Expression, Purification, Characterization, and Reconstitution of the Large and Small Subunits of Yeast Acetohydroxyacid Synthase†

Siew Siew Pang and Ronald G. Duggleby*

Centre for Protein Structure, Function and Engineering, Department of Biochemistry, The University of Queensland, Brisbane QLD 4072, Australia

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ABSTRACT: Acetohydroxyacid synthase (AHAS, EC 4.1.3.18) catalyzes the first step in the biosynthesis of the branched-chain amino acids. In bacteria, the enzyme has a large subunit containing the catalytic machinery and a small subunit with a regulatory role. In eucaryotes, the evidence for a regulatory subunit is largely indirect and circumstantial. We investigated the possibility that the yeast open reading frame *YCL009c* is an AHAS small subunit. Analysis of the DNA sequence shows that it contains all the appropriate transcription, translation and regulatory signals. *YCL009c* was shown to be expressed in yeast and the protein localized in mitochondria where it undergoes removal of a transit peptide targeting sequence. This putative small subunit protein (*ilv6*) and the catalytic subunit of yeast AHAS (*ilv2*) were each overexpressed in *Escherichia coli* and purified to near homogeneity. Reconstitution studies showed that the *ilv6* protein stimulates the catalytic activity of the *ilv2* protein by up to 7-fold (from 6.8 ± 0.7 to 49.0 ± 1.8 U/mg) and confers upon it sensitivity to inhibition by valine (K_i = 0.16 ± 0.02 mM). Valine inhibition is partially reversed by ATP. The reconstitution is favored by high concentrations of potassium phosphate (∼1 M) and at neutral pH. Under optimal conditions for reconstitution, a dissociation constant for the subunits of 70 ± 7 nM was determined. Valine inhibition is partial, resulting in a specific activity that is similar to that of the *ilv2* protein alone. However, measurements of the K_m for substrate rule out the possibility that valine inhibition is accomplished by dissociation of the subunits.

Acetohydroxyacid synthase (AHAS, EC 4.1.3.18; also known as acetolactate synthase)[1] catalyzes the first step that is common to the biosynthesis of the branched-chain amino acids. The reaction involves the irreversible decarboxylation of pyruvate to a bound hydroxyethyl group that then condenses with either a second pyruvate molecule to form 2-acetolactate or with 2-ketobutyrate to form 2-aceto-2-hydroxybutyrate (*1*). The former product is the precursor for valine and leucine biosynthesis while the latter leads to isoleucine. In addition, 2-acetolactate is the precursor for diacetyl, which is responsible for an off-flavor in beer (*2*).

AHAS requires three cofactors for activity: thiamin diphosphate (ThDP), Mg^{2+} or another divalent metal ion, and flavin adenine dinucleotide (FAD). The first two cofactors are typical for enzymes that catalyze the decarboxylation of 2-ketoacids, as occurs in the first stage of the AHAS reaction. The requirement for FAD is unusual since the reaction involves no oxidation or reduction. However, it is not unprecedented, and a requirement for flavin nucleotides has also been described for glyoxylate carboligase (*3*), which is structurally related to AHAS, as well as the unrelated enzyme chorismate synthase (*4*). Much interest in AHAS was stimulated by the discovery that it is the target site of at least four structurally diverse families of herbicides, namely sulfonylureas, imidazolinones, triazolopyrimidines, and pyriminidinyl oxybenzoates (for herbicide reviews, see *5*−*8*).

AHAS activity is found in bacteria, fungi, and plants, contributed by one or more isozymes. The enterobacterial enzymes have been studied extensively in terms of their genetic regulation (reviewed in *9*), kinetic properties, susceptibility to end-product (valine) feedback regulation, and sensitivity to herbicidal inhibitors (*10*−*16*). There are at least three active AHAS isozymes in *Escherichia coli*, namely AHAS I, II, and III encoded within the *ilv*BN, *ilv*GMEDA, and *ilv*IH operons, respectively (*17*−*19*). Some cryptic genes have also been identified (*20*−*23*). The three functional AHAS genes from *E. coli* or *Salmonella typhimurium* have been cloned and the proteins purified to near homogeneity (*11, 13*−*15, 24*). Biochemical analysis reveals that the isozymes have a common $\alpha_2\beta_2$ tetrameric structure, consisting of two large (≈60 kDa) and two small (≈10−17 kDa) subunits. All studies agree that the main AHAS catalytic activity is contained in the large subunits, while the small subunits may affect feedback regulation (*13, 25*−*27*), enzymatic activity, and stability (*24, 26, 28*−*30*).

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* Correspondence: Ronald G. Duggleby, Centre for Protein Structure, Function and Engineering, Department of Biochemistry, The University of Queensland, Brisbane QLD 4072 Australia. Phone: +617 3365 4615. Fax: +617 3365 4699. E-mail: duggleby@biosci.uq.edu.au.

[1] Abbreviations: ADH, alcohol dehydrogenase; AHAS, acetohydroxyacid synthase; bp, base pairs; DTT, dithiothreitol; FAD, flavin adenine dinucleotide; GST, glutathione *S*-transferase; IMAC, immobilized metal affinity chromatography; ORF, open reading frame; PCR, polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; ThDP, thiamin diphosphate; UTR, untranslated region; YNB, yeast nitrogen base.

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In contrast to the bacterial enzyme, the structure and biochemical properties of AHAS from eucaryotes have not been extensively characterized, and in most cases the enzyme has been studied in crude cell extracts only. Fungal AHAS is localized in the mitochondria (*31, 32*) and plant AHAS in the chloroplast (*33*), although the genes may be found in the nuclear or in the organelle genome (*34−36*). In those cases where it is nuclear-encoded, the enzyme is transported to the appropriate subcellullar compartment. A number of AHAS genes have been cloned and characterized from plants including *Arabidopsis thaliana* (*37*), *Brassica napus* (*38*), *Gossypium hirsutum* (*39*), *Nicotiana tabacum* (*37*), and *Zea mays* (*40*) and the yeasts *Saccharomyces cerevisiae* (*41*) and *Schizosaccharomyces pombe* (*42*). The deduced amino acid sequences of AHAS from fungi and plants are collinear with each other and with the large subunit of bacterial AHAS, suggesting strongly that all are derived from a common ancestor. The principal sequence difference between the procaryotic and eucaryotic forms is that the latter have an additional N-terminal sequence (*37−42*). This additional sequence, which is poorly conserved among the enzymes, is presumed to target the enzyme to mitochondria (in fungi) or chloroplasts (in plants). A second difference between the bacterial and other forms of AHAS is that no eucaryotic small subunit gene has ever been cloned and characterized. This may be because the eucaryotic AHAS does not contain a second subunit or because this subunit is yet to be discovered. The latter is more likely to be the reason, as there is increasing evidence for the presence of eucaryotic AHAS small subunits.

Identification of the bacterial AHAS small subunit is not always easy, even with highly purified enzyme. On SDS−PAGE, it migrates rapidly as a diffuse band that stains weakly and can easily be missed. In most cases, the bacterial small subunit has not been observed directly. Rather, its existence is deduced from the presence of an open reading frame (ORF) immediately 3′ to, and sometimes overlapping, that for the large subunit. Due to the low abundance and high lability of the eucaryotic AHAS, purification of the enzyme from its native source is difficult and results in low yield (*43−45*). With the availability of the cloned genes (corresponding to the large subunit of bacterial AHAS), studies on the eucaryotic AHAS have been carried out with recombinant enzymes expressed in *E. coli* (*46−49*). Of course, these preparations will not contain a small subunit if the cloned gene lacks the appropriate sequence. In this context it may be significant that, unlike native eucaryotic AHAS activity, the recombinant enzymes are insensitive to feedback inhibition. This could be interpreted to indicate that a regulatory subunit is missing, given the demonstration that the small subunit of *E. coli* AHAS III is required for feedback inhibition (*27*).

An increasing number of eucaryotic small subunit candidate genes has been identified (*50*), benefiting from the expanding information in sequence databases. The *S. cerevisiae* candidate gene *YCL009c* maps to chromosome III (*51, 52*) and shows a considerable amino acid sequence similarity to the bacterial small subunit (*50*). Using homologous recombination (*53*), the *YCL009c* ORF has been deleted. Although the null mutant shows no distinct phenotype when grown on complex medium (*54*), AHAS assays using permeabilized mutant cells show that the activity is resistant to valine inhibition. This provides persuasive evidence that this ORF, which has been termed *ilv6* and will be referred to henceforth by that name, encodes the small subunit of yeast AHAS.

The information summarized above supports the general hypothesis that AHAS from all species is comprised of a large subunit that contains the catalytic machinery and a small subunit that is responsible for valine inhibition. In the absence of this regulatory subunit, AHAS activity is lower, but not usually absent. However, there is some evidence which suggests that valine inhibition of eucaryotic AHAS involves the large subunit, thereby throwing doubt upon the necessity, or even the existence, of a small subunit. For example, a number of herbicide-resistant mutations have been identified in the catalytic subunit (summarized in *55*), and there is evidence that branched-chain amino acids interact at the herbicide binding site (*56*). Indeed, it has been argued that some AHAS-inhibiting herbicides are valine analogues (*57*). In addition, a valine-resistance mutation of tobacco AHAS has been identified and located in the catalytic subunit (*58*). Thus, the question of whether there is a small subunit of eucaryotic AHAS is not yet resolved.

We concluded that it would be useful to test directly whether the product of the yeast *ilv6* gene acts as the small subunit of yeast AHAS. In this report, it is shown that the *ilv6* gene is expressed in *S. cerevisiae* and that the gene product is targeted to mitochondria. Also, for the first time, a putative eucaryotic small subunit gene has been cloned and expressed in *E. coli* and the protein purified. The catalytic subunit (the *ilv2* gene product, homologous to the bacterial AHAS large subunit) was separately expressed in *E. coli* and purified. Mixing of the purified small subunit with the pure yeast AHAS catalytic subunit results in reconstitution to give an enhanced AHAS activity that acquires high sensitivity to valine inhibition.

EXPERIMENTAL PROCEDURES

Materials. E. coli expression vectors and host cell BL21-(DE3) were obtained from Novagen, while the yeast episomal vector pYEX4T-1 and yeast cells were from AMRAD Biotech. The plasmid pTB15 (*59*) carrying *ilv2* was obtained from Danisco Biotechnology (Copenhagen, Denmark). The *ilv6* coding region is contained in the D8B clone and was obtained from the Manchester Biotechnology Centre, England. DNA restriction enzymes, DNA modifying enzymes, and other molecular biology reagents were purchased from Progen Industries, New England Biolabs, Boehringer Mannheim, or Bio-Rad. Unless otherwise stated, these were used in accord with the suppliers' recommendations. All other materials and chemicals were purchased from BDH, Ajax Chemicals, Boehringer Mannheim, Sigma Chemical Co., Calbiochem, Pharmacia, Bio-Rad, Merck, or Difco Laboratories.

Cloning of ilv2 and ilv6. The coding sequence of *ilv2* was excised from the plasmid pTB15 by *Hind*III and partial *Bcl*I digestion, and the fragment was cloned into the *Bam*HI/*Hind*III-digested pET30c(+) vector. These and other molecular biological and microbiological procedures followed those outlined by Sambrook et al. (*60*) unless indicated otherwise. This plasmid, termed pET.YLSU, encodes a fusion protein containing the entire sequence of *ilv2* and an

amino-terminal hexahistidine (6xHis) tag for purification by immobilized metal affinity chromatography (IMAC). The plasmid pET.YLSU(-tp) was constructed by removing part of the coding sequence for the presumed mitochondrial transit peptide of pET.YLSU by digestion with *Eco*RV and *Alw*NI.

The *ilv6* coding sequence was amplified from the D8B clone using the primers 5′-CTTTCGCATCGCCGAAGT-GCC-3′ and 5′-GCCACGGCCTCCTCGGCGAGGGGG-CC-3′. The PCR fragment was ligated into the pET30a(+) vector. The resultant plasmid pET.YSSU encodes the entire sequence of *ilv6* and an amino-terminal 6xHis tag. To construct pET.YSSU(-tp), the plasmid pET.YSSU was used as the template for PCR with 5′-GATGCACGGATCCG-CAACAAG-3′ and T7 terminator primers. The amplified PCR fragment was digested with *Bam*HI and *Msc*I and ligated into *Bam*HI/*Msc*I-digested pET.YSSU. The resulting plasmid pET.YSSU(-tp) has 120 bp removed from the 5′ end of the *ilv6* coding region.

The yeast episomal vector pYEX.YSSU(C/His) was constructed by introducing the entire sequence of *ilv6* and a carboxyl-terminus 6xHis tag (from the pET30a(+) vector) into the pYEX4T-1 vector. The pYEX4T-1 vector was first modified to remove its amino-terminal GST tag so that expression starts with the native ATG of *ilv6*.

Yeast Transformation and Diploid Cell Construction. Competent cells of the yeast strain DY150 (MATa, *ura3*-52*, leu2*-3, *leu2*-112, *trp1*-1*, ade2*-1*, his3*-11*, can1*-100) were prepared as described by Becker and Guarente (*61*). The yeast episomal vectors were transformed into the yeast cells by electroporation using a 0.2 cm gap disposable electroporation cuvette (Bio-Rad). The Bio-Rad pulse controller was set to 1.5 kV, 25 μF, and 200Ω. After transformation, the cells were resuspended in 1 mL of ice-cold 1 M sorbitol and selected on YNB medium agar plates supplemented with adenine, tryptophan, and histidine. The transformed yeast cells were crossed with the yeast strain DBY745 (MATα, *leu2*-3*, leu2*-112, *ade1*-100*, ura3*-52*, CUP^R*) as described by Martyn et al. (*62*). The diploid cells were selected on YNB medium containing adenine only. The plasmids carried by the transformed yeast cells were confirmed by back-transformation into *E. coli* (*63*).

Yeast Solid-Phase cDNA Library Construction and RT-PCR. The procedure for construction of a yeast cDNA library was modified from Faulkner and Minton (*64*), replacing the beads coupled with poly(dT) oligo-primers with streptavidin-coupled paramagnetic beads (Dynabeads M-280 Streptavidin). The Dynabeads (500 μg) were sterilized with an equal volume of 0.1 N NaOH containing 0.05 M NaCl followed by washing with 0.1 M NaCl, before resuspension in binding/wash buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 2 M NaCl). The beads were then combined with biotinylated oligo-$(dT)_{25}$ and washed thoroughly to remove unbound primers before mRNA isolation. After the first strand cDNA synthesis, beads to which the yeast cDNA library was immobilized were used directly in the first-round PCR reactions with specific forward and oligo-$(dT)_{16}$ primers. The *ilv2* and *ilv6* specific primers used were 5′-CAAGGTTGC-CAACGACACAGG-3′ and 5′-GATGCACGGATCCG-CAACAAG-3′, respectively. The resultant PCR fragments were cleaned to remove unused primers and then subjected to a second-round nested PCR reaction. The *ilv2* specific primers used were 5′-CAAGGTTGCCAACGACACAGG-3′ and 5′-GTTTTTGAAATTCAAAGCTTACCGCCTG-3′, and *ilv6* specific primers were 5′-AATGCCAACAGTGC-CATGGCATCCATCATTTAC-3′ and 5′-CGCAAGGC-CGAGAGACTAACCTAACC-3′. The amplified DNA fragments were cloned into the pGEM-T Easy vector (Promega) for sequencing.

Expression of ilv6 in Yeast and Isolation of Yeast Mitochondria. A single colony of yeast diploid cells carrying the plasmid pYEX.YSSU(C/His) was selected for *ilv6* expression. The procedures for protein expression in yeast were as described in the pYEX4T yeast expression vector system manual (AMRAD Biotech). Yeast mitochondria were isolated as detailed by Daum et al. (*65*), and a mitochondrial extract was prepared by sonication of the isolated mitochondria. Any 6xHis tagged proteins from the cytosolic and mitochondrial extracts were purified by IMAC as we have described previously (*24*).

Expression of ilv2 and ilv6 in E. coli and Protein Purification. The plasmids pET.YLSU, pET.YLSU(-tp), pET.YSSU, or pET.YSSU(-tp) were each used to transform *E. coli* strain BL21(DE3). The transformed cells were grown, induced, and harvested as described previously (*24*). Cell lysis and purification of the expressed 6xHis tagged recombinant proteins by IMAC was as detailed in Hill et al. (*24*).

Enzyme Assays and Protein Analyses. AHAS activity was measured by the colorimetric single-point method (*66*). The optimized assay contained 50 mM pyruvate, 1 mM ThDP, 10 mM $MgCl_2$, and 10 μM FAD in 1.15 M potassium phosphate buffer at pH 7.0. Variations on this assay mixture were as required for the particular experiment. After incubation at 30 °C for 20 min, the reaction was stopped by the addition of sufficient 50% H_2SO_4 to give a final concentration of 1%. Upon incubation at 60 °C for 15 min, the enzymatic product of AHAS activity, 2-acetolactate, is converted to acetoin. Acetoin was then quantified (*67*) by further incubation at 60 °C for 15 min in the presence of 0.15% creatine and 1.54% α-naphthol, and the color developed was measured at 525 nm. Using acetoin as the standard, the extinction coefficient (ϵ) of the colored complex produced was determined to be 16 164 $M^{-1}cm^{-1}$. One unit (U) of activity is defined as that producing 1 μmol of 2-acetolactate per minute under the above conditions. Protein concentration determination was carried out by the bicinchoninic acid assay (*68*). SDS−PAGE was performed as described by Laemmli (*69*).

DNA and Protein Sequence Determination. DNA sequencing was performed using the dye terminator cycle sequencing ready reaction kit (ABI Prism) and DNA sequencer 373A (Applied Biosystems). Protein N-terminal sequencing was conducted using protein sequencing system 473A (Applied Biosystems). Protein samples were prepared and transferred onto the Immobilon-P membrane (Millipore) as described in the manufacturer's user guide.

Kinetic Data Analysis. The appropriate equations were fitted to the kinetic data by nonlinear regression using the programs DNRPEASY (modified from DNRP53; *70*) and InPlot4 (GraphPAD Software).

RESULTS

Expression of the ilv6 ORF in Vivo. Analysis of the 5′ and 3′ untranslated regions (UTR) of the *ilv6* ORF reveals the presence of the essential elements required for its

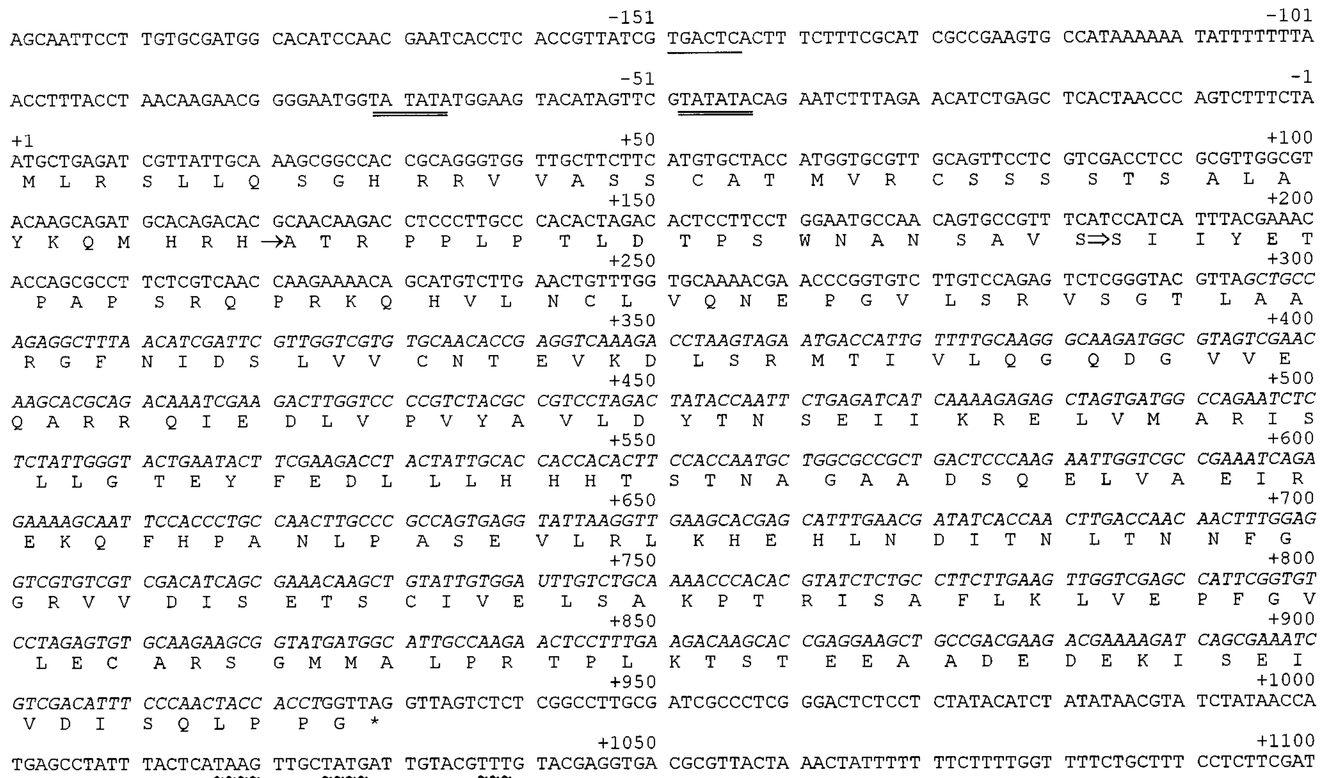

FIGURE 1: Nucleotide sequence of the *YCL009c* ORF and flanking regions. The ORF extends from the start codon (ATG), designated as +1, to the TAG stop codon ending at +930. The consensus *GCN4* binding core sequence (−150 to −145) is underlined, potential TATA box sequences (−72 to −67 and −49 to −44) are double underlined, and ∼∼∼ marks the possible transcription termination signal sequences starting at +1017. Bases shown in italics (+295 to +924) indicate the sequence obtained by RT-PCR. The U at +741 is G in our sequence but A in the sequence deposited in Genbank. The thick arrow (⇒) shows the position of the N-terminus after natural cleavage of the transit peptide, while the thin arrow (→) indicates the start of the *ilv6* protein in the *E. coli* expression vector pET.YSSU(-tp).

transcription, translation, and regulation. Figure 1 shows the nucleotide sequence which includes a continuous ORF of 930 bp, starting with ATG designated +1 and ending with TAG. Two potential TATA box sequences are located at nucleotides −49 to −44 and −72 to −67, which are positions that are typical for TATA boxes found in yeast. Potential transcription termination signal sequences (*71*) are located beyond the stop codon, beginning at bp +1017.

Expression of AHAS activity in yeast is controlled by two mechanisms; *GCN4*-dependent general amino acid control (*72*) and the poorly defined specific multivalent regulation occurring at high concentration of all three branched-chain amino acids (*73*). 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 (*74, 75*). This cis-acting element was previously identified upstream of the *ilv2* gene and shown to function both in vitro and in vivo. If *ilv6* is to be functional as the small subunit of yeast AHAS, we would expect its expression to be regulated by the same mechanism. Analysis of the 5′ UTR identified a DNA sequence located at nucleotides −150 to −145 that matches perfectly with the *GCN4* binding consensus sequence. Further experiments will be required to demonstrate that this sequence will bind *GCN4* and that this up-regulates *ilv6* expression.

The expression of the *ilv6* ORF was shown by RT-PCR. Poly-A tailed mRNA was isolated from a diploid strain of yeast (cross of the haploid strains DY150 and DBY745). This diploid strain is auxotrophic for leucine due to mutations in the *leu2* gene resulting in a deficiency of β-isopropylmalate dehydrogenase (EC 1.1.1.85), the enzyme catalyzing the third reaction that is unique to leucine biosynthesis. To prevent the repression of AHAS genes by exogenous leucine, the diploid yeast cells were transformed with the *E. coli*-yeast shuttle plasmid pYEX4T carrying a functional *leu2*-d gene. This allows the isolation of mRNA from cells grown without addition of any branched-chain amino acids.

A solid-phase cDNA library was constructed using superparamagnetic polystyrene beads coupled with poly(dT)$_{25}$ chains via streptavidin−biotin bridges. The cDNA library was first amplified with specific forward primer and an oligo-(dT)$_{16}$ reverse primer. This was followed by a second round of nested PCR using specific forward and reverse primers to enhance the signal. Specific primers for *ilv2* were included in the experiments as a positive control. As can be seen in Figure 2, a single distinct band of about 800 bp was obtained with *ilv6* specific primers, while the *ilv2* positive control yielded three bands in the range 330−630 bp. All the bands were cloned and sequenced. The sequencing results (not shown) confirmed that the *ilv6* ORF is transcribed in yeast. Of the three DNA fragments amplified in the positive control, only the largest is derived from *ilv2* mRNA.

Localization of ilv6 Gene Product in Yeast. The deduced amino acid sequence of the *ilv6* ORF consists of 309 residues. Multiple alignment of the deduced sequence with several bacterial AHAS small subunit sequences shows significant homology except for an additional 70 residues at the N-terminus and about 40 residues in the middle of the sequence (Figure 3). Since yeast AHAS activity is located

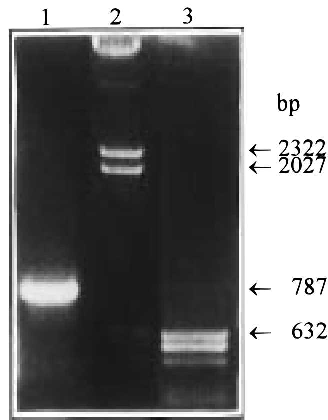

FIGURE 2: Agarose gel of DNA fragments obtained by RT-PCR. Lane 1: the 787 bp fragment obtained using *ilv6* primers. Lane 2: size markers. Lane 3: the three fragments (632, 496, and 337 bp) obtained using *ilv2* primers.

in the mitochondria (*32*) and the extra residues at the N-terminus of *ilv6* show the properties of a typical yeast mitochondrial transit peptide (*50, 54, 76, 77*), it may serve as a targeting signal sequence.

The *ilv6* ORF was cloned into a yeast episomal vector pYEX.YSSU(C/His) that will express the protein with an additional C-terminal 6xHis tag. In this construct, expression of the ORF in *S. cerevisiae* is under the control of the inducible CUP1 promoter. Mitochondria of the induced yeast cells were harvested and lysed by sonication, then the soluble cytosolic and mitochondrial lysates were purified using a Ni^{2+}-charged IMAC column to isolate any 6xHis tagged proteins. Figure 4A shows an SDS−PAGE analysis of the proteins purified from the cytosolic and mitochondrial fractions. In addition to the expected 6xHis tagged proteins, a contaminating yeast protein of about 40 kDa was observed using this purification method. N-terminal analysis gave the protein sequence SIPET that matches exactly that expected for yeast alcohol dehydrogenase (ADH), which has a calculated subunit molecular weight of 36.9 kDa. Copurification of ADH is not surprising because this enzyme contains zinc that is bound at two sites, and a recent paper (*78*) has reported the purification of ADH using a zinc-coupled affinity column. Being a cytosolic protein, the contaminating ADH acts as a useful marker protein. The deduced molecular weight of the *ilv6* gene product is 34.1 kDa. The purified mitochondrial fraction (lane 1), which is free of the cytosolic ADH marker, has 2 bands corresponding to the *ilv6* gene product. This shows that the 6xHis tagged *ilv6* gene product is targeted to the mitochondria as predicted. The two forms of *ilv6* protein, which may represent the processed and unprocessed gene products, were sequenced. The larger band yielded no sequence, suggesting that the N-terminus is blocked, while the smaller protein species gave a sequence of SI(V/I)YETPAPS. Except for some ambiguity at the third residue, the sequence matches exactly with the deduced *ilv6* amino acid sequence starting from residue 62 and is close to the start of homology with the bacterial small subunit sequences (Figure 3). The Ser61−Ser62 bond may be the transit signal sequence cleavage site of *ilv6* gene product. The purified cytosolic fraction (lane 3) also contains the *ilv6* proteins, possibly released by partial lysis of mitochondria during the isolation. Alternatively, since *ilv6* is being overexpressed in these cells, their capacity to keep pace with the rate of protein production may have been overloaded. The incomplete processing in mitochondria of *ilv6* to remove the transit peptide may also be explained in this way.

Expression of ilv2 and ilv6 in E. coli. The results presented above show that the *ilv6* gene product is expressed in yeast and transported to the mitochondria and that the transit peptide is removed. However, they do not show that it functions as the small subunit of yeast AHAS. Therefore, we attempted its reconstitution with the *ilv2* gene product and investigated the effect on enzymatic activity.

The entire sequences of *ilv2* and *ilv6* genes were cloned into the pET.30(+) series vector to yield pET.YLSU and pET.YSSU, respectively. The cloning procedure introduces N-terminal 6xHis tags to simplify purification. Overexpression in *E. coli* was successful but both recombinant proteins were found exclusively in the insoluble bacterial lysate (results not shown). Two further plasmids, pET.YLSU(-tp) and pET.YSSU(-tp), were constructed by removing part of the presumed transit peptide sequence. After the removal of 54 and 40 amino acids from *ilv2* and *ilv6*, respectively, expression in *E. coli* resulted in soluble recombinant proteins, and these were purified to near homogeneity (Figure 4B). In the case of the *ilv2*-encoded catalytic subunit, we recovered 35% of the activity and achieved a 53-fold purification.

Activity and Stability of the Bacterial-Expressed ilv2 and ilv6 Proteins. Enzymatic assay with the purified proteins shows that the truncated *ilv2*, but not the *ilv6*, protein has AHAS activity. It was observed that this activity is very labile, especially when the protein is dissolved in low ionic strength (20 mM Tris-HCl or 50 mM potassium phosphate) buffer (pH 8) containing 10 μM FAD, with or without 20% glycerol. Up to 80% of the activity is lost when stored at 4 °C overnight although it is stable for several months at −70 °C. Enzyme stability is improved (no loss after 24 h at 4 °C or four weeks at −70 °C) by storage in buffer of high ionic strength (0.5 M potassium phosphate, pH 7) with the addition of 10 μM FAD, 1 mM DTT, and 20% glycerol. The specific activity of the purified *ilv2* protein is approximately 7 U/mg. Like all eucaryotic AHAS expressed in bacterial systems (*46*−*49*), the enzymatic activity of the recombinant *ilv2* protein is insensitive to branched-chain amino acids up to 10 mM.

The *ilv6* protein has limited solubility in aqueous solution, with a maximum concentration of about 0.35 mg/mL attainable when stored under the conditions mentioned above. Similar low solubility has been observed with the small subunits of *E. coli* AHAS II (this laboratory, unpublished results) and AHAS III (*27*).

Reconstitution of ilv2 with ilv6 Protein. When assayed in a buffer of low ionic strength, addition of a large excess of the *ilv6* protein to the *ilv2* protein results in little or no observable change in the AHAS activity or valine sensitivity. Several papers on fungal AHAS, published around 1970 but now largely forgotten, offered an explanation for these observations. For example, Magee and de Robichon-Szulmajster (*79*) demonstrated that the sensitivity to valine inhibition of yeast AHAS activity in permeabilized cells and crude extracts is extremely labile with the activity being desensitized in a crude extract when solubilized from the mitochondria. Similar results were observed with the mitochondrially localized *Neurospora crassa* AHAS activity (*80*−*82*). Kuwana and Date (*83*) later reported the ability to

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Sce      MLRSLLQSG -HRRVVASS- ---------- -CATMVRCS- -SSSTSALAY    KQMHRHATRP PLPTLDTP-S WN-ANSAVSS IIYETPAPSR QPRKQHVLNC    82
Spa      MLRSLLQSG -HRRVAASS- ---------- -CATMVRCS- -SSSTSALAY    KQMHRHAARP PLPTLDTP-S WN-ANSAVSS IIYETPAPSR QPRKQHVLNC    82
Spo                                                                                                    MSTPFNRV QRPKRHVFNC    18
Ncr     MASRGLVMSA PLRRISAKAR DAAPVARQSL PAAIGVRYSS HSSSTSAIAY    KAIRRRHA-- PLPAADAPPA WSSAQAAVSN ILYETPSPSL APPKRHILNC    98
Cca                                                                                                               MKHTLSV     7
Cme                                                                                                               MKHTLSV     7
Gth                                                                                                               MKHTLSV     7
Ppu                                                                                                               MKHTLSV     7
Ssp                                                                                            MEF YPNGHRRSPS LPPMKHTLSV    23
Cgl                                                                                                       MAN SDVTRHILSV    13
Mav                                                                                                            MSPQTHTLSV    10
Mae                                                                                                             MKNTHIISV     9
Eco                                                                                                               MRRILSV     7
Con                                                                                                                  : :.

Sce     LVQNEPGVLS RVSGTLAARG FNIDSLVVCN TEVKDLSRMT IVLQGQDGVV    EQARRQIEDL VPVYAVLDYT NSEIIKRELV MARISLLGTE YFEDLLLHHH   182
Spa     LVQNEPGVLS RISGTLAARG FNIDSLVVCN TEVKDLSRMT IVLQGQDGVI    EQARRQIEDL VPVYAVLDYT NSEIIKRELV MARISLLGTE YFEDLLLHHH   182
Spo     LVQNEPGVLS RLSGILAARG FNIDSLVVCA TEVENLSRMT IVLRGADEVV    EQAKRQIEDI VSVWAVLDYT GTSMVERELL LAKVSLLGPD HFQEHFER--   116
Ncr     LVQNEPGVLS RVSGILAARG FNIDSLVVCN TEVDDLSRMT IVLTGQDGVV    EHARRQLEDL VPVWAVLDYS N????????? ?????????? ??????????   169
Cca     LVEDEAGVLT RIAGLFARRS FNIESLAVGP AEQVGISRIT MVVPGDDRTI    EQLMKQLYKF IPIFKVENLT Q--------- ---------- ----------    78
Cme     LVEDEAGVLT RIAGLFARRS FNIESLAVGP AEQAGISRIT MVVPGDDRTI    EQLMKQLYKL IPILQVENLT Q--------- ---------- ----------    78
Gth     LVEDESGVLT RIAGLFARRG FNIESLAVGP TEKLGISRIT MVVPSDERTI    EQLTKQLYKL VNILKVEDIT N--------- ---------- ----------    78
Ppu     LVQDEAGVLS RISGLFARRG FNIASLAVGP AEQIGVSRIT MVVQGDNRTI    EQLTKQLYKL VNILNVQDVT N--------- ---------- ----------    78
Ssp     LVEDEAGVLT RIAGLFARRG FNIESLAVGS AEQGDVSRIT MVVPGDENTI    EQLTKQLYKL VNVIKVQDIT E--------- ---------- ----------    94
Cgl     LVQDVDGIIS RVSGMFTRRA FNLVSLVSAK TETHGINRIT VVVDADELNI    EQITKQLNKL IPVLKVVRLD E--------- ---------- ----------    84
Mav     LVEAKPGVLA RVAALFSRRG FNIESLAVGA TEQKDMSRMT IVVSAEETPL    EQITKQLNKL INVIKIVELE D--------- ---------- ----------    81
Mae     LVLNKPGVLQ RISGLFTRRW YNISSITGGS TDSTDISRMT IVVKGDDKVV    EQVVKQLNKL IEVIKVIDLD E--------- ---------- ----------    80
Eco     LLENESGALS RVIGLFSQRG YNIESLTVAP TDDPTLSRMT IQTVGDEKVL    EQIEKQLHKL VDVLRVSELG Q--------- ---------- ----------    78
Con     *:     * : *: . :: *  :*: *:.    ::   :.*:* :   . :  :    *:  :*: .: : :  :

Sce     TSTNAGAADS QELVAEIREK QFHPANLPAS EVLRLKHEHL NDITNLTNNF    GGRVVDISET SCIVELSAKP TRISAFLKLV EPFG-VLECA RSGMMALPRT   281
Spa     TSTSSGGADA NELVAEIREK QFHPANLPAS EILRLKHEHL NDVTNLTNNF    GGRVVDISET SCIVELSAKP TRISAFLKLV EPFG-VLECA RSGMMALPRT   281
Spo     -------SEK VAESTNAKAK SDGEGVMNAN AALQLRASQL AAINQLTTLF    HGRVADISTE TIILELTATP DRVDNFLSLL RPYG-VLEAC RTGTSAMTRA   208
Ncr     ?????????G GEHSLEETAK DFHPSRLVAS EALRHKHEHL KSITYFTHQF    GGKVLDISTN SCIVEVSAKP VRIDSFLKLI APFG-ILESA RTGLMALPRS   259
Cca     ---------- ----VPCVER ELMLMKVKAN S------DTR REILDMANIF    RARIVDIAAE DLMLEVTGDP GKMVALEQVL AKFG-LVEVA RTGKIALKRS   157
Cme     ---------- ----VPCVER ELILMKVQAN S------QTR REILDMANIF    RARVVDISAD DVMLEVTGDP GKMVALEQIL TKFG-IVEVA RTGKIALKRS   157
Gth     ---------- ----LPTVER ELMLIKLRVS T------FER TEALDIINIF    RAKVVDLSED FLIIEVTGDP GKIVAIEQIL SKFT-ISEIV RTGKISLVRE   157
Ppu     ---------- ----IPSVER ELMLIKIQVN S------QNR IEALEIVKIF    RANVVDIAED LIIVEVTGDP GKIVAIEQLL TKFG-IIEIA RTGKISLVRT   157
Ssp     ---------- ----TPCVER ELMLVKVSAN A------PNR AEVIELAQVF    RARIVDISED TVTIEVVGDP GKMVAILQML AKFG-IKEVA RTGKIALVRE   173
Cgl     ---------- ----ETTIAR AIMLVKVSAD S------TNR PQIVDAANIF    RARVVDVAPD SVVIESTGTP GNSAHCLT                          142
Mav     ---------- ----GNSVSR ELALIKVRAD A------GTR SQVIEAVNLF    RAKVIDVSPE ALTIEATGDR GKIEALLRVL EPSV-SVRSS NREWCRCPGP   160
Mae     ---------- ----EECVER ELCLIKIYAP TE-----SSK SQVIQYANIF    RGNIVDLSQE SLTVQITGDK TKISAFIKLV KPMG-IKEIS RTGLTALMRG   160
Eco     ---------- ----GAHVER EIMLVKIQAS G------YGR DEVKRNTEIF    RGQIIDVTPS LYTVQLAGTS GKLSAFLASI RDVAKIVEVA RSGVVGLSRG   158
Con                                :          : .                    *     ..: *::       ::   .     .

Sce     PLKTSTEEAA DEDEKISEIV DISQLPPG                                                                                        309
Spa     PLKTSIEEAA DEDEKINEIV DISQLPPG                                                                                        309
Spo     PHSNEVTEEA ED----DVEV EEVFLPPG                                                                                        232
Ncr     PLYEHGEETH VK--EADDVV DASQLPPG                                                                                        285
Cca     SKVNTEWLR                                                                                                             166
Cme     SKVNTEWLR                                                                                                             166
Gth     SNVNTRSLLK EY                                                                                                         169
Ppu     SKINTEYLKD KVVAYNA                                                                                                    174
Ssp     SGVNTEYLKS LESKF                                                                                                      188
Cgl                                                                                                                           142
Mav     RGIGTAK                                                                                                               167
Mae     PKILKSNKA                                                                                                             169
Eco     DKIMR                                                                                                                 163
Con
```

FIGURE 3: Alignment of known and putative AHAS small subunit protein sequences. Sequences shown are for fungi (Sce, *Saccharomyces cerevisiae*; Spa, *Saccharomyces pastorianus*; Spo, *Schizosaccharomyces pombe*; Ncr, *Neurospora crassa*), algae (Cca, *Cyanidium caldarium*; Cme, *Cyanidioschyzon merolae*; Gth, *Guillardia theta*; Ppu, *Porphyra purpurea*), and bacteria (Ssp, *Synechocystis* sp. strain PCC6803; Cgl, *Cornebacterium glutamicum*; Mav, *Mycobaterium avium*; Mae, *Methanococcus aeolicus*; Eco, *Escherichia coli* isoenzyme III). Con indicates conserved residues denoted by (*) invariant; (:) strong conservation group (STA, NEQK, NHQK, NDEQ, QHRK, MILV, MILF, HY, FYW); (●) weaker conservation group (CSA, ATV, SAG, STNK, STPA, SGND, SNDEQK, NDEQHK, NEQHRK, FVLIM, HFY). The Ncr sequence is combined from two expressed sequence tags, and the question mark (?) indicates possible central residues that may be missing.

maintain a high specific activity and sensitivity to valine of the *N. crassa* AHAS when the mitochondrial extract was solubilized by sonication in a buffer containing 1.0 M potassium phosphate. Accordingly, high concentrations of potassium phosphate were tested for the reconstitution of the bacterially expressed *ilv2* and *ilv6* proteins. Under these conditions, a striking enhancement of the AHAS activity was observed.

Effect of Potassium Phosphate Concentrations on Reconstitution Activity and Valine Sensitivity. The effect of increasing concentrations of potassium phosphate on the AHAS activity of *ilv2* protein and the reconstituted activity was investigated in the presence and absence of 5 mM valine (Table 1). In the absence of this branched-chain amino acid, stimulation of AHAS activity was observed at potassium phosphate concentrations over the range 0.6−1.5 M. At the optimum potassium phosphate concentration of approximately 1 M, the specific activity is enhanced by as much as 6-fold. Upon addition of 5 mM valine, the stimulation of AHAS activity is largely abolished.

Effect of pH on Reconstitution Activity and Valine Sensitivity. In addition to showing a dependence on the potassium phosphate concentration in the buffer, the stimulation of AHAS activity and valine inhibition is pH sensitive (Table 1). At a pH of 6, little enhancement of activity is observed in the presence of *ilv6* protein. The enzymatic activity increases as the pH increases, with the optimum pH for the reconstituted activity between pH 7.0 and 7.5. In contrast, the AHAS activity of the *ilv2* protein alone is largely unaffected by pH. With the addition of 5 mM valine, reconstituted enzyme behaves in a way similar to that of the *ilv2* protein alone.

Association of ilv2 and ilv6 Proteins. The above experiments defined the optimum conditions for subunit reconstitution, and subsequent experiments were conducted in 1.15 M potassium phosphate buffer, pH 7.0. With increasing concentrations of the *ilv6* protein, the stimulation of activity follows an hyperbolic curve (Figure 5). In this experiment, the specific activity of *ilv2* protein alone is 6.8 ± 0.7 U/mg, while the extrapolated maximum specific activity of the

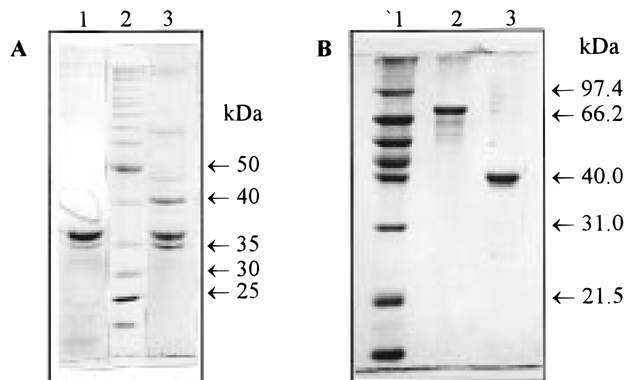

FIGURE 4: SDS–PAGE analysis of proteins obtained by expression of *ilv2* and *ilv6*. Panel A: expression of *ilv6* in yeast using the pYEX.YSSU(C/His) plasmid. After separation of the mitochondrial and cytoplasmic fractions, proteins were purified by IMAC: lane 1, purified mitochondrial fraction; lane 2, size markers; lane 3, purified cytoplasmic fraction. Panel B: purified *ilv2* and *ilv6* proteins. Proteins were separately expressed in *E. coli* then purified by IMAC: lane 1, size markers; lane 2, the *ilv2* protein obtained using the plasmid pET.YLSU(-tp); lane 3, the *ilv6* protein obtained using the plasmid pET.YSSU(-tp).

Table 1: Effect of Phosphate Concentration, pH, and ATP on the Activity and Valine Sensitivity of Yeast AHAS

	specific activity (U/mg of *ilv2*)			
	no added valine[a]		5 mM valine[a]	
tested effect	*ilv2*[b]	*ilv2* + *ilv6*[b]	*ilv2*[b]	*ilv2* + *ilv6*[b]
[phosphate], M[c]				
0.04	7.8	10.5	7.3	9.9
0.06	7.9	10.5	6.9	10.0
0.09	8.2	11.4	7.1	10.3
0.13	7.7	11.0	6.8	10.4
0.22	7.6	11.1	7.0	11.8
0.41	7.0	11.9	7.3	12.3
0.59	7.5	14.8	6.0	9.9
0.78	4.6	27.2	4.2	6.0
0.96	6.4	36.2	5.7	7.1
1.14	6.6	36.5	6.4	8.7
1.33	7.7	31.4	6.4	8.7
1.51	5.7	21.3	5.9	7.2
pH[d]				
6.0	7.3	11.2	6.6	8.6
6.5	7.2	30.1	7.0	7.9
7.0	7.6	41.0	6.5	9.1
7.5	7.1	41.2	6.1	7.6
8.0	5.0	31.0	3.8	5.6
additions[e]				
none	6.1	34.3	5.7	6.5
1 mM ATP	5.7	37.9	6.1	18.0

[a] Valine added. [b] Proteins added; *ilv2* = 5.09 nM, *ilv6* = 610 nM. [c] At pH 7.0. [d] Buffered with 1.15 M potassium phosphate. [e] At pH 7.0 buffered with 1.15 M potassium phosphate.

reconstituted enzyme is 49.0 ± 1.8 U/mg of *ilv2*. Using 5.09 nM of the *ilv2* protein, the concentration of *ilv6* protein required to give 50% of the maximum stimulation of activity is 69.8 ± 7.3 nM. We interpret this value as representing the dissociation constant of the complex between the two types of subunit.

Branched-Chain Amino Acid Inhibition and ATP Activation. The reconstituted activity generated by the mixing of the bacterially expressed *ilv2* and *ilv6* gene products is sensitive to valine inhibition (Figure 6) with an apparent K_i of 0.16 ± 0.02 mM. Not all AHAS activity is abolished in the presence of valine, even at a concentration of 5 mM,

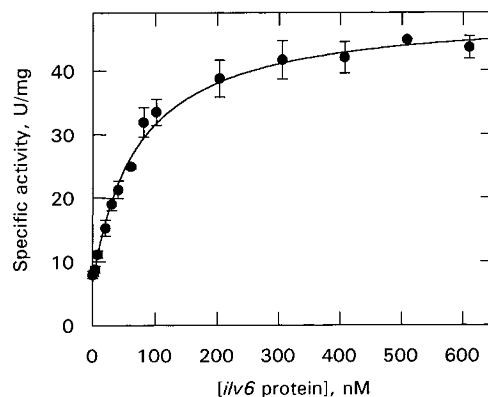

FIGURE 5: Reconstitution of AHAS by increasing concentrations of the *ilv6* protein. Assays contained 5.09 nM of the *ilv2* protein and varying concentrations of the *ilv6* protein as shown. Specific activity is expressed as U/mg of *ilv2* protein. The line represents the best fit to the data of an hyperbolic saturation curve defined by the parameters: *Y*-intercept = 6.8 ± 0.7 U/mg; *Y*-asymptote = 49.0 ± 1.8 U/mg; half-saturating concentration of the *ilv6* protein = 69.8 ± 7.3 nM.

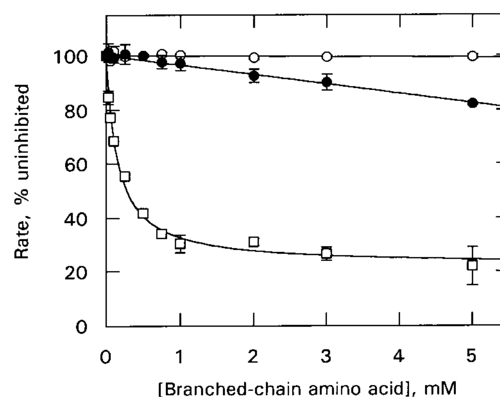

FIGURE 6: Inhibition of reconstituted AHAS by branched-chain amino acids. Activity, relative to the activity with no additions, is plotted against the concentration of added leucine (○), isoleucine (●), or valine (□). The line for valine inhibition represents the best fit to the data of an hyperbolic curve defined by a half-saturating concentration of 0.162 ± 0.022 mM valine and a *Y*-asymptote of 22.2 ± 1.7% activity. The concentrations of *ilv2* and *ilv6* were 5.09 and 610 nM, respectively.

with about 20% of the activity retained. This residual activity is similar to the basal AHAS activity of *ilv2* alone. The inhibitory effects of the other two branched-chain amino acids, leucine and isoleucine, were tested (Figure 6); 5 mM isoleucine inhibits by about 20%, while this concentration of leucine has no effect on the reconstituted activity.

It has been reported that AHAS activity in intact mitochondria of *Euglena gracilis* (*84*) and *Neurospora crassa* (*82*) is activated by ATP, but it was not established whether the effect was direct or mediated through a mitochondrial process. We therefore tested the effect of ATP on the purified proteins (Table 1). There is little effect on *ilv2* protein alone, with or without valine. The reconstituted *ilv2* plus *ilv6* AHAS activity is also unaffected by ATP in the absence of valine, but in its presence, the activity is stimulated 3-fold. Thus, ATP affects AHAS activity directly by partially reversing the inhibition by valine.

Substrate Saturation Curves. The *ilv2* protein alone, in the absence of valine, exhibits a K_m for pyruvate of 8.6 ± 1.4 mM, and this value does not change (8.3 ± 0.7 mM) if 5 mM valine is added. Reconstitution with the *ilv6* protein

causes an increase in the K_m to 18.1 ± 1.3 mM, together with the increase in the specific activity described earlier. Adding 5 mM valine to the reconstituted enzyme reduces the specific activity but this is not accompanied by a decrease in the K_m to the value found for the *ilv2* protein alone. Instead, there is a further small increase in the K_m to 23.0 ± 2.6 mM.

DISCUSSION

In this report it is demonstrated that *S. cerevisiae* AHAS requires another subunit, corresponding to the bacterial small subunit, for the activity to be regulated by end-product (valine) feedback inhibition as seen in the native enzyme. Besides conferring valine sensitivity, a substantial enhancement of the enzymatic activity is observed upon reconstitution. This is the first time that a eucaryotic AHAS small subunit has been cloned and shown to reconstitute with its large subunit.

Earlier work (*50, 54*) had suggested that the yeast ORF *YCL009c* (*ilv6*) might encode a regulatory subunit of AHAS. Here we have shown that the gene possesses all the transcription, regulation, and translation signals that would be consistent with this function (Figure 1) and that it is expressed in yeast (Figure 2). Further, the gene product is transported to mitochondria where it undergoes cleavage of the transit peptide (Figure 4A). The predicted amino acid sequence differs from procaryotic AHAS small subunits by possessing an insertion of approximately 40 residues (Figure 3). We considered the possibility that this extra sequence may not form part of the translated protein, even though there are no obvious intron-exon splice sites at its boundaries. However, there is no evidence that would support this conjecture. RT-PCR demonstrated a fragment that contains the extra sequence (Figure 2), and expressing the gene in yeast yielded a protein of a size consistent with translation of the entire ORF (Figure 4A). Finally, expression in *E. coli*, where mRNA splicing does not occur, yielded a protein that is competent in reconstitution with the yeast catalytic subunit (Figure 5).

Previously there had been some circumstantial evidence for a eucaryotic AHAS small subunit (*50, 85*) but little direct biochemical demonstration that it exists. Purified barley AHAS (*86*) showed only a single band in SDS−PAGE although the specific activity of this preparation (1.6 U/mg) is quite low, possibly due to the loss of a small subunit during the five-step purification. Southan and Copeland (*45*) observed a 15 kDa band in SDS−PAGE of purified wheat AHAS that they suggested could represent the small subunit. However, the very low activity of their preparation (0.06 U/mg) casts some doubt upon its integrity. Thus, the studies reported here constitute the first unequivocal biochemical evidence for a eucaryotic AHAS small subunit.

The *ilv2* protein alone, which is the homologue of the bacterial AHAS large subunit, exhibits some AHAS activity (Table 1). Expression of *ilv2* in *E. coli* and purification of the protein have been reported previously by Poulsen and Stougaard (*59*). After a six-step purification they obtained an enzyme of low specific activity (0.17 U/mg) although they speculated that, had they not lost up to 99% of the enzyme due to inactivation, a specific activity of 5.1 U/mg might be possible. This is comparable to the value of about 7 U/mg that we obtain. In contrast to the low recovery of AHAS obtained by Poulsen and Stougaard (*59*), we recovered 35% of the activity. The pure enzyme shows classical Michaelis−Menten kinetics with pyruvate, giving a K_m of 8.6 mM. Reconstitution results in a doubling of the K_m value, together with a substantially higher catalytic activity.

Previously, it has been reported that the valine feedback regulation of yeast and *N. crassa* AHAS is extremely labile (*79*−*82*). Taking a cue from the observation (*83*) that valine sensitivity of *N. crassa* AHAS is preserved in 1 M potassium phosphate buffer, we found that reconstitution of the bacterially expressed pure yeast AHAS subunits could be demonstrated at high ionic strength (Table 1 and Figure 5).

In addition to being sensitive to the concentration of the potassium phosphate buffer used, the reconstitution of the yeast AHAS subunits is pH dependent, with the optimum pH between 7.0 and 7.5. Under the optimal conditions for reconstitution, the apparent K_i for valine is 0.16 mM (Figure 6). These results are similar to those reported by Magee and de Robichon-Szulmajster (*79, 87*) using a cell-free yeast extract. Their AHAS activity had a optimum pH of 7.2 and was inhibited by valine with an apparent K_i of 0.05 mM.

The small subunit of *E. coli* AHAS III, in the absence of the large subunit, has been shown to bind valine with a K_d of 0.2 mM (*27*). The interaction of the valine-binding small subunit was suggested to induce conformation changes at the catalytic site of the large subunit. Although we have not demonstrated directly that the yeast small subunit binds valine, the observation that this subunit is required for the large subunit to exhibit valine sensitivity suggests that a similar mechanism may be operating.

At a saturating concentration of valine (5 mM), the activity of the yeast reconstituted AHAS is not totally inhibited, leaving a specific activity that is similar to the intrinsic AHAS activity of the *ilv2* protein (Table 1). Moreover, the presence of valine abolishes the response of AHAS to changes in pH and potassium phosphate concentration, properties that are shared with the activity of the *ilv2* protein. A possible interpretation of these results is that valine exerts its inhibitory action by inducing the dissociation of the large and small subunits. However, this mechanism is not consistent with the finding that the K_m for pyruvate of the reconstituted enzyme in the presence of valine (23.0 mM) is different from that of *ilv2* large subunit alone (8.6 mM). This result strongly suggests that the decrease in activity caused by adding valine is not due to dissociation of the *ilv6* and *ilv2* proteins. Thus it appears that the catalytic site of *ilv2* must be altered when the protein interacts with the *ilv6* protein and that valine exerts its inhibitory effect through conformational changes in the reconstituted enzyme, rather than by inducing dissociation.

The purified bacterially expressed *ilv6* gene product has low solubility and tends to precipitate at concentrations above 0.35 mg/mL. This is probably because the yeast small subunit has a hydrophobic surface that promotes self-aggregation. Reconstitution of the small subunit with the yeast AHAS large subunit to give valine-sensitive enzymatic activity occurs only in high ionic strength potassium phosphate buffer. Thus we conclude that hydrophobic interactions, which are promoted at high ionic strength, are involved in subunit association.

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