

Cloning and expression in yeast of a higher plant chorismate mutase

Molecular cloning, sequencing of the cDNA and characterization of the *Arabidopsis thaliana* enzyme expressed in yeast

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Chorismate mutase (EC 5.4.99.5) catalyzes the first step in the branch of the shikimate pathway which leads to the aromatic amino acids, phenylalanine and tyrosine. We have isolated a cDNA for this enzyme from the higher plant, *Arabidopsis thaliana*, by complementing a yeast strain (*aro7*) with a cDNA library from *A. thaliana*. This is the first chorismate mutase cDNA isolated from a plant. It encodes a protein of 334 amino acids. The identity of the deduced amino acid sequence is 41% to the chorismate mutase sequence from *Saccharomyces cerevisiae*. The N-terminal portion of the deduced amino acid sequence has no homology to the *S. cerevisiae* sequence but resembles known plastid-specific transit peptides. The *A. thaliana* chorismate mutase expressed in yeast revealed allosteric control by the three aromatic amino acids, as previously described for plastidic chorismate mutase isozymes.

Shikimate pathway; Chorismate mutase; *Arabidopsis thaliana*

1. INTRODUCTION

Many natural compounds containing one or more aromatic rings are derived from the shikimate pathway, the occurrence of which is restricted to bacteria, fungi and plants. In plants, besides the three proteinogenic aromatic amino acids, phenylalanine, tyrosine, and tryptophan, which are metabolites of primary metabolism, this large group of aromatic compounds contains thousands of secondary metabolites such as alkaloids, amines, phenolic compounds, flavonoids etc.

Chorismate mutase (EC 5.4.99.5) is the first enzyme of the branch of the shikimate pathway which leads to the aromatic amino acids phenylalanine and tyrosine (Fig. 1). It catalyzes what is formally a Claisen rearrangement of the enolpyruvyl side chain of chorismate to form prephenate and appears to be a very rare example of a pericyclic process in primary metabolism. Chorismate mutase activities have been characterized from many different bacteria and fungi and they differ remarkably from organism to organism. The enzymatic properties of some chorismate mutases have been studied in great detail, and some of the corresponding genes have been cloned. The enzyme from *Saccharomyces cerevisiae* is a monofunctional polypeptide, and the corre-

sponding gene has been cloned (for a review, see [1,2]). Considering the known extensive homologies between the sequences of some shikimate pathway enzymes from eukaryotic and prokaryotic organisms, it is remarkable to note that the *S. cerevisiae* sequence has no apparent similarity to prokaryotic sequences [2], whereas the prokaryotic sequences share a certain amount of homology with each other [3].

Chorismate mutase activity has also been found in a variety of higher plants, and frequently two isozymes (designated CM-1 and CM-2) could be separated. The enzymatic properties of the two sets of chorismate mutase isozymes have been studied in detail (for a review, see [4,5]). One of the chorismate mutase isozymes (CM-1) is allosterically activated by tryptophan and is inhibited by phenylalanine and tyrosine, whereas the other (CM-2) is insensitive to all three aromatic amino acids. The CM-1 activity has been shown to be localized in plastids, while the CM-2 activity was reported to be in the cytosol [5–7].

To analyze the different chorismate mutase isozymes in more detail, we are in the process of isolating the corresponding cDNAs. Here we describe the first cloning and characterization of a chorismate mutase cDNA from a higher plant, i.e. *Arabidopsis thaliana*.

2. MATERIALS AND METHODS

2.1. Materials

The two chorismate mutase mutants of *S. cerevisiae* (*aro7* and *aro7, ura3*) were a gift from Dr. G. Braus, ETH-Zürich, and the *A. thaliana*

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The nucleotide sequence reported in this paper has been submitted to the EMBL DataBank where it was allocated the accession number: Z26519.

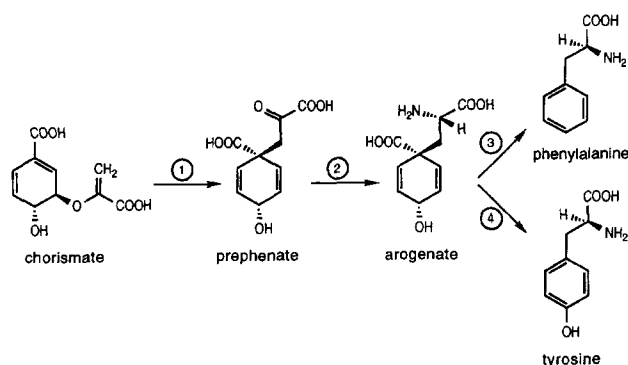


Fig. 1. The pathway from chorismate to phenylalanine and tyrosine in higher plants. The enzymes are: 1, chorismate mutase; 2, prephenate aminotransferase; 3, aroenate dehydratase; 4, aroenate dehydrogenase.

cDNA library in the vector pFL61 was a gift from Dr. M. Minet, Gif-sur-Yvette. All chemicals were of the highest grade available. Yeast cells were grown either in the rich medium, YPD (1% bacto-yeast extract, 2% bacto peptone, 2% glucose), or in the minimal medium, SD (0.67% yeast nitrogen base without amino acids, 1% glu-

cose). The vector pBluescript SK(+) was from Stratagene (Heidelberg) and the T7 sequencing kit from Pharmacia (Düßeldorf). Chorismic acid was prepared according to the procedure in [8].

2.2. Complementation assay

The preparation of competent yeast cells and their transformation with the *A. thaliana* cDNA library were performed according to [9]. Plasmid DNA was isolated from selected yeast colonies as described in [10] and used to transform *Escherichia coli* strain DH5 according to [11].

2.3. Molecular techniques

The basic molecular techniques were adopted from [9,12]. The sequence of both strands of the cDNA was determined by the dideoxy chain-termination method [13] using the nested deletion method [14] for one strand and defined oligonucleotides (16-mers) for the other strand. The sequence comparison was done with the program, BESTFIT (Genetic Computer Group Inc, GCG package, version 7).

2.4. Preparation of crude extracts and chorismate mutase assay

Yeast cells were grown and protein extracts prepared according to [15].

Chorismate mutase assays were performed in 100 mM potassium phosphate buffer (pH 8) using 1 mM chorismic acid as substrate. The assays were carried out as described in [16].

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1      100
CGCTCATTGTTGATGAGATCGTCTTGTGCTCCTCTGCGATTGGTGGGTCTTCGACCATCGACGTGAATTATCAACCTCAACACCCATTTCCTCTTC
      M R S S C C S S A I G G F F D H R R E L S T S T P I S T L L
      1      10      20      30
101    200
TTCCTCTTCCATCAACCAATCTTCTTTCTGTTCGTGTTCTCTCTCAGCCATCAAGCCACGCTCTGGAACCGCTCTGTTACGCGGTATGAC
      P L P S T K S S F S V R C S L P Q P S K P R S G T S S V H A V M T
      40      50      60
201    300
ACTCGCTGGATCGTTGACAGGGAAGAAACGAGTGAGTGGATGAGAGTTGACTCTTGAAGGTATTAGAACTCTTGATCCGTCAAGAGACAGCATT
      L A G S L T G K K R V D E S E S L L E G I R N S L I R Q E D S I
      70      80      90
301    400
ATATTTGGGCTATTGGAGAGAGCCAGTACTGTACAACTGCTGATCTTATGATCCTACTGCTTTTGACATGGATGGTTTCAATGGTTCTTTGGTTGAGT
      I F G L L E R A K Y C Y N A D T Y D P T A F D M D G F N G S L V E Y
      100     110     120     130
401    500
ACATGGTTAAAGGCACTGAGAAGCTTACGCTAAGGTTGGTAGGTTTAAAGAGTCTGATGAACATCCTTTCTTCCCTGATGATCTACCAGAGCCTATGTT
      M V K G T E K L H A K V G R F K S P D E H P F P P D D L P E P M L
      140     150     160
501    600
GCCTCCTCTTCAGTACCCAAAGGTGTTGCATTGCTGCTGATTCGATAAACATAAACAAGAAGATATGGAACATGTACTTCAGAGACCTTGTCCAAGA
      P P L Q Y P K V L H F A A D S I N I N K K I W N M Y F R D L V P R
      170     180     190
601    700
CTTGTGAAGAAAGGCGATGATGGTAACTACGGCTCAACAGCTGTCTGTGACGCTATCTGCCTTCAGTGTCTCTCAAAGAGAATCCATTACGGTAAATTTG
      L V K K G D D G N Y G S T A V C D A I C L Q C L S K R I H Y G K F V
      200     210     220     230
701    800
TTGCAGAAGCTAAATTTCAAGCCTCACCCGAAGCATACGAGTCCGCCATCAAGCACAAGATAAGGATCGACTGATGGATATGCTGACATCCCGACTGT
      A E A K F Q A S P E A Y E S A I K A Q D K D R L M D M L T F P T V
      240     250     260
801    900
GGAAGATGCGATAAAGAAGAGAGTTGAGATGAAAACCCGAACATACGGGCAAGAAGTGAAGTTGGGATGGAGGAGAAAGAAGAAGAAGAAGGG
      E D A I K K R V E M K T R T Y G Q E V K V G M E E K E E E E E G
      270     280     290
901    1000
AATGAATCTCATGTTTACAAAATCAGTCCGATCTAGTTGGTGACTTATATGGAGATTGGATCATGCCCTTAAACAAAAGAGTTCAAGTGGAGTACTTGC
      N E S H V Y K I S P I L V G D L Y G D W I M P L T K E V Q V E Y L L
      300     310     320     330
1001   1100
TCAGAAGACTGGACTGAGGCAACAACAAAATAACAATATGGCTTGGTAGTAGAGTAGAAGGTTTTTGAATGTTCTTTGGTTTTTTTTTTTACTTTA
      R R L D *
      1100     1200
1101   1200
CAATATTTCTAAACGTTGTTACACTATTATCCACTGTACAAAGCGTGCATGGTCAGTGGTATTGAAGAAGGGTAATTAGCCGTTACTCAAACGGTGTCG
      1201
TTTATGT (A)n

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Fig. 2. DNA sequence and deduced amino acid sequence of an *Arabidopsis thaliana* chorismate mutase-specific cDNA clone. The proposed cleavage site of the plastid-specific transit peptide is indicated by ▲.

3. RESULTS

A yeast mutant (*aro7*, *ura3*), which has no chorismate mutase activity, was complemented with a complete *A. thaliana* cDNA library in the yeast expression vector, pFL61 [17]. Transformants were selected to grow on minimal medium. Using 10 µg plasmid DNA a single colony was obtained. After testing the transformant by plating it on fresh minimal medium, plasmid DNA was isolated and used to transform *E. coli* by selecting for ampicillin resistance. Plasmid DNA was isolated from a single *E. coli* colony and used to again complement two distinct yeast mutants (*aro7* and *aro7*, *ura3*). The complementation frequency for both yeast strains was about 1×10^4 colonies per µg plasmid DNA. This experiment confirmed the original complementation experiment and proved, in principle, that a cDNA fragment encoding *A. thaliana* chorismate mutase had been isolated. The plasmid containing the *A. thaliana* chorismate mutase cDNA was called pAtCM.

The *NotI* fragment which contained the cDNA insert was isolated, ligated into the *NotI* site of the plasmid, pBluescript SK(+), and its sequence determined (Fig. 2). The cDNA without its poly(A) tail is 1,207 bp long and contains an open reading frame of 1,002 bp which has the capacity to code for a peptide of 334 amino acids with a calculated M_r of 37,679. The 5' untranslated region is 12 bp and the 3' untranslated region is 193 bp long. The deduced amino acid sequence exhibits a sequence identity with the chorismate mutase sequence of *S. cerevisiae* of 41% (Fig. 3). Hardly any similarity was found to known sequences of bacterial chorismate mutases (data not shown). The N-terminal region of the deduced *A. thaliana* chorismate mutase sequence is, however, not similar with the *S. cerevisiae* sequence, but does resemble known plastidic transit peptides [18]. The sequence V⁵⁸HA[V⁶¹] is similar to the consensus sequence (I/V)X(A/C)A for the cleavage site (I) of plastidic transit peptides. If the proposed processing site is indeed used in vivo, the mature chorismate mutase is expected to have a M_r of 31,328.

Table I

Allosteric effects of aromatic amino acids on *Arabidopsis thaliana* chorismate mutase activity expressed in yeast

Specific chorismate mutase activity (pkat · mg ⁻¹)			
Without effectors	+100 µM Tyr	+100 µM Phe	+100 µM Trp
187.5 ± 6.7	29.8 ± 5.3	76.8 ± 5.8	288.3 ± 11.2

Chorismate mutase activities were measured in a desalted crude extract from mutant yeast cells (*aro7*) harboring the plasmid, pAtCM, with or without the addition of aromatic amino acids (100 µM) to the assay. An extract from mutant yeast cells (*aro7*) without the plasmid, prepared as a control, did not contain detectable chorismate mutase activity.

At CM	1	MRSSCCSSAI	GGFFDHRREL	STSTPISTLL	PLPSTKSSFS	VRCSLPQPSK	50
At CM	51	PRSGTSSVHA	VMTLAGSLTG	KKRVDSESL	TLEGIRNSLI	ROEDSIIFGL	100
Sc CM							
At CM	101	LERAKYCINA	DTYDP..TAF	DMDGFNGSLV	EYMKVGTEKL	HAKVGRFKSP	148
Sc CM							
At CM	149	DEHPFFPDDL	PEPMLPPLQY	PKVLHFAADS	ININKKIWNM	YFRDLVPRLV	198
Sc CM							
At CM	199	KKGDDG..NY	GSTAVCDAIC	LQCLSKRIHY	GKFVAEAKFQ	ASPEAYESAI	246
Sc CM							
At CM	247	KAQDKDRLMD	MLTFPTVEDA	IKKRVMKTR	TYGQEVKVM	BEKEEEEEEG	296
Sc CM							
At CM	297	NESHVYKISP	ILVGDLYGDW	IMPLTKEVQV	EYLLRRLD*		334
Sc CM							

Fig. 3. Comparison of the deduced amino acid sequence of *Arabidopsis thaliana* chorismate mutase (At CM) with that of *Saccharomyces cerevisiae* (Sc CM). The numbers refer to the amino acid sequence of the *A. thaliana* chorismate mutase as shown in Fig. 2.

Chorismate mutase activity was only detectable in extracts from mutant yeast cells (*aro7*) transformed with the plasmid, pAtCM (Table I). The activity was inhibited by phenylalanine and tyrosine (both 100 µM) by 59% and 84%, respectively, and tryptophan (100 µM) stimulated the activity by 50%.

4. DISCUSSION

The putative N-terminal transit peptide, as well as the regulatory effects of the aromatic amino acids on the chorismate mutase activity of transformed yeast, clearly identify the cloned chorismate mutase from *A. thaliana* as a plastidic enzyme (CM-1 type). Since chorismate mutase activities have not been analyzed in *A. thaliana*, it is not known whether this plant also contains an unregulated, and presumably cytosolic, chorismate mutase isozyme (CM-2 type).

Expression of the enzyme in yeast in an active form suggests that the chorismate mutase precursor polypeptide is enzymatically active, as previously shown for precursors of other enzymes of the shikimate pathway, such as 5-enolpyruvylshikimate 3-phosphate synthase [19] and shikimate kinase [20]. As yet, we cannot exclude the possibility that yeast cells process the precursor to the mature form by an unknown mechanism. The *A. thaliana* enzyme expressed in yeast is regulated by the three aromatic amino acids (Table I) as has been reported for mature plant chorismate mutases of the CM-1 type.

Present efforts in our laboratory are now directed towards the identification of a cDNA coding for a CM-2 type chorismate mutase.

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