Isolation and kinetic properties of acetohydroxy acid isomeroreductase from spinach (*Spinacia oleracea*) chloroplasts overexpressed in *Escherichia coli*

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Acetohydroxy acid isomeroreductase catalyses a two-step reaction, an alkyl migration and a NADPH-dependent reduction, in the assembly of the carbon skeletons of branched-chain amino acids. Detailed investigations of acetohydroxy acid isomeroreductase aimed at elucidating the biosynthetic pathway of branched-chain amino acids and at designing new inhibitors of the enzyme having herbicidal potency have so far been conducted with the enzymes isolated from bacteria. To gain more information on a plant system, the gene encoding the mature acetohydroxy acid isomeroreductase from spinach (*Spinacia oleracea*) leaf chloroplasts has been used to transform *Escherichia coli* cells and to overexpress the enzyme. A rapid protocol is described that allows the preparation of large quantities of pure spinach chloroplast acetohydroxy acid isomeroreductase. Kinetic and structural properties of the plant enzyme expressed in *Escherichia coli* are compared with those reported in our previous studies on the native enzymes purified from spinach chloroplasts and with those reported for the corresponding enzymes isolated from *Escherichia coli* and *Salmonella typhimurium*. Both the plant and the bacterial enzymes obey an ordered mechanism in which NADPH binds first, followed by substrate (either 2-acetolactate or 2-aceto-2-hydroxybutyrate). Inhibition studies employing an inactive substrate analogue, 2-hydroxy-2-methyl-3-oxopentanoate, showed, however, that the binding of 2-hydroxy-2-methyl-3-oxopentanoate and NADPH occurs randomly, suggestive of some flexibility of the plant enzyme active site. The observed preference of the enzyme for 2-aceto-2-hydroxybutyrate over 2-acetolactate is discussed with regard to the contribution of acetohydroxy acid isomeroreductase activity in the partitioning between isoleucine and valine biosyntheses. Moreover, the kinetic properties of the chloroplast enzyme support the notion that biosynthesis of branched-chain amino acids in plants is controlled by light. As judged by analytical-ultracentrifugation and gel-filtration analyses the overexpressed plant enzyme is a dimer of identical subunits.

INTRODUCTION

Recent studies have demonstrated that several molecules with herbicidal potency act at the level of the first enzymes involved in the biosynthetic pathway of branched-chain amino acids. This is the case for the sulphonylurea, imidazolinone and triazopyridine or sulphanilide herbicides that inhibit acetolactate synthase (the first common enzyme in the pathway; EC 4.1.3.18). Other compounds such as HOE 704 (2-methylphosphinoyl-2-hydroxyacetic acid) and IpOHA (*N*-hydroxy-*N*-isopropyl-oxamate) have been shown to behave as very potent and selective inhibitors of acetohydroxy acid isomeroreductase (EC 1.1.1.86), the second common enzyme of the pathway, probably because these molecules bear some similarities to the rearrangement transition state (Schulz *et al.*, 1988; Aulabaugh & Schloss, 1990). The demonstration that both these compounds give rise to herbicidal effects has led to a renewed interest in the study of acetohydroxy acid isomeroreductase.

This enzyme catalyses a two-step reaction in the assembly of the carbon skeletons of branched-chain amino acids and is involved into two parallel synthetic pathways using as substrate either 2-acetolactate (ultimate products of biosynthetic pathway, valine and leucine) or 2-aceto-2-hydroxybutyrate (ultimate product, isoleucine). The substrate is converted by the enzyme via an alkyl migration and an NADPH-dependent reduction to give 2,3-dihydroxy-3-isovalerate (substrate 2-acetolactate) or

2,3-dihydroxy-3-methylvalerate (substrate 2-aceto-2-hydroxybutyrate). The reduction step requires the transfer of the pro-S hydrogen atom from NADPH (Arfin & Umbarger, 1969). In addition the enzyme activity requires the presence of a bivalent Mg²⁺ ion (Armstrong & Wagner, 1961). Most of our present knowledge on acetohydroxy acid isomeroreductase comes from studies conducted with the enzyme purified from prokaryotic cells. Although the latter has been considered as a model system to design new molecules with increased herbicidal potency and higher specificity (Schloss & Aulabaugh, 1990), it seems desirable to carry out mechanistic studies on the true target of herbicide action, that is, the acetohydroxy isomeroreductase from plants. To this end, we have previously purified to homogeneity this enzyme from the stroma of spinach leaf chloroplasts (Dumas *et al.*, 1989). In addition, we have isolated and characterized a full-length cDNA from a λ gt 11 spinach library encoding the complete acetohydroxy acid isomeroreductase protein precursor (Dumas *et al.*, 1991). The derived amino acid sequence from this open reading frame showed little identity with the deduced amino acid sequence of the corresponding enzymes from *Escherichia coli* and *Saccharomyces cerevisiae* (Dumas *et al.*, 1991).

We describe here the cloning of a synthetic gene encoding the 523 amino acid residues of mature spinach chloroplast acetohydroxy acid isomeroreductase into a plasmid vector (pKK223-3) that permits overproduction of this enzyme in *E. coli* as a full-length native protein without a leader peptide. We describe also

Abbreviation used: IPTG, isopropyl thiogalactoside.

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a rapid purification procedure allowing the preparation of large quantities of the enzyme from this source and compare the biochemical properties of the overexpressed protein with those of acetohydroxy acid isomeroreductase as obtained from spinach leaf chloroplasts (Dumas *et al.*, 1989), *Salmonella typhimurium* (Arfin & Umbarger, 1969; Shematek *et al.*, 1973; Hofler *et al.*, 1975) and *E. coli* (Chunduru *et al.*, 1989).

MATERIALS AND METHODS

Purification of acetohydroxy acid isomeroreductase from spinach chloroplasts

Preparation of soluble proteins from the stroma of spinach leaf chloroplasts was performed as described by Douce & Joyard (1982). Acetohydroxy acid isomeroreductase was purified as described by Dumas *et al.* (1989).

Construction of the gene

A *NarI*-*EcoRI*-purified fragment of 1799 bp containing a major part of the coding region of mature acetohydroxy acid isomeroreductase from spinach leaf chloroplasts minus 251 bp of the 5' sequence and plus 237 bp of the 3' untranslated sequence was excised from plasmid pUC19-AHRI (Dumas *et al.*, 1991). Two complementary synthetic oligonucleotides (5' AATTC-ATGGTTTCGG 3' and 5' CGCCGAAACCATG 3') were mixed in equal amounts and heated to 65 °C for 5 min before slowly cooling to 30 °C over 30 min. The hybridized oligonucleotides were ligated to the *NarI*-*EcoRI* fragment, which led (i) to the creation of an *EcoRI* site, (ii) to the creation of the ATG codon for the initiating methionine and (iii) to the restoration of the 5' sequence of the coding region of the mature protein. The ligation product was digested by *EcoRI*, purified and ligated in the *EcoRI* site of the expression vector pKK223-3 (Pharmacia), which yielded pKK-AHRI. The construct was controlled by restriction-mapping and sequence analyses (Sanger *et al.*, 1977). pKK-AHRI plasmids were transformed into competent *E. coli* strain JM105.

Expression of the gene in *E. coli*

For preliminary evaluation of the expression, wild and transformed strains were each grown at 28 °C in 250 ml of Luria-Bertani medium containing 100 µg of carbenicillin/ml to a density equivalent to an A_{600} of 0.5. Isopropyl thiogalactoside (IPTG) was added to a concentration of 1 mM and an amount of cells corresponding to 40 ml was sampled at various time periods after induction. The pelleted cells were resuspended in 10 ml of lysis buffer [15 mM-potassium phosphate (pH 7.5)/1 mM-EDTA/1 mM-dithiothreitol/1 mM-benzamidine] and sonicated for 10 min. For each sample, 300 µl were tested for acetohydroxy acid isomeroreductase activity and an amount of protein corresponding to 100 µg was used for analysis by SDS/PAGE.

For large-scale purification the cells were grown in a 2-litre Erlenmeyer flask. IPTG (1 mM) was added when the A_{600} was 0.5 and the cells were grown for 15 h. The cells were harvested by centrifugation. The pellet was resuspended in 100 ml of lysis buffer and sonicated in 100 pulses each of 3 s on power setting 5 on a Vibra-cell disruptor (Sonics and Materials, Danbury, CT, U.S.A.). The cell extract was centrifuged (20000 g for 30 min) to yield a cell-free supernatant. The unbroken bacteria were resuspended again in 25 ml of lysis buffer, sonicated as described above and centrifuged at 20000 g for 30 min. The two supernatants were combined, filtered and stored at -80 °C until purification could be performed.

Purification of acetohydroxy acid isomeroreductase expressed in *E. coli*

The above cell-free extract (125 ml, 1080 mg of protein) in lysis buffer was applied to a 2 cm × 18 cm column of Trisacryl M-DEAE (Sepracor), equilibrated in buffer A [25 mM-potassium phosphate (pH 7.5)/1 mM-EDTA/0.5 mM-dithiothreitol/1 mM-benzamidine]. Elution was performed with 50 ml of buffer B [50 mM-potassium phosphate (pH 7.5)/1 mM-EDTA/0.5 mM-dithiothreitol/1 mM-benzamidine] (flow rate 1 ml/min; fraction size 5 ml). Chromatographic fractions containing acetohydroxy acid isomeroreductase activity were concentrated to 4 ml by ultrafiltration (PM 30 membrane; Amicon). This extract (47 mg of protein) was loaded on to a Hiload 16/60 Superdex 200 column (Pharmacia) connected to a Pharmacia f.p.l.c. system and previously equilibrated in buffer B. The enzyme was eluted with 72 ml of buffer B (flow rate 1 ml/min; fraction size 1.5 ml). The fractions that contained the acetohydroxy acid isomeroreductase activity were dialysed and concentrated to 2 ml by ultrafiltration with a PM 30 membrane in buffer C (20 mM-potassium phosphate, pH 7.5). This extract (28 mg of protein) was then applied to a HiLoad 16/10 Q-Sepharose column (Pharmacia) previously equilibrated in buffer C. The column, connected to a Pharmacia f.p.l.c. system, was then washed with 10 ml of buffer C followed by elution of enzyme activity with a 100-ml gradient of 20–50 mM-potassium phosphate, pH 7.5 (flow rate 0.5 ml/min; fraction size 1 ml). Purified acetohydroxy acid isomeroreductase was stored at -80 °C in the elution buffer.

Activity measurements

Acetohydroxy acid isomeroreductase activity was assayed in 50 mM-Tris/HCl buffer (pH 8.2)/3 mM-MgCl₂/250 µM-NADPH, in a final volume of 1 ml. Reactions were usually initiated by adding either 2 mM-2-aceto-2-hydroxybutyrate or 2 mM-2-acetolactate, and the progress of the reaction was monitored by the decrease in absorbance of NADPH at 340 nm (measured in a Uvikon 860 spectrophotometer; Kontron) (Arfin & Umbarger, 1969). Enzyme activity was expressed as µmol of NADPH oxidized/mg of protein. Apparent kinetic parameters were calculated from linear least-squares analysis of double-reciprocal plots of velocity versus the appropriate substrate or cofactor concentration.

Electrophoresis

SDS/PAGE. Electrophoresis was carried out at room temperature in SDS/polyacrylamide slab gels containing a 7.5–15 % (w/v) linear acrylamide gradient, as described by Chua (1980).

Native gels. Gels consisting of acrylamide [5–10 % (w/v) linear gradient] were prepared as described by Laemmli (1970), except that detergent was omitted from the different buffers. Electrophoresis was performed at 10 mA/gel at 4 °C for 12 h.

Ultracentrifugation assays

These assays were performed at 20 °C with the aid of a Spinco Model E ultracentrifuge equipped with a double-sector Alu filled cell and an interferometric system for M_r determination (Yphantis, 1964).

Fluorescence analysis

Titration of acetohydroxy acid isomeroreductase with NADPH by fluorimetric techniques was conducted in a SFM 25 (Kontron) fluorimeter using a 1 ml cuvette and an excitation wavelength of 370 nm. Fluorescence was measured at 460 nm.

Protein determination

Protein was determined either with the Bio-Rad protein assay

Fig. 1. Construction of the plasmid pKK-AHRI

(a) A synthetic double-stranded oligonucleotide, 5' AATTCATGGTTTCGGG 3' / 3' GTACCAAAGCCGC 5', was ligated to the *NarI* site of the *NarI*-*EcoRI* fragment excised from pUC19-AHRI (see the Materials and methods section) to the *EcoRI* site of pKK223-3, which yielded pKK-AHRI. Abbreviations and symbols: TP, transit peptide; ptac, *tac* promoter of the plasmid; rbs, ribosome-binding site; □, non-coding cDNA; ■, coding sequence for spinach chloroplast acetoxy acid isomeroreductase; ■, sequence of the synthetic double strand. (b) SDS/PAGE of soluble proteins of *E. coli* crude extract. Proteins were separated on a 7.5–15% (w/v) gradient polyacrylamide slab gel and stained with Coomassie Brilliant Blue R250. Lane 1, soluble proteins of *E. coli* JM105, 100 µg; lane 2, soluble proteins of *E. coli* JM105 transformed with pKK-AHRI and induced by IPTG, 100 µg; lane 3, M_r markers. The arrow indicates the position of the acetohydroxy acid isomeroreductase protein.

(Bradford method) or by measuring the absorbance at 205 nm as described by Scopes (1974).

Synthesis of (2*S*)- and (2*R*)-2-acetolactate and of (2*S*)- and (2*R*)-2-aceto-2-hydroxybutyrate

For the synthesis of (2*S*)- and (2*R*)-acetolactate, the corresponding ethyl esters were prepared starting from L-(+)-lactic acid (Ortholand & Greiner, 1992). Enantiomeric purity, investigated by 250 MHz ^1H n.m.r. in the presence of the chiral-shift reagent tris-[3-(heptafluoropropyl)hydroxymethylene]-(+)-camphoratoeuropium(III), was shown to be greater than 99%. Sodium salts were obtained by alkaline hydrolysis in non-epimerizing conditions (Hill *et al.*, 1979; Crout *et al.*, 1984) followed by the addition of HCl to bring the final pH of the substrate solutions to 8.2. For the synthesis of (2*R*)- and (2*S*)-2-aceto-2-hydroxybutyrate, both ethyl esters were obtained by a reaction sequence similar to the preparation of (2*R*)- and (2*S*)-acetolactate ethyl esters, starting from either L-(+)- or D-(−)-lactic acid, after a stereoselective one-carbon homologation (Ortholand, 1991; a full description of the method is available from R. Douce on request). Enantiomeric purity was shown to be 97% for the (2*R*) isomer and over 99% for the (2*S*) isomer. Sodium salts were obtained by alkaline hydrolysis.

Synthesis of 2-hydroxy-2-methyl-3-oxopentanoate

Racemic 2-hydroxy-2-methyl-3-oxopentanoate was prepared by alkaline hydrolysis of racemic methyl 2-hydroxy-2-methyl-3-oxopentanoate (Aldrich Co.).

Synthesis of 2,3-dihydroxy-3-isovalerate

This compound was synthesized by the protocol of Van Rhee *et al.* (1973).

RESULTS

Cloning and overproduction

To express spinach leaf chloroplast acetoxy acid isomeroreductase in *E. coli*, a synthetic gene coding for the mature protein (Dumas *et al.*, 1991) was cloned into the *EcoRI* site of the expression vector PKK223-3. Fig. 1(a) depicts the strategy for cloning this DNA. The results of SDS/PAGE analyses of *E. coli* crude extracts show that a protein of the expected size (57 kDa) was present in the cytosolic fraction of the bacteria; this protein, which was much more abundant in the IPTG-induced bacteria than in non-transformed cells (Fig. 1b), was assigned to spinach acetoxy acid isomeroreductase by Western blotting using a specific polyclonal antibody directed against the native chloroplast enzyme (Dumas *et al.*, 1991) (results not shown). This revealed that the plant acetoxy acid isomeroreductase was produced at levels ranging from 2 to 5% of the soluble bacterial cell proteins, and that the enzyme was freely soluble within the bacterial cell and not packaged into inclusion bodies.

Purification of overexpressed enzyme

A three-step procedure was devised to purify the enzyme from extracts of *E. coli* cells overexpressing the plant acetoxy acid isomeroreductase. The procedure described in the Materials and methods section allowed us to purify the enzyme in good yield and at high level of purity in 3 working days. This procedure resulted in a 50-fold purification, and in an overall yield of enzyme activity of about 60%. Thus 13 mg of pure enzyme were obtained starting from 1080 mg of *E. coli* soluble proteins (Table 1). The proteins at different stages of the purification are shown in Fig. 2. The purified acetoxy acid isomeroreductase from this source could be stored frozen at -80°C for several months without noticeable loss of enzyme activity.

On the basis of SDS/PAGE, the purified plant protein migrated as a single band of M_r 57000 ± 2000 (Fig. 2). This value

Table 1. Purification procedure of acetohydroxy acid isomeroreductase expressed in *E. coli*

Protein was determined with the Bio-Rad protein assay (Bradford method), with γ -globulin as standard. Enzyme activity was measured in the Tris/HCl standard buffer, pH 8.2, with 3 mM-MgCl₂, 250 μ M-NADPH and 2 mM-2-aceto-2-hydroxybutyrate.

Purification stage	Total protein (mg)	Total activity (μ mol of NADPH oxidized/min)	Specific activity (activity/mg of protein)	Yield (%)
Crude extract of soluble proteins	1080	108	0.1	100
Trisacryl M-DEAE pool	47	108	2.3	100
HiLoad 16/60 Superdex 200 pool	28	95	3.4	88
HiLoad 16/10 Q-Sepharose pool	13	65	5	60

is nearly equivalent to that predicted by the nucleotide sequence of the cDNA encoding acetohydroxy acid isomeroreductase from spinach chloroplasts [the mature protein is predicted to consist of 523 amino acid residues, giving a protein of M_r 56853 (Dumas *et al.*, 1991)].

An apparent M_r value of 205000 was calculated from PAGE analysis under non-denaturing conditions (Fig. 3a). This therefore suggests that, as for the native enzymes isolated from spinach chloroplasts (Dumas *et al.*, 1989), the overexpressed plant acetohydroxy acid isomeroreductase is a tetramer composed of four subunits with an identical M_r of 57000. A tetrameric structure has also been described for the acetohydroxy acid isomeroreductase isolated from *S. typhimurium* (Hofler *et al.*, 1975). For this latter enzyme, the M_r , as determined by sedimentation-equilibrium ultracentrifugation, is approx. 220000 (Arfin & Umbarger, 1969). We previously reported that aceto-

Fig. 2. Documentation of purification procedure for acetohydroxy acid isomeroreductase by SDS/PAGE

Proteins were separated on a 7.5–15% (w/v) gradient polyacrylamide slab gel under denaturing conditions and stained with Coomassie Brilliant Blue R250. Lane 1, M_r markers; lane 2, soluble proteins of the *E. coli* crude extract (see Table 1), 75 μ g; lane 3, Trisacryl M-DEAE pool (see Table 1), 50 μ g; lane 4, HiLoad 16/60 Superdex 200 pool (see Table 1), 15 μ g; lane 5, HiLoad 16/10 Q Sepharose pool (see Table 1), 5 μ g.

hydroxy acid isomeroreductase activity from spinach leaf chloroplasts is resolved into three enzyme isoforms (isoforms 1, 2 and 3; see Dumas *et al.*, 1989) by Mono-Q HR anion-exchange chromatography, each of which exhibited an identical M_r of 57000 ± 2000 by SDS/PAGE. These forms were easily distinguishable after PAGE under non-denaturing conditions (the apparent M_r s of isoforms 1, 2 and 3 are respectively 235000, 220000 and 205000; see lanes 2, 3 and 4 of Fig. 3a) and by

Fig. 3. M_r determinations of purified acetohydroxy acid isomeroreductase

(a) PAGE analysis under non-denaturing conditions. Proteins were separated on a 5–10% (w/v) gradient polyacrylamide slab gel under non-denaturing conditions and stained with Coomassie Brilliant Blue R250. Lane 1, HiLoad 16/10 Q Sepharose pool (see Table 1 and Fig. 1) of acetohydroxy acid isomeroreductase expressed in *E. coli*, 5 μ g; lanes 2, 3 and 4, isoform 1 (5 μ g), isoform 2 (2 μ g) and isoform 3 (2 μ g) of acetohydroxy acid isomeroreductase purified spinach leaf chloroplast, respectively (Dumas *et al.*, 1989). Their corresponding apparent M_r values are indicated. (b) Gel filtration analysis. Proteins were subjected to molecular sieving on a HiLoad 16/60 Superdex 200 column (1.6 cm $\times$ 60 cm; Pharmacia). Elution was in a 50 mM-potassium phosphate buffer, pH 7.5, containing 1 mM-EDTA and 4 mM-MgCl₂. The flow rate was 1 ml/min. Abbreviations and symbols: V_e , elution volume; V_0 , void volume (45 ml); ●, thyroglobulin, apoferritin, β -amylase, albumin and carbonic anhydrase were used as M_r markers; ○, acetohydroxy acid isomeroreductase expressed in *E. coli*. (c) Ultracentrifugation analysis. The upper part shows the Rayleigh interference pattern resulting from high-speed equilibrium ultracentrifugation assays according to Yphantis (1964). In the lower part fringe displacement, Δy , is plotted (on a logarithmic scale) as a function of r^2 , the square of the radius. An M_r of 113785 ± 1748 was calculated from the slope of this graph. The run was for 30 h at 20000 rev./min and at 20 °C. The solution was 1.5 mg of enzyme/ml in 50 mM-potassium phosphate (pH 7.5)/1 mM-EDTA/0.1 mM-dithiothreitol.

Table 2. Steady-state kinetic parameters

Assays were performed at 30 °C in the Tris/HCl standard buffer, pH 8.2. Abbreviations: AL, 2-acetolactate; AHB, 2-aceto-2-hydroxybutyrate. The millimolar concentration of the fixed substrate and/or cofactor is given in parentheses. Protein was determined as described by Scopes (1974), with γ -globulin as standard.

Variable substrate	Fixed substrate	K_m (μ M)
AL (2S)	NADPH (0.25)/Mg ²⁺ (3)	10*
AHB (2S)	NADPH (0.25)/Mg ²⁺ (3)	10†
NADPH	AL (1.5)/Mg ²⁺ (3)	1.7*
NADPH	AHB (1.5)/Mg ²⁺ (3)	3.3†
NADH	AL (1.5)/Mg ²⁺ (3)	101‡
NADH	AHB (1.5)/Mg ²⁺ (3)	645§
Mg ²⁺	NADPH (0.25)/AL (1.5)	4.5*
Mg ²⁺	NADPH (0.25)/AHB (1.5)	9†

* $V_{\max.} = 1.4 \mu\text{mol}$ of NADPH oxidized/mg of protein.
† $V_{\max.} = 8.5 \mu\text{mol}$ of NADPH oxidized/mg of protein.
‡ $V_{\max.} = 1 \mu\text{mol}$ of NADPH oxidized/mg of protein.
§ $V_{\max.} = 5 \mu\text{mol}$ of NADPH oxidized/mg of protein.

isoelectric focusing. They may correspond either to the expression of two distinct allelic forms of a same gene or to the occurrence of post-transcriptional modifications before the condensation of the subunits into the final forms (Dumas *et al.*, 1991). Fig. 3(a) shows that, in contrast with what is observed for the acetoxy acid isomeroreductase activity isolated from spinach leaf chloroplasts, the pure overexpressed enzyme migrated as a single band

in native PAGE. It is clear from these data that the overexpressed protein corresponds to spinach acetoxy acid isomeroreductase isoform 3.

In contrast with the PAGE determination, gel-filtration experiments disclosed an M_r of the order of 115 000 for the enzyme expressed in *E. coli* (Fig. 3b), indicative of the existence of a dimer with identical subunits, rather than of a tetramer. This peculiar behaviour has also been encountered with the three isoforms of acetoxy acid isomeroreductase isolated from spinach leaf chloroplasts (Dumas, 1991). Determination of the M_r of the overexpressed enzyme was further assessed by equilibrium sedimentation (Yphantis, 1964). A plot of the observed Rayleigh fringe displacement (on a logarithmic scale) as a function of the square of the radius was linear, testifying that the enzyme preparation was homogeneous. Furthermore this plot provided an M_r of $113\,785 \pm 1748$ (Fig. 3c). We conclude that the plant enzyme is most likely a dimer. It must be stressed that M_r determinations from electrophoretic migrations in native gels are complicated by the fact that both the size and the net charge of the protein influence migration.

Some biochemical properties of acetoxy acid isomeroreductase expressed in *E. coli* were also examined and compared with those of the native isoforms from spinach leaf chloroplasts. It appeared that the enzymes were not differentiated by the substrate (either 2-acetolactate or 2-aceto-2-hydroxybutyrate) and cofactors (either NADPH and Mg²⁺) or by their pattern of pH-dependence (the pH of maximum enzymic activity was 8.2) (results not shown). Finally the purified enzyme from the recombinant bacteria had a specific activity of $8.5 \mu\text{mol/mg}$ in the presence of 2-aceto-2-hydroxybutyrate as substrate, which

Scheme 1. Steady-state kinetic mechanism for acetoxy acid isomeroreductase at saturating Mg²⁺ concentration

(a) Competition of 2-acetolactate (AL) and 2-aceto-2-hydroxybutyrate (AHB) for the active site of spinach chloroplast acetoxy acid isomeroreductase. Further abbreviations: E, enzyme; DHIV, 2,3-dihydroxy-3-isovalerate; DHIMV, 2,3-dihydroxy-3-methylvalerate. (b) Ordered mechanism for *E. coli* (Chunduru *et al.*, 1989) and *S. typhimurium* (Shematek *et al.*, 1973) acetoxy acid isomeroreductases. S, 2-acetolactate or 2-aceto-2-hydroxybutyrate; P, 2,3-dihydroxy-3-isovalerate (substrate 2-acetolactate) or 2,3-dihydroxy-3-methylvalerate (substrate 2-aceto-2-hydroxybutyrate). (c) Ordered mechanism for spinach chloroplast acetoxy acid isomeroreductase. The scheme is adapted from that shown in (b), to account for the inhibition patterns encountered with 2,3-dihydroxy-3-isovalerate (P) (Fig. 4) and with 2-hydroxy-2-methyl-3-oxopentanoate (OP), an inactive analogue of 2-aceto-2-hydroxybutyrate substrate (Fig. 5).

compares well with that of 7 $\mu\text{mol}/\text{mg}$ previously determined for the chloroplast isomeroreductase isoforms under the same experimental conditions (Dumas, 1991).

Kinetic properties

Kinetic experiments were carried out as described in the Materials and methods section at saturating Mg^{2+} (3 mM) and NADPH (250 μM) concentrations and with varied concentrations of 2-acetolactate or 2-aceto-2-hydroxybutyrate. Lineweaver-Burk plots of the data were linear and yielded the apparent K_m and $V_{\max}$ values listed in Table 2. We also analysed the effect of racemic substrates in comparison to chiral substrates on the steady-state kinetics (Table 2). With racemic substrates, apparent K_m values were nearly identical for both 2-acetolactate and 2-aceto-2-hydroxybutyrate. These values were of the order of 20 μM . For either 2-acetolactate or 2-aceto-2-hydroxybutyrate the (2*R*) enantiomer was completely inactive. Furthermore, the apparent K_m values for the (2*S*) enantiomers (10 μM) were nearly half those obtained with the racemic substrates. This shows that only the (2*S*) enantiomers act as substrates for the plant enzyme, as previously reported by Chunduru *et al.* (1989) for the enzyme from a recombinant *E. coli* that overexpresses the prokaryotic acetohydroxy acid isomeroreductase. In contrast with the K_m values, apparent $V_{\max}$ values were substantially different for the two substrates. Thus the $V_{\max}$ obtained with 2-acetolactate was 6 times smaller than that calculated with 2-aceto-2-hydroxybutyrate as the varied substrate (Table 2).

To investigate the possibility that the enzyme contains a single or different binding sites for the two hydroxy acid substrates, we have determined the steady-state rate of NADPH oxidation, v , under two protocols. In the first, reactions were carried out in the presence of 2-aceto-2-hydroxybutyrate as the varied substrate, at saturating Mg^{2+} (3 mM) and NADPH (250 μM). In the second, the assays were identical, except that, in addition to 2-aceto-2-hydroxybutyrate, they received 2-acetolactate in equimolar amounts with respect to 2-aceto-2-hydroxybutyrate. We observed that in the 1.9–489 μM -2-aceto-2-hydroxybutyrate concentration range both plots of v versus [2-aceto-2-hydroxybutyrate (AHB)] could be fitted satisfactorily to the simple hyperbolic relationship:

$$v = \frac{V_{\max}^{\text{app}} [\text{AHB}]}{K_m^{\text{app}} + [\text{AHB}]}$$

The apparent K_m value for the experiment with the mixtures of substrates [$K_m^{\text{mixture}} = 6.00 (\pm 0.55) \mu\text{M}$] was approximately half that obtained with 2-aceto-2-hydroxybutyrate as the single substrate [$K_m^{\text{AHB}} = 10.97 (\pm 0.84) \mu\text{M}$]. Furthermore, the apparent $V_{\max}$ calculated from the experiment carried out with the mixtures of substrates [$V_{\max}^{\text{mixture}} = 6.4 (\pm 1.6)$ units per mg of enzyme] was smaller than that determined with 2-aceto-2-hydroxybutyrate as the single substrate [$V_{\max}^{\text{AHB}} = 8.6 (\pm 1.9)$ units per mg of enzyme]. These findings indicate that 2-acetolactate and 2-aceto-2-hydroxybutyrate compete for the same binding site on the enzyme (Segel, 1975) (Scheme 1a).

Apparent K_m values for NADPH and Mg^{2+} are listed in Table 2. The enzyme exhibited a high affinity for both the nucleotide and metal-ion cofactor. It should be noted that apparent K_m values for NADPH and Mg^{2+} were significantly different when 2-acetolactate or 2-aceto-2-hydroxybutyrate was used as substrate. Both values were about 2 times smaller with 2-acetolactate compared with the corresponding values with 2-aceto-2-hydroxybutyrate (Table 2).

An inhibition of enzymic activity was observed by using fixed concentrations of Mg^{2+} lower than 3 μM and NADPH concentrations above 300 μM . Assuming that the value of the formation constant of a $\text{Mg} \cdot \text{NADPH}$ complex is of the order of 200 M^{-1}

Table 3. Product-inhibition pattern

All assays were performed at 30 °C in the Tris/HCl standard buffer, pH 8.2, containing 3 mM- MgCl_2 . Enantiomeric (2*S*)-2-acetolactate (AL) and (2*S*)-2-aceto-2-hydroxybutyrate (AHB), racemic 2,3-dihydroxy-3-isovalerate (DHIV) and 2-hydroxy-2-methyl-3-oxopentanoate (OP) were used in the inhibition studies. The pattern designation is non-competitive (NC), competitive (C) or uncompetitive (UC). The micromolar concentration of the fixed substrate is given in parentheses.

Variable substrate	Fixed substrate	Inhibitor	K_i (μM)	Pattern	
				Spinach	<i>E. coli</i> *
AL	NADPH (75)	NADP	330	NC	NC
AHB	NADPH (75)	NADP	225	NC	NC
NADPH	AL (200)	NADP	5.0	C	C
NADPH	AHB (200)	NADP	7.5	C	C
AL	NADPH (250)	DHIV	95	C	NC
AHB	NADPH (250)	DHIV	145	C	C
NADPH	AL (200)	DHIV	935	UC	UC
NADPH	AHB (200)	DHIV	1500	UC	UC
AL	NADPH (250)	OP	245	C	C
AHB	NADPH (250)	OP	240	C	C
NADPH	AL (200)	OP	1700	NC	NC
NADPH	AHB (200)	OP	3600	NC	NC

* Taken from Chunduru *et al.* (1989) for *E. coli* acetohydroxy acid isomeroreductase; a similar pattern was reported by Shematek *et al.* (1973) for acetohydroxy acid isomeroreductase from *S. typhimurium*.

(Chunduru *et al.*, 1989), we calculate that, under the above conditions, most of the NADPH should be chelated with Mg^{2+} . The observed behaviour implies that, as for the bacterial enzyme (Chunduru *et al.*, 1989), it is the uncomplexed metal and nucleotide that are the true substrates for the enzymic reaction catalysed by the plant acetohydroxy acid isomeroreductase.

As previously noted for the acetohydroxy acid isomeroreductase isoforms purified from spinach chloroplasts, the overexpressed enzyme was able to use NADH as an electron donor. Nevertheless, the apparent K_m for NADH was much higher than that for NADPH. As for NADPH, the apparent K_m for NADH was substantially smaller when calculated with 2-acetolactate than with 2-aceto-2-hydroxybutyrate as the substrate (Table 2). In contrast, $V_{\max}$ values were nearly the same with NADPH and NADH. Thus discrimination between NADPH and NADH occurs mainly at the initial binding step.

The results of product-inhibition studies are summarized in Table 3. The data in Fig. 4(b) established that NADP^+ competitively inhibited the reaction when NADPH was the varied substrate, in agreement with previous results obtained with the acetohydroxy acid isomeroreductase isoforms from spinach chloroplasts (Dumas *et al.*, 1989). This kinetic pattern was observed for both reactions carried out in the presence of 2-acetolactate and 2-aceto-2-hydroxybutyrate. When 2-aceto-2-hydroxybutyrate (Fig. 4a) or 2-acetolactate (results not shown) was the varied substrate, NADP^+ was found to behave as a non-competitive inhibitor. Fig. 4 also shows that 2,3-dihydroxy-3-isovalerate, the product of the enzymic reaction with 2-acetolactate as substrate, acted as a competitive inhibitor with respect to 2-acetolactate (Fig. 4c) and as an uncompetitive inhibitor with respect to NADPH (Fig. 4d).

Fig. 5 shows the effect of adding 2-hydroxy-2-methyl-3-oxopentanoate, an inactive analogue of 2-aceto-2-hydroxybutyrate substrate, on the steady-state kinetics. On the one hand, the analogue behaved as a competitive inhibitor with respect to 2-aceto-2-hydroxybutyrate (Fig. 5a), as expected. On the other

Fig. 4. Product inhibition of aceto-hydroxy acid isomeroreductase activity

Reactions were conducted at 30 °C in the standard Tris/HCl buffer, pH 8.2, containing 3 mM-MgCl₂. The enzyme was at 2.2 μg/ml (a,b) or at 9.5 μg/ml (c,d). *v*, Velocity of NADPH oxidation (μmol of NADPH oxidized/min per mg of protein). Linear least-squares analysis of the data in the form of Lineweaver-Burk plots yielded the kinetic parameter values listed in Table 3. (a) [2-Aceto-2-hydroxybutyrate] varied for the fixed [NADP⁺] (μM) indicated on the plots. (b) [NADPH] varied for the fixed [NADP⁺] (μM) indicated on the plots. (c) [2-Acetolactate] varied for the fixed [2,3-dihydroxy-3-isovalerate] (mM) indicated on the plots. (d) [NADPH] varied for the fixed [2,3-dihydroxy-3-isovalerate] (mM) indicated on the plots.

Fig. 5. Inhibition of aceto-hydroxy acid isomeroreductase activity by the substrate analogue 2-hydroxy-3-oxopentanoate

Reactions were conducted at 30 °C in the standard Tris/HCl buffer, pH 8.2, containing 3 mM-MgCl₂ and 2.2 μg of enzyme/ml. *v* is the rate of NADPH oxidation (μmol of NADPH oxidized/min per mg of protein). Linear least-square analysis of the data in the form of Lineweaver-Burk plots yielded the kinetic parameter values listed in Table 3. (a) [2-Aceto-2-hydroxybutyrate] or (b) [NADPH] varied for the fixed [2-hydroxy-3-oxopentanoate] (μM) indicated on the plots.

hand, it acted as a non-competitive inhibitor when NADPH was the varied substrate (Fig. 5b).

Spectroscopic properties of the enzyme

Since the sequence of the cDNA encoding chloroplast aceto-hydroxy acid isomeroreductase isoform 3 reveals the presence of several aromatic amino acid residues in the mature protein (Dumas *et al.*, 1991), we wished to characterize more directly the interaction between enzyme and substrate or coenzyme by conducting absorbance and fluorescence analyses of the over-expressed enzyme in the near-u.v. During these experiments and

with the enzyme alone, we observed slow changes in the spectroscopic properties of the enzyme upon irradiation with u.v. light. Thus the absorbance at 280 nm slowly increased, whereas upon excitation at wavelengths over 260–280 nm, the fluorescence emitted by the enzyme at 295 nm slowly decreased. Interestingly we observed that these spectral changes were associated with a progressive inhibition of the enzymic activity. It is clear, therefore, that this peculiar behaviour precluded the possibility to investigate the formation of a binary complex between enzyme and 2-acetolactate or 2-aceto-2-hydroxybutyrate by the above-mentioned spectroscopic techniques. It is emphasized, however, that the effectiveness of u.v. light in blocking aceto-hydroxy acid isomeroreductase was strongly reduced by NADPH. In contrast, neither Mg²⁺ nor 2-aceto-2-hydroxybutyrate protected the enzyme against such an inactivation (results not shown). In this connection we note that spinach leaf chloroplast aceto-hydroxy acid isomeroreductase contains a tryptophan residue at position 133 within the 'fingerprint' structural motif

Gly-Trp-Gly-Ser-Gln-Ala-Pro-Ala-Gln-Ala

previously assigned to the NADPH-binding domain (Dumas *et al.*, 1991).

NADPH binding

The formation of an NADPH·enzyme complex was assessed by fluorescence measurements. Titrations were carried out by monitoring the fluorescence of NADPH in the absence or presence of enzyme versus [NADPH] under the same ionic conditions as those used for the activity measurements (Tris/HCl buffer, pH 8.2). There was a marked enhancement of NADPH fluorescence in the presence of enzyme compared with the control experiment carried out in its absence (Fig. 6a), suggestive of the

Fig. 6. Binding of NADPH to acetohydroxy acid isomeroreductase

Fluorimetric titrations were effected at 30 °C in the standard Tris/HCl buffer, pH 8.2, using a 1 ml cuvette. The excitation wavelength was 370 nm and the emission wavelength was 460 nm. After stepwise addition of NADPH (0.2 μl additions of various concentrated NADPH solutions) to 200 μl solutions (standard buffer minus or plus 16 μM -enzyme), the emitted fluorescence F_{460} was recorded and the difference between the two curves

$$\Delta F_{460}^{\text{exper.}} = F_{460}^{\text{plus enzyme}} - F_{460}^{\text{minus enzyme}}$$

calculated. Measurements were corrected for the inner-filter effect as follows: the initial slope, S , of the plot of the experimental $F_{460}^{\text{minus enzyme}}$ versus [NADPH] was calculated by regression analysis, then the value of the ratio

$$R = S \cdot [\text{NADPH}] / F_{460}^{\text{minus enzyme}}$$

was calculated for each of the experimental [NADPH]; multiplying $\Delta F_{460}^{\text{exper.}}$ by R gave ΔF_{460} . (a) Plot of NADPH fluorescence versus total [NADPH] in the presence (●) or absence (○) of enzyme. (b) Plot of ΔF_{460} versus total [NADPH]. The continuous line was obtained by non-linear least-squares analysis of the data to eqn. (1) (Cleland, 1979) for the values of the best-fit parameters listed in Table 4. (c) Replot of the data in (b) in the 0–50 μM -[NADPH] range. (d) Statistical analysis of the data in (b) according to Mannervik (1981) and Bardsley (1986). Regression analyses of the experimental data to eqn. (1) were effected starting from different fixed values of e_0 , the total concentration of NADPH binding sites. When mathematical convergence was achieved, sums of residuals and of residual squares of the fits were calculated. These quantities were plotted as a function of e_0 . ○, Sum of residuals; ●, sum of residual squares. The best fit of the experimental data to eqn. (1) was obtained when $e_0 = e_0^{\text{theor.,min.}}$, i.e., for the value of e_0 yielding a sum of the residuals close to zero and the smallest value of the sum of the residual squares.

formation of a binary complex between enzyme and nucleotide. In this case emitted fluorescence at 460 nm with the enzyme-containing assays was stable upon excitation at 370 nm. Figs. 6(b) and 6(c) show a plot of the calculated difference between the two curves, ΔF_{460} versus [NADPH]. At 16 μM -enzyme, this quantity first increased upon increase of [NADPH] and then reached a plateau level at about 25 μM -NADPH. To characterize the stoichiometry of binding, i.e., the number of binding sites on the enzyme for NADPH, as well as the affinity of the binding

Table 4. Summary of binding constants for NADPH and acetohydroxy acid isomeroreductase

Fluorimetric titrations were effected and analysed as described in the Materials and methods section and in the legend to Fig. 6. Reactions were conducted at 30 °C in the standard Tris/HCl buffer, pH 8.2, or in a pH 7.5 buffer containing 50 mM-potassium phosphate, 1 mM-EDTA, and 0.1 mM-dithiothreitol. The excitation wavelength was 370 nm, and the emission wavelength 460 nm. Binding parameters (K_a , association constant of NADPH for enzyme; $\Delta F_{\text{max.}}$, maximum enhancement of NADPH fluorescence at saturation) were obtained through non-linear regression analyses of the experimental data to eqn. (1). e_0 is the total concentration of the NADPH-binding sites, assuming that the overexpressed enzyme contains one binding site per subunit. $e_0^{\text{theor.,min.}}$ is the theoretical concentration of the NADPH-binding sites for which the simulated curve using eqn. (1) yields the smallest value of the sum of the residual squares and a value of the sum of the residuals close to zero (see Fig. 6d). The best-fit parameters from the titration at pH 8.2 and eqn. (1) were used to calculate the curves shown in Figs. 6(b) and 6(c).

Conditions			Parameter values			
pH	Mg ²⁺ (5 mM)	AHB (500 μM)	e_0 (μM)	$e_0^{\text{theor.,min.}}$ (μM)	$\Delta F_{\text{max.}}$ (arbitrary units)	K_a (μM^{-1})
8.2	—	—	15.5	13.5	7.77 ± 0.06	0.54 ± 0.05
7.5	—	—	16.5	13.5	4.2 ± 0.05	0.57 ± 0.08
7.5	+	—	16.5	13	4 ± 0.05	0.36 ± 0.04
7.5	—	+	16.5	15	4.5 ± 0.03	0.4 ± 0.03

sites for the ligand, non-linear regression analyses of the data in Fig. 6 were performed (Cleland, 1979), assuming that the simple equilibrium occurred:

where E is the free enzyme, e_0 the total concentration of binding sites for ligand, l_0 the total concentration of added ligand, x the concentration of bound ligand, and by definition:

$$K_a = \frac{k_{+1}}{k_{-1}} = \frac{x}{(e_0 - x)(l_0 - x)}$$

Since saturation occurred at rather low [NADPH], the data in Fig. 6 were analysed on the basis of a tight-binding hypothesis. In addition to K_a , an other parameter to be fitted is $\Delta F_{\text{max.}}/e_0$, i.e. the maximum enhancement of fluorescence achieved at saturation. The number of NADPH-binding sites on the enzyme was determined as follows. Non-linear regression analyses and eqn. (1) were used to fit experimental data such as those in Fig. 6(b), starting from different fixed values of e_0 . Provided mathematical convergence was achieved, a set of the best-fit parameters together with the sum of residuals and of residual squares (Fig. 6d) were obtained, for any value of e_0 . By this method, one can find out the theoretical value of the total concentrations of binding sites, $e_0^{\text{theor.,min.}}$, giving a sum of the residuals close to zero and the smallest value of the residual squares (Mannervik, 1981; Bardsley, 1986). As Fig. 6(d) shows, the value of $e_0^{\text{theor.,min.}}$ was very close to the experimental value of e_0 , assuming that acetohydroxy acid isomeroreductase contains one NADPH-binding site per monomeric unit. Furthermore, since there was no significant deviations of the experimental data from the simulated curves derived from eqn. (1) (Figs. 6b and 6c) we conclude that all NADPH-binding sites within the dimeric structure of acetohydroxy acid isomeroreductase have same binding affinity. Similar results were obtained in a phosphate buffer at pH 7.5 and

in the absence or presence of Mg^{2+} or 2-aceto-2-hydroxybutyrate. Binding parameters are listed in Table 4. These data show that the values of K_a and ΔF_{max} were not significantly different when the assay contained either Mg^{2+} or 2-aceto-2-hydroxybutyrate, suggesting that the latter compounds do not interfere with the binding of NADPH to the enzyme.

DISCUSSION

In the present study we used a spinach cDNA to overexpress spinach acetohydroxy acid isomeroreductase in *E. coli* cells. The enzyme we obtain from the recombinant bacteria corresponds to isoform 3 previously purified from spinach leaf chloroplasts (Dumas *et al.*, 1989). The proposed purification procedure yields 13 mg of pure enzyme from a 2-litre cell culture. For comparison, our previous purification scheme yielded about 1 mg of acetohydroxy acid isomeroreductase isoform 3, starting from 3 kg of spinach leaves (Dumas *et al.*, 1989). The specific activity of the overexpressed enzyme compares well with that of the native enzymes isolated from leaves (Dumas *et al.*, 1989) and with that of the corresponding enzymes from prokaryotes (Shematek *et al.*, 1973; Chunduru *et al.*, 1989). For all enzymes the maximum rate of NADPH oxidation is 5–6-fold higher with 2-aceto-2-hydroxybutyrate as substrate compared with that with 2-acetolactate (Shematek *et al.*, 1973; Chunduru *et al.*, 1989; Dumas *et al.*, 1989).

The present kinetic and substrate binding studies allow one to compare some properties of plant and prokaryotic acetohydroxy acid isomeroreductases. Steady-state kinetic and product-inhibition studies carried out with bacterial enzymes established a sequential reaction mechanism in which NADPH binds first and 2-acetolactate or 2-aceto-2-hydroxybutyrate binds second at saturating bivalent-metal-ion concentrations (Shematek *et al.*, 1973; Chunduru *et al.*, 1989). These studies also established an ordered liberation of 2,3-dihydroxy-3-isovalerate and $NADP^+$ (Scheme 1b). Our present results essentially confirm the same basic mechanism with, however, some differences. Thus, as for the bacterial enzymes, the inhibition pattern for $NADP^+$ argues against ordered or random reaction mechanisms in which 2-acetolactate or 2-aceto-2-hydroxybutyrate binds to the free enzyme. However, the inhibition pattern for 2,3-dihydroxy-3-isovalerate cannot be simply explained according to the ordered model, as in this case 2,3-dihydroxy-3-isovalerate should behave as a non-competitive inhibitor with respect to 2-acetolactate at saturating [NADPH]. One possibility to account for the observed behaviour is to postulate a Theorell–Chance mechanism in which binding of 2-acetolactate or 2-aceto-2-hydroxybutyrate to the enzyme·NADPH complex, substrate rearrangement and reduction occur almost simultaneously. According to this hypothesis the concentrations of the two central ternary complexes enzyme·NADPH·substrate and enzyme·NADP⁺·2,3-dihydroxy-3-isovalerate are essentially zero at steady state. However, if this were the case 2,3-dihydroxy-3-isovalerate should behave as a mixed-type inhibitor with respect to NADPH, which is not observed. A tentative explanation of the results in Fig. 4 would be that 2,3-dihydroxy-3-isovalerate binds to the enzyme·NADPH complex, thereby forming a dead-end complex that prevents further binding of 2-acetolactate or 2-aceto-2-hydroxybutyrate (Scheme 1c). The competitive behaviour also indicates that, within the enzyme active site, there are some common amino acid residues that interact with both the 2-acetolactate substrate and the 2,3-dihydroxy-3-isovalerate product.

The validity of the ordered mechanism depicted in Scheme 1(b) was also evaluated by using a substrate analogue, 2-hydroxy-2-methyl-3-oxopentanoate. As expected, this molecule behaved as a competitive inhibitor of 2-acetolactate and 2-aceto-2-hydroxy-

butyrate. However, it acted as a non-competitive inhibitor with respect to NADPH. These findings suggest a model in which, at steady state, 2-hydroxy-2-methyl-3-oxopentanoate and NADPH combine independently of the enzyme to produce enzyme·2-hydroxy-2-methyl-3-oxopentanoate, enzyme·NADPH and enzyme·NADPH·2-hydroxy-2-methyl-3-oxopentanoate complexes (Scheme 1c). Thus the substitution of the 3-methyl group in the natural substrate 2-acetolactate for a 3-ethyl group alters the mechanism of substrate binding to the enzyme, from an ordered to a random addition, and alleviates the requirement for NADPH binding prior to that of hydroxy acid substrate.

A noticeable difference between the physicochemical properties of plant and bacterial acetohydroxy acid isomeroreductase is provided by the results of NADPH binding to the enzymes. The binding data in Fig. 6 and Table 4 can be simply explained by assuming that the two subunits of the plant enzyme contain identical NADPH-binding sites. This contrasts with the results reported by Shematek *et al.* (1973) for the acetohydroxy acid isomeroreductase from *S. typhimurium*: these authors observed the binding of four molecules of NADPH per molecule of enzyme, with binding of the first molecule ($K_d = 1.7 \times 10^{-6}$ M) affecting that of subsequent molecules ($K_d = 6 \times 10^{-6}$ M). Our interpretation is supported by the fact that the Lineweaver–Burk plots (1/velocity versus 1/[NADPH]) were linear at least up to 250 μ M. In this context it may also be worth pointing out that the bacterial enzyme is a tetramer of identical subunits (Hofler *et al.*, 1975), whereas the results of ultracentrifugation and gel-filtration analyses in the present study indicate that the overexpressed plant enzyme is a dimer.

There are also marked differences in the values of the kinetic parameters deduced for the plant and bacterial enzymes. Of particular importance is the finding that the plant enzyme displays a very high selectivity for NADPH: at saturating 2-aceto-2-hydroxybutyrate the K_m for NADH is about 200-fold higher than that for NADPH. Moreover, $NADP^+$ is a much more potent inhibitor of the plant enzyme (K_i of the order of 5 μ M) than of the bacterial enzymes (K_i is the order of 200 μ M; Shematek *et al.*, 1973; Chunduru *et al.*, 1989). Since it is well established that, in higher-plant chloroplasts, the NADPH/ $NADP^+$ concentration ratio is regulated by light (light conditions, high [NADPH]/[$NADP^+$]; dark conditions, low [NADPH]/[$NADP^+$]), the present study, together with our previous results showing that acetohydroxy acid isomeroreductase activity from spinach leaves is located exclusively within the chloroplasts (Dumas *et al.*, 1989), support the notion that biosynthesis of branched-chain amino acids in plants is controlled by light.

Another relevant aspect concerns the contribution of acetohydroxy acid isomeroreductase activity to the partitioning between the two parallel biosynthetic pathways leading to valine and isoleucine. We have observed that discrimination between the two hydroxy acids (2-acetolactate and 2-aceto-2-hydroxybutyrate) results from a competition of substrates for the active site of enzyme (Scheme 1a). Under steady-state conditions Scheme 1(a) allows one to express the ratio of the rates of utilization of 2-acetolactate (v_{AL}) and 2-aceto-2-hydroxybutyrate (v_{AHB}) as (Laidler, 1958):

$$\frac{v_{AL}}{v_{AHB}} = \frac{V_{max}^{AL} K_m^{AHB} [2\text{-acetolactate}]}{V_{max}^{AHB} K_m^{AL} [2\text{-aceto-2-hydroxybutyrate}]} \quad (2)$$

Since $K_m^{AL} = K_m^{AHB} = 10 \mu$ M and $V_{max}^{AL} = 0.17 V_{max}^{AHB}$ (Table 2), eqn. (2) leads to:

$$\frac{v_{AL}}{v_{AHB}} = 0.17 \left(\frac{[2\text{-acetolactate}]}{[2\text{-aceto-2-hydroxybutyrate}]} \right) \quad (3)$$

Therefore, if the kinetic mechanism as well as the kinetic

parameters deduced from the present study *in vitro* hold under conditions found *in vivo*, the ratio of rates between the two routes leading to the conversion of the acetohydroxy acids into the oxo analogues of isoleucine and valine will be proportional to the ratio of concentrations of 2-acetolactate and 2-aceto-2-hydroxybutyrate, i.e. the products of acetolactate synthase activity starting from pyruvate and 2-oxobutyrate respectively. It has been pointed out that acetolactase synthases from prokaryotic and eukaryotic organisms have evolved with high specificity to 2-oxobutyrate to provide comparable quantities of isoleucine and valine under conditions of low 2-oxobutyrate and high pyruvate concentrations (Gollop *et al.*, 1990). This suggestion is supported by the demonstration that 2-oxobutyrate can be toxic in bacteria (Daniel *et al.*, 1983; La Rossa *et al.*, 1987). On the basis of the observed preference for 2-aceto-2-hydroxybutyrate over 2-acetolactate, it appears that acetohydroxy acid isomeroreductase is also well adapted to fulfill the requirements for isoleucine synthesis under conditions of low 2-oxobutyrate supply.

In conclusion, the present work was undertaken to define better the properties of some of the protein components involved in the synthesis of valine from pyruvate and isoleucine from 2-oxobutyrate. To this end we have overexpressed a spinach leaf acetohydroxy acid isomeroreductase in *E. coli* cells. Substantial differences were noted between the structural and kinetic properties of the plant enzyme compared with those of its well-documented bacterial counterpart, which may reflect the fact that plant (Dumas *et al.*, 1991), yeast (Petersen & Holmberg, 1986) and prokaryotic (Wek & Hatfield, 1986; Aguilar & Grasso, 1991) acetohydroxy acid isomeroreductases show little sequence similarity. These differences highlight the need for precise information regarding the plant systems in order to understand better their regulation, notably at the level of the interactions between the nuclear and chloroplast genomes, and to develop efficient herbicide molecules acting at the level of these enzymes. The cloning of a plant acetohydroxy acid isomeroreductase gene and its expression in bacterial cells provide invaluable tools to investigate these points.

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REFERENCES

- Aguilar, O. M. & Grasso, D. H. (1991) *J. Bacteriol.* **173**, 7756–7764.
- Arfin, S. M. & Umbarger, H. E. (1969) *J. Biol. Chem.* **244**, 1118–1127.
- Armstrong, F. B. & Wagner, R. P. (1961) *J. Biol. Chem.* **236**, 2027–2032.
- Aulabaugh, A. & Schloss, J. V. (1990) *Biochemistry* **29**, 2824–2830.
- Bardsley, W. G. (1986) in *Dynamics of Biochemical Systems* (Damjanovich, Keleti, T. & Trón, L., eds.), pp. 267–281, Akadémiai Kiadó, Budapest.
- Chua, N. H. (1980) *Methods Enzymol.* **69**, 434–446.
- Chunduru, S. K., Mrachko, G. T. & Calvo, K. C. (1989) *Biochemistry* **28**, 486–493.
- Cleland, W. W. (1979) *Methods Enzymol.* **63A**, 103–138.
- Crout, H. D. G., Littlechild, J., Mitchell, M. B. & Morrey, S. M. (1984) *J. Chem. Soc. Perkin Trans. 1*, 2271–2276.
- Daniel, J., Dondon, L. & Danchin, A. (1983) *Mol. Gen. Genet.* **190**, 452–458.
- Douce, R. & Joyard, J. (1982) in *Methods in Chloroplast Molecular Biology* (Edelman, M., Hallick, R. B. & Chua, N. H., eds.), pp. 239–256, Elsevier Biomedical, Amsterdam.
- Dumas, R. (1991) Doctoral Thesis, University of Grenoble.
- Dumas, R., Joyard, J. & Douce, R. (1989) *Biochem. J.* **262**, 971–976.
- Dumas, R., Lebrun, M. & Douce, R. (1991) *Biochem. J.* **277**, 469–475.
- Gollop, N., Damri, B., Chipman, D. M. & Barak, Z. (1990) *J. Bacteriol.* **172**, 3444–3449.
- Hill, R. K., Sawada, S. & Arfin, S. M. (1979) *Bio-org. Chem.* **8**, 175–189.
- Hofler, J. G., Decedue, C. J., Luginbuhl, G. H., Reynolds, J. A. & Burns, R. O. (1975) *J. Biol. Chem.* **250**, 877–882.
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
- Laidler, K. J. (1958) *The Chemical Kinetics of Enzyme Action*, pp. 87–89, Clarendon Press, Oxford.
- LaRossa, R. A., Van Dyk, T. K. & Smulski, D. R. (1987) *J. Bacteriol.* **169**, 1372–1378.
- Mannervik, B. (1981) in *Kinetic Data Analysis* (Laszlo, L., ed.), pp. 235–270, Plenum Press, New York.
- Ortholand, J.-Y. (1991) Doctoral Thesis, University of Lyon.
- Ortholand, J.-Y. & Greiner, A. (1992) *Tetrahedron Lett.* **33**, 1897–1900.
- Petersen, J. G. L. & Holmberg, S. (1986) *Nucleic Acids Res.* **14**, 9631–9651.
- Sanger, F., Nicklen, S. & Coulson, A. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463–5467.
- Schloss, J. V. & Aulabaugh, A. (1990) in *Biosynthesis of Branch Chain Amino Acids* (Barak, Z., Chipman, D. M. & Schloss, J. V., eds.), pp. 345–356, VCH publishers, Weinheim and New York.
- Schulz, A., Spönemann, P., Köcher, H. & Wengenmayer, F. (1988) *FEBS Lett.* **238**, 375–378.
- Scopes, R. K. (1974) *Anal. Biochem.* **59**, 277–282.
- Segel, I. H. (1975) *Enzyme Kinetics: Behavior and Analysis of Rapid-Equilibrium and Steady-State Enzyme Systems*, pp. 113–118, John Wiley and Sons, New York.
- Shematek, E. M., Arfin, S. M. & Diven, W. F. (1973) *Arch. Biochem. Biophys.* **158**, 132–138.
- VanRheenen, V., Kelly, R. C. & Cha, D. Y. (1976) *Tetrahedron Lett.* **23**, 1973–1976.
- Wek, R. C. & Hatfield, G. W. (1986) *J. Biol. Chem.* **261**, 2441–2450.
- Yphantis, D. A. (1964) *Biochemistry* **3**, 297–317.

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