Properties of Subcloned Subunits of Bacterial Acetohydroxy Acid Synthases

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The acetohydroxy acid synthase (AHAS) isozymes from enterobacteria are each composed of a large and small subunit in an $\alpha_2\beta_2$ structure. It has been generally accepted that the large (ca. 60-kDa) subunits are catalytic, while the small ones are regulatory. In order to further characterize the roles of the subunits as well as the nature and the specificities of their interactions, we have constructed plasmids encoding the large or small subunits of isozymes AHAS I and AHAS III, each with limited remnants of the other peptide. The catalytic properties of the large subunits have been characterized and compared with those of extracts containing the intact enzyme or of purified enzymes. Antisera to the isolated subunits have been used in Western blot (immunoblot) analyses for qualitative and semiquantitative determinations of the presence of the polypeptides in extracts. The large subunits of AHAS isozymes I and III have lower activities than the intact enzymes: $V_{\max}/K_m$ is 20 to 50 times lower in both cases. However, for AHAS I, most of this difference is due to the raised K_m of the large subunit alone, while for AHAS III, it is due to a lowered $V_{\max}$. The substrate specificities, R , of large subunits are close to those of the intact enzymes. The catalytic activity of the large subunits of AHAS I is dependent on flavin adenine dinucleotide (FAD), as is that of the intact enzyme, although the apparent affinities of the large subunits alone for FAD are 10-fold lower. Isolated subunits are insensitive to valine inhibition. Nearly all of the properties of the intact AHAS isozyme I or III can be reconstituted by mixing extracts containing the respective large and small subunits. The mixing of subunits from different enzymes does not lead to activation of the large subunits. It is concluded that the catalytic machinery of these AHAS isozymes is entirely contained within the large subunits. The small subunits are required, however, for specific stabilization of an active conformation of the large subunits as well as for valine sensitivity.

The enteric bacteria encode at least three isozymes of acetohydroxy acid synthase (AHAS; EC 4.1.3.18), the enzyme catalyzing the first common step in the biosynthesis of the branched-chain amino acids (28, 29). The isozymes differ in kinetic properties and physiological functions (3, 6-8, 10, 11, 17, 25, 27, 29) but have similar structures. Each is composed of two kinds of polypeptides, with molecular masses of about 60 and 9 to 17 kDa (2, 13, 24), encoded on the same operon, with the large polypeptide proximal to the promoter. It is generally accepted that the ca. 60-kDa polypeptides, which share considerable sequence similarity (25, 30, 32), are the catalytic subunits, while the far more heterogeneous smaller polypeptides are involved in feedback control of the enzymes by valine (9, 14, 20, 26). These assumptions are largely based on genetic manipulation of the bacterial AHASs: deletions in or truncation of the small subunit gene which leave the large subunit intact result in a lowered AHAS activity which is feedback resistant (14, 20, 26). Deletions in the large subunit gene, on the other hand, result in complete loss of the enzyme's activity (14, 20, 26). For AHAS II or III, in vitro combination of extracts containing the intact large and small subunits has been shown to lead to reconstitution of enzymic activity similar to that of the original enzyme (20, 21, 26).

However, the intact large subunits alone which have been studied so far are each encoded by constructs retaining a sizable fragment of the N-terminal portion of the small subunit (40, 23, and 80% of the small subunit in the cases of AHAS I, II, and III, respectively [14, 20, 26]). Our preliminary studies of the enzymic properties of a crude extract of

bacteria carrying the plasmid pCV7 (26) suggested to us that the truncated small subunit of AHAS III encoded on this construct might still interact with the large subunit. To examine this possibility and to further study the properties of the large subunits, we have constructed plasmids encoding the large or small subunits of AHAS I (the *ilvB* or *ilvN* polypeptides) and AHAS III (the *ilvI* or *ilvH* polypeptides), each with very limited remnants of the other peptide. The enzymic properties of the large subunits suggest that all of the catalytic machinery of these enzymes is located in the large subunits. We have also quantitatively studied the reconstitution of AHAS I and III from their subunits and investigated whether subunits from the two different isozymes can complement one another.

MATERIALS AND METHODS

The microorganisms and plasmids used in this study and their sources are listed in Tables 1 and 2, respectively. The minimal medium for *Escherichia coli* strains was that of Vogel and Bonner (31), which contained 0.5% glucose and was supplemented with vitamin B₁ (5 μ g/ml) and the appropriate amino acids when required (50 μ g/ml each). Luria broth was used as the rich medium. Tetracycline (15 μ g/ml) and/or ampicillin (50 μ g/ml) was added when required.

Cloning and transformation procedures. Cleavage with restriction enzymes and ligations were carried out in accordance with the suppliers' recommendations. The alkaline lysis plasmid preparation method, restriction mapping, and transformations with plasmids were carried out according to the methods of Sambrook et al. (23) with slight modifications. Transformants were selected for the relevant antibiotic resistance.

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TABLE 1. Microorganisms used

Strain	Genotype	Relevant phenotype	Source	Reference
MF2000	<i>ilvB800::Mu-1 bgl-32 ilv-115 thi-1 argE3 rps L31</i> <i>Δ(ara leu ilvIH)863 mtl-1 xyl-5 lacY1 recA1</i>	IlvIH ⁻ IlvB ⁻	N. Yadav	34
CU1246	<i>ilvBN2102 ilvIH2202 ara thi rbs-221 srl::Tn10</i> <i>Δ(pro-lac) recA56</i>	IlvIH ⁻ IlvBN ⁻	H. E. Umbarger	20
DR103	F ⁻ <i>thr-1 ara-14 leu-1 aziR tonA gal-12 mal Str^r</i> <i>minA minB thi recA</i>	MinA ⁻ MinB ⁻	G. Cohen	12

The expression of polypeptides encoded on the plasmids was examined by the minicell method of Dougan and Kehoe (12), using minicell-forming strain DR103.

Enzymes. The purified isozyme AHAS I from *E. coli* (1) was a gift of J. V. Schloss. AHAS III was purified as described elsewhere (2). Crude enzyme extracts were prepared as previously described (16) from exponentially growing cultures of *E. coli* in a minimal medium containing glucose that were harvested at a turbidity of about 50 Klett units (filter no. 66). Cells were washed, concentrated 100-fold (to approximately 10¹⁰ bacteria per ml) in a disruption buffer (0.1 M potassium phosphate buffer [pH 7.6] containing 0.1 mM flavin adenine dinucleotide [FAD], 0.5 mM dithiothreitol, 10 mM EDTA, and 20% glycerol [vol/vol]), and lysed by ultrasonic disruption at 0°C for 1/2-min periods with 1/2-min interruptions over 10 min. Unbroken cells and debris were removed by centrifugation. The AHAS activities of extracts prepared from host strain MF2000 were negligible.

Protein concentration was determined by the dye binding method (5). Polypeptides in the lysates were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (23) on 10 to 20% gradient gels. Antisera against the subunits from each of the three isozymes were prepared in collaboration with John V. Schloss. The individual polypeptides were isolated by preparative SDS-PAGE of the purified enzymes (1, 2, 24). These were then provided to Hazleton Biotechnologies Co. (Denver, Pa.), which immunized rabbits with the polypeptides and prepared antisera. After adsorption of nonspecific antibodies with an extract of the background strain MF2000/pBR322, the antisera were used for identification of polypeptides on electrophoretic gels by immunoblotting with protein A-alkaline phosphatase conjugate together with 5-bromo-4-chloro-3-indolylphosphatet-Nitro Blue Tetrazolium for staining (23).

Enzyme activity measurements. The enzyme was assayed by determining the rate of acetolactate (AL) formation in the presence of 40 mM pyruvate in 0.1 M potassium phosphate buffer (pH 7.6) containing 0.025 mM FAD, 0.1 mM thiamine pyrophosphate, and 10 mM MgCl₂. Cell extracts (2 to 100 μl)

were diluted to 0.5 ml, and the reaction was started by the immediate addition of 0.5 ml of the concentrated reaction mixture at 37°C.

The amount of AL was determined by the method of Westerfeld (33), using acetoin as standard. Specific activity is expressed as nanomoles of product formed per minute per milligram of protein. The simultaneous synthesis of both AL and acetohydroxybutyrate in the presence of pyruvate and 2-ketobutyrate was studied by using the gas-liquid chromatography method of Gollop et al. (15).

RESULTS

Figure 1 shows the dependence of the specific activity of an extract from cells carrying plasmid pCV7 (26) on the concentration of the extract. This plasmid encodes the large *ilvI* subunit of AHAS III, together with a small *ilvH* subunit which is truncated by 30 C-terminal amino acids. The dependence of the activity of the pCV7 extract on concentration makes quantitative comparisons between the activities of this construct and the intact enzyme inappropriate. This behavior is apparently due to properties of the protein encoded by pCV7 and not to other components of the bacterial extract (data not shown) and suggests the possibility that there is a concentration-dependent interaction between the intact large subunit and the remnants of the small subunit. In order to avoid such effects, we chose to subclone each of the subunits of AHAS I and III. Figure 1 shows for comparison the behavior of an extract of cells carrying the plasmid pOI1, which encodes *ilvI* alone. The construction of this plasmid is described below. The lack of dependence of specific activity on concentration for this construct supports the suggestion that the truncated small peptide encoded by pCV7 can affect the activity of the large subunit and justifies our efforts.

Subcloning of individual subunits. Figure 2A outlines the procedure used to carry out the subcloning of *ilvI* alone from plasmid pCV88, which contains a complete *ilvIH* operon with a mutated enhanced promoter, *ilv-752* (18). The subcloning required several steps, as there are no restriction

TABLE 2. Plasmids used

Plasmid	Genotype	Source	Reference
pBR322	Amp ^r Tet ^r		4
pTB	Amp ^r <i>ilvBN</i>	N. Yadav	Personal communication
pTCN12	Amp ^r Tet ^r <i>ilvBN</i>		22
pCV7	Amp ^r <i>ilvI</i> <i>ΔilvH</i>	M. De Felice	26
pCV88	Amp ^r <i>ilv-752 ilvIH</i>	J. M. Calvo	19
pOI1	Amp ^r <i>ilv-752 ilvI</i>	This work	
pOH1	Amp ^r <i>ilv-752 ilvH ΔilvI</i>	This work	
pOB1	Amp ^r <i>ilvB ΔilvN</i>	This work	
pON1	Amp ^r Tet ^r <i>ilvN ΔilvB</i>	This work

FIG. 1. Dependence of specific activities of two constructs on the amount of extract used in an assay. Extracts of cells carrying plasmid pCV7 (open squares) or pOI1 (filled squares) were prepared and assayed for AHAS activity as described in the text, with different amounts of extract added to 1-ml assay mixtures. The apparent specific activity is expressed in nanomoles of AL formed per minute per milligram of protein.

sites in the 26-bp noncoding region between the end of *ilvI* and the start codon of *ilvH* and no unique sites near the 5' end of *ilvH*. The resulting plasmid, pOI1, contains the entire *ilvI* gene with its *ilv-752* promoter.

The construction of plasmids containing the genes *ilvH*, *ilvB*, *ilvN*, and *ilvM* was more straightforward. Plasmid pOH1 (Fig. 2B) contains 280 bp of *ilvI* between the *ilv-752* promoter and the intact *ilvH* gene. Only 42 amino acids of the *ilvI* polypeptide should be coded by this plasmid, since the cleavage and ligation lead to a frameshift in the gene fragment.

The genes coding for the two polypeptides of AHAS isozyme I were subcloned from different plasmids containing the *ilvBN* operon because of the location of convenient restriction sites on these plasmids. Plasmid pOB1 (Fig. 2C) bears the intact *ilvB* (large-subunit) gene behind its native promoter and encodes only the first 10 amino acids of the small subunit. Plasmid pON1 (Fig. 2D) contains the *ilvBN* promoter, 500 bp of *ilvB*, and the intact small subunit *ilvN*. If the filling-in reaction was complete, the *ilvB* gene would remain in frame, so that a peptide consisting of the 8 N-terminal and about 150 C-terminal amino acid residues of the *ilvB* polypeptide would be encoded.

The structures of all of these plasmids were verified by restriction analyses with a number of enzymes. The plasmids which encode the AHAS isozymes or their subunits were also transformed into minicell-producing strain DR103, and expression of the polypeptides in the minicells was examined (12, 19, 22). Each of the new plasmids led to expression of the expected intact subunit polypeptide only, with no other peptides detectable except the background products of the plasmid.

Expression of the plasmids in exponential cultures of an AHAS⁻ strain (MF2000) was also examined by Western blotting (immunoblotting), using antisera to the subunits. Each of these antisera was specific, so that there was no significant cross-reactivity when they were challenged on immunoblots with the three purified enterobacterial enzymes. The plasmids were each transformed into MF2000, and extracts of these cells were analyzed by SDS-PAGE

followed by immunoblotting and detection with the specific antisera. Each of the polypeptides from isozymes I and III was expressed as expected from the plasmids.

Activity of subcloned subunits of AHAS I and III and reconstitution of the enzymes. The activities of AHAS I or III large subunits alone (in extracts of MF2000/pOB1 and MF2000/pOI1) were examined and compared with those of extracts from cells expressing both subunits of the enzyme from related plasmids. In each case, we expected that the amount of the large subunit present in extracts of cells expressing the large polypeptide alone would be comparable to that in cells expressing the entire operon because of the similarity of the plasmid vector and promoter, the host strain, and the growth conditions. This expectation was supported by semiquantitative analysis of Western blots of the extracts. In contrast, the catalytic activity of the extract containing the large subunit of AHAS I was approximately 11% that of the complete enzyme, while the activity of the large subunit of AHAS III was only 3% that of the respective complete enzyme (Table 3).

The activities of the large subunits alone were also reflected *in vivo*. Cells expressing only the large subunit of AHAS isozyme I or III grew on a minimal medium without added branched-chain amino acids, although they grew more slowly than the strains expressing both subunits. As expected if small subunits are required for feedback regulation, these strains were valine resistant.

The activities of the large subunits were strongly enhanced *in vitro* complementation with extracts containing the appropriate small subunits (Fig. 3). The activity obtained upon complementation is linear with time, after a slight lag period (half-life = 1 to 1.5 min) which is independent of the time the two extracts were preincubated together. For AHAS III, complementation with the small subunits raises the calculated specific activity of the large subunits to that of the extract containing the intact enzyme (Table 3). For AHAS I, the activity obtained upon complementation of the large subunit with a saturating amount of the small subunit was still about 2.5-fold lower than that of an extract from cells expressing intact enzyme (Table 3). As expected, extracts containing only a small subunit of isozyme I or III showed no detectable AHAS activity by themselves (<1 nmol of AL formed [mg of protein]⁻¹ min⁻¹ under standard conditions).

Western blots allowed an estimate of the molar amounts of the AHAS III polypeptides in the extracts, with purified enzyme (2) serving as a standard (data not shown). The MF2000/pOH1 extract contained the small subunit at a level (in nanomoles per milligram of protein) about three- to fivefold higher than that of the large subunit in the MF2000/pOI1 extract. The reconstitution of the activity of AHAS III shown in Fig. 3 thus requires a 5- to 10-fold stoichiometric excess of small subunits to reach a plateau.

Comparison of the enzymatic properties of the large subunits and "complete" enzymes. The properties of the large subunits were compared with those of the holoenzymes by determining several enzymatic parameters for extracts containing large subunits and for presumably "complete" enzymes (Table 3). The apparent affinity for pyruvate, K_m , was determined under standard conditions in the absence of 2-ketobutyrate. K_m for the large subunits of AHAS I alone is nearly 1 order of magnitude larger than for the complete enzyme. The purified enzyme, cell extracts containing the complete enzyme, and enzyme reconstituted by complementation all have similar K_m s (Table 3). The difference between the K_m s of the large subunit alone and the complete enzyme

FIG. 2. Schemes for the subcloning of AHAS subunits. (A) Construction of plasmid pOI1, with the subcloned *ilvI* gene. Plasmid pCV88 was cleaved with *Bam*HI and *Hind*III to yield a 570-bp fragment which was cleaved again with *Hpa*II. The resulting 188-bp *Hpa*II-*Bam*HI fragment 1 contained the 3' end of *ilvI* and the five initial base pairs of *ilvH*. It was ligated into pBR322, which had been cleaved with *Bam*HI and *Cla*I. The new plasmid, pHF1, was amplified and then cleaved with *Pvu*II and *Bam*HI, and its major fragment (2,500 bp) was ligated with the 2,400-bp *Pvu*II-*Bam*HI fragment 2 from pCV88, which contains most of *ilvI* except the 3' end. (B) Plasmid pOH1, containing *ilvH*, was constructed from pCV88 by cleavage with *Bam*HI and *Bcl*II, isolation of the 4,700-bp fragment, and ligation. (C) Plasmid pOB1, containing *ilvB*, was constructed by cleavage of plasmid pTB with *Sac*I and ligation of the isolated major (4,900-bp) fragment. (D) Plasmid pON1, containing *ilvN*, was constructed by cleavage of pTCN12 (22) with *Mlu*I and *Hind*III, filling in of the sticky ends of the isolated 6,550-bp DNA fragment with Klenow fragment, and self-ligation.

TABLE 3. Enzymatic properties of large subunits and complete enzymes^a

Enzyme source(s) ^b	Composition ^c	Sp act ^d	K_m for pyruvate (mM) ^e	Specificity (R) ^f	% Inhibition by valine ^g	K_m for FAD (mM)
MF2000/pOI1	AHAS III LSU	54	17	33	0	
MF2000/pOI1 + xs MF2000/pOH1	AHAS III LSU + xs SSU	2,000	6	54	79	
MF2000/pCV88	AHAS III extract	1,860	9	40	85	
Purified enzyme	AHAS III	8,000	6	40	80	
MF2000/pOB1	AHAS I LSU	120	25	3.9	0	0.76
MF2000/pOB1 + xs MF2000/pON1	AHAS I LSU + xs SSU	400	3.7	1.5	87	
MF2000/pTB	AHAS I extract	1,100	2.6	2.1	87	0.062
Purified enzyme	AHAS I	30,000	1.5	2	90	

^a Experiments were carried out under standard conditions at pH 7.6 in 0.1 M phosphate buffer containing cofactors, as described in Materials and Methods.

^b Extracts containing 3 to 6 μ g of protein per ml were used in all experiments. Where excess (+ xs) small subunits were added, the quantity of the extract in question was shown to be saturating (i.e., at least 10-fold the quantity giving 50% of the complementation; Fig. 3 and 4), and the extracts were preincubated together for 10 min before the reaction was started.

^c LSU, large subunits; SSU, small subunits.

^d Nanomoles per minute per milligram of protein for the formation of AL in the presence of a saturating concentration of pyruvate. Where extracts containing small subunits were added, the specific activity was calculated on the basis of the protein contributed by the large subunit extract only.

^e In the absence of 2-ketobutyrate. Determined by nonlinear least-squares fit of data to the Michaelis-Menten equation.

^f Specificity ratio, R, for the two physiological reactions of AHAS, defined by $V_{\text{AHB}}/V_{\text{AL}} = R \times [2\text{-ketobutyrate}]/[\text{pyruvate}]$, where V is the rate of formation of the given product as determined by analysis of the dependence of the two rates on the concentration of 2-ketobutyrate in the presence of a constant pyruvate concentration, as described in reference 15.

^g Inhibition by 1 mM valine of the formation of AL in the presence of 40 mM pyruvate.

is much less significant for AHAS III than it is for isozyme I (Table 3).

Another important parameter of AHAS activity is the catalytic specificity of the enzyme for formation of the two alternative products, AL and acetohydroxybutyrate (AHB), in the presence of pyruvate and 2-ketobutyrate (3, 15). This can be defined as a specificity ratio, R, where $(V_{\text{AHB}}/V_{\text{AL}}) = R \times [2\text{-ketobutyrate}]/[\text{pyruvate}]$ (V is the rate of formation of a product). For either isozyme, the specificity of the large subunits alone is only slightly different from that of the complete or reconstituted enzyme (Table 3).

The large subunits alone are almost completely insensitive

to inhibition by valine, but valine sensitivity is completely reconstituted by complementation with the respective small subunits (Table 3).

When extracts were prepared from washed MF2000/pOB1 cells by sonic disruption in a buffer devoid of FAD, the activity of AHAS I large subunits alone was completely dependent on added FAD in the reaction mixture. Under the same conditions, the residual activity of an extract containing the complete enzyme was 10% of the activity obtained on saturation with FAD. The apparent K_m for FAD of the large subunit alone is 1 order of magnitude higher than that of the complete enzyme (Fig. 4 and Table 3). Such experiments were not carried out for AHAS III, since the tightly bound FAD can be removed from this isozyme only by rather drastic treatment (2).

FIG. 3. Complementation of the AHAS activities of large subunit polypeptides with small subunit polypeptides. Standard assays of AHAS activity were carried out in 1-ml reaction mixtures. *ilvB* polypeptide (AHAS I large-subunit polypeptide) was complemented with *ilvN* (small-subunit polypeptide) (Δ). Assays were carried out with 17.5 μ g of protein from an extract of MF2000/pOB1 (5 μ l of an extract with 3.5 mg of protein per ml) and various quantities of an extract of MF2000/pON1. Maximal activity was 7.2 nmol min⁻¹. *ilvI* polypeptide (AHAS III large-subunit polypeptide) was complemented with *ilvH* (small-subunit polypeptide) ($\square$). Assays were carried out with 22.5 μ g of protein from an extract of MF2000/pOI1 (5 μ l of an extract with 4.5 mg of protein per ml) and various quantities of an extract of MF2000/pOH1. Maximal activity was 35 nmol min⁻¹.

FIG. 4. FAD dependence of the activity of AHAS I or its large subunit after preparation of the extracts in the absence of FAD. Extracts containing the AHAS I large subunits alone (from MF2000/pOB1; $\bullet$) or the complete enzyme (from MF2000/pTB; $\square$) were prepared by ultrasonic lysis of the cells in a buffer devoid of FAD, and their activities were measured under standard conditions except for the presence of various concentrations of FAD. The reaction mixtures with large subunits and with complete enzyme contained about 72 and 6.3 μ g protein, respectively.

FIG. 5. Attempted complementation of the AHAS activities of large subunits with heterologous small subunits. Activities of large subunits from AHAS I (17.5 μ g of protein from MF2000/pOB1 extract) (A) and AHAS III (26.5 μ g of protein from MF2000/pOI1) (B) supplemented with 0 (open bars), 0.6 mg (light hatched bars), or 1.8 mg (dark hatched bars) of protein from extracts of the indicated strains are shown.

Heterologous complementation. We considered the possibility that, given the homology among the large subunits of the AHAS isozymes, small subunits might be able to interact with large subunits from different isozymes. We carried out experiments in which an extract containing the *ilvH* product was added to the large *ilvB* polypeptide of AHAS I (Fig. 5A) or an extract containing the *ilvN* product was added to the large *ilvI* polypeptide of AHAS III (Fig. 5B) in quantities nearly 1 order of magnitude greater than those required to saturate the complementation of the large peptide from the same isozyme. The effect of these heterologous additions on activity was no more significant than the very slight effect of addition of an extract from the untransformed host strain (Fig. 5). The heterologous subunits did not confer any valine sensitivity, either (data not shown).

DISCUSSION

Each of the plasmids we have constructed here bears a gene encoding a separate large or small polypeptide of one of the enterobacterial AHAS isozymes and only a minimal fragment of the gene for the peptide which normally complements it. Plasmids with deletions in the gene for one or the other of the polypeptides of each of the AHAS isozymes have been constructed previously (14, 20, 21, 26), and reconstitution of isozymes II and III from their subunits has been reported (20, 21). However, we chose to carry out somewhat more elaborate constructions before proceeding with the characterization of the properties of the subunits because of the possibility that truncated or chimeric polypeptides might still be capable of associating with their erstwhile partners and thus lead to complications. Indeed, the behavior of the products of plasmid pCV7, which encodes about two-thirds of the AHAS III small subunit

together with the intact large subunit (18, 21, 26), seems to show just such associations (Fig. 1), which affect the specific activity of the protein. In our constructs, the *ilvI* polypeptide is encoded on pOI1 with no trace of *ilvH*, the plasmids pOH1 and pOB1 encode less than 10% of the deleted polypeptides, and pON1 encodes the *ilvN* polypeptide and only 28% of *ilvB*. In each of these cases, no peptide larger than about 8 kDa could be detected on Western blot analysis with antisera to the deleted peptide. The specific activity of the extract from a cell carrying pOI1, in contrast to one carrying pCV7, is independent of protein concentration.

For AHAS I and III, each of the cleanly subcloned large subunits has catalytic activity, but the specific activities of extracts from cells expressing only a large subunit are considerably lower than those from cells expressing a complete enzyme (Table 3), despite the close similarity of the plasmids from which they are expressed. That the addition of an excess of small subunits to the extract from cells expressing only the AHAS III large subunit results in an activity essentially equivalent to that of the extract from cells bearing the parent plasmid pCV88 indicates that the large subunit is present in the pOH1 extract in an undiminished quantity. Western blot analysis of the extracts confirms this. On the other hand, the extract from cells expressing only the large subunit of AHAS I could be reconstituted to only 40% of the activity observed with cells expressing the complete enzyme (the first report of such a reconstitution). This suggests that in addition to having a lowered intrinsic activity, the large *ilvB* polypeptide might also be present in the extract in somewhat diminished quantity in the absence of small subunits.

We have characterized and compared the properties of the intact enzyme, the reconstituted enzyme, and the large subunit alone (Table 3) for both AHAS I and III. Within the limits of the techniques that can be applied to crude bacterial extracts, the properties of the reconstituted enzyme are indistinguishable from those of the enzyme in an extract from cells expressing both subunits or, indeed, from those of the purified enzyme. The rapidity and near completeness of the reconstitution suggest that the reconstitution process does not involve any major refolding of the polypeptides. More important, the facts that a large subunit alone has significant activity, shows substrate specificity very close to that of the intact enzyme, and remains FAD dependent (at least in the case of AHAS I) indicate that the catalytic machinery of these enzymes must lie entirely within the large subunits.

The small subunits of AHAS I and III are absolutely required for sensitivity of the enzyme activity to the feedback inhibitor valine and thus can be considered "regulatory" subunits. However, unlike most enzymes in which separate catalytic and regulatory subunits can be recognized, the small subunits also play an important role in enhancing the activity of the large subunits. For both of the enzymes, the parameter (V_{max}/K_m) is 20 to 50 times greater in the presence of the small subunit than it is in its absence, although the effect in AHAS I is largely on K_m and in AHAS III is largely on V_{max} . Thus, the small subunit specifically induces a catalytically competent conformation of the enzyme which interacts with and stabilizes the transition state. The small subunit of AHAS I also raises the apparent affinity of the large subunit for FAD, presumably by stabilization of specific conformation(s) of the protein.

Despite the functional similarities between the large subunits of AHAS I and AHAS III and the similarity in their amino acid sequences (more than 40% identity), their interactions with their small subunits are quite specific. In our attempts to reconstitute hybrid enzymes, no evidence for an

effect of the "wrong" small subunit on the large subunit could be detected, so that any possible interaction between heterologous subunits must be 2 orders of magnitude weaker than the interactions between complementary polypeptides. Since the different enterobacterial enzymes play different physiological roles (3, 7), this specificity is important.

Further characterization of the properties of the subunits and the interactions among them will require isolation and purification of these polypeptides in native form; the plasmids we have described here should make such a purification far simpler.

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