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Expression in *Escherichia coli* of a Putative Human Acetohydroxyacid Synthase

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A human gene has been reported that may encode the enzyme acetohydroxyacid synthase. Previously this enzyme was thought to be absent from animals although it is present in plants and many microorganisms. In plants, this enzyme is the target of a number of commercial herbicides and the use of these compounds may need to be reassessed if the human enzyme exists and proves to be susceptible to inhibition. Here we report the construction of several plasmid vectors containing the cDNA sequence for this protein, and their expression in *Escherichia coli*. High levels of expression were observed, but most of the protein proved to be insoluble. The small amounts of soluble protein contained little or no acetohydroxyacid synthase activity. Attempts to refold the insoluble protein were successful insofar as the protein became soluble. However, the refolded protein did not gain any acetohydroxyacid synthase activity. *In vivo* complementation tests of an *E. coli* mutant produced no evidence that the protein is active. Incorrect folding, or the lack of another subunit, may explain the data but we favor the interpretation that this gene does not encode an acetohydroxyacid synthase.

Keywords: Acetohydroxyacid synthase, Branched-chain amino acids, Herbicide inhibition, Heterologous expression, Protein refolding.

Introduction

Most insecticides, herbicides, antibiotics, and poisons depend for their action on their ability to interact with a specific biomolecular target. In many cases, this target is an enzyme for which the compound is a specific inhibitor.

An effective and safe herbicide should kill plants (or selected plants) while having little or no effect on animals, especially humans. While there are several ways in which this specificity can be brought about, a common strategy is to target a process that is found only in plants. Photosynthesis is the most obvious one (Kleczkowski, 1994); others are the metabolic pathways for the biosynthesis of amino acids that are not synthesized by animals and are therefore essential in their diet (Mazur and Falco, 1989). For example, N-(phosphonomethyl)-glycine (Glyphosate, the active ingredient of Zero and Roundup) interferes with the synthesis of aromatic amino acids by inhibiting enolpyruvoylshikimate 3-phosphate synthase, an enzyme that is found in plants but not in animals.

The present work concerns acetohydroxyacid synthase (AHAS; also known as acetolactate synthase, ALS), which has two principal biological roles (Fig. 1). It catalyses the first step in the synthesis of branched-chain amino acids (valine, leucine and isoleucine) and in some bacteria it also functions in the fermentation pathway that forms diacetyl and butanediol. The main interest in this enzyme stems from the fact that it is the target for several classes of herbicide (reviewed by Singh and Shaner, 1995) including Du Ponts sulfonylureas and American Cyanamids imidazolinones. These are widely used in agriculture and are generally considered safe because it is believed that animals do not synthesize branched-chain amino acids. If the target enzyme is

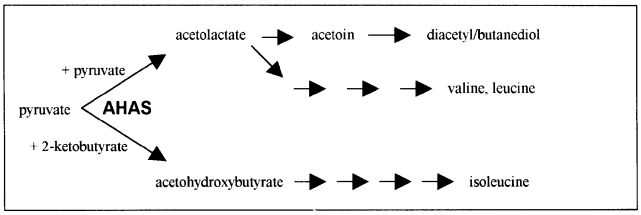

Fig. 1. Metabolic role of AHAS in the synthesis of branched-chain amino acid biosynthesis and in diacetyl/butanediol fermentation.

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not present, the herbicide should have no effect. AHAS activity and/or genes have been detected in a variety of plants, fungi and bacteria but until 1996 there was no evidence suggesting that the enzyme was present in any animal. The accidental discovery of what appears to be an AHAS gene in humans (Joutel *et al*., 1996) has important ramifications.

If this gene encodes a protein that is expressed, has AHAS activity, and is susceptible to inhibition by herbicides, then the safety of these compounds will need to be reassessed. The alleged safety is based largely on the lack of acute toxicity in rodents. Long-term studies have not been conducted in humans and, because these herbicides have only attained widespread use relatively recently (10-15 years ago), there may have been insufficient time for sub-acute effects to be seen in farm workers or the general population.

The available evidence suggests that this gene is expressed. Joutel *et al*. (1996) showed expression in several human tissues and identified 17 human expressed sequence tags in Genbank. Together these correspond to most of the mRNA that would encode the protein. Moreover, Southern blotting in a number of vertebrate species identified a similar gene.

Given that the gene is expressed, it may well be that the protein is not an AHAS, or that it is an AHAS which, like AHAS from some species (Xing and Whitman, 1987), is not inhibited by herbicides. It would be reassuring if one of these alternatives is true. However, if the gene is expressed as an active, herbicide-sensitive, AHAS then the use of these herbicides will have to be reevaluated. The purpose of this work is to express the protein and determine whether it has AHAS activity.

Material and Methods

Materials FAD, ThDP, BSA, dithiothreitol (DTT), α-naphthol, creatine hydrate, sodium pyruvate and Tris were obtained from Sigma Chemical Co. (St. Louis, USA). Ammonium sulphate, potassium chloride, magnesium chloride and potassium phosphate were purchased from Ajax (Auburn, Australia). Restriction enzymes, T4 DNA ligase and Vent DNA polymerase were purchased from New England Biolabs (Beverly, USA) or Progen Industries (Brisbane, Australia) while oligonucleotides (Table 1) were obtained from Pacific Oligos (Lismore, Australia).

The *E. coli* strain BL21(DE3) [*hsdS gal* (lc*Its*857 *ind*1 *S*am7 *nin*5 *lac*UV5-T7 gene 1)] was obtained from Novagen (Madison, USA), while strain CU1147 was kindly provided by Dr. H.E. Umbarger, Purdue University. The cloning and expression vectors were obtained as follows: pET30a(+), (Novagen); pBluescript SK(+), (Stratagene, La Jolla, USA); pProExHTa, (Gibco BRL, Gaithersburg, USA); and pGEX-4T-1, (Pharmacia, Uppsala, Sweden). The 306715 cDNA clone was purchased from the American Type Culture Collection (Rockville, USA) while the genomic clone R34018 was obtained from the Lawrence Livermore Laboratory (Livermore, USA). Dr Carol Wicking (Centre for Molecular and Cellular Biology, University of Queensland) provided a human heart cDNA library. For DNA sequencing, we used the Prism Ready Dye Deoxy Terminator Cycle Sequencing kit from Perkin Elmer Applied Biosystems (Norwalk, USA).

Bacterial growth and DNA manipulations Bacterial culture and molecular biology procedures generally followed the methods described by Sambrook *et al*. (1989). Restriction endonucleases, DNA ligase and DNA polymerase were used in accordance with the suppliers' recommendations.

***In vivo* complementation** Constructs containing human AHAS cDNA in pProExHTa were used to transform the *E. coli* strain CU1147 and individual colonies were replica-plated on to (a) LB medium; (b) minimal medium (M9 plus 0.4 mM thiamine and 0.4 mM proline); (c) minimal medium supplemented with valine (0.4 mM) and leucine (0.4 mM); and (d) minimal medium supplemented with isoleucine (0.4 mM). All plates contained 100 μg/ml ampicillin and expression was induced with 200 μl of 50 mM IPTG. As a positive control, the *Arabidopsis thaliana* AHAS cDNA clone pTrc99A-AHAS (Chang and Duggleby, 1997) was used. This positive control grows on all four plates, and the human AHAS clones grew on plate (a). Growth on the other plates indicated an AHAS activity (Fig. 1) capable of forming

Table 1. Sequences of oligonucleotide primers used for PCR

Forward primers	Sequence (5'→3')	Explanation
T3	ATTAACCCTCACTAAAGGGA	pT7T3D-pac upstream vector primer
F1	AGGTACCACCTCATGGAG	Bases -12 to 6
T23	CCTGCTCCTGGAATTCGGGATGCTGGTGGCCGCCTTG	Bases 51 to 87; introduced *Eco*RI site at 61 to 66
T47	TGCTGCACAAGGAATTCATGGCAAGCGTCCGGCATGGC	Bases 125 to 162; introduced *Eco*RI site at 136 to 141
F11	GGCAGAAGGAGCAGACCT	Bases 1280 to 1297
Reverse primers	Sequence (5'→3')	Explanation
R1A	TTACTCACCTTGTGCAGCAGCTG	Complement of bases 121 to 135 plus 8 bases of intron 2
Rstu	TCCCGATTACGATTGACG	Complement of bases 1116 to 1133
R15	GCAATGATGAGACTCCAA	Complement of bases 1958 to 1975

acetolactate (d), acetohydroxybutyrate (c), or both (b).

Protein and enzyme analyses AHAS activity was measured using the discontinuous colorimetric creatine/α-naphthol method (Singh *et al*., 1988), or by the continuous method of Schloss *et al*. (1985) under the conditions described previously (Kil and Chang, 1998; Lee *et al*., 1999). The colorimetric assay was performed in a 250 μl reaction containing 50 mM K_2HPO_4-KH_2PO_4 (pH 7.0), 50 mM Na-pyruvate, 10 mM $MgCl_2$, 1 mM ThDP, 10 μM FAD and enzyme. The reaction was incubated at 37°C for 30 min and then stopped with 25 μl of 10% H_2SO_4 and heated at 60°C for 15 min to convert acetolactate to acetoin. The acetoin formed was quantified by incubation with creatine (0.17% w/v) and α-naphthol (1.7% w/v) for 15 min at 60°C and the absorbance at A_{525} was measured ($\varepsilon_M = 22{,}700\ M^{-1}\cdot cm^{-1}$, determined using authentic acetoin). For the continuous assay, the consumption of pyruvate was monitored directly at 333 nm ($\varepsilon_M = 17.5\ M^{-1}\cdot cm^{-1}$) under the same assay conditions as those used in the colorimetric assay. SDS-PAGE was performed as described by Laemmli (1970) and protein concentration was determined using the bicinchoninic acid method (Smith *et al*., 1985).

Results

cDNA cloning Initially we attempted to isolate a full-length clone by PCR of a human heart cDNA library. Although we were able to show that the 3'-half was present (using primers F11 plus R15), PCR using the flanking primers (F1 plus R15) failed to yield the expected product. Consequently, we searched for other sources.

The gene isolated by Joutel *et al*. (1996) contains at least 15 introns. They reported that several Genbank expressed sequence tags contain sequences that correspond to exons of the gene that they identified. We noted that one of these expressed sequence tags (zb47b10.s1) is near to the 5'-end and found that the database contained another expressed sequence tag (zb47b10.r1) that is from the same clone and includes the 3'-end of the gene. Thus, it is of a sufficient size that it could contain most of the cDNA. This turned out to be the case; the corresponding IMAGE clone 306715 contained 96% of the full-length cDNA, but was missing approximately half of the 5'-end of exon 1. To obtain the rest of the sequence we used the genomic cosmid clone R34078. The strategy for constructing the full-length cDNA is illustrated in Fig. 2. The two regions were amplified by PCR using the primer pairs F1 plus R1A, or T3 plus R15. The amplified products were separately cloned into the *Sma*I site in the multiple cloning site of pBluescript SK(+), then joined at a unique *Nar*I site (GGCGCC) where they overlap to yield pBSK-AHAS.

cDNA sequencing The cDNA was fully sequenced to confirm the construct. The sequence obtained agreed exactly with that described by Joutel *et al*. (1996) except at 4 bases. At position 596, we find a G as the second base in the codon GGC that Joutel *et al*. (1996) reported as N. This allows identification of the predicted amino acid at position 199 as glycine. We also identified three silent differences at nucleotides 639 (G instead of T), 1404 (C instead of T) and 1462 (C instead of T). However, Joutel *et al*. (1996) mentioned several unspecified silent variants in different individuals that may correspond to those that we observed. Finally, a non-silent polymorphism at nucleotide 575 (C or T) was identified in our sequence as the more common form, C.

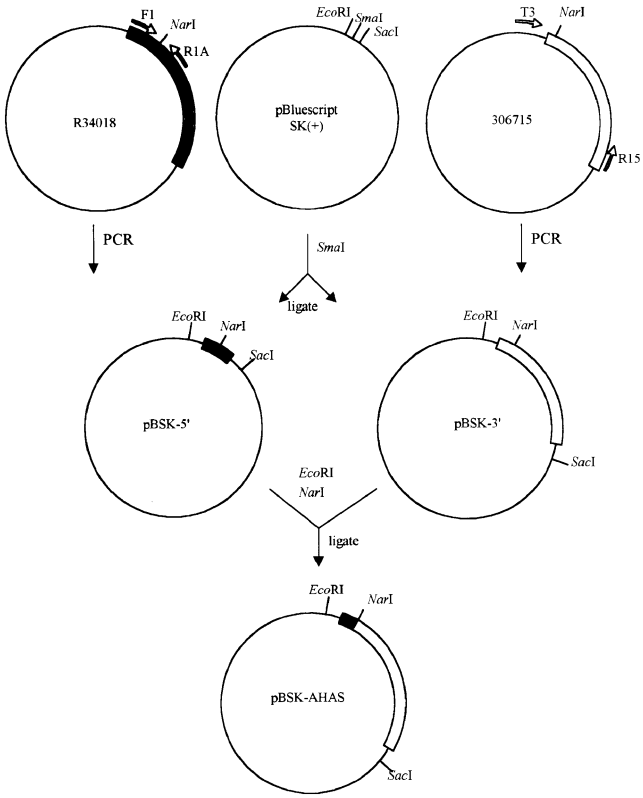

Fig. 2. Cloning strategy for the construct of a full-length cDNA clone for the putative human AHAS.

Expression in *E. coli* BL21(DE3) For expression the cDNA was excised from pBSK-AHAS and cloned into the multiple cloning site of the vector pET30a(+) using the flanking *Eco*RI and *Sac*I sites. This construct (pET-AHAS) expresses the full-length of the protein with an N-terminal fusion peptide that contains a hexahistidine tag to enable purification by immobilized-metal affinity chromatography (IMAC). Very good expression was observed with the predominant protein band observed in SDS-PAGE of cell extracts having an estimated molecular weight of 74.4 kDa (expected, 73.5 kDa). However, the soluble material from this extract contained very little of the protein. Nevertheless, purification was attempted by IMAC and, after elution with a linear gradient (0 to 0.2 M) of imidazole, a partially purified protein fraction was obtained. This had a trace of AHAS activity, but the specific activity was less than 0.05 U/mg. For comparison, the specific activity of AHAS from various species is usually in the range of 1-50 U/mg.

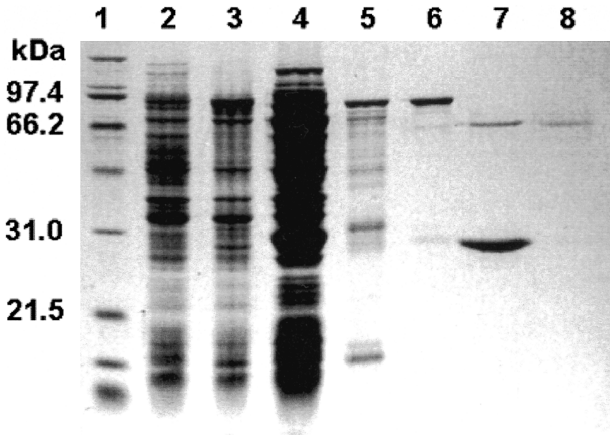

Fig. 3. Purification of the GST-AHAS fusion protein obtained after transformation of *E. coli* BL21(DE3) cells with the plasmid pGEX-AHAS. After 4 h induction of the *tac* promoter using 0.3 mM IPTG, *E. coli* BL21(DE3) harboring the plasmid pGEX-AHAS were harvested and lysed, and the GST-AHAS fusion protein was purified as described in Results. Samples were electrophoresed on 10% polyacrylamide gels containing SDS. Lane 1: molecular weight markers; lane 2: total cell lysate before GST-AHAS induction; lane 3: total cell lysate after GST-AHAS induction; lane 4: supernatant after centrifugation; lane 5: pellet after centrifugation; lane 6: purified GST-AHAS after solubilization and refolding; lane 7: GST-AHAS after treatment with thrombin; lane 8: purified AHAS.

A second construct was made to express the cloned insert as a fusion with glutathione S-transferase (Smith and Johnson, 1988). The cDNA was excised from pBSK-AHAS and cloned into the multiple cloning site of the *tac* promoter-based vector pGEX-4T-1, using the flanking *Eco*RI and *Sac*I sites. This vector incorporates a linker and thrombin cleavage site between these two domains in order to allow cleavage and the removal of the fusion partner after purification of the expressed protein using glutathione (GSH) agarose column chromatography. The resulting expression vector pGEX-AHAS was used to transform the *E. coli* strain BL21(DE3). Induction by 0.3 mM IPTG of the putative human AHAS fused with GST was demonstrated by analyzing crude bacterial extracts on SDS-PAGE as shown in Fig. 3, lane 3. A 94 kDa band corresponding to the fusion protein composed of GST (26 kDa) and the human AHAS (68 kDa) was detected. Western blot analysis of the crude extract indicated that the fusion protein does not react with antibodies against the recombinant tobacco AHAS (Chang *et al*., 1997) (data not shown). The fusion protein was insoluble (Fig. 3, lane 4) and was found in the pellet of the extract after centrifugation (Fig. 3, lane 5).

Subcloning into pProExHTa *E. coli* BL21(DE3) was used as the host for the expression of AHAS from the pET30a(+) vector because it requires the DE3 lysogen as a source of the gene for T7 RNA polymerase. The difficulty with this host is that the cells themselves contain several AHAS isoforms. Moreover, we have shown (Hill *et al*., 1997) that one of these *E. coli* isoforms, AHAS II, will bind to an IMAC column despite the absence of a hexahistidine tag. Thus, the activity that we observed could be due to *E. coli* AHAS II. The *E. coli* strain CU1147 is AHAS , but it cannot be used for expression of proteins cloned into the pET30a(+) vector because it does not possess the gene for T7 RNA polymerase. Therefore, the complete cDNA was subcloned into the multiple cloning site of a different vector (pProExHTa) using the flanking *Eco*RI and *Sal*I sites. This construct (pPEH-AHAS) expresses the full-length of the protein with an N-terminal fusion peptide that contains a hexahistidine tag under the control of a *trc* promoter.

Expression in *E. coli* CU1147 Good expression was observed, but as with expression in *E. coli* BL21(DE3), most of the protein was insoluble. A small amount of AHAS activity was observed in both the total cell extract and the soluble phase, but this was barely greater than the background in cells containing the empty vector.

Solubilization and refolding We attempted to refold the insoluble histidine-tagged protein after dissolution in the 6 M urea. The solubilized protein was bound to the IMAC column then allowed to refold over 400 minutes while washing with a decreasing linear gradient (6 to 0 M) of urea. The refolded protein was then eluted with a linear gradient (0 to 0.2 M) of imidazole. Traces of AHAS activity were observed, but these did not correlate well with the position where the refolded protein was observed.

We also attempted to refold the insoluble GST-tagged protein. The inclusion bodies were washed with water then solubilized in a 50 mM Tris-HCl buffer (pH 8.6) containing 6 M guanidinium-HCl by gently stirring at 4°C for 24 h. This solubilized protein was diluted in 50 mM Tris-HCl buffer (pH 7.5) containing 6 M urea, 1.25 mM reduced glutathione, 0.25 mM oxidized glutathione, and 100 mM NaCl, and then stirred at 4°C for 24 h. The solution was dialyzed against 50 mM Tris-HCl buffer (pH 7.5) containing 2 M urea and 100 mM NaCl, and then against 50 mM Tris-HCl buffer at pH 7.5. The refolded protein was applied to a GSH-Sepharose affinity chromatography, and GST-AHAS was purified to homogeneity as shown in Fig. 3, lane 6. Purification of the fusion protein resulted in the recovery of 2 mg GST-AHAS per liter of bacterial culture with a specific activity of less than 0.0006 U/mg. For comparison, the specific activity of the tobacco AHAS fused with GST was 0.9-2.5 U/mg (Kil and Chang, 1998).

This purified GST-AHAS was subjected to a 16 h-digestion at 4°C in the presence of thrombin. An aliquot of the reaction mixture was analyzed by SDS-PAGE; a band at 26 kDa, corresponding to GST, and a band at 68 kDa, a corresponding to AHAS, was obtained as shown in Fig. 3, lane 7. The cleaved AHAS enzyme was purified to homogeneity by GSH-

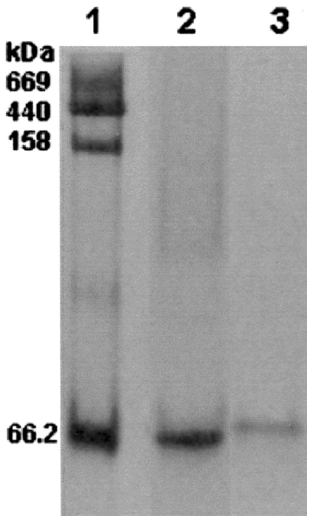

Fig. 4. Nondenaturing gel electrophoresis of the putative AHAS under 10% acrylamide concentrations. Lane 1: molecular weight markers; lane 2: putative human AHAS; lane 3: tobacco AHAS (Kil and Chang, 1998). A molecular weight of the purified putative human AHAS was calculated as 67 kDa.

Sepharose affinity chromatography as shown in Fig. 3, lane 8.

The purified AHAS enzyme was analyzed further by nondenaturing gel electrophoresis in order to determine its molecular weight (Fig. 4, lane 2). Protein motilities are calculated as the R_f value (distance of protein migration divided by distance of migration of the dye front). A semilogarithmic plot of the R_f relative to the acrylamide concentration provided a line with a slope of -7.1. Proteins of known molecular weight were electrophoresed under the same conditions and the slopes were generated to define a linear relationship with the molecular weight. The molecular weight of the purified AHAS was extrapolated from the data with the molecular weight standards. A molecular weight of the purified putative human AHAS was calculated as 67 kDa. For comparison, the purified tobacco AHAS was also run under nondenaturing gel electrophoresis to determine its molecular weight (Fig. 4, lane 3). A molecular weight of the purified putative human AHAS was calculated as 63 kDa. Previously, we have shown that the purified tobacco AHAS is fully active (Kil and Chang, 1988). These results suggest that the purified putative AHAS is successfully refolded.

N-terminal truncations AHAS from eukaryotes (plant and fungi) differs from its bacterial counterpart by being located in organelles (chloroplasts and mitochondria, respectively). Transfer to the appropriate organelle relies on a transit peptide at the N-terminus that is thought to be cleaved once the destination is reached (Mazur *et al*., 1987). The putative human AHAS has an N-terminal sequence of 40-50 residues that resembles typical mitochondrial signals, so it is likely that this protein is located in the mitochondrion. Previous studies in which active eukaryotic AHAS has been expressed in bacteria (Wiersma *et al*., 1990; Chang and Duggleby, 1997; Chang *et al*., 1997; Dumas *et al*., 1997; Pang and Duggleby, 1999) have involved constructs that have had part or all of this transit peptide removed. Consequently, we made some additional expression vectors with 23 and 47 amino acids deleted from the N-terminus.

PCR was used to amplify approximately half of the cDNA, using as forward primer either T23 or T47 together with the reverse primer, Rstu (Table 1). Each of the forward primers contains an introduced *Eco*RI site, while the reverse primer is beyond a unique *Stu*I site in pPEH-AHAS. Each of the PCR products was digested with *Eco*RI and *Stu*I and then used to replace the corresponding fragment in pPEH-AHAS that was digested with these two enzymes. Expression of these truncated forms in *E. coli* CU1147 did not result in any significant quantity of AHAS activity and the expressed protein was largely insoluble.

***In vivo* complementation** The experiments that have been described provided no convincing evidence that the protein is an AHAS, although there was some indication that it could have a small activity. In order to investigate this further, *in vivo* complementation tests were undertaken.

As described earlier, *E. coli* CU1147 is AHAS and therefore cannot grow unless it is supplied with all three branched-chain amino acids. This requirement can be overcome when the cells contain a plasmid that expresses AHAS and this allows a very sensitive test for AHAS activity (Chang and Duggleby, 1998; Hill and Duggleby, 1998). Cells expressing either the full-length protein or one of its N-terminal truncated forms were unable to grow in the absence of the branched-chain amino acids.

We considered the possibility that the human protein might be unable to form acetohydroxybutyrate (see Fig. 1), so isoleucine may need to be added for these complementation experiments. This proved not to be the case as cells were unable to grow when supplemented with this amino acid. Similarly, supplementation with valine plus leucine did not allow the cells to grow. Cells grew only when all three branched-chain amino acids were supplied, or when cells contained a plasmid that expresses a known AHAS sequence (Chang and Duggleby, 1998; Hill and Duggleby, 1998).

Discussion

AHAS is an enzyme that is required for the synthesis of branched-chain amino acids in plants and many microorganisms. Until recently, AHAS was thought to be absent from animals and this is why these amino acids are considered to be essential in the human diet. However, it is possible that dietary intake is needed to supplement the endogenous supply derived from the activity of an AHAS. This possibility was given some support by the discovery (Joutel *et al*., 1996) of a DNA sequence that is homologous to

those of known AHAS. In this context it is of interest that the clone isolated by Joutel *et al*. (1996) was from a fetal brain cDNA library. Although they showed that the corresponding mRNA could be detected in a variety of adult human tissues, this does not rule out the possibility that some fetal tissues may be fully capable of branched-chain amino acid biosynthesis and that this ability is partially lost sometime later in life. If this is true, exposure to AHAS inhibitors such as the sulfonylurea and imidazolinone herbicides might be particularly undesirable during pregnancy. Successful demonstration that this gene expresses an active and herbicide-sensitive AHAS would result in the requirement that the safety of these herbicides be reassessed and their continued use in agriculture monitored with utmost care.

In this report we describe the construction of various expression vectors for this putative human AHAS. Expression of the protein yielded a predominantly insoluble product and the small amounts of soluble protein present had little or no AHAS activity. Refolding of the insoluble protein failed to produce an active enzyme. The expressed product was also unable to complement the growth of an AHAS strain of *E. coli*.

Although these results suggest that the protein is not an AHAS, some alternatives must be considered. First, it may be that the protein is not folding correctly; the fact that most of the protein was insoluble lends some credence to this explanation. Although it was possible to refold the protein into a soluble form, this does not guarantee that it was folded into the native structure. A second possibility involves the fact that bacterial AHAS is comprised of a catalytic subunit and a smaller regulatory subunit (see review by Chipman *et al*., 1998; Duggleby and Pang, 2000). Although the catalytic subunit alone is partially active in most cases, at least one form (*E. coli* AHAS II; Hill *et al*., 1997) has an absolute requirement for its small subunit. Thus, it is possible that the human protein has similar characteristics to *E. coli* AHAS II; the cDNA expressed here would correspond to the catalytic subunit only. Although the evidence for a small subunit of eukaryotic AHAS was circumstantial until recently (Duggleby, 1997), the successful demonstration of a small subunit (Pang and Duggleby, 1999) for yeast AHAS suggests that the presence of a small subunit may be universal.

At this stage the function of the human gene identified by Joutel *et al*. (1996) is uncertain. However, it is clearly expressed as judged from their Northern blots, the existence of several Genbank expressed sequence tags, and our demonstration that the sequence is present in a human cDNA library. Moreover, a related sequence is present in the genome of several vertebrates (Joutel *et al*. 1996) and there is a homolog in the invertebrate, *Caenorhabditis elegans* (Genbank accession number AAC17553).

The putative human AHAS has an amino acid sequence homology to other enzymes such as oxalyl-CoA decarboxylase and pyruvate decarboxylase, and the latter is known to have weak AHAS-like activity. Although neither of these enzymes has been described in animal tissues, it may be that the sequence identified by Joutel *et al*. (1996) encodes a related enzyme that is not an AHAS. Further studies are needed in order to investigate this possibility.

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