JOURNAL OF BACTERIOLOGY, Jan. 2004, p. 570–574 Vol. 186, No. 2
0021-9193/04/$08.00+0 DOI: 10.1128/JB.186.2.570–574.2004

Cloning and Characterization of Acetohydroxyacid Synthase from *Bacillus stearothermophilus*

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Received 20 June 2003/Accepted 3 October 2003

Five genes from the *ilv-leu* operon from *Bacillus stearothermophilus* have been sequenced. Acetohydroxyacid synthase (AHAS) and its subunits were separately cloned, purified, and characterized. This thermophilic enzyme resembles AHAS III of *Escherichia coli*, and regulatory subunits of AHAS III complement the catalytic subunit of the AHAS of *B. stearothermophilus*, suggesting that AHAS III is functionally and evolutionally related to the single AHAS of gram-positive bacteria.

The first step common to the biosynthesis of branched-chain amino acids, catalyzed by acetohydroxyacid synthase (AHAS) (EC 4.1.3.18), is the condensation of pyruvate with either 2-ketobutyrate (the precursor of isoleucine) or pyruvate (the precursor of valine) (4, 26). Bacterial AHASs are composed of large (60-kDa) catalytic and small (9- to 18-kDa) regulatory subunits. Isolated catalytic subunits have lower activity than the holoenzymes but are similar to them in their cofactor dependence and specificity towards the two different substrates (10, 27, 28). The sensitivity of AHAS to feedback inhibition is completely dependent on the small subunit.

Many bacteria and archaea apparently contain a single AHAS enzyme. In most gram-positive bacteria, the genes for the first two enzymes in the pathway are located in the same operon (*ilvBNC*) (5, 9, 13, 15, 30), often together with the *leu* genes (*ilvBNC-leuACBD*) (17, 25, 30). The enterobacteria contain three isozymes of AHAS, encoded by distinct and differently regulated operons (3, 4).

To investigate the AHAS of *Bacillus stearothermophilus* ($AHAS_{Bst}$), we cloned the genes for this holoenzyme (*ilvBN*) and its large (*ilvB*) and small (*ilvN*) subunits to allow sequencing and overexpression. The screening for these genes was conducted with a genomic cosmid library for *B. stearothermophilus* ATCC 7954, created by H. Ewis (unpublished data), with a digitonin-labeled 1,100-bp probe that is highly conserved (50 to 75% amino acid identity) among AHASs (7) and only slightly conserved in other thiamine diphosphate (ThDP)-dependent enzymes, such as pyruvate oxidase (30%) and catabolic acetolactate synthase (25%) of *Bacillus subtilis*. This probe was amplified from the *B. stearothermophilus* ATCC 12980 genome by using two degenerate oligonucleotide primers: 5′(C/T/A)GGNACNGA(T/C)GCNTT(T/C)CA(A/G)GA and 5′ T(C/G)(C/T)TGCCA(C/T)(T/G)NACCAT.

The gene order in the insert of the AHAS-positive cosmid, as determined by coding analysis of its sequence (Fig. 1), seems similar to that of the *B. subtilis ilv-leu* operon (16, 30). The 5′ end of *ilvB* was absent in the cosmid-cloned fragment. This region was added to the clone, as shown in Fig. 1, from a PCR-amplified fragment obtained from the genome of *B. stearothermophilus* ATCC 7954 by using primers that were identical to the T-box element of the *ilv-leu* operon from *B. subtilis* (14) (GGGTGGTACCGCGG) and to a sequenced 3′ region of *ilvB* from *B. stearothermophilus* (GGCGGATTTGC CAATGGTTCGGC).

The DNA sequences of the *ilv-leu* operon of *B. stearothermophilus* (NCBI accession no. AY083837) and the deduced amino acid sequences of its encoded proteins show 67% and 68 to 74% identity, respectively, with those of *B. subtilis* for this region.

The purification of AHAS for biochemical characterization required the subcloning of its genes into the expression vector pT7-6 (Table 1), as illustrated in Fig. 1. In the purification of the holoenzyme [from *E. coli* HMS174(DE3)/pT7-6-ilvBN] and its separately expressed subunits [from HMS174(DE3)/pT7-6-ilvB or HMS174(DE3)/pT7-6-ilvN], we took advantage of the thermophilic properties of the enzyme and precipitated most of the mesophilic proteins of the host by heat denaturation in the first step of this process (Fig. 2; Table 2). After two further steps, we achieved more than 90% purity for the polypeptides (Fig. 2). The inferred molecular masses of the polypeptides encoded by the *ilvB* gene (63.3 kDa) and by *ilvN* (18.7 kDa) were confirmed. The N-terminal amino acid sequence of the putative large subunit (AKMNVEEQTKTKMSGSMM) also agrees with that deduced from the *ilvB* gene, when initiated from the fourth in-frame AUG triplet, after cleavage of the initial *N*-formylmethionine. The *ilvN* product showed no AHAS activity, as

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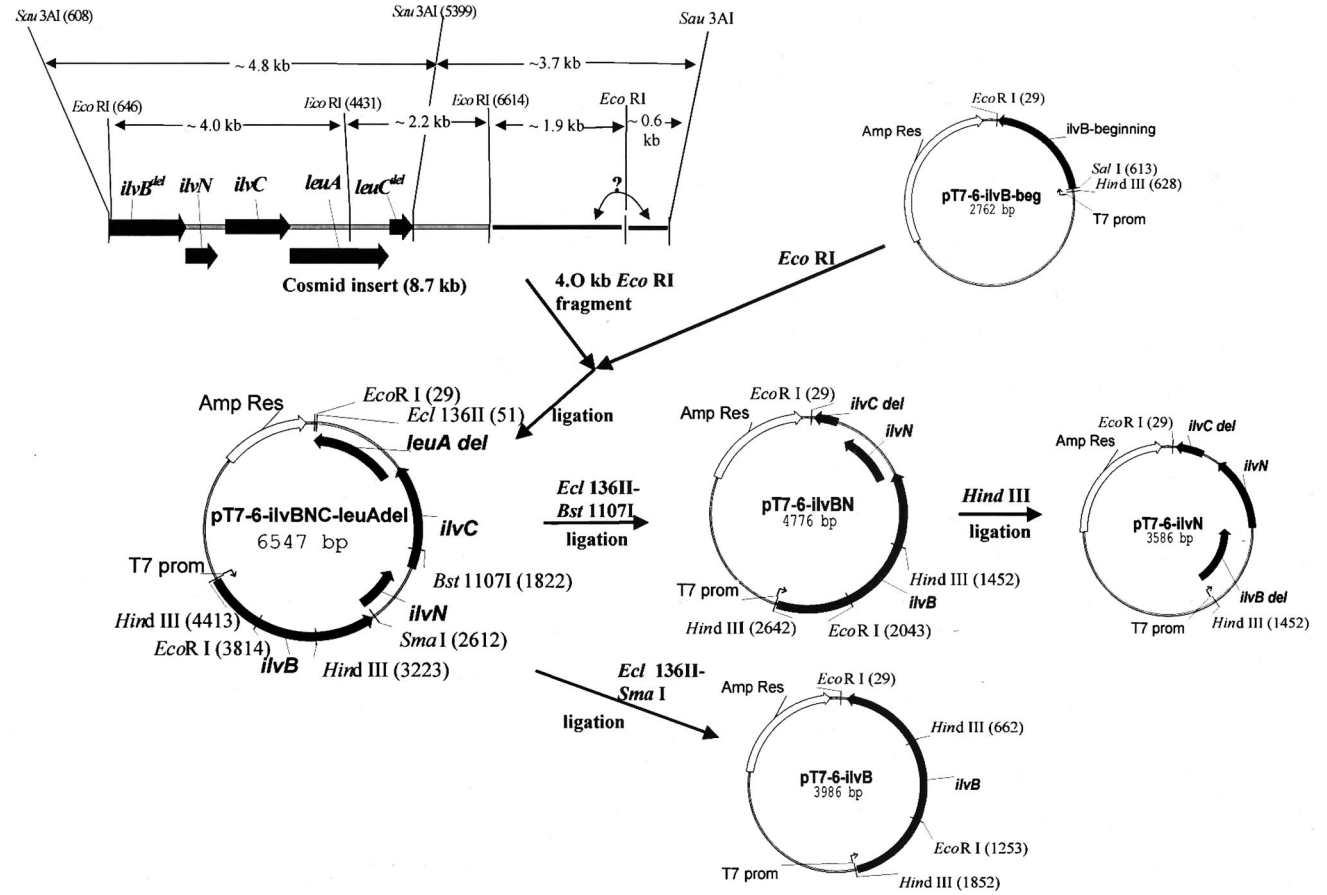

FIG. 1. Restriction map and schematic representation of the insert of the AHAS-positive cosmid (upper-left diagram) and construction of plasmids. Plasmid pT7-6-ilvB-beg was constructed by ligating the DNA fragment of the beginning of *ilvB* (see text) (which was initially introduced into the pGEM-T Easy plasmid, creating pGEM-T Easy-beg-ilvB-777) into the *Sal*I site of the pT7-6 expression vector. Plasmid pT7-6-ilvBNC-leuAdel was constructed by inserting the 4.0-kb *Eco*RI fragment, obtained from the cosmid containing the putative 3′ end of the *ilvB* gene, into the *Eco*RI site of pT7-6-ilvB-beg. The pT7-6-ilvBN plasmid was constructed by deleting a 1,771-bp *Ecl*136II-*Bst*1107I DNA fragment from the pT7-6-ilvBNC-leuAdel plasmid and by a self-ligation of the rest of the plasmid. The pT7-6-ilvB plasmid was constructed by deleting a 2,561-bp *Ecl*136 II-*Sma*I fragment from pT7-6-ilvBNC-leuAdel and by a self-ligation of the remaining plasmid. The pT7-6-ilvN plasmid was constructed by deleting a 1,190-bp *Hin*dIII fragment from pT7-6-ilvBN and by a self-ligation of the rest of the plasmid. Genes and their directions of transcription are marked by black arrows. Overlapping genes are shown below the line for clarity. The order of the last two *Eco*RI fragments in the cosmid insert was not determined.

TABLE 1. Bacterial strains and plasmids used in this work

Strain or plasmid	Purpose or description	Reference or source[b]
Strains		
B. stearothermophilus ATCC 12980	Used for preparation of *ilvB* probe	ATCC catalog no. 12980
B. stearothermophilus ATCC 7954	Used for preparation of genomic library and PCR amplification of 5′ end of *ilvB*	ATCC catalog no. 7954
E. coli HMS174(DE3)	Used for overexpression of cloned genes	Novagen (Madison, Wis.)
Plasmids		
pGEM-T Easy	Used for cloning of PCR products	pGEM-T Easy Vector Systems; Promega (Madison, Wis.)
pT7-6	Used for overexpression of cloned genes under T7 promoter	1
pUC57-clone 4	pUC57 that contains the 1,100-bp PCR fragment (partial putative *ilvB* gene from *B. stearothermophilus* ATCC 12980)	This work
pGEM-T Easy-beg-ilvB-777	pGEM-T Easy with 777-bp amplified fragment from 5′ end of *ilvB* gene of *B. stearothermophilus* ATCC 7954	This work
pT7-6-ilvB-beg	pT7-6 with 580 bp from 5′ end of *ilvB* gene[a] inserted between the *Sal*I and *Eco*RI sites	This work
pT7-6-ilvBNC-leuAde1	pT7-6 with *ilvB*, *ilvN*, *ilvC*, and half of *leuA*[a] inserted between the *Sal*I and *Eco*RI sites	This work
pT7-6-ilvBN	pT7-6 with *ilvB*, *ilvN*, and the beginning of *ilvC*[a] inserted between the *Sal*I and *Eco*RI sites	This work
pT7-6-ilvB	pT7-6 with *ilvB*[a] inserted between the *Sal*I and *Eco*RI sites	This work
pT7-6-ilvN	pT7-6 with 3′ end of *ilvB* and all of *ilvN*[a] inserted between the *Hin*dIII and *Eco*RI sites	This work

[a] The inserts are derived from *B. stearothermophilus* ATCC 7954.
[b] ATCC, American Type Culture Collection.

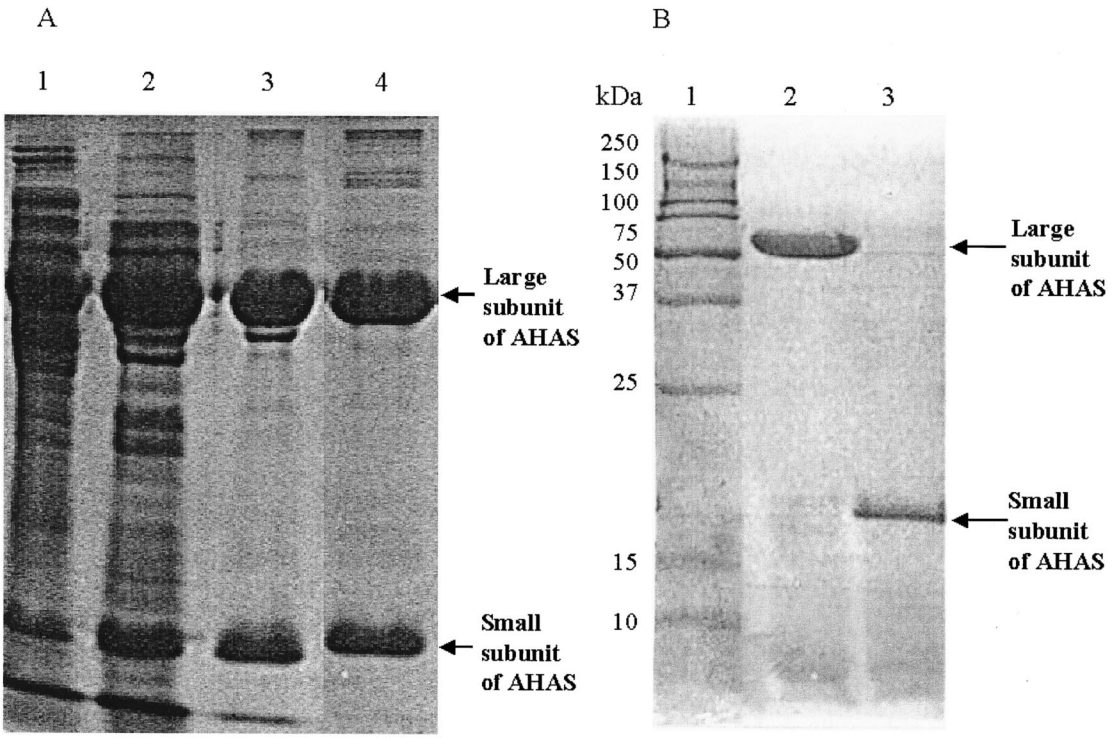

FIG. 2. SDS-PAGE of $AHAS_{Bst}$ and its subunits. (A) Results for the steps of purification of the holoenzyme (lanes 1 to 4) as summarized in Table 2. Forty micrograms of protein was loaded in each lane. (B) Purified large subunit (lane 2) and small subunit (lane 3), 10 μg each. Protein size markers are in lane 1.

expected for the regulatory subunit, but was capable of activating the large subunit.

$AHAS_{Bst}$ is the first thermophilic AHAS to be isolated and characterized. Its optimal temperature for activity is about 55°C. At 65°C, the enzyme loses its activity with a half time of 5 min, and at 70°C, it does so in less than a minute. Although none of the *Escherichia coli* AHAS isozymes is as thermostable as $AHAS_{Bst}$, the latter is less thermophilic (Table 3) than one might expect for an enzyme from an organism whose optimal growth temperature is 55°C. Interestingly, the optimal temperature for AHAS activity is nearly 65°C in crude extracts of *B. stearothermophilus* ATCC 12980 (21), suggesting that interactions with other factors in the cytosol might contribute to the thermostability of this enzyme.

The specific activity and substrate affinity of $AHAS_{Bst}$ under optimal conditions are quite similar to those of *E. coli* AHAS III (Table 3), except that the former shows substrate inhibition at high pyruvate concentrations (Fig. 3). The enzyme has a moderately high preference for ketobutyrate over pyruvate as the second substrate ($R = 22$). $AHAS_{Bst}$ is more sensitive to the inhibitor sulfometuron methyl than is *E. coli* AHAS III (Table 3) but is less sensitive than the AHAS II of *E. coli* or plant enzymes (22, 24).

The isolated large subunit has about one-third the activity of the holoenzyme, but it can be reconstituted to nearly complete activity by the addition of purified small subunits (Table 3; Fig. 4). It also shows a low apparent affinity for the enzyme's cofactors (ThDP, Mg^{2+}, and flavin adenine dinucleotide [FAD]).

TABLE 2. Purification of holoenzyme and large subunit of AHAS

Purification step	Holoenzyme[a]				Large subunit[b]			
	Protein[c] (mg)	Total activity[d] (μmol min^{-1})	Sp act (μmol mg^{-1} min^{-1})	Yield (%)	Protein (mg)	Total activity[d] (μmol min^{-1})	Sp act (μmol mg^{-1} min^{-1})	Yield (%)
1. Crude extract	4,030	4,071	1.01	100	2,313	579	0.25	100
2. Heat	618	2,934	4.75	72	1,427	757	0.53	130
3. Ammonium sulfate	175	1,426	8.17	35	1,150	702	0.61	121
4. Phenyl Toyopearl	63.4	581	9.16	14	136	424	3.12	73

[a] The preparation began with 33.1 g of wet cell paste.
[b] The preparation began with 14.4 g of wet cell paste.
[c] The amount of protein was determined by the dye-binding method (6) with bovine serum albumin as the standard.
[d] Enzyme assays were carried out under standard conditions (55°C, 10 mM pyruvate, 0.1 mM ThDP, 10 mM $MgCl_2$, 0.025 mM FAD, and 0.5 mM dithiothreitol in 0.1 M HEPES buffer at pH 7.9).

TABLE 3. Comparison of catalytic activity of $AHAS_{Bst}$ (holoenzyme, isolated large subunit, and reconstituted enzyme) to that of AHAS III from *E. coli*[a]

Enzyme	Sp act (μmol mg^{-1} min^{-1})	K_m for pyruvate (mM)	Substrate specificity (R)[b]	Valine inhibition K_i (μM) [max inhibition (%)]	Optimum pH	Sulfometuron methyl inhibition K_i (μM)	$K_{0.5}$ for ThDP (μM)	$K_{0.5}$ for $MgCl_2$ (μM)	Optimum temp (°C)
$AHAS_{BST}$									
Holoenzyme[c]	9.2	8.8 ± 1.2[d]	22 ± 2	18 (24)[e]	7.6	11.7	5.5 ± 0.8	20 ± 3	55
Large subunit[f]	3	12 ± 2	14 ± 2[g]	ND[h]	NT	NT	240 ± 40	1,300 ± 180	50
Reconstituted enzyme[i]	10–12	3.0 ± 0.7	13 ± 1	4 (40)[h]	NT	NT	3.7 ± 0.9	NT	55
AHAS III from *E. coli*	7.3[j]	6[k]	40[k]	5 (80)[l]	8.5[m]	310[n]	NR	NR	43[o]

[a] NT, not tested; NR, not reported; $K_{0.5}$, concentration of the added cofactor leading to 50% activity. K_ms, substrate specificities, and $K_{0.5}$s are given as means ± standard deviations.
[b] R is defined by $V_{AHB}/V_{AL} = R \times$ [2-ketobutyrate] / [pyruvate], where V_{AHB}/V_{AL} is the relative rate of formation of the two alternative products acetohydroxybutyrate and acetolactate in competition experiments.
[c] Experiments with the holoenzyme were carried out under standard conditions (see Table 2, footnote *d*).
[d] Pyruvate dependence of the holoenzyme was examined at 60°C in 0.1 M Tricine buffer, pH 8.0.
[e] Valine inhibition of the holoenzyme was examined at 60°C in 0.1 M Tricine buffer, pH 8.0, with 1 mM pyruvate.
[f] Unless otherwise stated, parameters for isolated large subunits were measured under conditions of 20 mM pyruvate, 0.2 mM ThDP, 2.5 mM $MgCl_2$, and 0.1 mM FAD.
[g] Substrate specificity (R) of the large subunit was determined at 50°C.
[h] Measured under standard conditions, except for the presence of 0.3 mM pyruvate and 1.25 mM $MgCl_2$. ND, not detected.
[i] Parameters for the reconstituted enzyme were measured under conditions of 20 mM pyruvate, 0.1 mM ThDP, 2.5 mM $MgCl_2$, and 0.1 mM FAD.
[j] Data from reference 2.
[k] Data from reference 11.
[l] Calculated with data from reference 19.
[m] Data from reference 12.
[n] Data from reference 22.
[o] Data from present study; determined with the crude extract of *E. coli* MF2000/pCV88 (18, 29) under standard conditions (23).

The feedback regulation of $AHAS_{Bst}$ by valine is dependent on the presence of the small subunits (Table 3). The inhibition at saturation with valine is incomplete (Table 3), as has been shown for other AHASs (19, 31), and depends on the substrate concentration; at 1.0 mM pyruvate, for instance, the inhibition was 24% with a valine concentration for half of this inhibition ($K_{0.5}$) of 4 μM, while at 10 mM pyruvate, there was no inhibition. We suggest that at physiological levels of pyruvate (0.1 to 0.5 mM), feedback inhibition by valine may play a significant role in modulating branched-chain amino acid biosynthesis. Leucine and isoleucine have only very small effects on the enzyme (data not shown).

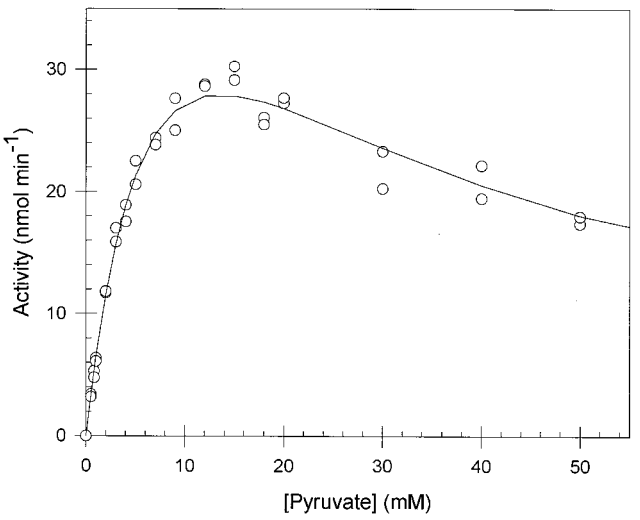

FIG. 3. Pyruvate dependence of AHAS holoenzyme. The reaction was carried out at 60°C in 0.1 M Tricine, pH 8.0, with 5 mg of purified enzyme ml^{-1} in the presence of 0.1 mM ThDP, 10 mM Mg^{2+}, and 0.025 mM FAD. Data were fitted to the empirical equation $V = (v_{max}[Pyr])/(K_m + [Pyr] + [Pyr]^2/K_2)$, where v is the measured reaction velocity, V_{max} is the maximum velocity at substrate saturation, and Pyr is pyruvate.

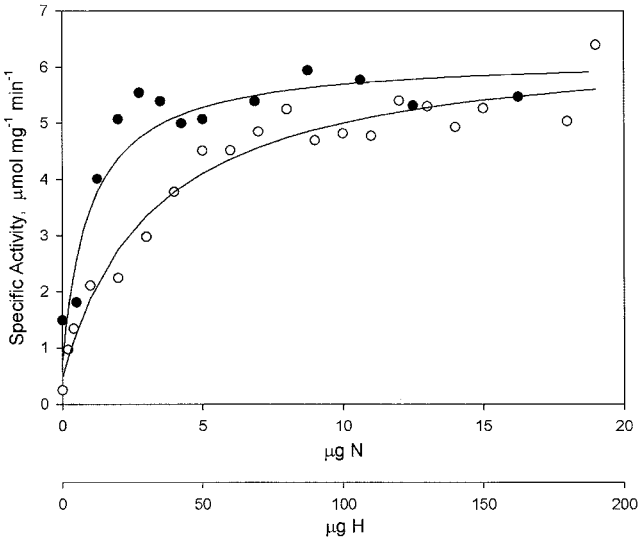

FIG. 4. Reconstitution titration of the purified AHAS large subunit with purified small subunits. Large subunits (4.2 μg/ml) were preincubated for 15 min at 55°C with varied amounts of purified small subunits of $AHAS_{Bst}$ (●) (upper abscissa; micrograms of N) in the standard reaction buffer. Note that the large and small subunits are calculated to be equimolar at 1.25 μg of small subunit. The reaction was initiated by the addition of pyruvate (20 mM), stopped after 20 min, and analyzed. In a parallel reaction, the large subunits were incubated at 40°C with purified small subunits of *E. coli* AHAS III (○) (lower abscissa, micrograms of H).

It is interesting that the purified small subunits of *E. coli* AHAS III can also activate the *B. stearothermophilus* large subunits, if the reaction is carried out at a temperature at which the AHAS III small subunits are stable (40°C). The concentration that is required for half activation (Fig. 4) is about four to five times higher than that required for the activation of the homologous subunit. The activity of this heterologous large- and small-subunit combination is sensitive to valine inhibition. In this case, at a pyruvate concentration of 0.3 mM, valine leads to 48% inhibition of activity at saturation, with an apparent $K_{0.5}$ of about 150 μM (data not shown). In contrast, there is no heterologous activation or conferral of valine sensitivity when regulatory and catalytic subunits from different *E. coli* isozymes (23, 27, 28) or from *E. coli* AHAS III and *Saccharomyces cerevisiae* (20; R. Duggleby, personal communication; M. Vyazmensky, unpublished results) are combined.

In conclusion, $AHAS_{Bst}$ is quite similar to isozyme III from *E. coli* (2, 11, 19, 23) (Table 3) and to AHASs from other gram-positive bacteria with a single AHAS (8, 15, 31). The evolutionary and functional connections between the single AHASs of gram-positive bacteria and isozyme III of *E. coli* can be seen in the sequences of the regulatory subunits, the tendencies of the regulatory subunits to dissociate, and the heterologous complementation described above. Another hint of such a relationship is the regulation of the expression of *E. coli ilvIH* by leucine and the encoding of a typical gram-positive AHAS by an *ilvBNC-leu* operon.

Nucleotide sequence accession number. The sequence data reported here have been deposited in the NCBI database under accession no. AY083837.

This research was supported in part by grant 93-00233 from the U.S.-Israel Binational Science Foundation and by seed grants from the Vice President for Research and Development of Ben-Gurion University.

I.P. thanks the students and technical staff who assisted her during the period she spent at Georgia State University, particularly Debby Walthall and Hosam Ewis.

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