Isolation and Characterization of Subunits of Acetohydroxy Acid Synthase Isozyme III and Reconstitution of the Holoenzyme†

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ABSTRACT: The separately cloned large and small subunits of AHAS isozyme III from *Escherichia coli* have been isolated and purified. The essentially pure small subunit (17 kDa *ilvH* product) was obtained by a procedure exploiting its low solubility. The large, catalytic subunit (62 kDa *ilvI* product) was isolated by standard techniques, to ≥95% purity. The large subunit has low catalytic activity relative to holoenzyme (∼5%) but shows similar substrate specificity and qualitatively similar cofactor dependence and inhibition by a sulfonylurea herbicide. Its activity is insensitive to valine, and the protein does not bind valine. The small subunit binds valine with $K_d = 0.2$ mM. Reconstitution of the holoenzyme from its subunits leads to a complex with the properties of the native protein, including valine inhibition of activity with $K_i = 12$ μM. Reconstitution titrations confirm the 1:1 stoichiometry of subunit assembly and a tendency to dissociation (about 50% dissociation near 0.1 μM subunit). Size exclusion HPLC indicates that either subunit alone is largely monomeric, and that assembly of the holoenzyme (two large + two small subunits, 150−160 kDa) requires FAD. On the basis of its homology with pyruvate oxidase and pyruvate decarboxylase, we suggest that the active sites of AHAS III are located at the interface of a dimer of catalytic subunits. Our experiments suggest that such a dimer is not stable except in the presence of the small subunits. The association of valine with sites on the regulatory subunits presumably influences the active sites by an allosteric conformational effect.

Acetohydroxy acid synthases (AHAS,[1] EC 4.1.3.18) are biosynthetic enzymes which catalyze thiamin pyrophosphate (TPP) dependent decarboxylation of pyruvate and condensation of the "active acetaldehyde" thus formed with a second ketoacid, to produce acetohydroxy acids. AHAS isozyme III from *Escherichia coli*, like other bacterial AHASs, is composed of two kinds of subunits: a large, catalytic subunit and a small, "regulatory" subunit (DeFelice et al., 1974; Squires et al., 1981, 1983), in an equimolar ratio (Barak et al., 1988). The large subunit of AHAS III, encoded by the *ilvI* gene, has a molecular mass of 62 kDa, while the small subunit encoded by *ilvH* has a mass of 17 kDa. The enzymatic properties of crude extracts from bacteria expressing only *ilvI* suggest that the large subunit contains all the catalytic machinery of the enzyme (Weinstock et al., 1992). However, such an extract does not show any of the normal feedback inhibition of AHAS III activity by valine and has low total activity. Reconstitution of the enzyme by combining extracts containing the small and large subunit restores valine sensitivity and increases the enzyme activity by 30−50-fold (Weinstock et al., 1992).

Studies of the purified AHAS III revealed an unexpected property of the enzyme: its specific activity is dependent on its concentration, suggesting that the enzyme has a significant tendency to dissociate (Sella et al., 1993). Preliminary studies indicated that the two kinds of subunits readily dissociate from one another, and that the association is important both for full catalytic competence of the enzyme and for interaction with valine.

In the present study we have isolated and purified the AHAS III large and small subunits separately and studied their properties. We were particularly interested in the oligomeric state of the subunits when they are separated from one another and the kinetic properties of the isolated large subunit.

MATERIALS AND METHODS

Materials. Sodium pyruvate, FAD, TPP, amino acids, vitamin B_1, ampicillin, tetracycline, DTT, EDTA, creatine, citric acid, and SDS were obtained from Sigma Chemical Co. (St. Louis, MO). Fractogel TSK DEAE-650M, acrylamide, *N*′,*N*′-methylenebisacrylamide, ammonium peroxydisulfate, D-glucose, and ammonium sulfate were purchased from Merck (Darmstadt, Germany). 1-Naphthol was obtained from BDH (Poole, U.K.). Yeast extract, peptone, and agar were from Biolife (Milan, Italy). L-[U-^{14}C]Valine was obtained from Amersham (U.K.). SMM was the gift of Dr. J. V. Schloss, then of E. I. duPont and Company Central R&D Department. All other materials were of analytical grade.

Growth of Bacterial Cells. Plasmids pOI1 (carrying the subcloned gene for the AHAS III large subunit, *ilvI*) or pOH1 (carrying the subcloned small subunit gene *ilvH*) (Weinstock et al., 1992) were transformed into *E. coli* K12 strain BUM1 (M. Ibdah, unpublished results). The latter strain is a *RecA* mutant of strain CU9090 (Sella et al., 1993; Harms et al., 1985) created by bacteriophage P1 transduction from strain WBM535 (Cohen-Fix & Livneh, 1992). The cells were grown in the minimal medium of Vogel and Bonner (1956) with tetracycline (10 mg/L) and ampicillin (100 mg/L) in

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[1] Abbreviations: AHAS, acetohydroxy acid synthase; SMM, sulfometuron methyl.

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shaker flasks (500 mL in 2 L flasks at 200 rpm). Growth was followed spectrophotometrically and cultures were harvested at an OD of about 1 at 660 nm. Cells were harvested by centrifugation and washed twice with 0.1 M phosphate buffer (pH 7.6). The intact cells were stored at −20 °C if not immediately used.

Preparation of Cell Extracts. Solution A (containing 0.1 mM FAD, 10 mM EDTA, 1 mM DTT in an 0.1 M potassium phosphate buffer at pH 7.6) was used as disruption buffer for the preparation of small subunit and for preincubation of reconstituted mixtures. Solution B (containing 0.02 mM FAD, 10 mM EDTA, 1 mM DTT in a 0.1 M Tris-HCl buffer at pH 7.8) was used as the disruption buffer in the preparation of large subunit. A Model XL2015 ultrasonic liquid processor (Heat Systems Inc., New York) with a 1.3 cm flat tip probe was used for cell disruption. The cell suspension (35−40 mL) was cooled in an ice bath and subjected to 12 cycles of full power sonication at 70% duty cycle for 20 s with 40 s pauses. The disrupted cells were centrifuged at 27 000*g* for 1 h to remove cell debris.

Enzyme Assay. In the standard assay for AHAS activity, the rate of acetolactate formation was determined at 37 °C in a 1 mL reaction mixture containing 40 mM pyruvate, 0.1 mM TPP, 10 mM $MgCl_2$, 0.075 mM FAD, 0.5 mM DTT, and 5 mM EDTA in a 0.1 M potassium phosphate buffer, pH 7.6. The enzyme was generally preincubated for 15 min in solution A, before initiation of the reaction by adding a concentrated mixture of the above factors to bring it to final reaction conditions. The reaction was stopped after 20 min by addition of one-tenth volume of 50% H_2SO_4 and analyzed for acetoin by the Westerfeld method as previously described (Westerfeld, 1945). One unit (U) of activity is defined as that producing 1 μmol of acetolactate min^{-1} under the above conditions.

The activity of the small subunit was determined from its ability to activate the large subunit. In the standard assay for following the purification of the small subunit, aliquots of the small subunit solution were added to an aliquot of an extract of BUM/pOI1 containing approximately 0.002 U of AHAS activity. The mixture was diluted to 0.5 mL in the preincubation buffer described above. After 15 min of incubation at 37 °C, the reaction was initiated by addition of concentrated reaction mixture. The specific activity of the small subunit is defined as the slope of a plot of observed AHAS activity as a function of the amount of small subunit protein added.

Specificity for the second substrate, "R", was determined under standard conditions, except that 2-ketobutyrate was included in the reaction mixtures at concentrations from 0.1 to 2.0 mM. The assay for the two alternative products acetolactate (AL) and acetohydroxybutyrate (AHB) was carried out by catalyzed oxidative decarboxylation and subsequent assay of the diketones formed by gas-liquid chromatography, as previously described (Gollop et al., 1988, 1989).

Size Exclusion Chromatography. Size exclusion chromatography was carried out on a 7.5 × 300 mm TSK-gel G3000SW column (Tosohaas, Philadelphia, PA), at a flow rate of 1.0 mL/min, on a Waters HPLC system (Waters-Millipore, Milford, MA) with M-45 pump, Rheodyne 7125 syringe-loading sample injector valve, 600E controller, and 484 absorbance detector set at 280 nm. Samples of about 100 μg of purified large subunit or 50 μg of purified small subunit, or mixtures, were loaded on a 50 μL loop. The column was calibrated with Gel Filtration Molecular Weight marker kit MW-GF-200 (Sigma, St. Louis, MO), including standards from 12.4 to 200 kDa.

Equilibrium Dialysis. Equilibrium dialysis was carried out in 1 mL polymethyl methacrylate cells, with Spectra/Por MWCO:3,500 molecularporous membranes (Spectrum Medical Industries, Houston, TX). Because of the low solubility of the isolated small subunit under the standard assay conditions, the buffer on each side of the membrane in the dialysis experiments generally contained 0.9 M tricine, 0.25 M $MgCl_2$, and 10 μM FAD, at pH 8.5. Protein was included on one side and [C^{14}]valine (approximately 1×10^6 cpm μmol^{-1}) on the other, and the cells were rotated at 4 rpm for about 16 h at 25 °C. The half-time for equilibration of valine was about 100 min under these conditions. Samples (20 μL) were taken and counted in UltimaGold LSC cocktail (Packard Instrument Co., Meriden, CT) in a Packard 1600TR TriCarb liquid scintillation counter.

Other Analyses. Protein concentration was determined by the dye-binding method of Bradford (1976), with bovine serum albumin as standard. This method does not necessarily yield correct absolute concentrations, but we find the relative concentrations of purified large and small subunits determined by the Bradford and Lowry methods agree within 5%. FAD was determined spectrophotometrically, assuming $\epsilon_{450} = 11\,300$ (Dawson et al., 1969). Electrophoretic analyses of protein fractions in the course of purification was carried out by SDS−PAGE on 11% polyacrylamide gels by the method of Laemmli (Sambrook et al., 1989). Molecular weight standards (MW range 14 400−97 400, cat no. 161-0304) were obtained from Bio-Rad Laboratories (Richmond, CA).

Ammonium sulfate solutions (2 M) for chromatography were purified by passing them through an iminodiacetic acid resin (Sigma) chelating column prior to use. Ammonium sulfate concentrations were determined by titration of the acid produced by formalin alkylation. Formalin solution (5 mL) adjusted to pH 7.0 with NaOH was added to 0.5 mL of solution containing ammonium sulfate. The acid produced was titrated to the phenolphthalein endpoint with 0.5 N NaOH.

RESULTS

Purification of AHAS III Large Subunits. Frozen cell paste of *E. coli* BUM/pOI1 (8 g) was suspended at room temperature in 80 mL of solution B, and a cell extract was prepared by sonication as described in Materials and Methods. The results of the isolation of the large subunit from this extract are summarized in Table 1. Ammonium sulfate (20.5 g) was added to 82 mL of extract containing 840 mg of protein, to yield a final ammonium sulfate concentration of 250 g L^{-1}. After incubation for 30 min at 4 °C, the precipitate was collected by centrifugation at 27 000*g* for 20 min. The pellet was redissolved in 27 mL of solution B and the ammonium sulfate concentration determined (about 0.2 M).

The protein was prepared for hydrophobic chromatography by adding the calculated amount (about 45 mL) of 1 M ammonium sulfate in solution B, to bring the mixture to exactly 0.7 M in ammonium sulfate. The protein (280 mg) was loaded on a 1.5 × 30 cm column of Toyopearl Phenyl

Table 1: Purification of AHAS III Large Subunit from *E. coli* BUM1/pOI1[a]

purification step	protein mg	total activity[b] (μmol/min)	specific activity (nmol mg^{-1} min^{-1})	yield (%)
(1) crude extract	840	25.2	30	100
(2) ammonium sulfate	280	19.6	70	78
(3) phenyl Toyopearl column	120	16.8	140	67
(4) DEAE Fractogel column	60	13.8	230	55

[a] The preparation began with 8 g of wet cell paste. [b] The enzyme assay was carried out under standard conditions as described in Materials and Methods. Pyruvate was not saturating for the reaction with large subunit alone.

650M (Tosohaas, Philadelphia, PA) pre-equilibrated with 0.7 M ammonium sulfate in solution B, and the column was washed with 600 mL of the same buffer. The bound protein was then eluted at 1 mL min^{-1} with a 100 mL linear gradient from the starting buffer to 0.01 M Tris-HCl buffer, pH 6.7, containing 1 mM EDTA and 1 mM DTT, followed by 100 mL more of the limit solution. The AHAS activity, which peaked at about 40−50 mL of the limiting eluent, was collected (120 mg of protein with a specific activity of 140 nmol mg^{-1} min^{-1}).

The pooled peak from the above column was diluted 5-fold in 0.025 M Tris-HCl buffer, pH 7.8, containing 1 mM EDTA and 1 mM DTT, and applied to a 1 × 12 cm column of Fractogel TSK DEAE-650M previously equilibrated in the same buffer. The column was eluted at 1 mL min^{-1} with a 100 mL linear gradient from the starting buffer to a solution containing 0.4 M KCl, 10 mM EDTA, 1 mM DTT, and 0.1 mM FAD in 0.1 M Tris-HCl buffer, pH 7.8. The fractions containing enzyme activity, which eluted after about 30 mL (60 mg of protein, specific activity 230 nmol mg^{-1} min^{-1}) were combined and concentrated to 2 mL by dialysis overnight against 50% glycerol in 0.1 M Tris-HCl buffer, pH 7.8, containing 10 mM EDTA, 1 mM DTT, and 0.1 mM FAD. The large subunit could be stored in this solution at −20 °C for at least one month without loss of activity.

Figure 1 shows an SDS−PAGE analysis of the protein at various stages of the purification. Densitometric analysis suggested that the band at a position equivalent to M_R 60 kDa contains 95% of the protein.

Purification of AHAS III Small Subunit. Frozen cell paste of *E. coli* BUM/pOH1 (8 g) was suspended at room temperature in 35 mL of solution A, and a cell extract was prepared as above. The isolation of the small subunit from this extract was based on the limited solubility of this protein, which is highly dependent on the nature of the buffer and its ionic strength. The procedure is summarized in Table 2. Ammonium sulfate (6.3 g) was added to 35 mL of the extract containing 602 mg of protein in phosphate buffer A, to obtain an ammonium sulfate concentration of 180 g L^{-1}. After 10 min at room temperature, the precipitate was removed by centrifugation for 10 min at 27 000*g*. Ammonium sulfate (3.3 g) was then added to the supernatant (final concentration 270 g L^{-1}). After 30 min at room temperature, the second precipitate was collected by centrifugation 20 min at 27 000*g*.

This precipitate dissolved on suspension in 6 mL of 25 mM Tris-HCl buffer, pH 8.0, containing 1 mM EDTA and 1 mM DTT. On removal of ammonium sulfate by dialyzing

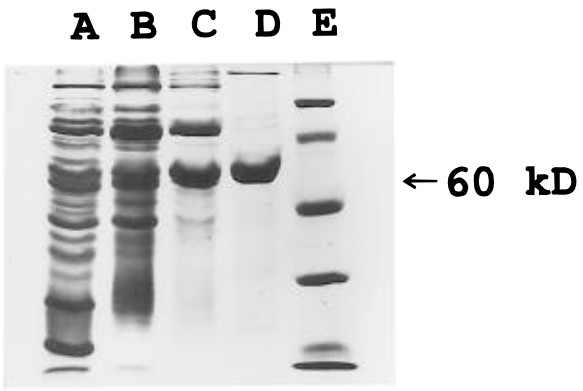

FIGURE 1: SDS−PAGE analysis of the stages of the purification of the AHAS III large subunits on 11% polyacrylamide gels by the method of Laemmli. Lane A, 50 μg of protein from the crude extract from *E. coli* BUM1/pOI1; lane B, 50 μg of protein from the ammonium sulfate step; lane C, 20 μg of protein from phenyl-Toyopearl column step; lane D, 10 μg of protein from the final, DEAE Fractogel chromatography step; lane E, molecular weight markers from 14 400 to 97 400 (Bio-Rad cat. no. 161-0304).

Table 2: Purification of AHAS III Small Subunit from *E. coli* BUM1/pOH1[a]

purification step	protein (mg)	total activity[b] (μmol/min)	specific activity (nmol mg^{-1} min^{-1})	yield (%)
(1) crude extract	602	175	0.29	100
(2) first ammonium sulfate supernatant	466	172	0.37	99
(3) final ammonium sulfate precipitate	247	158	0.64	91
(4) final low salt precipitation	15.5	167	10.8	96

[a] The preparation began with 8 g of wet cell paste. [b] The activity was determined from the activation of the large subunits, described in Materials and Methods, and is defined as the slope of a plot of observed AHAS activity as a function of the amount of small subunit protein added.

the mixture (in Spectra/Por MWCO:3500 molecularporous membrane tubing) against the same buffer overnight at 4 °C, the protein precipitated. The final low salt precipitate was collected by centrifugation 10 min at 27 000*g* and washed twice in about 1 mL of the same buffer. The small subunit protein could be redissolved in 1 M Tris-HCl buffer, pH 9.0, containing 0.1 M $MgCl_2$, diluted 2-fold with glycerol and stored at −20 °C.

Figure 2 shows the SDS−PAGE analysis of this protein, which appears extremely pure. The summary of the isolation of the small subunit (Table 2) suggests that it comprises about 3% of the protein in the original extract.

Reconstitution of the Holoenzyme from the Isolated Subunits. Figure 3 shows a typical set of reconstitution titrations carried out with a single preparation of each of the subunits. The isolated large subunits have low AHAS activity which is strongly activated by addition of small subunits, in a saturable fashion. As can be seen from the data, the activation does not behave as expected for very high affinity association of the large and small subunits. If the interaction were nearly irreversible, the observed activity would increase linearly with the amount of small subunit added and reach a sharp endpoint, proportional to the amount of large subunit in the reaction mixture. The dotted lines, which show the least-squares fit to such a very high affinity model, clearly do not fit the data well. The solid lines, which

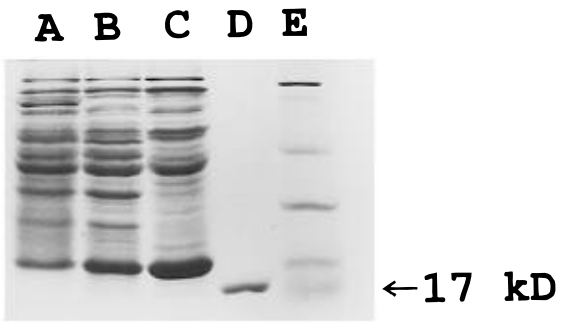

FIGURE 2: SDS−PAGE analysis of the stages in the isolation of the AHAS III small subunits. Lane A, 50 μg of protein from the crude extract from BUM1/pOH1; lane B, 50 μg of protein from the first ammonium sulfate supernatant; lane C, 50 μg of protein from the second ammonium sulfate precipitate; lane D, 5 μg of protein from the final step; lane E, molecular weight markers. Experimental details are as in Figure 1.

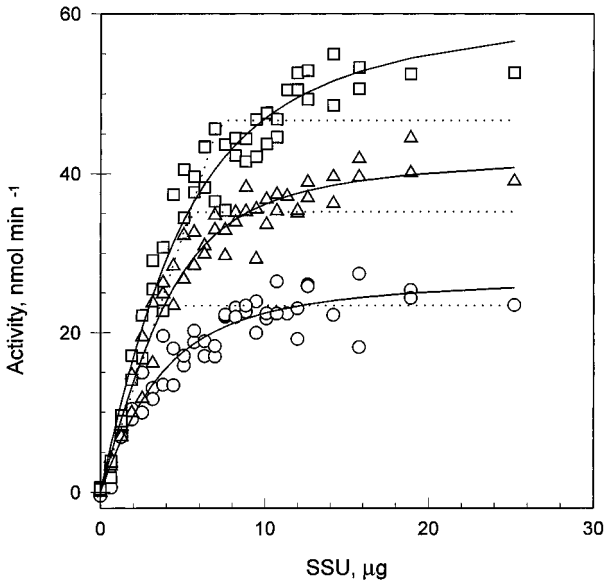

FIGURE 3: Reconstitution titration of AHAS III large subunits with small subunits. Purified AHAS III large subunit (8.9 μg, ○; 13.4 μg, △; or 17.8 μg, □) was preincubated 15 min at 37 °C with the indicated quantities of isolated small subunit as described in Materials and Methods. The standard AHAS assay was then carried out as described. The solid lines are the best fit of the data to the model described in the legend to Table 3, with reversible, finite dissociation of small subunit and large subunit. Dotted lines are best fit of the data assuming an infinitely low dissociation constant K_d for interaction of small subunit and large subunit.

are least squares fits to a simplified model assuming dissociation of a complex of large and small subunits, appear to be more appropriate. The calculated parameters for these fits are given in Table 3, and the equations used are described in the notes to the table. Since protein concentrations were determined by the Bradford (1976) dye-binding method, the *absolute* molar amounts of protein have some uncertainty. However, since the *relative* concentrations of purified large and small subunits determined by the Bradford and Lowry methods agree within 5%, the stoichiometry of the reconstitution is far less uncertain. The equivalent molecular weight calculated for the small subunit from the titration, 19 000 ± 2800, is within experimental error of the assumed molecular mass of the polypeptide, 17 kDa. The calculated turnover number for the large subunit in this experiment, in the presence of saturating amounts of the small subunit, is 3.5 s^{-1}, equivalent to an activity of 2.6 μmol mg^{-1} min^{-1}

Table 3: Results of Fits of the Reconstitution Titrations of AHAS III Large Subunit with Small Subunit (Figure 3) to a Dissociation Model[a]

quantity of LSU (μg)	k_{cat} for LSU at saturation with SSU (s^{-1})	F, calc'd equiv molecular weight for SSU	calc'd K_d (nM)
8.9	3.29	21 300	82
13.4	3.41	20 600	58
17.8	3.74	15 000	150
average	3.5 ± 0.2	19 000 ± 2800	97 ± 39

[a] LSU and SSU are the large and small subunits, respectively. Each titration experiment, with a different quantity of purified large subunit, was carried out as described in Materials and Methods. The data are shown in Figure 3. The data were fit to a model assuming (1)

$$\frac{(I_t - x)(H_t - x)}{x} = K_d$$

Large and small subunit form a 1:1 complex with a dissociation constant K_d, where x is the concentration of the complex and I_t and H_t are the total molar concentrations of large and small subunits. (2) I_t is determined by (apparent weight concentration of LSU)/62000 and H_t is determined by (apparent weight concentration of SSU)/F. If both proteins were pure, their realtive weight concentrations were correctly determined and activity required a stoichiometric equivalence of large subunit and small subunit, F would theoretically be 17 000. (3) $V = k_{cat}(0.05I_t + 0.95x)$. The catalytic activity of the large subunits in the presence of a saturating amount of small subunit is k_{cat}, while the large subunits alone have an activity of only 0.05 k_{cat}. (The fit to the data is quite insensitive to the choice of values between 0.03 and 0.08 k_{cat} for the activity of the large subunits alone.) These equations, with k_{cat}, K_d, and F as parameters, were fit to each data set by the nonlinear least-squares method, and the results given in the table.

for the reconstituted holoenzyme (equivalent weight 62 + 17 kDa). The activity of the holoenzyme purified from bacteria expressing the complete *ilvIH* operon has been found to be 2.7 μmol mg^{-1} min^{-1} using the assay conditions of this paper (Gollop et al., 1989) and 7.3 μmol mg^{-1} min^{-1} under slightly different conditions (Barak et al., 1988). The stoichiometry and specific activity found for reconstitution suggest that both subunits have been successfully purified in active, fully reconstitutable form.

Enzymatic Properties of the Isolated Large Subunit. The isolated large subunit of AHAS III has low, but real AHAS activity. This activity was, necessarily, examined using relatively high concentrations of the protein (50−250 μg of protein mL^{-1}). The kinetic properties of the large subunit are summarized in Table 4 and compared with those of the reconstituted enzyme and the intact enzyme. At saturation with pyruvate and the required cofactors, the activity of the large subunit without added small subunit is about 4−7% that of the reconstituted holoenzyme. The apparent affinity of the large subunit for the substrate pyruvate is nearly an order of magnitude weaker than that of the reconstituted or intact enzyme. The apparent affinity of the large subunit for the inhibitory herbicide SMM is also weaker than that of the holoenzyme, by about 6-fold. On the other hand, the apparent K_ms for the cofactors TPP and Mg^{2+} are only slightly altered (Table 4). The specificity of the reaction catalyzed by the large subunit for the second, acceptor ketoacid, is also very close to that of the holoenzyme. The pH dependence of the activity of the isolated large subunit is similar to that of the holoenzyme, with a pH optimum between 7.5 and 8.5 (not shown).

It is interesting to note that the activity of the isolated large subunit is much less sensitive to the nature of the buffer than

Table 4: Kinetic Parameters for Isolated AHAS III Large Subunits and Reconstituted Holoenzyme[a]

parameter	isolated large subunit[b]	reconstituted enzyme[b]	intact enzyme (literature)
V_{max}, (μmol mg^{-1} min^{-1})[c]	0.37	2.6	2.7 (Gollop et al., 1989) 7.3 (Barak et al., 1988)
K_m for pyruvate (mM)[d]	86 ± 14	11.5 ± 1.4	6 (Gollop et al., 1989)
K_m for TPP (μM)[d,e]	29 ± 7	18 ± 3	
K_m for Mg^{2+} (mM)[d,e,f]	3.1 ± 0.9	3.3 ± 0.8	
K_m for FAD (μM)[g]	nd	2.2 ± 0.5	
R (specificity)[h]	36 ± 4	53 ± 8	40 (Gollop et al., 1989)
K_i for valine (μM)[i]	∞	12.7 ± 0.14	7 (Sella et al., 1993)
K_i for SMM (mM)	1.2 ± 0.1	0.19 ± 0.03	0.3 (Schloss et al., 1988)

[a] Except where indicated, reactions were carried out under standard conditions as described in Materials and Methods at 37 °C. The data were fit to hyperbolic saturation kinetics . [b] Measurements with the large subunit alone were carried out with 50−200 μg mL^{-1}. For the kinetics of the reconstituted enzyme, large subunit concentrations were 5−10 μg mL^{-1}, and a 2-fold stoichiometric excess of small subunit was added. Under these conditions >95% reconstitution appears to be achieved. [c] Calculated V_{max} at saturation with pyruvate, in presence of standard concentrations of cofactors. [d] EDTA was omitted from the preincubation buffer for measurements of K_m for pyruvate, TPP, and Mg^{2+}. The maximum EDTA concentration (from the large subunit prep) in the reaction was 1 mM. [e] Reactions were carried out in the presence of 400 mM pyruvate. [f] The Mg^{2+} dependence may deviate from hyperbolic saturation. Fits to the Hill equation (Fersht, 1985), showing apparent Hill coefficients of about 2 for both isolated large subunit and reconstituted enzyme, had smaller and less biased deviations than did fits to the Michaelis−Menten equation. [g] The residual FAD in the large subunit preparation did not allow determination of the affinity of the large subunit for FAD. Because of the much lower quantity of large subunit preparation necessary for measurements with reconstituted enzyme, the residual FAD was only 0.5 μM and K_m could be determined. [h] The specificity is defined as $R = (V_{AHB}/V_{AL})/([2\text{-ketobutyrate}]/[\text{pyruvate}])$ and was determined as described in Gollop et al. (1989). [i] Inhibition of AHAS III by valine is incomplete at saturation. The data were fit to an empirical equation which assumes K_i is the apparent dissociation constant for an inhibitory effect leading to a final activity V_f (Sella et al., 1993). For the reconstituted enzyme, V_f is 13 ± 2% of V_0 under standard conditions (pH 7.6 in phosphate buffer).

is that of the reconstituted holoenzyme; e.g., for the holoenzyme the activity is some 15-fold lower in bistrispropane than in potassium phosphate, whereas for the isolated large subunit the difference was less than 2-fold (not shown). Examination of the buffer dependence of the reconstituted holoenzyme showed that the effect is due to activation in the presence of potassium phosphate rather than inhibition by bistrispropane. KCl and sodium phosphate also strongly increase the activity of the holoenzyme, by about 16- and 7-fold, respectively, at saturation.

An important difference between the activity of the isolated large subunit and the holoenzyme is, of course (Squires et al., 1981; Weinstock et al., 1992), the complete lack of sensitivity to valine of the catalytic activity of the large subunit in the absence of the small subunit. The reconstituted enzyme shows a K_i for valine and extrapolated residual activity at saturation similar to that observed for the intact enzyme under standard conditions (Table 4). In order to compare the inhibitory effects of valine with valine binding measured by equilibrium dialysis, we also measured valine

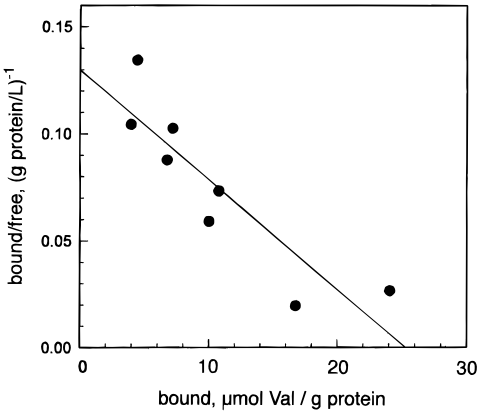

FIGURE 4: Scatchard plot of valine binding to purified AHAS III small subunit. Equilibrium dialysis experiments were carried out as described, with purified small subunit (7−10 mg mL^{-1}) and [U-^{14}C]valine (0.1−2 mM) in tricine buffer, pH 8.5. The line is the least-squares fit, with calculated $K_d = (1.9 \pm 0.4) \times 10^{-4}$ M and valine binding at saturation of $(2.5 \pm 0.6) \times 10^{-5}$ mol (g of protein)$^{-1}$.

inhibition of the activity of the reconstituted enzyme in tricine buffer, at the optimum pH of 8.5. Under these more alkaline conditions, K_i was 6.0 ± 0.8 μM, about 2-fold lower than under standard conditions, and V_f was 29 ± 2% of the initial activity.

Valine Binding. Valine binding was studied by equilibrium dialysis. Because of the relatively low affinity of these proteins for valine, rather high concentrations were required for determination of the valine dissociation constant. In order to achieve adequate solubility of the small subunit, a higher pH was necessary. Figure 4 shows a Scatchard analysis of valine binding to the small subunit, at pH 8.5 in a tricine buffer containing 10 mM Mg^{2+}. Magnesium was necessary for sufficient solubility of the small subunit, but it should be noted that this Mg^{2+} level is the same as that used for the activity assay. The apparent dissociation constant, K_d, of the valine−small subunit complex under these conditions was 190 ± 40 μM, more than an order of magnitude higher than the K_i for valine under comparable conditions (6 μM; see above). The extrapolated valine binding stoichiometry at saturation, $(2.5 \pm 0.6) \times 10^{-5}$ mol of valine (g of protein)$^{-1}$, was about half that expected for a 1:1 complex with a pure peptide of molecular weight 17 000. Practical limitations on the protein concentrations made measurements of valine binding to the large subunit or reconstituted enzyme even more problematic. Within the limits of the technique, the large subunit alone did not bind valine ($K_d \geq 10$ mM). In the same pH 8.5 tricine/10 mM $MgCl_2$ buffer in which the experimental K_i is 6 μM, reconstituted enzyme (1:1 large and small subunit, with or without the addition of TPP) bound valine with a dissociation constant on the order of 100−200 μM (not shown).

Binding of valine to a mutant small subunit was also studied. A spontaneous *E. coli* K12 mutant, BUC4, was selected for valine resistance in AHAS III; the mutant *ilvH* gene was cloned by PCR and sequenced in parallel with the wild-type gene (Squires et al., 1983) and found to have a single amino acid sequence mutation, G14 → D (C. Sella, unpublished results). The mutant gene product was isolated and purified by the same method as the wild-type small subunit. We could detect no valine binding to a purified mutant small subunit by equilibrium dialysis, under condi-

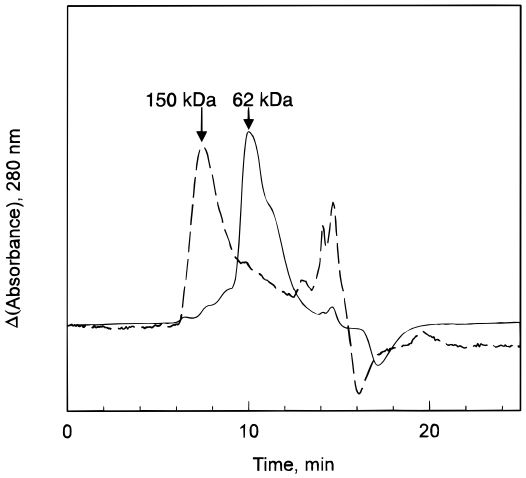

FIGURE 5: Chromatographic traces from size exclusion HPLC analyses of isolated AHAS III large subunit (full line) and a 1:2 mixture of large subunit and small subunit (broken line). Samples were separated on 7.5 × 300 mm TSK-gel G3000SW column at a flow rate of 1.0 mL/min, eluted with a 0.1 M potassium phosphate buffer, pH 7.6, containing 50 μM FAD, 100 μM TPP, 10 mM $MgCl_2$, and 40 mM sodium pyruvate. The arrows indicate the expected positions of proteins with apparent molecular sizes of 62 and 150 kDa. The depression at 16−18 min is due to depletion of pyruvate.

tions identical to those used for the wild-type small subunit. This mutant peptide was found to activate the large subunit to the same extent as the wild-type small subunit (data not shown), but this active, reconstituted complex showed no valine sensitivity.

Apparent Molecular Size. The association of the subunits in quaternary complexes was studied using size exclusion HPLC on a TSK-gel G3000SW column. The isolated subunits, or mixtures of the large subunit with a stoichiometric excess of small subunit, were preincubated for 15 min in potassium phosphate buffer at pH 7.6, containing various combinations of the cofactors and substrate of AHAS, and chromatographed using buffers of various compositions. Under all conditions we studied, including preincubation and chromatography in the complete reaction mixture with pyruvate, the isolated small subunit behaved as a monomer of apparent molecular size near 17 kDa (not shown), and the isolated large subunit (at initial concentrations up to 2 μg/μL) behaved as a monomer of apparent molecular size near 60 kDa (e.g., Figure 5). In the presence of a 2-fold molar excess of the small subunit, the major protein peak eluted under appropriate conditions at a position equivalent to an apparent molecular size of 150−160 kDa (Figure 5), as expected for an $\alpha_2\beta_2$ complex of 17 and 62 kDa subunits (Barak et al., 1988; Schloss et al., 1985). So long as the HPLC elution buffer contained FAD (0.05 mM), this 150−160 kDa peak appeared as the major protein peak. It should be noted that the protein mixture was always preincubated with about 0.03 mM FAD, present in the purified subunit preparations. On the other hand, when FAD was absent from the elution buffer, but present in the preincubation mixture, major protein peaks for the mixture of subunits appeared at intermediate elution volumes, equivalent to molecular sizes of 80−100 kDa.

We examined the possible influence of valine on the quaternary structure of the enzyme by carrying out the chromatography of a mixture of large and small subunits with the addition of 5 mM valine to preincubation and elution buffers containing FAD. Valine had no effect, and the major protein peak appeared at the position expected for a holoenzyme of 150−160 kDa, as in the control in the absence of valine.

Previous experiments showed that native AHAS III releases its bound FAD very slowly (Barak et al., 1988). We examined FAD binding to the reconstituted enzyme by combining the large subunit with excess small subunit, in the presence of FAD, TPP and Mg^{2+} in phosphate buffer, and chromatographing the mixture in buffer without cofactors. The major protein peak was collected and assayed for protein and for FAD. It contained 13.8 pmol of FAD per mg of protein, equivalent to 2.2 mol of FAD for each mole of the presumed 158 kDa $\alpha_2\beta_2$ complex. Surprisingly, on chromatography the large subunit alone also carried with it FAD from the preincubation stage, although less than a stoichiometric amount (0.38 mol of FAD per mol of large subunit).

DISCUSSION

We have isolated the large and small subunits of AHAS isozyme III of *E. coli* from bacteria expressing the separate, subcloned genes (Weinstock et al., 1992). The subunits, which were isolated at greater than 95% purity, maintained the capacity to undergo reconstitution to form a holoenzyme, whose activity was close to that of the purified "native" enzyme and which had kinetic properties quantitatively similar to those of the native enzyme (Table 4).

Preliminary work from our laboratory (Weinstock et al., 1992), based on the properties of crude extracts from bacteria expressing only the subcloned large subunit of AHAS III, had provided strong evidence that, despite the significant activation of the large subunit observed on addition of small subunit, the large subunit is by itself the catalytic unit of the enzyme. The purification of the large subunit enables us to confirm this proposal. The properties of the purified large subunit, including substrate specificity, requirements for cofactors, and inhibition by the herbicide SMM, are all similar enough to those of the holoenzyme (Table 4) to make it fairly clear that all of the catalytic machinery of AHAS III is contained in the large subunit.

Crystal structures have recently been published for pyruvate decarboxylase (Dyda et al., 1993) and pyruvate oxidase (Muller et al., 1994; Muller & Schulz, 1993), two TPP-dependent enzymes with peptide sequences homologous to those of AHAS large subunits (Muller et al., 1994; Chang & Cronan, 1988; Green, 1989). Further, since the first step in the reactions catalyzed by all of these enzymes involves decarboxylation of TPP-bound pyruvate, it is very reasonable to assume that AHAS III is structurally similar to the two enzymes of known three-dimensional structure. The active forms of both pyruvate oxidase and pyruvate decarboxylase are homotetramers in which pairs of subunits form a pair of active sites at the interfaces between the monomers. We therefore expected that a dimer of large subunits would be the minimal active form of AHAS III and would be intrinsically stable even in the absence of small subunit. The tendency of the isolated large subunit, which has low but significant catalytic activity, to behave as though it exists chiefly in the form of the monomer (as measured by size exclusion chromatography) in all combinations of buffer, cofactors, and substrates was thus somewhat unexpected.

This can be rationalized if some small fraction of the large subunit exists at equilibrium as active dimers, which are responsible for the observed residual activity of the isolated catalytic subunits. Such a dimerization equilibrium would only lead to a small shift in the apparent molecular size of the large subunit on size exclusion chromatography.

The purified small subunit, the *ilvH* gene product, is sparingly soluble, which makes its isolation relatively simple (Table 2) but complicates studies of its properties. The soluble small subunit also appears to behave as a monomer in solution, suggesting that strong interactions between the small subunits are not directly responsible for the quaternary structure of AHAS III holoenzyme. The small subunit binds valine, as expected if it is truly the regulatory subunit of the enzyme (Weinstock et al., 1992), but the apparent dissociation constant for the small subunit−valine complex is some 20-fold higher than K_i under similar conditions (Figure 4). The measured binding capacity at saturation was about half of the expected value, and it is not clear whether this is due to experimental error, incomplete purification, or loss of binding capacity by partial denaturation. As expected, we could not detect valine binding to the isolated large subunit.

The reconstituted enzyme has an apparent molecular size near 150 kDa, as expected for an $\alpha_2\beta_2$ complex of the large subunit and small subunit. The presence of FAD is required for the assembly of the complex. By analogy with the structure of pyruvate oxidase (Muller et al., 1994), in which FAD is completely buried within one subunit, one would expect this coenzyme to have a profound effect on the conformation of the subunit. The behavior of the system on size exclusion HPLC suggests that the catalytic subunit can rapidly bind FAD and form the $\alpha_2\beta_2$ complex but that dissociation of FAD from the enzyme is slow.

Activity titrations of the large subunit with small subunit (Figure 3 and Table 3) confirm the 1:1 stoichiometry previously suggested for this enzyme (Barak et al., 1988). These titrations also confirm the fairly large dissociation tendency of the enzyme (Sella et al., 1993) and imply that the enzyme would be dissociated into separate subunits to a significant extent at concentrations of 0.1 μM (15 μg/mL) and below. Valine does not appear to promote this dissociation; the presence of 5 mM valine does not effect the apparent molecular size of the holoenzyme on size exclusion chromatography.

Although experimental limitations prevent us from determining the valine binding properties of the reconstituted enzyme with accuracy, the holoenzyme clearly binds valine (at equilibrium in the absence of substrate turnover) with an affinity at least an order of magnitude lower than that implied by K_i. One possible explanation is that substrate turnover increases the affinity of the enzyme for the inhibitor. Since the enzyme has a "ping-pong" kinetic mechanism (Gollop et al., 1989; Tse & Schloss, 1993), it is not unreasonable that, e.g., binding of the first substrate (pyruvate) increases the affinity for an inhibitor. [Experiments with slow-binding inhibitors of AHAS II indicate that the binding of the first substrate is in fact required for tight inhibitor binding in these cases (Schloss & Aulabaugh, 1990; Schloss et al., 1988; Hawkes et al., 1989). We do *not*, however, suggest that the feedback inhibitor valine and the herbicide-like inhibitors are bound analogously.] Unfortunately, equilibrium dialysis experiments cannot be carried out under substrate turnover conditions, and we have not found a workable alternative method for measuring valine binding.

The relevance of the observed valine binding to the small subunit to the physiological regulation of the holoenzyme by valine is strongly supported by the behavior of the *ilvH* G14 → D mutant. The purified mutant small subunit fully activates the large subunit, but the enzyme thus reconstituted is insensitive to valine inhibition. The purified G14 → D small subunit itself shows no detectable valine binding.

The results presented here imply that the assembly of the subunits of AHAS III into the fully active $\alpha_2\beta_2$ complex must involve mutual effects of the large and small subunits on one anothers conformations. The association of valine with a site on a regulatory subunit lowers the activity of the catalytic sites, presumably at the large subunit dimer interface, by an allosteric conformational effect. Other bacterial AHASs [and perhaps even eukaryotic AHASs (Singh & Shaner, 1995)] may well have quite similar structural and functional arrangements. Minor differences in the nature of subunit−subunit contacts among the different AHASs might be responsible for the different extents to which the large subunits are stable or active alone and for different tendencies to dissociation.

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