Regulation of yeast acetohydroxyacid synthase by valine and ATP

Siew Siew PANG and Ronald G. DUGGLEBY[1]

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

The first step in the common pathway for the biosynthesis of branched-chain amino acids is catalysed by acetohydroxyacid synthase (AHAS; EC 4.1.3.18). The enzyme is found in plants, fungi and bacteria, and is regulated by controls on transcription and translation, and by allosteric modulation of catalytic activity. It has long been known that the bacterial enzyme is composed of two types of subunit, and a similar arrangement has been found recently for the yeast and plant enzymes. One type of subunit contains the catalytic machinery, whereas the other has a regulatory function. Previously, we have shown [Pang and Duggleby (1999) Biochemistry **38**, 5222–5231] that yeast AHAS can be reconstituted from its separately purified subunits. The reconstituted enzyme is inhibited by valine, and ATP reverses this inhibition. In the present work, we further characterize the structure and the regulatory properties of reconstituted yeast AHAS. High phosphate concentrations are required for reconstitution and it is shown that these conditions are necessary for physical association between the catalytic and regulatory subunits. It is demonstrated by CD spectral changes that ATP binds to the regulatory subunit alone, most probably as MgATP. Neither valine nor MgATP causes dissociation of the regulatory subunit from the catalytic subunit. The specificity of valine inhibition and MgATP activation are examined and it is found that the only effective analogue of either regulator of those tested is the non-hydrolysable ATP mimic, adenosine 5′-[β,γ-imido]triphosphate. The kinetics of regulation are studied in detail and it is shown that the activation by MgATP depends on the valine concentration in a complex manner that is consistent with a proposed quantitative model.

Key words: acetolactate synthase, branched-chain amino acids, kinetic model, reconstitution, subunit interactions.

INTRODUCTION

Plants, fungi and bacteria can synthesize valine, leucine and isoleucine, with all three of these branched-chain amino acids being derived via a common pathway [1]. The enzymes involved in this process are regulated in a number of ways, including transcription, translation and allosteric modulation of catalytic activity [2]. Such regulation occurs at several steps, but one of the most important of these is that catalysed by acetohydroxyacid synthase (AHAS; EC 4.1.3.18). This enzyme [3] converts 2 mol of pyruvate into 1 mol of 2-acetolactate as the first step in the synthesis of valine and leucine. It also converts 1 mol each of pyruvate and 2-ketobutyrate into 1 mol of 2-aceto-2-hydroxybutyrate, the precursor of isoleucine.

In bacteria, such as *Escherichia coli*, the allosteric modulation is complex due to the existence of three isoforms with somewhat different properties with respect to their relative abilities to produce acetolactate and acetohydroxybutyrate, and their sensitivity to feedback inhibition [3]. Two of these isoenzymes (AHAS I and AHAS III) are inhibited by valine, whereas AHAS II is not. Inhibition by valine, but not the other branched-chain amino acids, seems to be a general characteristic of bacterial AHAS. The exceptions (apart from enterobacterial AHAS II) are the catabolic AHAS enzymes found in bacteria that possess the butanediol fermentation pathway. This form of AHAS, which differs in many respects from the biosynthetic enzyme [1], is not the focus of the present study.

AHAS from most, if not all, bacteria is composed of two types of subunit. The larger one contains the active site and the cofactors [thiamin diphosphate (ThDP), Mg^{2+} and FAD] and is usually [4–6], but not always [7], active alone. The smaller subunit modulates the activity of the catalytic subunit by increasing its activity and conferring upon it sensitivity to valine inhibition [4]. At least in the case of *E. coli* AHAS III, it has been shown that the regulatory subunit contains the valine-binding site [6,8].

In fungi, there is a single isoform [9–11] that, like its bacterial counterpart, is inhibited by valine. However, unlike the bacterial system the inhibition is partial so that a saturating concentration of valine does not abolish the activity. We showed recently, by *in vitro* reconstitution, that yeast AHAS is also composed of catalytic and regulatory subunits [12]. That work constituted the first unequivocal demonstration of a regulatory subunit for any eukaryotic AHAS; previously the existence of this subunit had been speculated upon [13], but never demonstrated directly. As in most bacterial systems, the catalytic subunit alone is active but insensitive to valine inhibition. Combination with the regulatory subunit stimulates activity 7–10-fold and makes it sensitive to valine inhibition. It was also shown that the valine-inhibited reconstituted yeast AHAS is stimulated by ATP. This property of fungal AHAS was first described [14] in a system that was too crude to be sure that there was a direct effect of ATP on AHAS. Another unusual feature of the fungal enzyme is that reconstitution requires a very high (approx. 1 M) phosphate concentration, again in agreement with earlier studies in crude systems, which showed that a loss of regulatory properties could be prevented by these high phosphate concentrations [15].

Plant AHAS is inhibited by all three branched-chain amino acids [16,17], usually in the order of potency leucine > valine > isoleucine. There is also a pronounced synergy between leucine and valine so that this combination is effective at far lower

Abbreviations used: AHAS, acetohydroxyacid synthase; p[NH]ppA, adenosine 5′-[β,γ-imido]triphosphate; DTT, dithiothreitol; ϵ-ATP, 1,N^6-ethenoATP; ThDP, thiamin diphosphate.

[1] To whom correspondence should be addresssed (e-mail duggleby@biosci.uq.edu.au).

concentrations than either amino acid alone. As with yeast AHAS, inhibition is partial. A possible regulatory subunit has been isolated for tobacco AHAS [18], although it is of some concern that this fails to make the catalytic subunit sensitive to inhibitors. More recently, an *Arabidopsis thaliana* AHAS regulatory subunit has been isolated [19], and in this case the reconstituted enzyme is inhibited by branched-chain amino acids. Moreover, the order of potency and the synergy between leucine and valine, match those of the native enzyme. The plant regulatory subunit contains a sequence duplication of approximately 180 amino acids, and it has been suggested [19] that these provide separate but interacting binding sites for leucine and valine.

Yeast and bacterial AHAS regulatory subunits contain only one copy of the repeat mentioned above [1], consistent with the finding that only valine inhibits. However, the yeast sequence contains a 50-residue insert that might provide the ATP-binding site. This hypothesis must be tempered by the fact that it has not been demonstrated that the regulatory subunit is the site of ATP binding.

In the present study we further examine the mechanism of regulation of yeast AHAS reconstituted from its purified subunits. It is demonstrated that ATP binds to the regulatory subunit alone, most probably as MgATP. The requirement for high phosphate concentrations, and the effects of MgATP and valine, are examined in terms of their effects on the quaternary structure. It is shown that the reconstituted enzyme contains equal amounts of the two subunits. Neither valine nor MgATP causes dissociation of the catalytic subunit from the regulatory subunit. The specificity and kinetics of valine inhibition and MgATP activation are examined in detail and it is shown that the activation by MgATP depends on the valine concentration in a complex manner. A quantitative model is proposed to explain the data.

EXPERIMENTAL

Materials

Most materials were obtained from standard commercial suppliers, as described previously [12]. The valine derivatives *N*-acetyl-L-valine, *N*-methyl-D,L-valine and L-valinamide, and ATP analogues AMP, ADP, GTP, CTP, UTP and adenosine 5′-[β,γ-imido]triphosphate (p[NH]ppA) were purchased from Sigma (St Louis, MO, U.S.A.), whereas 1,N^6-ethenoATP (ϵ-ATP) was obtained from Molecular Probes (Eugene, OR, U.S.A.).

E. coli growth and protein expression

Plasmids based on the pET30(+) series of vectors containing cDNA for the catalytic and regulatory subunits of yeast AHAS have been described previously [12]. These were used to transform the BL21(DE3) strain of *E. coli* (Novagen, Madison, WI, U.S.A.). Transformed cells were grown at 37 °C in Luria–Bertani medium containing 50 μg/ml kanamycin, and protein expression was induced by the addition of 1 mM isopropyl β-D-thiogalactoside when D_{600} (1 cm pathlength) reached 0.8–1.0. Expression was for 5 h at approx. 22 °C. Cells were harvested by centrifugation at 5000 ***g*** for 10 min at 4 °C. The cell pellet was washed once with ice-cold water, and the intact cells were stored at −20 °C if they were not used immediately.

Cell lysis and protein purification

All reagents and solutions containing the enzyme were kept at 4 °C unless otherwise stated, and with minimal exposure to light if FAD was added. The recombinant yeast AHAS subunits are fusion proteins with hexahistidine tags, which facilitate purification by immobilized metal affinity chromatography. All buffers contained 20 mM Tris/HCl (pH 7.9), 0.5 M NaCl and imidazole as specified. For purification of the catalytic subunit, 10 μM FAD was also added. A 6 ml column was packed with the Novagen His·Bind metal chelation resin (binding capacity approx. 45 mg of protein), and washed, charged and equilibrated with binding buffer (5 mM imidazole) as detailed by the manufacturer.

The frozen pellet of cells from a 1 litre expression culture was thawed on ice and resuspended in a minimum volume (approximately 5 ml) of ice-cold binding buffer. Lysozyme (10 mg/g of cells) was added, the suspension was incubated on ice for 30 min and the cells were subsequently lysed by sonication (Branson sonifier cell disruptor B-30; John Morris Scientific, Chatswood, Australia). The cell suspension was clarified by centrifugation and filtration (0.45 μm membrane) before loading on to the equilibrated column. Unbound proteins were removed by rinsing the column using 3 vol. of binding buffer, followed by 4 vol. of wash buffer (25 mM imidazole) to remove weakly bound proteins. The desired proteins were displaced from the column using elution buffer (200 mM imidazole). Fractions (3 ml) were collected in tubes that contained 3 μl of 1 M dithiothreitol (DTT). For the purification of the catalytic subunit, 0.5 ml of 1.5 M potassium phosphate (pH 6.0) was added to each fraction immediately after elution. This increased the phosphate concentration in the collected fractions to approx. 0.2 M, in order to maintain the enzyme activity. The protein elution profile was monitored by both bicinchoninic acid protein assays [20] and SDS/PAGE [21] analysis. Fractions containing the subunit protein ($\geqslant$ 0.1 mg/ml) were pooled and concentrated to 2.5 ml by ultrafiltration though a Millipore 30000 NMWL ('nominal molecular weight limit') regenerated cellulose membrane (Millipore, Bedford, MA, U.S.A.). Finally, the purified yeast AHAS subunits were exchanged into the appropriate storage buffer using a Pharmacia PD-10 desalting column (Amersham Pharmacia Biotech, Castle Hill, Australia). The storage buffer for the catalytic subunit was 0.2 M potassium phosphate (pH 6.0) containing 10 μM FAD, 1 mM DTT and 20% (v/v) glycerol, whereas the regulatory subunit was equilibrated into 0.1 M potassium phosphate (pH 7.0) containing 1 mM DTT and 20% (v/v) glycerol. The AHAS subunits were stored in small aliquots at −70 °C.

AHAS activity assay

All AHAS activity measurements were carried out using the colorimetric single point method [22]. The assays were conducted at 30 °C in reaction mixture containing 200 mM pyruvate, 1 mM ThDP, 10 mM $MgCl_2$ and 10 μM FAD in 1 M potassium phosphate buffer at pH 7.0. In a typical assay, the enzyme was preincubated in 225 μl of reaction mixture without the substrate pyruvate at 30 °C for approx. 10 min. To start the reaction, 25 μl of 2 M pyruvate solution was added to the reaction mixture. The reaction was stopped after 20 min by the addition of 35 μl of 50% (v/v) sulphuric acid. Upon incubation at 60 °C for 15 min the enzymic product of AHAS activity, acetolactate, is converted into acetoin. Acetoin was then quantified [23] by the addition of 400 μl of 0.5% (w/v) creatine and 400 μl of 5.0% (w/v) α-naphthol (dissolved in 4 M NaOH), and further incubation at 60 °C for 15 min. The colour developed was then measured by its absorbance at 525 nm. Since acetolactate is chemically unstable, acetoin was used as the standard to determine the absorption coefficient of 20400 $M^{-1}\cdot cm^{-1}$ for the coloured complex

produced. One unit of enzymic activity is defined as that producing 1 μmol of acetolactate/min under the above conditions. Specific activity is expressed as enzyme units/mg of catalytic subunit as determined by the bicinchoninic acid protein assay, unless otherwise stated.

Apoenzyme preparation

The catalytic and regulatory subunits were purified as described above, except that the catalytic subunit was stored in 0.5 M potassium phosphate buffer rather than 0.2 M. To ensure good recovery of the AHAS activity, both subunits were maintained at relatively high concentrations of 8 (catalytic subunit) and 4 (regulatory subunit) mg/ml. The purified catalytic subunit and the reconstituted enzyme were tested for the absence of bound ThDP by conducting activity assays in reaction mixtures without addition of this cofactor.

As FAD is routinely added to the purification and storage buffer of the catalytic subunit, this cofactor had to be removed using activated charcoal before the apoenzyme could be obtained. The activated charcoal was washed several times and resuspended in 0.5 M potassium phosphate (pH 7.0) containing 1 mM DTT and 20% (v/v) glycerol. This was mixed with the catalytic subunit solution to give a final charcoal concentration of 0.5% (w/v) and incubated on ice for approx. 5 min. The charcoal was then removed by centrifugation at 4 °C. Usually, the treatment had to be repeated several times to remove all of the bound FAD. The extent of removal of the cofactor was tested by activity measurements in the absence of added FAD. Since FAD was not used in the purification of the yeast AHAS regulatory subunit, this charcoal treatment was not necessary.

Gel-filtration chromatography

Gel-filtration chromatography experiments were carried out at approx. 22 °C on a Superdex HR 10/30 column (Pharmacia) connected to an FPLC System (Pharmacia). All running buffers were filtered twice through a 0.45 μm membrane (Millipore), and protein samples were centrifuged briefly before use. The column was equilibrated with the experimental buffers, then protein samples were loaded on to the column via a 50 μl sample loop and eluted at a flow rate of 0.5 ml/min. The elution of proteins was monitored by following the absorbance at 280 nm.

The column was calibrated with six protein standards (Sigma): bovine thyroglobulin (669 kDa), horse apoferritin (443 kDa), sweet potato β-amylase (200 kDa), yeast alcohol dehydrogenase (150 kDa), BSA (66 kDa) and bovine carbonic anhydrase (29 kDa). The protein standards were dissolved, either singly or in combination, and separated on the column. A molecular mass calibration curve was constructed by plotting the logarithm of the protein molecular mass against the ratio of the elution volume to void volume.

Yeast AHAS subunits were separated by gel-filtration chromatography under various equilibration conditions. Preparations (100 μl) of the catalytic subunit alone (35.5 μM), the regulatory subunit alone (70.1 μM), or a mixture of the two were equilibrated with the desired buffer by dialysis using Slide-A-Lyzer mini dialysis units (Pierce, Rockford, IL, U.S.A.) at 4 °C for at least 6 h before application on to the column. The dialysed protein samples were centrifuged for approx. 5 min to remove any precipitate before the supernatant was used for AHAS activity measurements and gel-filtration chromatography. All eluted peak fractions were collected, and analysed by AHAS activity measurements in the presence and absence of 1 mM L-valine, and by SDS/PAGE. Owing to the interference of high phosphate with SDS/PAGE, the peak fractions were first desalted by dialysis against water. Protein content in the fractions was determined by the Bradford [24] protein assay (Bio-Rad, Regents Park, Australia), which was calibrated using BSA.

Tryptophan fluorescence measurements

Fluorescence spectrometry was carried out using a Jasco PF-770 spectrofluorimeter at approx. 22 °C. The excitation wavelength was 300 nm and emission spectra were recorded from 315 to 450 nm. The catalytic subunit solution at a concentration of 20 μg/ml (0.27 μM) and the regulatory subunit solution at a concentration of 20 μg/ml (0.57 μM) were used in the measurements. In addition, catalytic subunit plus regulatory subunit mixtures at the above protein concentrations were prepared. All the subunits were diluted and resuspended in buffer containing 0.2 or 1.0 M potassium phosphate (pH 7.0), 10 μM FAD and 1 mM DTT. The protein spectra were corrected for the spectrum of the buffer.

CD spectrometry

The regulatory subunit protein solution was diluted to a concentration of 0.2–0.4 mg/ml in buffer containing 0.2 M potassium phosphate (pH 7.0), 1 mM DTT and other additives. The diluted protein samples (approx. 300 μl) were loaded into a 1 mm pathlength cell (Jasco cylindrical quartz cell). CD spectra were recorded using a Jasco J-710 spectropolarimeter at approx. 22 °C. The instrument was set at 100 mdeg sensitivity, 1 nm resolution, 0.5 nm band width, 2 s response and a scanning speed of 100 nm/min.

Cross-reaction with the *A. thaliana* AHAS catalytic subunit

The recombinant *A. thaliana* AHAS catalytic subunit was purified to homogeneity in the laboratory of R.G.D. [25] and was supplied by Ms Yu-Ting Lee. The AHAS activity assays were conducted at 30 °C at two different phosphate concentrations. The low phosphate reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.0), 1 mM ThDP, 10 mM $MgCl_2$, 10 μM FAD and 200 mM pyruvate, whereas the high phosphate reaction mixture contained 1.1 M potassium phosphate buffer (pH 7.0) and other components as above. The three branched-chain amino acids, each at 1 mM, and 1 mM ATP were also added in some experiments to test the reconstituted activity. The regulatory subunit of yeast AHAS was added in an excess of 8-fold or more compared with the molar concentration of the catalytic subunit in the reactions. The AHAS assays were carried out as described above.

Data analysis

Kinetic parameters were determined by fitting the appropriate equation to the data by non-linear regression using GraFit (Erithacus Software, Staines, U.K.) or Inplot (GraphPad Software, San Diego, U.S.A.). The best fit values and standard errors obtained from these analyses are reported.

RESULTS

Subunit interactions

Previously [12] we have shown that yeast AHAS can be reconstituted by combining its two separately purified subunits, resulting in stimulation of the activity of the catalytic subunit and acquisition of sensitivity to valine inhibition. Reconstitution

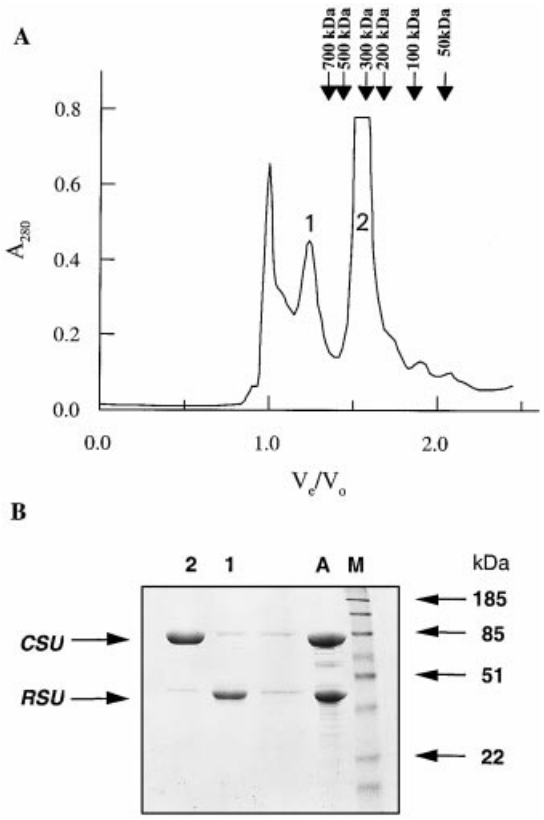


Figure 1 Gel-filtration chromatography of yeast AHAS subunits in 0.2 M potassium phosphate buffer

A mixture of the catalytic subunit (CSU; 35.5 μM) and the regulatory subunit (RSU; 70.1 μM) was dialysed against buffer containing 0.2 M potassium phosphate (pH 7.0), 0.1 mM ThDP, 0.1 mM $MgCl_2$, 10 μM FAD and 1 mM DTT. The sample was applied on to a Superdex HR 10/30 column equilibrated with the same buffer and the elution profile (**A**) was monitored at 280 nm. Elution volumes (V_e) are expressed relative to the void volume (V_0). The peaks labelled 1 and 2 were collected and analysed by SDS/PAGE (**B**), together with an aliquot of the applied sample (lane A) and molecular mass markers (lane M).

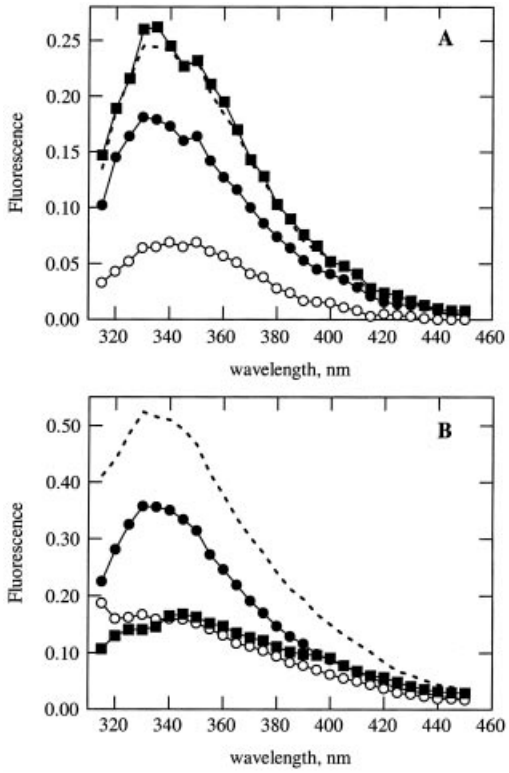


Figure 2 Tryptophan fluorescence emission spectra of yeast AHAS subunits

Fluorescence emission spectra were recorded of solutions containing 0.27 μM catalytic subunit (●), 0.57 μM regulatory subunit (○), or both (■). In (**A**) the buffer is 0.2 M potassium phosphate (pH 7.0) containing 10 μM FAD and 1 mM DTT. In (**B**) the buffer is the same except that the potassium phosphate concentration is 1.0 M. The broken lines show the expected spectra for the mixed subunits if they were not interacting, calculated by summing the spectra of the individual subunits.

is maximal at pH 7.0–7.5, in high concentrations (approx. 1 M) of potassium phosphate. The salt specificity has now been examined (results not shown). Sodium or potassium phosphates are equally effective, while sodium or potassium sulphate allows partial reconstitution. Neither KCl nor sodium nitrate allows significant reconstitution, and the latter is inhibitory. In all subsequent experiments, potassium phosphate was used.

The high phosphate concentration could be a requirement for physical association between the subunits. Alternatively, the subunits could associate in both low and high phosphate, but the effects of the regulatory subunit on the activity of the catalytic subunit may only be manifested at high phosphate concentrations. These two alternatives were investigated by gel-filtration chromatography. When a mixture of the two subunits was applied on to a Superdex HR 10/30 column at low (0.2 M) phosphate concentration, two protein peaks were eluted (Figure 1A). SDS/PAGE analysis of the peaks (Figure 1B) showed that one is composed predominantly of the catalytic subunit and the other is the regulatory subunit. Surprisingly, each eluted at a position much earlier than would be expected based on the subunit molecular mass. The regulatory subunit (molecular mass of 39.4 kDa) eluted before the largest standard used to calibrate the column (thyroglobulin, 669 kDa), whereas the catalytic subunit (molecular mass of 73.5 kDa) eluted with an apparent molecular mass of 303 kDa. If the elution positions observed during gel filtration are indicative of the molecular masses, then the catalytic subunit is tetrameric, whereas the regulatory subunit is an oligomer of 17 or more subunits.

A similar experiment conducted in 1 M phosphate again gave two protein peaks (results not shown). However, in this case the first peak (apparent molecular mass $>$ 669 kDa) contained both subunits and activity measurements showed that it was sensitive to valine inhibition. The second peak (apparent molecular mass of 182 kDa) contained only the catalytic subunit. Similar results were obtained in the presence or absence of ThDP plus Mg^{2+}. Thus the effect of the high phosphate concentration is to allow the physical association of the two subunits.

The physical association was substantiated by measurements of fluorescence of the proteins due to the tryptophan residues that they contain. In 0.2 M phosphate, the spectrum of a mixture of the subunits is very similar to that expected from a sum of the spectra of the individual proteins (Figure 2A). However, at 1.0 M phosphate, the fluorescence of the mixture is substantially lower than that of the sum of the individual spectra (Figure 2B), which we interpret to be indicative of association between the two proteins.

The subunit composition of the reconstituted enzyme was investigated further by quantitative densitometry after gel-filtration chromatography and SDS/PAGE. Each of the subunits

Table 1 Effect of valine and MgATP on the Michaelis constant of yeast AHAS

CSU, catalytic subunit; RSU, regulatory subunit.

Subunits	Effectors	K_m (mM)
CSU	None	2.1 ± 0.3
CSU	1 mM valine	2.5 ± 0.1
CSU	5 mM MgATP	2.6 ± 0.4
CSU	5 mM MgATP + 5 mM valine	2.7 ± 0.3
CSU + RSU	None	13.3 ± 1.6
CSU + RSU	1 mM valine	18.6 ± 1.3
CSU + RSU	5 mM MgATP	13.3 ± 1.8
CSU + RSU	5 mM MgATP + 5 mM valine	16.2 ± 1.8

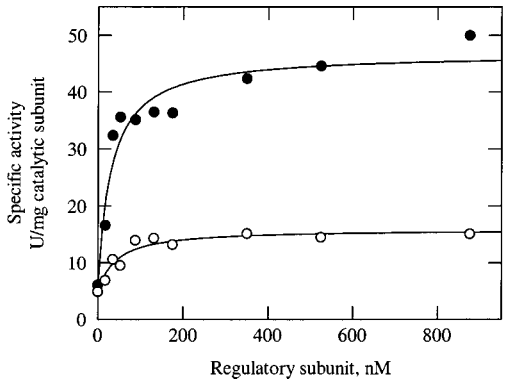


Figure 3 Effect of valine on the stimulation of yeast AHAS catalytic activity by its regulatory subunit

Assays contained 5.2 nM catalytic subunit and various concentrations of the regulatory subunit as shown. The two curves represent results obtained in the absence (●) and presence (○) of 1 mM L-valine. The lines represent the best fit to the data by hyperbolic saturation curves. The concentrations of the regulatory subunit required to give 50% of the maximum stimulation of activity are 39.9 ± 11.1 and 39.7 ± 12.8 nM in the absence and presence of L-valine respectively. U, units.

showed a linear relationship (results not shown) between the peak area on the stained gel, measured by densitometry, and the amount of protein, as measured by the alkaline hydrolysis–ninhydrin method [7]. The ratio of catalytic to regulatory subunit in the reconstituted enzyme was estimated to be 1.06, 1.13 and 0.96 in three separate experiments. We interpret this to mean that the reconstituted enzyme contains equal numbers of the two types of subunit. However, the observed elution position in gel-filtration chromatography (apparent molecular mass > 669 kDa) is inconsistent with the $\alpha_2\beta_2$ structure observed for bacterial AHAS [26]. It should be noted that most studies of eukaryotic AHAS [27–33] have reported apparent molecular masses that are much higher than would be expected for a heterotetramer.

Valine inhibition

As described previously [12], reconstituted yeast AHAS is inhibited by valine. The specificity of the enzyme with respect to this modulator was investigated, as well as its effect on subunit association.

Valine inhibits with an apparent K_i of approximately 0.1 mM. No inhibition was observed with 0.1 or 1 mM leucine or isoleucine, and slight inhibition (13%) was only found with 5 mM isoleucine. Thus the enzyme distinguishes well between amino acids with similar side-chains. To investigate the recognition of other parts of the valine molecule, *N*-acetylvaline,

Table 2 Effect of ATP and its analogues on the activity of yeast AHAS

Activity is expressed as the percentage of the reconstituted activity with no added ligands. CSU, catalytic subunit; RSU, regulatory subunit; n.d., not determined.

	Activity (%)			
	− Valine		+ Valine	
Addition	CSU	CSU + RSU	CSU	CSU + RSU
1 mM Valine				
None	17	(100)	17	22
1 mM ATP	18	106	18	89
1 mM ADP	18	99	18	28
1 mM AMP	18	105	17	24
1 mM ATP + 1 mM ADP	19	113	17	98
1 mM ATP + 1 mM AMP	18	111	18	93
1 mM ADP + 1 mM AMP	18	103	16	30
1 mM ATP + 1 mM ADP + 1 mM AMP	18	111	16	86
10 mM ATP	17	110	16	104
10 mM GTP	16	99	17	25
10 mM CTP	17	103	17	25
10 mM UTP	16	101	17	28
0.5 mm Valine				
None	14	(100)	14	32
0.5 mM ATP	n.d.	114	n.d.	103
0.5 mM ϵ-ATP	n.d.	108	n.d.	23
0.5 mM ATP + 0.5 mM ϵ-ATP	n.d.	n.d.	n.d.	98
0.5 mM p[NH]ppA	n.d.	113	n.d.	87
0.5 mM ATP + 0.5 mM p[NH]ppA	n.d.	n.d.	n.d.	106

N-methylvaline and valinamide were tested at 0.1 and 1 mM. None of these compounds inhibited, and none interfered with the inhibition by 0.1 mM valine. Thus the ineffectiveness of these compounds can be ascribed to their inability to bind to AHAS.

The inhibition by valine is partial, leaving a residual activity of 10–15 % at a saturating concentration ($\geqslant$ 1 mM) of valine. The residual activity is similar to that of the catalytic subunit alone, suggesting that valine might exert its inhibitory effect by causing dissociation of the regulatory and catalytic subunits. One line of evidence against this proposal comes from measurements of the kinetic properties with respect to substrate and cofactors of the catalytic subunit and the reconstituted enzyme, with and without valine. The half-saturating concentrations of ThDP (ranging from $26.9 \pm 3.4\ \mu$M to $35.2 \pm 3.0\ \mu$M) and FAD (24.1 ± 4.5 nM to 35.0 ± 3.2 nM) show little or no change under all conditions. However, the K_m for pyruvate is clearly higher for the reconstituted enzyme than for the catalytic subunit alone (Table 1). Addition of valine alters the K_m of the reconstituted enzyme, but not to a value resembling that of the catalytic subunit; rather, it causes a further increase. This result is inconsistent with the idea that valine causes subunit dissociation.

A second line of evidence bearing on this question comes from measurements of the activity versus [regulatory subunit] saturation curve, with and without valine (Figure 3). The half-saturating concentrations are 39.9 nM (without valine) and 39.7 nM (1 mM valine). Thus the affinity between the two subunits is unaffected by valine.

The two lines of evidence mentioned above are indirect, in the sense that they rely on measurements of activity rather than examining the protein itself. Consequently, gel-filtration chromatography experiments were undertaken, using the protocol described previously. These experiments showed that in the presence of 1 mM valine, AHAS elutes as a protein peak containing both subunits, thereby demonstrating that valine does not cause subunit dissociation. The reconstituted enzyme elutes later from the column than is observed in the absence of valine, at an apparent molecular mass of 577 kDa. We cannot say whether this change represents a reduction in oligomerization or an alteration in shape. However, it is clear that valine causes a structural change and this is, presumably, related to its effect on catalytic activity.

ATP activation

As described previously [12], valine inhibition of the reconstituted yeast AHAS is reversed by ATP. This activation was investigated further.

A variety of ATP analogues were tested for their ability to mimic the effects of ATP (Table 2). Neither ADP nor AMP is effective, and neither interferes with the effect of ATP, suggesting that only ATP binds. Other nucleoside triphosphates (GTP, CTP and UTP) are also ineffective, as is the fluorescent ATP derivative, ϵ-ATP. However, the non-hydrolysable analogue p[NH]ppA is capable of stimulating the valine-inhibited enzyme, thereby ruling out the possibility that the effect of ATP is mediated by autophosphorylation. Gel-filtration chromatography experiments showed that the reconstituted enzyme remains intact in the presence of 1 mM ATP. It was also observed that the regulatory subunit alone, in the presence of ATP, elutes with an apparent molecular mass of 377 kDa in 1 M phosphate buffer. In the absence of ATP, the regulatory subunit is almost insoluble in this high phosphate buffer. Thus ATP apparently increases the solubility of the regulatory subunit. These findings suggest that ATP interacts directly with the regulatory subunit, and this idea was investigated further by fluorescence and CD spectrometry.

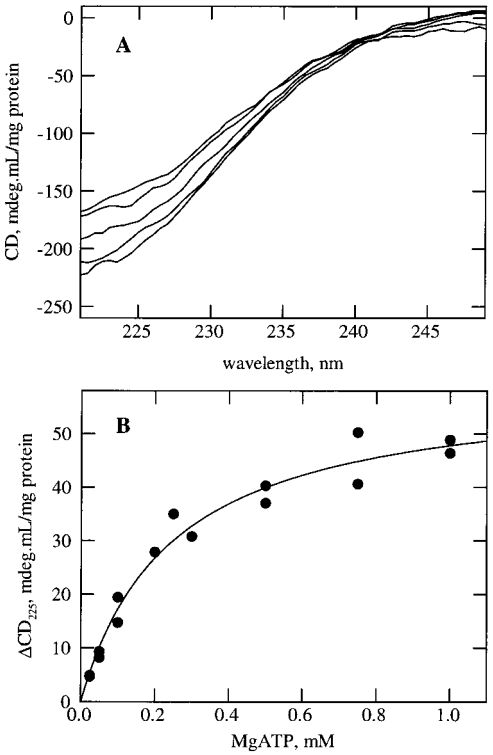


Figure 4 Analysis of MgATP binding to the yeast AHAS regulatory subunit by CD spectroscopy

The regulatory subunit protein solution was diluted in buffer containing 0.2 M potassium phosphate (pH 7.0), 1 mM DTT and MgATP concentrations as shown. CD spectra were recorded at approx. 22 °C. (**A**) The spectra obtained at 0, 0.05, 0.2, 0.5 and 1 mM MgATP (top to bottom). (**B**) The change in CD at 225 nm, relative to the control with no added MgATP. The line represents the best fit to the data by a hyperbolic saturation curve with a half-saturating MgATP concentration of 0.24 ± 0.05 mM.

ATP does not affect the tryptophan fluorescence spectrum of the individual subunits or the reconstituted enzyme. However, the CD spectrum of the regulatory subunit is affected and the change depended on the concentration of ATP (Figure 4A). Mg^{2+} is required for this ATP-dependent spectral change and the change can be prevented by the inclusion of EDTA (results not shown). These results confirm that ATP binds directly to the regulatory subunit and suggest that the active species is MgATP. The CD change at 225 nM shows a hyperbolic dependence on the ATP concentration (Figure 4B) with a half-saturating concentration of 0.24 ± 0.05 mM. Mg^{2+} alone, or in combination with valine, does not alter the CD spectrum and the MgATP-dependent change is unaffected by added valine (results not shown).

Kinetics of valine inhibition and MgATP activation

The specific activity of the reconstituted enzyme was measured as a function of MgATP concentration, over a series of ten valine concentrations spanning 0 to 5 mM (Figure 5A). Even in the absence of valine, MgATP causes a small degree (approx. 20 %) of activation. MgATP does not affect the K_m for pyruvate, irrespective of whether valine is present or absent (Table 1).

Each of these activation curves is hyperbolic and allows estimation of three quantities: V_o, the specific activity in the

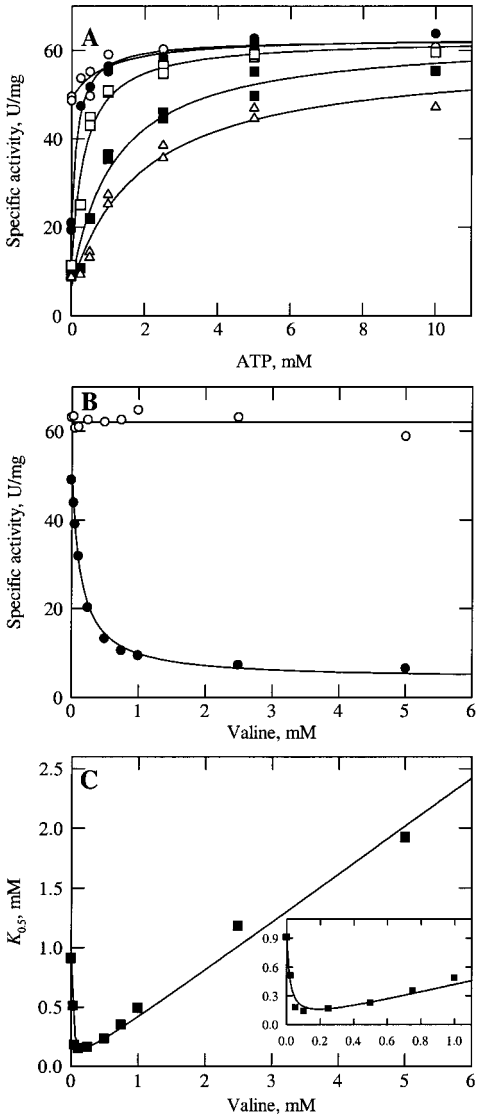


Figure 5 Effects of valine and ATP on the kinetic properties of yeast AHAS

The enzymic activity was measured over a range of valine and ATP concentrations. (**A**) ATP activation curves obtained at 0 (○), 0.25 (●), 0.75 (□), 2.5 (■) and 5 mM (△) valine. A hyperbolic curve was fitted to each data set to obtain values for the specific activity in the absence of ATP (V_o) and at saturating ATP (V_m), and the ATP concentration that gives 50% activation ($K_{0.5}$). (**B**) The values of V_m (○) and V_o (●) as a function of the valine concentration. The upper line represents a specific activity of 61.3 units/mg, whereas the lower line is the best fit of a partial inhibition curve corresponding to an apparent K_i of 142 ± 12 μM, an uninhibited specific activity of 50.1 ± 0.8 units/mg, and a residual activity of 8.2 ± 1.3% at saturating valine. (**C**) The values of $K_{0.5}$ as a function of the valine concentration, with the inset illustrating an expanded view of the region from 0 to 1 mM valine. The line represents that predicted by the model described in the text and illustrated in Scheme 1.

absence of MgATP; V_m, the extrapolated specific activity at a saturating concentration of MgATP; and the activation constant, $K_{0.5}$, which is the half-saturating MgATP concentration.

Values for the specific activities (V_o and V_m), as a function of the valine concentration, are shown in Figure 5(B). The V_o values show a hyperbolic decrease in specific activity from 50.1 ± 0.8 units/mg that levels off at 8.2 ± 1.3% residual activity. The apparent K_i for valine is 142 ± 12 μM, similar to the value (162 ± 22 μM) reported previously [12]. The values for V_m are constant, averaging 61.3 ± 1.7 units/mg, and show that saturating with MgATP completely overcomes valine inhibition.

More complex behaviour was shown by $K_{0.5}$ (Figure 5C); this starts at 0.92 mM in the absence of valine, reaches a minimum of 0.14 mM at 0.1 mM valine, and increases thereafter in an approximately linear fashion as the valine concentration increases. A quantitative model to explain these data will be presented later.

Cross-reaction with the *A.thaliana* AHAS catalytic subunit

The yeast AHAS regulatory subunit was tested for its ability to activate the catalytic subunit of *A. thaliana* AHAS. Like all AHAS catalytic subunits, the recombinant *A. thaliana* AHAS has a low specific activity and is insensitive to end-product feedback inhibition [34]. This clearly suggests a missing regulatory subunit and a strong candidate for this subunit has been recently identified [19].

Reconstitution of the yeast AHAS had been shown to require high concentrations (approx. 1 M) of potassium phosphate, whereas the reconstitution of *A. thaliana* AHAS occurs in low (0.05–0.4 M) phosphate buffer [19]. Consequently, both high (1.1 M) and low (0.05 M) potassium phosphate concentrations were tested in the reconstitution experiments. In addition, the three branched-chain amino acids and ATP were added into the activity assays to test whether these effectors regulate the reconstituted activity. The enzymic activity of the *A. thaliana* AHAS catalytic subunit was observed to be stimulated by approx. 2–3-fold in the presence of the yeast regulatory subunit. This cross-reconstitution occurs at both low and high phosphate concentrations, and the stimulated activity is inhibited by branched-chain amino acids, but not affected appreciably by ATP.

DISCUSSION

The catalytic and regulatory subunits of yeast AHAS, separately purified and combined *in vitro*, associate in 1:1 stoichiometry. The formation of this complex is associated with a substantial enhancement of the catalytic activity and the acquisition of sensitivity to inhibition by valine. The association between the subunits requires a high concentration of phosphate ions, although sulphate is also partially effective. Monovalent anions, such as chloride and nitrate, are without effect, although the latter is strongly inhibitory, which may mask the effects of reconstitution on activity.

Although the reconstituted enzyme contains equimolar amounts of the two subunits, it is not a simple $\alpha\beta$ heterodimer. Indeed, such a structure would not be expected to be catalytically active, based on the known three-dimensional structure of related enzymes, such as pyruvate oxidase [35], pyruvate decarboxylase [36], and benzoylformate decarboxylase [37]. Although these enzymes contain a single type of subunit only, which is homologous to the catalytic subunit of AHAS, in all cases the active site is formed at the interface between two of the subunits. It would be expected that a similar arrangement would exist in AHAS, so that an $\alpha_2\beta_2$ heterotetramer would be the minimal active unit. In gel-filtration chromatography, the enzyme migrates as if it is a much larger species, possibly $\alpha_6\beta_6$. Further investigation is needed to determine whether the structure is really as complex as this, or whether it is merely showing anomalous behaviour in gel-filtration chromatography.

The reconstituted enzyme is inhibited by valine, and ATP reverses this inhibition. Neither effector causes dissociation of the regulatory subunit. There is substantial specificity with respect to these two regulators. Thus modification of the valine amino

group (*N*-methylvaline and *N*-acetylvaline), the carboxy group (valinamide), or the side chain (leucine and isoleucine) results in a loss of the inhibitory effect. Similarly, the activation by ATP is not mimicked by other purines (GTP and ϵ-ATP) or pyrimidines (CTP and UTP), or when one or both phosphate groups are removed (ADP and AMP). The only effective ATP analogue is p[NH]ppA, demonstrating that the activation does not involve an autophosphorylation reaction.

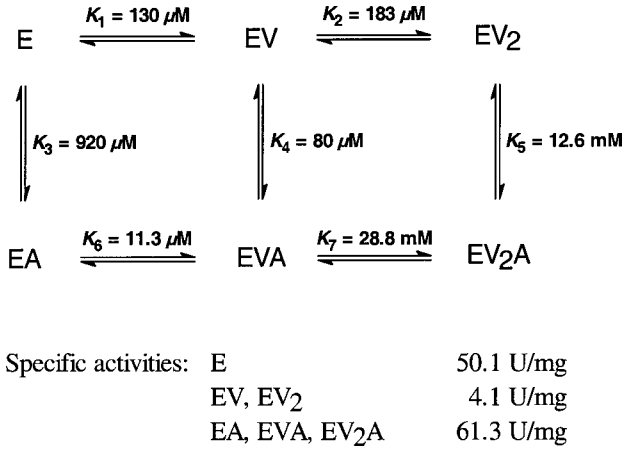
No direct evidence was adduced to show that valine binds to the regulatory subunit, although this has been demonstrated for *E. coli* AHAS III. It would be very surprising if a similar situation did not exist for the yeast enzyme. For ATP, it was shown by CD measurements that the regulatory subunit binds this ligand and that Mg^{2+} is required, presumably because the active ligand is MgATP. The site of ATP binding in the regulatory subunit is not known, but we note that there is an extra sequence of approximately 50 residues in this subunit that is not found in the bacterial [1] or plant [18,19] homologues. Since ATP does not activate the bacterial or plant enzymes, we suggest that the extra 50 residues in the yeast subunit might contain the MgATP binding site. However, this sequence does not contain classical ATP-binding sites described in the Prosite database.

The yeast regulatory subunit is capable of activating the *A. thaliana* catalytic subunit and this cross-reconstitution occurs at both high and low phosphate concentrations. The resulting interspecies hybrid enzyme is inhibited by branched-chain amino acids, but MgATP does not reverse this inhibition. This latter finding was somewhat unexpected given that the MgATP binding site appears to be located on the regulatory subunit. Apparently, the interaction between these fungal and plant subunits is imperfect and the full range of regulatory effects cannot be transmitted from one to the other.

At all valine concentrations, saturation with MgATP completely overcomes the inhibition (Figure 5B). One interpretation of this result is that MgATP and valine compete for the same binding site. However, we feel that this interpretation is unlikely, as it would require that the site recognizes two compounds with radically different structures while being able to distinguish accurately between analogues of valine and of ATP. The alternative explanation, which we favour, is that the effect on specific activity of occupancy of the valine binding site is entirely reversed by binding of MgATP at its own allosteric site. This hypothesis is elaborated on below.

The kinetics of valine inhibition and MgATP activation are complex, particularly the relationship between the MgATP activation constant ($K_{0.5}$) and the valine concentration. These data cannot be accommodated by a model in which the reconstituted AHAS is composed of a single regulatory subunit combined with a single catalytic subunit. Moreover, as was discussed earlier, the enzyme contains several copies of each subunit, although there are equal numbers of each. In the present study we propose a model based on a heterotetramer; models based on larger oligomers would also explain the data, but we have chosen the heterotetramer as it is the simplest structure that is compatible with the combined effects of valine and MgATP.

A general model for binding of both valine and MgATP to an $\alpha_2\beta_2$ protein would involve nine different enzyme species, with 0, 1 or 2 mol of each regulator bound to the heterotetramer. However, there is little indication in the data (Figure 5A) that the MgATP activation curves depart from rectangular hyperbolae. Therefore we can simplify the model by eliminating the three enzyme species containing 2 mol of bound MgATP. We are not proposing that such species do not exist; rather we suggest that either (1) they are not formed in kinetically detectable amounts in this experiment or (2) MgATP binding to each $\alpha\beta$ heterodimer is independent of the other, and the three pairs of enzyme species with 1 or 2 mol of bound MgATP are kinetically equivalent.



Scheme 1 Model for the interactions between AHAS, valine and ATP, and their effects on the activity of yeast AHAS

See the text for details. E, enzyme; V, valine; A, ATP; U, units.

This simplified model is shown in Scheme 1 and will now be analysed in more detail. It should be understood that, while seven equilibrium constants are shown, only five of these are independent, whereas the other two may be obtained from the relationships $K_1K_4 = K_3K_6$ and $K_2K_5 = K_4K_7$. Using this model, we may calculate [38] the dependence of the specific activity (SA) on the concentrations of valine ([V]) and MgATP ([A]) as:

$$SA = \frac{S_E F_E + S_{EV} F_{EV} + S_{EV_2} F_{EV_2} + S_{EA} F_{EA} + S_{EVA} F_{EVA} + S_{EV_2A} F_{EV_2A}}{F_E + F_{EV} + F_{EV_2} + F_{EA} + F_{EVA} + F_{EV_2A}} \quad (1)$$

where S_X is the specific activity of enzyme species X, and the various F factors are given by:

$$F_E = K_1K_2K_3K_4K_5$$
$$F_{EV} = K_2K_3K_4K_5[V]$$
$$F_{EV_2} = K_3K_4K_5[V]^2$$
$$F_{EA} = K_1K_2K_4K_5[A]$$
$$F_{EVA} = K_2K_3K_5[A][V]$$
$$F_{EV_2A} = K_3K_4[A][V]^2$$

Some of these parameters are readily estimated from the data, such as the specific activity of the unregulated enzyme ($S_E = 50.1$ units/mg) and the activation constant for MgATP in the absence of valine ($K_3 = 0.92$ mM). As noted earlier, the specific activity at saturating MgATP is unaffected by the valine concentration, from which it may be deduced that S_{EA}, S_{EVA} and S_{EV_2A} are each equal to 61.3 units/mg. The specific activity at saturating valine in the absence of MgATP is estimated to be 4.1 units/mg and, for the purposes of our model, we assume that this represents the value of both S_{EV} and S_{EV_2}.

The data in Figure 5(C) show that the half-saturating MgATP concentration ($K_{0.5}$) first decreases as the valine concentration increases, followed by a region where there is an approximately linear increase. Such behaviour is exactly as would be expected from the model if K_4 is small, and K_5 is large, in comparison with K_3. At low valine concentrations some of the enzyme species E would be converted into EV, which has a higher affinity for

MgATP. However, high valine concentrations would push the equilibria towards the low affinity enzyme species EV_2.

Thus the dependence of $K_{0.5}$ upon the valine concentration can be used to estimate the remaining parameters (K_1, K_2, K_4 and K_5):

$$K_{0.5} = \frac{K_1K_2 + K_2[V] + [V]^2}{K_1K_2/K_3 + K_2[V]/K_4 + [V]^2/K_5} \quad (2)$$

It should be noted that this expression is independent of any assumptions about the values of the specific activity of each enzyme species. As shown by the line in Figure 5(C), this equation gives an accurate representation of the data when K_1–K_5 are assigned the values shown in Scheme 1. However, we are conscious that other sets of values can yield a similar relationship between $K_{0.5}$ and [V], so we do not claim that these estimates are a unique solution. Nevertheless, they do illustrate that the model in Scheme 1 is entirely consistent with the behaviour of the enzyme towards its regulators MgATP and valine.

A more complete understanding of the way in which valine and MgATP interact with the regulatory subunit of yeast AHAS, and modulate the activity of the catalytic subunit, must await the determination of its three-dimensional structure. We are actively pursuing this goal and have found, but not yet optimized, crystallization conditions for the reconstituted enzyme. We have also crystallized the catalytic subunit and the structure has now been solved (S. S. Pang, L. W. Guddat and R. G. Duggleby, unpublished work).

This work was supported by grant number A09937067 from the Australian Research Council. We are grateful to Ms Yu-Ting Lee for providing a sample of the *A. thaliana* AHAS catalytic subunit.

REFERENCES

1 Duggleby, R. G. and Pang, S. S. (2000) Acetohydroxyacid synthase. J. Biochem. Mol. Biol. **33**, 1–36

2 Umbarger, H. E. (1996) Biosynthesis of the branched chain amino acids. In *Escherichia coli* and *Salmonella typhimurium*: Cellular and Molecular Biology (Neidhardt, F. C., Curtiss, III, R., Ingraham, J. L., Lin, E. C. C., Low, K. B., Magasanik, B., Reznikokk, W. S., Riley, M., Schaechter, M. and Umbarger, H. E., eds.), pp. 442–457, American Society for Microbiology, Washington, D.C.

3 Chipman, D., Barak, Z. and Schloss, J. V. (1998) Biosynthesis of 2-aceto-2-hydroxy acids: acetolactate synthases and acetohydroxyacid synthases. Biochim. Biophys. Acta **1385**, 401–419

4 Weinstock, O., Sella, C., Chipman, D. M. and Barak, Z. (1992) Properties of subcloned subunits of bacterial acetohydroxy acid synthases. J. Bacteriol. **174**, 5560–5566

5 Sella, C., Weinstock, O., Barak, Z. and Chipman, D. M. (1993) Subunit association in acetohydroxy acid synthase isozyme III. J. Bacteriol. **175**, 5339–5343

6 Vyazmensky, M., Sella, C., Barak, Z. and Chipman, D. E. (1996) Isolation and characterization of subunits of acetohydroxy acid synthase isozyme III and reconstitution of the holoenzyme. Biochemistry **35**, 10339–10346

7 Hill, C. M., Pang, S. S. and Duggleby, R. G. (1997) Purification of *Escherichia coli* acetohydroxyacid synthase isoenzyme II and reconstitution of active enzyme from its individual pure subunits. Biochem. J. **327**, 891–898

8 Mendel, S., Elkayam, T., Sella, C., Vinogradov, V., Vyazmensky, M, Chipman, D. M. and Barak, Z. (2001) Acetohydroxyacid synthase: a proposed structure for regulatory subunits supported by evidence from mutagenesis. J. Mol. Biol. **307**, 465–477

9 Polaina, J. (1984) Cloning of the *ilv2*, *ilv3* and *ilv5* genes of *Saccharomyces cerevisiae*. Carlsberg Res. Commun. **49**, 577–584

10 Jarai, G., Yagmai, B., Fu, Y.-H. and Marzluf, G. A. (1990) Regulation of branched-chain amino acid biosynthesis in *Neurospora crassa*: cloning and characterization of the *leu-1* and *ilv-3* genes. Mol. Gen. Genet. **224**, 383–388

11 Bekkaoui, F., Nadin-Davis, S. A. and Crosby, W. L. (1993) Isolation and structure of an acetolactate synthase gene from *Schizosaccharomyces pombe* and complementation of the *ilv2* mutation in *Saccharomyces cerevisiae*. Curr. Genet. **24**, 544–547

12 Pang, S. S. and Duggleby, R. G. (1999) Expression, purification, characterization and reconstitution of the large and small subunits of yeast acetohydroxyacid synthase. Biochemistry **38**, 5222–5231

13 Duggleby, R. G. (1997) Identification of an acetolactate synthase small subunit gene in two eucaryotes. Gene **190**, 245–249

14 Takenaka, S. and Kuwana, H. (1972) Control of acetohydroxy acid synthetase in *Neurospora crassa*. J. Biochem. (Tokyo) **72**, 1139–1145

15 Kuwana, H. and Date, M. (1975) Solubilization of valine-sensitive acetohydroxy acid synthetase from *Neurospora* mitochondria. J. Biochem. (Tokyo). **77**, 257–259

16 Miflin, B. J. (1971) Cooperative feedback control of barley acetohydroxyacid synthetase by leucine, isoleucine, and valine. Arch. Biochem. Biophys. **146**, 542–550

17 Stidham, M. A. and Singh, B. K. (1991) Imidazolinone-acetohydroxyacid synthase interactions. In The Imidazolinone Herbicides (Shaner, D. L. and O'Connor, S. L., eds.), pp. 71–90, CRC Press, Boca Raton

18 Hershey, H. P., Schwartz, L. J., Gale, J. P. and Abell, L. M. (1999) Cloning and functional expression of the small subunit of acetolactate synthase from *Nicotiana plumbaginifolia*. Plant Mol. Biol. **40**, 795–806

19 Lee, Y.-T. and Duggleby, R. G. (2001) Identification of the regulatory subunit of *Arabidopsis thaliana* acetohydroxyacid synthase and reconstitution with its catalytic subunit. Biochemistry. **40**, 6836–6844

20 Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. and Klenk, D. C. (1985) Measurement of protein using bicinchoninic acid. Anal. Biochem. **150**, 76–85

21 Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **277**, 680–684

22 Singh, B. K., Stidham, M. A. and Shaner, D. L. (1988) Assay of acetohydroxyacid synthase. Anal. Biochem. **171**, 173–179

23 Westerfeld, W. W. (1945) A colorimetric determination of blood acetoin. J. Biol. Chem. **161**, 495–502

24 Bradford, M. M. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**, 248–254

25 Lee, Y.-T., Chang, A. K. and Duggleby, R. G. (1999) Effect of mutagenesis at serine 653 of *Arabidopsis thaliana* acetohydroxyacid synthase on the sensitivity to imidazolinone and sulfonylurea herbicides. FEBS Lett. **452**, 341–345

26 Schloss, J. V., Van Dyk, D. E., Vasta, J. F. and Kutny, R. M. (1985) Purification and properties of *Salmonella typhimurium* acetolactate synthase isoenzyme II from *Escherichia coli* HB101/pDU9. Biochemistry **24**, 4952–4959

27 Glatzer, L., Eakin, E. and Wagner, R. P. (1972) Acetohydroxy acid synthetase with a pH optimum of 7.5 from *Neurospora crassa* mitochondria: characterization and partial purification. J. Bacteriol. **112**, 453–464

28 Tanaka, H. and Kuwana, H. (1984) A basal unit of valine-sensitive acetolactate synthase of *Neurospora crassa*. Biochem. Biophys. Res. Commun. **123**, 418–423

29 Muhitch, M. J., Shaner, D. L. and Stidham, M. A. (1987) Imidazolinones and acetohydroxyacid synthase from higher plants. Properties of the enzyme from maize suspension culture cells and evidence for the binding of imazapyr to acetohydroxyacid synthase *in vivo*. Plant Physiol. **83**, 451–456

30 Durner, J. and Böger, P. (1988) Acetolactate synthase from barley (*Hordeum vulgare* L.): purification and partial characterization. Z. Naturforsch., C: Biosci. **43**, 850–856

31 Durner, J. and Böger, P. (1990) Oligomeric forms of plant acetolactate synthase depend on flavin adenine dinucleotide. Plant Physiol. **93**, 1027–1031

32 Singh, B. K. and Schmitt, G. K. (1989) Flavin adenine dinucleotide causes oligomerization of acetohydroxyacid synthase from Black Mexican Sweet corn cells. FEBS Lett. **258**, 113–115

33 Southan, M. D. and Copeland, L. (1996) Physical and kinetic properties of acetohydroxyacid synthase from wheat leaves. Physiol. Plant. **98**, 824–832

34 Chang, A. K. and Duggleby, R. G. (1997) Expression, purification and characterization of *Arabidopsis thaliana* acetohydroxyacid synthase. Biochem. J. **327**, 161–169

35 Muller, Y. A., Schumacher, G., Rudolph, R. and Schulz, G. E. (1994) The refined structures of a stabilized mutant and of wild-type pyruvate oxidase from *Lactobacillus plantarum*. J. Mol. Biol. **237**, 315–335

36 Dobritzsch, D., König, S., Schneider, G. and Lu, G. (1998) High resolution crystal structure of pyruvate decarboxylase from *Zymomonas mobilis*. Implications for substrate activation in pyruvate decarboxylases. J. Biol. Chem. **273**, 20196–20204

37 Hasson, M. S., Muscate, A., McLeish, M. J., Polovnikova, L. S., Gerlt, J. A., Kenyon, G. L., Petsko, G. A. and Ringe, D. (1998) The crystal structure of benzoylformate decarboxylase at 1.6 Å resolution: diversity of catalytic residues in thiamin diphosphate-dependent enzymes. Biochemistry **37**, 9918–9930

38 Varón, R., García-Sevilla, F., García -Moreno, M., García-Cánovas, F., Peyró, R. and Duggleby, R. G. (1997) Computer program for the equations describing the steady-state of enzyme reactions. Comput. Appl. Biosci. **13**, 159–167

Received 22 February 2001/17 April 2001; accepted 8 May 2001