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Interactions between Large and Small Subunits of Different Acetohydroxyacid Synthase Isozymes of *Escherichia coli*[†]

Maria Vyazmensky, Yuri Zherdev, Alex Slutzker, Inna Belenky, Olga Kryukov, Ze'ev Barak, and David M. Chipman*

Department of Life Sciences, Ben-Gurion University of the Negev, Beer-Sheva 84105, Israel

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ABSTRACT: The large, catalytic subunits (LSUs; ilvB, ilvG and ilvI, respectively) of enterobacterial acetohydroxyacid synthases isozymes (AHAS I, II and III) have molecular weights ~60 kDa and are paralogous with a family of other thiamin diphosphate dependent enzymes. The small, regulatory subunits (SSUs) of AHAS I and AHAS III (ilvN and ilvH) are required for valine inhibition, but ilvN and ilvH can only confer valine sensitivity on their own LSUs. AHAS II is valine resistant. The LSUs have only ~15, ≪1 and ~3%, respectively, of the activity of their respective holoenzymes, but the holoenzymes can be reconstituted with complete recovery of activity. We have examined the activation of each of the LSUs by SSUs from different isozymes and ask to what extent such activation is specific; that is, is effective nonspecific interaction possible between LSUs and SSUs of different isozymes? To our surprise, the AHAS II SSU ilvM is able to activate the LSUs of all three of the isozymes, and the truncated AHAS III SSUs ilvH-Δ80, ilvH-Δ86 and ilvH-Δ89 are able to activate the LSUs of both AHAS I and AHAS III. However, none of the heterologously activated enzymes have any feedback sensitivity. Our results imply the existence of a common region in all three LSUs to which regulatory subunits may bind, as well as a similarity between the surfaces of ilvM and the other SSUs. This surface must be included within the N-terminal βαββαβ-domain of the SSUs, probably on the helical face of this domain. We suggest hypotheses for the mechanism of valine inhibition, and reject one involving induced dissociation of subunits.

Acetohydroxyacid synthases (AHASs;[1] EC 2.2.1.6) catalyze the first common step in the biosynthesis of the branched-chain amino acids: valine, isoleucine and leucine. Prokaryotic AHASs are composed of two kinds of subunits, catalytic and regulatory in function, and it appears that at least some eukaryotic AHASs also have separate regulatory subunits (*1*−*3*). The three AHAS isozymes in the enterobacteria (AHAS I, II and III) are encoded by genes *ilvBN*, *ilvGM* and *ilvIH*, respectively, and differ in catalytic activity, kinetic properties and feedback inhibition by valine (*4*−*9*). In *Escherichia coli* or *Salmonella typhimurium* these isozymes apparently have overlapping but slightly different physiological roles (*8*, *10*−*17*). The earliest investigations of the roles of these subunits, based largely on bacterial genetics, molecular biology and physiology, demonstrated that the large subunits (LSUs; ilvB, ilvG and ilvI) have implied molecular weights near 60 kDa and are homologous in sequence with a large family of other thiamin diphosphate (ThDP) dependent enzymes (*18*). The small regulatory subunits (SSUs) of AHAS I and AHAS III (ilvN, ilvH) appeared to be required for valine inhibition of the activity (*4*, *19*), but ilvN and ilvH can only confer valine sensitivity on their own catalytic subunit partners (*9*). The isozyme II is resistant to valine (*20*, *21*). It is of interest that the LSU polypeptides ilvB, ilvG and ilvI have only ~15, ≪1 and ~3%, respectively, of the molar activity of their respective holoenzymes when isolated (*22*−*24*), but under appropriate conditions the holoenzymes can be reconstituted from their subunits with essentially complete recovery of activity. The valine sensitivity of isozymes AHAS I and III is also recovered on reconstitution from subunits.

The SSUs of AHASs all share a domain of roughly 80 amino acids, although the sequences of these subunits have fairly low identity overall (15−20%). These domains are examples of the widely distributed ACT family of small effector molecule binding domains (*25*). The valine-binding sites in AHASs appear to be localized to a characteristic interface between the ACT domains of dimeric SSUs (*25*−*27*). The SSUs of isozymes I, II and III (ilvN, ilvM and ilvH) have molecular weights 11, 9 and17 kDa, respectively, and we noted in previous work that isolated, modified ilvH peptides with large truncations of the C-terminus are able to fully activate ilvI, but do not confer any valine sensitivity (*28*). The nomenclature "ilvH-Δ*x*" will refer here to a modified ilvH peptide with *x* amino acid residues truncated from the C-terminus. The crystallographic structure of the ilvH dimer (*27*) shows clearly that it has a second domain with a rather different fold. The ilvH peptides with 80 to 89 residues truncated from the C-terminus (ilvH-Δ80, -Δ86, -Δ89) effectively include the N-terminal ACT domain only.

In the course of our efforts to understand the molecular basis for the effects of the SSUs on LSUs, and the mechanism of the transfer of the signal from the valine binding sites to the active sites of AHAS, we considered whether the activation of AHAS catalytic subunits by the SSUs is isozyme-specific in the same way

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*Address correspondence to this author at Dept. of Life Sciences, Ben-Gurion University of the Negev, P.O. Box 653, Beer-Sheva 84105, Israel. Tel: [+972]-8-647 2646. Fax: [+972]-8-646 1710. E-mail: chipman@bgu.ac.il.

[1]Abbreviations: AHAS, acetohydroxyacid synthase; LSU, large [catalytic] subunit; SSU, small [regulatory] subunit; ThDP, thiamin diphosphate; ACT domain, small effector molecule binding domain related to that in 3-phosphoglycerate dehydrogenase; Ni-NTA, Nickel nitrilotriacetate.

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as is the conferral of valine sensitivity. Is any effective nonspecific interaction possible between LSUs and SSUs of different isozymes? To answer this question we have now examined the effects of each of the isolated SSUs on all of the isolated LSUs. To our surprise, the AHAS II SSU ilvM is able to activate the LSUs of all three of the isozymes, and the truncated AHAS III SSUs ilvH-Δ80, ilvH-Δ86 and ilvH-Δ89 are able to activate the LSUs of both AHAS I and AHAS III. However, none of the heterologously activated enzymes have any feedback sensitivity.

MATERIALS AND METHODS

Restriction enzymes and other molecular biology reagents were from BioLab Inc. (Beverly, MA). The FailSafe PCR Selection Kit was purchased from EPICENTRE Biotechnologies (Madison, WI). Yeast extract, peptone and agar were obtained from Difco (Detroit, MI). Sodium pyruvate, ThDP, DTT, FAD, ampicillin, tetracycline, amino acids and creatine were obtained from Sigma Chemical Co. (St. Louis, MO). EDTA was purchased from Merck (Darmstadt, Germany), SDS was obtained from BDH Chemicals Ltd. (Pool, U.K.), and 1-naphthol was from Carlo Erba, Reagents (Milano, Italy). Ni-NTA agarose resin was obtained from Qiagen (Hilden, Germany).

All other materials were of analytical grade.

Cloning, Mutagenesis and Protein Isolation. Site-directed mutagenesis, bacterial growth, gene expression and purification of AHAS I (*24*) and AHAS III (*28*, *29*) large and small subunits were carried out as described previously. IlvI, the LSU of AHAS III without any additions, was amplified and expressed in XL-MRF and purified as described by Vyazmensky et al. (*29*). IlvH, the regulatory SU of AHAS III with an N-terminal hexahistidine fusion, was grown in BL21 and purified on a Ni-NTA agarose column (*28*). Both subunits of AHAS I had N-terminal hexahistidine fusions and were grown in XL-MRF (*24*).

The truncated AHAS III small subunit ilvH-Δ80 was prepared by introducing a stop codon into the expression plasmid construct pNH6-ilvH, whose N-terminal six-histidine tag allowed us to purify the products on a Ni-NTA column under nondenaturing conditions (*28*). The truncated peptides ilvH-Δ86 and ilvH-Δ89 were grown in XL-MRF for reasons of convenience, and prepared similarly except that the NH6-ilvH coding region was transferred to pUH (*29*).

The genes coding for the isolated LSU (ilvG) and SSU (ilvM) of AHAS II were subcloned from pQEV-GM (*30*) by PCR and inserted into the inducible expression plasmid pHIS.parallel2 (*31*) between the sites *Bam*HI and *Eco*RI, to yield the plasmids pHIS-ilvG and pHIS-ilvM, respectively.

Plasmid pAM, encoding the wild type AHAS II SSU (ilvM) after the native promoter of *ilvGMEDA*, was created from plasmid pAH29 (*32*), a gift of G. W. Hatfield, for use in experiments in vivo. Fragment *Hin*dIII−*Nco*I, containing the ilvGMEDA promoter, was prepared by PCR amplification with pAH29 as template, using a primer which introduced the *Nco*I site in place of the first ATG codon of *ilvG*. The second fragment, *Nco*I−*Nhe*I, containing *ilvM*, was prepared in the same way, inserting the *Nco*I site in the place of the first ATG of *ilvM*. After restriction enzyme digestion, the two fragments were inserted into the pBR322 vector (Fermentas, MD) between the *Hin*dIII and *Nhe*I sites. Plasmid pOH-Δ86, encoding the truncated AHAS III SSU without an N-terminal histidine affinity tag, was constructed for in vivo experiments by introducing a stop codon into plasmid pOH (*9*).

The ilvG protein with an N-terminal hexahistidine tag was purified as described for the ilvB protein (*24*, *33*). We found that insoluble protein frequently precipitated out during the purification unless all the solutions used, from the cell disruption step onward, contained 20% glycerol.

The ilvM protein with the same leader sequence was purified under denaturing conditions using Ni-NTA agarose (*28*). In the last step of the purification the protein was eluted from the resin with 8 M urea, 0.1 M sodium dihydrogen phosphate and 0.4 M imidazole in 10 mM Tris-HCl buffer, pH 8.0. The ilvM protein was always used immediately after purification.

Reconstitution of AHASs from Isolated Subunits and Enzyme Assay. The enzymes were reconstituted by incubation of the different LSUs with a 10- to 300-fold molar excess of the SSUs, as previously described (*28*) in a solution containing 0.1 M potassium phosphate buffer (pH 7.6), 50 mM pyruvate, 0.1 mM ThDP, 0.075 mM FAD, 5 mM EDTA, 10 mM $MgCl_2$, 0.5 mM DTT, except where otherwise indicated. Enzymatic acetolactate production activity was determined using the Westerfeld colorimetric assay as previously described (*30*, *34*)

Experiments in Vivo. Plasmids pOH (encoding the *ilvH* wild type SSU), pOH-G14D (*23*) and pOH-Δ86 (encoding two valine resistant proteins ilvH variants), pBR322 and pAM were each transformed into *E. coli* K12 strain MM294. The transformed cells were grown in the minimal medium VB with ampicillin (100 mg/L) and glutamine (40 mg/L) in 100 mL shaker flasks. Growth was followed spectrophotometrically, and 20 mg/L valine was added to cultures at the point indicated.

Molecular Modeling and Graphics. Homology models were prepared using SWISS-MODEL (*35*, *36*).

Molecular graphics images were produced using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIH P41 RR-01081) (*37*).

RESULTS

Interaction between Different LSUs and SSUs. IlvM (the AHAS II SSU) is able to activate the LSUs from either of the other AHAS isozymes of *E. coli*. It specifically activates ilvB and ilvI to 100% of the maximum measured catalytic activity of the holoenzymes AHAS I (ilvB-ilvN) and AHAS III (ilvI-ilvH), respectively (Figures 1, 2 and Table 1). The pyruvate dependences of the hybrid proteins ilvB-ilvM and ilvI-ilvM are very similar to those of the respective homologous holoenzymes (Table 2). In contrast, ilvG (the AHAS II LSU) could not be activated within the sensitivity of the colorimetric assay, by either of the SSUs from the other isozymes (Figure 3). It is important to note, however, that the concentration of ilvM peptide required to activate ilvI is an order of magnitude higher than the concentration of ilvM required to activate ilvG (or ilvB), and 5-fold higher than the concentration of ilvH needed to activate ilvI (Table 1). While ilvM activates ilvG to half of its maximal activity at a molar ratio of 0.72:1, it requires a 8.5- or 25-fold excess of ilvM to half-activate ilvB or ilvI.

IlvB (the AHAS I LSU) could be activated by its homologous SSU (ilvN) or by ilvM. It could also be very weakly (2.5%) activated by the AHAS III SSU (ilvH) (Figure 1 and Table 1). Significantly, the mutant ilvH-Δ80, truncated so that it contains only the N-terminal domain and is roughly similar in size to the

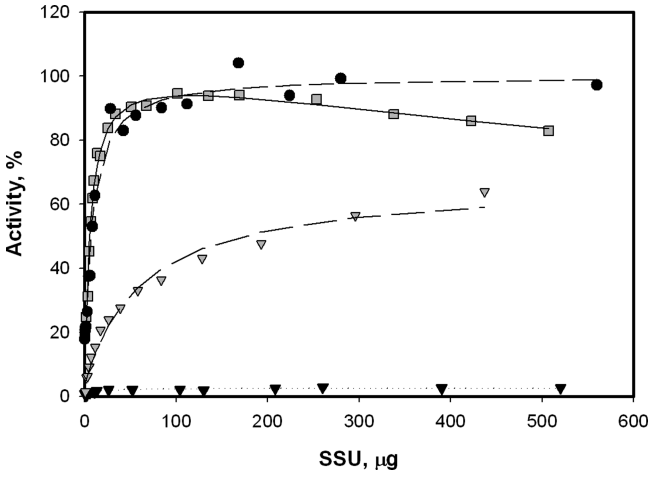

FIGURE 1: Activation of the AHAS I LSU ilvB (3.5 μg in assay) by different SSUs: ilvB + ilvN (black ●), ilvB + ilvM (gray ■), ilvB + ilvH (black ▼), ilvB + ilvH-Δ86 (gray ▼). Reactions were carried out in the presence of 50 mM pyruvate at pH 7.6, as described in Table 1. The activities were normalized to the extrapolated maximum for ilvB + ilvN as 100%.

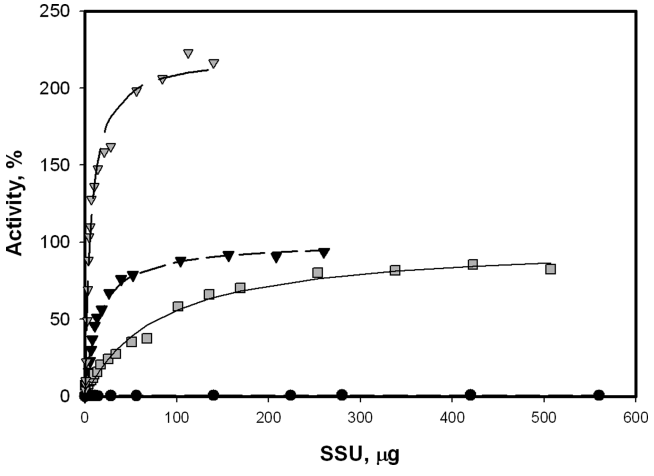

FIGURE 2: Activation of the AHAS III LSU ilvI (15.6 μg in assay) by different SSUs: ilvI + ilvH (black ▼), ilvI + ilvM (gray ■), ilvI + ilvN (black ●), ilvI + ilvH-Δ86 (gray ▼). Reactions were carried out in the presence of 50 mM pyruvate at pH 7.6, as described in Table 1. The activities were normalized to the extrapolated maximum for ilvI + ilvH as 100%.

ilvM or ilvN proteins, is capable of activating ilvB to some 63% of the activity of the normal holoenzyme. The same truncated peptide can activate the AHAS III LSU to more than 100% of the activity of the "normal" holoenzyme (Figure 2 and Table 1). The truncated ilvH mutants, ilvH-Δ80 and ilvH-Δ89, also activate ilvB to 50−65% (data not shown).

Valine Inhibition of Reconstituted AHAS Proteins. It became clear early on in the study of regulation of bacterial AHASs that AHAS I and AHAS III are inhibited by valine, while AHAS II is resistant to valine inhibition (*38*). The observation of ("heterologous") activation of catalytic subunits by SSUs from other AHASs raises the question of whether the hybrid enzymes are sensitive to valine inhibition. We thus examined the valine sensitivity of activated hybrids ilvB-ilvM (Figure 4), ilvI-ilvM (Figure 5) and ilvB-ilvH-Δ86, as well as of the natural, homologously reconstituted enzymes (Table 2). Inhibition by other amino acids, particularly leucine, was examined as a control. The homologously reconstituted enzymes behaved here as we have previously reported (Table 2) (*26*, *33*). On the other hand, none of the hybrid enzymes showed significant inhibition by valine. The enzyme reconstituted from ilvB and ilvM appeared to be very slightly inhibited by valine (Figure 4), but this effect is apparently not specific, because ilvB-ilvM was inhibited by threonine and methionine in the same way (data is not shown). The activated constructs ilvI-ilvH-Δ80, -Δ86 and -Δ89 were also completely insensitive to valine inhibition, as we previously reported for ilvH-Δ80 (*28*).

Two alternative structural−mechanistic schemes can be proposed to account for valine inhibition of AHASs and the major observations described above, one based on valine-induced dissociation of the regulatory subunits from the holoenzyme and the other on inhibitor-dependent allosteric shifts between two different conformations of the holoenzymes. In either scheme, the catalytic subunits are in a state with low activity in the absence of SSUs. Interactions with the corollary regulatory SSU, with the ilvM peptide, or with the truncated ilvH peptide can all stabilize the normal active state, implying that the LSU−SSU interfaces of the three isozymes have common elements. The possible nature of the interface is discussed below.

As the association−dissociation of AHAS holoenzymes is in equilibrium on a time scale of minutes (*39*), the valine-induced dissociation mechanism predicts that the observed valine inhibition constant of a valine-dependent construct should be increased in the presence of excess SSU. Experiments with AHAS III

Table 1: Reconstitution of AHAS Activity of LSUs with Different SSUs[a,b]

	catalytic SU					
	ilvB		ilvG		ilvI	
regulatory SU	sp act., U mg^{-1} (% of homol)	$K_{0.5}$, μg mL^{-1} (SSU/LSU)	sp act., U mg^{-1} (% of homol)	$K_{0.5}$, μg mL^{-1} (SSU/LSU)	sp act., U mg^{-1} (% of homol)	$K_{0.5}$, μg mL^{-1} (SSU/LSU)
ilvN	50.8 ± 1.9 (100%)	6.8 ± 1.3 (9.7)	0		0.06 (< 1%)	
ilvM	49.8 ± 1.5 (~100%)	5.7 ± 1.1 (8.5)	60.4 ± 1.5 (≡ 100%)	3.0 ± 0.2 (0.72)	9.3 ± 0.4 (~100%)	79.0 ± 1.0 (25)
ilvH	1.3 ± 0.1 (2.5%)	7.9 ± 2.3 (11.5)	0		9.4 ± 0.2 (≡100%)	14.3 ± 0.9 (3.7)
ilvH-Δ86	32.0 ± 1.7 (63%)	46 ± 7 (83)	0		20.9 ± 0.5 (220%)	6.3 ± 0.6 (2.3)

[a]AHAS activity in the standard assay. The rate of acetolactate formation was determined at 37 °C in 1 mL reaction mixture containing 50 mM pyruvate, 0.1 mM ThDP, 10 mM $MgCl_2$, 75 μM FAD, 0.5 mM DTT and 5 mM EDTA in a 0.1 M potassium phosphate buffer, pH 7.6. The concentration of the catalytic subunits in the reaction mixtures was 3.5 μg/mL for ilvB, 20 μg/mL for ilvG and 15.6 μg/mL for ilvI. [b]Specific activity of the holoenzyme reconstituted from its homologous subunits was normalized to 100% for the extrapolated maximum for LSU + SSU. $K_{0.5}$ is defined as the concentration of the SSU required for half-maximum of AHAS activity. $K_{0.5}$ is also given in terms of the ratio of SSU to LSU at half-maximum activation.

Table 2: Kinetic Parameters of AHASs Reconstituted from LSU and SSU of Different Isozymes[a]

	pyruvate dependence[b]		valine inhibition[c]
proteins	V_{max}, U mg^{-1}	K_m, mM	K_i, μM
	AHASI		
ilvB alone	9.9 ± 0.4	59.3 ± 5.2	resistant
ilvB + ilvN	49.4 ± 2.1	10.8 ± 1.8	37.0 ± 4.0
ilvB + ilvM	47.7 ± 1.1	5.9 ± 0.6	130 ± 19
ilvB + ilvH-Δ86	nd	nd	>2000
	AHASIII		
ilvI alone	1.1 ± 0.1	44.4 ± 9.2	resistant
ilvI + ilvH	8.3 ± 0.3	15.0 ± 0.4	8.3 ± 0.8
ilvI + ilvM	7.8 ± 0.2	18.1 ± 1.9	>300
ilvI + ilvH-Δ86	18.7 ± 0.4	8.5 ± 0.5	>2000

[a]Reactions were carried out as described in Table 1. [b]AHAS activity was measured with a 20- to 100-fold molar excess of SSUs. [c]Reactions were carried out in the presence of 10 mM pyruvate.

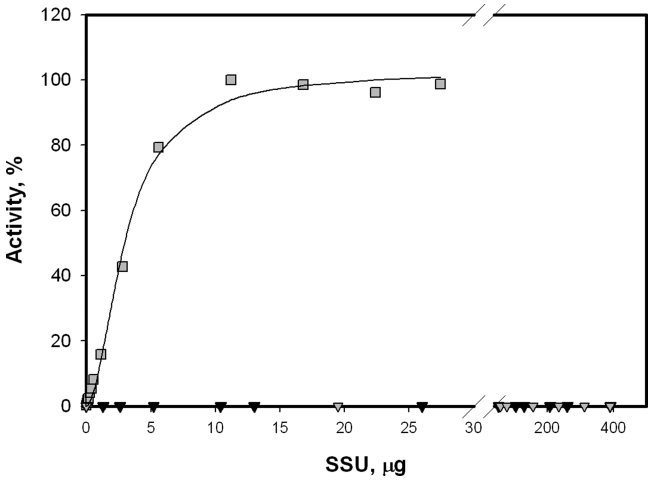

FIGURE 3: Activation of the AHAS II LSU ilvG (20 μg in assay) by different SSUs: ilvG + ilvM (gray ■), ilvG + ilvN (black ●), ilvG + ilvH (black ▼), ilvG + ilvH-Δ86 (gray ▼). Reactions were carried out in the presence of 50 mM pyruvate at pH 7.6, as described in Table 1. The activities were normalized to the extrapolated maximum for ilvG + ilvM as 100%.

showed that the addition of a large excess of crude ilvH peptide had very little effect on the apparent K_i for valine (*39*). We have now re-examined the effects of ilvH on AHAS III using purified holoenzyme and SSU, and confirmed this observation (data not shown). We also examined the behavior of the AHAS I holoenzyme in the absence and presence of a 300-fold excess of its SSU, ilvN. We examined the reactions rates in the presence of 2 substrate concentrations (2 or 20 mM pyruvate) over a range of valine concentrations, and over a range of pyruvate concentrations with or without 0.3 mM valine in each case. The data could be successfully fit by least-squares analysis of all the data for by the same modified MWC mechanism suggested previously (*33*), with very similar best fit parameters for the enzyme with and without added ilvN except for the apparent V_{max} (e.g., Figure 6). In particular, the same apparent valine dissociation constant, allosteric equilibrium and relative affinities of the protein for substrate and valine in two allosteric states gave acceptable fits to the data in the two cases.

In Vivo Complementation. The presence of valine inhibits the growth of *E. coli* K-12 strains on minimal medium, because both of the AHAS isozymes (AHAS I and III) expressed in K-12 strains are inhibited by valine leading in turn to starvation for isoleucine. However, under the standard conditions of growth used here with the *E. coli* K-12 strain MM294, some of the strains carrying plasmids which constitutively express valine-insensitive AHAS SSUs are resistant to 20 μg/mL valine in the medium (Supplementary Figure 1 in the Supporting Information). This in vivo complementation effect is seen with plasmids expressing valine-resistant ilvH mutants (ilvH-G14D and ilvH-Δ86; Supplementary Figures 1E and 1F in the Supporting Information) or valine-resistant ilvN missense mutants (G. Haran, unpublished results) but *not* with normal ilvN, ilvH (Supplementary Figure 1D in the Supporting Information) or ilvM (Supplementary Figure 1C in the Supporting Information). The AHAS II regulatory subunit ilvM is in fact expressed and can be detected in SDS−PAGE analyses (Supplementary Figure 2 in the Supporting Information).

DISCUSSION

There are as yet no structures of AHAS holoenzymes available, and the quaternary structures of these enzymes have yet to be unambiguously determined. The three enterobacterial isozymes are quite different in apparent physiological function (*12*, *40*−*43*), substrate specificity, and regulatory responses to valine (*26*, *33*). One might assume that the catalytic subunits associate specifically and, perhaps, exclusively with the regulatory subunits with which they are expressed. The amino acid sequence of ilvM is different from that of the SSUs of other isozymes in positions particularly characteristic of the ACT regulatory small-molecule-binding domain (*26*, *44*) and is not recognized as an "ACT domain" by the PSI-BLAST algorithm. The isolated AHAS II subunits are also relatively unstable when separated, and the conditions for reconstituting AHAS II were only worked out after Duggleby and colleagues prepared the individual subunits as N-terminal fusion proteins with oligohistidine sequences and purified them by immobilized-metal-affinity chromatography (*22*).

We were thus initially surprised by the observation that ilvM could activate the catalytic subunits ilvB and ilvI just as well as could the normal SSUs of each catalytic subunit (Table 1). We found, further, that the hybrid enzymes reconstituted from ilvB + ilvM or from ilvI + ilvM had substantially the same the kinetic parameters, V_{max} and K_m for pyruvate, as the reconstituted normal enzymes AHAS I (ilvB + ilvN) and AHAS III (ilvI + ilvH), respectively (Table 2). These observations imply the existence of a region common to the catalytic subunits of the three isozymes to which regulatory subunits may bind, as well as a similarity between the surface structures of ilvM and the other regulatory subunits. The data also imply that the ilvM subunits have a high affinity for all the catalytic subunits. This affinity is not affected by the presence of valine because ilvM apparently lacks the canonical small-molecule binding site of ACT domains, even though it may well have the characteristic $\beta\alpha\beta\beta\alpha\beta$ topology of such domains. Without an appropriate valine binding site, the enzymes reconstituted from ilvB + ilvM and ilvI + ilvM are valine resistant.

Despite the firm evidence that the binding site for valine in the AHAS III regulatory subunit (ilvH) is located in the N-terminal domain (*26*, *27*), when the AHAS III catalytic subunit (ilvI) is

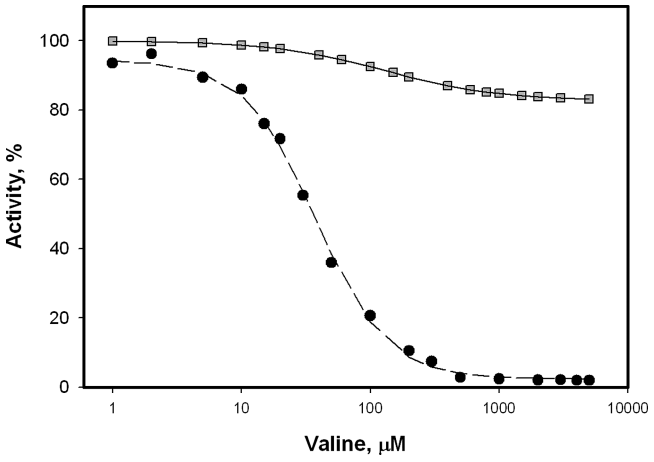

FIGURE 4: Valine inhibition of AHAS I reconstituted from ilvB and ilvN (●), and of the heterologous combination ilvB + ilvM (gray square). Reactions were carried out as described in Table 1 in the presence of 10 mM pyruvate.

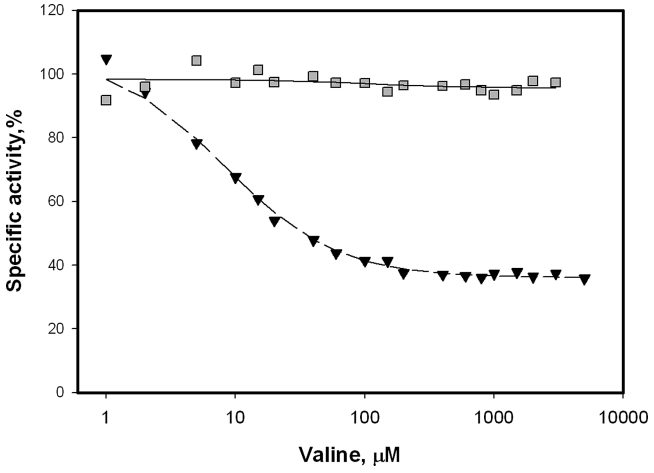

FIGURE 5: Valine inhibition of AHAS III reconstituted from ilvI and ilvH (▼) and of the heterologous combination ilvI + ilvM (gray square). Reactions were carried out as described in Table 1, in the presence of 10 mM pyruvate.

activated by truncated ilvH peptides apparently including the entire N-terminal domain, the resulting active enzyme is not valine sensitive (*28*). We have repeated and confirmed this observation here. In addition, the truncated constructs ilvH-Δ86 and -Δ89 activate AHAS I catalytic subunits (ilvB) to a significant extent (Table 1). In this case as well, the activated hybrid constructs show no valine sensitivity.

However, the activation of both ilvB and ilvI, by the isolated N-terminal domain of the ilvH peptide, implies that the normal interaction surface must be in the N-terminal domain of ilvH. In the three-dimensional structure of the ilvH dimer (Figure 7A) (*27*), the 8-stranded β-sheet of its ACT domain is largely buried between the *N*- and *C*-terminal domains, implying by elimination that the other face of the domain, with four parallel α-helices, is in contact with the catalytic subunits. The SSUs of the three isozymes have a degree of sequence homology (Figure 7B) which suggests that the helical faces of ilvM (Figure 7C) and ilvN (not shown) may have sufficient similarity in distribution of polar and nonpolar groups to explain the formation of hybrid complexes. Interestingly, the AHAS I catalytic subunit (ilvB) is not significantly activated by the normal, full-length AHAS III regulatory subunit (ilvH). The AHAS III catalytic subunit (ilvI) is also not activated to any

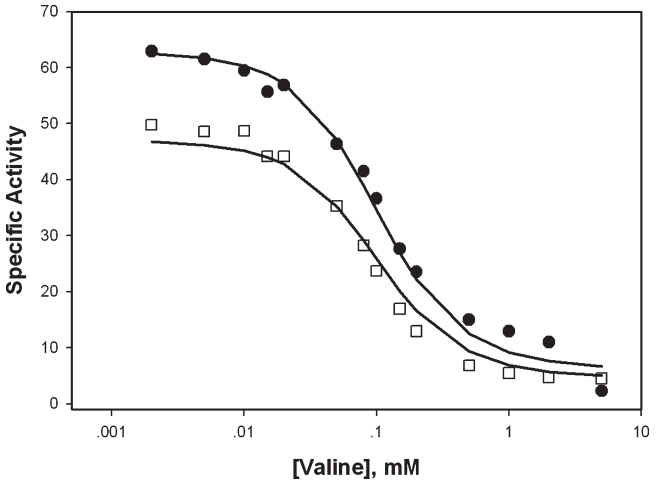

FIGURE 6: Valine inhibition of AHAS I with (●) and without (□) a 300-fold excess of added ilvN. Reactions were carried out as described in Table 1, in the presence of 20 mM pyruvate. The data in each case was fit by a modified MWC mechanism (*33*), with parameters *L* (the conformational equilibrium [T]/[R]) = 0.15; K_i (valine dissociation constant from the R state) = 280 ± 48 μM; c_i (the ratio $K_{i,R}/K_{i,T}$) = 19 ± 4 and cs ($K_{s,R}/K_{s,T}$) = 0.37 ± 0.04. The apparent V_{max} in the presence of excess ilvN small subunits is about 33% higher than for the holoenzyme alone.

significant extent by the AHAS I regulatory subunit (ilvN). A comparison of the putative interaction surfaces of the three SSUs does not provide a simple explanation for the apparent relative affinities (Table 1) of the SSUs, and further experiments involving crystallography, mutagenesis and multidimensional NMR (*45*) and other methods will be needed to fully understand this system.

The results of experiments carried out in the presence of excess regulatory subunits (e.g., Figure 6) seem to rule out the valine-induced dissociation mechanism for valine inhibition of either AHAS I or AHAS III. The alternative regulatory mechanism is an allosteric conformational shift in the holoenzymes on valine binding. Any such mechanism would imply that the holoenzymes of AHAS I or III each can exist be in a state with lower activity and/or affinity for substrate, stabilized by the valine-bound conformational state of the regulatory subunit. The binding of valine to the ACT domain of the regulatory subunit changes its conformation in a manner that is complementary to the change in the catalytic subunit associated with its shift to the inhibited state. The changes in ilvN and ilvH on valine binding must be dissimilar, however, so that the effects of valine binding can not be transmitted effectively in the hybrid constructs. Further experimental data on the structures of the holoenzymes and on the thermodynamics of subunit associations and valine binding, as well as the behavior of mutant subunits, will be required in order to test this scheme and flesh out its details.

The function and properties of the C-terminal domain of ilvH remain an unsolved problem. The interactions between the very end of this domain and the N-terminal domain probably buttress the conformation of the latter, as point mutations in the last dozen or so residues at the C-terminus, in addition to the major truncations, lead to loss of the valine response (A. Slutzker et al., in preparation). The native ilvH subunit may constrict the conformational freedom of ilvI, so that the catalytic activity of the enzyme reconstituted from ilvI and the "unbuttressed" truncated ilvH is almost double that of the native AHAS III holoenzyme.

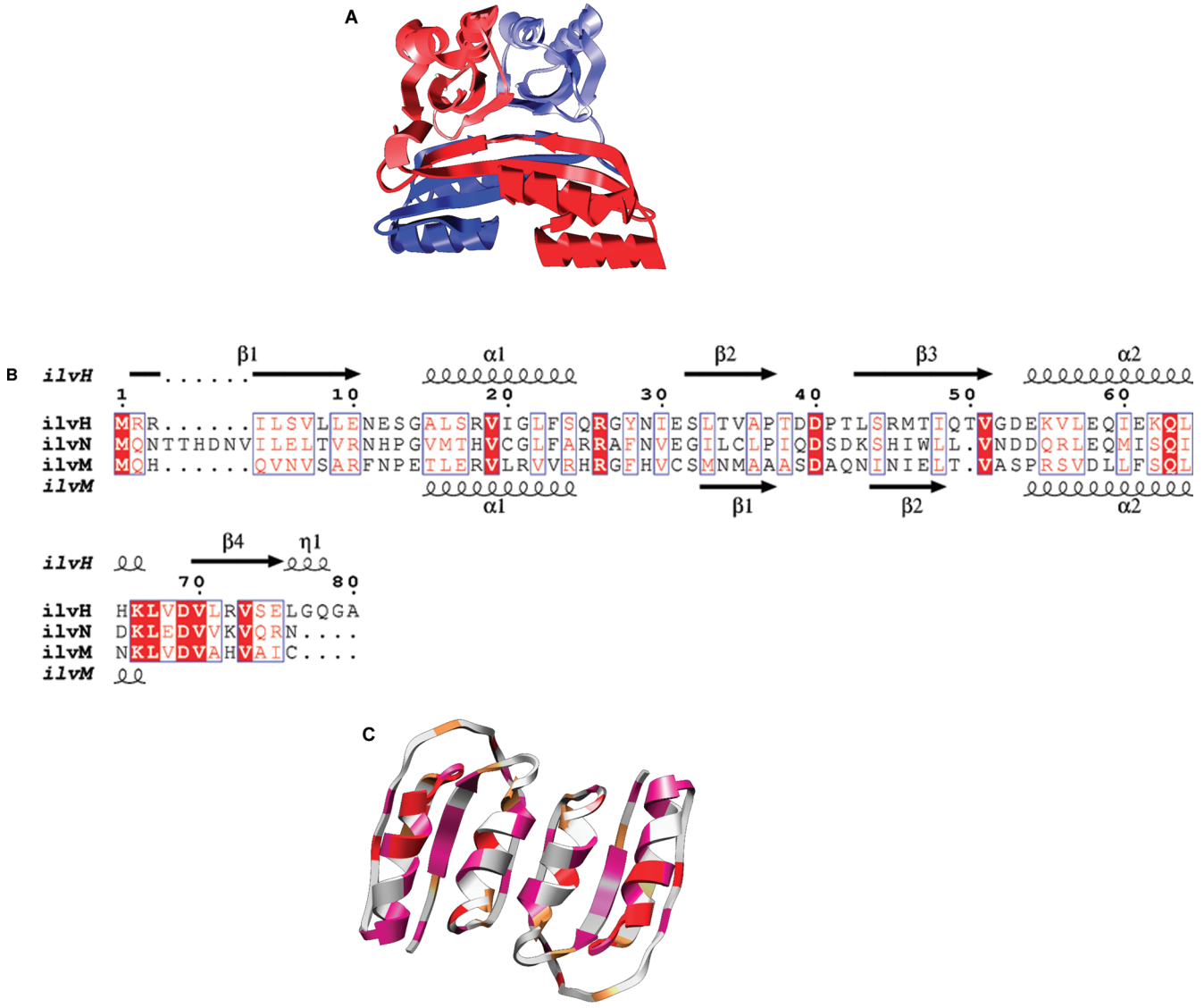

FIGURE 7: Possible structural relationship among AHAS small, regulatory subunits. (A) The structure of the ilvH dimer, as determined by X-ray crystallography (*27*). In this ribbon depiction of the secondary structure, one monomer is in red and the other in blue, with the N-terminal domains in bright colors and the *C*-termini in darker colors. Note the dimer structure is slightly asymmetric, probably due to asymmetric crystal contacts. (B) Multiple structure-based sequence alignment of the first 80 residues of ilvH, ilvN and ilvM from *E. coli*, prepared with ESPript (*46*) using default parameters. The secondary structure symbols in the first row and sequence numbers in the next refer to ilvH. (C) Suggested secondary structure of an ilvM dimer, based on its homology with the N-terminal domain of the ilvH dimer. The model was prepared using SWISS-MODEL (*35*, *36*). The ribbons are colored according to the degree of sequence conservation: Positions where all three SSUs have the identical residue are in red, positions with conservative homology are in dark rose, and positions of weak conservation where ilvM is most different are in sandy brown. Positions with no obvious sequence homology are in light gray.

The cross-activation of catalytic subunits by small subunits from other isozymes does not appear to be of high significance in vivo. In the presence of concentrations of valine sufficient to inhibit cell growth, ilvM expressed constitutively from a multicopy plasmid does not effect cell growth in the minimal medium (Supplementary Figure 1 in the Supporting Information). Apparently the levels of ilvM produced (Supplementary Figure 2 in the Supporting Information) are not sufficiently high to compete with the normal, genomically encoded valine-sensitive regulatory subunits. The only significant in vivo effect we observed was that of valine-resistant ilvH variant G14D, and to a lesser extent with ilvH-Δ86, which can apparently compete with the genomically encoded valine-sensitive SSUs of AHAS III, to allow growth in the minimal medium in the presence of valine. This phenomenon can be exploited in an in vivo selection scheme, for isolating random mutants of *ilvH* and *ilvN* (A. Slutzker et al., in preparation).

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SUPPORTING INFORMATION AVAILABLE

Growth curves illustrating the effects of various SSU constructs on the valine response and SDS−PAGE electrophoretograms of growing cells. This material is available free of charge via the Internet at http://pubs.acs.org.

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