* strains; (b) Baker's[5] preliminary investigations of chorismate mutase in *Neurospora crassa*; (c) Giles[6,7] and Gaertner's[8] investigations of the early enzymes (Enzymes 2–6, Fig. 1) of the shikimate pathway in *N. crassa* and

Abbreviation: DAHP, 3-deoxy-D-arabinoheptulosonic acid.

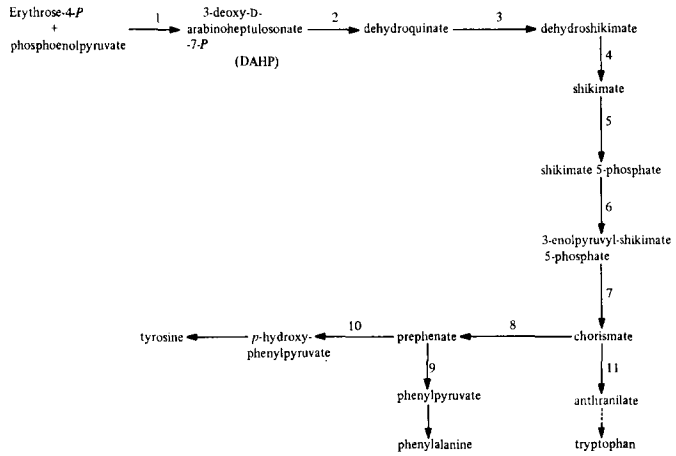

Fig. 1. The shikimic acid pathway for aromatic amino acid biosynthesis. The enzymes of specific interest are: (1) DAHP synthase; (5) shikimate kinase; (8) chorismate mutase; (9) prephenate dehydratase; (10) prephenate dehydrogenase.

a variety of *Phycomycetes*, *Ascomycetes* and *Basidiomycetes*; and (d) Doy's[9] studies of DAHP synthetase in *N. crassa*.

Little work has been done to detail the shikimic acid pathway, its isozyme patterns or control points in other fungi. It was with this purpose in mind that we undertook to compare the chorismate mutase isozyme pattern in two species of *Penicillium* with those already established in *N. crassa* and higher plants.[10,11] *N. crassa* was chosen as we have found it relatively easy to grow and Baker[5] has already established the presence of only one mutase in *N. crassa* sensitive to all three aromatic amino acids. We chose to study chorismate mutase, the enzyme which commits the shikimate pathway to production of phenylalanine and tyrosine as previous reports indicated its isozyme, aggregate and control pattern varies significantly among various organisms studied (see Table I, ref. 11).

MATERIALS AND METHODS

Growth of cultures

N. crassa was maintained on *Neurospora* agar (Difco). Material from one slant was gently agitated into sterile distilled water and transferred asceptically to a 2-l erlenmeyer flask containing 1 l of HB67 (ref. 12) media which was supplemented with 5.0 mg/l biotin and 4.0% sucrose. The culture was incubated for 2–3 days at 30 °C on a New Brunswick incubating rotary shaker set at 350 rev./min and describing a 1-inch circle.

Penicillium chrysogenum and *Penicillium duponti* were maintained on Citrate No. 3 agar.[13] Material from one to two slants was transferred as above to a 500-ml erlenmeyer flask containing 100 ml of citrate No. 3 media containing 4.0% glucose and 1.0 g/l Na_2SO_4. Unless otherwise noted, cultures were incubated for two days at 30 °C (*P. chrysogenum*) and 40 °C (*P. duponti*) respectively on a New Brunswick incubating rotary shaker operating at 350 rev./min and describing a 1-inch circle.

All fungi produced dense fluffy mycelia with no balling. The mycelia were collected by suction filtration on a Buchner funnel. In all cases the media (filtrates) were clear, indicating no bacterial or yeast contamination. Mycelial pads were frozen and either used immediately or stored at −20 °C for future use.

Preparation of extracts

Frozen mycelia were broken into small pieces and ground (either with a mortar and pestle or in a Waring blender) with liquid nitrogen until well powdered. After thawing, the powder was extracted three times with 0.1 M potassium phosphate buffer, pH 6.5 at a ratio of 2 g powder to 1 ml buffer. The extracts were clarified by centrifugation for 20 min at 16 000 rev./min in the SS34 head of a Sorvall refrigerated centrifuge (30 900 × *g*). All operations, including electrophoresis and elution were performed at 4 °C.

Electrophoresis

Electrophoresis was performed on 6% polyacrylamide gels prepared according to Ornstein and Davis[14] with the exception that no spacer or sample gels were employed. Bromophenol blue was employed as the tracking dye. 50 µl of the first mycelial extraction made 10% in sucrose was applied gently under buffer to each of six gels. Each run lasted about 15 h, with two sets of six gels, each approximately 9.0 cm long, subjected to a current of 3–4 mA/tube. Immediately at the end of each run five gels of each set were frozen in liquid nitrogen and stored overnight at −20 °C. One gel of each set was stained for protein by immersion for 12 h in 2.5% Amido Schwarz. These were destained by repeated flushing with 7% acetic acid.

Gel extraction and assay

The frozen gels were lined up with dye fronts matching and cut into sections 3 mm long. Each section was put in a 1.2 cm × 7.5 cm test tube to which was added 1.0 ml of 0.1 M potassium phosphate buffer, pH 6.5. Gels and buffer were incubated for 4–6 h with occasional hand shaking. When the dye band was evenly distributed between gel and buffer, we assumed protein elution was complete. Appropriate aliquots were used for assays of chorismate mutase, using a slight variation of a method developed by Nishioka and Woodin[15]. Assay mixtures consisting of 0.6 ml of 0.1 M phosphate buffer, pH 6.5, containing 1 µmole chorismic acid and an appropriate aliquot of gel eluate were incubated at 37 °C for 30 min. The reaction was stopped by adding 0.2 ml of 20% trichloroacetic acid. After 15 min incubation at room temperature, 2.4 ml of 1.25 M borate–2.8 M phosphate, pH 6.5, were added. Absorbance at 300 nm was read after 30 min, using a Coleman-Hitachi Model III single beam spectrophotometer. One unit of enzyme activity represents 1 µmole of product produced in 1 min.

Prephenate dehydratase was determined according to Cotton and Gibson[16]. Protein concentration was determined by the biuret method[17]. Proteins in active fractions of the gel were determined by the method of Lowry *et al.*[18].

Chemicals

Chorismic acid was prepared according to Gibson[19]. All other chemicals were purchased as the purest grade available and used without further purification.

Chorismic, caffeic, ferulic, and 3,4-dimethoxycinnamic acid were chromatographed on Brinkmann thin-layer plates (Cellulose NM300 UV_{254}) in methanol–butanol–benzene–water (2:1:1:1, by vol.). Each exhibited only one ultraviolet absorbing spot.

RESULTS

Relative levels of chorismate mutase in each fungus

Data presented in Table I and Table II indicate there may be an inverse relationship between the level of chorismate mutase present in the fungal extracts and the number of mutase isozymes separable from such extracts. Previous results in our laboratory indicate that when assayed between 20 and 40 °C, levels of mutase in extracts of *P. duponti* are consistently lower than those from *P. chrysogenum*[20].

However, the data in Table I were obtained under a specific set of conditions such as pH, temperature and substrate concentration, which may have favored expression of *N. crassa* mutase and so cannot be construed as definitive.

TABLE I

ACTIVITY LEVELS OF CHORISMATE MUTASE IN CRUDE EXTRACTS

Extracts were prepared as outlined in Materials and Methods. To obtain dry weight equivalents, a weighed aliquot of mold nitrogen powder was dried for 24 h at 100 °C and percent dry wt of each sample calculated. Enzyme assays were performed at 37 °C as detailed in Materials and Methods.

Species	*Units/g wet wt*	*Units/g dry wt*	*Units/mg protein*
P. duponti	270	—	17
P. chrysogenum	300	2000	39
N. crassa	940	5700	77

Separation of mutase isozymes by electrophoresis

As can be seen in Fig. 2, chorismate mutase does not appear to be associated with any particular protein band. The method employed resulted in separation of a series of proteins and complete separation of each form of mutase. From Fig. 2 and Table II, it can be seen that one isozyme of chorismate mutase is found in *N. crassa*

TABLE II

RELATIVE DISTRIBUTION OF CHORISMATE MUTASE ISOZYMES IN THE THREE FUNGI

Extraction, electrophoresis and assay procedures are detailed in Materials and Methods. Figures in parenthesis represent the R_F of the peak fraction of activity for each isozyme.

Species	*% of total mutase activity*			*% recovery of total activity added*
in:	CM_1	CM_3	CM_2	
N. crassa	100 (0.42)	0	0	87–91
P. chrysogenum	68 (0.45)	32 (0.17)	0	86–97
P. duponti	59 (0.33)	31 (0.19)	10 (0.10)	60–100

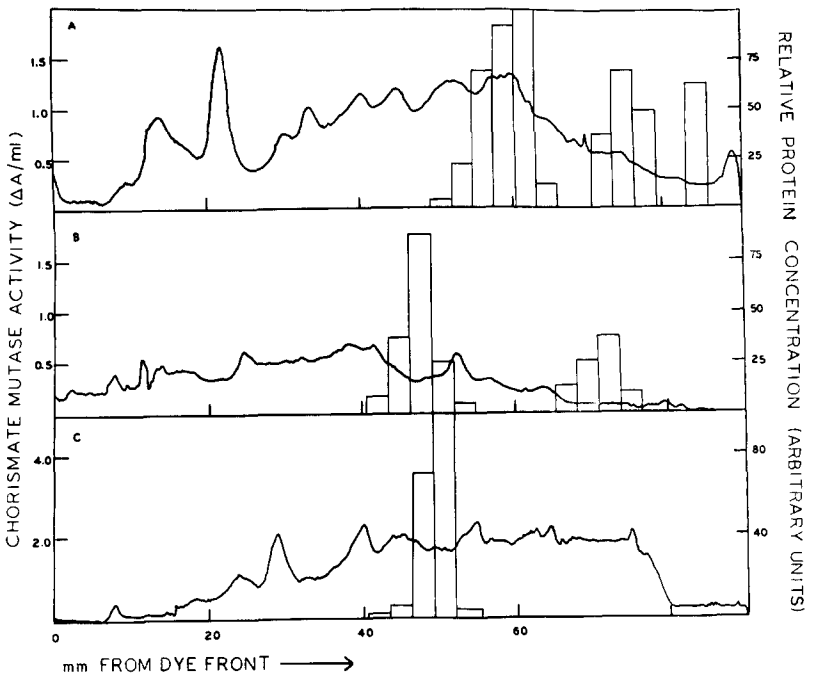

Fig. 2. Protein and chorismate mutase activity patterns of fungal enzymes subjected to gel electrophoresis. (A) *P. duponti*; (B) *P. chrysogenum*, and (C) *N. crassa*. Extraction, electrophoresis, protein staining and assay procedures are detailed in Materials and Methods; ———, relative protein concentration as determined by recording the absorbance of a stained gel using a Densicord Recording Electrophoresis Densitometer. The bar graphs represent the level of chorismate mutase present in each fraction.

which agrees with Baker's[5] results, two in *P. chrysogenum* and three in *P. duponti*. The difference in mobility of the mutase isozymes from *P. chrysogenum* and *P. duponti* indicate slight differences in charge, size or both may exist between comparable (on the basis of effector sensitivity) forms of mutase in these fungi. Occasionally, as indicated in Table II, we experienced poor recovery of activity. However, in all cases (whether total recovery was 40 or 100%), the relative levels of each isozyme remained constant (to within a 2% variation). This, *plus* results of similar work in our laboratory using plant extracts, implies that poor recovery is usually due to inadequate protein elution rather than instability of any or all mutase isozyme(s). In order to assess the effects of buffer extraction and gel properties on the appearance of multiple forms of mutase, *P. duponti* was (1) extracted as detailed in materials and Methods but with 0.1 M Tris–HCl buffer, pH 8.5, and then electrophoresed as detailed in Materials and Methods, or (2) extracted as detailed in Materials and Methods but electrophoresed in the buffer system described by Smith and Hedrick[21], again with the omission of spacer and sample gels. In both cases, number and relative levels of mutase isozymes were as reported in Table II. Extracts from *P. duponti* grown at 40 °C for 64 h gave essentially the same results as those detailed here in regard to specific activity, units/g wet wt and relative percentage of each mutase present. Specific activity of *P. duponti* chorismate mutase enzymes 1 and 2 (CM_1 and

CM_2) as eluted from the gel are also given as an indication of the relative purification afforded by electrophoresis (approx. 15-fold).

Gel fractions of *P. duponti* exhibiting high levels of mutase were also assayed for the presence of prephenate dehydratase. Results were negative indicating that as with *N. crassa*[5], prephenate dehydratase and chorismate mutase of *P. duponti* do not exist as a complex.

Characterization of each isozyme

As indicated by Table III, the different isozymes of chorismate mutase are distinguishable on the basis of effector sensitivity as well as by their electrophoretic mobility. The effector sensitivity of the three mutase isozymes in *P. duponti* parallels that found in alfalfa[11]. The mutase isozymes are designated CM_1, CM_2 or CM_3 (corresponding to those reported in alfalfa) based on their effector sensitivity and relative electrophoretic mobility.

None of the substances tested was an effector of all three isozymes, though any two isozymes investigated showed common effectors, *e.g.* all three aromatic amino

TABLE III

EFFECTOR SENSITIVITY OF FUNGAL CHORISMATE MUTASE ISOZYMES

Assays were performed as detailed in Materials and Methods, except that reaction mixtures contained only 0.5 μmole chorismate and the indicated concentration of effector. Reference cuvettes for each assay included enzyme and inhibitor but no chorismate and were treated as a regular assay. In addition, each assay was corrected for endogenous absorbance of chorismate at 300 nm.

Effector	*Original activity in the presence of effector (%)*					
	CM_1			CM_3		CM_2
from:	*N. crassa*	*P. chrysogenum*	*P. duponti*	*P. chrysogenum*	*P. duponti*	*P. duponti*
Phenylalanine	50	46	50	65	40	100
Tyrosine	50	35	50	60	50	100
Tryptophan	140	150	180	130	150	105
3,4-Dimethoxycinnamic acid	100	100	100	130	180	100
Caffeic acid	50	35	40	100	120	40
Ferulic acid	95	—	92	—	60	100
Spec. act.	—	—	260	—	270	—

acids affect CM_1 and CM_3, but the C_6C_3 aromatic compound caffeic acid affects only CM_1 and CM_2; CM_3 is unique in its sensitivity to 3,4-dimethoxycinnamic acid. It is unlikely that the observed inhibition and activation patterns are due to an artifact built into the assay, or that we are seeing an effect of a common contaminant as: (1) the three isozymes are affected differently by each compound; (2) each effector compound moved as one band on thin-layer chromatography; (3) of the three effectors studied, only caffeic acid exhibited any change in $A_{300\text{ nm}}$ when incubated with the mutase isozyme sensitive to it (a $A_{300\text{ nm}}$ of -0.05 unit); and (4) adequate controls were established to compensate for any change in $A_{300\text{ nm}}$ due to chemically or enzymatically produced changes in effector under assay conditions.

DISCUSSION

Detailed investigations of the enzymes in a fungal shikimic pathway have been done only in such *Pyrenomycetes* as *Claviceps paspali* and *N. crassa*. In both organisms there are three isozymes of DAHP synthetase[4,9], each isozyme sensitive to feedback inhibition by a different aromatic amino acid (tyrosine, tryptophan or phenylalanine). In *N. crassa*[6] and a variety of *Phycomycetes*, *Ascomycetes* and *Basidiomycetes*, the succeeding enzymes of this pathway (Enzymes 2–6) exist as an aggregate. Gaertner *et al.*[8] have found that this aggregate is much more efficient at catalyzing the overall pathway than any one enzyme in it is at catalyzing the reactions specific to it. One form of chorismate mutase (inhibited by phenylalanine and tyrosine and activated by tryptophan) has been found in *N. crassa*[5] and *Claviceps* SD58[3]. *Claviceps* Pb 156 contains an additional mutase isozyme sensitive only to tryptophan[3].

The same general pattern as that in *N. crassa* has been found for *S. Saccharomyces cerevisiae* with the exception that the mutase of this organism is not sensitive to inhibition by phenylalanine[22].

The latter enzymes (Enzymes 6–10) of the shikimate pathway have not been investigated in any species of the *Plectomycetes* subclass of *Euascomycetes*. The differing patterns of chorismate mutase isozymes in *Penicillium* species detailed above are the first reported instance of any yeast or fungal species significantly diverging from the *N. crassa* pattern.

Considering the wide diversity of chorismate mutase types in bacteria, it is not surprising to see variations among fungal species. Adequate assessment of the significance of these mutase isozyme variations must await characterization of other key fungal shikimate pathway enzymes such as DAHP synthetase, shikimate kinase, prephenate dehydrogenase, prephenate dehydratase and the related transaminases. The ratio of the three forms of mutase in *P. duponti* as well as their effector sensitivities agree substantially with those found in corn, tobacco, cauliflower, and alfalfa.

The studies of effector sensitivities are particularly interesting. The pattern of coupled compensatory activation by tryptophan and feedback inhibition by phenylalanine and tyrosine has been well documented in the chorismate mutases of *Neurospora*, *Claviceps*, *Euglena* and *Saccharomyces*. However, the differential effects of the C_6C_3 phenolics, ferulic, 3,4-dimethoxycinnamic and caffeic acid have not previously been reported, though we have noted them in mutase isozymes from higher plants (Nishioka and Woodin[11]). There have been recent reports of C_6C_3 compounds either accumulating in fungal culture media or being used to synthesize common fungal metabolites such as the alkaloids.

Aspergillus niger has been reported to convert added cinnamic acid to 4-hydroxycoumarin[23], and 3,4-dihydroxybenzoic acid[24]. Packter[25] presents evidence for incorporation of [U-^{14}C]tyrosine into 4-methoxytoluquinol by the basidiomycete *Lentinus degener* and postulates one mode of biosynthesis may be *via* the tyrosine ammonialyase reaction leading to *p*-cinnamic, *p*-methoxycinnamic and then isoferulic acids. Methyl-substituted coumarins and methoxybenzoquinone have recently been isolated from culture filtrates of the basidiomycete *Lenzites thermophilia*[26].

In addition, fungi may encounter C_6C_3 compounds as a consequence of growth on or in the vicinity of decaying plant material high in lignin content. It has been shown that a variety of white-rot fungus are capable of degrading lignin both to lig-

nans and their C_6C_3 components[27–29]. Components isolated from lignin-enriched culture media containing white-rot fungus includes *p*-hydroxycinnamic acid, alcohol and aldehyde, ferulic acid, coniferyl aldehyde and alcohol, 4-hydroxy-3-methoxyphenylpyruvate and their C_6C_1 counterparts such as vanillin. It is intriguing to speculate that fungi growing in rich media such as on plant material might utilize these C_6C_3 lignin breakdown products for synthesis of their aromatic amino acids, thus decreasing the necessity for their biosynthesis from simpler components *via* the shikimic acid pathway.

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