

Review

Acetohydroxyacid Synthase

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Acetohydroxyacid synthase (EC 4.1.3.18) catalyses the first reaction in the pathway for synthesis of the branched-chain amino acids. The enzyme is inhibited by several commercial herbicides and has been subjected to detailed study over the last 20 to 30 years. Here we review the progress that has been made in understanding its structure, regulation, mechanism, and inhibition.

Keywords: acetohydroxyacid synthase, branched-chain amino acids, enzyme regulation, FAD, herbicide inhibition, herbicide resistance, thiamin diphosphate, subunits.

Introduction

Plants and many microorganisms are able to synthesize from inorganic precursors all of the metabolites needed for their survival. In contrast, animals must obtain many compounds, such as vitamins, essential fatty acids and certain amino acids, from their diet. This is because they lack the full biosynthetic machinery, so there are metabolic pathways and their component enzymes that are not found in animals.

These metabolic differences are the basis for the action of various selectively toxic compounds. For example, some sulfonamide compounds interfere with the synthesis of folic acid in many bacteria; since this vitamin is not made by animals and must be obtained from their diet, these sulfonamides have proved useful as antibiotics. The pathway for the synthesis of particular amino acids is another potential target of bioactive compounds and several herbicides act in this way (Mazur and Falco, 1989). One such pathway is for the synthesis of the branched-chain amino acids valine, leucine and isoleucine. Several enzymes in this pathway are inhibited by commercial and experimental herbicides (Singh and Shaner, 1995). In this review we shall focus on the first enzyme in this pathway, acetohydroxyacid synthase (AHAS; EC 4.1.3.18). First we will describe its biochemical properties

then move on to discuss the herbicides that inhibit it. For a somewhat different perspective on the biochemical properties, the reader is referred to the recent review of Chipman *et al.* (1998).

Metabolic role

AHAS has two distinct metabolic roles. In most organisms where it is found, its function is in the biosynthesis of the branched-chain amino acids. However, in certain microorganisms it has another function, in the fermentation pathway that forms butanediol and related compounds. The existence of these two enzymes has given rise to some confusing nomenclature because the AHAS that is involved in the catabolic process of butanediol fermentation differs in several respects from its anabolic counterpart in branched-chain amino acid biosynthesis. For this reason, some authors refer to the enzymes by different names. Thus the catabolic enzyme has been referred to in the older literature as the pH 6 acetolactate-forming enzyme and, more recently, as α -acetolactate synthase. Gollop *et al.* (1989) suggested that the anabolic enzyme should be known as acetohydroxyacid synthase (or acetohydroxy acid synthase), while the name acetolactate synthase (abbreviated ALS) would be reserved for the catabolic enzyme. The rationale for these suggested names is that the catabolic enzyme is capable of forming acetolactate only while the anabolic enzyme will form either of two acetohydroxyacids: acetolactate and acetohydroxybutyrate. Unfortunately, this nomenclature has not been widely adopted and many publications continue to use the name acetolactate synthase for the anabolic enzyme. Here, to distinguish the two, we shall describe them as we have done so far; that is, as the anabolic and catabolic AHAS.

Branched-chain amino acid biosynthesis Valine, leucine and isoleucine are synthesized by a common pathway in microorganisms and plants (Fig. 1). One unusual feature of this pathway is the employment of parallel steps leading to the formation of valine and isoleucine. These parallel steps involve four enzymes, namely the anabolic AHAS, ketol-acid

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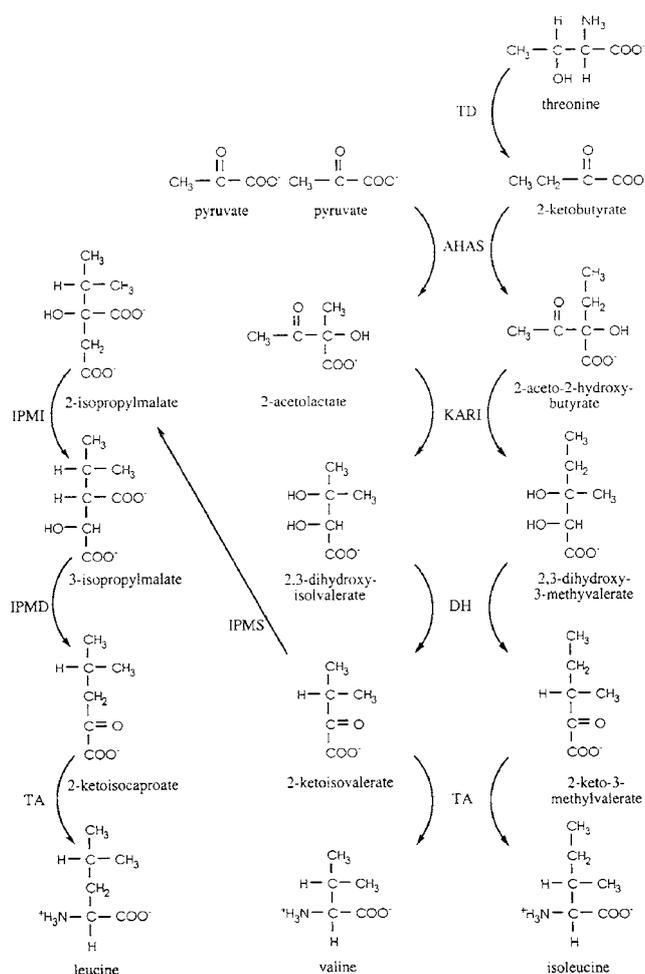


Fig. 1. Pathway for the synthesis of the branched-chain amino acids. Abbreviations used are: TD, threonine deaminase; KARI, ketol-acid reductoisomerase; DH, dihydroxyacid dehydratase; TA, transaminase; IPMS, 2-isopropylmalate synthase; IPMI, isopropylmalate isomerase; IPMD, 3-isopropylmalate dehydrogenase.

reductoisomerase, dihydroxyacid dehydratase, and a transaminase, each of which is capable of catalyzing two slightly different reactions. The common precursor for these amino acids is the central metabolite pyruvate, hence these form a subset of the pyruvate-derived amino acids. In addition, isoleucine also requires a second precursor, 2-ketobutyrate. The source of 2-ketobutyrate is by deamination of threonine, catalysed by threonine deaminase.

The anabolic AHAS catalyzes the first of the parallel steps and is at a critical branch point in the pathway because its reactions will determine the extent of carbon flow through to the branched-chain amino acids. The reactions involve the irreversible decarboxylation of pyruvate and the condensation of the acetaldehyde moiety with a second molecule of pyruvate to give 2-acetolactate, or with a molecule of 2-ketobutyrate to yield 2-aceto-2-hydroxybutyrate. Each of the products is then converted further in three reactions, catalyzed

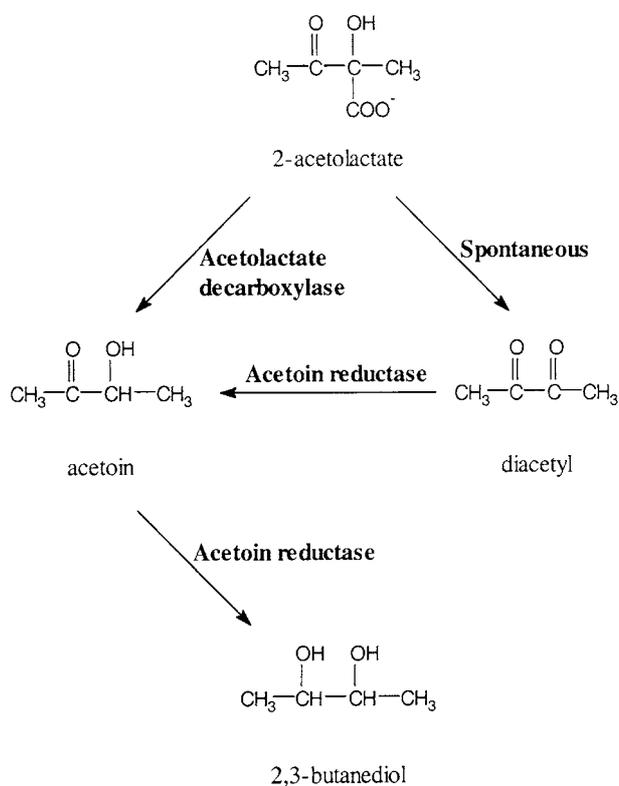


Fig. 2. Reactions of the butanediol fermentation pathway.

by ketol-acid reductoisomerase, dihydroxyacid dehydratase and a transaminase to give valine and isoleucine respectively. For leucine biosynthesis, four additional enzymes are required (see Fig. 1) using the valine precursor 2-ketoisovalerate as the starting point for synthesis.

The regulation of the biosynthesis of the branched-chain amino acids is complex and carefully controlled. This regulation is essential, not only to ensure a balanced supply of the amino acids within cells, but also because its intermediates interact with other cellular metabolic pathways. Even through microbes and plants share the common branched-chain amino acid pathway, its regulation may vary among organisms and is not fully understood. Most studies on the regulation have been conducted using *Salmonella typhimurium* and *Escherichia coli* (Umbarger, 1987; Umbarger, 1996). Regulation involves the presence of multiple isozymes, different mechanisms controlling the expression of the enzymes, allosteric effects on activity such as end-product feedback inhibition, and compartmentalization of the biosynthetic pathway in the case of eukaryotes. In later sections, the regulation of the anabolic AHAS will be discussed in more detail.

Butanediol fermentation In some bacteria, under certain fermentation conditions, pyruvate can be channeled through acetolactate into the production of 2,3-butanediol. In fact, this ability to produce butanediol has been used in microbiology laboratories for bacterial identification in the VP (Voges-Proskauer) test, which detects acetoin formation. Organisms

possessing this pathway include the enteric bacteria *Enterobacter*, some *Klebsiella* and *Serratia* species, certain lactic acid bacteria (*Lactococcus sp.* and *Leuconostoc sp.*) and *Bacillus subtilis*. Three enzymes are involved in this fermentation pathway (Fig. 2). The first step is identical to that in valine biosynthesis, but is catalyzed by the catabolic AHAS. The product, acetolactate, can either be decarboxylated by the second enzyme acetolactate decarboxylase to acetoin or undergo spontaneous conversion to diacetyl in the presence of oxygen. The last enzyme, acetoin reductase, reduces acetoin in a reversible reaction to form 2,3-butanediol. Acetoin reductase is also involved in the reduction of diacetyl to acetoin.

The butanediol fermentation pathway is activated in bacteria by low external pH (5.5-6.5), low oxygen levels, the presence an excess of acetate (Störmer, 1968a; Störmer, 1977; Johansen *et al.*, 1975; Blomqvist *et al.*, 1993; Mayer *et al.*, 1995) and/or pyruvate (Tsau *et al.*, 1992) and during the stationary phase (Renna *et al.*, 1993). It has been argued that the pathway prevents intracellular acidification by diverting metabolism from acid production to the formation of the neutral compounds acetoin and butanediol (Johansen *et al.*, 1975; Tsau *et al.*, 1992). The relative amounts of NAD⁺ and NADH within the cell may be regulated by the balance of acetoin and butanediol through the reversible reaction catalyzed by acetoin reductase. Hence, the significance of this pathway includes the maintenance of pH homeostasis, removal of excess pyruvate not used in biosynthesis, and regulating the NADH:NAD⁺ ratio within the cells. In addition, it has been shown that in *Lactococcus lactis* subsp. *lactis*, the activity of acetolactate decarboxylase is activated allosterically by leucine (Phalip *et al.*, 1994), and its gene is located downstream of, and co-transcribed with, the branched-chain amino acid gene (*leu-ilv*) cluster (Chopin, 1993). The regulation of acetolactate decarboxylase activity and this genetic linkage suggest the importance of coordination between the butanediol fermentation pathway and branched-chain amino acid biosynthesis (Monnet *et al.*, 1994; Goupil *et al.*, 1996; Goupil-Feuillerat *et al.*, 1997).

Despite the similarity of the reactions catalyzed by the catabolic and anabolic AHAS, these enzymes can be distinguished easily. The catabolic AHAS has been purified from its native source, and genes cloned and characterized. The purified catabolic enzyme is composed of a single subunit of about 60 kDa. It differs from the anabolic AHAS by having a low pH optimum of about 6.0, is stimulated by acetate, does not require FAD, is not inhibited by the branched-chain amino acids and has no regulatory subunit (Störmer, 1968a; Störmer 1968b; Holtzclaw and Chapman, 1975; Snoep *et al.*, 1992; Phalip *et al.*, 1995). This differentiation is further supported by genetic characterization. The gene that encodes the catabolic AHAS is found within the butanediol operon, no regulatory subunit gene is located downstream of the gene, and the up-regulation of the operon corresponds to the conditions that activate the butanediol pathway (Blomqvist *et*

al., 1993; Renna *et al.*, 1993; Mayer *et al.*, 1995).

Later we will discuss the regulation, FAD requirement, and subunit composition of the anabolic AHAS. However, at this point we will mention that the two types of subunit found in the anabolic AHAS will be referred to below as the catalytic and regulatory subunits. In much of the existing literature on AHAS, these are described as the large and small subunits, respectively. We prefer to name the subunits according to their function rather than their size, particularly because the most recently described regulatory subunit (Hershey *et al.*, 1999) is relatively large and there may be regulatory subunits yet to be discovered that exceed the size of their corresponding catalytic subunits.

Occurrence and genetics

Anabolic AHAS is found in bacteria, fungi, algae and plants, and hence these organisms are autotrophic for the branched-chain amino acids. The activity is contributed by one or more isozymes.

Bacteria Among the bacteria, enzymes from the enterobacteria are the most extensively studied both genetically and biochemically. At least three active AHAS isozymes have been demonstrated in *E. coli* and *S. typhimurium*, namely AHAS I, II, and III encoded within the *ilvBN* (Wek *et al.*, 1985), *ilvGMEDA* (Lawther *et al.*, 1987) and *ilvIH* (Squires *et al.*, 1983a) operons, respectively. In wild-type *E. coli* K-12 and *S. typhimurium* LT2, only two of these isozymes are expressed. The former does not have AHAS II due to a frame-shift mutation (Lawther *et al.*, 1981), and the latter is missing an active AHAS III due to a mutation that creates a premature stop codon (Ricca *et al.*, 1991) within the coding region of the catalytic subunit. Other cryptic genes have also been identified in *E. coli* (Jackson *et al.*, 1981; Robinson and Jackson, 1982; Alexander-Caudle *et al.*, 1990; Jackson *et al.*, 1993). Due to the differences in their kinetic properties, substrate specificity, sensitivity to allosteric regulators, and hence the physiological functions of the various enterobacterial AHAS isozymes, their expression is differently regulated (for reviews see Umbarger, 1987; Umbarger, 1996).

Expression of the *ilvBN* operon is regulated by two mechanisms; negative control via attenuation by the excess of valyl- and leucyl-tRNA, and positive control by cAMP and the cAMP receptor protein (Sutton and Freundlich, 1980; Friden *et al.*, 1982). Genes coding for the subunits of AHAS II are located within the gene cluster *ilvGMEDA*. As mentioned earlier, AHAS II is cryptic in *E. coli* K-12 due to a frameshift that leads to a premature stop codon in the middle of the catalytic subunit gene, *ilvG*. Expression can be restored by a frameshift mutation known as the *ilvO* mutation (Lawther *et al.*, 1981). The translational stop codon of *ilvG* overlaps the regulatory subunit gene (*ilvM*) initiation codon in the four base sequence ATGA. A similar feature is also observed in the

AHAS subunit genes (*ilvBN*) of *Lactococcus lactis* subsp. *lactis*, which have an 8 bp overlap (Godon *et al.*, 1992). Such overlaps have also been observed in genes specifying different polypeptides which are associated in multi-subunit enzyme complexes, presumably to ensure translational coupling leading to equimolar expression of the subunits (Oppenheim and Yanofsky, 1980; Das and Yanofsky, 1984). The expression of AHAS II is controlled by multivalent attenuation in which its expression is inhibited by the presence of all branched-chain amino acids (Harms *et al.*, 1985). Lastly, the production of AHAS III in *E. coli* is limited by excess leucine, mediated via the leucine-responsive regulatory protein (Wang and Calvo, 1993).

AHAS genes have been isolated from many bacteria by complementation of AHAS-deficient bacteria or the use of heterologous AHAS probes. Most, if not all, are arranged within an operon consisting of the catalytic and regulatory subunit genes, sometimes together with genes for other enzymes involved in branched-chain amino acid biosynthesis (Tarleton and Ely, 1991; Godon *et al.*, 1992; Milano *et al.*, 1992; Inui *et al.*, 1993; Keilhauer *et al.*, 1993; De Rossi *et al.*, 1995; Gusberti *et al.*, 1996; Bowen *et al.*, 1997).

Fungi A single *Saccharomyces cerevisiae* AHAS gene, designated *ilv2*, has been identified and cloned by complementation of an *ilv* yeast mutant (Polaina, 1984), and by its ability to confer low level resistance to the herbicidal inhibitor sulfometuron methyl in host cells when carried on a high copy number plasmid (Falco and Dumas, 1985). The *ilv2* gene has been mapped to the right arm of chromosome XIII (*YMR108w*) (Petersen *et al.*, 1983). Other fungal AHAS genes, which correspond to the catalytic subunit of the bacterial enzymes, have also been cloned (Jarai *et al.*, 1990; Bekkaoui *et al.*, 1993). In contrast to *E. coli*, fungi have only one AHAS isozyme, and no regulatory subunit gene has been found downstream of the cloned genes. A candidate regulatory subunit gene had been discovered in the yeast genome sequencing project and mapped to chromosome III of *S. cerevisiae* (*YCL009c*) (Oliver *et al.*, 1992). The identification was based on its considerable amino acid sequence similarity to the bacterial AHAS regulatory subunit (Bork *et al.*, 1992; Duggleby, 1997) and functional analysis studies (Cullin *et al.*, 1996). Recently its gene product, termed ILV6, has been confirmed biochemically to function as an eukaryotic AHAS regulatory subunit (Pang and Duggleby, 1999). Other open reading frames within the DNA sequence and EST databases have been suggested to function as AHAS regulatory subunits (Duggleby, 1997; Nelson *et al.*, 1997). Most fungal AHAS genes do not contain introns. Two exceptions identified to date include the *Magnaporthe grisea* catalytic subunit gene that has four introns (Sweigard *et al.*, 1997), and the *Schizosaccharomyces pombe* putative regulatory subunit gene with one intron. While the genes are nuclear-encoded, the protein is localized in the mitochondria (Cassady *et al.*, 1972; Ryan and Kohlhaw, 1974).

Expression of AHAS in yeast is controlled by two mechanisms; the GCN4-dependent general amino acid control (Xiao and Rank, 1988) and the poorly-defined specific multivalent regulation occurring at high concentration of all three branched-chain amino acids (Magee and Hereford, 1969). General amino acid control regulates the expression of unlinked genes in several amino acid biosynthetic pathways and is mediated by the binding of the GCN4 transcription activator to the cis-acting TGACTC element (Donahue *et al.*, 1983; Arndt and Fink, 1986). Upon starvation of any one of a number of different amino acids, the transcription of the biosynthetic genes is up-regulated from their basal level. Upon GCN4-mediated derepression, *ilv2* transcription and AHAS activity increase by approximately 1.6 fold (Xiao and Rank, 1988). Even through the expression of the regulatory subunit gene has not been demonstrated to be regulated by a similar mechanism, the GCN4 binding consensus sequence is also found upstream of the gene (Pang and Duggleby, 1999).

Plants The identification of AHAS as the site of action of sulfonyleurea (Chaleff and Mauvais, 1984; LaRossa and Schloss, 1984; Ray, 1984) and imidazolinone (Shaner *et al.*, 1984) herbicides greatly advanced our understanding of the enzyme and the biosynthetic pathway in which it functions in plants. The first two plant genes were isolated by Mazur *et al.* (1987) from *Arabidopsis thaliana* and *Nicotiana tabacum* using the yeast gene *ilv2* as a heterologous hybridization probe. Since then, a number of plant AHAS genes have been cloned and characterized. These include those from *Brassica napus* (Rutledge *et al.*, 1991), *Zea mays* (Fang *et al.*, 1992), *Gossypium hirsutum* (Gruha *et al.*, 1995) and *Xanthium* sp. (Bernasconi *et al.*, 1995). These organisms vary in having a single AHAS allele (*A. thaliana* and *Xanthium* sp.), two copies of AHAS (*N. tabacum* and *Z. mays*) to complex gene families (*B. napus* having five genes, and *G. hirsutum* having six). The deduced amino acid sequence of the plant genes are collinear with each other, and with the catalytic subunit of the bacterial and yeast AHAS, except for the N-terminal transit peptide sequence (see below). Possible plant AHAS regulatory subunit sequences have been identified in the EST databases (Duggleby, 1997) and recently Hershey *et al.* (1999) have cloned and expressed a probable AHAS regulatory subunit of *Nicotiana glauca*. The plant catalytic subunit genes identified to date contain no introns, and are encoded in the nuclear genome, while the expressed enzymes are transported to function in the chloroplast (Jones *et al.*, 1985; Bascomb *et al.*, 1987).

In all plant species examined, at least one AHAS gene is expressed in a constitutive manner, even though the level of expression may vary between tissues and developmental stages. The highest level of AHAS transcription and activity is found in the metabolically active meristematic tissues (Schmitt and Singh, 1990; Ouellet *et al.*, 1992; Keeler *et al.*, 1993). These constitutive genes are also known as the housekeeping AHAS genes. In the cases of *N. tabacum*, *B.*

napus and *G. hirsutum*, all being allotetraploids, the presence of multiple AHAS genes is partly the result of the combination of genomes derived from their diploid parents (Lee *et al.*, 1988; Rutledge *et al.*, 1991; Grula *et al.*, 1995). These plants have two housekeeping AHAS genes, each expressed at about similar levels. In addition to these constitutive genes, *B. napus* and *G. hirsutum* also have another AHAS gene that is expressed in a tissue-specific manner and the mRNA of these functionally distinct AHAS genes is detected only in reproductive tissues. The specific function and regulation of these genes are unknown.

Algae AHAS genes have also been cloned from algae, the more primitive representatives of the Kingdom Plantae. In contrast to those of higher plants, these genes are, in some cases, found to be located in the plastid genome (Reith and Munholland, 1993). With the availability of the complete nucleotide sequence of several algae plastid genomes, the genes for AHAS regulatory subunits have also been identified (Reith and Munholland, 1995; Ohta *et al.*, 1997). As expected, the gene products of the organelle-localized genes do not contain the transit peptide sequence (Pang and Duggleby, 1999). Because of their location and sequence homology with those of cyanobacteria, it has been proposed that the AHAS genes of the algae were acquired during the endosymbiosis of the bacteria which formed the chloroplast (Bowen *et al.*, 1997). However the localization of the AHAS genes in plastid genomes is not universal to all algae (Ohta *et al.*, 1997). In these cases it is probable that the genes have been moved to the nuclear genome.

Animals It has long been known that mammalian tissues have the ability to produce acetoin (Juni, 1952; Schreiber *et al.*, 1963). Indeed, the most commonly-used method to assay AHAS activity is based on a colorimetric method developed by Westerfeld (1945) for the determination of acetoin in blood. One of the possible mechanisms for the formation of acetoin is the breakdown of the chemically unstable acetolactate. However this putative acetolactate-forming enzyme has never been isolated. Therefore, acetoin formation may be the result of a condensation reaction between acetaldehyde and/or pyruvate, and the hydroxyethyl-enzyme intermediate formed during catalysis by the pyruvate dehydrogenase complex (Alkonyi *et al.*, 1976; Baggetto and Lehninger, 1987).

Nevertheless, a gene proposed to be the human homolog of the bacterial AHAS catalytic subunit has been cloned (Joutel *et al.*, 1996). This gene was isolated accidentally in the process of mapping for the gene responsible for the condition known as CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy). Positional cloning mapped the gene to human chromosome 19. Expression analysis showed that this gene is an ubiquitous and abundantly transcribed gene, but

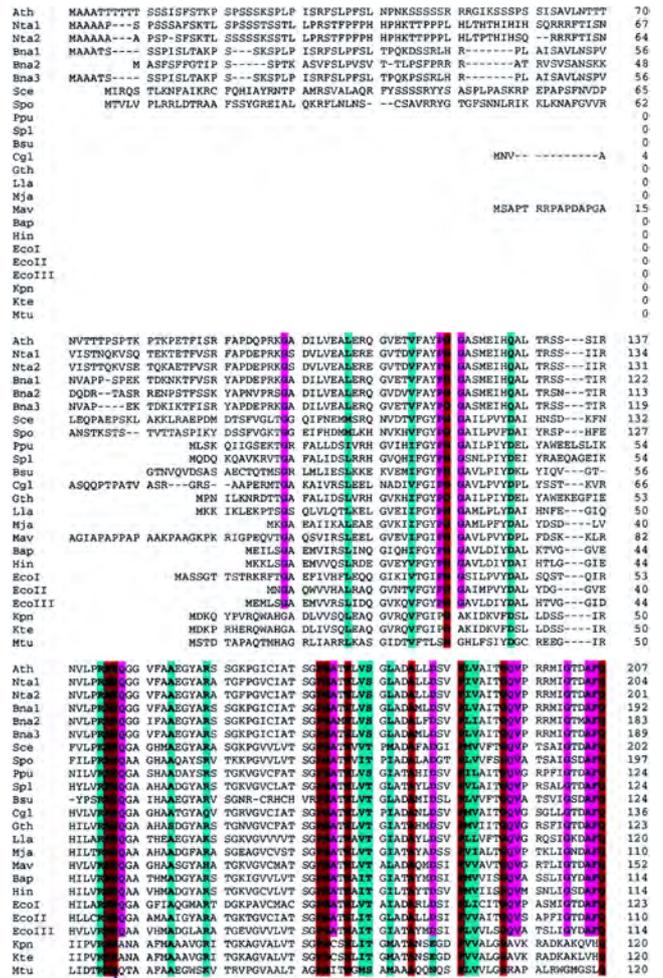


Fig. 3. Alignment of 24 AHAS catalytic subunit protein sequences from selected plant, fungal, algal and bacterial species. Sequences were obtained from GenBank and aligned using the ClustalW program (Thompson *et al.*, 1994). The abbreviations for the organisms are: Ath, *Arabidopsis thaliana*; Nta, *Nicotiana tabacum*; Bna; *Brassica napus*; Sce, *Saccharomyces cerevisiae*; Spo, *Schizosaccharomyces pombe*; Ppu, *Porphyra purpurea*; Spl, *Spirulina platensis*; Bsu, *Bacillus subtilis*; Cgl, *Corynebacterium glutamicum*; Gth, *Guillardia theta*; Lla, *Lactococcus lactis*; Mja, *Methanococcus jannaschii*; Mav, *Mycobacterium avium*; Bap, *Buchnera aphidicola*; Hin, *Haemophilus influenzae*; Eco, *Escherichia coli*; Kpn, *Klebsiella pneumoniae*; Kte, *Klebsiella terrigena*; and Mtu, *Mycobacterium tuberculosis*. Arabic (1, 2, 3) and Roman (I, II, III) numerals indicate different isozymes from one species. Residues highlighted in brown are identical across all sequences shown, while pink shows residues that are identical in the first 21 proteins, excluding Kpn, Kte and Mtu. Residues in all sequences that belong to the same strong conservation group (STA, NEQK, NHQK, NDEQ, QHRK, MILV, MILF, HY, FYW) are shown in turquoise. Other features that are described in more detail in the text are: the blue triangle which identifies the catalytic glutamate; the green bar which delineates the ThDP-binding motif; and the purple bar which corresponds to the Prosite signature PS00187.

Ath	TPVVEVRS	ITRNHYLMD	V-EIDPRIVR	EAPFLATSG	GPVLYVVR	IIQQ-QLAIP	NWQPMRLFG	275	Ath	LDIDSAEIG	NKTPHVSVOG	WVKLALQGN	KVLNRAEEL	-KLDQVWRN	ELN--VQKQ	FPLSK--TF	458	
Nca1	TPVVEVRS	ITRNHYLMD	V-EIDPRIVR	EAPFLATSG	GPVLYVVR	IIQQ-QLAIP	DWQPMRLFG	272	Nca1	LDIDSAEIG	NKTPHVSVOG	WVKLALQGN	KVLNRAEEL	-KLDQVWRN	ELN--VQKQ	FPLSK--TF	455	
Nca2	TPVVEVRS	ITRNHYLMD	V-EIDPRIVR	EAPFLATSG	GPVLYVVR	IIQQ-QLAIP	NWQPMRLFG	269	Nca2	LDIDSAEIG	NKTPHVSVOG	WVKLALQGN	KVLNRAEEL	-KLDQVWRN	ELN--VQKQ	FPLSK--TF	452	
Bna1	TPVVEVRS	ITRNHYLMD	V-EIDPRIVR	EAPFLATSG	GPVLYVVR	IIQQ-QLAIP	NWQPMRLFG	260	Bna1	LDIDSAEIG	NKTPHVSVOG	WVKLALQGN	KVLNRAEEL	-KLDQVWRN	ELN--VQKQ	FPLSK--TF	443	
Bna2	TPVVEVRT	ITRNHYLME	V-EIDPRIVR	EAPFLATSV	GPVLYVVR	IIQQ-QFAIF	NWQPMRLFL	251	Bna2	LDIDSAEIG	NKTPHVSVOG	WVKLALQGN	KVLNRAEEL	-KLDQVWRN	ELN--VQKQ	FPLSK--TF	433	
Bna3	TPVVEVRS	ITRNHYLMD	V-EIDPRIVR	EAPFLATSG	GPVLYVVR	IIQQ-QLAIP	NWQPMRLFG	257	Bna3	LDIDSAEIG	NKTPHVSVOG	WVKLALQGN	KVLNRAEEL	-KLDQVWRN	ELN--VQKQ	FPLSK--TF	440	
Scs	ADAVGVSRS	CTNNVMVKS	V-EELPLAIN	EAPFIATSG	GPVLYVVR	IIQQ-QLAIP	NWQPMRLFL	270	Scs	FVSPKNIIM	VVQVQIAGV	BATNIGDM	SKIPFVKE	---	RSEVFA	QIN--KWKKE	YFVAMHEPT	467
Spo	ADAVGVSRS	CTNNVMVKS	V-EELPLAIN	EAPFIATSG	GPVLYVVR	IIQQ-QLAIP	NWQPMRLFL	265	Spo	FVSPKNIIM	VVQVQIAGV	BATNIGDM	SKIPFVKE	---	RSEVFA	QIN--KWKKE	YFVAMHEPT	460
Kpn	ADAVGVSRS	CTNNVMVKS	V-EELPLAIN	EAPFIATSG	GPVLYVVR	IIQQ-QLAIP	NWQPMRLFL	192	Kpn	FVSPKNIIM	VVQVQIAGV	BATNIGDM	SKIPFVKE	---	RSEVFA	QIN--KWKKE	YFVAMHEPT	377
Bsu	ADAVGVSRS	CTNNVMVKS	V-EELPLAIN	EAPFIATSG	GPVLYVVR	IIQQ-QLAIP	NWQPMRLFL	190	Bsu	FVSPKNIIM	VVQVQIAGV	BATNIGDM	SKIPFVKE	---	RSEVFA	QIN--KWKKE	YFVAMHEPT	382
Cgl	ADAVGVSRS	CTNNVMVKS	V-EELPLAIN	EAPFIATSG	GPVLYVVR	IIQQ-QLAIP	NWQPMRLFL	202	Cgl	FVSPKNIIM	VVQVQIAGV	BATNIGDM	SKIPFVKE	---	RSEVFA	QIN--KWKKE	YFVAMHEPT	377
Gth	ADAVGVSRS	CTNNVMVKS	V-EELPLAIN	EAPFIATSG	GPVLYVVR	IIQQ-QLAIP	NWQPMRLFL	191	Gth	FVSPKNIIM	VVQVQIAGV	BATNIGDM	SKIPFVKE	---	RSEVFA	QIN--KWKKE	YFVAMHEPT	376
Lla	ADAVGVSRS	CTNNVMVKS	V-EELPLAIN	EAPFIATSG	GPVLYVVR	IIQQ-QLAIP	NWQPMRLFL	186	Lla	FVSPKNIIM	VVQVQIAGV	BATNIGDM	SKIPFVKE	---	RSEVFA	QIN--KWKKE	YFVAMHEPT	367
Mja	ADAVGVSRS	CTNNVMVKS	V-EELPLAIN	EAPFIATSG	GPVLYVVR	IIQQ-QLAIP	NWQPMRLFL	179	Mja	FVSPKNIIM	VVQVQIAGV	BATNIGDM	SKIPFVKE	---	RSEVFA	QIN--KWKKE	YFVAMHEPT	362
Mav	ADAVGVSRS	CTNNVMVKS	V-EELPLAIN	EAPFIATSG	GPVLYVVR	IIQQ-QLAIP	NWQPMRLFL	182	Mav	FVSPKNIIM	VVQVQIAGV	BATNIGDM	SKIPFVKE	---	RSEVFA	QIN--KWKKE	YFVAMHEPT	363
Bap	ADAVGVSRS	CTNNVMVKS	V-EELPLAIN	EAPFIATSG	GPVLYVVR	IIQQ-QLAIP	NWQPMRLFL	217	Bap	FVSPKNIIM	VVQVQIAGV	BATNIGDM	SKIPFVKE	---	RSEVFA	QIN--KWKKE	YFVAMHEPT	406
Hin	ADAVGVSRS	CTNNVMVKS	V-EELPLAIN	EAPFIATSG	GPVLYVVR	IIQQ-QLAIP	NWQPMRLFL	192	Hin	FVSPKNIIM	VVQVQIAGV	BATNIGDM	SKIPFVKE	---	RSEVFA	QIN--KWKKE	YFVAMHEPT	368
EcoI	ADAVGVSRS	CTNNVMVKS	V-EELPLAIN	EAPFIATSG	GPVLYVVR	IIQQ-QLAIP	NWQPMRLFL	181	EcoI	FVSPKNIIM	VVQVQIAGV	BATNIGDM	SKIPFVKE	---	RSEVFA	QIN--KWKKE	YFVAMHEPT	364
EcoII	ADAVGVSRS	CTNNVMVKS	V-EELPLAIN	EAPFIATSG	GPVLYVVR	IIQQ-QLAIP	NWQPMRLFL	178	EcoII	FVSPKNIIM	VVQVQIAGV	BATNIGDM	SKIPFVKE	---	RSEVFA	QIN--KWKKE	YFVAMHEPT	348
EcoIII	ADAVGVSRS	CTNNVMVKS	V-EELPLAIN	EAPFIATSG	GPVLYVVR	IIQQ-QLAIP	NWQPMRLFL	182	EcoIII	FVSPKNIIM	VVQVQIAGV	BATNIGDM	SKIPFVKE	---	RSEVFA	QIN--KWKKE	YFVAMHEPT	369
Kpn	ADAVGVSRS	CTNNVMVKS	V-EELPLAIN	EAPFIATSG	GPVLYVVR	IIQQ-QLAIP	NWQPMRLFL	186	Kpn	FVSPKNIIM	VVQVQIAGV	BATNIGDM	SKIPFVKE	---	RSEVFA	QIN--KWKKE	YFVAMHEPT	367
Kte	ADAVGVSRS	CTNNVMVKS	V-EELPLAIN	EAPFIATSG	GPVLYVVR	IIQQ-QLAIP	NWQPMRLFL	187	Kte	FVSPKNIIM	VVQVQIAGV	BATNIGDM	SKIPFVKE	---	RSEVFA	QIN--KWKKE	YFVAMHEPT	369
Mcu	ADAVGVSRS	CTNNVMVKS	V-EELPLAIN	EAPFIATSG	GPVLYVVR	IIQQ-QLAIP	NWQPMRLFL	186	Mcu	FVSPKNIIM	VVQVQIAGV	BATNIGDM	SKIPFVKE	---	RSEVFA	QIN--KWKKE	YFVAMHEPT	357

Fig. 3. Continued.

sequence analysis showed that it is not implicated in the CADASIL disorder. Because of the importance of the gene, as shown by its expression in all tissues and animals that were examined, Joutel *et al.* (1996) went on to predict the possible function of its gene product. The deduced amino acid sequence shows the highest homology (25% identity) with the bacterial AHAS catalytic subunit throughout the entire length while the next most similar sequence is that of a bacterial oxalyl-coenzyme A decarboxylase. Hence it was concluded to be a human AHAS.

This putative human AHAS gene, which is interrupted by several introns, has been cloned from a cDNA library, and examined for AHAS activity in this laboratory. The cloned gene failed to complement AHAS-deficient *E. coli*. The protein, expressed in *E. coli*, exists exclusively in the insoluble fraction and no AHAS activity can be detected (Duggleby *et al.*, 2000). These results, together with the fact that animals are not believed to be capable of synthesizing the branched-chain amino acids, weaken the suggestion that this gene encodes a human AHAS. However, the possibility that it catalyzes an AHAS-like reaction and functions in an as yet unknown pathway in animals cannot be excluded.

Fig. 3. Continued.

Amino acid sequences

Conserved residues As described above, AHAS genes have been identified and sequenced in a variety of plant, fungal, algal and bacterial species. In some, and perhaps all, species the enzyme is composed of a catalytic subunit and a smaller regulatory subunit. These regulatory subunits will be discussed in a later section. An alignment of the deduced amino acid sequences, for a selection of 24 of these catalytic subunits, is shown in Fig. 3. For any given pair, the calculated similarity score (Thompson *et al.*, 1994) ranges from 99% (Bna1 versus Bna3) to 17% (Ppu versus Mtu) with this latter pair showing 122 identities and 127 conservative substitutions. However, the overall alignment of all 24 sequences reveals only 27 identities. In part, the low number of absolutely conserved residues is due to a few sequences that differ substantially from the majority. For example, if the Kpn, Kte and Mtu proteins are excluded from the alignment, the number of identities rises from 27 to 73 (Fig. 3).

A phylogenetic analysis (Fig. 4) indicates that the Kpn, Kte and Mtu sequences form a separate group that are well separated from the remaining proteins and similar results have

Ach	QEDRIFFNML LFAAACIIPA ARVTKKADLR EATQMLDLD	--PGPYLDDV ICPHQEHLV MI PS GTFND	659
Nta1	NEAEI FPNML KFAEACVIPA ARVTRHDDL AATQMLDLD	--DGPYLLDV IVPHQEHVLI MI PS GAFKD	656
Nta2	NEAEI FPNML KFAEACVIPA ARVTRHDDL AATQMLDLD	--DGPYLLDV IVPHQEHVLI MI PS GAFKD	653
Bna1	RENEI FPNML QFAAGACIIPA ARVTKKEELR EATQMLDLD	--DGPYLLDV ICPHQEHLV MI PS GTFKD	644
Bna2	NFEAVFPNML LFAASCIIPA ARVTRREDLR EATQMLDLD	--DGPYLLDV VCPHQEHVLI MI PS GTFKD	634
Bna3	RENEI FPNML QFAAGACIIPA ARVTKKEELR EATQMLDLD	--DGPYLLDV ICPHQEHLV MI PS GTFKD	641
Scs	-----PDFI KLAEMALGK LRVKQOEELD AKLKEFVSTK G	---PVLDEV EVDKKVPVL MVAGSGLLD	663
Spo	-----PNFV KLADAMTKA LRVEKRELA KKKKEFLSTK G	---PVLDEV LVAQKEHVY FVPGKALHQ	661
Ppu	-----ADNFQ KLADAMTKA FVYNNQNM SSKADAMKY	--DGPYLLDV QVTEHENCY MVAPKSNAD	570
Sp1	-----TFEFA KLADVYIEA HNVYRQKIV RRPKALSH	--DGPYLLDV RVTRDEDCY MVAPKSNAD	573
Bsu	-----PDFV KLSEAYIKG IRISSEAEK EKLEALTSR	---EPVVIVD RVASEEKVF MVAPKGLR	565
Cgl	-----GYMFPVF TLSEGLACVA IRVTKAEVL PAVQKAREIN	--DRPVVIVD IVGEDAQV MVAPKSNAD	586
Gth	-----APNFT KVAEAFILRS LRKISRNDLK LRKIBALDY	--DGPYLLDV QVIADENCY MVAPKSNAD	569
Lla	-----PNFQ LLAEAYIKH VKLDNFKTLA DDKIITED	---EPMLDEV LISKSEHLV MI PALREND	559
Mja	-----PDFV KLAESYVKA DRISIPDEIK EKIBALIS	--NEPYLDDI VIDP-ALAL MVPPKRLTW	560
Mav	-----LAPMFPVF KLAELACVY LRCREEDNV DVINAARAIN	--DRPVVIAF IVGADAQV MVAAKTSND	602
Bap	-----PDFV KLSEYVHIG LKVKTRERLE EKILALKKL	SEGNLVFDI QIDDESHVY MQIQGGNN	562
Hin	-----PDFA KLAESYVGVG IKIATPDELE SKLQEAFSIK	--NKLVPVDI NVDESEHVY MQIRGANN	562
EcoI	--K---INFM QIAAGFLET CLANHEADPQ ASLQEIINR	---DGPALSHV RIDAEKVVY MVPPKAAFT	558
EcoII	--N---PDFL KLASAFVHG QHITRQDVE AALDFMLNS	--DGPYLLDV SIDENVWV LVPPKSNSE	542
EcoIII	-----PDFV KLAESYVGVG IQIHFHELE SKSEALEQV	RNNRVYVDF VVQDESEHVY MQIRGQDME	565
Kpn	-----MDFK VYAESFAKQ FAVESAEALE PTKRAAMDV	--DGPVAVAI PVDYRD--N LLAGQLHLSQ	557
Kte	-----VDFK VYAESFAKQ FAVESAEALE PTKRAAMDV	--DGPVAVAI PVDYRD--N LLAGQLHLSQ	557
Mtu	-----TRDY EVVRAIYGHG ELVSVFAELR PALERAFAS	--GLPAVAVV LTDP-SVAYI RRSNLA	547
Ach	VITEGDGRK Y		670
Nta1	VITEGDGRSS Y		667
Nta2	VITEGDGRSS Y		664
Bna1	VITEGDGRTK Y		655
Bna2	IIV		637
Bna3	VITEGDGRTK Y		652
Scs	FIMDFEYER QQTELRRHRT GGH		687
Spo	FILHESLS		669
Ppu	MGIAKPRGQ TASNYVSRNI		590
Sp1	MGLSS		579
Bsu	MGVKVP		571
Cgl	IQYALGLRFP FQDGEAAED PADIHEAVSD IDAAVESTEA		626
Gth	MGNINS		575
Lla	MIGLHPTDKN EEDINA		575
Mja	IVQPIRVEPK IKKPFDEIK KIRDMAVKE F		591
Mav	IQAARGIRPL FD-DETEGTP		621
Bap	MWLRK-KEVS		571
Hin	MILSKPQETT N		573
EcoI	MVGR		562
EcoII	MLEKLS		548
EcoIII	MWLSK-TERT		574
Kpn	IL		559
Kte	IL		559
Mtu	IL		547

Fig. 3. Continued.

been reported by Bowen *et al.* (1997). As mentioned earlier, AHAS has two distinct metabolic roles, in branched-chain amino acid biosynthesis and in butanediol fermentation. It appears that these anabolic and catabolic functions are performed by different forms of AHAS that may be distinguished genetically (see earlier) and by their cofactor requirements (see later). At least for the two *Klebsiella* proteins (Kpn and Kte), it is clear (Peng *et al.*, 1992; Blomqvist *et al.*, 1993) that they belong to the catabolic type. Although there are a number of sequence differences between the anabolic and catabolic types, no distinctive motif has been identified that can reliably place any given protein into one of the two types.

Glycine or proline residues constitute 15 of the 27 residues that are identical in all 24 proteins and it is probable that these are at the boundaries of important secondary structural elements. Of the remaining absolutely conserved residues, the function of none of these has been tested directly although the probable role of a few can be deduced by analogy with related enzymes. One of these residues is the catalytic glutamate that is usually contained within the subsequence RHEQ in AHAS; to facilitate residue identification and comparison with some structures to be shown later, we will number residues according to the *E. coli* AHAS II sequence wherever possible. Table 1 provides a cross-referencing of the important residues of AHAS from *A. thaliana*, yeast and *E. coli* (isozyme II). Under the *E. coli* AHAS II numbering system, the catalytic glutamate is residue 47. A second conserved element in all sequences is the cofactor-binding motif (GDGX₂₄₋₂₇LN) described by Hawkins *et al.* (1989) that spans residues 427-455. These two features are described in other sections, of this review.

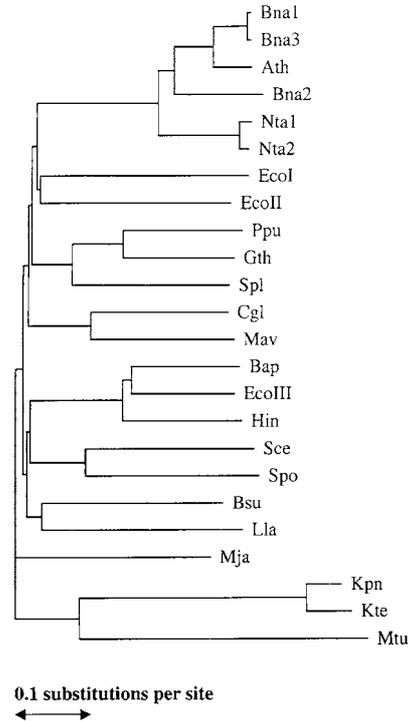


Fig. 4. Phylogeny of AHAS catalytic subunits, as calculated using the ClustalW program (Thompson *et al.*, 1994). Abbreviations are as defined in Fig. 3.

Table 1. Important residues and their role in AHAS from *A. thaliana*, yeast and *E. coli* (isozyme II).

<i>A. thaliana</i>	<i>S. cerevisiae</i>	<i>E. coli</i> II	Role
G121	G116	G25	Herbicide resistance
A122	A117	A26	Herbicide resistance
M124	L119	M28	Herbicide resistance
E144	E139	E47	Catalysis
V196	V191	V99	Herbicide resistance
P197	P192	S100	Herbicide resistance
R199	S194	P102	Herbicide resistance
A205	A200	A108	Herbicide resistance
K256	K251	K159	Herbicide resistance
G350	G353	G249	FAD binding
M351	M354	M250	Herbicide resistance
D376	D379	D275	Herbicide resistance
W491	W503	W381	FAD binding
M513	M525	M403	ThDP conformation
D538	D550	D428	Mg ²⁺ binding
N565	N557	N455	Mg ²⁺ binding
H567	E579	R457	Mg ²⁺ binding
M570	M582	M460	Herbicide resistance
V571	V583	V461	Herbicide resistance
W574	W586	W464	Herbicide resistance
F578	F590	F468	Herbicide resistance
S653	G657	P536	Herbicide resistance

Transit peptide The six plant (Ath, Nta and Bna) and two fungal (Sce and Spo) sequences are all substantially longer than the other proteins due to an N-terminal extension. It will be recalled from an earlier section that in eukaryotes, AHAS is located in plastids (plants) or mitochondria (fungi). Since nuclear genes encode the enzyme, it must be moved to these organelles after synthesis and it is probable that the N-terminal extension is involved in this intracellular trafficking. The unusual composition of these N-terminal regions, particularly the preponderance of serine residues, is typical of chloroplast and mitochondrial transit peptides (von Heinje *et al.*, 1989).

The transit peptide targets the protein to the appropriate organelle and it is usually assumed that this transit peptide is cleaved during or after translocation. It is probable that the cleavage site is close to the region where homology with prokaryotic AHAS sequences begins. The main evidence for cleavage is that the size of the mature protein in a variety of plant species appears to be approximately 65 kDa (Singh *et al.*, 1991) or less, which is significantly smaller than that expected for the plant sequences shown in Fig. 3. From this information, Rutledge *et al.* (1991) have proposed that the cleavage involves removal of the first 70, 61 and 67 residues of Bna1, Bna2 and Bna3, respectively, so that each mature protein begins with the sequence TFXS[K/R][F/Y]AP that is common to all the plant AHAS sequences shown in Fig. 3.

There is some experimental evidence from expression of various eukaryotic AHAS in bacteria to support a cleavage site in this region. For example, deletion of the first 64 residues of Bna2 results in a protein that is active when expressed in *S. typhimurium* but deletion of a further 8 residues abolishes this activity (Wiersma *et al.*, 1990). Similarly, deletion of the first 85 residues of Ath AHAS (up to but not including the TFXS[K/R][F/Y]AP sequence) gives a protein that is active when expressed in *E. coli* and deletion of a further 16 residues abolishes this activity (Chang and Duggleby, 1997). Other work has established that deletion of the first 80 residues of Ath (Dumas *et al.*, 1997), 65 residues of Nta1 (Chang *et al.*, 1997), and 54 residues of Sce (Pang and Duggleby, 1999) AHAS each results in an active protein when expressed in *E. coli*.

While a consistent picture emerges from these experiments, it should be noted that expression of a truncated protein in bacteria is a somewhat different situation from cleavage of a larger protein in mitochondria or chloroplasts. It is therefore conceivable that a truncated protein that is not active in an expression system might well be active when formed in its native milieu. A relevant observation in this context is that while the 64 residue deletion in Bna2 is active in *S. typhimurium* as mentioned above, a 56 residue deletion is not (Wiersma *et al.*, 1990). At most these experiments delineate what regions of the N-terminal extension are non-essential for AHAS activity, but they do not identify the actual site of cleavage in the appropriate organelle. Thus, the site of cleavage has not yet been established for any AHAS protein. Experimental identification of the cleavage site would require

isolation of the mature protein and N-terminal sequence determination.

It is of interest that the two eukaryotic algal sequences (Ppu and Gth) lack this transit peptide although it would be expected that AHAS would be located in the chloroplast. However, this observation is fully consistent with the finding that the gene is located on the plastid genome (Douglas and Penny, 1999); thus in these organisms the protein is synthesized within the chloroplast and no intracellular trafficking is required.

Assay and catalytic properties

Assay The validity of any measurement of the catalytic properties of any enzyme is reliant upon a suitable activity assay. In the vast majority of studies on AHAS, the enzyme activity is measured using a discontinuous colorimetric assay based on that described by Singh *et al.* (1988). In this method, samples containing the enzyme, pyruvate, and other additives are incubated for a fixed time that is usually between 30 minutes and 2 hours. The reaction is then terminated by adding sulfuric acid and heated at 60° for 15 minutes to convert acetolactate to acetoin, which is then estimated by converting it to a colored product of unknown structure ($\epsilon_M \approx 2 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ at 525 nm), by reaction with creatine and α -naphthol (Westerfeld, 1945). The great advantage of this assay is its excellent sensitivity that allows activities of 10^{-4} units of enzyme to be measured routinely. This is invaluable when working with tissue extracts due to the low abundance of AHAS in its natural sources. The major disadvantage is the discontinuous nature of the assay; if the formation of product is not linear with time, the rate measured will be an average of the changing rate over the assay period. Since it is known that both herbicide inhibition and cofactor activation are time-dependent processes, this assay is not well suited for studying the kinetics of these compounds.

A continuous assay was described by Schloss *et al.* (1985), based on the decrease in absorbance at 333 nm due to pyruvate ($\epsilon_M = 17.5 \text{ M}^{-1} \text{ cm}^{-1}$). This assay is about 1000-fold less sensitive than the discontinuous assay and is only suitable for purified enzymes that are available in large amounts. Nevertheless, it is the only assay that is reliable for studying the kinetics of herbicide inhibition and cofactor activation.

In principle, there is a third assay that would combine the advantages of a continuous assay and high sensitivity. The next enzyme in the pathway of branched-chain amino acid biosynthesis is ketol-acid reductoisomerase, which catalyses the reduction of acetolactate or acetohydroxybutyrate by NADPH ($\epsilon_M \approx 6.2 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ at 340 nm). The potential for using *E. coli* ketol-acid reductoisomerase in a coupled assay has been explored by Hill and Duggleby (1999); unfortunately, ketol-acid reductoisomerase has an intrinsic lactate dehydrogenase activity that results in NADPH oxidation by pyruvate in the absence of AHAS. Although the rate of this side-reaction is low, the high concentration of

ketol-acid reductoisomerase required for a coupled assay results in a degree of interference that renders the assay impractical. Until conditions are found that abolish this side-reaction, or a source of ketol-acid reductoisomerase is found that lacks this lactate dehydrogenase activity, the great potential of this assay cannot be realized.

Specificity and kinetic properties After the initial decarboxylation step, AHAS is capable of utilizing either pyruvate or 2-ketobutyrate as the second substrate. One of the important characteristics that distinguishes the isozymes of bacterial AHAS is the specificity for the second substrate. Measurement of this property requires the simultaneous monitoring of the rates of formation of acetolactate (V_{AL}) and of acetohydroxybutyrate (V_{AHB}) in the presence of both substrates (Gollop *et al.*, 1987; Delfourne *et al.*, 1994). The preference of the enzyme for either pyruvate or 2-ketobutyrate in the second phase is defined by the specificity constant, R (Barak *et al.*, 1987).

$$R = (V_{AHB}/V_{AL})/([2\text{-ketobutyrate}]/[\text{pyruvate}])$$

A wide range of substrate concentrations, pH, or the presence of inhibitors (valine or herbicides) do not affect this constant, which is an intrinsic property of the enzyme. Enzymes with a high R value (>10) have a greater specificity for 2-ketobutyrate, while a value of less than 1 favors acetolactate synthesis.

Among the three enterobacterial enzymes, only AHAS I has a relatively low R factor of 2, which means that it has an almost equal preference for the two substrates. AHAS II and III each have high R values of 65 and 40, respectively. Determination of this characteristic in a variety of organisms has revealed the presence of at least one AHAS activity with high specificity for acetohydroxybutyrate formation (Gollop *et al.*, 1990; Delfourne *et al.*, 1994). This is consistent with the fact that the intracellular concentration of the major metabolic intermediate pyruvate is higher than that of 2-ketobutyrate. For example, *S. typhimurium* LT2 (which lacks AHAS III) grown on glucose contains more than 80 times as much pyruvate as 2-ketobutyrate (Epelbaum *et al.*, 1998). Thus, having an AHAS with a high R will allow production of comparable amounts of the precursors for both valine/leucine and isoleucine biosynthesis. The presence of the low R value AHAS I in enterobacteria was suggested to allow special adaptation of the organism for growth on certain poor carbon sources, which results in a drop in intracellular pyruvate concentrations (Dailey and Cronan, 1986). The importance of multiple isozymes in enterobacteria is further supported by quantitative studies on the branched-chain amino acid biosynthesis. The analysis showed that none of the AHAS isozymes could be adequate for the varied conditions that the bacteria encounter (Epelbaum *et al.*, 1998). Besides AHAS I, the catabolic AHAS has extremely low R values (<0.1) (Gollop *et al.*, 1990). This is not surprising as the catabolic

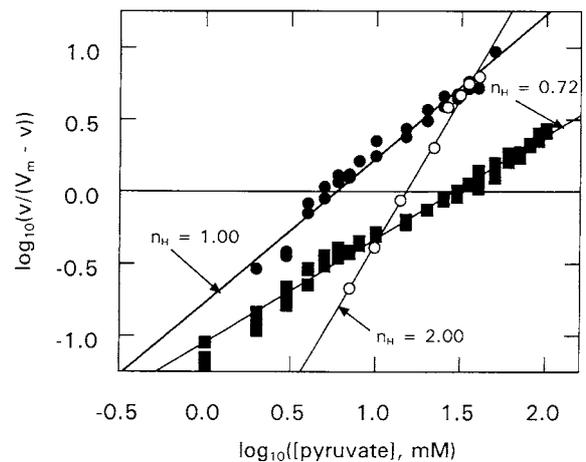


Fig. 5. Substrate saturation kinetics of AHAS from various of species. Data are shown in the form of a Hill plot and illustrate: Michaelis-Menten kinetics (*E. coli* AHAS II, $n_H = 1.00$; taken from Lee and Duggleby, 2000); negative cooperativity (*A. thaliana* AHAS n, $n_H = 0.72$; taken from Lee *et al.*, 1999); positive cooperativity (*S. marcescens* AHAS \circ , $n_H = 2.00$; taken from Yang and Kim, 1993).

AHAS catalyzes exclusively the condensation of pyruvate to acetolactate, the precursor for the butanediol fermentation pathway.

As mentioned above, it is of physiological importance for most AHAS to select 2-ketobutyrate over pyruvate as the second substrate. In order to favor a larger substrate over a smaller one, it requires adaptation of the catalytic site to accommodate the extra methyl group of 2-ketobutyrate (Gollop *et al.*, 1989). Using a structural model of *E. coli* AHAS (discussed below) and site-directed mutagenesis, W464 was shown to play a part in the second substrate recognition and specificity (Ibdah *et al.*, 1996). Replacement of W464 lowers the specificity constant of AHAS II by at least an order of magnitude without any major effect on most other properties of the enzyme.

The most detailed study of the substrate kinetics is that described by Gollop *et al.* (1989) who examined the effect of simultaneous variation of the concentrations of pyruvate and 2-ketobutyrate using *E. coli* AHAS III. However, most authors have confined themselves to studying the effect of pyruvate alone. In the absence of 2-ketobutyrate, the AHAS reaction requires two moles of pyruvate and it might be expected that the substrate saturation curve would be sigmoidal. However, as will be seen in the catalytic cycle described later, CO_2 release intervenes between the binding of the first and second pyruvate. Unless a very high CO_2 concentration is present, this step will be irreversible and the substrate saturation curve would be expected to be hyperbolic. This prediction agrees with the findings of most studies (e.g. *E. coli* AHAS II, Fig. 5) and K_m values in the range 1 to 20 mM are usually reported (Table 2).

There have been occasional reports of negative

Table 2. Values of K_m for pyruvate for AHAS from various species.

Enzyme	K_m (mM)	Reference
Bacteria		
<i>E. coli</i> I	1.5	Eoyang and Silverman, 1988
<i>E. coli</i> II	5.0	Ibdah <i>et al.</i> , 1996
<i>E. coli</i> II	2.6	Hill <i>et al.</i> , 1997
<i>E. coli</i> III	12.0	Barak <i>et al.</i> , 1988
<i>Lactococcus lactis</i>	8.3	Benson <i>et al.</i> , 1996
<i>Rhizobium leguminosarum</i>	7.2	Royeula <i>et al.</i> , 1998
<i>S. typhimurium</i> II	10.6	Schloss <i>et al.</i> , 1995
Fungi		
<i>N. crassa</i>	3.2	Glatzer <i>et al.</i> , 1972
Yeast	8.2	Magee and de Robichon-Szulmajster, 1968a
Yeast (recombinant)	18.1	Pang and Duggleby, 1999
Plants		
<i>A. thaliana</i>	11.2	Wu <i>et al.</i> , 1994
<i>A. thaliana</i>	6.3	Mourad <i>et al.</i> , 1995
<i>A. thaliana</i> (recombinant)	2.3	Singh <i>et al.</i> , 1991
<i>A. thaliana</i> (recombinant)	3.0	Dumas <i>et al.</i> , 1997
Barley	5.5	Durner and Böger, 1988
Cocklebur	3.2-6.6	Bernasconi <i>et al.</i> , 1995
Cotton	2.5-6.0	Subramanian <i>et al.</i> , 1990
<i>Datura innoxia</i>	0.9	Rathinasabapathi and King, 1991
Maize	5.0	Singh <i>et al.</i> , 1988
<i>Lactuca serriola</i>	7.1	Eberlein <i>et al.</i> , 1997
Soybean	63-74	Nemat Alla and Hassan, 1996
<i>Sonchus oleraceus</i>	16	Boutsalis and Powles, 1995
Tobacco	6.5-9.6	Subramanian <i>et al.</i> , 1990
Tobacco (recombinant)	16.8	Kim and Chang, 1995
Tobacco (recombinant)	12.1	Chang <i>et al.</i> , 1997
Tobacco (recombinant)	20.8	Chong <i>et al.</i> , 1999

cooperativity in the pyruvate saturation curve. Phalip *et al.* (1995) reported a Hill coefficient of 0.84 for the catabolic AHAS from *Leuconostoc mesenteroides* and Chang and Duggleby (1997) observed a somewhat lower value (0.6) for recombinant *A. thaliana* AHAS. Subsequent work on this *A. thaliana* enzyme (Chang and Duggleby, 1998; Lee *et al.*, 1999) and recombinant tobacco AHAS (Kil and Chang, 1998) have confirmed that the enzyme does not follow Michaelis-Menten kinetics (Fig. 5). For these plant enzymes, the negative cooperativity was ascribed to interactions between the active sites of the dimer and it has been pointed out by Chang and Duggleby (1997) that earlier published data on both native (Mourad *et al.*, 1995) and recombinant (Kim and Chang, 1995) *A. thaliana* AHAS show deviations from Michaelis-Menten kinetics.

Given that subunit interactions can occur, it would not be surprising to observe positive cooperativity also, and several papers have reported such kinetics for various forms of AHAS. These include the enzyme from the barley (Mifflin, 1971), *N. crassa* (Kuwana *et al.*, 1968), the bacterial anabolic AHAS from *L. lactis* (Snoep *et al.*, 1992), *Serratia marcescens* (Yang and Kim, 1993; see Fig. 5), and *M. aeolicus* (Xing and Whitman, 1994) and the catabolic enzyme from *A. aerogenes* (Störmer, 1968a). It should be noted that the *L. lactis* enzyme was later reported (Benson *et al.*, 1996) to show hyperbolic kinetics but this difference may be due to different assay conditions. In this context, it has been shown that the positive cooperativity of *M. aeolicus* AHAS increases markedly on addition of a low (5 mM) concentration of Na_2SO_4 while that of the *A. aerogenes* varies with the buffer.

Cofactor requirements The reaction catalyzed by AHAS involves an initial decarboxylation of pyruvate. The acetaldehyde that is formed remains bound and condenses with the second substrate (pyruvate or 2-ketobutyrate) to form the acetohydroxyacid product. In common with related enzymes that catalyze the decarboxylation of pyruvate and other 2-ketoacids, the enzyme requires thiamin diphosphate (ThDP, formerly known as thiamin pyrophosphate and abbreviated TPP) and a divalent metal ion as obligatory cofactors (Halpern and Umbarger, 1959). An unexpected observation, given that the reaction involves no oxidation or reduction, is that AHAS also requires FAD (Störmer and Umbarger, 1964). It was later shown that some forms of AHAS have no FAD requirement (Störmer, 1968b; Peng *et al.*, 1992) and we now recognize that the latter form corresponds to the catabolic enzyme described earlier. The role of each of the cofactors is described in the following three sections.

ThDP

It is believed that ThDP is required by AHAS from all species although this has not been tested in many cases. More often than not, ThDP is included in assays for the enzyme at concentrations of 50 μM or more without regard for whether a requirement has actually been demonstrated. However, in those cases where care has been taken to remove any ThDP that might be bound to the enzyme, the activity can be reduced greatly or abolished entirely, and is fully restored upon adding back ThDP. There is a hyperbolic dependence of activity upon ThDP concentration; from such experiments an activation constant or K_m can be estimated (Table 3) and values ranging from less than 1 μM to greater than 200 μM have been reported. In general, the eukaryotic enzymes have a higher K_m than AHAS from bacteria. However, Ortéga *et al.* (1996) have shown that the activation of *E. coli* AHAS II by ThDP is a slow process taking tens of minutes to reach equilibrium, while for barley AHAS some hours are required. Since most studies of ThDP activation have used the discontinuous assay (see above), the reliability of the K_m

Table 3. Values of K_m for cofactors for AHAS from various species.

Enzyme	K_m for cofactor (μM)			Reference
	ThDP	Mg^{2+}	FAD	
<i>E. coli</i> I	-	-	0.06	Weinstock <i>et al.</i> , 1992
<i>E. coli</i> II	0.6	-	0.09	Ibdah <i>et al.</i> , 1996
<i>E. coli</i> II	1.1	3.8	0.2	Hill and Duggleby, 1998
<i>S. typhimurium</i> II	1.5	22	0.8	Schloss <i>et al.</i> , 1985
<i>E. coli</i> III	18	3300	2.2	Vyazmensky <i>et al.</i> , 1996
<i>S. cerevisiae</i> (recombinant)	110	280	0.3	Poulsen and Stougaard, 1989
<i>P. sativum</i>	11 - 21	-	-	Shimizu <i>et al.</i> , 1994
<i>G. hirsutum</i>	32 - 49	-	-	Subramanian <i>et al.</i> , 1990
<i>N. tabacum</i>	20 - 42	-	-	Subramanian <i>et al.</i> , 1990
<i>N. tabacum</i> (recombinant)	80 - 210	-	0.5 - 2.6	Kil and Chang, 1998
<i>A. thaliana</i> (recombinant)	25	64	1.5	Chang and Duggleby, 1998

values may be suspect. Only the values for isozyme II of *E. coli* and *S. typhimurium*, and *A. thaliana* AHAS, were determined using the continuous assay.

Even for organisms where AHAS has been identified only as a DNA sequence, without any biochemical work on the protein, it can be assumed with reasonable confidence that the enzyme requires ThDP based on the presence of an amino acid sequence first identified by Hawkins *et al.* (1989). This motif usually begins with the triplet GDG and ends with NN, separated by 24-27 other amino acids which contain features that are conserved to a lesser extent. For example, the six residues preceding the NN are usually predominantly apolar residues. It is now known that there is a large family of ThDP-dependent enzymes and, without exception, all contain this motif (Candy and Duggleby, 1998) which is clearly seen in the alignment shown as Fig. 3. It should be noted that the Prosite database also includes a signature sequence for ThDP-dependent enzymes (see Fig. 3) that overlaps with, but is not identical to, the motif of Hawkins *et al.* (1989).

It came as a surprise, when the first three-dimensional structure for a ThDP-dependent enzyme was published (Lindqvist *et al.*, 1992), that this motif does not interact with the thiamin region of the coenzyme. Rather, it is involved in binding to a metal ion that itself is coordinated to the phosphate groups of the coenzyme. This is discussed further in the next section.

The reactions catalyzed by almost all (see Khaleeli *et al.* (1999) for an exception) ThDP-dependent enzymes can be written as involving cleavage of a carbon-carbon bond that is adjacent to a keto group. One of the products is released while the other may be released, or remain bound to the enzyme where it undergoes further conversion. The AHAS reaction is therefore typical; the bond broken is that linking the keto and carboxyl carbons of pyruvate and the product released is carbon dioxide (or bicarbonate). The second product is not released as acetaldehyde but is condensed with the 2-ketoacid substrate.

The conventional view of the catalytic cycle is shown in

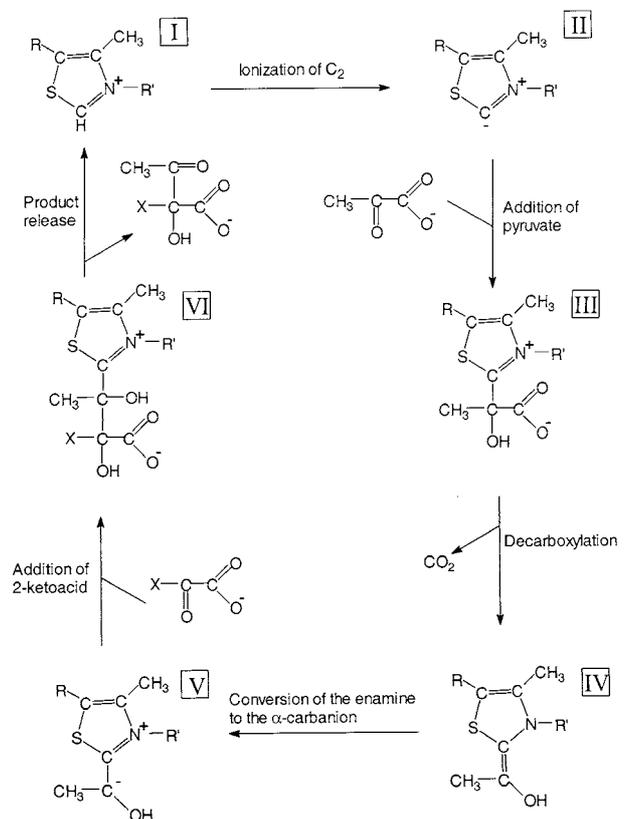


Fig. 6. Proposed catalytic cycle of AHAS. Intermediates I to VI are discussed in the text. R represents the hydroxyethyl diphosphate group while R' is the methyl aminopyrimidine ring of ThDP.

Fig. 6. Initially, ThDP is in its protonated form (I) that then ionizes to the reactive ylide (II); this then attacks a pyruvate molecule giving the lactyl-ThDP intermediate (III). After decarboxylation, the enamine of hydroxyethyl-ThDP (IV) undergoes charge separation giving the α -carbanion (V) that can now react with the 2-ketoacid substrate to give the product complex (VI). Finally, the product is released and ThDP is

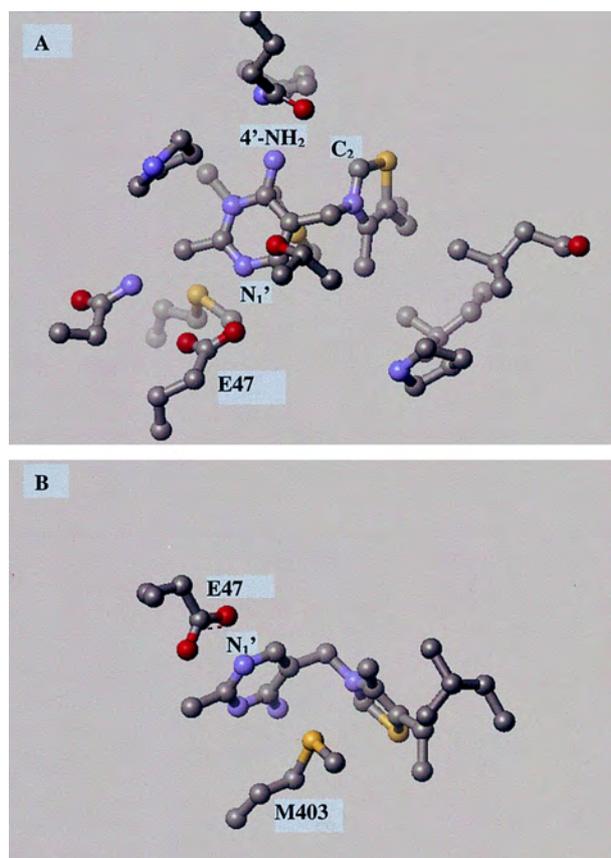


Fig. 7. Structure of *E. coli* AHAS II around the ThDP cofactor. **Panel A** shows the proximity of E47 of the enzyme, and C₂ of the thiazole ring, to N₁' and the 4'-NH₂ group of the pyrimidine ring, respectively. The side view in **Panel B** again shows E47 as well as the location of M403 in maintaining ThDP in the V-conformation. Coordinates of *E. coli* AHAS II were calculated by Ibdah *et al.* (1996).

regenerated.

The ultimate source of this hypothetical catalytic cycle stems from the work of Breslow (1958) and derives from studies of non-enzymatic, thiamin-catalyzed reactions. A key step in this process is the initial ionization of the thiazole C₂ proton. However, it has been shown (Washabaugh and Jencks, 1988) that the pK_a for this step is very high (17-19) so very little of the ylide (**II**, Fig. 6) would be present at neutral pH. However, it is possible that enzyme-bound ThDP might have a much lower pK_a, consistent with the observation that enzyme-catalyzed reactions can be more than 10¹²-fold faster than their non-enzymatic counterparts (Alvarez *et al.*, 1991).

The conformation, structure and location of ThDP provide a possible mechanism for lowering the pK_a. In all ThDP-dependent enzymes whose structure have been determined (Muller *et al.*, 1993; Hasson *et al.*, 1998; Dobritzsch *et al.*, 1998; Chabrière *et al.*, 1999; Ævarsson *et al.*, 1999), the coenzyme is in the V-conformation which brings the 4'-amino group into reasonably close proximity to C₂. Although the structure of no AHAS has been determined, homology models

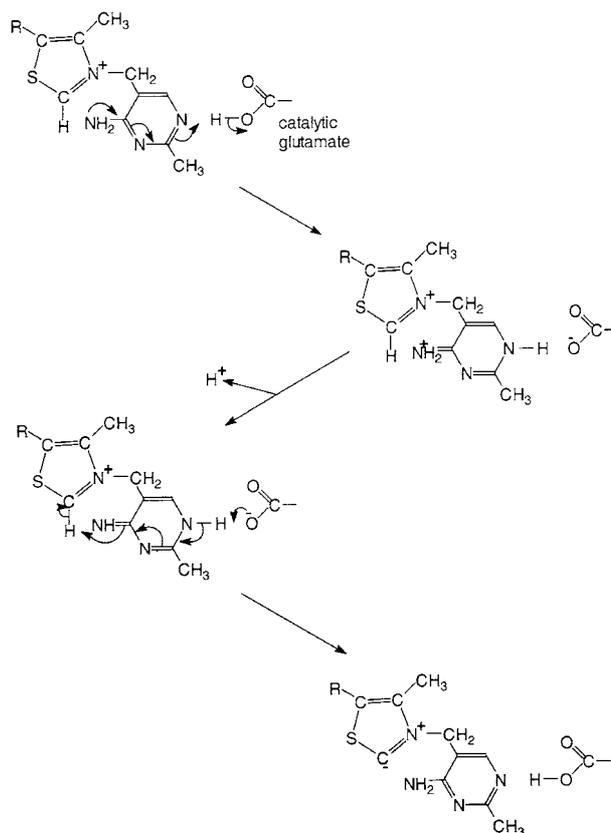


Fig. 8. Proposed role of the catalytic glutamate (E47 in *E. coli* AHAS II) in promoting the ionisation of C₂ of ThDP in AHAS and in other ThDP-dependent enzymes. R is as described in Fig. 6.

of *A. thaliana* AHAS (Ott *et al.*, 1996) and *E. coli* AHAS II (Ibdah *et al.*, 1996) have been constructed based on the structure (Muller and Schulz, 1993; Muller *et al.*, 1994) of pyruvate oxidase (POX). Here we will use the coordinates of the *E. coli* AHAS II model to illustrate the key features of the structure (Fig. 7). The V-conformation is best seen when ThDP is viewed from the side (Fig. 7B) where it is clear that M403 lies between the thiazole and pyrimidine rings and is responsible for maintaining the coenzyme in this conformation.

It is evident from Fig. 7 that N₁' is very close to the carboxyl group of a glutamate residue (E47 in *E. coli* AHAS II) which corresponds to a glutamate that is conserved in all ThDP-dependent enzymes. Mutagenesis of the residue greatly decreases the activity of these enzymes (Wikner *et al.*, 1994; Candy *et al.*, 1996; Killenberg-Jabs *et al.*, 1997; Fang *et al.*, 1998). Further, it is known that substitution of ThDP with analogs lacking N₁' or the 4'-amino group results in a loss of enzymatic activity (Golbik *et al.*, 1991). Putting these observations together, we may propose the scheme shown in Fig. 8. Initially, the 4'-amino group acts as a base resulting in the formation of a protonated imine with the glutamate carboxyl donating a proton to N₁'. After ionization of this imine, the glutamate carboxylate acts as a base and the proton

is removed from C_2 .

Some authors have attempted to measure the ionization state of C_2 . Although no measurements have been on AHAS, experiments performed on pyruvate decarboxylase are relevant because the mechanism is expected to be similar up to the point of formation of the α -carbanion (V, Fig. 6). Kern *et al.* (1997) incorporated ^{13}C -labelled ThDP into pyruvate decarboxylase and observed the ^{13}C -NMR spectrum at pH 6.0. They suggest that this spectrum is no different from that of ThDP in solution and concluded that ThDP is fully protonated. However, it is not clear whether these experiments could have detected a small fraction of the ionized form. For example, if 10% of the ionized form is present, this implies a $\text{p}K_a$ of 7, which is 10-12 pH units lower than that of free ThDP. This is a massive change that would have profound implications for the mechanism of ThDP-dependent enzymes. More recently, Jordan *et al.* (1999) have attempted to measure the $\text{p}K_a$ directly and have argued that it is shifted by about 9 pH units. However, it should be noted that the value obtained is for the protonation of an analog of the α -carbanion (V, Fig. 6) rather than for ThDP itself. Thus the $\text{p}K_a$ and ionization state of enzyme-bound ThDP remains unresolved.

One possibility that has not been widely considered is that the reaction between ThDP and pyruvate may involve a concerted mechanism, as illustrated in Fig. 9. This scheme would avoid a discrete ylide and allow the lactyl-ThDP intermediate (III, Fig. 6) to be formed directly. The base involved could be an amino acid side chain although the ionized imine (Fig. 8) would serve equally well and would be perfectly positioned to perform this function. This concerted mechanism involves a different route to the lactyl-ThDP intermediate from that which is accepted for non-enzymatic thiamin-catalyzed reactions. However, it should be remembered that the latter are extremely slow and it may be that ThDP-dependent enzymes owe much of their catalytic power to their ability to by-pass formation of the ylide.

Metal ions

In common with all other ThDP-dependent enzymes, AHAS requires a metal ion for activity. There is no great specificity in this requirement and any of Mn^{2+} , Mg^{2+} , Ca^{2+} , Cd^{2+} , Co^{2+} , Zn^{2+} , Cu^{2+} , Al^{3+} , Ba^{2+} and Ni^{2+} are active (Tse and Schloss, 1993) giving between 133% (Mn^{2+}) and 50% (Ni^{2+}) of the activity with Mg^{2+} . The metal ion requirement is usually satisfied with

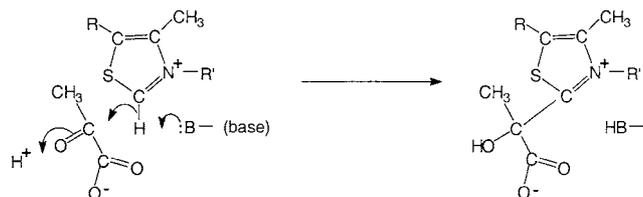


Fig. 9. Proposed concerted mechanism for ThDP-dependent enzymes that bypasses the ionization of C_2 of ThDP. R and R' are as described in Fig. 6.

Mg^{2+} , generally at a concentration of 0.1 to 10 mM. However, it is not always easy to demonstrate that the metal ion is absolutely required due to the difficulty of removing all metal ions from both the enzyme and the assay solutions. In those cases where care has been taken to remove all sources of metal ions, the activity can be reduced to a small fraction of the initial value, but is completely restored upon adding back Mg^{2+} (Schloss *et al.*, 1985; Poulsen and Stougaard, 1989; Vyazmensky *et al.*, 1996; Chang and Duggleby, 1998; Hill and Duggleby, 1998). There is a hyperbolic dependence of activity upon Mg^{2+} concentration; from such experiments an activation constant can be determined. There are very large variations in the values reported (Table 3) that range from 3.8 μM (*E. coli* isozyme II; Hill and Duggleby, 1998) through 280 μM (yeast; Poulsen and Stougaard, 1989) to 3.3 mM (*E. coli* isozyme III; Vyazmensky *et al.*, 1996). It is not clear whether these variations represent true differences between AHAS from various species or result from subtle differences in the assay conditions.

As mentioned earlier, the structure of no AHAS has been determined at the atomic level, but those of six other ThDP-dependent enzymes have been solved (Muller *et al.*, 1993; Hasson *et al.*, 1998; Dobritzsch *et al.*, 1998; Chabrière *et al.*, 1999; Åvarsson *et al.*, 1999). In all cases, the role of the metal ion is the same; it acts as an anchor, holding the ThDP in place by coordinating to two of the phosphate oxygen atoms and two amino acid side-chains. These two amino acids constitute part of the ThDP-motif (Hawkins *et al.*, 1989 and Fig. 3) mentioned earlier; the aspartate in the GDG sequence and the second of the two asparagines.

As described in the previous section concerning ThDP, a model of *E. coli* AHAS II has been constructed by Ibdah *et al.* (1996). Part of this model surrounding the metal ion is

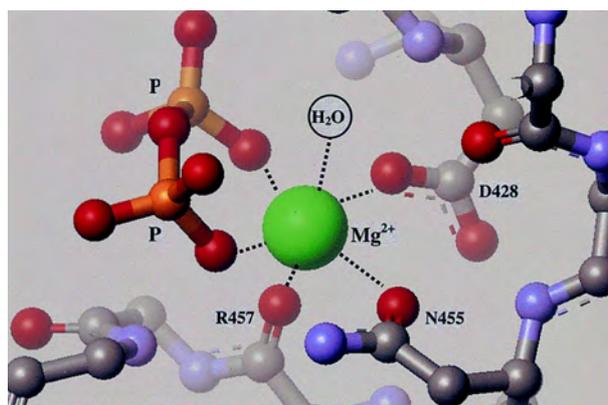


Fig. 10. Structure of *E. coli* AHAS II around the Mg^{2+} cofactor. The six coordinating ligands are one oxygen from each of the phosphate groups of ThDP, the backbone carbonyl oxygen of R457, the amide oxygen of N455, one of the carboxyl oxygens of D428, and a water molecule. D428 and N455 are contained within the ThDP-binding motif (Fig. 3). Coordinates of *E. coli* AHAS II were calculated by Ibdah *et al.* (1996).

illustrated in Fig. 10. The two ligands that form part of the ThDP motif are the side-chains of D428 and N455 (*E. coli* AHAS II numbering). The remaining four ligands are the two phosphate oxygen atoms, the backbone oxygen of R457, and a water molecule. From the structure of POX it is not possible to say with certainty whether the asparagine is ligated through the oxygen or the nitrogen of the side-chain amide. However, we think that binding through the oxygen is more likely based on the fact that some sequences contain an aspartate rather than an asparagine at this position (Fig. 3). This conclusion is supported by the finding that mutagenesis of this asparagine to aspartate in the related enzyme pyruvate decarboxylase (Candy and Duggleby, 1994) results in no change in its ability to bind Mg^{2+} .

The close relatives of AHAS for which the three-dimensional structure is known each has the active site formed at the dimer interface, with contributions from different domains of each subunit. However, it should be noted that the more distantly-related pyruvate:ferredoxin oxidoreductase (Chabrière *et al.*, 1999) shows a somewhat different organization with the active site between two domains of the same polypeptide chain. It therefore appears likely that the minimal active unit of AHAS would be the dimer, although a similar arrangement to that seen in pyruvate:ferredoxin oxidoreductase cannot be ruled out.

FAD

The reaction catalyzed by AHAS does not involve any oxidation or reduction so there is no obvious reason why the enzyme should require FAD and indeed, the activity of the catabolic AHAS is independent of this cofactor (Störmer, 1968b). Other enzymes that contain flavins with no redox role are rare but not unknown. One of these is glyoxylate carboligase (Chang *et al.*, 1993) which catalyzes a reaction that parallels that of AHAS. Rather than using two molecules of pyruvate, two molecules of glyoxylate are converted to tartronic semialdehyde and CO_2 . This similarity, together with the extensive amino acid sequence homology to AHAS, suggest that glyoxylate carboligase and AHAS share a common evolutionary ancestor and that glyoxylate carboligase may be considered to be an AHAS with unusual substrate specificity. Other examples of non-redox flavin-dependent enzymes include chorismate synthase (Macheroux *et al.*, 1999) and hydroxynitrile lyase (Wajant and Effenberger, 1996) but in these cases, neither resembles AHAS in structure nor in the reaction catalyzed. In an interesting parallel to AHAS, there are two forms of hydroxynitrile lyase only one of which requires FAD (Wajant and Effenberger, 1996).

The role of FAD in the anabolic AHAS has been speculated upon but there is, as yet, no clear-cut answer. Initially it was suggested that there might be a cyclic oxidation and reduction during the catalytic reaction (Störmer and Umbarger, 1964) but this possibility is now considered unlikely. The main evidence against a cyclic oxidation and reduction stems from the work of Schloss *et al.* (1988) who reported little effect on

the enzyme activity when FAD is replaced with $FADH_2$, or analogs that have different redox potentials from FAD ($E_0' = -209$ mV) such as 5-deaza-FAD ($E_0' = -311$ mV) and 8-chloro-FAD ($E_0' = -146$ mV). We do not regard this evidence as particularly strong, particularly because Schloss and Aulabaugh (1988) have proposed a role for FAD that appears to be inconsistent with it being replaceable by $FADH_2$ (see later).

There are two main schools of thought on the function of FAD, both reliant on the observation that AHAS is structurally related to POX (Chang and Cronan, 1988). In addition to a convincing amino acid sequence homology, the first stage of the reactions catalyzed by the two enzymes each involves the decarboxylation of pyruvate, mediated by bound ThDP. Thereafter the two reactions diverge and in POX the hydroxyethyl intermediate is oxidized to acetate using an acceptor such as ubiquinone. FAD is an essential cofactor for POX and serves to transfer electrons to ubiquinone. Given these similarities it is possible that AHAS has evolved from a POX-like ancestor and this leads to two hypotheses of why the anabolic AHAS has retained its requirement for FAD.

The first suggested function is that FAD is required for purely structural reasons; that is, unless FAD is present the active site cannot attain the correct geometry for substrate binding and/or catalysis to occur. The second hypothesis (Schloss and Aulabaugh, 1988) is that the FAD plays a protective role in the catalytic cycle. Under this proposal, it is necessary to prevent protonation of the α -carbanion (V, Fig. 6) during the step where the 2-ketoacid substrate binds, when the active site would be open to solvent. This is achieved by allowing the enamine (IV, Fig. 6) to form a reversible adduct with FAD, as illustrated in Fig. 11. The difficulty with this hypothesis is that a similar adduct could not be formed by $FADH_2$ and yet replacing FAD with the reduced form of this cofactor actually leads to a 10% increase in AHAS activity. Schloss and Aulabaugh (1988) have argued that this increased activity is due to elimination of the non-productive adduct

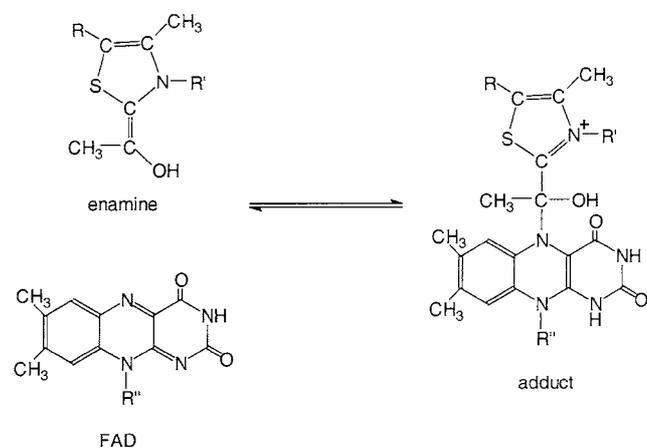


Fig. 11. Structure of the enamine-FAD adduct of AHAS proposed by Schloss and Aulabaugh (1988).

formation but this seems inconsistent with the need to protect the α -carbanion from protonation. Perhaps there are subtleties in the argument that we have not understood. Moreover, it provides no explanation of how the catabolic AHAS is able to function without FAD. Thus, a definitive statement on the role of FAD is not yet possible.

Two studies have reported mutants of AHAS that have lost the ability to bind FAD. These have involved changing G249 of *E. coli* AHAS II to alanine, valine or glutamate (Hill and Duggleby, 1998) or a tryptophan (equivalent to W381 of *E. coli* AHAS II) of tobacco AHAS to phenylalanine (Chong *et al.*, 1999). In each case, the loss of FAD binding is accompanied by a total abolition of enzymatic activity and, for the *E. coli* enzyme, it was shown by circular dichroism spectroscopy that the mutants have unaltered folding.

Apart from FADH₂ and the FAD analogs mentioned above, little work has been done on the specificity with respect to this cofactor. However, Schloss *et al.* (1985) have shown that fragments of FAD such as FMN, adenosine, AMP, ADP-ribose or pyrophosphate, either alone or in combination, are not able to activate *S. typhimurium* AHAS II. In contrast, the enzyme from the archaebacterium *Methanococcus aeolicus* is 75% more active with FMN than with FAD, while riboflavin plus phosphate results in an activity that is more than twice that observed with FAD (Xing and Whitman, 1994). It will be of interest to investigate whether the *M. aeolicus* enzyme is unique in this respect or whether the AHAS from other organisms exhibits a similar ability to use these alternatives.

In most studies, the FAD requirement is satisfied by adding this cofactor at a concentration of 2 to 100 μ M, although it is not uncommon for it to be omitted entirely. Presumably, this is not because the enzyme does not require FAD; rather it is bound sufficiently tightly that it is not lost from the enzyme during extraction and purification. Indeed, removal of FAD may require special action such as treatment with activated charcoal (e.g. Hill and Duggleby, 1998). Once this is done, the enzyme shows little or no activity but can be made fully active by adding back FAD. The hyperbolic reactivation curve yields an activation constant and the values that have been determined for various forms of AHAS are listed in Table 3. In all cases these are in the high nM to low μ M range and these high affinities are consistent with the fact that the enzyme is frequently found to be active in the absence of added FAD.

Feedback regulation AHAS is the key control point within the branched-chain amino acid biosynthetic pathway. Because of the critical role to ensure a balanced supply of the amino acids, AHAS expression and enzymatic activity within cells are tightly controlled by various mechanisms. One of the mechanisms involves the control of the enzyme at the transcriptional level, which has been dealt with in earlier. The other mechanism, as in many biosynthetic pathways, regulates AHAS activity by end-product feedback inhibition. The activities of all AHAS, except AHAS II of *E. coli* and *S.*

typhimurium, are inhibited by at least one of the branched-chain amino acids. The inhibition is described as non-competitive in relation to pyruvate (Magee and de Robichon-Szulmajster, 1968b; Glatzer *et al.*, 1972; Takenaka and Kuwana, 1972; Proteau and Silver, 1991). In all the cases examined in bacteria (Arfin and Koziell, 1973; Barak *et al.*, 1988; Proteau and Silver, 1991; Yang and Kim, 1993), fungi (Magee and de Robichon-Szulmajster, 1968b; Glatzer *et al.*, 1972; Takenaka and Kuwana, 1972; Pang and Duggleby, 1999), and algae (Oda *et al.*, 1982; Landstein *et al.*, 1993), valine is clearly the most potent inhibitor amongst the branched-chain amino acids. The reported apparent K_i for valine ranges from 4.4 μ M to 1.4 mM. In contrast, AHAS from higher plants is regulated slightly differently from the enzymes in lower organisms. In plants, leucine is an equally good, and sometimes better, inhibitor than valine (Mifflin, 1971; Durner and Böger, 1988; Singh *et al.*, 1988; Southan and Copeland, 1996). In addition, the inhibitory effect of the branched-chain amino acids used in combination is greater than the additive effect of them added singly (Mifflin, 1971; Durner and Böger, 1988). Such synergism has not been reported for AHAS of bacteria, yeast or algae.

It is well known that eukaryotic AHAS is very labile. Attempts to purify the enzyme from its native source results in rapid loss of activity and this is also often accompanied by the desensitization of the enzyme to inhibition by the branched-chain amino acids. Comparing the plant and fungal AHAS, the latter is more unstable. In fungi and some algae, the biosynthetic enzyme is localized in mitochondria and it was observed in *S. cerevisiae* (Magee and de Robichon-Szulmajster, 1968b), *Neurospora crassa* (Glatzer *et al.*, 1972; Takenaka and Kuwana, 1972) and *Euglena gracilis* (Oda *et al.*, 1982) that the AHAS activity is almost totally desensitized to valine inhibition when it is released from the mitochondria. Neither the substrate nor the cofactors can act as effective protectants. As a result, all early studies involving the feedback regulation were conducted with solvent-permeabilized cells or isolated intact mitochondria. In addition to this necessity for the enzyme to remain in the organelle, the allosteric effect is pH dependent with the maximum inhibition observed at pH 7.0-7.5. It was later discovered that AHAS from *N. crassa* can be solubilized while maintaining a high activity and sensitivity to valine by inclusion of high concentrations of potassium phosphate in the extraction buffer (Kuwana and Date, 1975). The significance of this unusual requirement for stability will be explained further below. For plant AHAS, the situation is similar. Usually the end-product feedback sensitivity is maintained after extraction, but is lost progressively on storage, freeze/thaw cycles, heat treatment and passage through chromatography columns. Inclusion of cofactors, pyruvate, branched-chain amino acids, or high concentrations of glycerol and polyvinylpyrrolidone have been reported to provide some stabilization (Relton *et al.*, 1986; Muhitch *et al.*, 1987; Durner and Böger, 1988; Southan and Copeland, 1996).

In addition to the regulation by its biosynthetic end-products, the activity of the fungal AHAS may be also controlled by ATP, which reverses the inhibition of the enzymatic activity by valine. This effect has been demonstrated in both intact mitochondria (Takenaka and Kuwana, 1972) and with highly purified reconstituted yeast AHAS (Pang and Duggleby, 1999). Similar activation has been demonstrated with the mitochondrial-localized algal *E. gracilis* AHAS (Oda *et al.*, 1982). The activation is sigmoidal and very specific to ATP; it can be partially replaced by ADP but not by AMP, CTP, GTP, UTP or ϵ -ATP (Pang and Duggleby, unpublished results). The reversal of inhibition does not appear to involve the phosphorylation of the enzyme as the non-hydrolyzable ATP analog AMP-PNP is also effective (Pang and Duggleby, unpublished results). These results suggest an interaction between the amino acid biosynthetic pathway and the availability of metabolic energy. Indeed, it had been shown previously with *N. crassa* mitochondria that valine and isoleucine biosynthesis is dependent on the metabolic state of the organelle (Bergquist *et al.*, 1974). ATP has not been reported as an effector in bacterial and plant AHAS. It would be interesting to see if a similar mechanism operates in all AHAS or if it is connected with the specific organelle localization of the enzyme.

Subunits

The subunit composition and quaternary structure of AHAS from different sources appears to vary. Most studies agree that the enzyme functions as a multimeric protein, but the types of subunit present in the eukaryotic enzyme remains to be resolved. It was not until recently that the issue has been clarified in yeast (Pang and Duggleby, 1999) and a higher plant (Hershey *et al.*, 1999). The following three sections will describe studies on AHAS structure, subunit functions and interactions in various organisms.

Bacteria The functional genes of AHAS isozymes of *E. coli* and *S. typhimurium* have been cloned and characterized, and each consists of two structural genes arranged within an operon (Squires *et al.*, 1983a; Squires *et al.*, 1983b; Wek *et al.*, 1985; Lawther *et al.*, 1987). Each set of genes encodes a large subunit of about 60 kDa and a smaller (about 10-17 kDa) subunit, with both subunits essential for full AHAS activity. AHAS purified from *S. marcescens*, is also composed of two types of subunit although in this case the smaller subunit (35 kDa) is considerably larger than that from the enteric bacteria (Yang and Kim, 1995).

The physical association of the large and small subunits was demonstrated initially for *E. coli* AHAS I by the co-precipitation of both subunits from a crude cell extract using an antibody against the large subunit (Eoyang and Silverman, 1984). The three active isozymes have been purified to near homogeneity (Schloss *et al.*, 1985; Barak *et al.*, 1988; Eoyang and Silverman, 1988; Hill *et al.*, 1997). Both subunits are

consistently purified together with the AHAS activity. Furthermore, studies including quantitative analysis of SDS-PAGE (Eoyang and Silverman, 1984), carboxymethylation and gel filtration (Schloss *et al.*, 1985), association kinetics and radioactive labeling (Sella *et al.*, 1993) have revealed that the two polypeptides are in 1:1 stoichiometry. The native size of the isozymes is between 140-150 kDa leading to the conclusion that the enzyme has an $\alpha_2\beta_2$ quaternary structure consisting of two large and two small subunits.

Studies on the properties of the subunits have been carried out by alteration of the subunit genes by mutation (De Felice *et al.*, 1974; Eoyang and Silverman, 1986; Lu and Umbarger, 1987; Ricca *et al.*, 1988; Vyazmensky *et al.*, 1996), expression of individual subunits and their reconstitution (Weinstock *et al.*, 1992; Sella *et al.*, 1993; Vyazmensky *et al.*, 1996; Hill *et al.*, 1997), or by chemical inactivation (Silverman and Eoyang, 1987). The mutation studies were done by the complementation of AHAS-deficient strains of bacteria and activity measurements in bacterial crude extract, while the remaining studies have been carried out using either crude extracts or purified subunits. All reports agree that the main AHAS catalytic machinery resides in the large subunits and we now prefer to describe these as the catalytic subunits. Except for AHAS II (Lu and Umbarger, 1987), transformants carrying high copy number plasmids expressing the catalytic subunits of AHAS I (Eoyang and Silverman, 1986; Weinstock *et al.*, 1992) and III (Weinstock *et al.*, 1992) are prototrophic for branched-chain amino acids, and the slow growth is valine resistant. These results agree with the AHAS activity assays of cell extracts. The highly purified AHAS III catalytic subunit, in the absence of the small subunits, exhibits only about 5% of the activity of the intact enzyme (Vyazmensky *et al.*, 1996). In addition, the cofactor requirements and substrate specificity of the catalytic subunit are similar to those of the intact enzyme. Consistent with its failure to complement AHAS-deficient *E. coli*, purified AHAS II catalytic subunits alone have little or no detectable activity (Hill *et al.*, 1997). Further supporting evidence for the role of the catalytic subunit was provided by the alkylation of AHAS I of *E. coli* with the reactive substrate analog [¹⁴C]3-bromopyruvate (Silverman and Eoyang, 1987). More than 95% of the radioactivity was associated with the catalytic subunits and the enzymatic activity for both acetolactate and acetoxybutyrate synthesis was totally lost with the incorporation of one mole label per mole of ILVB polypeptide.

The pure AHAS II catalytic subunit exists predominantly as dimers (Hill *et al.*, 1997). In contrast, the catalytic subunits of AHAS III were observed to migrate as monomers in gel filtration, but the authors express some surprise at this result given that these subunits are catalytically active (Vyazmensky *et al.*, 1996). As mentioned earlier, the homology-modeled structures of AHAS (Ibdah *et al.*, 1996; Ott *et al.*, 1996) have the active site of the enzyme located at the interface of two catalytic subunits. Thus it is expected that the minimal requirement for an active form of AHAS is a dimer of the

catalytic subunits, and Vyazmensky *et al.* (1996) propose that AHAS III catalytic subunits may dimerise to a small extent that is not detected in gel filtration experiments.

The AHAS small subunit has been implicated in conferring valine sensitivity on the enzyme (De Felice *et al.*, 1974; Eoyang and Silverman, 1986; Weinstock *et al.*, 1992; Sella *et al.*, 1993) and we therefore refer to it as the regulatory subunit. The residual activities of the catalytic subunit of AHAS I and III are completely valine insensitive. On reconstitution with the regulatory subunits, valine sensitivity is restored. The regulatory role of the small subunit was further confirmed by Vyazmensky *et al.* (1996) with highly purified subunits of AHAS III. Using equilibrium dialysis, each regulatory subunit was shown to bind one valine molecule with a K_d value of 0.2 mM. Valine binding was not observed for the catalytic subunit. Thus it was suggested that the restoration of valine sensitivity upon reconstitution is due to an allosteric conformational effect at the interface of the catalytic subunit dimer mediated by the valine-binding regulatory subunits.

It is known that the enterobacterial AHAS II isozyme is insensitive to valine feedback regulation but curiously it has an absolute requirement for its regulatory subunit. The specific activity of the catalytic subunit is massively enhanced upon reconstitution with the regulatory subunits (Hill *et al.*, 1997). This association apparently stabilizes the active conformation of the catalytic subunit leading to an increase in the turnover number. The interaction is highly specific in that reconstitution with non-matching subunits between isozymes does not occur (Weinstock *et al.*, 1992). The reconstitution of the catalytic subunit with its regulatory subunit follows simple saturation kinetics in AHAS III (Vyazmensky *et al.*, 1996), but is positively cooperative in AHAS II (Hill *et al.*, 1997). In both cases an excess of regulatory subunits is required to fully reconstitute the catalytic subunits. In summary, the regulatory subunits of bacterial AHAS control AHAS activity by conferring upon it end-product feedback inhibition, and by increasing substantially the enzymatic activity.

One apparent exception to this rule is AHAS from *Methanococcus aeolicus*. The purified enzyme contains no detectable regulatory subunit although the enzyme is sensitive to inhibition by valine and by isoleucine (Xing and Whitman, 1994). However, Bowen *et al.* (1997) later showed a genetic arrangement similar to that in other bacteria with a probable regulatory subunit open reading frame just downstream of the catalytic subunit gene. Thus, it seems likely that the regulatory subunit was present, but not detected, in the purified enzyme although this proposition has yet to be verified experimentally.

Fungi In contrast to the bacterial enzymes, the structure and biochemical properties of AHAS from eukaryotes are not well characterized, and in most cases the enzyme has been studied in crude extracts only. A few fungal AHAS genes have been cloned (Polaina, 1984; Falco *et al.*, 1985; Jarai *et al.*, 1990; Bekkaoui *et al.*, 1993) by complementation, and the deduced amino acid sequences (except for the N-terminal transit

peptide) are collinear with those of bacterial AHAS catalytic subunits. As mentioned earlier, fungal AHAS activity is extremely labile and no purified enzyme from its native source has ever been reported. On the other hand, the entire AHAS gene of *S. cerevisiae* (*ilv2*) was cloned and over-expressed in *E. coli* (Poulsen and Stougaard, 1989). The highly purified dimeric enzyme, which is also very unstable, is insensitive to valine and exhibits only a very low specific activity (0.17 U/mg). For comparison, *E. coli* AHAS II has a specific activity of approximately 50 U/mg (Hill *et al.*, 1997). By analogy with the bacterial enzyme, the lack of valine inhibition could be because the regulatory subunit is missing.

The first evidence for a fungal AHAS regulatory subunit came from the identification of a *S. cerevisiae* nuclear open reading frame (*YCL009c*) whose hypothetical gene product shows significant sequence homology to that of bacterial regulatory subunits (Bork *et al.*, 1992; Duggleby, 1997). Further supporting evidence came from functional studies of the null mutant of *YCL009c*. Enzyme assays using permeabilized mutant cells showed that AHAS activity is insensitive to added valine (Cullin *et al.*, 1996). Thus, this open reading frame (termed *ilv6*) appeared to be the yeast AHAS regulatory subunit. The gene product of *ilv6* (the mature protein is about 30 kDa) is about twice as large as the bacterial regulatory subunit due mainly to an extra segment of approximately 50 residues in the middle of the sequence. Unlike the situation observed in bacterial studies, the *ilv6* null mutant cells were reported to show no physiological differences from wild-type yeast. This is probably because the comparison was performed using a complex medium where AHAS activity may not be essential.

Both the catalytic (*ilv2*) and regulatory (*ilv6*) subunits of *S. cerevisiae* have been over-expressed in *E. coli*, purified, and reconstituted (Pang and Duggleby, 1999). These experiments provided the first conclusive biochemical proof that *ilv6* encodes an eukaryotic AHAS regulatory subunit. The conditions required for the reconstitution of subunits are unusual. Optimum reconstitution of the subunits, which is characterized by a 7- to 10-fold increase in specific activity and the restoration of valine sensitivity, occurs in 1 M potassium phosphate (which can be partially replaced by sulfate) and at a pH of 7.0 to 7.5. All kinetic properties of the reconstituted AHAS activity are comparable to those reported with permeabilized yeast cells (Magee and de Robichon-Szulmajster, 1968b). Under the low phosphate conditions (50 to 100 mM) used to study bacterial AHAS, no significant reconstitution is observed. The yeast reconstitution conditions are very similar to those reported by Kuwana and Date (1975) for the stabilization of the valine-sensitive *N. crassa* AHAS. Thus, phosphate is required for maintaining the integrity of the quaternary structure of fungal AHAS. High phosphate concentration is the major determinant of reconstitution (Pang and Duggleby, unpublished results) and may reflect the native environment within the mitochondria where the enzyme resides.

Valine inhibition of the reconstituted enzyme can be reversed by another effector, ATP (Pang and Duggleby, 1999). Using circular dichroism spectroscopy, ATP has been shown to interact directly with the yeast regulatory subunit alone (Pang and Duggleby, unpublished results). This interaction is magnesium dependent, and has a K_d value of approximately 0.2 mM. Binding of valine to the yeast regulatory subunit has not yet been demonstrated because this ligand does not affect its circular dichroism spectrum, but it is assumed to interact directly with this subunit. Hence in yeast, the regulatory subunit of AHAS controls activity via binding of both valine and ATP.

Yeast has only a single AHAS enzyme, and its activity is valine-sensitive. Unlike wild-type *E. coli* K-12 and higher plants, growth of yeast cells in minimal medium is not inhibited by including high concentrations of valine (Meuris, 1969). Thus, instead of having multiple AHAS isozymes as in *E. coli*, yeast may use ATP activation of AHAS to provide a mechanism similar to that of having the valine-insensitive isozyme AHAS II.

Plants As with fungi, the plant AHAS genes that have been cloned over the past decade correspond to the catalytic subunits of the bacterial enzyme. These genes encode a polypeptide with a molecular mass of about 72 kDa, which is about 10 kDa larger than the bacterial catalytic subunit. As expected, the extra 10 kDa is contributed by an N-terminal organelle-targeting sequence. This transit sequence has been shown *in vitro* to be cleaved upon translocation into the chloroplast (Bascomb *et al.*, 1987), and *in vivo* by Western blotting with anti-AHAS antisera (Singh *et al.*, 1991; Bekkaoui *et al.*, 1993). In the latter experiments, the antisera cross-react with a 65 kDa protein found in a wide variety of monocotyledonous and dicotyledonous plants. In contrast, SDS-PAGE analysis of the purified AHAS from barley (Durner and Böger, 1988) and wheat (Southan and Copeland, 1996) revealed that the large subunits have molecular masses of 57-58 kDa. The reason for the apparently smaller molecular mass of the large subunit of the purified enzyme is unknown, although it was argued that for barley AHAS it is due to misidentification of the appropriate band from the several that were seen in SDS-PAGE (Singh *et al.*, 1991).

From the few reports where a plant AHAS had been purified from its native sources, the presence of a regulatory subunit has not been demonstrated conclusively. This is due mainly to the low yield of the enzyme and the presence of multiple, and possibly contaminating, protein bands when the preparation was analyzed by SDS-PAGE. Durner and Böger (1988) concluded that barley AHAS is composed of one polypeptide only, while wheat AHAS may contain a second subunit of 15 kDa (Southan and Copeland, 1996). However, the possibility that the latter is a contaminating protein cannot be ruled out since the purified enzyme has a very low specific activity (0.06 U/mg) and is almost completely insensitive to the branched-chain amino acids.

The quaternary structure of the plant AHAS varies with the experimental conditions. Separation of purified barley AHAS by size exclusion chromatography reveals two activity peaks proposed to be a high molecular mass 440 kDa aggregate and a smaller form of 200 kDa (Durner and Böger, 1988). FAD was demonstrated to promote the association into the high molecular mass aggregate, while pyruvate counteracts the effect of FAD (Durner and Böger, 1990). The presence of multiple forms of barley AHAS is interpreted as different oligomeric state of a basic AHAS unit rather than different isozymes, as both forms exist in equilibrium with each other. The different AHAS forms are similar in their kinetic properties as well as inhibition by branched-chain amino acids and herbicides. AHAS from corn also exhibits a similar aggregation pattern (Stidham, 1991). Besides having what was called the "tetramer" and "dimer" aggregation states, there is also an additional "monomer" peak. The monomeric AHAS differs from the higher aggregation states in that its activity is valine insensitive, and only appeared in aged plant extracts when the tetrameric and dimeric AHAS peaks are mostly abolished. These results were interpreted to mean that the branched-chain amino acid binding site is formed by oligomerization of the AHAS, but an alternative explanation could involve the loss of the plant regulatory subunits. As mentioned earlier, all the close relatives of AHAS have their active site formed by the interface between catalytic subunits so it appears unlikely that AHAS could be active as a monomer.

Several plant AHAS genes, corresponding to the bacterial catalytic subunit genes, have been cloned and over-expressed in *E. coli* (see later). Consistent with the results obtained with bacterial and yeast catalytic subunits, these purified recombinant plant enzymes have low (0.6-7.8 U/mg) AHAS activity (Chang and Duggleby, 1997; Chang *et al.* 1997), are dimeric (Singh *et al.* 1992; Chang and Duggleby, 1997), and valine-insensitive (Singh *et al.* 1992; Chang and Duggleby, 1997; Chang *et al.* 1997). These results suggest some difference from the native enzymes, such as a missing regulatory subunit. Supporting evidence for the existence of a plant regulatory subunit has come from *in vivo* studies with transgenic plants that were transformed with AHAS catalytic subunit genes. Placing the genes under the control of a strong promoter and using a high gene dosage, the transgenes were highly expressed. However the resultant massive increase in the mRNA level produced relatively small increases in the specific activity of AHAS, suggesting that some post-translational modification might be limiting the enzyme activity (Odell *et al.* 1990; Ouellet *et al.* 1994). In addition, the over-expression of the introduced AHAS gene also confers resistance to valine in the transgenic plants (Tourneur *et al.* 1993). These observations may be explained by invoking a missing regulatory subunit as the limiting factor in the transgenic plants.

Consistent with the above analysis, a recent paper reports the isolation of the first plant AHAS regulatory subunit cDNA

(Hershey *et al.*, 1999). Like the yeast regulatory subunit gene, the plant gene was identified by the homology of the deduced amino acid sequence with that of the bacterial regulatory subunit. Like all reported plant catalytic subunits, the isolated *Nicotiana plumbaginifolia* regulatory subunit cDNA has a deduced N-terminal sequence that strongly resembles a chloroplast transit peptide. Surprisingly, the plant regulatory subunit is approximately twice as large as most bacterial regulatory subunits. The reason for this is that this plant subunit is composed of two similar "domains" with each domain collinear with a copy of the bacterial regulatory subunit. This *N. plumbaginifolia* regulatory subunit was over-expressed in *E. coli* and partially purified, and its function tested by mixing with purified AHAS catalytic subunits from either *N. plumbaginifolia* or *A. thaliana*. The addition of the plant regulatory subunit results in an increase in specific activity and stability of the catalytic subunit. Unlike *E. coli* AHAS isozymes, there is cross-reaction between subunits from different plant species in that the *N. plumbaginifolia* regulatory subunit stimulated the activity of the *A. thaliana* catalytic subunit. However, the enhanced activity is not sensitive to end-product feedback regulation. This may suggest that the conditions for the reconstitution of the plant enzyme are not optimal or that some other factor is still missing. We have observed cross-reconstitution between the yeast regulatory subunit and the *A. thaliana* catalytic subunit (Pang and Duggleby, unpublished results). Regardless the phosphate concentration used (0.05 or 1.0 M), mixing of these highly purified subunits results in a 2- to 3-fold increase in activity. The enhanced activity is valine-sensitive but is not affected by ATP.

Purification

In order to conduct thorough and accurate enzyme characterization, a large amount of relatively pure AHAS is required. The enzyme can be obtained either from its native source or by over-production in a heterologous expression system. Purification from its native source will be difficult if the enzyme constitutes only a very small proportion of the total protein and this is the case for plant AHAS. For example, Haughn *et al.* (1988) report measurements that correspond to a specific activity of 1.8×10^{-3} U/mg for AHAS in *N. tabacum* extracts while Chong *et al.* (1999) obtained a specific activity for the purified enzyme of 2.1 U/mg. On the basis of these data, AHAS constitutes less than 0.1% of the total protein. The low amounts of enzyme protein reflect the low steady state levels of AHAS mRNA. In *B. napus*, Ouellet *et al.* (1992) found values in the range of 0.001-0.01% of the total mRNA for transcripts of three of the genes in this plant. The difficulty of enzyme purification is further exacerbated by the high lability of the enzyme. As a result, AHAS has been purified from very few plants. More success has been achieved by over-expression in *E. coli*. However, this strategy can only be applied if the structural gene(s) of the AHAS have

been cloned. A second potential drawback is that the prokaryotic host cells might not provide the correct machinery for any post-translational modification and processing that might occur in the native cells.

Natural sources AHAS from bacteria, and particularly the *E. coli* isozymes, are purified more easily than eukaryotic AHAS. In *E. coli*, due to the presence of multiple isozymes, mutant strains that have only one active AHAS are usually used as the starting material. Griminger and Umbarger (1979) reported the purification of AHAS I from such a mutant. A relatively low yield of 8% was achieved after five purification steps, even though a fairly high specific activity (34 U/mg) was obtained. Such mutants are not required if the bacterial species have only one AHAS (Arfin and Koziell, 1973; Proteau and Silver, 1991; Xing and Whitman, 1994), or the multiple AHAS activities can be separated easily during purification (Yang and Kim, 1993). Yields as high as 27% (Arfin and Koziell, 1973) or as low as 1% (Xing and Whitman, 1994) have been reported, depending on the complexity of purification and lability of the enzyme. To simplify the purification and to improve the yields, the level of AHAS can be raised by the introduction of AHAS genes in high copy number plasmids (Barak *et al.*, 1988; Eoyang and Silverman, 1988) or under the control of a strong promoter (Hill *et al.*, 1997).

The use of conditions that limit enzyme lability are also important. In this laboratory, we routinely restrict exposure to light, which has proved to be very successful. Although no detailed studies have been performed, we believe that the prevention of FAD-mediated photo-oxidation may be a crucial factor.

Isolation of any eukaryotic AHAS from its natural source is much more difficult. In addition, the kinetic properties of the enzyme are usually noticeably changed during the course of purification. No fungal AHAS has ever been purified from its native source. Attempts to isolate the biosynthetic enzyme from *N. crassa* led to a complete desensitization to branched-chain amino acids and the partially purified enzyme behaved differently to the enzymatic activity in intact mitochondria (Glatzer *et al.*, 1972). Based on our current knowledge of the enzyme, these changes are almost certainly due to the dissociation of the regulatory subunit. Even though the presence of high (1 M) potassium phosphate concentrations were shown to stabilize the enzyme (Kuwana and Date, 1975), no follow-up purification has been reported, probably due to the incompatibility of these conditions with purification methods such as ion-exchange chromatography.

AHAS has been purified from a few plants. The level of AHAS activity varies considerably among plant tissues and the best sources are metabolically active or meristemic tissues. Generally, the yield of the plant enzyme is low (1.5-11%) and the final product has a low specific activity of 0.06-1.59 U/mg (Muhitch *et al.*, 1987; Durner and Böger, 1988; Southan and Copeland, 1996). Even though the purified enzyme is still

sensitive to feedback inhibition, the inhibition is usually partial and easily lost. Unless conditions for effective stabilization can be found, the purification of AHAS from plant tissues is unlikely to progress.

Recombinant systems *E. coli* is the most commonly used host cell for the over-production of foreign proteins because it is genetically and biochemically well-characterized, and easily handled. The enterobacteria have been used successfully to express AHAS from other bacteria (Schloss *et al.*, 1985; De Rossi *et al.*, 1995), as well as active wild-type and mutant AHAS from fungi and plants (Poulsen and Stougaard, 1989; Smith *et al.*, 1989; Wiersma *et al.*, 1990; Singh *et al.*, 1992; Bernasconi *et al.*, 1995; Kim and Chang 1995; Ott *et al.*, 1996; Chang and Duggleby, 1997; Chang *et al.*, 1997; Dumas *et al.*, 1997; Chang and Duggleby, 1998; Lee *et al.*, 1999; Pang and Duggleby, 1999). In contrast to the bacterial AHAS, most eukaryotic enzymes have an N-terminal extension of 60 to 90 residues that functions *in vivo* as an organelle transit peptide. In most cases, the removal of part or all of the transit peptide sequence is crucial for the expressed enzyme to remain in solution and be functional. Full-length eukaryotic AHAS genes that have been cloned and expressed in *E. coli* were observed to be processed into proteins of molecular masses similar to those of mature AHAS in plants, and active enzyme translocated to the periplasmic space (Smith *et al.*, 1989; Singh *et al.*, 1992; Chang and Duggleby, 1997). The specificity and requirements for such processing of the foreign protein in *E. coli* are unknown.

The recombinant AHAS gene is very often constructed so as to generate a fusion protein. The fusion partners that are commonly used are the glutathione S-transferase (GST) domain and the hexahistidine (6XHis) tag, usually introduced at the N-terminus. The usefulness of these fusion partners is to greatly improve the recovery of the highly unstable AHAS activity since purified protein usually can be obtained in a single chromatographic step. These GST and 6XHis tags can be removed if appropriate protease sites are available, but their presence has been shown not to alter the enzymatic activity (Chang *et al.*, 1997; Hill *et al.*, 1997). In addition, the catalytic and regulatory subunits of bacterial, yeast and plant AHAS have been expressed independently from each other in *E. coli* (Vyazmensky *et al.*, 1996; Hill *et al.*, 1997; Pang and Duggleby, 1999; Hershey *et al.*, 1999). Mixing of the purified subunits under the right conditions results in reconstitution of the intact enzyme. This allows the separate characterization of individual subunits.

Herbicidal inhibitors of AHAS

In the mid-1970's DuPont scientists discovered that various sulfonylurea derivatives are potent herbicides (Levitt, 1978) and since then many hundreds of such compounds have been identified. However, it was a further six years before the action of these compounds was shown to be due to inhibition of

AHAS in both bacteria (LaRossa and Schloss, 1984) and plants (Chaleff and Mauvais, 1984). In an interesting coincidence, at about the same time American Cyanamid developed the unrelated imidazolinone series of herbicides, which also act by inhibiting AHAS (Shaner *et al.*, 1984). Armed with the knowledge that AHAS inhibitors will act as herbicides, several other chemically unrelated compounds have been developed subsequently.

Structures and activity of herbicides

Sulfonylureas

The basic structure of the sulfonylurea herbicides is X-SO₂-NH-CO-NH-Y, where X is usually a substituted phenyl group and Y is a substituted pyrimidine or triazine ring. Some representative examples of the sulfonylurea herbicides are shown in Fig. 12 and Brown and Kearney (1991) give a more extensive group. It can be a source of confusion that a second group of bioactive compounds, also known as sulfonylureas, are used as anti-diabetic drugs. A typical example is

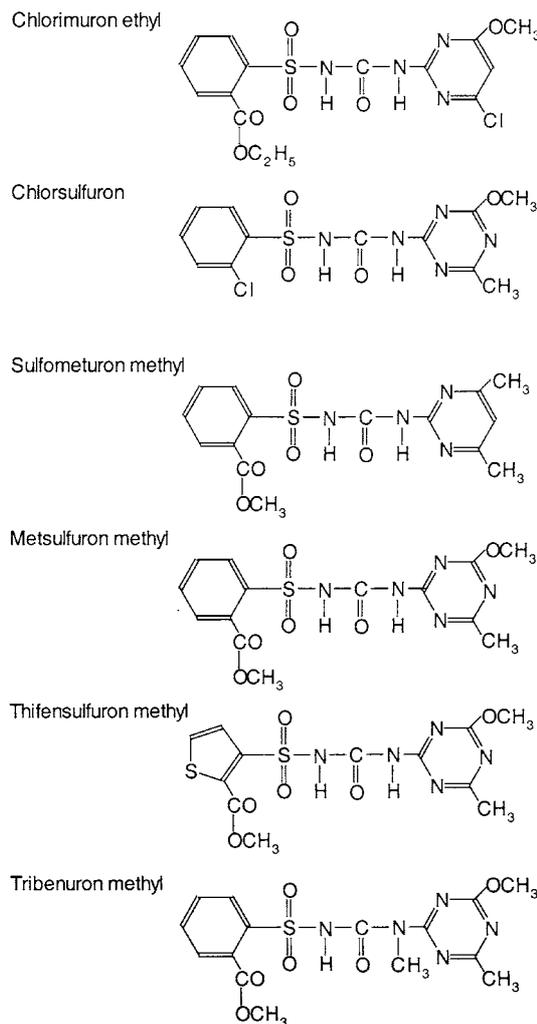


Fig. 12. Structures of several sulfonylurea herbicides.

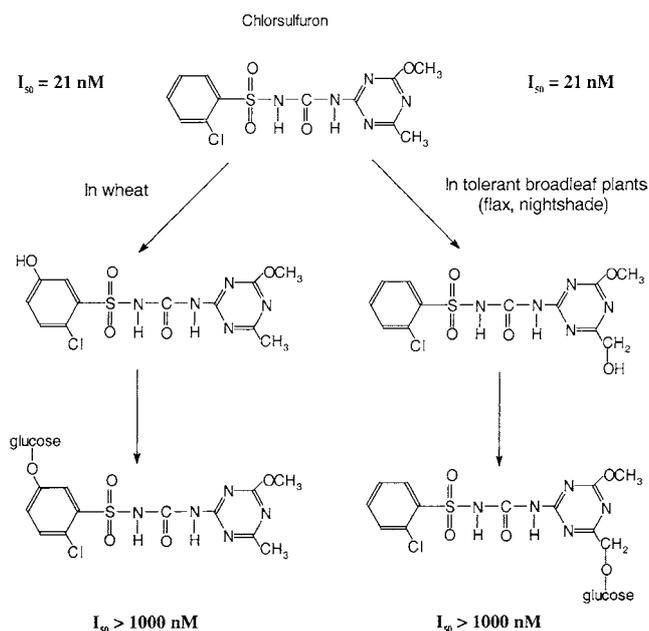


Fig. 13. Routes of detoxification of chlorsulfuron in wheat (left) and in chlorsulfuron-resistant weeds (right). After an initial oxidation reaction that differs between the two types of plant, the modified herbicide is glucosylated at the newly-introduced hydroxyl group to form derivatives that are unable to inhibit AHAS. Adapted from Brown (1990).

carbutamide ($X = 4$ -aminophenyl; $Y = n$ -butyl). However, there does not seem to be any cross-reactivity between the two groups of sulfonylureas in that the anti-diabetics are not herbicides while the herbicides have no anti-diabetic activity.

The biological activity of the sulfonylurea herbicides is extremely high with typical field application rates of 10 to 100 g per hectare. This high potency is reflected in their *in vitro* effects on AHAS that usually requires concentrations in the nM range for inhibition. In marked contrast, their toxicity to animals is extremely low with very high LD₅₀ values (e.g. chlorsulfuron in rats; approx. 6 g per kg body weight). This combination of high potency against plants and low toxicity to animals makes these compounds very effective and safe herbicides. Moreover, they are rapidly degraded in soil by a combination of non-enzymatic hydrolysis and microbial degradation (see Brown and Kearney, 1991).

Many of the sulfonylureas that are used commercially show substantial crop selectivity. For example, wheat is resistant to chlorsulfuron while soybeans are resistant to chlorimuron ethyl. This natural resistance is not due to any difference in AHAS, which is equally susceptible to inhibition in both sensitive and resistant plants (Ray, 1986). Instead, different plant species vary in their ability to convert different herbicides to non-toxic derivatives. These detoxification mechanisms involve a variety of hydroxylation, conjugation, hydrolytic and cleavage reactions; the proposed routes of detoxification of chlorsulfuron in wheat and some resistant weeds are illustrated in Fig. 13 (adapted from Brown, 1990).

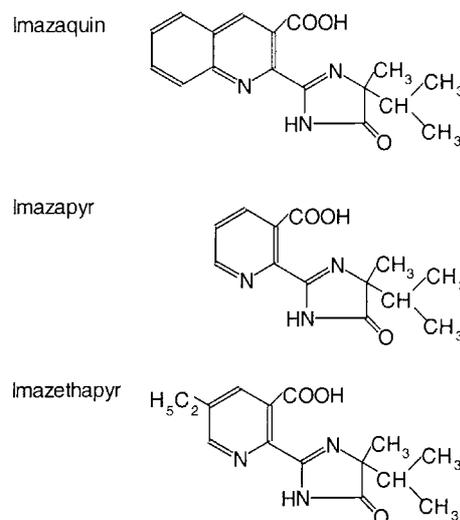


Fig. 14. Structures of typical imidazolinone herbicides.

In addition to these pre-existing resistance mechanisms, sulfonylurea-insensitive variants of AHAS have been identified in a number of crops and weeds. These AHAS mutants are discussed in later.

Imidazolinones

The imidazolinone family of herbicides consists of a 4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl nucleus linked at the 2-position to an aromatic (and usually heterocyclic) ring system. Typical members of this family are shown in Fig. 14.

Application rates are typically in the range 100 to 1000 g per hectare making the imidazolinones approximately 10-fold less potent than the sulfonylureas. This difference is not entirely explicable in the difference in sensitivity of AHAS, for which imidazolinone inhibition usually requires concentrations in the μ M range. Presumably the effectiveness of the imidazolinones in the field results from a combination of facile uptake, low metabolism, and soil persistence. Their toxicity to animals is similar to that of the sulfonylureas.

As with the sulfonylureas, certain plants show natural resistance due to their ability to metabolize the herbicides to non-toxic derivatives. Superimposed on this pre-existing resistance, imidazolinone-insensitive variants of AHAS have been identified and these AHAS mutants will be discussed in a later section.

It has been suggested that the imidazolinones were designed to resemble branched-chain amino acids and to act by binding at the feedback-regulatory site for these ligands. While there is a superficial resemblance between the common nucleus of the imidazolinones and valine, there are several lines of evidence which suggest that these compounds inhibit by binding at separate locations. First, the herbicidal activity of the imidazolinones was discovered before AHAS was identified as their site of action. Thus it is not obvious to us how a similarity to valine could have been part of the design strategy. Second, there does not seem to be a strict correlation between

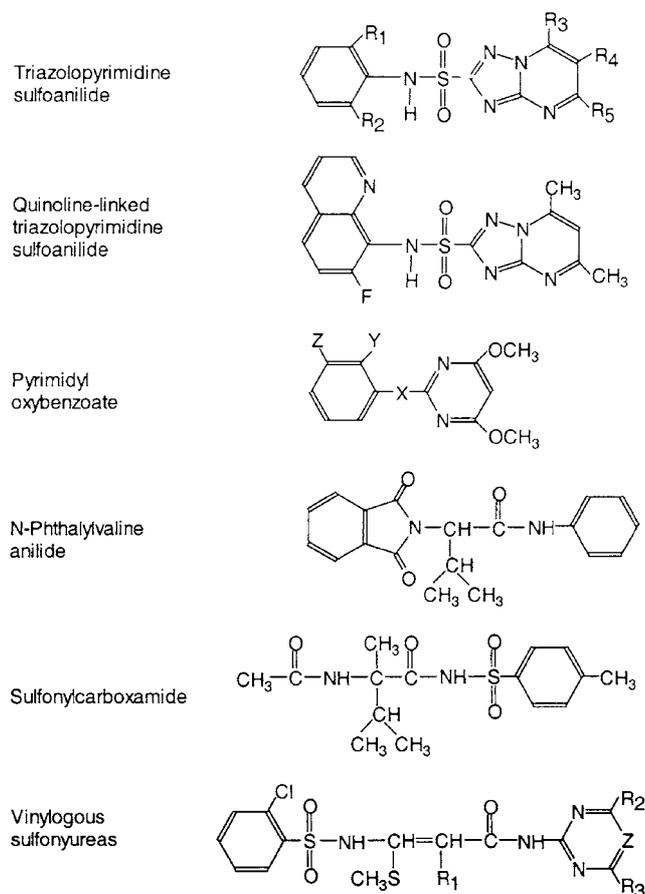


Fig. 15. Structures of various AHAS inhibitors.

inhibition by herbicides and by branched-chain amino acids in certain AHAS mutants. For example, Landstein *et al.* (1993) showed that a *Chlorella emersonii* AHAS mutant is resistant to imazapyr while its sensitivity to valine and leucine is unaffected. In contrast, Subramanian *et al.* (1991) showed that herbicide-resistant mutants of several plants were also resistant to valine. Third, it has been shown that the catalytic subunit of *A. thaliana* (Chang and Duggleby, 1997) and yeast AHAS (Pang and Duggleby, 1999; Pang and Duggleby, unpublished), and intact *E. coli* AHAS II (Hill *et al.*, 1997), are totally unaffected by branched-chain amino acids but all are inhibited by herbicides. Thus, the weight of evidence indicates that any similarity in structure between imidazolinones and valine may be no more than coincidental.

Other inhibitors

Spurred by the success of the sulfonylureas and imidazolinones as safe and effective herbicides, many groups have synthesized new AHAS inhibitors and a selection of such compounds is shown in Fig. 15.

In the triazolopyrimidine sulfoanilides (Kleschick *et al.*, 1990) electron-withdrawing substituents, such as NO₂, CF₃, F or Cl, at the R₁ and R₂ positions result in more active compound than electron-donating groups. Higher levels of

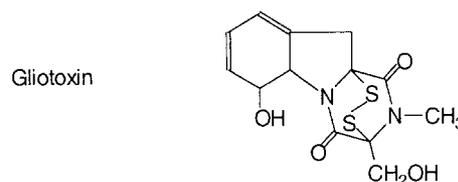


Fig. 16. Structure of the AHAS inhibitor, gliotoxin.

AHAS inhibition are observed with alkyl (e.g. methyl) or alkoxy (e.g. methoxy) substituents at the R₃ and R₅ positions than with halo or haloalkyl groups. One such compound (flumetsulam; R₁ = R₂ = F, R₃ = R₄ = H, R₅ = CH₃) has been reported to give 50% inhibition of AHAS at about 30 nM (Namgoong *et al.*, 1999). Recently, a new group of triazolopyrimidine sulfoanilides have been synthesized in which the aromatic ring is replaced by a quinoline derivative (Namgoong *et al.*, 1999); the example shown in Fig. 15 inhibits AHAS in the low nM concentration range.

The pyrimidyl oxybenzoates generally have a carboxylate or aldehyde moiety as the Y substituent, while the group at the Z position can be an alkyl, alkoxy, or halo group. The bridging atom X is an oxygen in the true pyrimidyl oxybenzoates although it can be replaced by a sulfur atom as in the pyrimidyl thiobenzoate herbicide pyrithiobac (Y = COOH; Z = Cl) that is a very potent inhibitor of maize AHAS (Wright and Penner, 1998).

N-Phthalylvaline anilide and sulfonylcarboxamide were each designed, as analogs of branched-chain amino acids, to inhibit by binding to the regulatory site (Huppertz and Casida, 1985; Stidham, 1991). There has been little published work on the inhibitory properties of these compounds.

McFadden *et al.* (1993) synthesized the vinylogous sulfonylureas as variations on the normal sulfonylureas. In active compounds, R₁ may be an ethyl or propyl ester of a carboxyl group, or a cyano group, while R₂ and R₃ are usually methyl or methoxy substituents. The heterocyclic ring can be either a pyrimidine (Z = CH) or a triazine (Z = N) as in the normal sulfonylureas.

In addition to these synthetic compounds, natural sources have been explored to find possible AHAS inhibitors. One compound found in this way is gliotoxin (Fig. 16), a metabolite of the fungus *Aspergillus flavus* (Haraguchi *et al.*, 1992; Haraguchi *et al.*, 1996). It is an inhibitor of plant growth and of AHAS activity at similar concentrations (μM) suggesting a connection between the two effects.

Summary

It will be evident that a wide variety of compounds can inhibit AHAS. Although there are some superficial similarities between the various families of inhibitor, any shared structural elements are also found in a vast range of compounds that do not inhibit AHAS. One interpretation of these facts is that there are distinct, but perhaps overlapping, sites on the enzyme that binds these inhibitors. The structure and nature of

the inhibitor binding site(s) is discussed later.

Mechanism and kinetics of herbicide action The AHAS-inhibiting herbicides interfere with the growth of both microorganisms and higher plants. Death of plants can take several weeks, with the meristemic tissues dying first followed by slow necrosis of the mature tissues. The physiological changes that follow the application of the herbicide include the accumulation of the cytotoxic AHAS substrate 2-ketobutyrate or derivatives (LaRossa *et al.*, 1987; Rhodes *et al.*, 1987), amino acid content imbalance (Höfgen *et al.*, 1995), inhibition of DNA synthesis (Stidham, 1991) and cell division, and reduction of assimilate translocation (Kim and Vanden Born, 1996). The direct cause(s) for the herbicide-induced growth stasis is not understood, but it is not solely due to the accumulation of 2-ketobutyrate (Shaner and Singh, 1993; Höfgen *et al.*, 1995; Epelbaum *et al.*, 1996). The effects of the herbicides can be reversed by supplementation with the branched-chain amino acids (Chaleff and Mauvais, 1984; LaRossa and Schloss, 1984; Ray, 1984).

The inhibition of AHAS is complex and not well understood. Out of the three enterobacterial isozymes, AHAS II is the most sensitive to the inhibition of herbicides (LaRossa and Schloss, 1984). However, this isozyme is less sensitive than the plant enzymes. Both reactions catalyzed by the enzyme are inhibited equally (Delfourne *et al.*, 1994). In general, the sulfonylureas are better inhibitors (apparent K_i values in the nM to μ M range) than the imidazolinones (apparent K_i values in the μ M to mM range). Inhibition of the AHAS reaction is a time-dependent, biphasic process with an initial weak inhibition followed by a slow transition into a final steady-state where the inhibition is more potent (LaRossa and Schloss, 1984; Ray, 1984; Muhitch *et al.*, 1987; Chang and Duggleby, 1997). Typical results are shown in Fig. 17 for the inhibition of *A. thaliana* AHAS by a sulfonylurea (chlorsulfuron) and an imidazolinone (imazapyr). The initial amount of inhibition, the final inhibition and the rate constant for the transition are all dependent on the concentration of the inhibitor. Since many of studies of AHAS inhibition employ the discontinuous assay method (see above) with a long incubation time (30 min to 2 hours) the results obtained may not be reliable because true rates are not measured. Due to the complex nature of the inhibition, it is preferable to follow the reaction continuously and, where this has been done, most inhibition studies have concentrated only on the initial weaker inhibition. In addition to the slow biphasic inhibition, inhibition by some sulfonylureas is also complicated by tight-binding effects because the concentrations of inhibitor added to the reaction are comparable to that of the enzyme (Hill *et al.*, 1997; Chang and Duggleby, 1997). As a result, the concentration of free inhibitor will be less than the total amount added (Szedlacsek and Duggleby, 1995).

Sulfonylureas have been reported to act as non-competitive (Durner *et al.*, 1991; Hill *et al.*, 1997) and nearly competitive (Schloss, 1984; Schloss, 1990; Ahan *et al.*, 1992) inhibitors

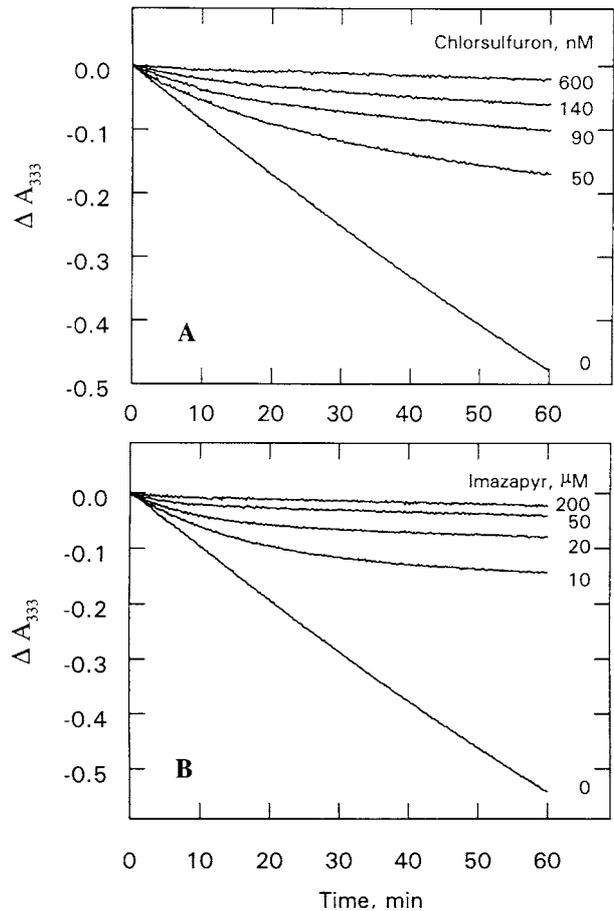


Fig. 17. Time-dependent inhibition of AHAS. After an initial, and relatively weak inhibition, the rate of catalysis becomes progressively slower in the presence of sulfonylureas (chlorsulfuron, **Panel A**) or imidazolinones (imazapyr, **Panel B**). The reaction is followed by measuring the consumption of pyruvate at 333 nm. The curves are for *A. thaliana* AHAS and are redrawn from Chang and Duggleby (1997).

with respect to the substrate pyruvate. The affinity of the enzyme for the herbicides increases under turnover conditions (Schloss *et al.*, 1988). In contrast, imidazolinone herbicides have been reported as non-competitive (Schloss *et al.*, 1988; Ahan *et al.*, 1992) and uncompetitive inhibitors (Shaner *et al.*, 1984; Durner *et al.*, 1991; Chang and Duggleby, 1997). The inconsistencies between these results may have arisen from the differences in the enzyme assay methods and the analysis of the results. The variations in the kinetics of inhibition and structural diversity among the different classes of herbicides favor the conclusion that these inhibitors bind to distinct sites on the enzyme. However competition studies have shown that their binding to the enzyme is mutually exclusive (Schloss *et al.*, 1988; Shaner *et al.*, 1990; Durner *et al.*, 1991; Landstein *et al.*, 1993). In addition, mutant enzymes that are resistant to one class of herbicides are often cross-resistant to other herbicide classes. On the other hand, mutations that affect the binding of only one class of herbicides have also been isolated

(next section). These results imply that the structurally diverse classes of AHAS inhibitors bind to different, but overlapping sites.

The interpretations of the inhibition kinetics are based on models in which the inhibition by the herbicides is reversible. Using gel filtration and equilibrium dialysis, it has been reported that the binding is reversible and the bound inhibitors can be completely, albeit slowly, separated from the enzyme (LaRossa and Schloss, 1984; Muhitch *et al.*, 1987; Schloss *et al.*, 1988; Durner *et al.*, 1991). However, the recovery of enzymatic activity after inhibitor removal is variable. Almost complete reversal of inhibition on removal of the inhibitor by desalting, dilution or ammonium sulfate precipitation was observed in some cases (LaRossa and Schloss, 1984; Ortéga *et al.*, 1996; Southan and Copeland, 1996; Ortéga and Bastide, 1997). In others, irreversible inactivation of the enzyme by both sulfonylureas and imidazolinones has been reported, even though all of the herbicide has been removed (Muhitch *et al.*, 1987; Durner *et al.*, 1991; Ortéga *et al.*, 1996). Similar irreversible inactivation has also been observed *in vivo* with imidazolinones herbicides where the amount of extractable AHAS activity is drastically reduced on incubation of the plant tissues or cells with the herbicide prior to isolation (Muhitch *et al.*, 1987; Stidham and Shaner, 1990). The level of inhibition is dependent on the time of incubation and the amount of herbicide. This irreversible loss of the extractable AHAS activity can be prevented by the sulfonylurea sulfometuron methyl (Shaner *et al.*, 1990), and is not due to specific degradation of the enzyme containing bound herbicide (Shaner and Singh, 1991).

The irreversible inactivation of AHAS by herbicides has been explained by the oxygenase side-reaction of the enzyme (Schloss, 1994). The binding of the herbicide to the enamine form of the hydroxyethyl-ThDP enzyme intermediate stabilizes the complex in an oxygen-sensitive state and retards the release of a peroxide product. Thus, with time, the retention of the peroxide leads to oxidative inactivation of the enzyme. However this explanation does not account for the observations of Ortéga *et al.* (1996). Barley AHAS and *E. coli* AHAS II inhibited by the sulfonylurea thifensulfuron methyl is apparently permanently inactivated if the inhibitor is removed by gel filtration or by dilution. However, if the inhibitor is removed by precipitation by ammonium sulfate, it leads to a full recovery of activity for the bacterial enzyme but not for the barley enzyme. In addition, the inactivation is observed only if the enzyme is incubated in the presence of ThDP and Mg^{2+} regardless of whether pyruvate is added.

One way to rationalize these observations is to invoke dissociation of the regulatory subunit of AHAS II from its catalytic subunit. It is known that the bacterial and yeast AHAS require the regulatory subunit for the enzymes to exhibit high activity (see above). Thus, the interaction of the subunits stabilizes the active site to ensure a high turnover rate of the intact enzyme. It has been shown indirectly that the herbicidal inhibitors bind close to the active site and their

binding to the enzyme might destabilize subunit interactions leading to regulatory subunit dissociation. Reconstitution of the bacterial subunits is dependent on the subunit concentrations (Sella *et al.*, 1993; Hill *et al.*, 1997). High subunit concentrations and/or an excess of the regulatory subunit are required for reconstitution to occur. Thus, this might be the reason why the removal of inhibitor by ammonium sulfate precipitation, which is a concentrating process, but not gel filtration and dilution leads to the recovery of the bacterial AHAS II activity. The failure to reactivate the barley enzyme might be due to the inherent instability of the plant enzyme. In addition, this phenomenon might also resolve another issue. It is known that the herbicidal inhibitors act by binding to the catalytic subunit, while the allosteric inhibitor valine interacts with the regulatory subunit (Vyazmensky *et al.*, 1996). However, competition studies suggest that the binding of both inhibitors to AHAS is mutually exclusive (Subramanian *et al.*, 1991; Landstein *et al.*, 1993). The herbicide-induced AHAS regulatory subunit dissociation might explain this observation, as well as the observed stronger final steady-state inhibition.

Herbicide-resistance mutations As mentioned in the above sections, the herbicidal inhibitors of AHAS do not act as analogs of the substrates or of cofactors, and the mechanism of inhibition is complex. Mutations resulting in resistance have been isolated and in some cases characterized genetically, biochemically, or both. In most reported cases, the resistance is due to a dominant or semi-dominant mutation in the catalytic subunit gene of AHAS. In contrast to the complexity of the inhibition mechanism, the molecular basis for most of the characterized herbicide resistance is due to a single amino acid change from the wild-type enzyme sequence. These changes result in an AHAS activity that is less sensitive to herbicide inhibition. Some recessive mutations have also been documented but the mechanism has not been identified and some might be caused by an alteration in the permeability of the mutant cells to the toxic herbicides (Falco and Dumas, 1985).

Bakers yeast was one of the first, and remains the most extensively characterized, model organism for studies of AHAS mutation. Spontaneous yeast resistant mutants were isolated by their ability to grow on minimal medium containing normally lethal concentrations of sulfometuron methyl, a sulfonylurea (Falco and Dumas, 1985). Out of 41 independent mutant yeast catalytic subunit genes isolated, 24 different amino acids substitutions occurring at ten different sites along the protein sequence were identified (Falco *et al.*, 1989; Mazur and Falco, 1989). These ten mutation sites are scattered throughout the whole sequence of the gene, and are at positions that are highly conserved amongst known natural herbicide-sensitive AHAS enzymes. The mutation sites were further characterized for other possible substitutions by site-directed mutagenesis. The results revealed that the residue at some sites could be replaced by a wide variety of amino acids

Table 4. Mutations in AHAS that confer herbicide resistance^a

Species	Mutations	<i>E. coli</i> II	Resistance	References
Yeast	G116[NS]	G25	SU	b
Yeast	A117X	A26	SU	b, c
<i>E. coli</i>	A26V	A26	SU	c, d
<i>A. thaliana</i>	A122V	A26	IM	e
Cocklebur	A100T	A26	IM	f
<i>A. thaliana</i>	M124[EI]	M28	SU, IM	g
<i>E. coli</i>	V99M	V99	SU	d
<i>A. thaliana</i>	P197S	S100	SU, TP	h, i
Yeast	P192[AELQRSVWY]	S100	SU	b, j
Tobacco	P196[QS]	S100	SU, IM	k, l
<i>A. thaliana</i>	R199[AE]	P102	IM	g
Yeast	A200[CDERTVWY]	A108	SU	b
<i>E. coli</i>	A108V	A108	SU	d
Yeast	K251[DENPT]	K159	SU	b
Yeast	M354[CKV]	M250	SU	b
Yeast	D379[EGNPSVW]	D275	SU	b
<i>E. coli</i>	M460[AN]	M460	SU	m
Yeast	V583[ACNY]	V461	SU	b
Yeast	W586X	W464	SU	b
<i>E. coli</i>	W464[AFLQY]	W464	SU	m
<i>A. thaliana</i>	W574[LS]	W464	SU, IM	e
Oilseed rape	W557L	W464	SU, IM, TP	n
Tobacco	W573L	W464	SU	l
Cocklebur	W552L	W464	SU, IM, TP, PB	f
Cotton	W563[CS]	W464	SU, IM	o
Yeast	F590[CGLNR]	F468	SU	b
<i>A. thaliana</i>	S653[FNT]	P536	IM, PB	e, h, p, q, r

a.X in the second column indicates that almost any substitution gives rise to herbicide resistant; where several substitutions are effective, these are listed in square brackets. The third column shown the equivalent residue in the *E. coli* AHAS II sequence. Where resistance to a particular group of herbicides is not shown, this may mean either that there is no resistance or, more commonly, that resistance has not been determined. The abbreviations to the herbicide families are: IM, imidazolinone; PB, pyrimidyl oxybenzoates; SU, sulfonyleurea; TP, triazolopyrimidine sulfoanilide.

b.Falco *et al.*, 1989; c.Yadav *et al.*, 1986; d.Hill and Duggleby, 1998; e.Chang and Duggleby, 1998; f.Bernasconi *et al.*, 1995; g.Ott *et al.*, 1996; h.Haughn *et al.*, 1988; i.Mourad and King, 1992; j.Xie and Jiménez, 1996; k.Harms *et al.*, 1992; l.Lee *et al.*, 1988; m.Ibdah *et al.*, 1996; n.Hattori *et al.*, 1995; o.Rajasekaran *et al.*, 1996; p.Hattori *et al.*, 1992; q.Sathasivan *et al.*, 1991; r.Lee *et al.*, 1999

to yield a herbicide-resistant enzyme, while others are limited to certain substitutions (Table 4).

Herbicide-resistant mutations from plants and bacteria have also been obtained by induced or spontaneous mutation under laboratory or field conditions, and by deliberate mutations based on structural models of AHAS (Table 4). Most of these mutations correspond to one of the ten mutation sites in yeast AHAS. The most commonly encountered natural mutations involve the residues A122, P197, W574 and S653 (*A. thaliana* AHAS numbering). Below is a summary of what is known about these mutations.

It is worth mentioning that the definition of herbicide resistance is extremely vague and no exact dividing line can be drawn between what might be described as reduced herbicide sensitivity and true resistance. No doubt the problem

of definition is further complicated by the difficulty of comparing systems that may range from *in vitro* activity assays of pure enzymes to field measurements on whole plants. Overall, it appears that *in vivo* selection using normally toxic amounts of a herbicide is capable of isolating mutants that, when AHAS is studied *in vitro*, can have reductions in herbicide sensitivity of as little as 4- to 5-fold. However, mutant enzymes that are 10,000-fold more resistant than wild-type have also been found in this way. There are many factors operating in a whole organism that can result in a continuum of responses to applied herbicides, and only one of these factors is the intrinsic sensitivity of AHAS to inhibition. We offer no prescription of what is and is not herbicide resistance but caution the reader that it can mean different things to different authors and in different contexts.

A122

The A122 mutation was first identified in *E. coli* (A26V of AHAS II, Yadav *et al.*, 1986) and yeast (A117[DPTV], Falco *et al.*, 1989) as spontaneous mutants that grow in the presence of a sulfonyleurea. Site-directed mutagenesis studies with the yeast enzyme showed that this residue can be replaced by almost any amino acid and still yield active herbicide-resistant enzymes. Enzymatic studies with the *E. coli* A26V mutant in crude cell extracts revealed that the mutation causes a 75% reduction in specific activity and a slight resistance (4-fold) to sulfometuron methyl compared to the wild-type activity (Yadav *et al.*, 1986).

The same mutation has also been isolated by hydroxylamine-induced random mutagenesis of the *E. coli* AHAS II catalytic subunit gene. AHAS-deficient *E. coli* cells transformed with a plasmid carrying this mutation have the ability to grow in the presence of the sulfonyleurea chlorimuron ethyl (Hill and Duggleby, 1998). This mutation has been studied in detail with the availability of the highly purified mutant enzyme. It has only half of the activity of the wild-type enzyme, and a 15-fold reduction in the affinity for the ThDP cofactor. No major effect on the affinity for pyruvate or the other cofactors was observed. In addition, this single mutation does not seem to affect subunit association, as revealed by SDS-PAGE of the purified protein. The mutation confers only weak resistance to different sulfonyleureas (6- to 20-fold), and does not result in cross-resistance to imidazolinones. These kinetic results with the purified mutant AHAS II are consistent with the observations obtained with *E. coli* mutant crude extracts.

A mutation (A100T) at the equivalent site of cocklebur AHAS has also been identified in a herbicide-resistant field isolate (Bernasconi *et al.*, 1995). In contrast to the *E. coli* mutant, growth of this mutant plant and its AHAS activity are resistant to imidazolinones (> 7.4-fold). An identical mutation is also found in the commercial mutant corn ICI 8532 IT. Enzymatic studies with partially purified mutant enzyme show a normal K_m value for pyruvate and unchanged leucine sensitivity.

A similar mutation at the corresponding position (A122V) of *A. thaliana* AHAS has also been studied extensively with highly purified recombinant enzyme (Chang and Duggleby, 1998). The purified *A. thaliana* A122V mutant enzyme, compared to the wild-type AHAS catalytic subunit, is less stable, has a 4-fold reduction in specific activity, an unaffected K_m value, and shows reduced affinities (5- to 20-fold) for all three cofactors. Mutation also results in 1000-fold resistance to imidazolinones with only weak resistance to sulfonyleureas (up to 4-fold). The weak resistance to sulfonyleureas is similar to that observed with the equivalent *E. coli* AHAS II mutant. However, as mentioned earlier the latter does not show resistance to the imidazolinones. This is not too surprising as the bacterial enzyme is naturally more resistant to the imidazolinones (with K_i values in the mM range) than the eukaryotic enzymes.

It should be pointed out here that all studies to date of plant AHAS mutants using recombinant systems have been performed on the catalytic subunit only. It is only recently that a plant AHAS regulatory subunit has been discovered (Hershey *et al.*, 1999) and the influence of plant regulatory subunits in modulating the effects of herbicide-resistance mutations are yet to be determined.

P197

One of the more commonly described mutation sites leading to herbicide resistance is P197. In the yeast enzyme, this residue can be replaced by at least nine other amino acids (Table 4). This well-known mutation site has been used in transgenic plant studies, but thorough characterization of the purified mutant enzyme has never been reported and enzymatic studies have been conducted with crude extracts only. AHAS activity of the yeast (P192S, Yadav *et al.*, 1986), *A. thaliana* (P197S, Haughn and Somerville, 1986; Mourad *et al.*, 1995) and tobacco (P196Q and P196A of the *SuRA* and *SuRB* loci, respectively, Lee *et al.*, 1988; Creason and Chaleff, 1988) mutants show unaltered specific activity and affinity for pyruvate. The mutation results in 100- to 1000-fold resistance to sulfonyleureas and triazolopyrimidines, but confers no significant resistance to imidazolinones or pyrimidyl oxybenzoates (Haughn and Somerville, 1986; Haughn *et al.*, 1988; Mourad and King, 1992). Similar degrees of resistance to the different classes of herbicide are observed in callus growth of the mutant and at the whole plant level. It has also been reported that substitution of this proline residue with serine slightly desensitizes the enzyme to branched-chain amino acid inhibition (Yadav *et al.*, 1986; Mourad *et al.*, 1995). The involvement of this site in herbicide resistance is further supported by results with *E. coli* AHAS II. AHAS II naturally contains a serine (S100) at this position, and mutation to a proline increases herbicide sensitivity (Mazur and Falco, 1989; Lee and Duggleby, 2000). Enhanced sulfonyleurea resistance and cross-resistance to imidazolinones and pyrimidyl oxybenzoates can be achieved by the combination of this mutation with mutations at other sites (see below; Lee *et al.*, 1988; Creason and Chaleff, 1988; Hattori *et al.*, 1992). Similar results are obtained by increasing the mutant gene expression through gene amplification (Harms *et al.*, 1992) or by over-expressing the gene using a strong (CaMV 35S) promoter (Odell *et al.*, 1990; Tourneur *et al.*, 1993).

W574

Until recently, W574 was the only single site mutation reported to confer resistance to multiple herbicides including sulfonyleureas, imidazolinones and triazolopyrimidines (Hattori *et al.*, 1995). In yeast, this residue (W586) can be substituted by almost any amino acid (Falco *et al.*, 1989). However, Bernasconi *et al.* (1995), using the recombinant cocklebur GST-AHAS fusion protein expressed in *E. coli*, reported that replacement with leucine is the only mutation

that results in active enzyme. This residue is highly conserved in all known AHAS sequences except the herbicide-resistant isozyme I of *E. coli*, which has a glutamine at this position. Conversion of this glutamine residue in AHAS I to a tryptophan results in increased sensitivity to herbicides (Mazur and Falco, 1989). In addition, as mentioned earlier, this residue is also implicated in the recognition of the second substrate 2-ketobutyrate in preference to pyruvate (Ibdah *et al.*, 1996). Alteration of this tryptophan residue in *E. coli* AHAS II by site-directed mutagenesis to other amino acids results in sulfonyleurea-resistant mutant enzymes that have lower specificity (by an order of magnitude) for 2-ketobutyrate.

Initially, this mutation was isolated together with the P196A mutation in the tobacco *SuRB* locus (Lee *et al.*, 1988; Creason and Chaleff, 1988). As mentioned previously, mutation at this tryptophan residue is thought to lead to cross-resistance to different classes of herbicides, unlike the P196 mutation alone. Spontaneous mutants of W574 alone have been reported in AHAS of *B. napus* (W557L of AHAS 3, Ouellet *et al.*, 1994; Hattori *et al.*, 1995), cocklebur (W552L, Bernasconi *et al.*, 1995) and cotton (W563C and W563S of the A5 and A19 gene products respectively, Rajasekaran *et al.*, 1996). Using crude plant extracts, these mutations were shown to result in AHAS activity with enhanced resistance (10- to 10,000-fold) to the different classes of herbicides. Similar levels of resistance are also observed in plant growth. Furthermore, it was observed that different substitutions at this residue cause different herbicide-resistance phenotypes in cotton (Rajasekaran *et al.*, 1996). The W563C mutation results in AHAS activity and callus growth that are about 8-fold more resistant to primisulfuron than the W563S substitution. The mutation of this tryptophan residue to leucine does not seem to affect the apparent sensitivity of AHAS to branched-chain amino acid inhibition (Hattori *et al.*, 1995; Bernasconi *et al.*, 1995). Using highly purified *A. thaliana* AHAS catalytic subunit, it was shown that different mutations at this site result in enzymes with different levels of herbicide resistance as well as varying kinetic properties (Chang and Duggleby, 1998). The W574 residue of the *A. thaliana* AHAS was mutated to either a serine or leucine. The W574S mutant is characterized as having only 50% of the wild-type activity and 2-fold reduction in the affinity for pyruvate. On the other hand, the W574L mutant has a specific activity double that of the wild-type but a 5-fold increase in the K_m value for pyruvate. Both mutants also differ in their resistance to sulfonyleurea and imidazolinone herbicides. In general, the W574S mutation results in an enzyme that is more resistant to herbicides and especially the imidazolinones. Differences in kinetic properties and herbicide sensitivity are also observed in mutants of *E. coli* AHAS II (Ibdah *et al.*, 1996).

S653

A mutation at S653 was first discovered by Hattori *et al.*

(1992) in imidazolinone-resistant *A. thaliana* (Haughn and Somerville, 1990). This residue is not totally conserved across species; it is a serine residue in most wild-type plant AHAS but an alanine in the cocklebur enzyme, glycine in the yeast enzyme and in *E. coli* AHAS III, and a proline in *E. coli* AHAS I and II (Sathasivan *et al.*, 1991; Bernasconi *et al.*, 1995). In addition to resistance to imidazolinones, mutation at this site is characterized by cross-resistance to pyrimidyl oxybenzoates but not to sulfonyleureas and triazolopyrimidines (Sathasivan *et al.*, 1991; Mourad and King, 1992). Both the growth and AHAS activity of the *A. thaliana* S653N mutant is 100- to 1000-fold resistant to imidazolinones and pyrimidyl oxybenzoates. This residue was not reported as one of the ten resistance sites in yeast AHAS (Falco *et al.*, 1989). This is not surprising as the latter mutations were selected by their resistance to a sulfonyleurea.

This mutation has been studied in detail using highly purified mutant *A. thaliana* catalytic subunit (Chang and Duggleby, 1998; Lee *et al.*, 1999). The serine residue was mutated to different amino acids with varying size ranging from alanine to phenylalanine. All the purified mutant enzymes resemble the wild-type enzyme in terms of their kinetic properties. Herbicide inhibition studies suggested that the β -hydroxyl group of the wild-type serine is not required for imidazolinone binding, and the size of the amino acid side chain at this position determines resistance. The alanine mutant remains sensitive to sulfonyleureas and imidazolinones, while S653T, S653N and S653F mutations result in enzymes with 100-fold or more resistance to imidazolinones and the last mutant also shows weak resistance (5- to 10-fold) to sulfonyleureas. Chimeric *A. thaliana* genes containing both the P197S and S653N mutations have also been constructed (Hattori *et al.*, 1992; Mourad *et al.*, 1994). The double mutant shows cross resistance to the four classes of herbicides mentioned in Table 4. While each mutation by itself has little or no effect on branched-chain amino acid sensitivity, the chimeric mutant shows a reduction in the end-product inhibition, demonstrated in both AHAS activity assays of crude extracts and in plant growth (Mourad *et al.*, 1995).

Herbicide-binding site The structure and natural function of the herbicide-binding site of AHAS is still largely a mystery. Residues that, when mutated, result in herbicide-resistance are highly conserved in almost all wild-type enzymes, but none of them appear to be involved in the catalytic mechanism or regulation of AHAS. The site where herbicides bind is capable of binding a wide variety of compounds of unrelated structure, resulting in inhibition of the enzymatic reaction. One explanation for the presence of such a site is that first proposed by Schloss *et al.* (1988) based on the similarities between AHAS and POX. As discussed earlier, this is also a ThDP-dependent enzyme, it has a primary sequence which shows significant homology (25-30%) to that of AHAS (Chang and Cronan, 1988), and the first step in the reaction of both enzymes (decarboxylation of

pyruvate) is the same. Unlike AHAS, POX requires as an additional substrate ubiquinone-40 (Q_8), which acts as an electron acceptor. The herbicide-binding site of AHAS was thus proposed to be an evolutionary vestige of the quinone-binding site. Experimental data supporting this notion include the observation that the ubiquinone homologs Q_0 and Q_1 are fairly good inhibitors of *E. coli* AHAS II (K_i in low mM range), and these compounds compete with sulfonyleurea and imidazolinone herbicides for binding to the enzyme. This evolutionary relationship between the two enzymes also provides an explanation for the FAD requirement in the non-redox reaction catalyzed by AHAS.

Due to the lack of an experimental three-dimensional crystal structure of AHAS, it is very difficult to confirm the nature of the herbicide-binding site, and design rationally more potent herbicidal inhibitors or better herbicide-resistant mutants of AHAS. However the crystallographic structure of POX from *Lactobacillus plantarium* had been solved with high resolution (Muller and Schulz, 1993, Muller *et al.*, 1994). With this information, attempts have been made to construct three-dimensional models of bacterial (Ibdah *et al.*, 1996) and plant (Ott *et al.*, 1996) AHAS. As POX does not have a subunit equivalent to the AHAS small subunit, these models consist of the catalytic subunit dimer only. These models were used to predict residues involved in catalysis and to locate those involved in herbicide binding. All of the ten of the yeast sulfonyleurea-resistance mutation sites (Falco *et al.*, 1989) are found to coalesce to form a proposed herbicide-binding site as illustrated in Fig. 18 for the *E. coli* AHAS II model. The models suggest that the herbicides binds near to the entry site for the substrates and in close proximity to the bound cofactors ThDP and FAD (Ott *et al.*, 1996). Furthermore, it has been shown experimentally that sulfometuron methyl binds near to the FAD binding site. Binding of the inhibitor to *E. coli* AHAS II causes spectral perturbation of the enzyme-bound FAD (Schloss, 1984; Schloss, 1990).

Although the presumed herbicide-binding site in these models is not remote from the active site, it is our opinion that inhibition by herbicides is not a result of any direct interference with the active site. We note that the herbicide-binding site is at the catalytic subunit interface, as is the active site. Thus we suggest that when herbicides bind this perturbs the subunit interface and this, in turn, alters the geometry of the active site leading to the observed loss of catalytic activity.

Conclusions and future directions

Over the past 20 to 30 years, AHAS has risen from relative obscurity to become a major focus of research. To a large extent, this interest has been driven by the discovery that the enzyme is the target site of several commercial herbicides. Nevertheless, despite this intensive study, the inhibition of the enzyme by herbicides, and many other features of its structure, mechanism and regulation, are not properly understood. One factor that has impeded our understanding is

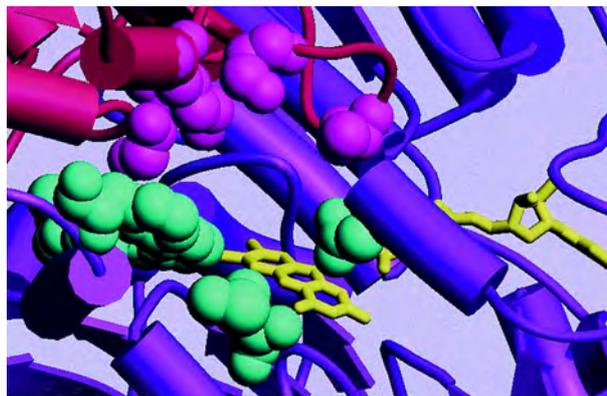


Fig. 18. Structure of *E. coli* AHAS II around the proposed herbicide-binding site. Sections of the secondary structure derived from neighboring catalytic subunits are shown in red and purple. The residues where mutation results in herbicide resistance (Table 1) are shown as pink (red subunit) or green (purple subunit) spheres. FAD is shown in yellow. Coordinates of *E. coli* AHAS II were calculated by Ibdah *et al.* (1996).

the lack of an experimentally-determined three-dimensional structure of the enzyme from any source. In part this is due to the difficulty in obtaining significant quantities of the protein from its native source, coupled with its inherent instability. The development of systems for over-expression of recombinant AHAS is beginning to overcome the former problem, while a better understanding of the instability is now allowing the enzyme to be maintained in an active form.

There now appears to be no fundamental reason why the enzyme cannot be crystallized and its three-dimensional structure determined. This will then allow answers to many remaining questions.

What is the basic enzyme structure and to what extent does it resemble related enzymes such as pyruvate oxidase and pyruvate decarboxylase?

Which residues are present in the active site and what does this tell us about the catalytic mechanism?

What is the role of the obligatory cofactor, FAD?

How do the catalytic and regulatory subunits interact to promote the catalytic activity?

Where on the enzyme do sulfonyleurea herbicides bind, and how does occupancy of this binding site result in enzyme inhibition?

Is the imidazolinone herbicide-binding site coincident with, overlapping, or entirely separate from the sulfonyleurea-binding site?

How and where do feedback inhibitors such as valine bind, and how does binding result in inhibition?

How and where does ATP bind and what is the mechanism by which ATP reverses valine inhibition?

We look forward to learning answers to these questions over the next few years.

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