Mutations in the regulatory subunit of yeast acetohydroxyacid synthase affect its activation by MgATP

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Isoleucine, leucine and valine are synthesized via a common pathway in which the first reaction is catalysed by AHAS (acetohydroxyacid synthase; EC 2.2.1.6). This heterotetrameric enzyme is composed of a larger subunit that contains the catalytic machinery and a smaller subunit that plays a regulatory role. The RSU (regulatory subunit) enhances the activity of the CSU (catalytic subunit) and mediates end-product inhibition by one or more of the branched-chain amino acids, usually valine. Fungal AHAS differs from that in other organisms in that the inhibition by valine is reversed by MgATP. The fungal AHAS RSU also differs from that in other organisms in that it contains a sequence insert. We suggest that this insert may form the MgATP-binding site and we have tested this hypothesis by mutating ten highly conserved amino acid residues of the yeast AHAS RSU. The modified subunits were tested for their ability to activate the yeast AHAS CSU, to confer sensitivity to valine inhibition and to mediate reversal of the inhibition by MgATP. All but one of the mutations resulted in substantial changes in the properties of the RSU. Unexpectedly, four of them gave a protein that required MgATP in order for strong stimulation of the CSU and valine inhibition to be observed. A model to explain this result is proposed. Five of the mutations abolished MgATP activation and are suggested to constitute the binding site for this modulator.

Key words: acetohydroxyacid synthase, branched-chain amino acid, catalytic subunit, kinetic model, MgATP activation, regulatory subunit.

INTRODUCTION

Valine is synthesized in four steps of an anabolic pathway that starts with two molecules of pyruvate (Scheme 1). The synthesis of isoleucine follows a parallel pathway, starting with one molecule each of pyruvate and 2-ketobutyrate. The third branched-chain amino acid, leucine, is formed by a side-branch after the third step of the valine pathway. Thus three enzymes are common to the synthesis of all three amino acids: AHAS (acetohydroxyacid synthase; EC 2.2.1.6; see [1] for a review), ketol-acid reductoisomerase (EC 1.1.1.86) and dihydroxy-acid dehydratase (EC 4.2.1.9). This pathway and its component enzymes are not found in animals, which must obtain the branched-chain amino acids from their diet. Therefore these enzymes have attracted attention as possible targets for bioactive compounds. This potential has been successfully realized for AHAS, which is inhibited by several, widely used herbicides such as the sulphonylurea and imidazolinone families. Moreover, AHAS is the primary regulated step in this biosynthetic pathway. Therefore there is great interest in understanding the basic catalytic and regulatory processes that are employed by this enzyme.

A variety of mechanisms are used by different species to regulate AHAS, including control of transcription, of translation and of enzyme activity. The latter is most commonly mediated through valine inhibition although the plant enzyme is sensitive to all three amino acids, with leucine acting synergistically with either of the other two [2]. The fungal enzyme is also inhibited by valine but, uniquely, this inhibition is reversed by ATP [3,4].

AHAS contains two types of subunit. There is a CSU (catalytic subunit) of 60–70 kDa that is usually active alone, and an RSU (regulatory subunit) that varies in size from 9 to 54 kDa, depending on the species and isoform. The inhibition of AHAS activity by branched-chain amino acids is dependent on the presence of this RSU [2,5,6], which also serves to stimulate the activity of the CSU.

Comparison of AHAS RSU amino acid sequences (Figure 1A) reveals that the eukaryotic proteins begin with a signal peptide that targets AHAS to chloroplasts in plants or mitochondria in fungi. This peptide is linked to a common core of approx. 140 residues with several highly conserved amino acids. Some of these residues have been shown, by mutagenesis studies, to be required for sensitivity to inhibition by branched-chain amino acids [5,7–10]. Following the core, there is a C-terminal tail that varies in length and sequence between species. The plant AHAS RSU contains a duplication [2,11] of this core and this has been shown to provide dual inhibitor binding sites that are needed for synergistic inhibition [2,10]. The fungal protein is unique in having an insert of 38–55 residues that contains several conserved residues (Figure 1B).

Previously, we have shown that yeast AHAS can be reconstituted from its separately isolated pure subunits [6]. This results in a stimulation of the activity of the CSU by 7–10-fold, and the enzyme becomes sensitive to valine inhibition. ATP, as its Mg^{2+} complex, reverses this inhibition by binding to the RSU [4]. Even in the absence of valine, there is a small ($\sim$20 %) stimulation of the reconstituted enzyme activity by MgATP.

MgATP activation is observed for the fungal enzyme only. Since the fungal RSU is unique in containing the insert shown in Figure 1(B), this invites the suggestion that the two observations are connected. That is, the insert contains the site where MgATP binds. Therefore we have made mutations in this insert focusing on residues that are well conserved across species. Several mutants were identified in which MgATP activation was nearly or completely abolished. Unexpectedly, four mutants were found to be strongly dependent on MgATP. The activity stimulation that occurs upon reconstitution of the enzyme from its subunits is very

Abbreviations used: AHAS, acetohydroxyacid synthase; CSU, catalytic subunit; RSU, regulatory subunit.

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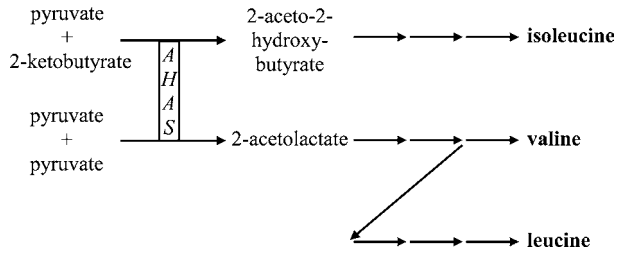


Scheme 1 The branched-chain amino acid biosynthetic pathway

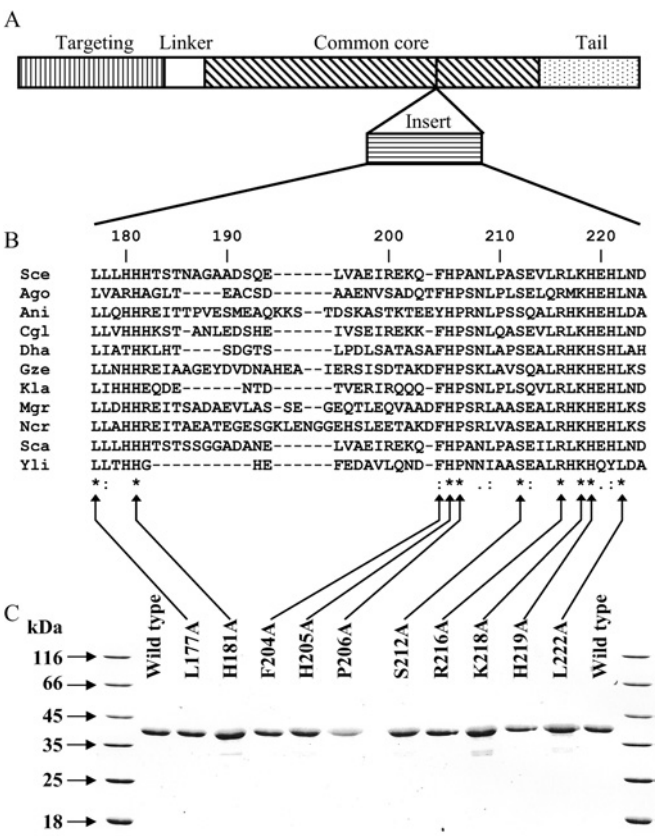


Figure 1 The AHAS RSU

(**A**) A schematic representation of the AHAS RSU amino acid sequence. The amino acid sequence of the insert that is characteristic of fungal AHAS RSUs is shown in (**B**) for 11 known or putative RSUs. Numbering is according to the yeast (*Saccharomyces cerevisiae*, Sce) protein sequence. The other abbreviations used are: Ago, *Ashbya gossypii*; Ani, *A. nidulans*; Cgl, *Candida glabrata*; Dha, *Debaryomyces hansenii*; Gze, *Gibberella zeae*; Kla, *Kluyveromyces lactis*; Mgr, *Magnaporthe grisea*; Ncr, *Neurospora crassa*; Sca, *S. carlsbergensis*; Yli, *Yarrowia lipolytica*. The star symbol indicates fully conserved, while ':' and '·' indicate strongly and weakly conservative substitutions respectively. The ten residues marked with an arrow were mutated to alanine and an SDS/PAGE of the purified proteins is shown in (**C**). The lanes flanking the ten mutants show the wild-type RSU and molecular mass markers.

small unless MgATP is present. In a complete reversal from the normal situation, MgATP activation is suppressed by valine. A model to explain these results is suggested.

EXPERIMENTAL

Materials were obtained from local commercial suppliers and agents, as described previously [6]. Standard laboratory procedures were employed.

Table 1 Oligonucleotide primers used for mutagenesis of the RSU of yeast AHAS

In each case, the mutated residue was replaced by alanine, using the codon GCT (boxed). Lower-case letters indicate bases that differ from the wild-type sequence.

Mutant	Sequence (5' → 3')
L177A	TACTTCGAAGAC**gct**CTATTGCACCAC
H181A	CTACTATTGCAC**gct**CACACTTCCACC
F204A	AGAGAAAAGCAA**gct**CACCCTGCCAAC
H205A	GAAAAGCAATTC**gct**CCTGCCAACTTG
P206A	AAGCAATTCCAC**gcT**GCCAACTTGCCC
S212A	AACTTGCCCGCC**gcT**GAGGTATTAAGG
R216A	CAGTGAGGTATTA**gct**TTGAAGCACGAGC
K218A	GTATTAAGGTTG**gct**CACGAGCATTTG
H219A	TTAAGGTTGAAG**gct**GAGCATTTGAACG
L222A	AAGCACGAGCAT**gct**AACGATATCACC

Escherichia coli growth, protein expression and purification

Plasmids based on a pET30 vector (Novagen, Madison, WI, U.S.A.) containing cDNA for the CSU or the RSU of yeast AHAS were as described previously [6]. Protein expression was performed in the BL21(DE3) strain of *E. coli* (Novagen) and also followed the method published previously [6]. Protein purification was based on immobilized nickel affinity chromatography using the His_6 tags on the recombinant yeast AHAS subunits. The Novagen His • Bind metal chelation resin was used in this procedure. The purified yeast CSU was exchanged into a storage buffer of 0.2 M potassium phosphate (pH 6.0) containing 10 μM FAD, 1 mM dithiothreitol and 20 % (v/v) glycerol and stored in small aliquots at −70 °C. For the wild-type and mutant RSUs, the storage buffer was 0.1 M potassium phosphate (pH 7.0) containing 1 mM dithiothreitol and 20 % glycerol. Protein concentrations were estimated using the bicinchoninic acid assay.

Mutagenesis

Mutations were introduced by PCR by the megaprimer method [12] using the oligonucleotide primers listed in Table 1 and a template consisting of the yeast AHAS RSU expression plasmid [6]. For the first PCR, reactions contained Vent DNA polymerase (New England Biolabs, Ipswich, MA, U.S.A.), template, mutant primer, the T7 terminator primer and buffer as recommended by the enzyme supplier. Cycle conditions were 96 °C for 1 min, then 30 cycles of 96 °C for 1 min, 50 °C for 1 min and 72 °C for 40 s, followed by 7 min at 72 °C. The PCR product was isolated and used as the megaprimer in the second PCR, which contained Vent, buffer, template, megaprimer and a pET30a(+) flanking primer with the sequence 5′-TGCCGGCCACGATGCGTCC-3′. Cycle conditions were 96 °C for 1 min, then 30 cycles of 96 °C for 1 min, 60 °C for 1 min and 72 °C for 2 min, followed by 7 min at 72 °C. The PCR product was digested with NdeI and XhoI, and ligated into the yeast AHAS RSU expression plasmid that had been digested with the same pair of restriction enzymes. The mutations were confirmed by DNA sequencing using the Prism Ready Dye Terminator Cycle Sequencing kit and DNA Sequencer 373A (PerkinElmer Applied Biosystems, Norwalk, CT, U.S.A.).

AHAS activity assay

All AHAS activity measurements were carried out using the colorimetric assay proposed by Singh et al. [13]. The assay

reaction mixture contained 200 mM pyruvate, 1 mM thiamin diphosphate, 10 mM $MgCl_2$, 10 μM FAD and wild-type yeast AHAS CSU in 1 M potassium phosphate buffer at pH 7.0. The reaction mixture also contained wild-type or mutant RSUs, at various concentrations for reconstitution experiments. To test the effects of MgATP and/or valine, these ligands were added at the appropriate concentrations, together with a saturating concentration of the required RSU. Typically, the 225 μl reaction mixture was preincubated without pyruvate at 30 °C for 15 min and the reaction was started by addition of 25 μl of 2 M sodium pyruvate solution. The reaction was stopped after 20 min by the addition of 35 μl of 50 % (v/v) sulphuric acid and incubated at 60 °C for 15 min to convert acetolactate into acetoin. Acetoin was then quantified [14] by the addition of 400 μl of 5.0 % (w/v, freshly prepared) α-naphthol dissolved in 4 M sodium hydroxide and 400 μl of 0.5 % (w/v) creatine. After further incubation at 60 °C for 15 min and cooling to room temperature ($\sim$22 °C), the colour developed was measured at 525 nm. One unit of enzymatic activity is defined as that producing 1 μmol of acetolactate per minute based on a molar absorption coefficient of 20400 $M^{-1} \cdot cm^{-1}$ for the coloured complex.

Data analysis

Kinetic parameters were determined by fitting the appropriate equation to the data by nonlinear regression using GraFit (Erithacus Software, Staines, U.K.) or Prism (GraphPad Software, San Diego, CA, U.S.A.). The best fit values and standard errors obtained from these analyses are reported. For subunit reconstitution experiments or MgATP activation experiments, eqn (1) was used while eqn (2) was used to analyse valine inhibition experiments:

$$v = v_0[1 + (F - 1)[A]/([A] + K_a)] \quad (1)$$

$$v = v_0[R + (1 - R)K_i/([I] + K_i)] \quad (2)$$

In these equations, v is the measured reaction rate and v_o is the reaction rate with no added RSU or MgATP (A; eqn 1), or valine (I; eqn 2). In eqn (1), K_a is the concentration of A that gives half-maximal activation, while F is the activation factor at saturating A (i.e. $v = Fv_o$ at $[A] = \infty$). In eqn (2), K_i is the concentration of I that gives half-maximal inhibition, while R is the fractional residual rate at saturating I (i.e. $v = Rv_o$ at $[I] = \infty$).

RESULTS AND DISCUSSION

Ten mutations were made in the insert region of the yeast AHAS RSU. The residues mutated include all nine sites that are totally conserved across the 11 fungal species shown in Figure 1(B) plus Phe^{204}, which is a tyrosine in the *Aspergillus nidulans* subunit only. In each case, the natural amino acid was replaced by alanine.

All mutations were introduced successfully by PCR mutagenesis, and the altered proteins were expressed and purified (Figure 1C). Each was then tested for its ability to stimulate the activity of the yeast AHAS CSU, to confer sensitivity to valine inhibition, and whether this inhibition can be reversed by MgATP. The standard buffer for reconstitution (1 M potassium phosphate, pH 7.0) could not be used for H205A, which precipitates under these conditions. Lowering the potassium phosphate concentration to 0.7 M overcame this problem for H205A, but these conditions may not be optimal.

Only one mutant RSU, H181A, shows properties that are similar to those of the wild-type yeast AHAS RSU. It stimulates the activity of the CSU by 7.1-fold with a K_a of 79 nM (Figure 2A), compared with the wild-type values of 7.2-fold and 69 nM (see Table 2 for summary of all mutants). The reconstituted enzyme is sensitive to valine inhibition ($K_i = 58$ μM; Figure 2B) and the inhibition can be reversed by MgATP ($K_a = 711$ μM; Figure 2C). As is observed for the wild-type RSU, valine inhibition is partial with a residual activity of approx. 10 % of the uninhibited rate (Figure 2B). The only substantial difference from wild-type is that H181A is 3-fold more sensitive to valine (Table 2).

We were hoping to identify amino acid residues involved in MgATP binding by finding mutants that were normal in all

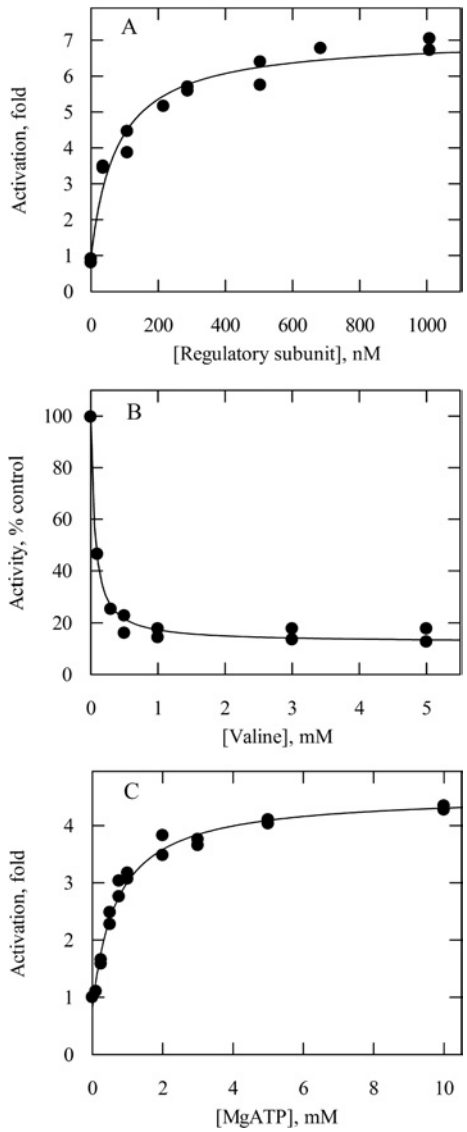


Figure 2 Properties of the H181A mutant of the yeast AHAS RSU

(**A**) Activation of 7.1 nM CSU by addition of the indicated concentration of mutant RSU. The data were fitted using eqn (1) to yield the parameter values shown in Table 2. For illustration, the data have been normalized to a relative activity of 1.0 in the absence of the RSU. (**B**) Inhibition by valine of the reconstituted enzyme (7.1 nM CSU and 1.08 μM RSU). The data were fitted using eqn (2) to yield the K_i value shown in Table 2. For illustration, the data have been normalized to a relative activity of 100 % in the absence of valine. (**C**) Activation by MgATP of the reconstituted, valine-inhibited enzyme (7.1 nM CSU, 1.08 μM RSU and 248 μM valine). The data were fitted using eqn (1) to yield the K_a value shown in Table 2. For illustration, the data have been normalized to a relative activity of 1.0 in the absence of MgATP.

Table 2 Properties of the wild-type and mutants of the yeast AHAS RSU

In reconstitution experiments with each protein, the parameters F and K_a (eqn 1) for the RSU are reported. The number in parentheses is the concentration of MgATP in these assays. In valine inhibition experiments, the inhibition constant (K_i; eqn 2) is reported and the number in parentheses is again the added concentration of MgATP. The last column shows the results of MgATP activation experiments (K_a; eqn 1) and the number in parentheses is the added concentration of valine.

	RSU F ([MgATP], mM)	RSU K_a (nM) ([MgATP], mM)	Val K_i (μM) ([MgATP], mM)	MgATP K_a (μM) ([Val], mM)
Wild-type	7.21 ± 0.90* (0.0)	69.1 ± 6.6* (0.0)	177 ± 25 (0.0)	622 ± 185 (1.0)
L177A	9.83 ± 3.77 (2.5)	29.3 ± 5.8 (2.5)	16.2 ± 1.6 (0.55)	279 ± 53 (0.0)
H181A	7.07 ± 0.25 (0.0)	78.7 ± 14.1 (0.0)	58.3 ± 7.0 (0.0)	711 ± 83 (0.25)
F204A	10.7 ± 0.52 (1.0)	144.0 ± 28 (1.0)	25.6 ± 7.5 (0.23)	115 ± 28 (0.0)
H205A†	1.69 ± 0.09 (0.0)	63.0 ± 19.8 (0.0)	19.6 ± 9.4 (0.0)	Slight activation (0.1)
P206A	9.95 ± 0.20 (2.5)	31.0 ± 2.7 (2.5)	39.2 ± 4.1 (0.55)	287 ± 109 (0.0)
S212A	9.17 ± 0.34 (6.0)	312 ± 32 (6.0)	15.6 ± 5.3 (1.0)	605 ± 26 (0.0)
R216A	1.76 ± 0.68 (0.0)	126 ± 107 (0.0)	24.6 ± 10.4 (0.0)	Nil (0.1)
K218A	2.41 ± 0.49 (0.0)	889 ± 615 (0.0)	5.99 ± 2.41 (0.0)	Nil (0.0 or 1.0)
H219A	1.89 ± 0.22 (0.0)	473 ± 366 (0.0)	n.d.‡	Nil (0.0)
L222A	3.28 ± 0.38 (0.0)	311 ± 123 (0.0)	2.91 ± 0.51 (0.0)	Nil (0.07)

*From [6].
†At 0.7 M potassium phosphate (pH 7.0).
‡n.d., not determined.

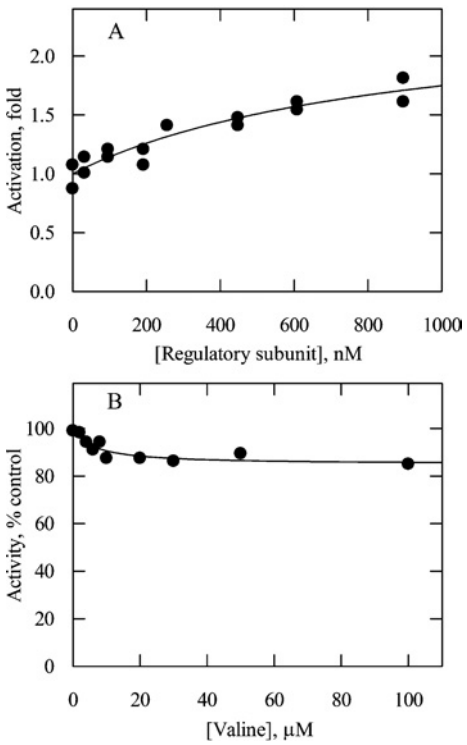


Figure 3 Properties of the K218A mutant of the yeast AHAS RSU

(**A**, **B**) are as for Figure 3, except that the reconstituted enzyme in (**B**) contains 4.8 μM RSU.

respects except that they could not be activated by this ligand. The mutation that came closest to meeting this expectation is L222A (Table 2), although it is not as effective as the wild-type RSU in enzyme activation. The extent of activation is halved, while the K_a for activation is increased by more than 4-fold. Remarkably, valine inhibition is shifted to very low concentrations, characterized by a K_i of 2.9 μM. Adding MgATP does not reverse valine inhibition.

Three of the mutants (R216A, K218A and H219A) show very much decreased ability to stimulate the activity of the CSU, and one example (K218A) is illustrated in Figure 3(A). The extrapolated fully activated enzyme shows less than 2.5 times the activity of the CSU alone. This reconstituted enzyme can be inhibited only slightly (~15 %) by valine (Figure 3B) and we were unable to detect any effect of MgATP. H205A is also similar, except that there is a very weak activation by MgATP that is too small to quantify properly. For all four mutants, the small changes in activity made it difficult to define accurately the K_a for activation or the K_i for inhibition.

The final four mutants (L177A, F204A, P206A and S212A) appeared initially to be similar to the previous four, showing only weak stimulation of the CSU activity (e.g. L177A; Figure 4A, closed circles). However, during the course of characterizing the enzyme reconstituted with these mutants, we discovered that each of them is strongly activated by MgATP ($F = 7.2$ for L177A) even in the absence of valine (Figure 4C). Unlike the enzyme in the absence of MgATP, this activated enzyme is strongly inhibited by valine (Figure 4B). The stimulation of the CSU by the RSU is greatly enhanced in the presence of MgATP (Figure 4A, open circles).

In an earlier report, we described a detailed kinetic study of the regulation of yeast AHAS by valine and MgATP [4]. We reported a slight stimulation, by approx. 20 %, of the reconstituted enzyme by MgATP. The stimulation is more easily observed when the enzyme is first inhibited by valine because MgATP will completely reverse this inhibition. The kinetic model that was proposed does not anticipate variants such as L177A, which shows substantial stimulation by MgATP in the absence of valine (Figure 4C), together with accentuated stimulation during reconstitution, and inhibition by valine, in the presence of MgATP. In order to account for these results, we propose a new model that is shown in Scheme 2. This model is reminiscent of the classical Monod–Wyman–Changeux [15] allosteric model, except that a monomeric protein is assumed. This is undoubtedly a simplification and the model that we proposed previously [4] requires a dimer. However, the current model is sufficient to explain the results presented here.

The basic premise is that the RSU exists in two conformations, H and L, which are in equilibrium with one another. Both can bind equally well (or are already bound) to the CSU but when wild-type H is bound it causes substantial stimulation (~10-fold) of activity, while the L conformation causes little or no stimulation. MgATP binds to H and at saturation will convert all of the RSU into the H conformation. For the wild-type RSU, the equilibrium constant is approx. 0.2 (Scheme 2) so that in the absence of MgATP the H conformation is favoured by a factor of 5:1; therefore the effect of MgATP on the reconstituted enzyme activity is small. Valine binds to L and therefore inhibits by decreasing the proportion of H. In the presence of valine, MgATP has a large activating effect by shifting the balance between H and L back towards H. That is, valine inhibition is reversed by MgATP. The conformational difference between the H and L forms is probably quite subtle and we have found no change in the CD spectrum upon addition of valine to the wild-type RSU [4].

For the L177A mutant (as well as F204A, P206A and S212A), the initial equilibrium between H and L favours the latter (estimated $K_{eq} \sim 5$, see Scheme 2). Therefore reconstitution in the absence of regulatory ligands causes only a small increase in activity. Valine has only a small effect, because the predominant conformation is L even when no valine is present. However, addition

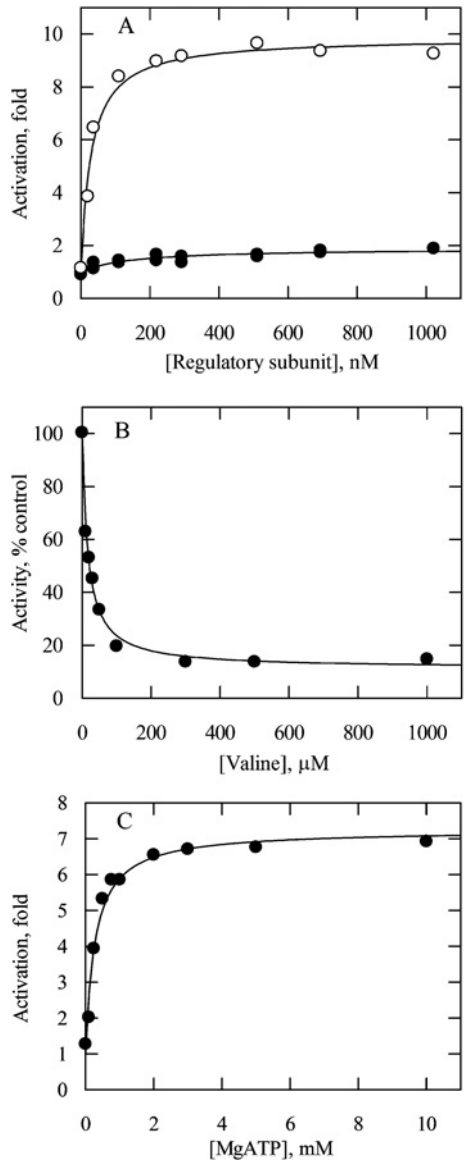
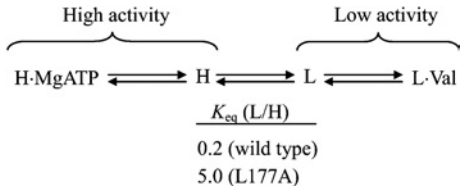


Figure 4 Properties of the L177A mutant of the yeast AHAS RSU

(**A**–**C**) are as for Figure 3, except for the following. For (**A**), the experiments represented by the open and filled symbols were performed in the absence and presence of MgATP (2.5 mM). For (**B**), the reconstituted enzyme contains 1.6 μM RSU and 0.55 mM MgATP. For (**C**), the reconstituted enzyme contains 1.6 μM RSU but no added valine.

of MgATP displaces the equilibrium towards H, and much greater stimulation of activity can be observed upon adding the RSU. For the same reason, addition of MgATP to the reconstituted enzyme causes a substantial increase in activity. Finally, in the presence of MgATP, valine inhibition is accentuated. It should also be observed that the K_i for valine (measured at an MgATP concentration that is twice its K_a) is substantially lower than that of wild-type (with no MgATP) for all four of these mutants (Table 2). The wild-type K_i reflects the multiplied effects of valine binding and displacement of the conformational equilibrium from H to L. In the experiments with these mutants, where L will still dominate (due to the relatively non-saturating MgATP concentration), equilibrium displacement will be less of an impediment, explaining the lower K_i values. Although these



Scheme 2 Model for the action of the yeast AHAS RSU

mutants display properties that differ markedly from those of the wild-type RSU, the difference in K_{eq} values shown in Scheme 2 correspond to a change in the standard free energy change ($\Delta G^{\circ\prime}$) for the conformational equilibrium of approx. 8 kJ · mol^{-1}. Loss of a few van der Waals interactions due to side-chain truncation, together with a hydrogen bond for the Ser^{212} mutation, is sufficient to account for such a change.

The properties of a second group of mutants (H205A, R216A, K218A and H219A) are also explained by this model. They also have an altered equilibrium between H and L and the main difference with this group is that they have lost the ability to bind MgATP. Therefore they show only a small degree of activation upon reconstitution, are insensitive to MgATP and exhibit minimal valine inhibition but relatively low K_i values. The fact that these mutations abolish sensitivity to MgATP is not surprising and this is precisely the type of change that we intended to introduce by mutating the fungal AHAS insert sequence. The altered equilibrium between H and L tends to disguise the important nature of the change, by allowing only a small degree of valine inhibition. L222A is also similar but the shift from the wild-type in the conformational equilibrium is smaller, allowing greater valine inhibition so that the lost sensitivity to MgATP is observed more readily.

In conclusion, we have mutated ten highly conserved amino acid residues in an insert sequence that is unique to the fungal AHAS RSU. Nine of these mutations are proposed to affect the equilibrium between two conformational states of the protein. Five of the mutations appear to abolish MgATP activation that is mediated by this subunit and we conclude that His^{205}, Arg^{216}, Lys^{218}, His^{219} and Leu^{222} constitute the binding site for this regulatory ligand.

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