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Evidence for Two Catalytically Different Magnesium-Binding Sites in Acetohydroxy Acid Isomeroreductase by Site-Directed Mutagenesis†

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ABSTRACT: Alignment of procaryotic and plant acetohydroxy acid isomeroreductase (EC 1.1.1.86) reveals five conserved regions designated domains I, II, III, IV, and V. Domain I has been previously proposed to correspond to the NADPH-binding site [Dumas *et al.* (1991) *Biochem. J. 277,* 469−475] and domain III to a putative magnesium-binding site [Sista & Bowman (1992) *Gene 120,* 115−118]. The binding and the function of this cation are of particular importance. First, Mg^{2+} is essential for the two-step reaction catalyzed by this enzyme: an isomerization followed by an NADPH-dependent reduction. Second, the plant acetohydroxy acid isomeroreductase exhibits K_d and K_m values for Mg^{2+} of 5 μM and 6 μM, respectively. Such values correspond to the strongest affinity known between an enzyme and the metal ion. To determine if domain III of acetohydroxy acid isomeroreductase is effectively involved in magnesium binding, and with the goal to assign a function to the other conserved domains, site-directed mutagenesis was performed on each charged or polar conserved amino acids of domains II−V of the spinach acetohydroxy acid isomeroreductase. The results demonstrate that mutation of each of these amino acids leads to a partial or complete inactivation of enzyme activity. Steady-state kinetic analysis and equilibrium binding experiments show that both domains III and IV are directly involved in the binding of magnesium. Also, they suggest that magnesium bound to domain III plays a role in the reductive half-reaction, whereas, magnesium bound to domain IV is involved in the isomerization half-reaction.

Acetohydroxy acid isomeroreductase (EC 1.1.1.86) is involved in the biosynthetic pathway leading in plants and microorganisms to branch-chain amino acids. Because animals lack this biosynthetic capability, this pathway has raised strong interest for herbicide development. Initial investigations showed that transition state analogues such as 2-(dimethylphosphinoyl)-2-hydroxyacetic acid (Hoe 704)[1] and *N*-hydroxy-*N*-isopropyloxamate (IpOHA), which behave as selective inhibitors of acetohydroxy acid isomeroreductase, exhibit herbicidal activity (Schulz *et al.*, 1988; Aulabaugh & Schloss, 1990; Dumas *et al.*, 1994a). The plant enzyme has been purified from the stroma of spinach (*Spinacia oleracea*) chloroplasts (Dumas *et al.*, 1989) and from barley (*Hordeum vulgare*) (Durner *et al.*, 1993). It is a homodimer of molecular mass 114 000 Da that contains one NADPH-binding site per monomeric unit (Dumas *et al.*, 1992). Its primary structure has been determined from spinach (Dumas *et al.*, 1991) and *Arabidopsis thaliana* cDNA (Curien *et al.*, 1993) and its gene sequenced from *A. thaliana* (Dumas *et al.*, 1993). The cDNA encoding the mature polypeptide sequence from spinach was further used to overexpress the enzyme in *Escherichia coli* (Dumas *et al.*, 1992). Recently (Dumas *et al.*, 1994b), we have crystallized the overexpressed enzyme as a complex with NADPH, magnesium, and a transition state analogue [either IpOHA (Aulabaugh & Schloss, 1990) or Hoe 704 (Schulz *et al.*, 1988)], and the search for heavy-atom derivatives is in progress.

Beside this potential agrochemical importance, acetohydroxy acid isomeroreductase presents several unique catalytic features. This enzyme catalyzes an unusual two-step reaction: an alkyl migration in which the substrate, either 2-acetolactate (AL) or 2-aceto-2-hydroxybutyrate (AHB), is converted to 3-hydroxy-3-methyl-2-oxobutyrate or 3-hydroxy-3-methyl-2-oxopentanoate, respectively, followed by an NADPH-dependent reduction to give 2,3-dihydroxy-3-isovalerate or 2,3-dihydroxy-3-methylvalerate, respectively (Figure 1). Studies from bacterial (Armstrong *et al.*, 1974, 1983; Hill *et al.*, 1979), fungi (Hawkes & Edwards, 1990), and plant enzymes (Dumas *et al.*, 1992) have demonstrated that the 2*S* isomers of AL and AHB are the true substrates for the reaction, and that their diol products have respectively a configuration (2*R*) and (2*R*, 3*R*) (Hill & Yan, 1971; Crout & White-House, 1972). The reduction step requires the transfer of the *pro-S* hydrogen atom from NADPH (Arfin & Umbarger, 1969). The enzyme catalyzed reaction obeys an ordered mechanism in which NADPH and magnesium bind first and independently, followed by acetohydroxy acid

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[1] Abbreviations and Nomenclature: AL, 2-acetolactate; AHB, 2-aceto-2-hydroxybutyrate; KP, ketopantoate; Hoe 704, 2-(dimethylphosphinoyl)-2-hydroxyacetic acid; IpOHA, *N*-hydroxy-*N*-isopropyloxamate; IPTG, isopropyl β-D-thiogalactoside. Description of mutants was made as follows: II-[R259K], for example, refers to mutant enzyme in which the wild-type arginine 259, which belongs to the domain II of the spinach acetohydroxy acid isomeroreductase, has been replaced by a lysine. The definition of domains I−V is given in Figure 2.

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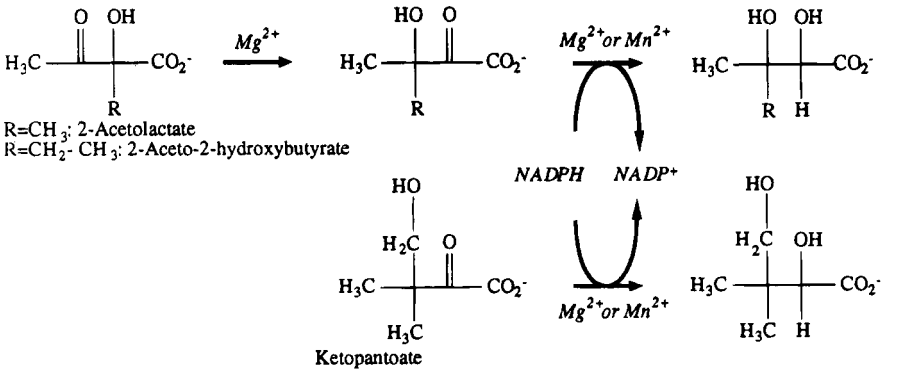

FIGURE 1: Reactions catalyzed by acetohydroxy acid isomeroreductase.

Table 1: Oligonucleotides Used for Mutagenesis[a]

enzyme domain[b]	mutation	oligonucleotide
II	R259K	5′-GGAATGGGGCCTTCTGTTAAAAGGTTGTATGTTCAAGG-3′
III	E311D	5′-CTCTTGAGCAGGACTATAAGAGTGACATCTTTGGGG-3′
III	D315E	5′-ATATAAGAGTGAGATCTTTGGGG-3′
III	E319D	5′-GTGACATCTTTGGGGACCGAGGTATCTTGCTTGG-3′
IV	E488D	5′-GGTCACTCGTACTCGGACATCATCAACGAGAGTGTG-3′
IV	E492D	5′-CTCGGAGATCATCAACGACAGTGTGATTGAAGCAG-3′
V	S518T	5′-GGTTGACAACTGCACAACCACAGCAAGGCTTGG-3′
V	T520S	5′-GACAACTGCTCAACCTCAGCAAGGCTTGGATCAAT-3′

[a] The underlined bases were mutated from wild-type spinach acetohydroxy acid isomeroreductase. [b] For definition of the enzyme domains, see Figure 2.

substrate binding (Chunduru *et al.*, 1990; Dumas *et al.*, 1992). Also, acetohydroxy acid isomeroreductase has been shown to catalyze efficiently the reduction of ketopantoate (KP) (an intermediate in pantothenate biosynthesis) in the presence of a bivalent metal cofactor and NADPH (Primerano & Burns, 1983; Julliard, 1994), thus providing a means to investigate the reductive half-reaction independently of the isomerization reaction. As Figure 1 shows, KP shares structural homology with the reaction intermediate of acetohydroxy acid isomeroreductase.

The two steps of the enzymatic reaction catalyzed by the *E. coli* acetohydroxy acid isomeroreductase (rearrangement and hydride transfer) can be distinguished by their metal ion requirements (Chunduru *et al.*, 1989). The conversion of acetohydroxy acid substrate (AL or AHB) to the diol product shows an absolute requirement for Mg^{2+}. In contrast, the reduction of the reaction intermediate is less specific since either Mg^{2+} or Mn^{2+} can be effective for catalysis. It is not yet known whether this difference in metal ion specificity reflects the existence of two separate metal ion binding sites on the enzyme, each being involved in a distinct half-reaction of the overall process (Figure 1), or whether the enzyme contains a single metal binding site that exhibits different specificities during catalysis of the isomerization and reduction steps. There are considerable differences in the affinity of acetohydroxy acid isomeroreductase from various sources for the divalent metal. Thus, the spinach enzyme displays an extremely high affinity for Mg^{2+}, with a K_m value of the order of 5 μM (Dumas *et al.*, 1992), which corresponds to the strongest affinity reported between an enzyme and this metal ion. In contrast, the bacterial enzyme has a much lower affinity (K_m of 420 μM) for Mg^{2+} (Chunduru *et al.*, 1989).

To identify essential domains or residues of the enzyme that participate in metal ion cofactor binding, the predicted amino acid sequences of plant acetohydroxy acid isomeroreductase have been aligned to the known sequences of corresponding enzymes from fungi (Petersen & Holmberg, 1986; Sista & Bowman, 1992) and bacteria (Blazey & Burns, 1984; Wek & Hatfield, 1986; Aguilar & Grasso, 1991; Godon *et al.*, 1992; Rieble & Beale, 1992) (Figure 2). This sequence comparison indicated five regions of identity that have been designated in this paper as domains I–V. Domain I is similar to the fingerprint region of the NADPH-binding site found in a large number of NADPH-dependent oxidoreductases (Dumas *et al.*, 1991). So, the role of this domain in magnesium binding was not considered in the present study. Domain III is a likely candidate for metal ion binding to the enzyme since Sista and Bowman (1992) have pointed out that this domain is very similar to a putative magnesium-binding site found in the catalytic subunit of vacuolar ATPases and F-type ATPases (Yoshida *et al.*, 1981, 1982). The function of domains II, IV, and V has not yet been assigned.

In this work, we have assessed the possibility that domain III and/or other domains (II, IV, and V) of acetohydroxy acid isomeroreductase are involved in the binding of magnesium by carrying a systematic site-directed mutagenesis of charged or polar conserved amino acids of the spinach enzyme. Our general finding is that both domains III and IV are involved in the binding of magnesium. Furthermore, our results suggest that magnesium bound to domain III plays a role in the reductive half-reaction, whereas magnesium bound to domain IV is involved in the isomerization step.

MATERIALS AND METHODS

Materials. Racemic 2-aceto-2-hydroxybutyrate and 2-acetolactate were prepared as described by Krampitz (1948). Ketopantoate was prepared from commercial oxopantoyl lactone according to King *et al.* (1974).

Bacterial Strains and Plasmids. All bacterial strains used in this study are derivatives of *E. coli* K-12. *E. coli* strain

```
S. oleracea     MAATAATTFS LSSSSSTSAA ASKALKQSPK PSALNLGFLG SSSTIKACRS      50
A. thaliana     ----MAAATS SIAPSLSCPS PSSSSKTLWS SKARTLALPN IGFLSSSSKS      46
S. cerevisiae   ---------- ---------- ---------- -------MLR TQAARLICNS      13
N. crassa       ---------- ---------- ---------- --MAARNCTK ALRPLARQLA      18
L. lactis       ---------- ---------- ---------- ---------- ----------
Synechocystis sp. ---------- ---------- ---------- ---------- ----------
R. meliloti     ---------- ---------- ---------- ---------- ----------
E.coli          ---------- ---------- ---------- ---------- ----------
Consensus       ---------- ---------- ---------- -------... ..........      50

S. oleracea     LKAARVLPSG ANGGGSALSA QMVSAPSINT PSATTFDFDS SVFKKEKVTL     100
A. thaliana     LRSLTATVAG NGATGSSLAA RMVSSSAVKA P--VSLDFET SVFKKEKVSL      94
S. cerevisiae   RVITAKRTFA LATRAAAYSR PAARFVKP-M ITTRGLKQIN FGGTVETV--      60
N. crassa       TPAVQRRTFV AAASAVRASV AVKAVAAPAR QQVRGVKTMD FAGHKEEV--      66
L. lactis       ---------- ---------- ---------- ---------- ----------
Synechocystis sp. ---------- ---------- ---------- ---------- ----------
R. meliloti     ---------- ---------- ---------- ---------- ----------
E.coli          ---------- ---------- ---------- -------MAN YFNTLNLRQQ      13
Consensus       .......... .......... ........-. .--..... .........--     100

                                                  Domain I
S. oleracea     SGHDEYIVRG GRNLFPLLPD AFKGIKQIGV IGMGSQAPAQ AQNLKDSLTE     150
A. thaliana     AGYEEYIVRG GRDLFKHLPD AFKGIKQIGV IGMGSQGPAQ AQNLRDSLVE     144
S. cerevisiae   --YE----RA DWPREKLL-D YFKN-DTFAL IGMGSQGYGQ GLNLRD----      98
N. crassa       --HE----RA DWPAEKLL-D YFKN-DTLAL IGMGSQGHGQ GLNLRD----     104
L. lactis       ------MAVT MYYEDDVEVS ALAG-KQIAV IGMGSQGHAH AQNLRD----      39
Synechocystis sp. -------MAR MYYDQDANLD LLAG-KTVAI IGMGIQGHAH ALNLKD----      38
R. meliloti     --------MR VYYDRDADLN LIKS-KKVAI IGMGSQA-AH ALNLKD----      36
E.coli          LAQLGKCRFM GRDEFADGAS YLQG-KKVVI VGCGAQGLNQ GLNMRD----      58
Consensus       --..--.... .........D ..KG-K..A. IGMGSQG.AQ ALNLRD----     150

S. oleracea     AKSDVVVKIG LRKGSNSFAE ARAAGFSEEN GTLGDMWETI SGSDLVLLLI     200
A. thaliana     AKSDIVVKIG LRKGSRSFEE ARAAGFTEES GTLGDIWETI AGSDLVLLLI     194
S. cerevisiae   --NGLNVIIG VRKDGASWKA AIEDGWVPGK -NLFTVEDAI KRGSYVMNLL     145
N. crassa       --NGLNVIVG VRKNGKSWED AIQDGWVPGK -NLFDVDEAI SRGTIVMNLL     151
L. lactis       --SGHNVIIG VRHG-KSFDK AKEDGFETFE -----VGEAV AKADVIMVLA      81
Synechocystis sp. --SGVNVVVG LYSGSKSVAK AEGAGLKVLS -----VAEAA KAADLIMILL      81
R. meliloti     --SGAQNVAI ALKSGSATAK KAEADGFKVM ----TVAEAA AWADLMMMAT      80
E.coli          --SGLDISYA LRKEAIAEKR ASWRKATENG FKVGTYEELI PQADLVINLT     106
Consensus       --SG..V..G .RK...S... A...G..... -....V.EAI ...DLVM.L.     200

S. oleracea     SDSAQADN-Y EKVFSHMKPN SILGLSHGFL LGHLQSLGQD FPKNISVIAV     249
A. thaliana     SDAAQADN-Y EKIFSHMKPN SILGLSHGFL LGHLQSSGLD FPKNISVVAV     243
S. cerevisiae   SDAAQSET-W PAIKPLLTKG KTLYFSHGFS PVFKDLTHVE PPKDLDVILV     194
N. crassa       SDAAQSET-W PHIKPQITKG KTLYFSHGFS PVFKDLTKVE VPTDVDVILV     200
L. lactis       PDELQQSIYE EDIKPNLKAG SALGFAHGFN IHFGYI-KV- -PEDVDVFMV     128
Synechocystis sp. PDEVQKTVYE AEIAPNLVAG NVLLFAHGFN INFAQI--VP -PADVDVVMA     128
R. meliloti     PTSCRPTSTR PTSPETSATA RQSPLRTAST STSASSSRRL GRRRND----     126
E.coli          PDKQHSDV-V RTVQPLMKDG AALGYSHGFN IVEVGEQIRK DITVV---MV     152
Consensus       .D..Q...-. ..I.P....G ..L..SHGF. .......... .P...DV..V     250

                Domain II
S. oleracea     CPKGMGPSVR RLYVQGKEVN GAGINSSFAV HQDVDGRATD VALGWSIAL-     298
A. thaliana     CPKGMGPSVR RLYVQGKEIN GAGINASFAV HQDVDGRAAD VRLGWSVAL-     292
S. cerevisiae   APKGSGRTVR SLFKEGRGIN -----SSYAV WNDVTGKAHE KAQALAVAI-     238
N. crassa       APKGSGRTVR SLFREGRGIN -----SSFAV YQDVTGKAKE KAVALGVAV-     244
L. lactis       APKAPGHLVR RTYTEGFGTP AL-----FVS HQNASGHARE IAMDWAKGI-     172
Synechocystis sp. APKGPGHLVR RTYEQGQGVP AL-----FAV YQDASGQARD YAMAYAKGI-     172
R. meliloti     RSEGPGHTVR GEYQKGGGVP CLV-----AV HQDASGNALD LALSYACGV-     170
E.coli          APKCPGTEVR EEYKRGFGVP TLI-----AV HPENDPKGEG MAIAKAWAAA     197
Consensus       APKG.G..VR ..Y..G.G.. ...--..FAV HQD..G.A.. .A...A.A.-     300

                                Domain III
S. oleracea     ---GSPFTFA TTLEQEYKSD IFGERGILLG AVHGIVECLF RRYTESGMSE     345
A. thaliana     ---GSPFTFA TTLEQEYRSD IFGERGILLG AVHGIVESLF RRYTENGMSE     339
S. cerevisiae   ---GSGYVYQ TTFEPEVNSD LYGERGCLMG GIHGM----- ----------     270
N. crassa       ---GSGYLYE TTFEKEVYSD LYGERGCLMG GIHGM----- ----------     276
L. lactis       -GCARVGIIE TTFKEETEED LFGEQAVLCG ---------- ----------     201
Synechocystis sp. -GGTRAGILE TTFREETETD LFGEQVVLCG ---------- ----------     201
R. meliloti     -GGGRSGIIE TNFKEECETD LFGEQVVLCG ---------- ----------     199
E.coli          TGGHRAGVLE SSFVAEVKSD LMGEQTILCG MLQAGSLLCF DKLVEEGTDP     247
Consensus       -..G.....E TTF..E..SD LFGE...L.G .....----- ----------     350
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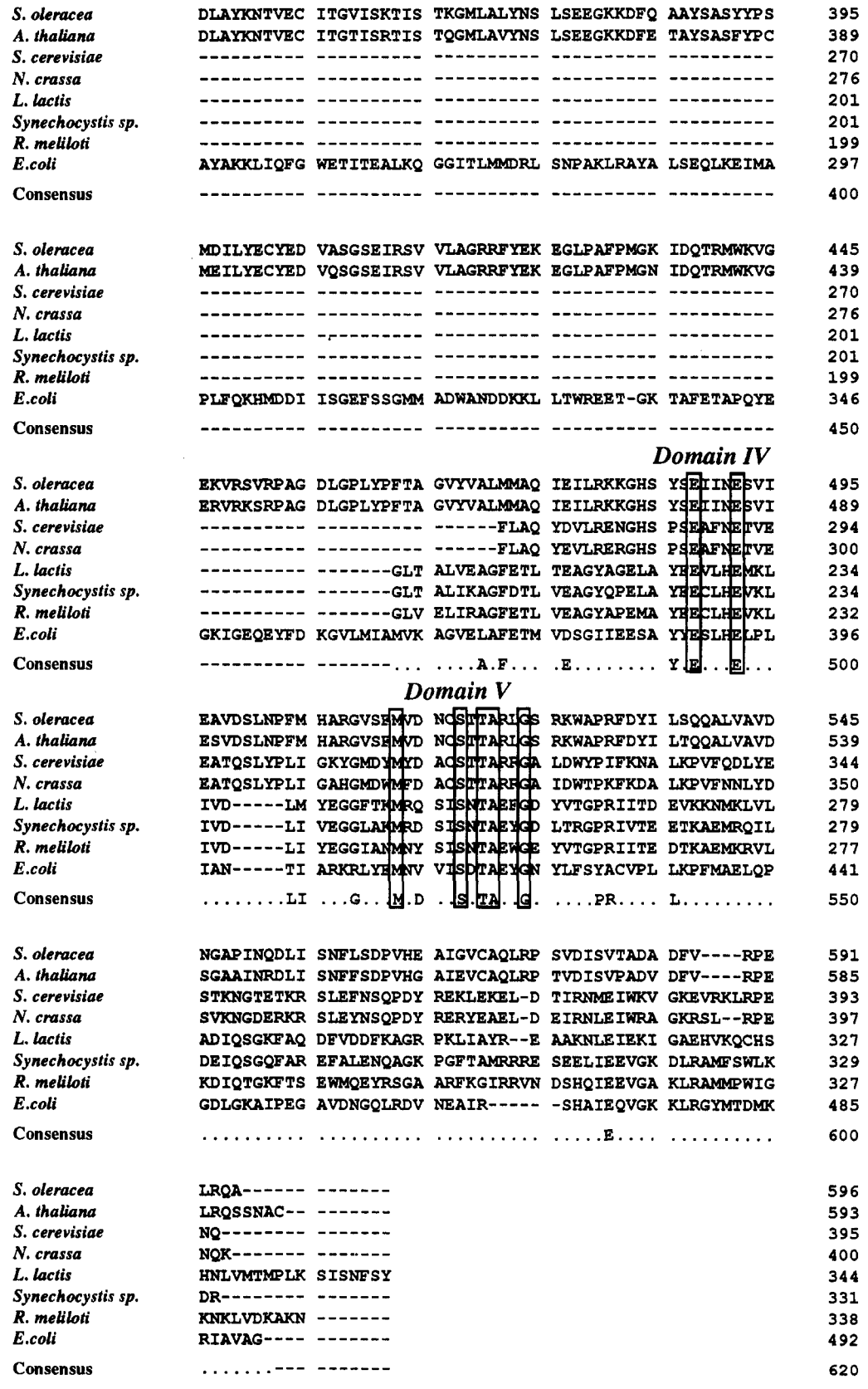


FIGURE 2: Comparison of the predicted amino acid sequences of acetohydroxy acid isomeroreductase. Boxes indicate sequence similarity between the acetohydroxy acid isomeroreductases from *S. oleracea* (Dumas *et al.*, 1991), *A. thaliana* (Curien *et al.*, 1993), *Saccharomyces cerevisiae* (Petersen & Holmberg, 1986), *Neurospora crassa* (Sista & Bowman, 1992), *Lactobacillus lactis* (Godon *et al.*, 1992), *Synechocystis sp.* (Rieble & Beale, 1992), *Rhizobium meliloti* (Aguilar & Grasso, 1991), and *E. coli* (Wek & Hatfield, 1986). They define the conserved domains I−V. A conserved sequence not found in the *R. meliloti* sequence was underlined.

BMH-71-18 *mut*S was supplied with the unique site elimination (U.S.E.) mutagenesis kit (Pharmacia) and was used during mutagenesis of the spinach acetohydroxy acid isomeroreductase cDNA. All experiments were conducted with the plasmid pKK-AHRI that contains the sequence of the mature spinach cDNA (Dumas *et al.*, 1992). Overexpression of the

plant enzyme was further realized in *E. coli* JM 105.

Site-Directed Mutagenesis. Mutagenesis was carried out as described by Deng and Nickoloff (1992), using the U.S.E. mutagenesis kit (Pharmacia) that utilizes a two-primer system to generate site-specific mutations. The first primer (*Pvu*II−*Not*I) is directed to the vector sequence of pKK-AHRI and transforms a unique and nonessential site of restriction enzyme (*Pvu*II) into another unique restriction site (*Not*I). The second primer (target mutagenic primer) is directed to the sequence of the mature spinach cDNA and carries the desired mutation. The sequences of the 23−35-base oligonucleotide primers leading to the point mutations R259L, E311D, D315E, E319D, E488D, E492D, S518T, and T520S are listed in Table 1. The two primers (25 pmol each, phosphorylated) and circular plasmid pKK-AHRI (0.025 pmol) were mixed in a 20 μL annealing buffer (20 mM Tris acetate, 20 mM magnesium acetate, 100 mM potassium acetate; pH 7.5), incubated 5 min at 100 °C, and chilled on ice for 5 min. Following a 30 min incubation at 25 °C, the reaction medium was brought to 30 μL by adding 7 μL of buffer containing the nucleotide mix (2.86 mM each of dATP, dCTP, dGTP, dTTP; 4.34 mM ATP) and 3 μL of reaction mix [T_4 DNA ligase (3 units), T_4 DNA polymerase (3 units), and T_4 Gene 32 protein (0.6 μg)]. After incubation at 37 °C for 1 h, synthesis and ligation were stopped by heating to 85 °C for 15 min. In order to linearize parental plasmid, the reaction medium was incubated 2 h with 15 units of *Pvu*II, and the resultant mixture was used to transform electrocompetent *E. coli* BMH-71-18 *mut*S cells. Following 2 h incubation at 37 °C in 1 mL of SOC medium (Maniatis *et al.*, 1982) with shaking at 180 rpm, 3 mL of SOC medium containing 100 μg of carbenicillin/mL was added to the transformed cells. Incubation was carried out overnight at 37 °C with shaking at 250 rpm. Plasmid DNA from transformed *E. coli* BMH-71-18 *mut*S cells was prepared using standard alkaline lysis procedures and subjected to a second round of selection by *Pvu*II. The reaction product was used to transform electrocompetent *E. coli* JM105. The transformed cells were platted on LB plates supplemented with 100 μg of carbenicillin/mL and 25 μg of streptomycin/mL. After incubation overnight at 37 °C, individual transformant colonies were picked and grown in 3 mL of 2× YT (Maniatis *et al.*, 1982). Plasmid DNA was isolated and assayed for the presence of the newly created restriction site *Not*I. *Not*I sensitive plasmid DNA was sequenced using the dideoxynucleotide method of Sanger *et al.* (1977) to verify that the desired mutation was introduced within the acetohydroxy acid isomeroreductase sequence.

Bacterial Growth Conditions. E. coli JM105 cells expressing the pKK-AHRI plasmid containing either the wild-type or a mutant form of acetohydroxy acid isomeroreductase were grown at 28 °C in 1.5 L of LB medium supplemented with 25 μg of streptomycin/mL and 100 μg of carbenicillin/mL (Maniatis *et al.*, 1982). 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. The cells were further grown for 15 h at 28 °C.

Purification of Wild-Type and Mutant Enzymes. Preparation of the soluble protein extract, which contains acetohydroxy acid isomeroreductase, was carried out according to Dumas *et al.* (1992). Acetohydroxy acid isomeroreductase, wild-type and mutant derivatives, overexpressed in *E. coli* JM105, was purified from the respective soluble protein extracts as previously described for the wild-type enzyme (Dumas *et al.*, 1992), except that the purification on HiLoad 16/60 Supedex 200 (Pharmacia) was carried out in 50 mM Tris-HCl, pH 7.5 (instead of 50 mM phosphate, pH 7.5), and after chromatography on HiLoad 16/10 Q-Sepharose (Pharmacia).

In Vitro Assays of Acetohydroxy Acid Isomeroreductase. Except where otherwise noted, acetohydroxy acid isomeroreductase activity was assayed in 50 mM Tris-HCl (pH 8.2), containing 5 mM $MgCl_2$ and 250 μM NADPH in a final volume of 1 mL. Reactions were initiated by adding 0.67 mM AHB or 2.5 mM KP. 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·mg protein), using a molar extinction coefficient of 6250 M^{-1} cm^{-1} for NADPH. The wild-type and all active mutant enzymes obeyed Michaelis−Menten kinetics with respect to substrate (either AHB or KP), NADPH, and metal ion cofactor (either Mg^{2+}, Mn^{2+} or Co^{2+}). The respective K_m and V_m values were calculated from nonlinear regression analyses of the experimental rate data to the Michaelis−Menten equation by using the Kaleidagraph program (Abelbeck Software) working on a Macintosh IIcx.

Fluorescence Studies. Fluorescence measurements were carried out at 460 nm in a SFM 25 (Kontron) fluorimeter, using a 1 mL cuvette and an excitation wavelength of 370 nm (Dumas *et al.*, 1992). The binding of magnesium to the acetohydroxy acid isomeroreductase−NADPH complex was measured through the variations of the emitted fluorescence of the enzyme−NADPH complex upon Mg^{2+} addition. Assays were carried out in a 200 μL solution containing 50 mM Tris-HCl, pH 8.2, 70 μM NADPH, and 14 μM enzyme. The enzyme concentration was given on a per monomeric unit basis.

For the wild-type enzyme and some mutants, the data were analyzed in terms of an enzyme−NADPH−magnesium equilibrium:

$$\text{E−NADPH} \overset{\underset{K_d}{Mg^{2+}}}{\rightleftharpoons} \text{E−NADPH−magnesium}$$

Under a tight-binding hypothesis the observed variations of fluorescence, F_{460}^{exp}, may thus be described by the following equation:

$$F_{460}^{exp} = F_0 + \Delta F_{460}^{max} \frac{(c_0 + l_0 + K_d) - \sqrt{(c_0 + l_0 + K_d)^2 - 4c_0 l_0}}{2c_0} \quad (1)$$

where c_0 and l_0 are the total concentrations of enzyme−NADPH complex and magnesium respectively, F_0 is the initial fluorescence, and ΔF_{460}^{max} is the maximum change of fluorescence achieved at saturation. On the other hand, when $K_d \gg c_0$ the experimental data were fitted to a simpler hyperbola equation:

$$F_{460}^{exp} = F_0 + \Delta F_{460}^{max} \frac{l_0}{K_d + l_0} \quad (2)$$

For some of the mutant enzyme forms, the experimental data were analyzed in terms of a more complex equilibrium, which involves tight binding of a magnesium to a strong

affinity binding site (dissociation constant, K_{1d}; maximal fluorescence change at 460 nm, ΔF^1_{460}), and binding of another magnesium to a lower affinity binding site on the enzyme−NADPH complex (dissociation constant, K_{2d}; maximal fluorescence change at 460 nm, ΔF^2_{460}):

$$\text{E−NADPH} \underset{K_{1d}}{\overset{Mg^{2+}}{\rightleftharpoons}} \text{magnesium−E−NADPH} \underset{K_{2d}}{\overset{Mg^{2+}}{\rightleftharpoons}}$$

$$\text{magnesium−E−NADPH−magnesium}$$

In this case:

$$F^{exp}_{460} = F_0 + \Delta F^1_{460}\frac{(c_0 + l_0 + K_{1d}) - \sqrt{(c_0 + l_0 + K_{1d})^2 - 4c_0l_0}}{2c_0} + \Delta F^2_{460}\frac{l_0}{K_{2d} + l_0} \quad (3)$$

For the determination of the dissocation constants of the complexes of enzyme (either wild-type or mutants) and NADPH with magnesium ligand, all calculations were performed by using the Kaleidagraph program (Abelbeck Software), by nonlinear regression analyses of the experimental fluorescence data to the appropriate binding equations (eq 1, 2, or 3).

RESULTS

Characterization of the Wild-Type Enzyme with Respect to Substrate Specificity and Divalent Cation Requirements. In the presence of an acetohydroxy acid substrate like AHB, the reaction catalyzed by acetohydroxy acid isomeroreductase includes two steps of substrate modification: an isomerization followed by a NADPH-dependent reduction (Figure 1). The overall reaction was thus analyzed through the ability of the enzyme to transform such a substrate to give 2,3-dihydroxy-3-methylvalerate. On the basis of previous reports (Primerano & Burns, 1983; Julliard, 1994), the NADPH-dependent reductive half-reaction was, on the other hand, examined independently of the isomerization step through the ability of the enzyme to reduce KP. The present study was carried out by using the mature form of wild-type spinach enzyme (i.e., without transit peptide), which is overexpressed in *E. coli* using the cDNA encoding this protein (see Materials and Methods). Previously, we showed that the cloned gene product exhibited the same physical properties as the enzyme from the native source, particularly concerning the subunit size (57 000 Da) as determined SDS/PAGE analysis, and the fact that the active enzyme is a homodimer (Dumas *et al.*, 1992). Also, the kinetic mechanism and kinetic parameters (K_m and V_m) for acetohydroxy acid substrate, NADPH coenzyme, and Mg^{2+} cofactor were unchanged for the cloned enzyme. These previous studies demonstrated that the two natural acetohydroxy acid substrates, AHB and AL, compete for the same site on the plant enzyme (Dumas *et al.*, 1992). By using the competition plot test described by Chevillard *et al.* (1993), we have observed that AHB and KP also compete for the same site (results not shown). Thus, the active site for isomerization and that for reduction are in close proximity in the catalytic pocket of the enzyme.

Table 2: Steady-State Kinetic Results for Wild-Type Enzyme and Mutants III-[E311D] and IV-[E488D][a]

	V_m[b]	K_m^{AHB}	K_m^{NADPH}	$K_m^{Mg^{2+}}$
wild-type	6.6	20	3.5	6
III-[E311D]	1.1	19.5	5	117
IV-[E488D]	3.2	10	4	82

[a] Kinetic parameters were calculated as indicated in Materials and Methods, by varying the concentration of either AHB, NADPH, or Mg^{2+}, while maintaining constant the other parameters. When AHB was varied (1 μM−2 mM), the concentrations of NADPH and Mg^{2+} were held constant at 0.25 and 5 mM, respectively. When NADPH was varied (1−250 μM), the concentrations of AHB and Mg^{2+} were held constant at 0.67 and 5 mM, respectively. When Mg^{2+} was varied (1 μM−1 mM), the concentrations of NADPH and AHB were held constant at 0.25 and 0.67 mM, respectively. [b] V_m values have units of μmol of NADPH/(min·mg of protein), and K_m values are in μM.

Table 3: Relative Activity of Mutant and Wild-Type Acetohydroxy Acid Isomeroreductases with Various Divalent Metal Ions at Saturating Concentrations of AHB or KP[a]

		divalent metal ion[b]		
	substrate	Mg^{2+}	Mn^{2+} [c]	Co^{2+}
wild-type	AHB	100	3.4	3.0
	KP	11	32.5	16.9
III-[E311D]	AHB	16.7	0.3	1.0
	KP	7.9	7.5	8.2
III-[D315E]	AHB	0	0	1.0
	KP	0	37.8	1.4
III-[E319D]	AHB	0	0	1.1
	KP	0	0	0.6
IV-[E488D]	AHB	48.2	2.0	0.9
	KP	4.2	14.1	6.9
IV-[E492D]	AHB	1.7	0	1.1
	KP	23.5	83.7	100

[a] All activities were expressed as percentage values of the specific activity determined with the wild-type enzyme (35 μg) in the presence of NADPH (250 μM), AHB (0.67 mM) as substrate, and Mg^{2+} (5 mM) as cofactor (100% = 6.6 μmol of NADPH oxidized/(min·mg of protein)). [b] All activities were determined with 35 μg of enzyme (wild-type or mutants), and with either 5 mM Mg^{2+}, 5 mM Mn^{2+}, or 1 mM Co^{2+}. [c] K_m values for Mn^{2+} in the reductive half-reaction (KP as substrate) with the wild-type enzyme, mutant III-[D315E], and mutant IV-[E492D] were determined to be 1.5 μM, 3 mM, and 10 μM, respectively.

In agreement with previous results (Dumas *et al.*, 1992), the wild-type enzyme exhibited an extremely high affinity for Mg^{2+} ($K_m^{Mg^{2+}}$ of 6 μM) in the overall reaction carried out in the presence of AHB as substrate (Table 2). Furthermore, Mg^{2+} was the preferred bivalent cation in the overall reaction: very low activity was found with Mn^{2+}, Co^{2+} (3% of maximal activity in both cases), or Cu^{2+} (1.5% of maximal activity) (Table 3). Also, with AHB as substrate, the enzyme was not active in the presence of either Ni^{2+}, Ca^{2+}, or Zn^{2+} (results not shown).

On the contrary, in the reductive half-reaction carried out with KP as substrate, Mn^{2+} was the most efficient bivalent cation, a finding that contrasts with the metal ion specificity observed in the overall reaction (Table 3). Thus, Mg^{2+}, Mn^{2+}, Co^{2+}, Cu^{2+}, and Ni^{2+} were all found to be effective for catalysis (Table 3). Furthermore, in the presence of Mn^{2+} the specific activity of the enzyme was 3 times higher compared to that with Mg^{2+}. As for Mg^{2+} in the overall reaction, the K_m value determined for Mn^{2+} in the reductive half-reaction was extremely low ($K_m^{Mn^{2+}}$ of 1.2 μM) (Table 3). Thus, as for the bacterial enzyme (Chunduru *et al.*, 1989),

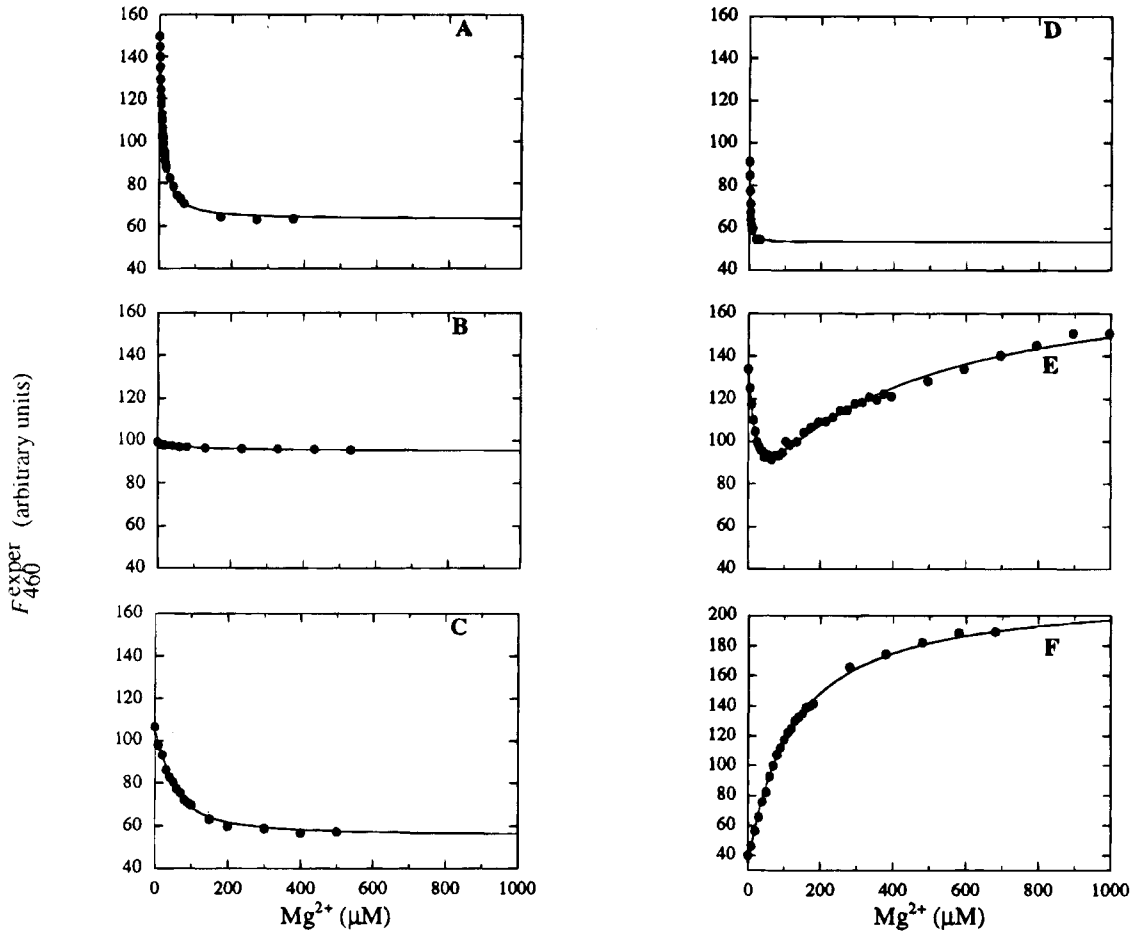


FIGURE 3: Fluorimetric characterization of the interaction between wild-type acetohydroxy acid isomeroreductase or mutant derivatives and Mg^{2+}: (A) wild-type, (B) III-[E311D], (C) III-[D315E], (D) III-[E319D], (E) IV-[E488D], and (F) IV-[E492D]. Assays were carried out in 200 μL of 50 mM Tris-HCl, pH 8.2, containing 70 μM NADPH and 14 μM enzyme. The excitation wavelength was 370 nm, and the emission wavelength was 460 nm. After stepwise addition of Mg^{2+} (0.2 μL additions of different Mg^{2+} stock solutions), the emitted fluorescence, F^{exp}_{460} was recorded. For all experiments, Mg^{2+} varied between 0 and 5 mM. Only part of the data and best fit curves are shown, up to 1 mM. For the experiments in panels A−D, the binding data were analyzed on the basis of a tight-binding hypothesis, assuming binding of a single magnesium per monomeric unit of the enzyme (eq 1, see Materials and Methods). The data corresponding to mutant IV-[E492D] (panel F) were fitted to eq 2, assuming binding of a single magnesium per monomeric unit of the enzyme to a low affinity site (see Materials and Methods). The data corresponding to mutant IV-[E488D] (panel E) were fitted to eq 3, assuming binding of two magnesium ions per monomeric unit of the enzyme (see Materials and Methods). The continuous lines were obtained by nonlinear least-squares analysis of the experimental data to eq 1 (panels A−D) or eq 2 (panel F) or eq 3 (panel E) for the following values of K_d: wild-type enzyme, 5.5 ± 0.4 μM; mutant III-[E311D], 78.5 ± 22.7 μM; mutant III-[D315E], 15.3 ± 1.2 μM; mutant III-[E319D], 1.2 ± 0.2 μM; mutant IV-[E488D], 14.1 ± 2.1 μM (from the decrease in fluorescence) and 369 ± 28.8 μM (from the increase in fluorescence); mutant IV-[E492D], 90.4 ± 5.3 μM.

Mg^{2+} is the only bivalent cation competent for both isomerization and reduction catalyzed by the plant enzyme, whereas several divalent cations (Mn^{2+}, Co^{2+}, Cu^{2+}, and Ni^{2+}) can serve as the metal cofactor in the reductive half-reaction catalyzed by this enzyme.

Binding of Mg^{2+} to the wild-type acetohydroxy acid isomeroreductase was also assessed directly by fluorescence measurements. Previously, we showed that there was a marked enhancement of NADPH fluorescence in the presence of enzyme, indicating the formation of a binary complex between NADPH and enzyme (Dumas *et al.*, 1992). When Mg^{2+} was added to this binary complex, there was a decrease in the emitted fluorescence. A saturation behavior was observed, indicating the formation of a ternary complex between enzyme, NADPH, and Mg^{2+} (Figure 3A). The value of K_d for Mg^{2+} calculated from the data in Figure 3A was 5 μM (see Materials and Methods), thereby confirming the strong affinity of the plant enzyme for Mg^{2+}. The K_m (6 μM) and K_d (5 μM) values for Mg^{2+} are very close, owing to the very slow turnover rate of the enzyme: from the data in Table 2, k_{cat} equals 6.3 s^{-1} with AHB as substrate. In agreement with the mechanism deduced from steady-state kinetic studies (Dumas *et al.*, 1992), the fluorescence titration data also indicate that Mg^{2+} can bind to the complex enzyme−NADPH prior to the addition of acetohydroxy acid substrate.

In the following, the different enzyme mutants have been characterized on the basis of substrate specificity, bivalent cation requirement, and fluorescence behavior and their properties compared to those of the wild-type enzyme.

Preliminary Characterization of Mutants. The pKK-AHRI plasmid carrying the cDNA for the mature form of spinach chloroplast acetohydroxy acid isomeroreductase was mutagenized as described under Materials and Methods so as to produce single mutations within domains II−V (Table 1; Figure 2). Under the conditions described in Materials and Methods, the wild-type spinach enzyme was expressed to a level of about 5% of total soluble proteins of *E. coli*. All

mutant proteins were also expressed in bacterial cells equally. To classify the mutants and to assess a role for the domains II−V, the mutant proteins were purified to homogeneity by the same method used for the wild-type protein, and their specific activity was determined with AHB as substrate in the presence of NADPH and Mg^{2+} as the metal ion cofactor. Mutations on the amino acid residues Asp-315, Glu-319, Glu-492, Ser-518, and Thr-520 led to a complete inactivation of enzyme activity. Under the same conditions, mutants II-[R259K], III-[E311D], and IV-[E488D], respectively, exhibited specific activities 2, 6, and 2 times lower than that of the wild-type enzyme. This decrease in enzyme activity was not due to a shift of the pH optimum of the enzyme catalyzed reaction since the pH−rate profiles obtained with all active mutants were identical with that determined for the wild-type enzyme; in all cases, the optimum in enzyme activity was at pH 8.2 (not shown). SDS/PAGE and gel filtration analyses were also carried out to determine whether there were any gross changes in the overall structures of the various mutants compared to the wild-type enzyme. In all cases, the enzyme subunit exhibited a molecular mass of the order of 57 000 Da. Furthermore, each of these mutants behaved as a dimer upon gel filtration on Superdex 200, as previously determined for the wild-type enzyme (Dumas *et al.*, 1992). Thus, partial or total loss of enzyme activity in the mutants was not due to a failure of the mutated subunits to associate within homodimers.

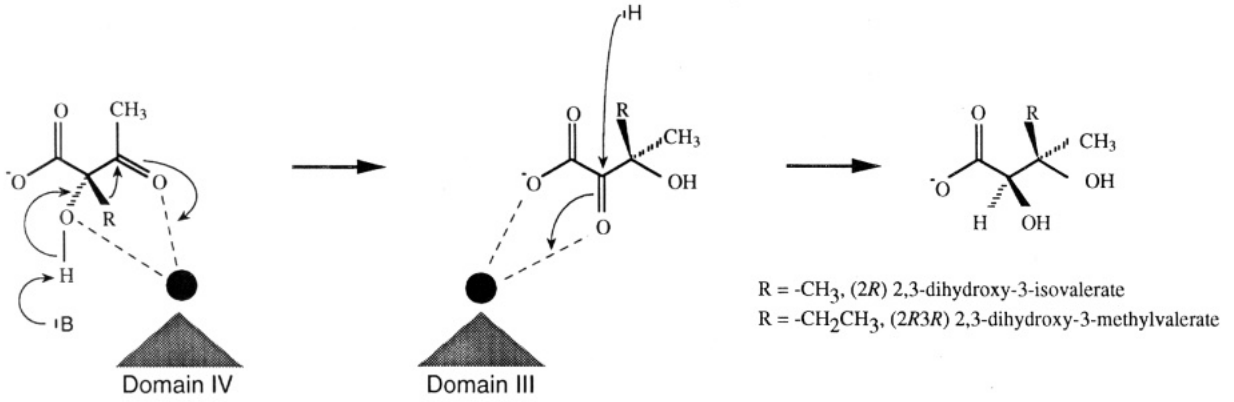
Identification of Domains III and IV as Putative Magnesium-Binding Sites by Steady-State Kinetic Analyses. Kinetic properties of mutants that were able to catalyze the overall reaction were compared first. Mutant II-[R259K] displayed an apparent K_m for Mg^{2+} (6 μM) identical with that determined for the wild-type enzyme (Table 2). By contrast, the affinity of mutants III-[E311D] and IV-[E488D] for Mg^{2+} was strongly reduced since these two mutants exhibited $K_m^{Mg^{2+}}$ values of 117 and 82 μM, respectively (Table 2). This strong diminution in Mg^{2+} affinity was not correlated with a modification in substrate or coenzyme interaction with enzyme molecules since for mutants III−[E311D] and IV−[E488D] the K_m values for NADPH and AHB were similar to those seen with the wild-type enzyme (Table 2). These results strongly suggest that, unlike domain II, both domains III and IV have a major role in metal cofactor binding.

Role of Domains III and IV. To assign a role to domains III and IV, mutants that proved unable to catalyze the overall reaction (i.e., the overall transformation of AHB) in the presence of Mg^{2+} were further examined for their ability to catalyze the second half of the overall reaction (i.e., the NADPH-dependent reduction of KP). With Mg^{2+} as cofactor and KP as substrate, mutants III-[D315E] and III-[E319D] were totally inactive (Table 3). By contrast, a mutation on domain IV (E492D), which led to a strong impediment of the overall reaction, did not reduce the rate of the reduction step (Table 3). Thus, the two half-reactions of the overall reaction catalyzed by the enzyme involve two distinct domains on the enzyme. Furthermore, it appears that Asp-315 and Glu-319 which belong to the domain III (Figure 2) participate in the reduction step, whereas Glu-492 which belongs to the domain IV (Figure 2) participates in substrate isomerization.

To further test this hypothesis, we have examined the ability of these mutants to reduce KP in the presence of either Mn^{2+} or Co^{2+}. As Table 3 shows, mutant III-[E319D] was virtually unable to reduce KP with either Mn^{2+} and Co^{2+}. Mutant III-[D315E] was also unable to reduce this substrate in the presence of Co^{2+}. Although with Mn^{2+} as cofactor this latter mutant was as active as the wild-type enzyme, its K_m for Mn^{2+} was dramatically increased (by a factor of 2500) compared to that of wild-type enzyme. Therefore, these results lend further credence to the possibility that the metal ion cofactor bound to domain III is involved in the reductive half-reaction. On the other hand, a mutation on domain IV (E492D) did not abolish the reduction of KP in the presence of Mn^{2+} and Co^{2+}, but even led to an enhancement of the NADPH-catalyzed reduction of KP (Table 3). This latter mutant had V_m values with these metal ion cofactors very similar to that determined for the wild-type enzyme in the overall reaction, i.e., in the presence of AHB and Mg^{2+}. Also, its K_m value for Mn^{2+} in the reduction of KP was not strongly modified compared to wild-type enzyme, and thus this domain IV mutant behaved differently than mutant III-[D315E] (Table 3). Since with Mg^{2+}, Mn^{2+}, and Co^{2+} mutant IV-[E492D] was unable to catalyze the overall reaction, we conclude that magnesium bound to domain IV participates in the isomerization step.

Probing Domains III and IV by Fluorescence Analyses. Binding of Mg^{2+} to the binary complex enzyme−NADPH was also investigated with the mutants of domains III and IV. For each of these, modification of fluorescence upon addition of Mg^{2+} was recorded and the K_d determined. Addition of Mg^{2+} to these mutants led to marked modifications of the fluorescence properties compared to those of the wild-type enzyme (Figure 3). These mutants displayed two distinct types of behaviors. For the domain III mutants, these modifications concerned mainly the $K_d^{Mg^{2+}}$ values: mutants III-[E311D] (Figure 3B), III-[D315E] (Figure 3C), and III-[E319D] (Figure 3D) had $K_d^{Mg^{2+}}$ values of 78 μM, 15 μM, and 1.2 μM, respectively, compared to a $K_d^{Mg^{2+}}$ value of 5 μM for the wild-type enzyme (Figure 3A). Yet, as for the wild-type enzyme, all of these mutants showed a decrease in emitted fluorescence upon addition of Mg^{2+}, thereby suggesting a similar perturbation of the NADPH environment upon metal ion binding. Furthermore, comparison of these results with the enzyme activity data in Table 3 indicated that Mg^{2+} binding to mutants III-[D315E] (Figure 3C) and III-[E319D] (Figure 3D) was unproductive since these two mutants were unable to reduce KP. In marked contrast to the domain III mutants and wild-type enzyme, addition of Mg^{2+} to domain IV mutants led to strong alterations in both $K_d^{Mg^{2+}}$ values and fluorescence behavior (Figure 3). Thus, for mutant IV-[E488D] the plot of emitted fluorescence as a function of the concentration of added Mg^{2+} exhibited two saturation curves (Figure 3E). A first decreasing curve defining a site of high affinity for the metal ion with a $K_d^{Mg^{2+}}$ value of 14 μM was followed by a second increasing curve defining a second binding site with lower affinity for the metal ion and exhibiting a $K_d^{Mg^{2+}}$ value of 369 μM. Even more striking was the behavior of mutant IV-[E492D], where addition of Mg^{2+} to the complex enzyme−NADPH led to an increase of the emitted fluorescence, which could be well fitted by a hyperbolic saturation function, giving a $K_d^{Mg^{2+}}$ value of 90 μM (Figure 3F). Thus, as judged by the sign of the fluorescence change, the NADPH

Scheme 1: Proposed Role of Acetohydroxy Acid Isomeroreductase Domains III and IV in Coordination of Magnesium Required for the Two Steps of the Reaction Catalyzed by This Enzyme (Isomerization and Reduction)[a]



R = -CH_3, (2*S*) 2-acetolactate
R = -CH_2CH_3, (2*S*) 2-aceto-2-hydroxybutyrate

R = -CH_3, (2*R*) 2,3-dihydroxy-3-isovalerate
R = -CH_2CH_3, (2*R*3*R*) 2,3-dihydroxy-3-methylvalerate

[a] The figure emphasizes that magnesium bound to domain IV is involved in the rearrangement half-reaction and that magnesium bound to domain III is involved in the reductive half-reaction.

environment in the active site was altered differently upon Mg^{2+} binding to the domain IV mutants compared to the domain III mutants and wild-type enzyme. Note however that such an altered metal ion binding to the domain IV mutants was productive since these mutants had substantial activity in reduction of the KP substrate (Table 3). Note also that mutant IV-[E488D], which exhibited a biphasic titration behavior with respect to Mg^{2+} (Figure 3E), retained substantial activity with AHB as substrate (48% of wild-type activity; Table 3), suggesting the involvement of two metal ions in the overall reaction catalyzed by this enzyme form. These experiments further demonstrate that (i) domains III and IV are involved in the binding of Mg^{2+}, (ii) Mg^{2+} binds with a strong affinity to the enzyme prior to the addition of substrate, and (iii) domains III and IV can be differentiated on the basis of the fluorescence behavior of their respective mutants.

DISCUSSION

Alignment of the predicted amino acid sequence of acetohydroxy acid isomeroreductase from plants, fungi, and bacteria has revealed five conserved regions designed domains I−V. To assess a role to these domains, the charged or polar conserved amino acids have been individually mutated by site-directed mutagenesis carried out on the spinach acetohydroxy acid isomeroreductase cDNA contained in the expression vector (pKK-AHRI). Replacement was made such that a minimum of structural disturbance occurs: glutamate was always replaced by an aspartate, lysine by an arginine, serine by a threonine, and vice versa. All these mutations led to a partial or a complete inactivation of enzyme activity, indicating that these conserved amino acids are essential for enzymatic activity, being most probably involved in the catalytic pocket of acetohydroxy acid isomeroreductase.

Steady-state kinetic analyses and fluorescence experiments on the mutants of domains III and IV clearly indicate that each of these two domains is involved in the binding of magnesium. For domain III, three conserved acidic amino acids (Glu-311, Asp-315, Glu-319) interact with magnesium, in agreement with previous suggestions of Sista and Bowman (1991). Domain IV, on the other hand, possesses two conserved acidic amino acids (Glu-488, Asp-492) where coordination of magnesium can occur. It is conceivable that other amino acids of the plant enzyme, proximal to domains III and IV, can interact with magnesium. For example, we note that amino acids Glu-309, His-484, and Glu-496 are conserved in the four eucaryotic acetohydroxy acid isomeroreductase sequences and that amino acid Asp-499 is conserved in the two plant enzymes. Also, by performing a Kyte and Doolittle (1982) analysis of the plant amino acid sequence (results not shown), we observed that domains III and IV define hydrophilic regions surrounded by hydrophobic regions, consistent with the finding that metals bind in proteins at centers of high hydrophobicity contrast (Yamashita *et al.*, 1990). Furthermore, by assessing the ability of enzyme mutants to use AHB and/or KP as substrates, we have shown that a mutation within domain IV (E492D) impeded specifically isomerization without diminution of the rate of the reduction process. Thus, this mutation transformed the enzyme from naturally behaving as an isomeroreductase to solely an NADPH-dependent dehydrogenase. On the other hand, two mutations within domain III (D315E and E319D) were associated with a strong impediment of the overall reaction, and importantly of the reductive half-reaction. Altogether, these results strongly suggest that domain III plays a role in the binding of a magnesium involved in the reduction step, whereas domain IV binds a magnesium involved in the isomerization step.

Based on these results, a working model may be proposed (Scheme 1) to account for the role of domains III and IV in the catalytic functions of acetohydroxy acid isomeroreductase. The model proposes that domains III and IV function as two different binding sites for magnesium and that the two metals bound to acetohydroxy acid isomeroreductase act as Lewis acids to promote substrate isomerization by transposition, and reaction intermediate reduction by hydride transfer (Scheme 1). Specifically, in the transposition reaction, magnesium bound to domain IV can polarize the carbonyl group at C3, thus inducing a partial positive charge on C3. A protein base (or a hydroxyl coordinated to magnesium) may also participate in the rearrangement process by removing a proton from the substrate hydroxyl group. This deprotonation would be facilitated by magne-

sium coordinated to the oxygen, thus increasing the acidity of the corresponding proton. This sequence of events is consistent with previous suggestions on the rearrangement reaction catalyzed by bacterial acetohydroxy acid isomeroreductase (Schloss, 1989; Aulabaugh & Schloss, 1990; Schloss & Aulabaugh, 1990). Similarly, coordination of the reaction intermediate carbonyl by the magnesium bound to domain III would polarize it, thus creating a partial positive charge on C2 and facilitating hydride transfer from NADPH to allow completion of the reaction (Scheme 1).

There are several possibilities to account for the present observation that the two distinct domains III and IV are involved in binding of catalytic magnesium. One possibility would be that the enzyme simultaneously binds two magnesium ions, the one to domain III, the other to domain IV, as suggested by the results obtained with mutant IV-[E488D] (Figure 3E). Yet, only the metal bound to domain IV may be involved in substrate binding and rearrangement, while the other, bound to domain III, may only participate to the final reduction step once substrate rearrangement is completed. An alternative to this is that, in the initial enzyme–NADPH–acetohydroxy acid complex, domain III is free while domain IV binds a magnesium. This metal ion would then migrate from domain IV to domain III as catalysis progresses from substrate isomerization to NADPH-dependent reduction of the reaction intermediate. A one-to-one stoichiometry between magnesium and monomeric subunit of the enzyme would be consistent with the observation that enzyme kinetics in the overall reaction do not deviate from Michaelis–Menten behavior when Mg^{2+} concentration is varied at fixed concentrations of acetohydroxy acid substrate and NADPH (Dumas *et al.*, 1992; the present study), and also with the fluorimetric titration data in Figure 3A that indicate the binding of a single magnesium to the enzyme–NADPH complex. Attempts to fit the fluorimetric data in Figure 3A–D,F with more complex equations than eqs 1 and 2 (one-site model, see Materials and Methods), such as eq 3 (two-site model, see Materials and Methods), were unsatisfactory (Mannervik, 1981; Bardsley, 1986), since mathematical convergence could only by obtained with largely undetermined values of the binding parameters. However, the present data do not preclude the possibility that the two magnesium ions bound at domains III and IV have close K_m values and that the binding of only one of these two metal ions produces the fluorescence change observed in Figure 3A, the binding of the other being silent with respect to perturbation of NADPH fluorescence. Finally, we shall consider the possibility that a magnesium is bound to each of the domains III and IV and that both metals participate in acetohydroxy acid substrate binding. As mentioned above, this would imply that both magnesium ions have similar K_m values and that binding of only one of the two metal ions perturbs NADPH fluorescence in the active site. In this case, magnesium bound to domain III may help, through coordination of C1 and C2 oxygens, to stabilize the acetohydroxy acid substrate inside the active site and as such would play a role not only in the reduction step but also in the isomerization step. Further work is needed to decipher whether or not domain III participates in the isomerization reaction supported by the natural substrate, since our activity measurements were based on a NADPH reduction assay, and therefore, it was not possible to investigate the isomerization step independently of the reduction step. Since AHB and KP compete for the same site on the enzyme, we note that domains III and IV should be in close three-dimensional proximity, although the two domains are separated by an intervening sequence of more than 140 amino acids for the acetohydroxy acid isomeroreductases from plants and *E. coli* (Figure 2). In this context, it is worth mentioning that for the acetohydroxy acid isomeroreductases from fungi and most of the bacteria only about 20 amino acids separate these two domains (Figure 2).

There is an interesting parallel between the reactions catalyzed by D-xylose isomerase and acetohydroxy acid isomeroreductase. Thus, the proposed catalytic role of magnesium bound by domain IV of acetohydroxy acid isomeroreductase (Scheme 1) is formally identical with that recently established for D-xylose isomerase (Allen *et al.*, 1994a,b; Lavie *et al.*, 1994). Furthermore, D-xylose isomerase utilizes two magnesium atoms bound at separate sites on the enzyme to catalyze the interconversion between glucose and fructose (Allen *et al.*, 1994,b; Lavie *et al.*, 1994). Another typical feature shared by the two enzymes is their very slow turnover rates. Thus, for D-xylose isomerase and glucose as substrate, $k_{cat} = 1\ s^{-1}$ (Allen *et al.*, 1994a). From the V_m value in Table 2, $k_{cat} = 6.3\ s^{-1}$ for acetohydroxy acid isomeroreductase and AHB as substrate. For the AL substrate k_{cat} is even slower, on the order of $1\ s^{-1}$, since the V_m value for this substrate (obtained at saturating concentrations of Mg^{2+} and NADPH) is about 5 times smaller than that with AHB (Dumas *et al.*, 1992). It has been pointed out that D-xylose isomerase is a very slow enzyme (Lavie *et al.*, 1994; Allen *et al.*, 1994a,b) compared to the well-studied glycolytic enzyme triosephosphate isomerase, which operates at the diffusion-controlled limit (Blaclow *et al.*, 1988). This rate difference has been attributed to differences in reaction mechanisms. Thus, for D-xylose isomerase (Allen *et al.*, 1994a,b; Lavie *et al.*, 1994), and presumably for acetohydroxy acid isomeroreductase (Chunduru *et al.*, 1989; Aulabaugh & Schloss, 1990; Scheme 1), the isomerization reaction is dependent on the chemistry of metal ion cofactors, whereas triosephosphate isomerase, which does not require metal cofactors for catalysis, utilizes general acid–base catalysis and a proton transfer mechanism involving a single base on the enzyme to promote substrate isomerization (Reider & Rose, 1959; Albery & Knowles, 1976). For the plant acetohydroxy acid isomeroreductase, it is worth noting the very low K_m values for acetohydroxy acid, NADPH, and metal ion (Table 2). Since all of them are in the micromolar range (Table 2), the specificity parameters, k_{cat}/K_m (Fersht, 1985), are of the order of $10^6\ M^{-1}\ s^{-1}$, taking into account that only the 2*S* isomer of AHB acts as substrate (Dumas *et al.*, 1992). This high value of the specificity constants may have physiological significance for orientating the flux of the metabolic pathway from pyruvate and 2-oxobutyrate precursors (i.e., the substrates of acetolactate synthase) toward the synthesis of isoleucine and valine products, respectively. Presumably, the catalytic properties of acetohydroxy acid isomeroreductase ensure that, under light conditions, the enzyme is always fully saturated by the metal ion and coenzyme, thus allowing an efficient trapping of AL and AHB (i.e., the products of acetolactate synthase), because an acetohydroxy acid substrate can only bind to the enzyme–NADPH–magnesium complex (Dumas *et al.*, 1992).

In conclusion, the present data demonstrate the participation of two distinct domains of acetohydroxy acid isomer-

oreductase in metal cofactor binding, each of them being involved in a distinct half-reaction of the overall reaction. Further investigation is required, however, to determine the exact number of magnesium ions bound to domains III and IV of the enzyme. Another question that is left unanswered concerns the strong difference in Mg^{2+} affinity between the plant and bacterial enzymes. As outlined above, this distinctive feature of the plant enzyme may have physiological significance for regulation of the branch-chain amino acid pathway within chloroplasts. Elucidation of the identity of the ligands with the metal ion(s) by crystallographic analyses of the plant enzyme will provide further insight into the role of the magnesium sites and lead to a better understanding of the mechanism of action of acetohydroxy acid isomeroreductase.

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