FEBS Letters 349 (1994) 397–402

FEBS 14324

The characterisation of the shikimate pathway enzyme dehydroquinase from *Pisum sativum*

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Received 2 June 1994; revised version received 29 June 1994

Abstract

Peptides accounting for 157 residues of the bifunctional shikimate pathway enzyme, dehydroquinase/shikimate dehydrogenase, of *Pisum sativum* were sequenced. Three of the peptides were homologous to regions in *Escherichia coli* dehydroquinase and two to *E. coli* shikimate dehydrogenase. The pea dehydroquinase activity was inhibited by treatment with dehydroquinate plus sodium borohydride, establishing it as a type I dehydroquinase. Synthetic oligonucleotides designed from the amino acid sequence were used as PCR primers to amplify fragments of *P. sativum* cDNA. DNA sequence analysis showed that these amplified products were derived from dehydroquinase/shikimate dehydrogenase cDNA. The complete amino acid sequence of the dehydroquinase domain has been defined; it is homologous to all other type I dehydroquinases and is N-terminal.

Key words: Type I dehydroquinase; Shikimate dehydrogenase; *Pisum sativum*

1. Introduction

Dehydroquinase (3-dehydroquinate dehydratase EC 4.2.1.10) catalyses the conversion of dehydroquinate to dehydroshikimate, the third step of the biosynthetic shikimate pathway [1–4]. Recently it has been established that there are two types of biosynthetic dehydroquinase in microorganisms [5]. The type I enzymes, which have been characterised in detail from *Escherichia coli* [6–13] and *Neurospora crassa* [12,14–16] and studied in other species such as *Aspergillus nidulans* [17], *Saccharomyces cerevisiae* [18] and *Salmonella typhi* [19,20] are heat labile, have a mechanism involving an imine intermediate and use a histidine residue as a general base [13]. Some of these type I enzymes, for example those from *E. coli* and *S. typhi* [19,20], occur as monofunctional enzymes while others, for example those from *N. crassa, A. nidulans* and *S. cerevisiae*, occur as the fourth domain of the pentafunctional *arom* protein [14–16,17,18]. It has been shown that the stereochemistry for the type I dehydroquinase-catalysed reaction is *syn*, unlike the *trans* stereochemistry normally observed for such elimination reactions [21]. It has recently been found that in several bacterial species, including *Streptomyces coelicolor* [22], *Mycobacterium tuberculosis* [23] and *Amycolatopsis methanolica* [24], the dehydration of dehydroquinate is catalysed by heat-stable enzymes, which show no sequence homology with the type I dehydroquinases, but are instead very similar to the inducible catabolic type II dehydroquinases first characterised in fungi [25,26]. The mechanism of these type II dehydroquinases is not known. However, it has been established that, unlike the type I enzymes, they do not work through an imine intermediate [5] and that at least in the case of the *A. nidulans* enzyme the stereochemistry is *trans* [27].

The question of whether higher plants contain either a type I or a type II dehydroquinase has not been addressed since no plant dehydroquinase has yet been characterised at the mechanistic or sequence level. However, it has been established in several plant species that dehydroquinase occurs as a bifunctional polypeptide with shikimate dehydrogenase [28–31]. Here we report that the *Pisum sativum* (pea) dehydroquinase is a borohydride-sensitive, type I enzyme. Micro-scale protein chemistry has been used to obtain 157 residues of amino acid sequence from 5 peptides of the bifunctional enzyme and the information used to design PCR primers and amplify fragments of cDNA which encode the complete dehydroquinase domain and a portion of the shikimate dehydrogenase domain. The deduced amino acid sequence is similar to that of *E. coli* dehydroquinase and confirms that the plant enzyme is type I. The dehydroquinase activity resides at the N-terminus of the bifunctional polypeptide chain.

2. Experimental

Pisum sativum dehydroquinase-shikimate dehydrogenase was purified to near homogeneity from 1 kg batches of 15-day-old pea shoots as previously described [31]. Final purification for microsequencing was carried out by SDS PAGE on a 10% gel and protein bands visualised by staining with Coomassie Blue R-250. The band corresponding to dehydroquinase-shikimate dehydrogenase was excised, neutralized by washing with 125 mM Tris-HCl, pH 6.8, 0.1% SDS, 1 mM dithiothreitol, loaded onto a second 18% SDS gel, and the protein digested with V8 proteinase (Glu-C, Boehringer) during electrophoresis [32]. Peptide fragments were electroblotted onto polyvinylidene difluoride

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SSDI 0014-5793(94)00710-1

(PVDF) membrane and subjected to microsequencing [33] using an Applied Biosystem 477A protein sequencer. Electroblotted intact protein was sequenced by the same method.

Dehydroquinase and shikimate dehydrogenase activities were determined as described previously [34] as was the inactivation of dehydroquinase-shikimate dehydrogenase with DEPC [13]. The imine intermediate was trapped by treating 100 μl samples of dehydroquinase-shikimate dehydrogenase in 20 mM Tris-HCl, pH 7.5, 1 mM benzamidine, 0.5 mM dithiothreitol at 20°C with 1 mM ammonium dehydroquinate for 5 min followed by the addition of 10 μl of freshly prepared $NaBH_4$ (20 mg/ml) in 40 mM NaOH.

Total RNA, extracted from freshly harvested 11-day-old pea shoots by a phenol extraction method [35], was used as template for an oligo d(T)-primed synthesis of single stranded cDNA [36] using a Pharmacia kit.

From the primary sequence data of dehydroquinase-shikimate dehydrogenase, two pairs of degenerate oligonucleotides were designed for use as PCR primers. A fifth primer was based on part of the 'anchor' portion of the Pharmacia *Not*I oligo $d(T)_{18}$ bifunctional primer. All the primers had restriction sites to facilitate cloning of PCR products (Table 1). Oligonucleotides were synthesised on an Applied Biosystems model 381A DNA Synthesiser. The PCR reaction was performed as described by Saiki et al. [37]; the PCR reaction mixtures (100 μl) contained: 10 mM Tris-HCl, pH 8.3, 3.5 mM $MgCl_2$, 0.01% Triton X-100, each dNTP at 0.2 mM and each primer at 2.0 μM together with pea cDNA. 2.5 units of *Taq* polymerase (Promega) were added before addition of 100 μl paraffin oil. Sometimes 'nested' PCR was done to improve the yield and specificity: in this case the PCR was performed for 30 cycles with primers-III and -6 (with a 50°C annealing temperature), then on 1% of the material an additional 30 cycles were done using a second primer pair (I and IV) internal to the amplified DNA. During nested PCR a higher annealing temperature (58°C for 2 min) was used. The position of the primers used in PCR and for DNA sequencing are shown in Fig. 1. PCR products were purified by electrophoresis on low melting point agarose gels, and cloned into M13 mp18 and 19. Clones containing inserts were sequenced on both strands using Sequenase Version 2.0 (USB). Specialized sequencing primers (SP1 and SP2 in Table 1) were used as well as the universal M13 sequencing primer. dITP was used to eliminate the compressions in GC rich regions.

The following sequences were used in the multiple alignment: type I dehydroquinases from *E. coli* [12], *S. typhi* [19], *A. nidulans* [17], *S. cerevisiae* [18]; shikimate dehydrogenases from *E. coli* [38] and *S. cerevisiae* [18]; the multiple sequence alignments were performed as described previously [39].

3. Results and discussion

3.1. Microsequencing of protein and V8-peptides

Peptides generated from in situ proteolysis of dehydroquinase-shikimate dehydrogenase were transferred onto PVDF membrane (Fig. 2). 157 amino acid residues were sequenced from five V8 peptides. The amino acid sequences of the peptides are shown in Table 2. The sequences of three peptides showed homology to other type I dehydroquinases and two to shikimate dehydrogenases (data not shown). The protein was not blocked at its N-terminus and peptide-I, which matched the N-terminal sequence of the intact protein, was homologous to the N-termini of all the other type I dehydroquinases for which sequence information is available. The first 40 residues from the N-terminus of the *P. sativum* enzyme are shown in the multiple alignment in Fig. 4. These results established that the dehydroquinase domain of the plant bifunctional protein is N-terminal.

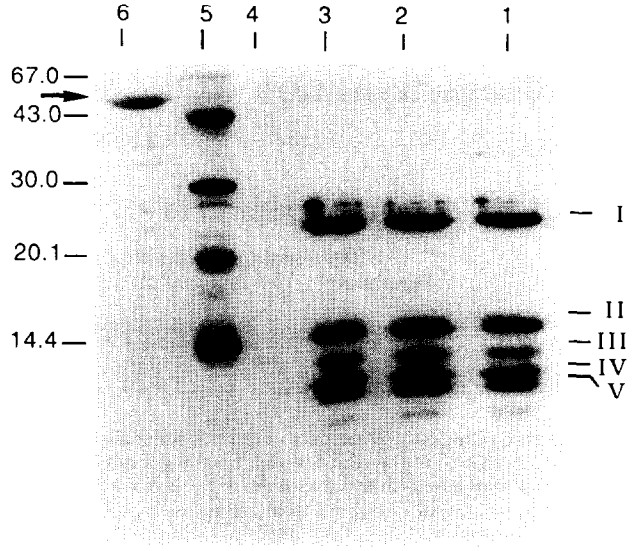

Fig. 2. V8 digestion fragments of dehydroquinase-shikimate dehydrogenase (Photograph of PVDF-bound protein/peptides). Gel slices containing 25–30 μg of DHQase-SHDase were digested with 2 μg of V8-protease and transferred electrophoretically onto PVDF membrane. Lanes 1–3, V8-digest; Lane 4, 2 μg V8 as control; Lane 5, 10 μg molecular mass standards; Lane 6, approx. 10 μg of intact protein (arrow mark). The V8 fragments are numbered I, II, III, IV and V according to their mobility.

3.2. Properties of the plant dehydroquinase-shikimate dehydrogenase activities

The K_m value determined for plant dehydroquinase is 37 μM, higher than the value for the type I dehydroquinase from *E. coli* (16 μM) [5]. Both these values are much lower than that reported for the type II dehydroquinase from *A. nidulans* (150 μM) [5]. The plant dehydroquinase activity, like that of *E. coli*, was > 99% inactivated upon borohydride treatment, while control experiments showed no loss of activity. The shikimate dehydrogenase activity was unaltered by the borohydride treatment. Inactivation by borohydride in the presence of substrate is characteristic of type I dehydroquinases indicating that

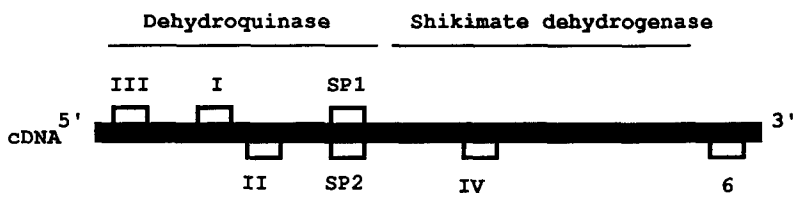

Fig. 1. Schematic representation of the positions of primers used for amplification and sequencing of dehydroquinase-shikimate dehydrogenase cDNA (see also Table 1 and Fig. 4).

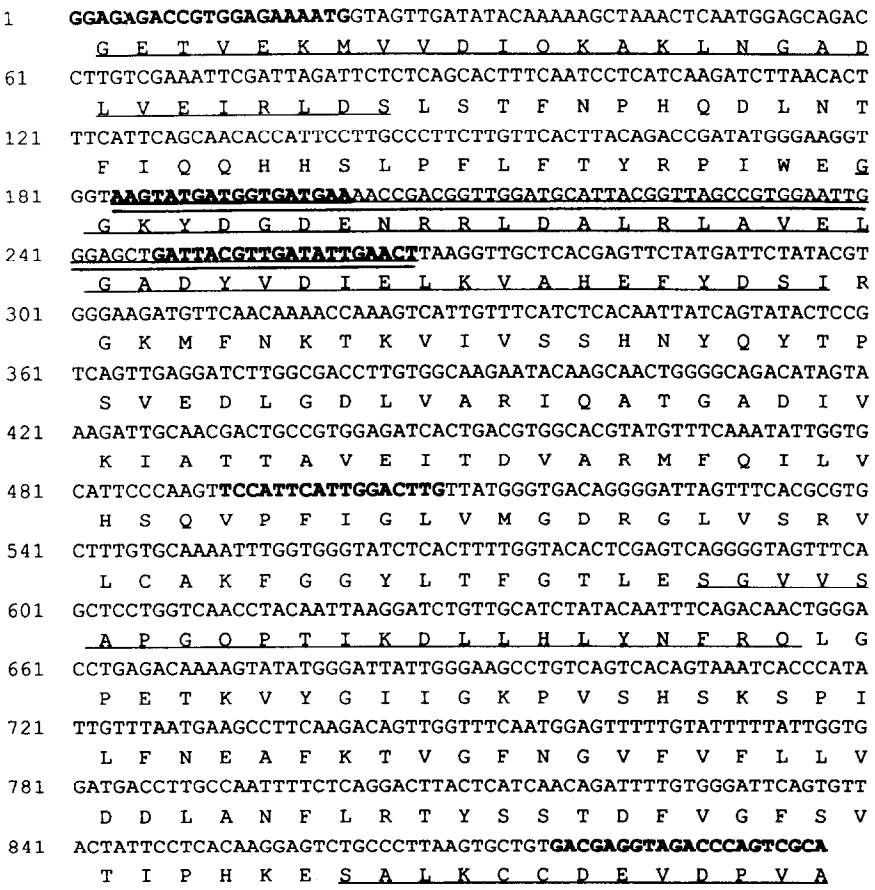

Fig. 3. Nucleotide and predicted amino acid sequence of cDNA encoding part of pea dehydroquinase-shikimate dehydrogenase. The nucleotide sequence represents two overlapping (shown **double underlined**) cDNA clones of 266 and 714 bp. The predicted amino acid sequence is also shown. The regions of sequence shown in **bold face** represent the sequences of the primers used for PCR amplification of cDNA. The amino acid sequences corresponding to those determined for the V8 peptides are **underlined**. The nucleotide sequence has been deposited in the EMBL Data Library (Accession Number Z34521).

the pea dehydroquinase belongs to this family, where an enzyme–substrate/product imine intermediate can be trapped by reduction. The dehydroquinase and shikimate dehydrogenase activities of the plant bifunctional protein are both inactivated by DEPC; in both cases inactivation followed pseudo first order kinetics. Substrates protected both activities against DEPC inactivation, indicating the presence of histidine residue(s) at or near both active sites. These results are similar to those obtained when the two corresponding monofunctional

Table 1
Primers for amplification and sequencing of pea cDNA

	*Bam*HI
Primer-I	5′-CCGGATCC AA(AG)TA(TC)GA(TC)GGNGA(TC)GA(AG)AA-3′
	*Sal*I
Primer-II	5′-CCGTCGACA(GA)(TC)TC(AGT)AT(AG)TCNAC(AG)TA(AG)TC-3′
	*Eco*RI
Primer-III	5′-CCGAATTC GGNGA(AG)ACNGTNGA(AG)AA(AG)ATGGT-3′
	*Eco*RI
Primer-IV	5′-CCGAATTC GCNACNGG(AG)TCNAC(TC)TC(AG)TC-3′
	*Cla*I
Primer-6	5′-CGATCGAT AACTGGAAGAATTC-3′
Primer-SP1	5′-TCCATTCATTGGACTTG-3′
Primer-SP2	5′-CAAGTCCAATGAATGGA-3′

Primers I and III: sense; II, IV and 6: antisense; SP1 and SP2: specialized sequencing primers. N indicates that a mixture of all 4 bases was used; brackets indicate where a simpler mixture of bases was used.

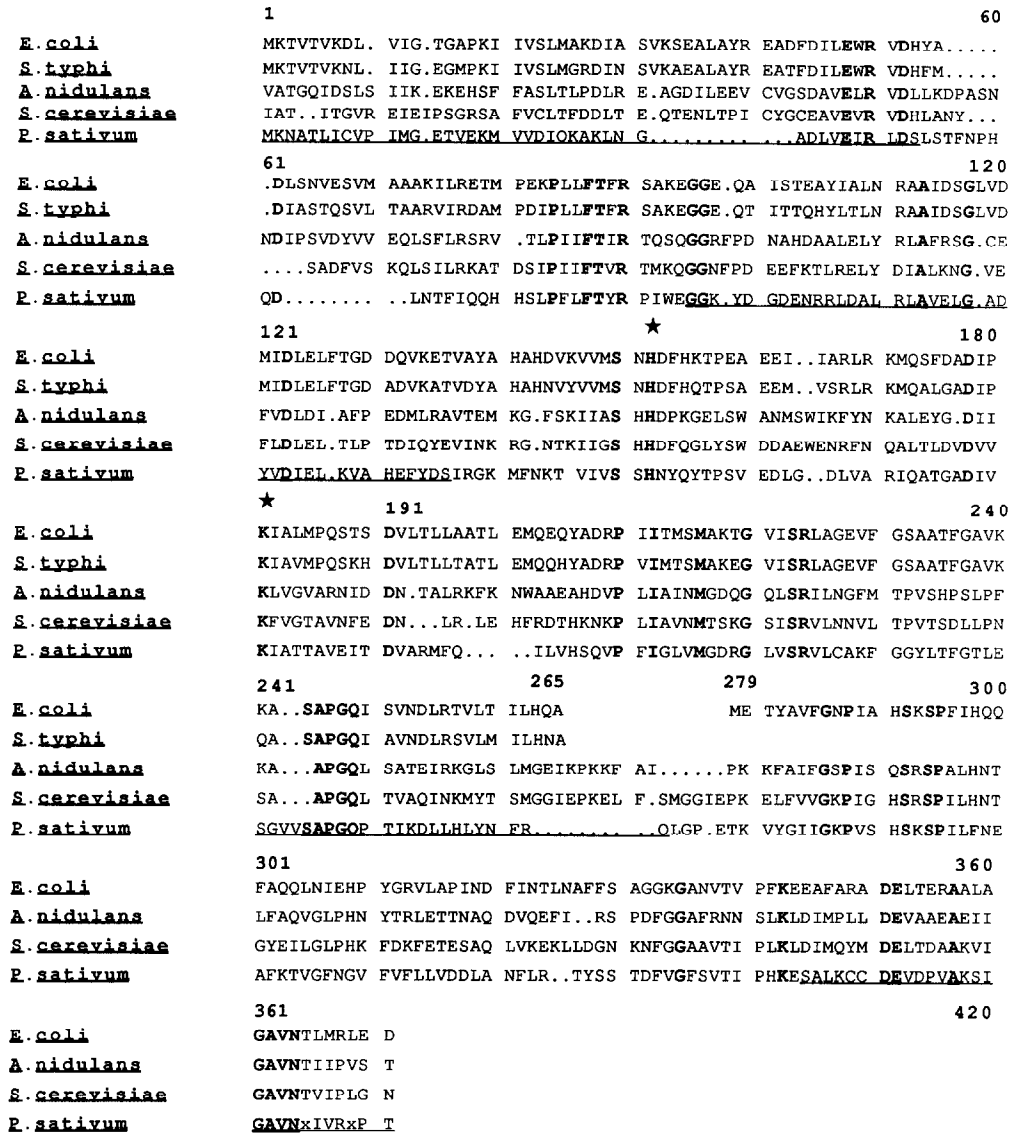

Fig. 4. Multiple alignment of the deduced amino acid sequence of the pea dehydroquinase region, and of part of the shikimate dehydrogenase region, with other type I dehydroquinases and shikimate dehydrogenases. The first 12 and last 15 residues (shown **double underlined**) of the pea sequence were from peptide sequences only. The amino acid sequences corresponding to those determined for the V8 peptides are **underlined**. The numbers run from the N-terminus of the *P. sativum* bifunctional enzyme. Gaps indicated by dots were introduced for optimal alignment. Amino acids numbered from 1 to 265 are from the dehydroquinase region; the *E. coli* enzyme has 252 residues [12] and 13 gaps are required to give the best alignment. Residues numbered 266–278 correspond to the linker region [18] between the domains of the multifunctional enzymes. The incomplete shikimate dehydrogenase region begins at residue 279. Conserved amino acid residues are shown in **bold face**. ★'s refer to the active site residues of *E. coli* dehydroquinase (His^{143} and Lys^{170}). X refers to residues not identified during peptide sequencing.

E. coli enzymes are treated with DEPC [8,13] (R. Syme, S. Chackrewarthy and J.R. Coggins, unpublished data).

To confirm the domain order and the identification of the dehydroquinase domain as being of the type I family it was necessary to clone and sequence a cDNA encoding at least the dehydroquinase region of the bifunctional pea dehydroquinase-shikimate dehydrogenase.

3.3. *Amplification, cloning and sequencing of plant dehydroquinase cDNA*

Using PCR, two cDNA segments of the expected size were amplified (data not shown). Primer pairs III/II and I/IV (see Fig. 1) gave fragments of 266 bp and 714 bp, respectively, which were then cloned into M13 for sequence analysis. Precautions were taken against the possibility of sequencing a mutant fragment arising from errors by either the reverse transcriptase or the *Taq* polymerase. The sequence of the 714 bp region was determined initially on both strands. It was checked by further sequencing of an independent clone (in M13) derived from a separate PCR experiment which used cDNA from a different synthesis to that used for the

Table 2
Amino acid sequences obtained from microsequence analysis

Peptide-I	MKNATLI?VPIMGETVEKMVVDIQKAKLNGADLVEIRLDD
Peptide-II	GGKYDGDENRRLDALRLAVELGADYVDIELKVAHEFYDSI
Peptide-III	SALK??DEVDPVAKSIGAVN-IVR-PTDG(AKL)KL-Y
Peptide-IV	SGVVSAPGQP(TP)(NI)(IK)(DK)(DL)LHLYNFRQ
Peptide-V	DGMILANTTSIGMQPKVDET

Amino acid residues within brackets represent ambiquities at a particular position. '–' represents residues not identified. '?' indicates a peak whose retention time did not match any of the standard amino acids (later this peak was identified as a cysteine derivative). Independent sequence analysis of the intact protein established that peptide-I is at the N-terminus of dehydroquinase-shikimate dehydrogenase.

initial PCR template. For the 266 bp fragment the initial sequence was checked by sequencing a second clone. The sequence of this clone confirmed that of the first except for an intron of 80 bp. Although this second clone came from the same PCR reaction as the first, the presence of the intron fortuitously marks this clone as having an independent origin. The second clone must have originated either from an independent cDNA derived from an aberrantly spliced mRNA or conceivably from contaminating genomic DNA in the total RNA preparation.

The 266 and 714 bp fragments overlapped by 40 bp (excluding primers). The 897 bp sequenced encode 299 amino acid residues (Fig. 3). The N-terminal amino acid sequence (peptide-I in Table 2) of the pea dehydroquinase-shikimate dehydrogenase overlaps with the start of the amino acid sequence deduced from the cDNA sequences. Only the amino acid sequence deduced from the cDNA sequences is shown in Fig. 3. The sequence right from the N-terminus of the protein is shown in the multiple alignment (Fig. 4). Multiple alignment showed that the N-terminal region of the polypeptide was homologous to the type I dehydroquinases and the C-terminal region to the shikimate dehydrogenases. This confirmed our earlier conclusion that the dehydroquinase domain of the plant bifunctional protein is N-terminal. It is interesting to note that this domain order is the same as that observed in the *arom* proteins where the dehydroquinase and shikimate dehydrogenase domains occur at the C-terminal end of the pentafunctional polypeptide chain [17,18].

There is evidence for isozymic forms of dehydroquinase-shikimate dehydrogenase in diverse plants including peas [31,32]. In the final FPLC Mono Q ion exchange step of the purification three activity peaks were seen (in a ratio of about 8:1:1) [31]. Only the major peak was taken for peptide sequencing. There is a chance that the dehydroquinase cDNA sequence is chimaeric with respect to isozymic sequences. However, we think this is unlikely for three reasons. Firstly, there is an exact match between the actual peptide sequences and those deduced from the DNA. Secondly, in the 40 bp overlap region (excluding primers) both PCR products have identical sequences (including the third base positions). Thirdly in both parts of the sequence (the 266 and 714 bp fragments) there is unequivocal homology to the other type I dehydroquinases.

The amino acid residues which are known to occur at the active site of the *E. coli* type I dehydroquinase, Lys^{170} [12] and His^{143} [13], are conserved in all the type I dehydroquinases. Lys^{170} is the active site residue involved in imine formation [12,40] and His^{143} is the general base [13]. The conservation of these residues in the *P. sativum* dehydroquinase reported here, confirms that this enzyme is an imine-forming, type I enzyme (Fig. 4).

All the shikimate pathway enzymes previously cloned from higher plants have been shown to have transit peptides and are believed to be synthesized in the cytosol and then transported into chloroplasts where the transit peptide is cleaved giving rise to the mature enzyme [41]. It is very likely that the situation for at least the major isoenzyme form of dehydroquinase/shikimate dehydrogenase is similar, although the present work does not address this question since the PCR primers were designed to amplify a cDNA species lying within the coding region of the mature enzyme.

Acknowledgements: We thank Dr. David Mousdale for guidance on the enzyme purification, Dr. John Miles for advice on the use of PCR and Miss Fiona McLeod who carried out some preliminary experiments. We also thank John Greene for assisting with the enzyme purification and Dr. V. Math for oligonucleotide synthesis. R.K.D. is a visiting scholar funded by the Government of Assam, India. This work was supported by the Science and Engineering Research Council of Great Britain.

References

[1] Haslam, E. (1974) The Shikimate Pathway, Butterworth, London.
[2] Haslam, E. (1993) Shikimic Acid: Metabolism and Metabolites, Wiley, Chichester.
[3] Weiss, U. and Edwards, J.H. (1980) The Biosynthesis of Aromatic Compounds, Wiley, New York.
[4] Bentley, R. (1990) CRC Crit. Rev. Biochem. Mol. Biol. 25, 306–384.
[5] Kleanthous, C., Deka, R., Davis, K., Kelly, S.M., Cooper, A., Harding, S.E., Price, N.C., Hawkins, A.R. and Coggins, J.R. (1992) Biochem. J. 282, 687–695.
[6] Mitsuhashi, S. and Davis, B.D. (1954) Biochim. Biophys. Acta 15, 54–615.
[7] Duncan, K., Chaudhuri, S., Campbell, M.S. and Coggins, J.R. (1986) Biochem. J. 238, 475–483.

[8] Chaudhuri, S., Lambert, J.M., McColl, L.A. and Coggins, J.R. (1986) Biochem. J. 239, 699–704.
[9] Kleanthous, C., Campbell, D.G. and Coggins, J.R. (1990) J. Biol. Chem. 265, 10929–10934.
[10] Kleanthous, C. and Coggins, J.R. (1990) J. Biol. Chem. 265, 10935–10939.
[11] Kleanthous, C., Reilly, M., Cooper, A., Kelly, S., Price, N.C. and Coggins, J.R. (1991) J. Biol. Chem. 266, 10893–10898.
[12] Chaudhuri, S., Duncan, K., Graham, L.D. and Coggins, J.R. (1991) Biochem. J. 275, 1–6.
[13] Deka, R.K., Kleanthous, C. and Coggins, J.R. (1992) J. Biol. Chem. 267, 22237–22242.
[14] Lumsden, J. and Coggins, J.R. (1977) Biochem. J. 161, 599–607.
[15] Smith, D.D.S. and Coggins, J.R. (1983) Biochem. J. 213, 405–415.
[16] Lambert, J.M., Boocock, M.R. and Coggins, J.R. (1985) Biochem. J. 226, 817–829.
[17] Charles, I.J., Keyte, J.W., Brammar, W.J. and Hawkins, A.R. (1985) Nucleic Acids Res. 13, 8119–8128.
[18] Duncan, K., Edwards, R.M. and Coggins, J.R. (1987) Biochem. J. 246, 375–386.
[19] Servos, S., Chattfield, S., Hone, D., Levine, M., Dimitriadis, G., Pickard, D., Dougan, G., Fairweather, N. and Charles, I.A. (1991) J. Gen. Microbiol. 137, 147–152.
[20] Moore, J.D., Hawkins, A.R., Charles, I.G., Deka, R., Coggins, J.R., Cooper, A., Kelly, S.M. and Price, N.C. (1993) Biochem. J. 295, 277–285.
[21] Hanson, K.R. and Rose, I.A. (1963) 50, 981–988.
[22] White, P.J., Young, J., Hunter, I.S., Nimmo, H.G. and Coggins, J.R. (1990) Biochem. J. 265, 735–738.
[23] Garbe, T., Servos, S., Hawkins, A.R., Dimitriadis, G., Young, D., Dougan, G. and Charles, I.G. (1990) Mol. Gen. Genet. 228, 385–392.
[24] Euverink, G.J.W., Hessels, G.I., Vrijbloed, J.W., Coggins, J.R. and Dijkhuizen, L. (1992) J. Gen. Microbiology 138, 2449–2457.
[25] Giles, N.H., Partridge, C.W.H., Ahmed, S.I. and Case, M.E. (1967) Proc. Natl. Acad. Sci. USA 58, 1930–1937.
[26] Giles, N.H., Case, M.E., Baum, J., Geever, R., Huiet, L., Patel, V. and Tyler, B.M. (1985) Microbiol. Rev. 49, 338–358.
[27] Harris, J., Kleanthous, C., Coggins, J.R., Hawkins, A.R. and Abell, C. (1993) J. Chem. Soc. Chem. Commun. 1080–1081.
[28] Koshiba, T. (1978) Biochim. Biophys. Acta 522, 10–18.
[29] Polley, L.D. (1978) Biochim. Biophys. Acta 526, 259–266.
[30] Fiedler, E. and Schultz, G. (1985) Plant Physiol. 79, 212–218.
[31] Mousdale, D.M., Campbell, M.S. and Coggins, J.R. (1987) Phytochemistry 26, 2665–2670.
[32] Cleveland, D.W., Fischer, S.G., Kirschner, M.W. and Laemmli, U.K. (1977) J. Biol. Chem. 252, 1102–1106.
[33] Matsudaira, P. (1987) J. Biol. Chem. 262, 10035–10038.
[34] Coggins, J.R., Boocock, M.R., Chaudhuri, S., Lambert, J.M., Lumsden, J., Nimmo, G.A. and Smith, D.D.S. (1987) Methods Enzymol. 142, 325–349.
[35] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York.
[36] Gubler, U. and Hoffman, B.J. (1983) Gene 25, 263–269.
[37] Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B. and Erlich, H.A. (1988) Science 239, 487–491.
[38] Anton, I.A. and Coggins, J.R. (1988) Biochem. J. 249, 319–326.
[39] Bell, L.H., Coggins, J.R. and Milner-White, E.J. (1993) Prot. Eng. 6, 683–690.
[40] Shneier, A., Kleanthous, C., Deka, R., Coggins, J.R. and Abell, C. (1991) J. Am. Chem. Soc. 113, 9416–9418.
[41] Schmid, J., Schaller, A., Leibinger, U., Boll, W. and Amrhein, N. (1992) Plant J. 2, 375–383.