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Branched-Chain Amino Acids

Miroslav Pátek

Institute of Microbiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, 14220 Prague 4, Czech Republic
patek@biomed.cas.cz

Abstract The branched-chain amino acids (BCAAs) leucine, isoleucine, and valine are synthesized by bacteria, fungi, and plants, but are essential for vertebrates including humans, who must receive them from their diet. The interest to construct overproducing industrial strains therefore stems from the need to supplement the food or feed with these amino acids to use them in medical treatment and as precursors in biochemical synthesis. Regulation of the biosynthesis pathways of branched-chain amino acids has many features, such as homologous reactions catalyzed by a single enzyme, branching of the pathways and multivalent regulation of both gene expression and enzyme activity, which make their analysis both interesting and challenging. The structural similarity of these three amino acids and their precursors causes their alternative binding to the proteins

as substrates, inhibitors, activators, and passengers in transporters with different affinities. Studies of threonine deaminase, the first enzyme specific for isoleucine biosynthesis, and of acetohydroxyacid synthase, the first common enzyme in the pathways of BCAA biosynthesis, promoted the discovery of feedback inhibition and may serve as paradigms for this regulatory mechanism. Regulation of the *Escherichia coli* operons *ilvGMEDA* and *ilvBN* provides examples of typical translation-mediated transcriptional termination (attenuation). Mechanisms of regulation by the seemingly similar structures found in *ilvBNC* operon and *leuA* gene of the industrial amino acid producer *Corynebacterium glutamicum* still have to be unveiled. A wide range of different specific and global regulatory mechanisms being gradually uncovered in various microorganisms will contribute to the knowledge of genetic control of BCAA biosynthesis.

1 Introduction

The branched-chain amino acids (BCAAs), leucine, isoleucine and valine, are the most hydrophobic amino acids due to their unsubstituted aliphatic chain with a branched alkyl group (Fig. 1). These BCAAs are often located in the core of the proteins and play a crucial role in determining the structures of globular proteins as well as in the interaction of the transmembrane domains of membrane proteins with phospholipid bilayers. In terms of protein secondary structure, valine and isoleucine exhibit a definite preference for the β-structure, whereas leucine has a higher preference for the α-helix. BCAAs, which are essential for human and other vertebrates, are synthesized de novo by most bacteria, fungi, and plants.

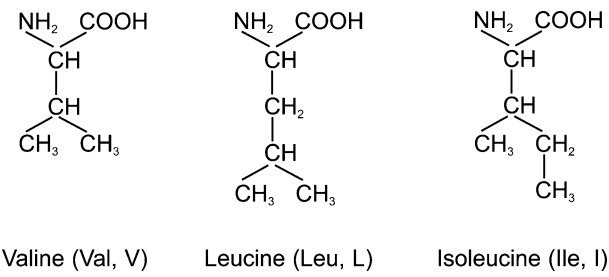

Fig. 1 Chemical structures of branched-chain amino acids

Biosynthesis pathways of isoleucine, valine and leucine (all in their L-forms) are closely tied, and run in most organisms as shown in Fig. 2. Valine and isoleucine are synthesized in parallel pathways in which homologous reaction steps are catalyzed by the same enzymes. Leucine is formed in a specific series of reactions branching off from the valine pathway, thus, sharing common precursors with valine. A complex regulatory network, which controls the metabolite flux through these pathways in bacteria, includes mul-

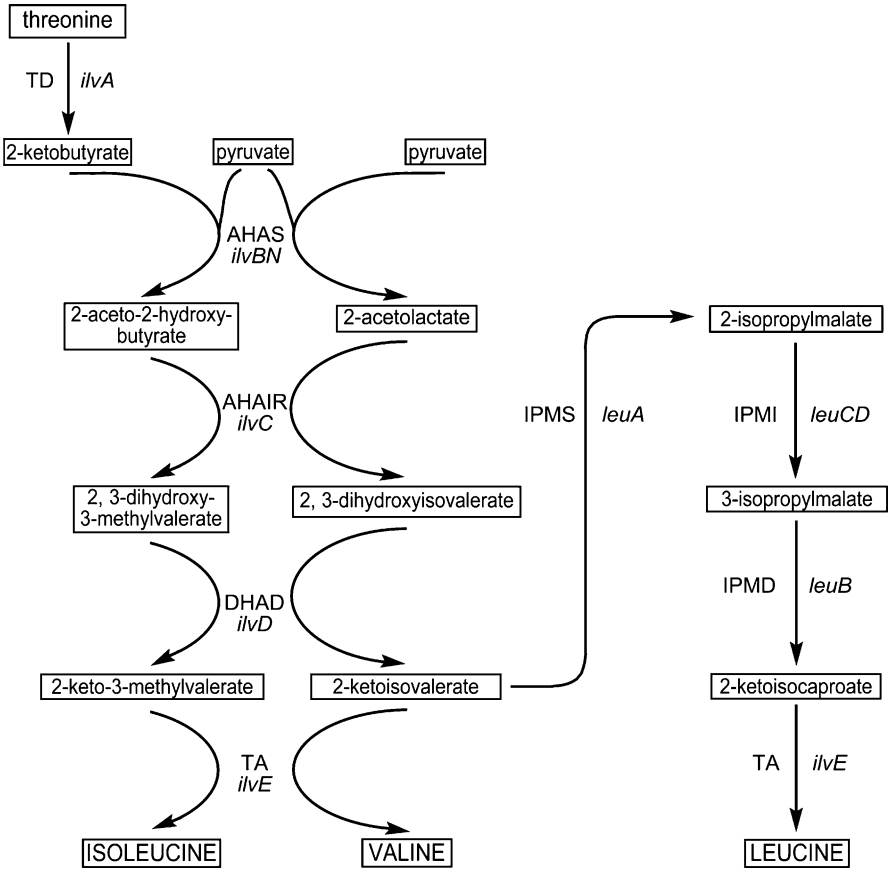

Fig. 2 Biosynthesis of isoleucine, valine, and leucine in bacteria. Enzymes and the corresponding genes: TD (*ilvA*), threonine deaminase; AHAS (*ilvBN*), acetohydroxyacid synthase; AHAIR (*ilvC*), acetohydroxyacid isomeroreductase; DHAD (*ilvD*), dihydroxyacid dehydratase; TA (*ilvE*), transaminase, IPMS (*leuA*), isopropylmalate synthase; IPMI (*leuCD*) isopropylmalate dehydratase; IPMD (*leuB*) isopropylmalate dehydrogenase. The genes are designated as those in *C. glutamicum*. The other AHAS isoenzymes are encoded by *ilvIH* and *ilvGM* in *E. coli*. Other transaminases active in BCAA pathways are encoded by *aroT*, *avtA* and *pdxR* in *C. glutamicum* and by *avtA* and *tyrB* in *E. coli*

tivalent regulation of both gene expression and enzyme activity. Regulatory circuits are also complicated by the structural similarity of branched-chain amino acids which mimic each other in their abilities to bind to the catalytic or regulatory sites of the proteins.

Binding of these near-isomorphic amino acids varies only with the different affinities of the respective proteins for the individual amino acids and this blurred recognition has a consequence in metabolic antagonism of the three BCAAs (Umbarger 1996). This review is focused on the synthesis of BCAAs

and its regulation in *Escherichia coli* (or *Salmonella enterica* serovar Typhimurium) and in *Corynebacterium glutamicum* as well as on their biotechnological production. *E. coli* is a model organism in which the regulation of the pathways has been most thoroughly described, whereas *C. glutamicum* is the industrial microorganism frequently used for manufacturing amino acids by fermentation processes.

2 Isoleucine and Valine

Since the structures of valine and isoleucine, as well as of the respective intermediates of their biosynthesis, differ only by a single methylene group, four reactions of their biosynthesis pathways are catalyzed by common enzymes.

2.1 Regulation of Enzyme Activity

The major roles in regulation of isoleucine and valine biosynthesis are played by threonine deaminase (converting threonine into 2-ketobutyrate), which is strongly inhibited by isoleucine and acetohydroxyacid synthase, which has higher affinity to 2-ketobutyrate than to pyruvate and thus favors the reaction within the isoleucine biosynthesis pathway. In this way, balancing the levels of valine and leucine on one side and isoleucine on the other, is ensured. The enzymes of the pathways have been studied particularly in plants and fungi as potential targets for herbicides and fungicides (McCourt and Duggleby 2006). Mutagenesis studies in yeast confirmed that the herbicides (which resemble neither the substrates nor feedback inhibitors) bind neither to the active site nor to the allosteric site (Duggleby et al. 2003). Structural and regulatory properties of the major enzymes of the pathways in gram-positive bacteria are similar to that from *E. coli* or *C. glutamicum* described here. The activity of the single acetohydroxyacid synthase in Gram-positive bacteria, such as in bacilli (Porat et al. 2004) or mycobacteria (Zohar et al. 2003), is essentially regulated as acetohydroxyacid synthase III from *E. coli*.

2.1.1 Threonine Deaminase (TD)

Escherichia Coli

The enzyme with the systematic name L-threonine ammonia-lyase (EC 4.3.1.19) is referred to as threonine deaminase or threonine dehydratase. This enzyme utilizes pyridoxal-5′-phosphate as a cofactor and catalyzes the con-

version of threonine to 2-ketobutyrate. It is the only enzyme specific for isoleucine synthesis (Fig. 2). The end product of the pathway, isoleucine, acts as an allosteric effector and exerts inhibition of TD activity, while valine, the end product of the parallel pathway, acts as its activator in both *E. coli* and *S. enterica* serovar Typhimurium (Changeux 1963; Eisenstein 1991). These heterotropic effectors bind to the site that is distinct from the active site in the molecule of the enzyme. Threonine deaminase can therefore serve as a useful model for structure-based mechanism of allostery. The early studies of threonine deaminase (Changeux 1961) actually influenced formation of a model of allosteric regulation of enzyme activity. Threonine deaminase of *E. coli* forms tetramers with identical subunits and cooperative binding of effectors (Eisenstein et al. 1994). The model assumed that binding of a single inhibitor or substrate or activator to a subunit would favor binding of an identical ligand molecule to the other subunits. This notion proposes equilibrium between the active and inactive form of the enzyme in which all subunits of the tetramer bind the same effector. Mutagenesis studies showed that the amino-terminal domain of TD is involved in catalysis whereas the carboxy-terminal domain is important for its regulation (Fisher and Eisenstein 1993).

Corynebacterium Glutamicum

TD from *C. glutamicum*, formed by four identical subunits of 47 kDa, is (according to its sequence) highly similar (59 to 78% identity) to TDs found in related nocardioform bacteria (corynebacteria, mycobacteria, nocardiae and rhodococci), but much less similar to TDs of Gram-negative bacteria like *E. coli*. While the N-terminal part shows 35% identity with that part of *E. coli* TD, the C-terminal part is shorter than its *E. coli* counterpart and differs substantially in sequence, although it is also involved in the allosteric regulation of the enzyme activity (Möckel et al. 1992). As in *E. coli*, *C. glutamicum* threonine deaminase is inhibited by isoleucine and activated by valine. The $K_{0.5}$ for threonine is 21 mM and it increases to 78 mM or decreases to 12 mM in the presence of isoleucine or valine, respectively.

The site-directed mutagenesis of the *ilvA* gene coding for TD resulted in the abolishment of feedback inhibition (Möckel et al. 1994). These mutations resided in the C-terminal part of the polypeptide, which represents the regulatory domain of the enzyme, spatially separated from the catalytic domain according to the 3D model of the threonine deaminase structure. Other random mutations decreasing the inhibitory effect of isoleucine as well as the basic enzyme activity were also located in the C-terminal part (residues within the region 266–349). The location of the mutations thus supported the assignment of the regulatory domain to the C-terminal part of the protein. However, the enzyme is apparently not of a modular structure and most probably interactions of the catalytic and regulatory domains are required for its proper function (Möckel et al. 1994).

2.1.2 Acetohydroxyacid Synthase (AHAS)

Acetohydroxyacid synthase (EC 2.2.1.6), also called acetolactate synthase, is the first common enzyme in the pathways of BCAA biosynthesis (Fig. 2). It catalyzes decarboxylation of pyruvate and its condensation either with another molecule of pyruvate to produce acetolactate (a precursor of valine and leucine) or with 2-ketobutyrate to produce acetohydroxybutyrate (a precursor of isoleucine). AHAS has been studied in plants as a target for herbicides (imidazolines and sulfonylureas), which has encouraged determination of the 3D structure of the protein (McCourt and Duggleby 2006). The AHAS catalytic subunit from *Arabidopsis thaliana* was crystallized (McCourt et al. 2005) and its structure was described in the presence of all three cofactors and each of five sulfonylurea herbicides (McCourt and Duggleby 2006). The crystal structure of the AHAS catalytic subunit from *Saccharomyces cerevisiae*, which is considered a suitable model for plant AHAS, was also analyzed (Pang et al. 2002). The structure and role of AHAS in biosynthesis of BCAAs in fungi and plants has recently been reviewed (McCourt and Duggleby 2006).

Escherichia Coli

Three AHAS isoenzymes, differing in their genetic determination, biochemical properties, and regulation of both biosynthesis and activity (Table 1), were described for wild-type strains of *E. coli* and *S. enterica* serovar Typhimurium (Umbarger 1996). The isoenzymes AHAS I, II, and III are composed of large subunits and small subunits encoded by the genes *ilvBN*, *ilvGM*, and *ilvIH*, respectively. The large subunits (IlvB, IlvG, and IlvI) are responsible for catalytic activity of the three isoenzymes, while the small subunits (IlvN, IlvM and IlvH) mediate their inhibition. The differences between the AHAS isoenzymes are based particularly on the size, sequence, and properties of regulatory subunits, whereas the catalytic subunits are much more similar to each other. The catalytic subunits exhibit about 40% sequence identity between any two isoenzymes and are of similar size (about 60 kDa), in contrast to the

Table 1 Characteristics of *E. coli* AHAS isoenzymes

Isoenzyme	Genes	Inhibition	R [a]	Repression
AHAS I	*ilvBN*	Val	2	Val, Leu
AHAS II	*ilvGM*	–	60	Val, Ile, Leu
AHAS III	*ilvIH*	Val, Ile, Leu	40	Leu

[a] A specific ratio of alternative products (acetohydroxybutyrate and acetolactate) formed in homologous reactions catalyzed by AHAS, defined in Eq. 1

regulatory subunits, which are far more diverse in sequence and size (11 kDa, 9.5 kDa, and 18 kDa, respectively) (Ibdah et al. 1996). Although the catalytic machinery is entirely contained within the large subunits, they have much lower activity (which is insensitive to inhibition), when separated from the small subunits (Weinstock et al. 1992). AHAS catalytic subunits thus require the presence of regulatory subunits for full activity and for regulation. Active holoenzymes may be reconstituted from the respective isolated subunits. Due to the specific interactions, the heterologous combinations of large and small subunits provide no detectable activity, despite their sequence similarities (Weinstock et al. 1992). Activity of AHAS depends on thiamine diphosphate (ThDP) and on flavin adenine dinucleotide (FAD). The large (catalytic) subunits show significant sequence similarities to pyruvate oxidase (EC 1.2.3.3) and other ThDP-dependent enzymes catalyzing reactions in which the first step is decarboxylation of a 2-ketoacid (Bar-Ilan et al. 2001).

The activities of AHAS I and AHAS III are inhibited by valine, while AHAS II is valine-resistant. A striking feature of AHAS II and AHAS III is their much higher affinity to 2-ketobutyrate than to pyruvate. In the presence of 2-ketobutyrate, synthesis of isoleucine is therefore preferred. To determine the rates of the formation of the alternative products (acetolactate and acetohydroxybutyrate), an analytical method for simultaneous assaying of the two separate reactions was developed (Gollop et al. 1987). A specific ratio of the two products was defined (Barak et al. 1987):

$$R = \frac{(\text{rate of acetohydroxybutyrate formation})/(\text{rate of acetolactate formation})}{[\text{2-ketobutyrate}]/[\text{pyruvate}]} . \qquad (1)$$

The parameter R is constant for a particular isoenzyme over a wide range of concentrations. However, it depends to some degree on the conditions of its experimental determination. The larger the value of R, the stronger the preference of the isoenzyme for 2-ketobutyrate (Table 2).

Many microorganisms possess only a single AHAS, which is predominantly similar to AHAS III (IlvIH) in its sequence. The respective orthologous genes should therefore be designated *ilvIH*, although they have been named *ilvBN* in some cases (e.g., in *C. glutamicum*). Orthologs of *ilvBN* encoding AHAS I are present only in enterobacteria. In the most commonly used strain of *E. coli*, K-12, the valine-insensitive isoenzyme (AHAS II) activity is missing due to a frameshift mutation in the *ilvG* gene (Lawther et al. 1981). This is the reason why the growth of K-12-derived strains is inhibited by higher concentrations of valine if isoleucine is missing in the medium.

AHAS I is markedly different from the other two AHAS isoenzymes in its sequence and, consequently, in its properties (Vinogradov et al. 2006). This isoenzyme shows only a two-fold preference for 2-ketobutyrate over pyruvate ($R \sim 2$), whereas AHAS II and III show 60-fold and 40-fold higher affinities to 2-ketobutyrate than to pyruvate, respectively (Gollop et al. 1989;

Vinogradov et al. 2006). AHAS I exhibited the highest activity of the three isoenzymes in vitro (ratio of specific activities AHAS I: AHAS II: AHAS III in $\mu mol\ min^{-1}\ mg^{-1}$ was 60 : 20 : 2.6) (Gollop et al. 1989; Vinogradov et al. 2006). However, AHAS I can be completely inhibited by valine even in the presence of high concentrations of pyruvate, whereas AHAS III is only partially inhibited by valine. Separated from the regulatory subunit IlvN, the catalytic subunit IlvB has 10 to 20% activity of the holoenzyme, in contrast to the large subunit IlvI, which has alone only 3 to 5% activity of the IlvI–IlvH holoenzyme (Vyazmensky et al. 1996). Synthesis of AHAS I is induced when the available carbon source is poor (e.g., acetate, succinate), since expression of the *ilvBN* operon is regulated by carbon catabolite repression (Sutton and Freundlich 1980; Tedin and Norel 2001). All these unique features suggest that AHAS I fulfils a special metabolic role in *E. coli* (Vinogradov et al. 2006). The AHAS I isoenzyme ensures the synthesis of valine and leucine under conditions, when the intracellular concentration of pyruvate is low (Dailey and Cronan 1986). Thus, AHAS I is important for growth on other substrates than glucose and provides *E. coli* and other enterobacteria with the ability to adapt to a wider range of carbon sources.

AHAS II is insensitive to inhibition by any of the BCAA (Lawther et al. 1987). This isoenzyme therefore sustains growth of the cells in the presence of excess valine, which inhibits activity of both AHAS I and AHAS III (McCourt and Duggleby 2006). AHAS isoenzymes have 26–30% sequence identity with pyruvate oxidases (EC 1.2.3.3) and an evolutionary relationship of these proteins may explain their peculiar requirement for FAD, which is not directly involved in catalysis. A 3D model of the catalytic subunit of AHAS II from *E. coli* could be created (Ibdah et al. 1996) by homology modelling on the basis of sequence similarity with pyruvate oxidase (Muller and Schulz 1993). The structural model suggests which parts of the protein dimer are involved in coenzyme binding and in substrate specificity. The replacement of Trp464 with other AA by site-directed mutagenesis confirmed its proposed role in preference of the isoenzyme for 2-ketobutyrate (Ibdah et al. 1996).

AHAS III, which has orthologs in those microorganisms possessing only a single AHAS, seems to represent an archetype of this enzyme. A structural model of its regulatory subunit (IlvH) is based on the mutagenesis of *ilvH* resulting in abolishment of the ability of IlvH to bind valine (Vyazmensky et al. 1996; Mendel et al. 2001). In addition to changes in valine binding, some mutants of the small subunit had altered affinity to the large subunit. The similarity search showed that the residues, whose mutation (e.g., Gly-14-Asp) abolished the valine-mediated inhibition of AHAS, are conserved in all regulatory subunits of AHAS in bacteria which possess only a single valine-sensitive AHAS. Mutation Asn-11-Ala (constructed on the basis of the IlvH model) resulted in a valine-resistant enzyme, as expected. IlvH of *E. coli* and a number of regulatory subunits from other microorganisms (e.g., from streptomycetes, corynebacteria, and yeast) belong, according to the se-

quence homology and folding, to the ACT sequence family, which includes regulatory domains of 3-phosphoglycerate dehydrogenase (EC 1.1.1.95) and aspartokinase (EC 2.7.2.2.4) (Mendel et al. 2001). It is noteworthy that IlvM, the regulatory subunit of the valine-insensitive AHAS II, is not a member of this family. Deletion studies of the regulatory subunit IlvH showed that its N-terminal portion (80 of 163 AAs) is sufficient for recognition and activation of the catalytic subunit. The C-terminal part is required for valine binding and inhibition (Mendel et al. 2003). The subunits IlvI and IlvH were isolated separately and the holoenzyme was reconstituted (Vyazmensky et al. 1996, 2000), which confirmed the stoichiometry of 1 : 1. The active sites in the holoenzyme (2 catalytic + 2 regulatory subunits) are located at the interface of a dimer of the catalytic subunits, which is stable only in the presence of the regulatory subunits (Mendel et al. 2001). Among the regulatory subunits of bacterial AHASs it is IlvH from *E. coli*, whose crystal structure was first described (Kaplun et al. 2006). The crystal structure of IlvH indicates that the positively charged C-terminal domain of the small subunit interacts with the negatively charged surface of the large subunit and that the valine-binding sites are located in two symmetrical positions in the interface between a pair of N-terminal ACT domains of the small subunits (Kaplun et al. 2006).

Enterobacteria living in a feast-or-famine style are equipped with three AHAS isoenzymes constituting a flexible set of enzymes with differences in (1) regulation of gene expression, (2) enzyme activity and its inhibition, (3) stability, (4) substrate affinity, and (5) response to stress and to carbon and nitrogen source availability. This set of isoenzymes allows the bacteria to cope with any growth conditions and produce the three amino acids prevalent in proteins in an appropriate level.

Corynebacterium Glutamicum

Only a single AHAS was found in *C. glutamicum* (Eggeling et al. 1987). As in other organisms, the active holoenzyme is composed of two large (catalytic) subunits and two small (regulatory) subunits. The subunits are encoded by the genes *ilvB* and *ilvN*, respectively, which form an operon together with *ilvC*, which codes for AHAIR (Keilhauer et al. 1993). The large subunit IlvB shows the highest degree of similarity (except for other corynebacteria) to the analogous proteins from nocardioform actinomycetes: *Nocardia farcinica*, *Rhodococcus* sp. and mycobacteria (69 to 65% identity). There is a 45% identity shared with the *E. coli* AHAS III large subunit (IlvI). The degree of identities shared with the large subunits of AHAS II (IlvG) and AHAS I (IlvB) from *E. coli* is 45 and 44%, respectively. As to the small (regulatory) subunit (172 AAs coded by *ilvN*) of *C. glutamicum* AHAS, the analogous proteins from nocardioforms show 51 to 48% identity. The AHAS III small subunit (IlvH) of *E. coli* exhibits 39% identity, while the much shorter AHAS I IlvN subunit (96

AAs) is identical at 30% of its residues. No obvious similarity could be recognized with the AHAS II IlvM subunit, which is only about half in size (86 AAs) and does not mediate sensitivity to valine.

Activity of *C. glutamicum* AHAS can be inhibited by any of the three BCAAs (Eggeling et al. 1987; Elišáková et al. 2005), with valine as the strongest inhibitor. The concentrations of the amino acids required for 50% inhibition (IC_{50}) were 0.9 mM for valine, 3.1 mM for isoleucine and 6.0 mM for leucine. In the presence of 5 mM amino acid, the inhibition was 56% (valine), 49% (isoleucine) and 48% (leucine), respectively. It should be noted that inhibition by any combination of two amino acids or by all three amino acids did not exceed 57% (Elišáková et al. 2005).

Similar to AHAS II and AHAS III from *E. coli* (Umbarger 1996), *C. glutamicum* AHAS has a much higher affinity to 2-ketobutyrate than to pyruvate (Eggeling et al. 1987). In the presence of 2-ketobutyrate, synthesis of isoleucine is therefore preferred, and synthesis of valine and leucine is reduced. Accordingly, high 2-ketobutyrate concentrations (100 mM) resulted in valine and leucine deficiency (Eggeling et al. 1987). The ratio *R* (Eq. 1), calculated on the basis of these results (Eggeling et al. 1987), was approximately 20 to 25, which is closest to the ratio for AHAS III from *E. coli* ($R = 40$).

Site-directed mutagenesis of the regulatory subunit was performed to remove the inhibition of AHAS in *C. glutamicum* (Elišáková et al. 2005). The bases within *ilvN* to be altered were selected according to the alignment of amino acid sequence of *C. glutamicum* IlvN with sequences of homologous subunits of *E. coli* and *Streptomyces cinnamonensis* mutants. Amino acid alterations in the mutants *E. coli ilvH** (Mendel et al. 2001) and *S. cinnamonensis ilvN** (Kopecký et al. 1999), which resulted in AHASs resistant to inhibition by valine, were found within the conserved N-terminus of the regulatory subunits. Alteration of three consecutive AAs in the N-terminus of *C. glutamicum* IlvN resulted in a mutant AHAS entirely resistant to inhibition by all three BCAAs (Elišáková et al. 2005). The results indicate that there is a single binding (allosteric) site for the three BCAAs at the enzyme molecule, which exhibits different affinities to the individual amino acids.

2.1.3 Acetohydroxyacid Isomeroreductase (AHAIR)

The active AHAIR (EC 1.1.1.86) of *E. coli* is a tetramer with identical 53-kDa subunits. It catalyzes the conversion of acetohydroxyacids into dihydroxyacids (Fig. 2). The reaction including alkyl isomeration and reduction requires Mg^{2+} and NADPH as the hydrogen donor. The enzyme is inhibited by valine and leucine in *C. glutamicum* ($IC_{50} = 7$ mM for both AAs) (Leyval et al. 2003). The protein encoded by the *ilvC* gene was found to display low ketopantoate reductase (EC 1.1.1.169) activity in *E. coli*, being able to substitute for the *panC* gene product (Elischewski et al. 1999) by converting

2-ketopantoate to pantoate. Moreover, IlvC is the only protein showing activity in this reaction of pantothenate synthesis pathway in *C. glutamicum* (Merkamm et al. 2003). Interestingly, the IlvC was identified in two variants differing by *pI* among the phosphorylated proteins of *C. glutamicum* in phosphoproteome analysis (Bendt et al. 2003). Similarly to AHAS, AHAIR is considered a promising target for the design of antituberculosis agents (Grandoni et al. 1998) and herbicides (Wang et al. 2005). New compounds that mimic the structure of intermediates metabolized by AHAIR are therefore designed and tested as competitive inhibitors (Wang et al. 2005).

2.1.4 Dihydroxyacid Dehydratase (DHAD)

The third step of valine and isoleucine biosyntheses, conversion of dihydroxyacids to 2-ketoacids, is catalyzed by dihydroxyacid dehydratase (EC 4.2.1.9) (Fig. 2). The *E. coli* enzyme encoded by the *ilvD* gene is a dimer of 66-kDa subunits. The apoprotein of *E. coli* DHAD, which contains a [4Fe – 4S] cluster essential for catalysis, was used after inactivation of the enzyme in vivo by hyperbaric oxygen to study Fe – S cluster biosynthesis involved in its reactivation (Flint et al. 1996). *C. glutamicum* DHAD is weakly inhibited by valine and leucine with IC_{50} values of 170 mM and 120 mM, respectively. For both DHAD and AHAIR, no cooperative inhibition was observed in the presence of two or all three BCAAs (Leyval et al. 2003).

2.1.5 Transaminases (TAs)

Transaminases typically catalyze the final step in biosynthesis of AAs. A number of these pyridoxal-5′-phosphate-dependent enzymes with both narrow and overlapping substrate specificities developed probably by the functional specialization of primeval enzymes with broad range of activities. In contrast to the other enzymes of BCAA biosynthesis, TAs also occur in animals where they participate in BCAA catabolism.

Transaminases B and C and aromatic transaminase (encoded by *ilvE*, *avtA* and *tyrB*, respectively) are active in BCAA synthesis in *E. coli*. Transaminase B (EC 2.6.1.6) is the main enzyme fulfilling this function for all three BCAAs in *E. coli*. According to the study of its crystal structure, the branched-chain amino acid TA is a trimer of dimers made up of six identical subunits of 32 kDa (Goto et al. 2003). Although the reactions catalyzed by transaminase B are reversible, the balance of the reaction greatly favors the direction to BCAA synthesis. The enzyme produces isoleucine most efficiently of the three BCAAs. Whereas transaminase B uses glutamate as an amino group donor, the alternative transaminase C (EC 2.6.1.66) uses alanine or 2-aminobutyrate (Umbarger 1996) in valine synthesis. This alanine-valine transaminase en-

sures sufficient synthesis of valine in *ilvE* mutants and even sufficient synthesis of isoleucine (and weak synthesis of leucine) when the *avtA* gene is present in high copy number (Berg et al. 1988). The third TA involved in BCAA synthesis, aromatic transaminase (EC 2.6.1.57), catalyzes efficient conversion of 2-ketoisocaproate to leucine (Powell and Morrison 1978; Vartak et al. 1991), in addition to its function in formation of the aromatic AAs.

Based on the complete *C. glutamicum* genome sequence, comprehensive knowledge of TAs in *C. glutamicum* has been gathered by the systematic analysis of all putative TA genes (McHardy et al. 2003) and of in vitro enzymatic activities of the respective isolated proteins (Marienhagen et al. 2005). Deletion studies (McHardy et al. 2003) confirmed that TA encoded by *ilvE* (Radmacher et al. 2002) is the main enzyme catalyzing the final step in biosynthesis of all three BCAAs (Leyval et al. 2003). Pairwise sequence comparisons of presumed protein family members with the motif-based approach resulted in identification of the *pdxR* gene product, which is active in valine biosynthesis (McHardy et al. 2003). In addition to the aminotransferase domain, the PdxR protein possesses a motif described in the MocR subfamily of GntR transcriptional regulators. According to its role in pyridoxine biosynthesis, the PdxR may function also as a transcriptional activator of the respective genes (McHardy et al. 2003). Assays of purified AvtA revealed that this protein provides a strong activity in valine synthesis in *C. glutamicum*, as in *E. coli*, and a low activity in isoleucine synthesis using alanine as the amino donor (Marienhagen et al. 2005). In another line of similarity to *E. coli*, corynebacterial aromatic TA encoded by *aroT* also showed detectable activity in leucine formation. In conclusion, only IlvE ensures sufficient synthesis of isoleucine and leucine to sustain significant growth, whereas both IlvE and AvtA are sufficient for substantial valine synthesis. The role of *pdxR* is not completely clear. The activities of other TAs involved in BCAA synthesis alone would not secure synthesis of any of the BCAAs in quantities sufficient for growth.

2.2 Regulation of Gene Expression

The enterobacteria possess three isoenzymes of AHAS, encoded by distinct and differently regulated operons (Umbarger 1996). Expression of the respective genes is subject to multivalent repression by branched-chain amino acids. In *E. coli*, expression of the operons *ilvGMEDA* and *ilvBN* is controlled by transcriptional attenuation, while transcription of *ilvIH* is regulated by the leucine-responsive protein. In most Gram-positive bacteria, the genes for the first two enzymes of the pathways (AHAS and AHAIR) belong to the same operon (*ilvBNC*), as in streptomycetes (De Rossi et al. 1995), corynebacteria or in mycobacteria (Gusberti et al. 1996), often together with the *leu* genes (*ilvBNC-leuACBD*), as in *Bacillus subtilis* (Zahler et al. 1991). A wide variety

of molecular machineries regulating expression of *ilv* and *leu* operons occur in microorganisms. These regulatory mechanisms include classical attenuation in proteobacteria (Vitreschak et al. 2004a), a modified transcriptional control in actinobacteria like *C. glutamicum* (Seliverstov et al. 2005), T-box transcriptional antitermination (Grandoni et al. 1992; Marta et al. 1996), expression control by global transcriptional regulators of nitrogen metabolism (Tojo et al. 2004) and carbon catabolite repression (Tojo et al. 2005) in *Bacillus subtilis*.

2.2.1 Regulation of the *ilvGMEDA* Operon in *Escherichia Coli*

The *ilvGMEDA* operon provides a unique example of a transcriptional unit whose expression depends on multivalent control by all three BCAAs (Lawther et al. 1987). Transcriptional attenuation of the whole operon is the main control mechanism, although fine tuning of expression of individual genes in the operon is ensured by transcription from internal promoters, differential stability of specific mRNA segments, and binding of regulatory proteins. Premature transcriptional termination depends on the formation of alternative secondary RNA structures, which is in turn affected by availability of the regulatory amino acids (Fig. 3). The leader peptide of 32 AAs encoded in the *ilvGMEDA* operon includes 15 BCAAs (Table 2). The regulatory mechanism depends on a coupling of transcription and translation. Depending on the availability of regulatory amino acids and the respective

Table 2 Amino acid sequences of the leader peptides of attenuators

Operon	Amino acid sequence[a]	Refs.
ilvGMEDA, *E. coli*	MTA**LL**R**VI**S**LVVI**S**VVVIII**PPCGAALGRGKA	(Lawther et al. 1987)
ilvBN, *E. coli*	MTTSMLNAK**LL**PTAPSAA**VVVV**R**VVVVV**GNAP	(Friden et al. 1982)
ilvBNC, *C. glutamicum*	MT**II**R**LVVV**TARRLP	(Keilhauer et al. 1993)
leuABCD, *E. coli*	MTHIVRFIG**LLLL**NASSLRGRRVSGIQH	(Wessler and Calvo 1981)
leuABCD, *S. enterica* serovar Typhimurium	MSHIVRFTG**LLLL**NAFIVRGRPVGGIQH	(Gemmill et al. 1979)
leuA, *C. glutamicum*	MTSRAN**LLLL**RRGGSQRS	(Pátek et al. 1994)

[a] Tandem amino acid residues supposed to exert the regulatory functions are underlined

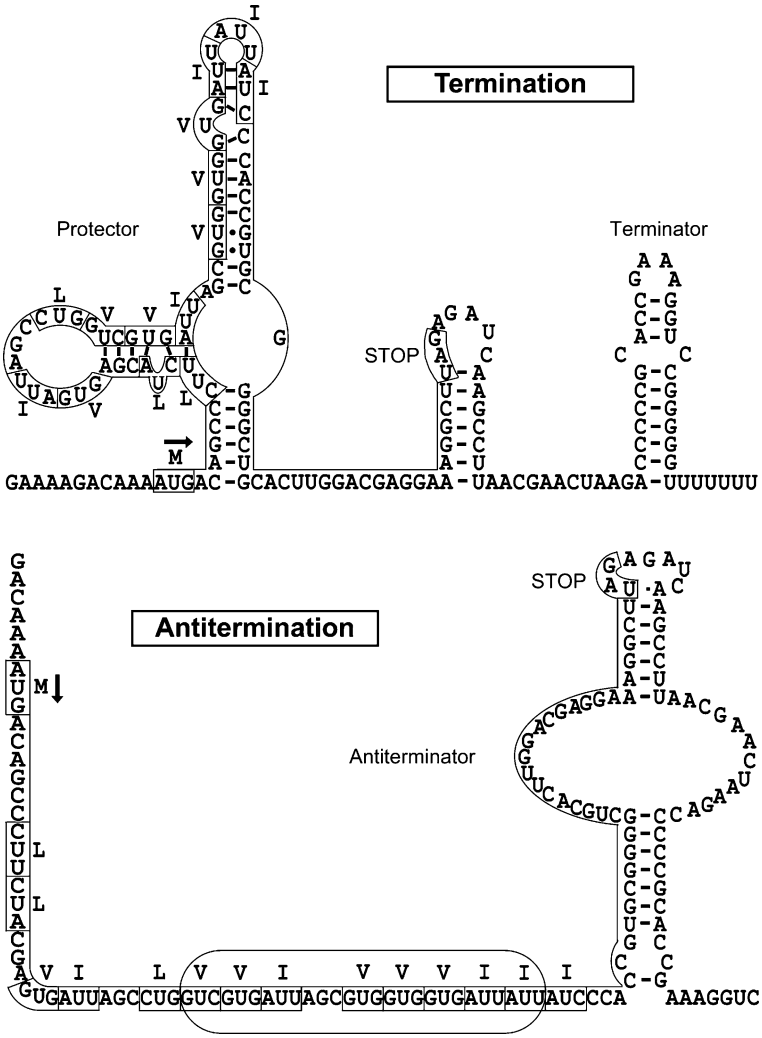

Fig. 3 Proposed alternative secondary RNA structures of the leader transcript of the *E. coli ilvGMEDA* operon. The *line* along the sequence marks the region encoding the leader peptide. The initial methionine codon (M), the tandem control codons (L, V and I) and the stop codon are *boxed*; the *oval* delimits the region masked by ribosome stalling at the central of three consecutive valine codons. Based on the results of Lawther et al. (1987) and Chen et al. (1991)

charged tRNAs, transcriptional terminator or antiterminator RNA structures are formed and transcription either is terminated or continues, accordingly. The concept of attenuation supposes the following mechanism (Chen et al. 1991): If any BCAA is limiting, the ribosome translating the leader transcript stalls at one of the control codons for BCAA, thus precluding formation of

the protector RNA structure (Fig. 3). In this case, the bases of the downstream nascent transcript are not covered by the ribosome and form an antiterminator RNA structure, which includes the upper arm of the potential terminator. The formation of antiterminator thus excludes formation of the terminator and transcription continues further into the *ilvGMEDA* operon (readthrough). Under conditions of excess of all BCAAs, translation does not stall but proceeds to the stop codon. The downstream bases in the leader, which may form secondary structure, are covered by the translating ribosome and can not interfere with formation of the protector, which includes the lower part of the potential antiterminator. Consequently, the terminator (typical rho-independent terminator hairpin) is formed, which results in transcriptional termination (Fig. 3). This description of the mechanism of attenuation is quite simplified, since additional transient secondary structures influencing the repression mechanism may be formed within the leader transcript.

There are also several BCAA-independent factors, which affect expression of the operon. The leucine-responsive regulatory protein (Lrp), known to affect expression of many operons and genes in *E. coli* (Willins et al. 1991; Brinkman et al. 2003), binds two sites in the DNA region between the attenuator and the *ilvG* gene and represses transcription of the operon about three-fold (Rhee et al. 1996; Hung et al. 2002). Another global regulator, the integration host factor (IHF), binds to two sites within the promoter region (Pereira et al. 1988). Upstream of the *ilvG* gene, two transcriptional start sites were detected, pertinent to the promoters P1-*ilvG* and P2-*ilvG*. One of the IHF-binding sites overlaps the P1-*ilvG* promoter, which suppresses transcription from this promoter, but activates transcription from the promoter P2-*ilvG*. This seven-fold activation of transcription from P2-*ilvG* may be accounted for by the enhanced DNA bending in the region, which itself exhibits intrinsic bending. P2-*ilvG* is an example of a promoter with a discriminator region that is probably responsible for a global regulation (activation) by the stringent response alarmone ppGpp during growth in AA-limited medium. Conversely, in rich medium, the expression of the operon is reduced, due to the low level of ppGpp. In addition to P1-*ilvG* and P2-*ilvG*, the weaker internal promoters P-*ilvE*, P-*ilvD* and P-*ilvA* were identified upstream of the genes *ilvE*, *ilvD* and *ilvA*, respectively (Huang et al. 1992). Promoter P-*ilvA* is preferentially active in cells grown under anaerobic conditions. Activity of P-*ilvE* depends neither on AA levels in the cell nor on the growth rate of the culture (Harms and Umbarger 1991). Transcription initiation at these promoters, potential transcriptional termination within the operon and processing (RNA decay) of the transcripts result in relatively stable transcripts covering *ilvEDA* and *ilvD* and unstable transcripts covering *ilvGMEDA* and *ilvDA* (Huang et al. 1992). Transcripts originated from different promoters and displaying different stability provide additional means for the cell to meet the momentary requirements of the cell metabolism.

Expression of the *ilvGMEDA* operon represents a complex transcriptional pattern due to a set of independent regulatory mechanisms of various nature and level that have gradually been uncovered over decades of investigations. A large number of overlapping regulatory signals encoded by a short DNA region demonstrates the capacity of DNA to code for exceptionally dense information.

2.2.2 Regulation of the *ilvIH* Operon in *Escherichia Coli*

Activation of the *ilvIH* operon by Lrp protein is one of the most thoroughly studied examples of regulation of gene expression by this transcriptional factor. Expression of the *ilvIH* genes was found to be repressed by leucine (Ricca et al. 1989). However, there is no sequence reminding any section of the attenuator structure upstream of *ilvIH*. Instead, six binding sites for Lrp were found within a 250-bp sequence upstream of the coding regions (Wang and Calvo 1993). Lrp directly activates the *ilvIH* promoter by enhancing the intrinsic bending of the region upstream of the promoter and thus positively regulates expression of the operon. Interaction dependent on distance and phasing between Lrp molecules bound to two sites centered at 231 (relative to the position of transcriptional start) and to four sites centered at 95 is essential for efficient activation of the promoter (Jafri et al. 2002; Chen et al. 2005). Activity of the *ilvIH* promoter was reduced more than 30-fold in the strain lacking Lrp (Platko et al. 1990). In the presence of leucine, the amount of the bound Lrp protein is reduced in the wild-type strain and, consequently, the activity of the *ilvIH* promoter decreased 5- to 10-fold (Jafri et al. 2002). In addition to regulation by Lrp, *ilvIH* was found to be repressed by the stringent response factor ppGpp under the conditions of amino acid starvation (Baccigalupi et al. 1995), in contrast to *ilvGMEDA* and *ilvBN*, which are activated by ppGpp.

2.2.3 Regulation of the *ilvBN* Operon in *Escherichia Coli*

In accordance with the unusual properties of AHAS I, which occurs exclusively in enterobacteria, regulation of the *ilvBN* operon expression exhibits unique features. It is regulated specifically by transcriptional attenuation and at the global level by carbon catabolite repression and by the stringent response to amino acid starvation.

The attenuator of *ilvBN* exhibits features quite similar to the features of other typical attenuators of amino acid biosynthesis operons. The leader transcript coding for a 32-AAs peptide (Table 2) contains a tandem of leucine codons and nine valine codons (runs of four and five), but lacks isoleucine codons (Friden et al. 1982; Hauser and Hatfield 1983). The operon is therefore

not regulated by isoleucine availability. The leader transcript may potentially form a number of mutually exclusive stem-loop RNA structures, however, due to the transient folding of RNA and different conceptions of their temporal appearance, the proposed secondary RNA structures differ in different models (Friden et al. 1982; Hauser and Hatfield 1983). On the basis of in vitro transcription, the termination site of the leader transcript (184 nt) was found downstream of the run of 5 U preceded by a G + C-rich region, which may fold into a typical terminator hairpin (Hauser and Hatfield 1983).

Another type of expression control, independent of attenuation, was found on the basis of the sequence of the *ilvBN* operon. A cAMP-receptor protein (CRP) binding site centered near position 65 upstream of the supposed transcriptional start point was recognized (Friden et al. 1982). The *ilvBN* operon is the only biosynthetic operon in *E. coli* induced by CRP, which is generally involved in carbon catabolite repression (Sutton and Freundlich 1980; Tedin and Norel 2001). Very little AHAS I activity was detected when the cAMP level in the cell was low. Addition of cAMP under these conditions increased *ilvBN* expression 24-fold. Activation of the *ilvBN* promoter through CRP thus probably permits efficient growth of *E. coli* on substrates different from glucose (e.g., acetate or oleate) (Dailey and Cronan 1986).

Similar to expression of *ilvGMEDA* and in contrast to expression of *ilvIH*, expression of *ilvBN* is stimulated by the alarmone of the stringent response, ppGpp (Freundlich 1977; Sutton and Freundlich 1980; Tedin and Norel 2001), although the mechanism of its action has not been described.

The recent finding that *ilvBN* is derepressed by the dephospho-form of enzyme IIA^{Ntr} (involved in nitrogen-metabolic phosphotransferase system), when the *E. coli* cells are transferred from BCAA-containing medium to a minimal medium (Lee et al. 2005) shows that not all factors influencing the expression of the long-studied genes have as yet been revealed.

2.2.4
Regulation of the *ilvBNC* Operon in *Corynebacterium Glutamicum*

The genes coding for the subunits of the single AHAS in *C. glutamicum* were named *ilvBN*, although they are orthologs of *ilvIH* of *E. coli*. The *ilvBNC* operon of *C. glutamicum* is transcribed into three different transcripts covering *ilvBNC*, *ilvNC* and the *ilvC* gene alone (Keilhauer et al. 1993). The leader region of *ilvB* (292 bp) is comprised of features typical for regulation by attenuation: (1) a short ORF with two Ile codons, three Val codons and two Leu codons, (2) a sequence with a potential to form alternative RNA stem-loop structures, (3) a sequence potentially forming a transcriptional terminator RNA structure (Morbach et al. 2000). Formation of a very short abundant RNA (about 200 bp) corresponding to the prematurely terminated *ilvBNC* transcript and increased formation of *ilvBNC* transcript under growth limitation by all BCAAs or by any one of them suggested that the

expression is regulated by transcriptional attenuation. Moreover, the necessity of translation of the leader peptide (Table 2) for the regulatory response was confirmed, although the transcriptional start and the supposed translation initiation codon are only 1 bp apart and a ribosome-binding site is thus missing. However, some results failed to consistently support the attenuation model: (1) the regulation was abolished by mutational alteration of valine codons, but not by alteration of isoleucine codons, and (2) evidence for the function of the putative terminator structure could not be obtained (Morbach et al. 2000). In a similar attenuator-like structure in *Streptomyces coelicolor*, replacement of the supposed control codons of the *ilvBNC* operon did not eliminate the regulatory effect of BCAAs. This led to the conclusion that some other regulatory mechanism is involved, although typical features of an attenuator are present in *S. coelicolor* (Craster et al. 1999). Induction of both the complete *ilvBNC* transcript and the short leader transcript by 2-ketobutyrate in *C. glutamicum* indicates that yet another independent regulation operates in control of *ilvBNC* expression. The *ilvNC* transcript was also induced by 2-ketobutyrate (Keilhauer et al. 1993). The *ilvN* promoter, which was repressed by valine, was localized about 300 bp upstream of the gene, within the *ilvB* coding region, but no sequences reminiscent of attenuators or other control elements were recognized in the untranslated region of the transcript (Pátek, unpublished results). The *ilvNC* transcript in *C. glutamicum* may ensure an excess of the regulatory subunit coded by *ilvN,* which is necessary for full AHAS activity and/or for efficient inhibition. Regulation of the most abundant *ilvC* transcript has not as yet been described. The complex expression pattern of the *ilvBNC* operon and the respective control mechanisms thus must still be revealed.

3 Leucine

The isopropylmalate pathway of leucine synthesis described here occurs in nearly all bacteria, fungi, and plants studied so far. An alternative isovalerate carboxylation pathway was found in some bacteria such as the anaerobic ruminal cellulose digester *Bacteroides ruminicola* (Allison et al. 1984).

3.1 Enzymes

Isopropylmalate synthase (LeuA; EC 4.1.3.12) catalyzing the first reaction in the leucine-specific biosynthesis pathway (Fig. 2) forms a homotetramer with subunits of about 50 kDa in *E. coli* and *S. enterica* serovar Typhimurium (Leary and Kohlhaw 1970). Proteins of the same type as LeuA from *E. coli* were identified in most γ-proteobacteria (e.g., *Erwinia* and *Vibrio*) and in

some α-proteobacteria (e.g., *Rhodobacter*), whereas enzymes of the LeuA2 type (sharing approximately 30% identity) were found particularly in fungi (e.g., *Saccharomyces*), in actinobacteria including *C. glutamicum* (Leary and Kohlhaw 1970; Pátek et al. 1994; Vitreschak et al. 2004a), in bacilli and in pseudomonads (Vitreschak et al. 2004a). Isopropylmalate synthase is sensitive to feedback inhibition by leucine in these bacteria (Ward and Zahler 1973) as well as in yeast (Hu and Kohlhaw 1995).

In both *S. enterica* serovar Typhimurium and *C. glutamicum*, inhibition of isopropylmalate synthase by leucine is the main regulatory mechanism on the level of enzyme activity in the leucine synthesis pathway (Leary and Kohlhaw 1970; Pátek et al. 1994). The apparent K_i value was 0.4 mM in *C. glutamicum* (Pátek et al. 1994). The inhibition is noncompetitive with respect to 2-ketoisovalerate and competitive with respect to acetyl-CoA. The inhibitor disaggregates the tetrameric form of the enzyme (Leary and Kohlhaw 1970).

Isopropylmalate dehydratase (LeuCD; EC 4.2.1.33) from *E. coli* consists of a heterodimer of 50-kDa and 23-kDa subunits encoded by *leuC* and *leuD*. The homologous genes of *C. glutamicum* encode proteins of 52 and 22 kDa, respectively. Isopropylmalate dehydratase from *Saccharomyces cerevisiae*, which was more deeply studied, is formed by a monomer of approximately 90 kDa (Kohlhaw 1988, 2003).

Isopropylmalate dehydrogenase (LeuB; EC 1.1.1.85) isolated from *S. enterica* serovar Typhimurium in its active form is a dimer of identical 35-kDa subunits (Parsons and Burns 1969). The *leuB* gene of *C. glutamicum* specifies a protein of 36 kDa (Pátek et al. 1998). It was cloned and overexpressed in the plasmid pEKEx2 (Eikmanns et al. 1991) providing 10-fold higher activity than the wild-type strain (Pátek et al. 1998). Knowledge of regulatory mechanisms, which govern the activity of Leu-enzymes is rather limited.

3.2 Regulation of Gene Expression

Leucine genes are clustered in operons in many organisms. In some γ-proteobacteria (e.g., *E. coli*, *Yersinia pestis* and *Vibrio cholerae*) the *leuABCD* operon is regulated by translation-mediated transcriptional attenuation (Wessler and Calvo 1981; Vitreschak et al. 2004a). In other groups of bacteria (e.g., *Agrobacterium tumefaciens*, *Rhodobacter sphaeroides* and *Caulobacter crescentus*), the leucine genes are located on different parts of the chromosome and only *leuA* is regulated by attenuation, although a typical terminator-like structure is missing within the attenuator sequence (Vitreschak et al. 2004a). In actinomycetes (e.g., corynebacteria, streptomycetes and mycobacteria) LEU elements were revealed upstream of the *leuA* gene, within the sequence with some features of an attenuator (Seliverstov et al. 2005). In *Bacillus subtilis*, the genes of leucine synthesis form together with the *ilv* genes a single operon, *ilvBNC-leuACBD* (Sect. 2.2).

3.2.1 Regulation of the Leucine Operon in *Escherichia Coli* and *Salmonella Enterica* Serovar Typhimurium

The *leuABCD* operon in *E. coli* (and probably in many other γ-proteobacteria) is regulated mainly by leucine-mediated transcriptional attenuation (Wessler and Calvo 1981). Expression of the leucine operon varies over a 40- to 50-fold range in both *E. coli* and *S. enterica* serovar Typhimurium depending on the intracellular concentration of leucine. There are four consecutive Leu codons in their leader transcripts (Table 2) and their crucial role in transcriptional attenuation in *S. enterica* serovar Typhimurium was proven by mutagenesis. Changing the three rare CTA codons to the more frequent CTG codons reduced the basal level of expression of *leuABCD* operon as well as the sensitivity of the response to leucine starvation (Carter et al. 1986). After converting the four Leu codons into four Thr codons by mutation, 90% regulation by leucine was abolished (Carter et al. 1985). Whether an additional type of regulation (e.g., Lrp control) is responsible for the remaining three-fold derepression of the operon needs to be solved. Surprisingly, the mutant with Thr codons responded significantly less to threonine starvation than the wild-type in which the regulation by threonine was unexpectedly found. Explanation of these observations may lay in modification of some tRNAs by threonine and in the presence of a single Thr codon upstream of the four Leu codons in the WT-leader (Carter et al. 1985). More generally, the lesson from mutagenesis studies of attenuators is that the results may differ from those anticipated from the models, and this is probably due to the involvement of both machineries of transcription and translation in the regulatory mechanism and due to the complex structure of the leader transcript in which codon-specific response and potential secondary structures cannot be altered independently.

3.2.2 Regulation of Leucine Genes in *Corynebacterium Glutamicum*

In *C. glutamicum*, the leucine biosynthesis genes (*leuA*, *leuB* and *leuCD*) are scattered over the chromosome. Upstream of the *leuA* gene, features of a typical attenuator are present. However, caution is needed in inferring a regulatory mechanism even if the DNA and the deduced AA sequences strongly suggest the presence of a standard attenuator, according to a study of a very similar structure upstream of *leuA* in the related actinomycete *S. coelicolor* (Craster et al. 1999). Replacement of three supposed leucine control codons within the leader transcript with threonine codons did not clearly abolish the regulation of *S. coelicolor leuA* by leucine. In *C. glutamicum*, the 18-AA leader peptide (Table 2) of *leuA* would contain four consecutive leucine residues as the *E. coli leu*-leader. In comparison to cells grown in medium

with leucine, the specific activity of isopropylmalate synthase encoded by *leuA* was about two-fold higher in cells grown in minimal medium without leucine (Pátek et al. 1994) and expression of a fusion of the *leuA* promoter region to the promoter-less chloramphenicol acetyltransferase gene was six-fold higher when the cells were starved for leucine (Pátek et al., unpublished). The regulatory effect of leucine was abolished when the AUG start codon of the supposed leader peptide was altered to AAG or when four consecutive leucine codons were exchanged for phenylalanine codons (Pátek et al., unpublished). These results indicate that (1) translation into the leader peptide is essential for the regulation, and (2) leucine codons play a crucial role in the regulatory mechanism. These lines of evidence suggest that it is indeed an attenuation mechanism as that in *E. coli*, which controls *leuA* expression in *C. glutamicum*.

However, according to the comparative analysis of RNA regulatory elements based on the genome sequences, *C. glutamicum* and a number of other actinomycete species (corynebacteria, mycobacteria, and streptomycetes) contain regulatory structures designated LEU elements in the leader regions of their *leuA* genes (Seliverstov et al. 2005). Although the LEU element resembles an attenuator, the transcript is supposed to fold into a pseudoknot with an additional stem at its base formed by pairing of the leucine control codons with the Shine-Dalgarno sequence of *leuA*. To accomplish the regulatory role, the same region might form an alternative hairpin with the same base stem. There is a similarity of the secondary structure to riboswitches (Vitreschak et al. 2004b), but binding of a small molecule to the LEU elements seems unlikely (Seliverstov et al. 2005). No experimental evidence that the element is a binding site of a protein has been provided. The role of ribosome stalling at the control Leu codons should also be taken into consideration. The actual nature and function of the *leuA* regulatory element thus remain to be elucidated.

Data on the regulation of the other *leu* genes in *C. glutamicum* are scarce. The *leuB* gene is transcribed into a monocistronic transcript and is repressed by leucine (Pátek et al. 1998). Upstream of *leuB* and *leuCD* no prominent sequences indicating the type of control are apparent.

The specific activity of isopropylmalate dehydratase (LeuCD) is about half in minimal medium with leucine as compared to medium without leucine and ten-fold lower in complex medium (Pátek et al. 1994). This suggests that expression of *leuCD* gene cluster is also subject to a complex regulation.

4 Use of Branched-Chain Amino Acids and their Biotechnological Production

BCAAs are used as additives to food and feedstuff for infusions and for synthesis of pharmaceuticals (Sahm et al. 1999). Sustained dynamic exer-

cise stimulates in human amino acid oxidation, chiefly of the BCAAs (Rennie and Tipton 2000). During endurance sport events, BCAAs (particularly leucine) attenuate the breakdown of muscle proteins, promote protein synthesis in skeletal muscle, and prevent premature muscle fatigue (Rennie et al. 2006; Shimomura et al. 2006). BCAAs in special dietary supplements for sport nutrition are therefore expected to promote maintaining the skeletal muscle and its regeneration. Since BCAAs are essential for immune cell functions and may improve immunity when administered to postsurgical or septic patients (Calder 2006), they are applied in nutritional or medical treatment.

Valine was found among the five most limiting amino acids in reduced protein corn-soybean meal diets for livestock (Mavromichalis et al. 1998). Isoleucine is more deficient than valine for pigs during both early and late finishing stages (Liu et al. 1999). It is expected that the decreasing price of the biotechnologically produced BCAAs, would permit wider application of BCAA in human and animal nutrition (Kelle et al. 1996).

BCAAs are mainly produced by fermentation, enzymatic catalysis and by extraction of protein hydrolyzates. The alternative to the production of pure AAs for nutrition purposes is to construct transgenic plants in which deficiency in some BCAAs (particularly isoleucine) is overcome by gene manipulations (Weaver et al. 2005). Isoleucine, having the most common use of BCAAs, is produced in quantities about 400 tons per year, with *C. glutamicum* and *E. coli* as the best described producers (Hermann 2003). A few hundred tons of L-valine are produced each year using enzyme membrane reactor (Leuchtenberger et al. 2005).

The productions of amino acid increases by about 10% annually (Hermann 2003) and BCAAs are particularly strong in the growing market. In response to the growing demand for BCAAs, the largest fermentation company in terms of volume, Ajinomoto, has opened a new plant in Brazil with initial production of four amino acid products, including valine, leucine and isoleucine for use in food and pharmaceutical industry.

4.1 Isoleucine Overproducing Strains

Isoleucine may be produced with microorganisms by conversion of precursors (e.g., 2-ketobutyrate) or by fermentation using mutant or recombinant strains (Eggeling et al. 1997). Biosynthesis of isoleucine, in which aspartate and pyruvate are the starting metabolites, consists of a number of reaction steps (Fig. 4). Along this pathway, several steps are rate-limiting and must be engineered to overcome the metabolic barriers. The strains were first derived particularly as mutants resistant to analogs of threonine and isoleucine-like hydroxynorvaline and methylthreonine, respectively. An isoleucine-producing strain of *Serratia marcescens* derived by transduction

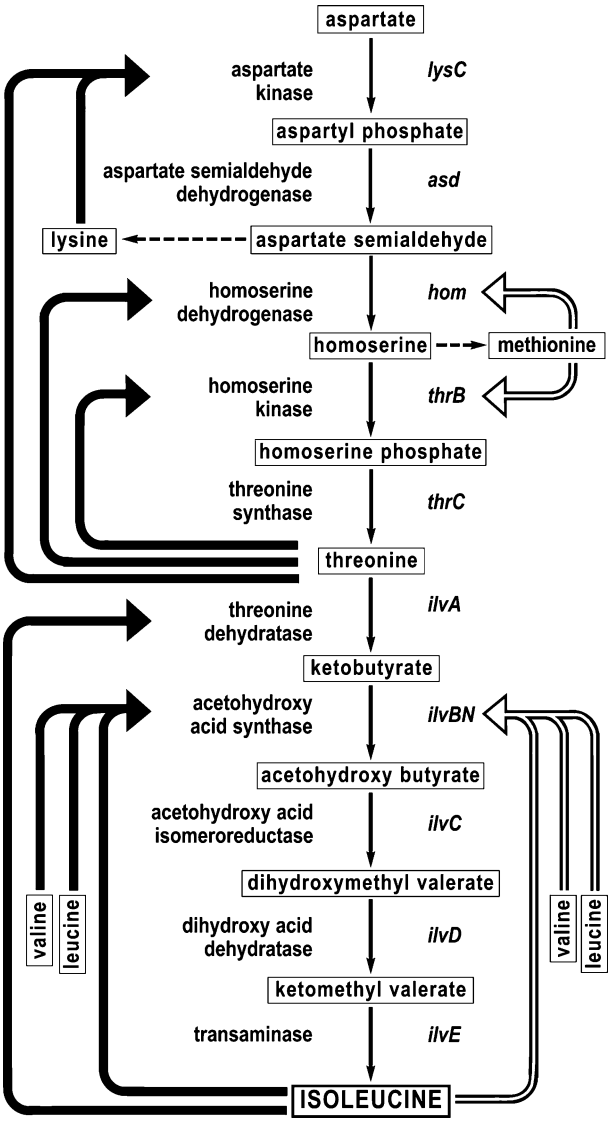

Fig. 4 Biosynthesis of isoleucine and its regulation by repression and inhibition in *C. glutamicum*. Feedback regulation by various amino acids is indicated by *thick arrows* for inhibition and by *empty arrows* for repression and/or attenuation. The genes encoding the respective enzymes are shown on the *right*

had six regulatory mutations for threonine and isoleucine biosyntheses and exhibited high specific activities of threonine deaminase and acetohydroxy-acid synthase, both of which were insensitive to feedback inhibition (Komatsubara et al. 1980). Methods of gene cloning permitted constructing recom-

binant isoleucine-producing strains of *E. coli* and *C. glutamicum*. The *E. coli* strain TVD5 (*ilvA*, *thrC*, thr^R, *tdh*::Tn5) contained two plasmids, one with the *thrA***BC* operon and the other with the *ilvGMEDA** operon (Hashiguchi et al. 1999b). Owing to the presence of the ThrA* protein, which is active both as aspartate kinase and as homoserine dehydrogenase and is resistant to inhibition by threonine and of TD (*ilvA*), which is resistant to inhibition by isoleucine, the strain produced isoleucine and valine. Since common enzymes synthesize both isoleucine and valine, the valine pathway cannot be shut down by metabolic block to achieve an isoleucine producer. When the *E. coli* strain with the *lysC** gene encoding the lysine-resistant aspartate kinase III was used as the host for the two plasmids, the resulting producer accumulated only isoleucine due to the increased 2-ketobutyrate supply (Hashiguchi et al. 1999a).

In *C. glutamicum*, at least five enzymes within the pathway from aspartate to isoleucine are inhibited by various AAs (Fig. 4): the end product (isoleucine), an intermediate (threonine), the product of a pathway which branches off the isoleucine pathway (lysine) and products of partially parallel pathways (valine and leucine). Repression of the genes *hom*, *thrB* and *ilvBN* involved in the pathway (Fig. 4) has also to be overcome in the construction of an isoleucine producing strain.

In a *C. glutamicum* strain with a feedback-resistant (FBR) aspartate kinase, genes coding for homoserine kinase (*thrB*), for FBR homoserine dehydrogenase (*hom*) and for FBR threonine deaminase (*ilvA*) were cloned in two plasmids, each with approximately 20 copies per chromosome (Morbach et al. 1996). Although high expression of *hom*(FBR) was found to be unstable in threonine-producing strains (Reinscheid et al. 1994), the *hom*(FBR) allele on a multicopy plasmid was stable in the presence of a highly expressed *ilvA*(FBR) allele. This finding documents the general necessity to first remove potential limitations within the downstream part of a biosynthesis pathway to avoid potentially deleterious accumulation of high intracellular concentrations of intermediates (Eggeling et al. 1997). Similar to threonine overproduction (Reinscheid et al. 1994), higher intracellular (110 mM) than extracellular (60 mM) isoleucine concentrations indicated that amino acid efflux has become the limiting step of isoleucine production in the engineered strain (Morbach et al. 1996). However, overexpression of *brnFE* encoding the exporter of BCAAs (Kennerknecht et al. 2002) did not increase isoleucine production (Eggeling 2005). Although in tryptophan and threonine producers the inactivation of the respective AA uptake systems improved their final yield (Ikeda and Katsumata 1995; Okamoto et al. 1997), deletion of *brnQ* coding for the isoleucine uptake carrier failed to prove beneficial (Eggeling 2005). Using another strategy of metabolic engineering, the *E. coli tdcB* gene coding for catabolic threonine deaminase was expressed together with *C. glutamicum thrB* and hom^{dr} (coding deregulated homoserine dehydrogenase) from a single plasmid vector harbored by *C. glutamicum* (Guillouet et al. 2001). Since

TdcB from *E. coli* converting threonine to 2-ketobutyrate is an isoleucine insensitive enzyme, its presence in *C. glutamicum* contributed substantially to the final yield of isoleucine.

The study of correlation of enzyme activity and mRNA abundance for *hom*, *thrB* and *ilvA* expression in *C. glutamicum* showed that *hom* mRNA and the level of homoserine dehydrogenase activity changed in profile and scale in a similar way, whereas in the case of *ilvA* and TD, increase in enzyme activity strongly exceeded the moderate increase in mRNA abundance. A third pattern emerged for *thrB* and homoserine kinase (ThrB): while the level of the transcript initially increased considerably and decreased later, HK activity showed a small but steady increase over the same time course (Glanemann et al. 2003). Since the relationship between the level of gene expression and product yield depends on a number of factors, including the rate of transcription initiation, the stability of the mRNA, the efficiency of translation and protein modification and stability, systems-level approaches (Wendisch et al. 2006), including monitoring mRNA abundances, enzyme activities, and metabolite concentrations over time are expected to provide deeper insights into the physiology of genetically engineered bacterial producers.

4.2 Valine Overproducing Strains

During the era of random mutagenesis of microorganisms and selection of AA producers, valine-producing strains of *C. glutamicum* (Tsuchida and Momose 1975) and *Serratia marcescens* (Kisumi et al. 1971) were isolated as mutants resistant to BCAA analogs such as norvaline or 2-aminobutyric acid. In these strains, deregulation of AHAS was probably the reason for valine overproduction (Tsuchida and Momose 1975). By the methods of directed metabolic engineering the valine-excreting strains *C. glutamicum ΔilvA ΔpanBC* (isoleucine and pantothenate auxotroph) carrying a plasmid with the cloned genes *ilvBNCD* or *ilvBNCE* were constructed (Sahm and Eggeling 1999; Radmacher et al. 2002). Deletion of *ilvA* coding for TD, the key enzyme of the isoleucine synthesis, seemed decisive for valine production. AHAS, which is active in both the isoleucine and valine pathways, is then available only for conversion of pyruvate to acetolactate (Fig. 2). When limiting amounts of isoleucine were used for supplementation of this isoleucine auxotrophic strain, the AHAS activity was not inhibited by isoleucine and the imposed growth limitation further improved valine production by this strain, probably by *ilvBNC* derepression under isoleucine starvation (Sahm and Eggeling 1999). Growth limitation of *C. glutamicum ilvA* strains, even in presence of sufficient levels of isoleucine in the medium, may also be induced by high concentrations of valine (Lange et al. 2003). Valine (50–200 mM) in the medium provokes unexpected effects: deficiency of isoleucine in the

cell, increased AHAS activity, and higher valine excretion. Genome-wide expression profiling using DNA microarrays in combination with proteomics and genetic experiments proved that these effects are caused by competition of valine and isoleucine for uptake by the carrier BrnQ, which transports all BCAAs (Lange et al. 2003). These consequences may also appear in fermentations by isoleucine auxotrophs at stages when a high level of valine is accumulated in the medium and the concentration of the supplemented isoleucine dropped to a critical value.

An AHAS protein resistant to inhibition by all end products of the pathways (i.e., all three BCAAs) was constructed by site-directed mutagenesis of regulatory AHAS subunit coded by *ilvN*, designed according to the respective DNA sequences of deregulated mutants of *E. coli* and *S. cinnamonensis* (Elišáková et al. 2005). The respective allele (*ilvN*M13) encoding three altered AAs in IlvN was introduced into the chromosome of *C. glutamicum ΔilvA ΔpanBC* harboring the plasmid with the *ilvBNC* operon. The resulting strain showed high AHAS activity and steadily produced valine in parallel with growth of the culture and during the stationary phase (Elišáková et al. 2005). As an alternative to *ilvA* deletion, which causes isoleucine auxotrophy and consequently a requirement for isoleucine supplementation, down-mutations in promoters of *ilvA* or *leuA* (P-*ilvA*, P-*leuA*) were constructed, which severely decreased expression of the respective genes and caused bradytrophy for isoleucine or leucine, respectively (Pátek at al. unpublished). Growth of the isoleucine or leucine bradytrophic strains was limited by the respective AA and provided the same valine yields as isoleucine auxotrophs or even higher. The leucine bradytroph displayed high excretion of valine but no production of isoleucine, although the unimpaired isoleucine pathway was expected to be favored over the valine pathway due to the much higher affinity of AHAS to 2-ketobutyrate than to pyruvate. This may be explained by the strict inhibition of wild-type TD activity by isoleucine or by the change of the role of valine at high concentrations from an activator of TD to its inhibitor, as observed in *E. coli* (Umbarger 1996). The concept of tuning the individual reaction rates in the biosynthesis by promoter strength modulation was followed also in enhancing the activity of promoters P-*ilvD* (100-fold) and P-*ilvE* (20-fold) by specific mutations, which further increased the carbon flux through the pathway. A plasmid-free *C. glutamicum* valine producer was constructed by introducing these mutations (*ΔpanB*, *ilvN*M13, P-*ilvA*M1, P-*ilvD*M7, P-*ilvE*M6) into the chromosome (Pátek et al. unpublished). The mutations present in this valine producer might also prove beneficial for isoleucine production when combined with deregulation of *ilvA* as well as for leucine production if the leucine-specific pathway is deregulated. Techniques of metabolic engineering within the chromosome (Vertes et al. 2005), which do not introduce plasmids, markers or heterologous DNA sequences into the cell, may provide more stable producer strains, which do not exhibit theoret-

ical hazards of horizontal transfer of genes with a potentially negative effect to the environment.

4.3 Leucine Overproducing Strains

Breeding of *C. glutamicum* leucine producers began as it did for other BCAAs by chemical mutagenesis and isolation of analog-resistant mutants. Mutants resistant to 2-azaleucine and 3-hydroxyleucine possessed isopropylmalate synthase and AHAS resistant to inhibition by leucine and by all three BCAAs, respectively (Tsuchida and Momose 1975; Tsuchida and Momose 1986). The repression of genes coding for AHAS could be overcome by a limiting supply of isoleucine. Leucine producers may be improved by genetic manipulation of *leu* genes (particularly *leuA*) in valine producing strains. In another approach, 2-ketoisocaproic acid was used as a precursor in continuous cultivations (Wichmann et al. 1990).

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