Purification of *Escherichia coli* acetohydroxyacid synthase isoenzyme II and reconstitution of active enzyme from its individual pure subunits

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The first step in the biosynthesis of branched-chain amino acids is catalysed by acetohydroxyacid synthase (EC 4.1.3.18). The reaction involves the decarboxylation of pyruvate followed by condensation with either a second molecule of pyruvate or with 2-oxobutyrate. The enzyme requires as cofactors thiamine diphosphate, a divalent metal ion and, usually, FAD. In most bacteria the enzyme is a heterotetramer of two large and two small subunits. *Escherichia coli* contains three active isoenzymes and the present study concerns isoenzyme II, whose large and small subunits are encoded by the *ilv*G and *ilv*M genes respectively. Cloning these genes into a plasmid vector and overexpression in *E. coli* allowed a two-step purification procedure for the native enzyme to be developed. The level of expression is considerably higher from a vector that introduces a 50 residue N-terminal fusion containing an oligohistidine sequence on the large subunit. Purification to homogeneity was achieved in a single step by immobilized-metal-affinity chromatography. The kinetic properties of the native and fusion enzyme are indistinguishable with respect to the substrate pyruvate and the inhibitor chlorsulfuron. The individual subunits were expressed as oligohistidine-tagged fusion proteins and each was purified in a single step. Neither subunit alone has significant enzymic activity but, on mixing, the enzyme is reconstituted. The kinetic properties of the reconstituted enzyme are very similar to those of the fusion enzyme. It is proposed that the reconstitution pathway involves successive, and highly co-operative, binding of two small subunit monomers to a large subunit dimer. None of the cofactors is needed for subunit association although they are necessary for the restoration of enzymic activity.

INTRODUCTION

Acetohydroxyacid synthase (AHAS; EC 4.1.3.18, also known as acetolactate synthase) catalyses the first step in the biosynthesis of the branched-chain amino acids. AHAS catalyses the decarboxylation of pyruvate and the condensation of the acetaldehyde moiety with a second molecule of pyruvate to give 2-acetolactate, as the first step in the biosynthesis of valine and leucine. It also catalyses a similar reaction where condensation with 2-oxobutyrate gives 2-aceto-2-hydroxybutyrate as the first step in the biosynthesis of isoleucine. Inhibition of the enzyme is the basis for the action of several, structurally diverse, classes of herbicide including the sulphonylureas, imidazolinones, triazolopyrimidines and the pyrimidyl-oxybenzoates (reviewed in [1–4]).

AHAS is present in bacteria, fungi, algae and plants. In bacteria there are two types of AHAS. The first requires FAD, thiamine diphosphate (ThDP) and a divalent metal ion such as Mn^{2+} or Mg^{2+} [5] for activity. This type is a heterotetramer containing two large and two small polypeptides with molecular masses of approx. 60 and 9–17 kDa respectively. The second type of AHAS does not require FAD, lacks a small subunit and probably supplies acetolactate for fermentation to butanediol [6,7]. The archaebacterium *Methanococcus aeolicus* contains an intermediate type; the enzyme requires FAD, although this can be replaced by FMN or by riboflavin plus phosphate and is claimed to contain no small subunit [8], although this latter property is now in question [9].

In eukaryotes, AHAS requires FAD but the difficulty in purifying the enzyme (see, for example, [10,11]) leaves open the question of whether there is a small subunit, although recent evidence [12,13] suggests that this subunit does exist.

AHAS from the enteric bacteria *Escherichia coli* and *Salmonella typhimurium* are the most thoroughly studied. There are three active isoenzymes, AHAS I, II and III, each encoded by a pair of linked genes for the two subunits: *ilv*BN, *ilv*GM and *ilv*IH respectively (reviewed in [14]). The genes encoding isoforms I and III from *E. coli* and II from *S. typhimurium* have been expressed in *E. coli* and the proteins purified to homogeneity [5,15–18]. AHAS I and III, but not II, are feedback-inhibited by valine.

The requirement for both subunits for *E. coli* AHAS II activity was established by mixing crude extracts of cells separately expressing the individual genes [19]. Subsequently, *E. coli* AHAS I and III activities were reconstituted from individually expressed (but not purified) subunits [20]. For these isoenzymes, the large subunit alone possesses some activity but is insensitive to valine. Later work, using purified subunits of *E. coli* AHAS III, showed that the small subunit is capable of binding valine [18]. Thus the principal catalytic machinery is contained within the large subunit while the small subunit has a regulatory role.

Reconstitution of *E. coli* AHAS II from its purified subunits has not previously been demonstrated. Here we describe the expression and purification of the enzyme in its native form and as a fusion protein containing an N-terminal oligohistidine tag. The large and small subunits of the enzyme have also been individually expressed as fusion proteins and then purified. Reconstitution of active enzyme is demonstrated and several physical and kinetic properties of the native and reconstituted enzyme are reported and compared.

MATERIALS AND METHODS

Materials

The plasmid pAH29 [21], containing *ilv*GM from the *ilv*G2096 allele within a 4.6 kb *Hin*dIII fragment, was obtained from Dr.

Abbreviations used: AHAS, acetohydroxyacid synthase; DTT, dithiothreitol; IMAC, immobilized-metal-affinity chromatography; ThDP, thiamine diphosphate.

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G. W. Hatfield (University of California, Irvine, CA, U.S.A.). Subcloning of the genes to yield the various expression vectors is described in the next section. BL21(DE3) *E. coli* cells and the plasmid pET-30a(+) were obtained from Novagen; pT7-7 was purchased from United States Biochemical Corp.

Restriction endonucleases, DNA-modifying enzymes and other molecular biology reagents were purchased from Boehringer-Mannheim, Bio-Rad, New England Biolabs or Progen Industries and, unless stated otherwise, were used in accordance with the manufacturers' instructions. Chlorsulfuron and chlorimuron ethyl were gifts from Dr. H. Brown (Du Pont Agricultural Products) while imazapyr was donated by Dr. B. K. Singh (American Cyanamid). Other chemicals were from Ajax Chemicals, BDH, Bio-Rad, Boehringer-Mannheim, Difco, Merck, Pharmacia or Sigma Chemical Co.

Construction of plasmids

An *Xba*I/*Hin*dIII double digest of pAH29 gave a 3.3 kb fragment containing *ilv*GM, which was cloned into pUC18 to yield the plasmid pCH2. Other plasmids used were constructed from pCH2.

pT7-GM

An *Nde*I restriction site overlapping the *ilv*G start codon was introduced by PCR, with pCH2 as the template. An *Nde*I/*Nsi*I double digest of the PCR product gave the 5′ 100 bp of *ilv*G, whereas the remaining 3′ region of *ilv*GM was obtained by an *Nsi*I/*Hin*dIII double digest of pCH2. The two restriction fragments were cloned into the plasmid pT7-7 to yield pT7-GM. The native form of the enzyme is expressed from this construct.

pET-GM

A *Bam*HI restriction site immediately 5′ to the *ilv*G start codon and an *Eco*RI restriction site immediately 3′ to the *ilv*M stop codon were introduced by PCR with pCH2 as the template. A *Bam*HI/*Eco*RI double digest of the PCR product gave a 1.9 kb fragment, which was cloned into the expression vector pET30a(+) to yield pET-GM. This construct expresses the large subunit with a 50-residue (5.43 kDa) N-terminal fusion that contains the oligohistidine tag, and the small subunit in its native form.

pET-G

One of the five *Bsp*HI sites in pET-GM overlaps the *ilv*G/*ilv*M junction. After partial digestion with this enzyme followed by digestion with *Eco*RI, the cohesive ends were filled in by T4 DNA polymerase, regenerating the *ilv*G stop codon. The fragments were circularized to yield pET-G among a mixture of constructs and the required construct was identified by the absence of a unique *Aat*II restriction site that is located within *ilv*M. The large subunit expressed from this construct is identical with that expressed from pET-GM.

pET-M

Digestion of pET-GM with *Bsp*HI and *Eco*RI yielded a 266 bp *Bsp*HI/*Eco*RI fragment that was cloned into the *Nco*I and *Eco*RI restriction sites of pET30a(+) to yield pET-M. This construct expresses the small subunit with a 44-residue (5.17 kDa) N-terminal extension that contains the oligohistidine tag.

Bacterial growth and protein expression

Native

A single colony of the *E. coli* strain BL21(DE3), transformed with the plasmid pT7-GM, was inoculated into 20 ml of 2YT medium [22] containing 100 μg/ml ampicillin. The culture was incubated overnight at 37 °C; the cells were harvested by centrifugation and washed with 20 ml of 0.9% NaCl. After collection of the cells by centrifugation they were resuspended in 2 ml of 0.9% NaCl and 0.5 ml of this suspension was used to inoculate each of four 500 ml volumes of 'terrific' broth [22] containing 100 μg/ml ampicillin; the cultures were incubated at 37 °C with shaking. When a D_{600} of 0.4 was reached, 0.5 ml of 1 M isopropyl β-D-thiogalactoside was added to each culture; these were then incubated at 37 °C for a further 2 h with shaking. The cells were harvested by centrifugation at 3000 ***g*** for 20 min at 4 °C. The cell pellet was frozen overnight at −70 °C.

Fusion enzyme and subunits

Bacterial growth conditions and expression of the fusion enzyme and subunits were identical with those for the native enzyme except that the medium contained 50 μg/ml kanamycin instead of ampicillin and the cultures were incubated at ambient temperature (approx. 22 °C) for 4 h after the addition of isopropyl β-D-thiogalactoside.

Purification

Fusion protein and subunits

The proteins expressed from pET-GM, pET-G and pET-M have an N-terminal oligohistidine tag that was used for purification by means of immobilized-metal-affinity chromatography (IMAC) on a metal-chelating column. The purity of the resultant proteins is illustrated in Figure 1.

Cell lysis was performed with minimal exposure to light. The frozen cell pellet was thawed, suspended in 30 ml of ice-cold binding buffer [20 mM Tris/HCl (pH 7.9)/500 mM NaCl/10 μM FAD) containing 5 mM imidazole and then treated with lysozyme (10 mg/g of cells for 30 min at 0 °C). The cells were disrupted by sonication, insoluble material was removed by centrifugation (27000 ***g*** for 20 min at 4 °C) and the supernatant was passed through a 0.45 μm filter.

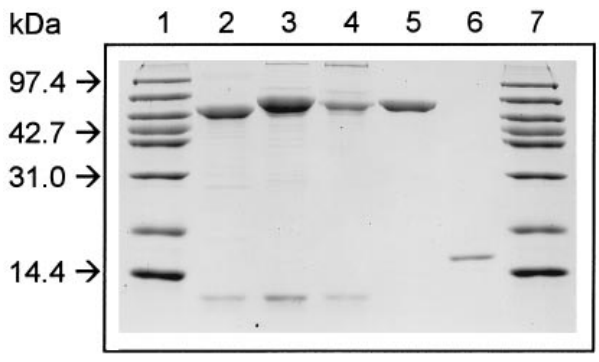

Figure 1 SDS/PAGE of purified *E. coli* AHAS II and its isolated subunits

Lanes 1 and 7, molecular mass markers; lane 2, native enzyme; lane 3, fusion enzyme before thrombin cleavage; lane 4, fusion enzyme after thrombin cleavage; lane 5, large subunit; lane 6, small subunit.

Table 1 Purification of fusion *E. coli* AHAS II from 9.7 g wet weight of cells

Total protein was determined with the bicinchoninic acid method. Thrombin cleavage and the second IMAC step were not performed routinely.

Fraction	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Yield (%)
Cleared lysate	7672	543	14	100
IMAC	5015	112	45	65
Thrombin	3997	80	50	52
IMAC	3531	67	53	46

Table 2 Purification of native *E. coli* AHAS II from 6.6 g wet weight of cells

Total protein was determined with the bicinchoninic acid method.

Fraction	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Yield (%)
Cleared lysate	361	620	0.58	100
IMAC	248	23	11	69
Blue Trisacryl	178	7.5	24	49

All subsequent chromatography steps were performed at 4 °C and where possible in darkness with the extra precaution of wrapping the apparatus in aluminium foil to minimize exposure to light. A 0.5 ml/min flow rate was maintained for each step. IMAC was performed with a 5 ml HiTrap Chelating column (Pharmacia) or a 1 cm × 7.7 cm column of His·Bind resin (Novagen); in each case these were charged by using 50 mM $NiSO_4$ then equilibrated with binding buffer containing 5 mM imidazole. The filtrate was applied, followed by 50 ml of binding buffer, then 30 ml of binding buffer containing 25 mM imidazole, and then 30 ml of binding buffer containing 100 mM imidazole. Fractions containing the desired protein were pooled, concentrated by ultrafiltration (Millipore 5000 or 30000 NMWL regenerated cellulose membrane) and desalted (Pharmacia PD-10 column) against 20 mM Tris/HCl buffer, pH 8.1, containing 1 mM EDTA, 10 μM FAD and 1 mM dithiothreitol (DTT). The eluate was snap-frozen in liquid nitrogen and stored at −70 °C. Alternatively the enzyme and the subunits were stored in a buffer of the same composition except that it contained 500 mM NaCl and no EDTA.

Thrombin treatment (see below) followed by IMAC to remove the cleaved peptide resulted in a small increase in specific activity, although 30% of the enzyme was lost in this process. The purification of fusion AHAS II is summarized in Table 1. As will be shown later, the kinetic properties of the fusion enzyme are very similar to those of the native enzyme; for this reason, thrombin cleavage of the fusion enzyme was not performed routinely.

Native

We were surprised to discover that the native form of the enzyme bound to the IMAC column. When fusion enzyme was bound to the column, the small subunit was not eluted by 6 M urea, although gel filtration experiments demonstrated that the subunits dissociate under these conditions. These results suggest that it is through the small subunit that native enzyme binds to the IMAC column, although it contains no oligohistidine sequence. This property was exploited in the purification of native AHAS II. Cell lysis and subsequent IMAC of the native enzyme are identical with those described for the fusion protein and subunits except for the following changes: (1) because the interaction between the chelating column and the native enzyme is weaker, the column was eluted with binding buffer containing 25 mM imidazole, omitting the wash step; and (2) the enzyme solution obtained from concentration by ultrafiltration was desalted against 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA, 20% (v/v) glycerol, 10 μM FAD and 1 mM DTT (buffer A).

The IMAC fraction was applied to a 1 cm × 21 cm Blue Trisacryl column equilibrated with buffer A. The column was washed with 30 ml of buffer A and eluted with 20 ml of 100 mM Tris/HCl buffer, pH 8.0, containing 1 mM EDTA, 20% (v/v) glycerol, 10 μM FAD and 1 mM DTT. A 0.2 ml/min flow rate was maintained for each step. The most active fractions were pooled, concentrated by ultrafiltration, desalted against 20 mM Tris/HCl buffer, pH 8.1, containing 1 mM EDTA, 10 μM FAD and 1 mM DTT, then snap-frozen in liquid nitrogen and stored at −70 °C. The purification of native AHAS II is summarized in Table 2.

Digestion with thrombin

The N-terminal fusion peptide of proteins expressed from the pET30a(+) vector contains a thrombin cleavage site on the C-terminal side of the oligohistidine tag, so digestion of the fusion protein with this protease removes the N-terminal 14 residues containing this tag from the expressed protein. Thrombin (0.5 unit/mg of protein) was mixed with 3.5 ml of purified fusion protein in 20 mM Tris/HCl buffer, pH 8.4, containing 150 mM KCl, 2.5 mM $CaCl_2$, 10 μM FAD and 1 mM DTT. The solution was incubated overnight at 20 °C, concentrated by ultrafiltration and desalted against binding buffer containing 5 mM imidazole, then the enzyme was separated from the oligohistidine peptide by IMAC (eluting with 25 mM imidazole).

Protein determination

Three different protein determination assays were employed. For routine protein concentration determinations the Bradford [23]

or bicinchoninic acid [24] assays were used. These methods give results that depend to some degree on the amino acid composition of the protein. To characterize the reconstitution of AHAS II, it was necessary to determine accurately the molar concentrations of the large and small subunits. For this purpose we calibrated the Bradford and bicinchoninic acid methods against a ninhydrin assay in which the α-amino group content of the protein was estimated after total alkaline hydrolysis. Nitrogen gas was bubbled through the ninhydrin reagent [75 % (v/v) methyl cellosolve/0.3 % hydrindantin/2 % (w/v) ninhydrin in 1 M sodium acetate (pH 5.5)] during preparation and just before use. The reagent was stored at 4 °C in the dark and used within 2 weeks. Protein samples were dried down and dissolved in 0.5 ml of 5 M NaOH before hydrolysis of peptide bonds by autoclaving at 121 °C for 20 min. After cooling, 1 ml of 30 % (v/v) acetic acid and 2 ml of ninhydrin reagent were added and mixed. Full colour development was achieved by heating the mixture at 100 °C for 20 min and, after cooling, the absorbance of the solution was measured at 570 nm. BSA was used as the standard in all protein assays.

Gel-filtration chromatography and SDS/PAGE

Gel-filtration experiments were performed on a Pharmacia Smart System fitted with a 3.2 mm × 300 mm Superose-12 column (Pharmacia). The column was calibrated with molecular mass markers in the range 1.35–670 kDa (Bio-Rad). Protein solutions (0.2–4.0 mg/ml) were applied at a flow rate of 40 μl/min to the column, which was equilibrated with 20 mM Tris/HCl buffer, pH 7.9, containing 500 mM NaCl. Samples eluted from the column were detected by monitoring the absorbance at 214 and 280 nm. Substrate (50 mM pyruvate) and cofactors (1 mM ThDP, 10 mM $MgCl_2$ and 10 μM FAD) were added, singly or in combination, as indicated. Under denaturing conditions 6 M urea was included in the running buffer. SDS/PAGE was performed as described by Laemmli [25].

Analytical ultracentrifugation

A solution of the small subunit was equilibrated with 50 mM potassium phosphate buffer, pH 7.8, by gel filtration on Superose-12 (Pharmacia) and diluted to an A_{214} of 0.2. This preparation was used in sedimentation equilibrium experiments on a Beckman Optima XL-1 instrument in an AN-60Ti rotor fitted with charcoal-filled Epon centrepieces (path length 11.42 mm). Centrifugation was performed at 5000, 16000 and 44000 ***g*** and 4 °C and the cells were scanned at 214 nm (for protein) and 360 nm (for baseline correction) after 12, 13, 14 and 15 h. The results were evaluated with Beckman Optima XL-A data analysis software and psi analysis [26,27] with a partial specific volume of 0.74 ml/g and a density of 1.0053 g/ml.

Sequence determinations

DNA sequencing was performed with the Dye Terminator Cycle Sequencing Ready Reaction Kit (ABI Prism) and DNA Sequencer 373A (Applied Biosystems). For the determination of N-terminal amino acid sequences, samples (10 pmol) were subjected to Edman degradation with an automated gas-phase sequencer (Applied Biosystems 470A).

Assay of AHAS

Two different methods were used to assay AHAS. A continuous assay [5] was conducted at 37 °C in which the disappearance of pyruvate was monitored at either 333 nm (ϵ 17.5 $M^{-1}\cdot cm^{-1}$) or 320 nm (ϵ 21.5 $M^{-1}\cdot cm^{-1}$). The assay contained 100 mM Tricine/NaOH or 50 mM potassium phosphate buffer, pH 7.8, 50 or 100 mM pyruvate, 1 mM ThDP, 10 mM $MgCl_2$ and 10 μM FAD. Where noted, a more sensitive single-point colorimetric assay [28] was used. The assay buffer composition was identical with that used for the continuous assay and the reaction was stopped after 30 min at 37 °C by the addition of 50 % (v/v) H_2SO_4 to give a final concentration of 1 %. The acetolactate was converted to acetoin by incubation at 60 °C for 15 min. Acetoin was then quantified [29] by measuring the absorbance at 525 nm after incubation at 60 °C for 15 min with 0.15 % creatine and 1.54 % (w/v) α-naphthol. The absorption coefficient of the coloured complex produced was quite sensitive to the assay conditions employed; the value used in the calculation of parameters reported here was 16164 $M^{-1}\cdot cm^{-1}$. Enzyme and assay buffers were preincubated at 37 °C for 5–15 min before the initiation of the reaction. One unit of activity is defined as that producing 1 μmol of acetolactate/min under the above conditions.

Reconstitution

Time course

Purified large and small subunit solutions were equilibrated at 37 °C. To initiate reconstitution, a 40-fold molar excess of small subunit was added to the solution of large subunit. Samples of the mixture were taken at intervals and assayed for AHAS activity with the colorimetric assay.

Saturation kinetics

These experiments were conducted at low (100.5 nM) and high (1.84 μM) concentrations of large subunit. Because of this difference, different AHAS assay methods were adopted for each experiment. The latter used the continuous assay, whereas the former required the more sensitive colorimetric method. A fixed concentration of the large subunit was incubated with various concentrations of small subunit (0–4.02 μM for low concentration and 0–7.89 μM for high concentration) at 37 °C for 15 min before the reaction was initiated.

CD spectroscopy

CD spectra were recorded over the range 185–350 nm on a Jasco J-710 spectropolarimeter set at 20 mdeg sensitivity, 1 nm resolution, 1 unit accumulation, 2 s response and a scanning speed of 200 nm/min. Protein solutions of approx. 250 μg/ml were measured in a 300 μl cylindrical quartz cell of 1 mm path length.

FAD spectra

Absorbance spectra were measured on a Molecular Devices SPECTRAmax-250 microplate spectrophotometer. Protein solutions (250 μl, at 0.4–4.0 mg/ml) were dispensed into the wells of a UV-transparent multi-well plate and the absorbance was measured over the range 350–500 nm at intervals of 1 nm. The baseline absorbance of the storage buffer was subtracted to obtain the spectrum of bound FAD.

Removal of FAD

A 25 % (w/v) suspension of activated charcoal was prepared in 20 mM Tris/HCl buffer, pH 7.9, containing 500 mM NaCl. The

suspension was added (0.5% final concentration) to an AHAS subunit solution (8.1 mg/ml large subunit or 0.2 mg/ml small subunit), mixed and incubated on ice for 5 min. The charcoal was removed by centrifugation at 4 °C for 5 min. Absorbance measurements at 454 nm (λ_{max} of FAD), and the absence of AHAS activity from solutions of the treated protein, confirmed the removal of FAD.

Kinetic data analysis

The appropriate equations were fitted to the kinetic data by nonlinear regression with the programs DNRPEASY (modified from DNRP53 [30]) and InPlot4.

RESULTS

Molecular mass

The expected molecular masses of the expressed large and small subunits were calculated from the pET30a(+), pT7-7 and *E. coli* *ilv*GM DNA sequences. The purified fusion enzyme was resolved into two components by SDS/PAGE (Figure 1). Estimates of the molecular masses of the two components for the fusion enzyme are 63.2 (expected 64.7) and 10.8 (expected 9.6) kDa. SDS/PAGE of the individually purified subunits revealed a single molecular species in each case (Figure 1). Estimates of the molecular masses gave values of 67.2 (expected 64.7) kDa for the large subunit and 16.2 (expected 14.7) kDa for the small subunit. The molecular mass of the large subunit was determined by gel filtration under non-denaturing conditions; this method gave a value of 130 kDa, consistent with that expected (129.4 kDa) for a dimeric structure. Estimation of the molecular mass of native AHAS II by gel filtration gave a value of 119 kDa, which is somewhat smaller than that expected (138.0 kDa) for a heterotetramer. Gel filtration of the small subunit yielded a molecular mass of 26 kDa, although there was some uncertainty and variability in this estimate. Gel filtration of the small subunit in 6 M urea also gave a molecular mass higher than that expected (14.7 kDa) for a monomer. Consequently it was unclear from these experiments whether the small subunit is an asymmetric monomer or a compact dimer. To clarify this issue, sedimentation equilibrium experiments were undertaken. The results were consistent with a preponderance of a species with a molecular mass of 12.5 kDa, together with some aggregates of much larger size (approx. 70 kDa). Thus we conclude that the small subunit is predominantly monomeric.

During purification of AHAS II and its subunits, care was taken to exclude light wherever possible. Deliberate exposure of intact AHAS II to light for several hours resulted in polymerization, as judged by the mobility of the protein under SDS/PAGE. We speculate that this process results from FAD-mediated photo-oxidation. Trace amounts of these polymers were present in the fusion enzyme purified by IMAC only (Figure 1, lanes 3 and 4).

N-terminal sequence

N-terminal sequencing of the large subunit expressed with the fusion peptide gave a methionine residue followed by exactly six blank cycles where histidine residues were expected, then a serine residue. Blank cycles have been observed when sequencing through oligohistidine regions of other proteins at this institution (R. Smith, personal communication). This sequence of amino acids corresponds to the first eight residues expected of this form of the large subunit. When the oligohistidine tag was removed from the large subunit by digestion with thrombin and the same analysis was performed, the expected seven-residue sequence GSGMKET was observed. Similarly three cycles of N-terminal sequencing on the large subunit expressed in the native form gave the expected MNG sequence.

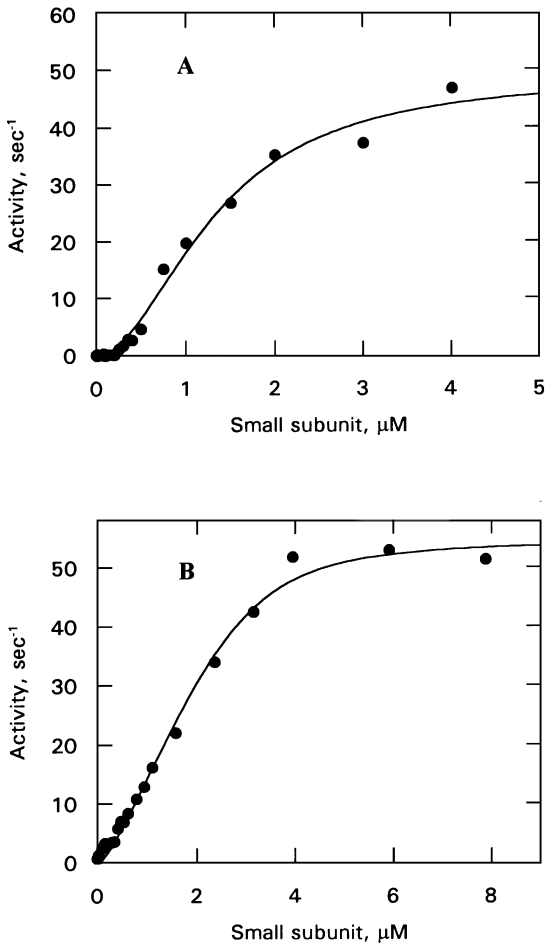

Figure 2 Reconstitution of *E. coli* AHAS II from its isolated subunits

Large subunit was present at 100.5 nM (**A**) or 1.84 μM (**B**). Activities are expressed in mol of acetolactate formed/s per mol of large subunit. The lines are the best fit of eqns. (2–6) with $K_1 = 40\ \mu$M, $k_{cat} = 49.3 \pm 1.8\ s^{-1}$ (**A**) or $54.6 \pm 1.2\ s^{-1}$ (**B**), and $K_2 = 38.3 \pm 4.6$ nM (**A**) or 17.2 ± 2.6 nM (**B**).

Reconstitution

The highly purified large and small subunits each exhibited a trace of AHAS activity; when these preparations were mixed there was a massive increase in this activity. The activity as a function of the small subunit concentration is shown in Figure 2. The difference between the two experiments is an 18-fold variation in the large subunit concentration, which necessitated the use of different activity assay methods. Figure 2(A) presents the data obtained at a low concentration of large subunit with the more sensitive single-point colorimetric assay, whereas the data of Figure 2B were obtained at a high concentration of large subunit with the continuous assay. Both curves are sigmoidal: this is particularly noticeable at the lower concentration of the large subunit (Figure 2A). Replotting the activity against the square of the small subunit concentration (results not shown) gave curves that were hyperbolic. Although this would suggest

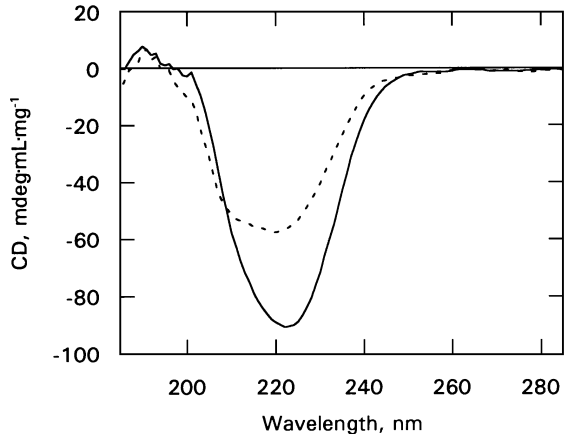

Figure 3 CD spectra of the *E. coli* AHAS II large subunit

Preparations that were active in reconstitution (solid line) show a different spectrum from an inactive preparation (broken line).

that reconstitution involves the combination of S_2 and L_2 dimers, the sedimentation equilibrium data suggested that the small subunit is monomeric. This contradiction is resolved if binding of the small subunits is sequential [eqn. (1)] and highly co-operative, with the dissociation constant for L_2S (K_1) much larger than that for L_2S_2 (K_2). This model is described by the system of simultaneous equations given in eqns. (2–6):

$$L_2 \overset{K_1}{\rightleftharpoons} L_2S \overset{K_2}{\rightleftharpoons} L_2S_2 \quad (1)$$

$$[L]_T = 2([L_2] + 2[L_2S] + [L_2S_2]) \quad (2)$$

$$[S]_T = [S] + 2[L_2S] + 2[L_2S_2] \quad (3)$$

$$K_1 = [L_2][S]/[L_2S] \quad (4)$$

$$K_2 = [L_2S][S]/[L_2S_2] \quad (5)$$

$$v = 2k_{cat}[L_2S_2] \quad (6)$$

Numerical solution of the above equations permitted the model to be fitted to the data; these fits describe the experimental data very well under the two different conditions used. It was not possible to estimate K_1 accurately, so a value of 40 μM was assumed. The best-fit values for k_{cat} in the two experiments were 49.3 ± 1.8 s^{-1} (Figure 2A) and 54.6 ± 1.2 s^{-1} (Figure 2B), whereas the values for K_2 were 38.3 ± 4.6 and 17.2 ± 2.6 nM respectively. Thus if the small subunit is monomeric, the binding of the first small subunit is about 1/1700 as strong as that of the second.

The time dependence of activity reconstitution was examined: nearly full activity was detected after 20 s and maximum activity was attained after about 8 min. The reconstitution reactions shown in Figure 2 were performed after preincubation of the mixture of subunits at 37 °C for 15 min. Some preparations of large subunits could not be reconstituted by addition of the small subunit. The reason for these occasional failures is not clear, although such preparations could be distinguished clearly from those that could be reconstituted by CD spectroscopy (Figure 3).

The cofactor and substrate requirements for the physical association of the subunits were examined by gel-filtration chromatography. The size differences between the large subunit dimer and small subunit monomer were great enough for them to be well separated by gel filtration, with retention times of 30.21 and 35.65 min respectively (results not shown). Unfortunately the large subunit dimer and the reconstituted enzyme were too similar in elution times to be distinguished clearly. The association of the subunits was monitored by the disappearance of the small subunit peak. Physical interaction of the subunits occurred irrespective of the addition of substrate, ThDP or Mg^{2+}. To test the requirement for FAD it was necessary to remove this cofactor, which was routinely added to the storage buffer. This was accomplished by treatment with activated charcoal. Gel filtration in the absence of FAD demonstrated that this cofactor is not necessary for the association of the subunits. After removal of FAD, no AHAS activity was detected on mixing of the large and small subunits. Addition of FAD allowed 95.7 % recovery of activity (results not shown). Spectral measurements (results not shown) indicated that the large subunit alone does not bind FAD, whereas the enzyme reconstituted in the presence of FAD contains this cofactor.

Table 3 Kinetic properties of *E. coli* AHAS II

Abbreviations: CE, chlorimuron ethyl; CS, chlorsulfuron; IM, imazapyr; n.d., not determined.

Parameter	Native	Fusion	Reconstituted
k_{cat} (s^{-1})	66.7 ± 0.7	47.4 ± 0.9	67.5 ± 1.3
K_m (mM)	2.6 ± 0.2[a]	2.9 ± 0.1*	
		5.2 ± 0.4†	4.7 ± 0.3†
K_i (app) CE (nM)	n.d.	24 ± 3	15 ± 3
K_{is} CS (μM)	0.30 ± 0.03	0.29 ± 0.02	n.d.
K_{ii} CS (μM)	1.07 ± 0.11	0.76 ± 0.04	n.d.
K_i (app) CS (μM)‡	0.42 ± 0.05	0.70 ± 0.05	0.55 ± 0.05
K_i (app) IM (mM)‡	2.5 ± 0.4	3.8 ± 0.4	6.1 ± 0.3

* Assayed in 100 mM Tricine/NaOH buffer, pH 7.8.
† Assayed in 50 mM potassium phosphate buffer, pH 7.8.
‡ Determined at [pyruvate] equal to K_m.

Catalytic properties

The results of the kinetic studies are summarized in Table 3. There are no major differences observed between the native, fusion and reconstituted AHAS II in terms of the catalytic properties investigated. Although the reaction catalysed involves two molecules of pyruvate, the saturation curve for this substrate is hyperbolic for both the intact forms and the reconstituted enzymes. The K_m values for intact and reconstituted forms are the same, although a dependence on the assay buffer was observed. The k_{cat} for the reconstituted enzyme is slightly higher than that for the intact form. This result demonstrates the effectiveness of reconstitution and suggests that the isolated 'intact' enzyme might not be fully saturated with small subunit. The activity of the intact AHAS was unaffected by the addition of the branched-chain amino acids.

AHAS activity is known to be inhibited by herbicides such as sulphonylureas and imidazolinones. Chlorimuron ethyl and chlorsulfuron belong to the former class; imazapyr is a member of the latter. Inhibition by each of these was examined. The continuous assay was used for the first two inhibitors and initial rates were measured to avoid complications arising from slow-binding inhibition [31]. For experiments with imazapyr, which has a significant absorbance at the concentrations necessary to inhibit AHAS II, the colorimetric assay was used with a relatively short assay time of 10 min.

Of the three herbicides tested (Table 3), chlorimuron ethyl was the most potent. However, the inhibited rate (v_i) as a function of the total chlorimuron ethyl concentration ($[I]_0$) did not follow a

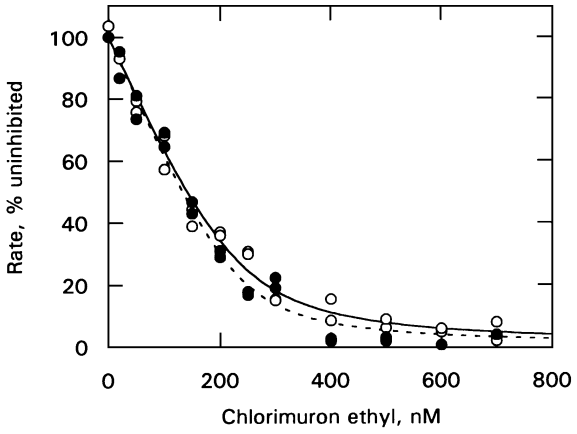

Figure 4 Inhibition of *E. coli* AHAS II by chlorimuron ethyl

Rates were measured as a function of the concentration of chlorimuron ethyl and the data were fitted to eqn. (7); $[E]_0$ was 234 nM. The fitted lines, scaled as a percentage of the uninhibited rate, are shown for the fusion (○, solid line; K_i (app) = 24 ± 3 nM) and the reconstituted (●, broken line; K_i (app) = 15 ± 3 nM) forms of the enzyme.

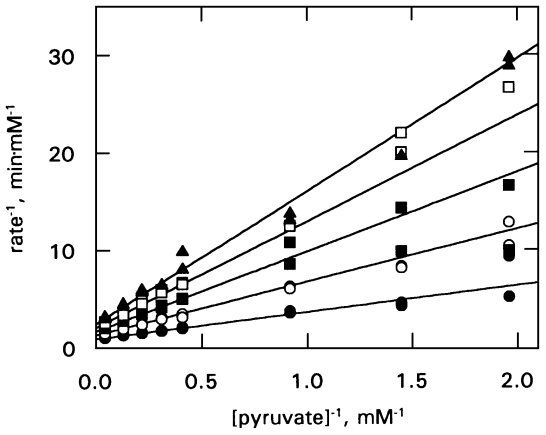

Figure 5 Inhibition of fusion *E. coli* AHAS II by chlorsulfuron

Rates, expressed as mM of acetolactate formed per min, were measured as a function of the concentration of pyruvate at fixed chlorsulfuron concentrations of 0 (●), 0.3 (○), 0.6 (■), 0.9 (□) and 1.2 (▲) μM. The data were fitted to eqn. (8) and are shown in the form of a double-reciprocal plot. The fitted lines are described by $V_{max} = 1.058 \pm 0.016$ mM/min, $K_m = 2.86 \pm 0.12$ mM, $K_{is} = 0.30 \pm 0.02$ μM and $K_{ii} = 0.76 \pm 0.04$ μM.

normal hyperbolic curve (Figure 4). The enzymic activity decreased almost linearly with increasing herbicide concentration and could be extrapolated to meet the abscissa at a concentration comparable to that of AHAS II active sites ($[E]_0$). In effect the enzyme was being titrated by the herbicide and the concentration causing 50% inhibition was not equivalent to the apparent inhibition constant [K_i (app)]. This tight-binding inhibition is described by eqn. (7); fitting this equation to the data gave similar values of K_i (app) for the fusion and reconstituted enzymes (Table 3):

$$v_i^2[E]_0 + v_0 v_i\{[I]_0 - [E]_0 + K_i\,(app)\} - v_0^2 K_i\,(app) = 0 \quad (7)$$

Chlorsulfuron is a non-competitive inhibitor for both the native (Figure 5) and fusion forms of AHAS II; the values of K_{is} and K_{ii} were determined by fitting eqn. (8) to the data:

$$v = V_{max}/\{1 + [I]/K_{ii} + (K_m/[A])(1 + [I]/K_{is})\} \quad (8)$$

Each form of the enzyme has similar values for the two inhibition constants (Table 3). K_{is} is similar to that reported for *S. typhimurium* AHAS II [31], whereas K_{ii} is approx. one-seventh of the reported value. The value of K_i (app) for chlorsulfuron at a pyruvate concentration equal to the K_m was also determined for the native, fusion and reconstituted enzymes. The three forms of the enzyme gave comparable results (Table 3). Also shown is the effect of imazapyr on the fusion and reconstituted enzymes; each was inhibited to a similar extent, as judged by comparison of the values for K_i (app).

As previously mentioned, the individual large and small subunits each exhibited a trace of AHAS activity although this was 1/2000 or less of that of the fusion enzyme. In each case, this trace activity exhibited a K_m for pyruvate that was similar to the value obtained for the fusion enzyme.

DISCUSSION

The purification of native AHAS II reported here is a significant improvement over that reported previously [5], requiring fewer steps but resulting in a similar final specific activity. However, the pT7-GM:BL21(DE3) vector:host combination does not seem to be optimal, producing approx. 1% of the soluble protein as AHAS II.

Production of the fusion enzyme from pET-GM:BL21(DE3) is 24-fold greater and allows pure enzyme to be obtained in a single chromatographic step, with 65% recovery. Although the product contains a non-native 50-residue N-terminal fusion on the large subunit, its kinetic properties are very similar to those of the native enzyme (Table 3). Part of this peptide can be removed by IMAC after digestion with thrombin, although there seems to be no real benefit from this procedure, which was not applied routinely.

The production of individual subunits from pET-G and pET-M allowed them to be isolated in pure form after a single chromatographic step; we have been able to demonstrate, for the first time, reconstitution of AHAS II from the pure subunits.

Each of the individual subunit preparations exhibited some AHAS activity but this is unlikely to have been due to contamination with enzyme from the host, as this would be eluted from the IMAC column during washing with 25 mM imidazole. We suggest that these trace activities are due to association of the expressed subunit with its partner from the host. This seems to be particularly likely for the small subunit, for which an intrinsic AHAS activity seems improbable. The observation that the K_m for pyruvate is, in each case, similar to that of the intact enzyme lends support to this interpretation. Irrespective of the source of these trace activities, they were so low that they were virtually undetectable and do not compromise the validity of the reconstitution experiments presented here.

Critical to our success in obtaining active preparations of the fusion enzyme and the individual subunits in high yields has been induction of the cells at room temperature, approx. 22 °C. Induction at higher temperatures resulted in a large proportion of the expressed proteins fractionating with the insoluble material. In this context it is of interest that active enzyme could not be reconstituted from some preparations of large subunits; these had clear differences in the CD spectrum. We suggest that correct folding of the large subunit is favoured only marginally and small variations in the induction conditions result in an altered conformation that cannot be converted to active enzyme in the presence of the small subunit.

Reconstitution seems to follow a pathway in which there is successive, and highly co-operative, binding of two small subunit molecules to the large subunit dimer. However, the results do not

rule out alternative routes; for example, an equally good fit to the data in Figure 2 is obtained with the model $L_2 \rightarrow L \rightarrow LS \rightarrow L_2S_2$. Although gel filtration experiments indicate that the large subunit is dimeric, an equilibrium mixture containing a small fraction of monomer cannot be excluded, which would make this alternative route for reconstitution feasible.

None of the cofactors (FAD, ThDP and Mg^{2+}) is required for reconstitution so evidently each can bind to the L_2S_2 tetramer; it would be of interest to determine whether there is a defined order for cofactor binding. Irrespective of the route of subunit and cofactor association, it is clearly an efficient process and results in an enzyme that is at least as active as the fusion enzyme and has very similar kinetic properties with respect to substrate and towards herbicidal inhibitors.

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