FEBS Letters 408 (1997) 156–160 FEBS 18555

Purification and characterization of a fusion protein of plant acetohydroxy acid synthase and acetohydroxy acid isomeroreductase

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Received 20 February 1997; revised version received 27 March 1997

Abstract The nucleotide sequence coding for the *Arabidopsis thaliana* acetohydroxy acid synthase was genetically fused in frame with the nucleotide sequence coding for the *Spinacia oleracea* acetohydroxy acid isomeroreductase and expressed in *Escherichia coli*. This construction allowed the production of large amounts of soluble fusion protein. The pure chimeric enzyme exhibits high acetohydroxy acid synthase and acetohydroxy acid isomeroreductase specific activities. Fusion and native enzymes exhibit similar K_m values for their substrates and for most cofactors. Furthermore, whereas native plant acetohydroxy acid synthase is highly unstable, the stability of this enzyme in the fusion has been increased. Thus, the chimeric enzyme appears to be a useful tool for the determination of kinetic and structural properties of plant acetohydroxy acid synthase.

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Key words: Acetohydroxy acid synthase; Acetohydroxy acid isomeroreductase; Fusion protein

1. Introduction

In plants and micro-organisms, acetohydroxy acid synthase (AHAS; EC 4.1.3.18) and acetohydroxy acid isomeroreductase (AHIR; EC 1.1.1.86) catalyse the first and the second step, respectively, in the common pathway leading to valine and isoleucine. Inhibition of either enzyme leads to plant death [1,2]. Noncompetitive inhibitors (sulfonylurea, imidazolinone) of acetohydroxy acid synthase have been shown to be powerful herbicides acting at low dose rate [for a review, see [3]]. However, only weak herbicides behaving as slow binding competitive inhibitors (IpOHA and HOE 704) have been described for acetohydroxy acid isomeroreductase [2,4,5].

Plant acetohydroxy acid synthase is highly unstable [6,7], and it is probably for this reason that purification to homogeneity of an intact enzyme exhibiting high activity has not been previously described from plant extracts. In order to produce large amounts of acetohydroxy acid synthase, the *Arabidopsis thaliana* gene was overexpressed in *Escherichia coli* [8–10]. The *A. thaliana* enzyme was finally purified from a fusion protein with glutathione *S*-transferase [10]. The purified enzyme exhibited a high level of activity (1.7 $\mu mol \cdot mg^{-1} \cdot min^{-1}$), but no data concerning enzyme stability, kinetic parameters or yield of purification were given [10].

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Abbreviations: AHAS, acetohydroxy acid synthase; AHIR, acetohydroxy acid isomeroreductase; DTT, dithiothreitol; FAD, flavin adenine dinucleotide; Hoe 704, 2-(dimethylphosphinoyl)-2-hydroxyacetic acid; IpOHA, *N*-hydroxy-*N*-isopropyloxamate; NADPH, nicotinamide adenine dinucleotide phosphate, reduced form; TPP, thiamine pyrophosphate

The next enzyme in the metabolic pathway, acetohydroxy acid isomeroreductase, has been purified to homogeneity from *Spinacia oleracea* [11] and *Hordeum vulgarum* [12]. The cDNA of the spinach enzyme has been overexpressed in *E. coli* and the kinetic parameters of the overproduced enzyme were determined [13–15]. The spinach enzyme was further co-crystallized with its cofactors (NADPH and magnesium) and IpOHA [16], and the structure of this complex has been solved at 1.65 Å resolution (Biou et al., EMBO J., in press).

In this work, we have genetically fused the nucleotide sequence encoding the *A. thaliana* acetohydroxy acid synthase (i.e. last 12 amino acids of the transit peptide followed by the mature amino acid sequence) in frame with the nucleotide sequence encoding the *S. oleracea* mature acetohydroxy acid isomeroreductase. The objective of this work is to determine (a) whether the fusion of such dimeric enzymes is possible, (b) if the fusion brings a kinetic advantage for these two enzymes and (c) if the stability of acetohydroxy acid synthase could be increased by the fusion.

2. Materials and methods

2.1. Materials

Restriction enzyme endonucleases, Pwo DNA polymerase, T4 DNA ligase and IPTG were supplied by Boehringer (Meylan, France). *E. coli* strain BMH-71-18 mutS was supplied with the unique site elimination (USE) mutagenesis Kit (Pharmacia) and was used during mutagenesis of the plasmid pKK-AHIR that contains the coding sequence for the mature spinach acetohydroxy acid isomeroreductase [14]. Overexpression of the fusion protein AHIR/AHAS was further realized in *E. coli* JM 105.

2.2. Insertion of a NcoI restriction site on pKK-AHIR by site-directed mutagenesis

Mutagenesis was carried out using the USE mutagenesis Kit (Pharmacia) that utilizes a two-primer system to generate site-specific mutations [17]. The first primer 5′-ACGCGCGAGGCGGCCGCGGTAAAGC is directed to the vector sequence and transforms a unique and non-essential site of restriction enzyme (*Pvu*II) into another unique restriction site (*Not*I). The second primer 5′-CCAGAGTTGCGCCAGCCATGGGTATTAGGCATGGAG is directed to the sequence of acetohydroxy acid isomeroreductase and transforms the sequence around the stop codon into a unique restriction site *Nco*I.

2.3. PCR amplification and cloning of the acetohydroxy acid synthase sequence into the NcoI restriction site of pKK-AHIR-NcoI

PCR amplification was carried from plasmid pUC19-AHAS which contains the *A. thaliana* acetohydroxy acid synthase gene [18] (gift from Dr. Michel Lebrun). Primers P1 5′-CCGCCGTGCTCAACCCATGGACCAATGTCACAACCACTCCC and P2 5′-CTGAGAGATGAAACCACCATGGATTATCAGAACCTT were used to amplify the coding sequence of the *A. thaliana* acetohydroxy acid synthase, devoid of a major part of the transit peptide. The amplification product was then digested by *Nco*I for further cloning into the *Nco*I site of pKK-AHIR-*Nco*I.

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PII S0014-5793(97)00410-9

2.4. Purification of the chimeric enzyme

E. coli JM105 cells expressing the pKK-AHIR/AHAS plasmid coding for the chimeric enzyme were grown at 28°C in 1.0 l of LB medium supplemented with 25 μg of streptomycin and 100 μg of carbenicillin per milliliter [19]. Isopropyl-β-D-thiogalactoside (IPTG) was added to a final concentration of 1 mM when bacterial growth was equivalent to an A_{600} of 0.5 and the cells were allowed to continue growing for 15 h at 28°C. The cells were harvested by centrifugation and the pellet was resuspended in 10 ml of lysis buffer (25 mM potassium phosphate (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol and 100 μM FAD) and sonicated in 100 pulses each of 3 s on a power setting of 5 by a Vibra-cell disruptor (Sonics and materials, Danbury, CT). The crude extract was centrifuged at 20 000×*g* for 30 min to yield a cell-free supernatant. At this step, nucleic acids were removed by addition of streptomycin-sulfate 0.1% (w/v) and further centrifugation at 20 000×*g* for 30 min. The soluble protein extract (600 mg), which contained the fusion protein, was applied to a 1×10 cm Source Q column (Pharmacia) connected to a Pharmacia FPLC system previously equilibrated in buffer A (25 mM potassium phosphate (pH 7.5), 1 mM EDTA). Elution was performed with a 300 ml gradient of 0–300 mM NaCl in buffer A (flow rate 1 ml/min; fraction size 2 ml). Chromatographic fractions containing the chimeric enzyme were concentrated to 2 ml by centrifugation at 5000×*g* in a Macrosep-30 unit (Filtron). This extract (40 mg protein) was then applied to a Hiload 16/60 Superdex 200 column (Pharmacia) connected to the Pharmacia FPLC system previously equilibrated in buffer B (25 mM potassium phosphate (pH 7.5), 1 mM EDTA, 150 mM NaCl, 100 μM FAD). The enzyme was eluted with 64 ml of buffer B (flow rate 1 ml/min; fraction size 1 ml). The chromatographic fractions containing the chimeric enzyme were concentrated by centrifugation at 5000×*g* in a Macrosep-30 unit (Filtron) and stored at −80°C until used. Just before the determination of kinetic parameters, the pure enzyme was applied to a PD 10 column (Pharmacia) previously equilibrated in buffer C (100 mM (Tris[hydroxymethyl]amino-methane)-HCl (pH 8.2), 150 mM NaCl) to remove phosphate, EDTA and FAD.

Electrophoresis was carried out at room temperature in SDS-polyacrylamide slab gels containing (SDS-PAGE) 7.5% (w/v) acrylamide [20]. Protein concentration was determined either with the Bio-Rad protein assay [21] during the purification steps or by measuring the absorbance at 205 nm [22] for the pure enzyme.

2.5. Enzyme assays

Acetohydroxy acid isomeroreductase activity was assayed in 100 mM Tris-HCl (pH 8.2), 150 mM NaCl, 10 mM $MgCl_2$ and 250 μM NADPH in a final volume of 1 ml at 30°C. Reactions were initiated by adding 1 mM 2-aceto-2-hydroxybutyrate or 2-acetolactate. Progress of the reaction was monitored by the decrease in absorbance of NADPH at 340 nm. Enzyme activity was expressed as μmol of NADPH oxidized·min^{-1}·mg^{-1} protein.

Acetohydroxy acid synthase activity was measured by estimation of 2-acetolactate after conversion to acetoin by acid decarboxylation [23]. The rate of 2-acetolactate formation was determined at 30°C in a 0.5 ml reaction mixture containing 100 mM Tris-HCl (pH 8.2), 150 mM NaCl, 10 mM $MgCl_2$, 20 mM pyruvate, 1 mM TPP and 0.1 mM FAD. The reaction was stopped by addition of 50 μl of 8.5% (v/v) H_2SO_4 and acetoin concentration was determined [23]. Enzyme activity was expressed as μmol of acetolactate formed min^{-1}·mg^{-1} protein.

The overall conversion of pyruvate to 2,3-dihydroxy-3-isovalerate catalyzed by the fused protein was assayed in 100 mM Tris-HCl (pH 8.2), 150 mM NaCl, 10 mM $MgCl_2$, 1 mM TPP, 0.1 mM FAD and 250 μM NADPH in a final volume of 1 ml at 30°C. Reactions were initiated by adding 20 mM pyruvate. Progress of the reaction was monitored by the decrease in absorbance of NADPH at 340 nm as described above. Enzyme activity was expressed as μmol of NADPH oxidized·min^{-1}·mg^{-1} protein.

Kinetic parameters were calculated by using the Kaleidagraph™ program (Abelbeck Software) working on a Macintosh IISI computer.

3. Results and discussion

3.1. Construction of the fusion protein

To obtain an expression vector encoding the fusion enzyme composed of acetohydroxy acid isomeroreductase and acetohydroxy acid synthase, the plasmid pKK-AHIR encoding the spinach acetohydroxy acid isomeroreductase [14] was modified by the insertion of an *Nco*I restriction site instead of its stop codon. The *A. thaliana* acetohydroxy acid synthase nucleotide sequence coding for the last 12 amino acids of the transit peptide followed by the mature amino acid sequence was amplified with two primers containing an *Nco*I restriction sequence. Thus, after digestion by *Nco*I restriction enzyme, the amplification product was cloned into the new *Nco*I restriction site of pKK-AHIR giving the plasmid pKK AHIR/AHAS encoding the chimeric enzyme.

The N-terminal sequence of the mature acetohydroxy acid synthase has never been determined. Nevertheless, Western blotting experiments carried out on an *A. thaliana* crude extract [9], have shown that acetohydroxy acid synthase is processed to a final molecular mass of 65 000 Daltons. From this data, the first amino acid of the mature *A. thaliana* acetohydroxy acid synthase amino acid sequence (without the transit peptide) corresponds approximately to proline-81 (nucleotide 522) [18]. The last 12 amino acids of the acetohydroxy acid synthase transit peptide (Trp–Thr–Thr–Asn–Val–Thr–Thr–Thr–Pro–Ser–Pro–Thr–Lys) were used as linker between the C-terminal of acetohydroxy acid isomeroreductase and the N-terminal of acetohydroxy acid synthase in order to avoid structural constraints between the two dimeric enzymes.

3.2. Characterization of the fusion protein

The protocol described in Section 2 allowed us to purify the chimeric enzyme from extracts of transformed *E. coli* with a good yield in two chromatographic steps (Table 1). Thus, 5 mg of pure fusion protein was obtained starting from 600 mg of *E. coli* soluble proteins (Table 1 and Fig. 1). The profile of protein at each step of purification is illustrated in Fig. 1. The purified chimeric enzyme from this source could be stored frozen at −80°C for several months without noticeable loss of enzyme activity.

Upon SDS-PAGE, the purified fusion protein migrated as a single band of M_r 120 000 (Fig. 1) which corresponds to the sum of the M_r of acetohydroxy acid isomeroreductase (57 000)

Table 1
Purification procedure of the fusion protein overexpressed in *E. coli*

Purification stage	Total protein (mg)	Total activity (μmol of NADPH oxidized·min^{-1})	Specific activity (activity·mg^{-1} protein)	Yield (%)
Crude extract of soluble proteins	605	41	0.068	100
Hiload 16/10 Source Q pool	22	18	0.8	44
Hiload 16/60 Superdex 200 pool	5	9.5	1.9	23

Protein was determined with the Bio-Rad protein assay during purification steps, or by detection at 205 nm for the pure enzyme. The fusion protein was detected by measuring the reaction catalyzed by acetohydroxy acid isomeroreductase activity at 30°C in the Tris-HCl buffer, pH 8.2, with 150 mM NaCl, 10 mM $MgCl_2$, 250 μM NADPH and 1 mM 2-aceto-2-hydroxybutyrate.

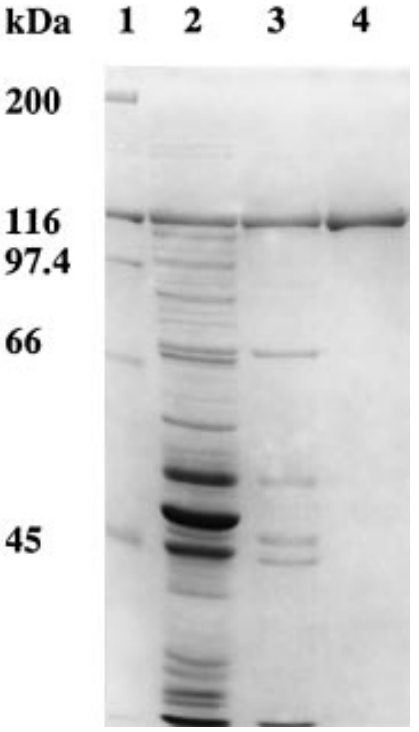

Fig. 1. Documentation of purification procedure for the fusion protein by SDS/PAGE. Proteins were separated on a 7.5% (w/v) polyacrylamide slab gel under denaturing conditions and stained with Coomassie Brillant Blue R250. Lane 1: M_r markers; lane 2, soluble proteins of the transformed *E. coli* crude extract (see Table 1), 100 μg; lane 3, Hiload 16/10 Source Q pool (see Table 1), 10 μg; lane 4, Superdex pool (see Table 1), 4 μg.

plus the M_r of the corresponding part of acetohydroxy acid synthase (66 000). During gel filtration chromatography, most of the protein (90%) eluted in fractions corresponding to a molecular mass of 240 kDa indicating that the chimeric protein behaves essentially as a dimer (not shown). However, some chimeric enzyme (10%) eluted in fractions corresponding to a molecular mass of a tetramer (480 kDa). These two oligomeric forms are not in equilibrium since re-chromatography on Superdex 200 of the dimer gives only a dimer. Similarly, re-chromatography of the tetramer gives only a tetramer. Surprisingly, unlike acetohydroxy acid synthase from barley [24] or from *E. coli* isozyme III [25], FAD does not play a function in the oligomerization state of the fusion enzyme since addition of FAD (0–100 μM) in the elution buffer does not alter the percentage of dimer and tetramer forms on gel permeation chromatography. In this paper, we report the kinetic characterization of the dimeric enzyme.

Optimum salt concentration and pH of the reaction buffer were initially determined. Without NaCl, acetohydroxy acid synthase has a broad pH optimum from 6.5 to 8.8, whereas at high NaCl concentration (150 mM), this enzyme is active in a shorter pH range between 7.0 and 8.2 (not shown). Furthermore, at pH 8.2, acetohydroxy acid synthase exhibits higher activity (1.4-fold) in 150 mM NaCl than in the absence of NaCl (not shown). Since acetohydroxy acid isomeroreductase activity is independent of NaCl concentration and its pH optimum is around pH 8.2, all kinetics were carried out in 100 mM Tris-HCl (pH 8.2) containing 150 mM NaCl.

3.3. Kinetics of acetohydroxy acid synthase in the fusion protein

As shown in Fig. 2, activation of acetohydroxy acid synthase in the fusion protein is a slow process. In order to determine if the observed latency depends upon pyruvate (Fig. 2A), FAD (Fig. 2B) or TPP (Fig. 2C) concentration, the data of Fig. 2 were fitted to the equation:

$$A = V{\cdot}t-(V/k){\cdot}(1-e^{-k{\cdot}t})$$

where A is the product formed, V the steady-state velocity, k a pseudo-first-order constant of activation and t the time of reaction. Plots of k values versus concentrations of pyruvate, FAD and TPP, allowed first, the determination of the dependency of enzyme activation on the fixation of pyruvate, FAD or TPP, and second, whether the fixation of these compounds involves one or two steps. From the data of Fig. 2A, k (0.0065 s^{-1}) is independent of pyruvate concentration. From the data of Fig. 2B, k is a linear function of FAD concentration indicating that FAD binds in one step to the enzyme with an association rate constant of 34 M^{-1} s^{-1} (not shown). From the data of Fig. 2C, k is a hyperbolic-function of TPP concentration indicating that the process involves two steps, a first step with a K_d value of 153 μM followed by a slow step with a rate constant k of 0.0065 s^{-1} (not shown). Finally, these results indicate that the activation of acetohydroxy acid synthase in the fusion protein is independent of the concentration of pyruvate but correlated with the concentration of TPP and FAD in the reaction buffer (Fig. 2). Furthermore, these results disclose that at saturating concentrations of pyruvate, FAD and TPP, the activity process is limited by the second step of TPP fixation characterized by a first-order rate constant k of 0.0065 s^{-1}. Thus, the half-time of formation of the active complex ($\tau = \ln 2/k$) is 1.8 min. Even with saturating concentration of FAD and TPP, or preincubation of the enzyme (30 min at 30°C or 37°C) with its cofactors, acetohydroxy acid synthase requires a minimum of approximately 4 min to reach its optimum activity. A possible explanation to account for this phenomenon is that the activity of the enzyme–TPP–FAD–pyruvate complex develops only slowly during catalysis, suggesting that different enzyme active forms are generated during the reaction, a behavior typical of histeretic enzymes [26]. Once activated, the specific activity of acetohydroxy acid synthase in the fusion protein is 1.5 $\mu mol{\cdot}min^{-1}{\cdot}mg^{-1}$ at 30°C (2.4 at 37°C). Referred to the mass of the native enzyme (2-fold smaller), the specific activity of acetohydroxy acid synthase should be 2-fold higher (3 $\mu mol{\cdot}min^{-1}{\cdot}mg^{-1}$). This last value appears slightly higher than those determined for barley (1.5 $\mu mol{\cdot}min^{-1}{\cdot}mg^{-1}$) [7] or *A. thaliana* (1.7 $\mu mol{\cdot}min^{-1}{\cdot}mg^{-1}$) [10].

At steady state, K_m for pyruvate, TPP, Mg^{2+} and FAD were determined to be 3 mM, 52 μM, 400 μM and 8 μM, respectively. Thus, K_m of acetohydroxy acid synthase for pyruvate (3 mM) in the fusion protein is similar to those determined for *A. thaliana* (2.3 mM) [9] and barley (5.5 mM) [7] native enzymes. On the other hand, K_m for TPP (52 μM) is slightly higher than that determined for the maize enzyme (12 μM) [27].

Acetohydroxy acid synthase in the fusion protein does not exhibit a tight interaction with FAD. Indeed, FAD readily dissociates from the fusion protein on gel permeation chromatography (Superdex 200), showing that FAD binding to

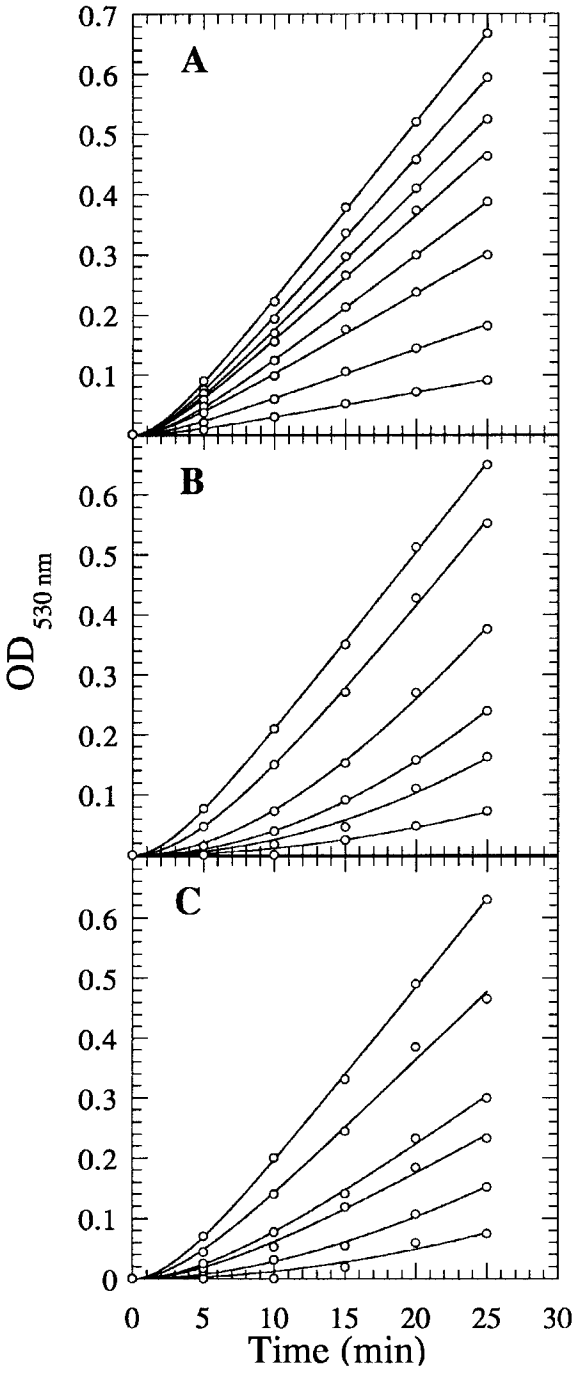

Fig. 2. Kinetics of actohydroxy acid synthase in the fusion protein with various concentration of pyruvate (A), FAD (B) and TPP (C). Reactions were effected at 30°C in 3 ml of 100 mM Tris-HCl, pH 8.2, containing 150 mM NaCl, 10 mM $MgCl_2$. Reaction were initiated by addition of the fusion enzyme (24 μg). A: Enzyme was incubated in the reaction buffer containing saturating concentration of FAD (100 μM), TPP (1 mM) and various concentrations of pyruvate (0.5, 1, 2, 3, 5, 7, 10, 20 mM). B: Enzyme was incubated in the reaction buffer containing saturating concentration of TPP (1 mM), pyruvate (20 mM) and various concentrations of FAD (2.5, 5, 10, 20, 80, 160 μM). C: Enzyme was incubated in the reaction buffer containing saturating concentration of FAD (100 μM), pyruvate (20 mM) and various concentrations of TPP (10, 25, 75, 100, 200, 400 μM). Every 5 min, an aliquot of 500 μl was taken and analyzed for acetolactate formation as described in Section 2. Acetoin was determined at 530 nm using known acetoin concentration as control.

the fusion enzyme is a reversible process. Furthermore, acetohydroxy acid synthase in the fusion protein shows no activity without added FAD.

Acetohydroxy acid synthase in the fusion protein is insensitive to leucine, valine and isoleucine (alone or mixed, at a concentration of 2 mM) as observed with the overexpressed native *A. thaliana* acetohydroxy acid synthase [9]. In contrast, the native enzyme from *A. thaliana* seedling [9], barley [24], black Mexican corn [28] and *E. coli* [25,29] are inhibited by branched-chain amino acids. Interestingly, bacterial acetohydroxy acid synthase isozyme II [29] and III [25] are made of two subunits, a large catalytic subunit and a small regulatory subunit. Alone, the large subunit is insensitive to inhibition by branched-chain amino acids but becomes sensitive upon reconstitution with the regulatory subunit [25]. Since intact acetohydroxy acid synthase exhibiting high activity has never been purified to homogeneity from plants, it is not known if such a complex exists in plants. Therefore, it is possible that such an additional regulatory subunit exists also in plants. Another possible explanation is that the sensitivity to branched-chain amino acids is related to a post-transcriptional modification.

3.4. *Kinetics of acetohydroxy acid isomeroreductase in the fusion protein*

Kinetic parameters of the spinach acetohydroxy acid isomeroreductase in the fusion protein were compared to those determined previously for the native spinach acetohydroxy acid isomeroreductase overexpressed in *E. coli* [14]. With 2-aceto-2-hydroxybutyrate and 2-acetolactate as substrates, the specific activity of acetohydroxy acid isomeroreductase in the fusion protein is 1.9 and 0.3 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ at 30°C, respectively. Taking into account the mass of the native enzyme (2-fold smaller), specific activity with 2-aceto-2-hydroxybutyrate (3.8 $\mu mol \cdot min^{-1} \cdot mg^{-1}$) is slightly lower than those determined for the native enzyme (8.5 $\mu mol \cdot min^{-1} \cdot mg^{-1}$).

K_m for 2-aceto-2-hydroxybutyrate, NADPH and Mg^{2+} were determined to be 10 μM, 7 μM and 500 μM, respectively. Thus, K_m of acetohydroxy acid isomeroreductase in the fusion enzyme for 2-aceto-2-hydroxybutyrate (10 μM) and NADPH (7 μM) are similar to those determined for the native enzyme (10 μM and 5 μM, respectively). However, K_m of acetohydroxy acid isomeroreductase in the fusion protein for Mg^{2+} (500 μM) has been increased considerably compared to the values determined for the native acetohydroxy acid isomeroreductase (5 μM). A possible explanation for this feature is a conformational modification of the acetohydroxy acid isomeroreductase in the fusion protein.

3.5. *Coupled reaction*

Upon addition of pyruvate, acetohydroxy acid synthase catalyses the formation of 2-acetolactate which is used by acetohydroxy acid isomeroreductase for synthesis of 2,3-dihydroxy-3-isovalerate. Therefore, after addition of pyruvate to the reaction mixture, the overall reaction can be monitored by following the absorbance of NADPH. When the reaction was performed by addition of pyruvate as described in Section 2, activity of acetohydroxy acid isomeroreductase increased slowly and reached its optimum (after 11 min in our test condition) at a 2-acetolactate concentration of 200 μM. Since the K_m value of acetohydroxy acid isomeroreductase for 2-acetolalactate is 10 μM and maximal activity is reached at a

concentration of 20× K_m (i.e., 200 μM), 2-acetolactate channeling does not take place between acetohydroxy acid synthase and acetohydroxy acid isomeroreductase in the fusion protein.

3.6. Conclusion

Finally, the chimeric enzyme appears to be a useful tool to determine kinetic and structural properties on plant acetohydroxy acid synthase. Also, using pyruvate or 2-aceto-2-hydroxybutyrate as substrate, both enzyme reactions can be measured by NADPH detection, thus providing an easy test for high throughput screening of inhibitors targeting acetohydroxy acid synthase and acetohydroxy acid isomeroreductase.

Acknowledgements: We are grateful to Michel Lebrun (Université de Montpellier, France) for the gift of pUC19-AHAS. We gratefully acknowledge Gilles Curien, Claude Alban, Michel Matringe, Matthew Rodgers, Stéphane Ravanel, and Jacqueline Martin for helpful discussions. We are also grateful to Dominique Job for his help in kinetic analysis. This study has been conducted under the BIO AVENIR program financed by Rhône-Poulenc with the Ministère de la Recherche et de l'Espace and the Ministère du Commerce Extérieur.

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