Purification and Properties of *Salmonella typhimurium* Acetolactate Synthase Isozyme II from *Escherichia coli* HB101/pDU9†

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ABSTRACT: A facile purification has been devised for recombinantly produced *Salmonella typhimurium* acetolactate synthase isozyme II. Purification of the enzyme was made possible by determining the complex set of factors that lead to loss of enzymic activity with this rather labile enzyme. When complexed with thiamin pyrophosphate, FAD, and magnesium, acetolactate synthase is subject to oxygen-dependent inactivation, a property not shared by the enzyme–FAD complex. When divorced from all of its tightly bound cofactors, losses of the enzymic activity are encountered at low ionic strength, especially at low protein concentrations. If purified and stored as the enzyme–FAD complex, acetolactate synthase is quite stable. The enzyme is composed of two types of subunits, a result that was not anticipated from previous studies of *ilvG* (the gene that codes for the large subunit of acetolactate synthase). These subunits were determined to be in equal molar ratio in the purified enzyme from the distribution of radioactivity between the two subunits after carboxymethylation with iodo[^{14}C]acetate and their respective amino acid compositions. Besides the expected *ilvG* gene product (59.3 kDa), purified acetolactate synthase contained a smaller subunit (9.7 kDa; designated here as the *ilvM* gene product). On the basis of sequence homology of the small subunit with that coded for by the corresponding *Escherichia coli* gene sequence [Lawther, R. P., Calhoun, D. H., Adams, C. W., Hauser, C. A., Gray, J., & Hatfield, G. W. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 922–925], it is encoded by the region between *ilvG* and *ilvE*, beginning at base-pair (bp) 1914 (relative to the point of transcription initiation). Amino-terminal sequence analysis of the large subunit of acetolactate synthase identifies the initial position of translation for *ilvG* as bp 271. The stop and start codons of *ilvG* and *ilvM* overlap. Overlapping stop and start codons have been observed for several other genes whose gene products are produced in equal amounts. Native acetolactate synthase has a molecular weight of about 140 000 and is thus composed of two large and two small subunits.

Acetolactate synthase (EC 4.1.3.18) catalyzes the first common step in the biosynthesis of branched-chain amino acids. Two molecules of pyruvate are condensed to form α-acetolactate and CO_2 in the first step of the valine and leucine pathway, or one molecule of pyruvate and one of α-ketobutyrate are condensed to form α-aceto-α-hydroxybutyrate as the first step of isoleucine biosynthesis. In *Escherichia coli*, as many as six isozymes (some cryptic) of acetolactate synthase, each encoded by a separate gene (*ilvB*, *ilvG*, *ilvIH*, *ilvJ*, which code for isozymes I, II, III, and IV, respectively, *ilvF*, and *ilvK*), have been described (Pittard et al., 1963; Ramakrishnan & Adelberg, 1964, 1965; Blatt et al., 1972; O'Neill & Freundlich, 1972, 1973; Guardiola et al., 1974, 1977; De Felice et al., 1974a,b, 1977, 1978, 1979, 1982; La Cara & De Felice, 1979; Favre et al., 1976; Lawther et al., 1981; Squires et al., 1981, 1983a,b; Abrescia et al., 1979; Robinson & Jackson, 1982; Jackson et al., 1981; Madu & Jackson, 1983; Alexander & Jackson, 1983). Of these six isozymes, only the *ilvB* gene product, also known as isozyme I, has been purified (Grimminger & Umbarger, 1979; Eoyang & Silverman, 1984). Recently, it was reported that isozyme I is composed of two dissimilar polypeptides (Eoyang & Silverman, 1984). The larger subunit (60 kDa) is presumably encoded by *ilvB*, and the smaller subunit (9.5 kDa) was not anticipated on the basis of previous work with *ilvB*.

Despite detailed studies on the genetics and regulation of acetolactate synthase [for review, see De Felice et al. (1982)], little progress has been made in understanding the mechanism of action of this rather unusual flavoenzyme. Acetolactate synthase is one of several enzymes that have an absolute requirement for flavin and yet catalyze a reaction involving no net oxidation or reduction. For this enzyme and other "nonredox" flavoenzymes, e.g., mandelonitrile lyase (EC 4.1.2.10, also known as oxynitrilase) (Becker et al., 1963; Jorns, 1979), chorismate synthase (EC 4.6.1.4) (Morell et al., 1967; Welch et al., 1974; Hasan & Nester, 1978), and tartronate-semialdehyde synthase (EC 4.1.1.47, also known as glyoxylate carbo-ligase) (Gupta & Vennesland, 1964, 1966; Chung et al., 1971; Cromartie & Walsh, 1976), there is no obvious mechanistic role for the flavin. One limitation to study of acetolactate synthase is the lack of a ready source of large quantities of purified enzyme. Although several of the bacterial isozymes have been cloned, extreme lability of the enzyme has hampered purification efforts (Grimminger & Umbarger, 1979).

An increased interest in the mechanism of action of acetolactate synthase has ensued with the realization that this enzyme is the site of action of two different new classes of herbicides. The sulfonylurea herbicides sulfometuron methyl and chlorsulfuron (marketed by Du Pont as the active ingredients in Oust and Glean, respectively) and the imidazolinone herbicides 2-(4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl)nicotinic acid and 2-(4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl)-3-quinolinecarboxylic acid (marketed by American Cyanamid as the active ingredients in Arsenal and Scepter, respectively) exhibit biologic activity by virtue of their inhibition of acetolactate synthase (LaRossa & Schloss, 1984; Ray, 1984; Chaleff & Mauvais, 1984; Falco

† A preliminary report of these results has been published (Kutny & Schloss, 1984). This article is dedicated to the late Dr. R. O. Burns of Duke University.

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& Dumas, 1984; Falco et al., 1984; Shaner et al., 1984a,b). In the case of sulfometuron methyl, inhibition appears to be active site directed as the inhibitor competes for the second pyruvate binding site (Schloss, 1984). A detailed understanding of the mode of action of these rather novel inhibitors requires a fuller understanding of the mechanism of action of acetolactate synthase than is currently available.

We describe here a new purification scheme for the *Salmonella typhimurium* acetolactate synthase isozyme II, starting with the recombinant source *E. coli* HB101/pDU9. This protocol yields large quantities (about 1 g) of fully active enzyme in high yield and employs commercially available chromatographic media. Several chemical, physical, and kinetic properties of the purified enzyme are reported.

Materials and Methods

Materials. FAD,[1] thiamin pyrophosphate, cytochrome *c*, malate dehydrogenase, NADH, Tris, Sephacryl S-200, phenyl-Sepharose CL-4B, Sephadex G-100, and sodium pyruvate were obtained from Sigma Chemical Co. Tricine and dithiothreitol were from Research Organics Inc. Puratronic potassium chloride, cadmium chloride, magnesium chloride, manganese chloride, cobaltous chloride, aluminum chloride, and nickel chloride were purchased from Alfa. Ultrapure ammonium sulfate was the product of Schwarz/Mann, and a 4 M stock solution was further purified by passage through Chelex 100 (Bio-Rad) prior to use. 1-Naphthol was obtained from MCB. Since the background color in the fixed-time assay of acetolactate synthase is primarily due to impurities in the naphthol, this reagent was further purified either by recrystallization from chloroform or by sublimation (the latter was kindly carried out by Dr. G. J. Sloan of Du Pont). Fractogel TSK DEAE-650M was purchased from EM Science. (Acetylmethyl)carbinol (acetoin), a standard used in the fixed-time assay, was from Aldrich Chemical Co. Bovine liver catalase was from Calbiochem. Ribulosebisphosphate carboxylases from *Rhodospirillum rubrum* and spinach were purified by published procedures (Schloss et al., 1982; Lorimer et al., 1976). Aldolase was purchased from Boehringer Mannheim.

Assay of Acetolactate Synthase. The fixed-time assay (Bauerle et al., 1964) was conducted at 37 °C as previously described (LaRossa & Schloss, 1984). Alternatively, a continuous assay was employed, in which the consumption of pyruvate was monitored directly at 333 nm. Although the extinction of pyruvate at 333 nm is extremely small (17.5 M^{-1} cm^{-1}), the equilibrium of this reaction strongly favors acetolactate formation, and acetolactate has negligible absorbance at 333 nm. The assay consisted of 0.1 M Tricine–NaOH, at its p*K* (pH 7.8 at 37 °C), 50 mM sodium pyruvate, 10 mM $MgCl_2$, 0.1 mM thiamin pyrophosphate, and 0.1 mM FAD. Most of the absorbance of pyruvate at 333 nm (≥94%) was consumed at equilibrium, and as expected, a 50 mM solution of pyruvate gave rise to 25 mM acetolactate (determined as acetoin after treatment with acid). Pyruvate decomposes in a base-dependent reaction to give a product with similar extinction (about 79%) at 333 nm (presumably an aldol condensation product). This product is not a substrate for acetolactate synthase and not a potent inhibitor of the enzyme. However, the residual absorbance of pyruvate solutions at 333 nm cannot be used for a reliable estimate of the equilibrium constant. Activity determinations with the direct assay were in perfect agreement with those made by the fixed-time assay. One unit of activity is the amount required to form 1 μmol of acetolactate (consume 2 μmol of pyruvate) per minute under the assay conditions described above. Protein concentrations were determined by the biuret procedure (Lyane, 1957).

Amino Acid Analyses and Sequencing. Total acid hydrolysis of purified acetolactate synthase and its resolved subunits was achieved in an evacuated (<50 μmHg) sealed tube with 6 N HCl–0.1% 2-mercaptoacetic acid at 110 °C for 21 h. Losses of amino acids due to destruction during hydrolysis are uncorrected. Tryptophan was determined after hydrolysis of samples with mercaptoethanesulfonic acid (Penke et al., 1974). Cysteine–cystine was determined as cysteic acid after hydrolysis with 6 N HCl–0.25 M dimethyl sulfoxide (Spencer & Wold, 1969) or as *S*-(carboxymethyl)cysteine from samples that had been carboxymethylated. Hydrolysates were analyzed on a Beckman Model 119CL amino acid analyzer with ninhydrin detection.

Before and after S-carboxymethylation, the enzyme was subjected to Edman degradation in an Applied Biosystem Inc. Model 470A protein sequencer using the manufacturer's program 01RUNT. Anilinothiazolinones obtained in Edman degradation were automatically converted to their corresponding phenylthiohydantoins (by use of 25% aqueous trifluoroacetic acid according to the manufacturer's instructions) and analyzed directly by HPLC (Glajch et al., 1985). All data reduction was via an HP3357 laboratory automation system, RTE-VI operation system.

S-Carboxymethylation. Before carboxymethylation, 1 mL of 20 mg/mL acetolactate synthase was dialyzed against 2 L of 0.1 M potassium phosphate (pH 8.1), 1 mM EDTA, and 0.1 mM dithiothreitol for 6 h at room temperature. To the dialyzed enzyme were added 25 μL 0.1 M dithiothreitol and 1.7 g guanidine hydrochloride. This solution was chilled to ice temperature, and a second addition of dithiothreitol (25 μL, 0.1 M) was made. Then, 186 mg of iodoacetic acid and 101 mg of $NaHCO_3$ were dissolved in 1 mL of water, and 140 μL of this solution was mixed with 20 μL of iodo[^{14}C]acetic acid (37.6 mM, nominally 29 500 dpm/nmol). The 160 μL of diluted radioactive iodoacetate was added to the chilled enzyme solution and allowed to incubate on ice for 20 min, at which time 20 μL of 2-mercaptoethanol was added. This solution was dialyzed (Spectrapor 6 membrane tubing, molecular weight cutoff 3500) against 2 L of 0.1 M potassium phosphate (pH 8.1), 1 mM EDTA, and 10 mM 2-mercaptoethanol for 4 h at 4 °C, followed by exhaustive dialysis (3 times vs. 2 L each) against 2.5 mM EDTA (pH 8) and 1 mM 2-mercaptoethanol.

Data Reduction. Kinetic data were fit to

$$v = VS/(K + S) \quad (1)$$

where V is maximum velocity, S is substrate or cofactor concentration, and K is the concentration giving half-maximal rate (Michaelis constant), or to

$$v = VS^2/(S^2 + K_1S + K_2) \quad (2)$$

where V and S are again maximum velocity and substrate concentration and K_1 and K_2 are the saturation constants for the two pyruvates involved in the reaction, using the MLAB data modeling program (Knott, 1979). Equation 2 corresponds to the expected rate equation for the mechanism

$$E \underset{k_2}{\overset{k_1S}{\rightleftharpoons}} ES \underset{k_4}{\overset{k_3}{\rightleftharpoons}} ES' \underset{k_6}{\overset{k_5S}{\rightleftharpoons}} ES'S \underset{k_8}{\overset{k_7}{\rightleftharpoons}} EP \xrightarrow{k_9} E + P$$

where $V = k_1k_3k_5k_7k_9E/[k_1k_3k_5k_7 + k_1k_3k_5(k_8 + k_9) +$

[1] Abbreviations: Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; Tris, tris(hydroxymethyl)aminomethane; FAD, flavin adenine dinucleotide; TPP, thiamin pyrophosphate; ALSII, acetolactate synthase isozyme II; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetate.

$k_1k_5k_7k_9]$, $K_1 = [k_1k_3k_6(k_8 + k_9) + k_1k_3k_7k_9 + k_1k_4k_7k_9 + k_1k_4k_6(k_8 + k_9) + k_2k_5k_7k_9 + k_3k_5k_7k_9]/[k_1k_3k_5k_7 + k_1k_3k_5(k_8 + k_9) + k_1k_5k_7k_9]$, and $K_2 = [k_2k_4k_7k_9 + k_2k_4k_6(k_8 + k_9)]/[k_1k_3k_5k_7 + k_1k_3k_5(k_8 + k_9) + k_1k_5k_7k_9]$. In this mechanism, k_3 represents the step in which decarboxylation of the first pyruvate takes place, and P is the product of the overall reaction, acetolactate. Such a mechanism would exhibit hyperbolic saturation by pyruvate (eq 1), if k_2 or k_4 were much smaller than the forward rate constants (e.g., if pyruvate was "sticky" or if decarboxylation was kinetically irreversible). The Michaelis constant for such a mechanism, when hyperbolic saturation by pyruvate is observed, would be that given for K_1 with those terms containing k_2, k_4, or k_2 and k_4 missing.

RESULTS

Purification of Acetolactate Synthase. To 600 g of frozen cell paste (LaRossa & Schloss, 1984) of *E. coli* HB101/pDU9 was added 1 L of 0.1 M Tris-HCl (at its p*K*, equal molar Tris and Tris-HCl), 10 mM NaEDTA (prepared from a 0.1 M stock of Na_2EDTA adjusted to pH 8 with NaOH at room temperature), 1 mM dithiothreitol, and 0.1 mM FAD (final buffer pH 8.1 at room temperature). This suspension was incubated in a 37 °C water bath until the cell paste had thawed and a uniform suspension was obtained. After the suspension was chilled to 4 °C, it was subjected to full-power sonication for 5 min at 70% duty cycle with a Model W-375 sonicator (Heat Systems-Ultrasonics, Inc.). This was performed on 350-mL aliquots in a rosette cell with the medium tip (1.3-cm diameter), until the entire sample had been treated 4 times. The cell debris was then centrifuged in a Sorvall GSA rotor at 7000 rpm for 5 h (centrifugation can be extended overnight without affecting recovery of enzymic activity). The supernatant (1060 mL, 36 700 units, 56.3 g of protein) was divided into two equal portions, and each was incubated for 30 min in a 50 °C water bath (600-mL beaker, with stirring). After heat treatment, the solution was centrifuged at 7000 rpm for 30 min. To the supernatant (930 mL, 30 800 units, 45.6 g of protein) was added 164 g of ammonium sulfate (176 g/L of supernatant), and the sample was again centrifuged for 30 min. To this supernatant (980 mL, 25 700 units, 39.1 g of protein) was added 267 g of ammonium sulfate (273 g/L), and the precipitate was collected by centrifugation at 7000 rpm in a GSA rotor for 3 h. The resultant pellet was suspended with a minimal volume (about 50 mL) of 0.1 M Tris-HCl, 10 mM EDTA, 1 mM dithiothreitol, and 0.1 mM FAD (prepared as described above) followed by dilution with additional buffer until a final volume of 150 mL had been added (it is important not to fully dissolve the pellet, which can be accomplished transiently at this point, as this results in a lower yield), and the pooled suspension was allowed to stir in the cold room for 30 min. The suspension was centrifuged for 30 min (7000 rpm, GSA rotor), and the resultant pellet was dissolved in 90 mL of suspension buffer. This solution (125 mL, 26 100 units, 7.3 g of protein) could be frozen and stored indefinitely without loss of enzymic activity. Subsequent column chromatography was conducted at 4 °C, with exposure to light minimized by covering columns and the fraction collector compartment with aluminum foil. All columns were eluted at 80 mL/h, and 480-drop fractions were collected. To the solution of the second ammonium sulfate pellet was added 0.17 volume (21.2 mL) of 4 M Chelex-treated ammonium sulfate. This solution was applied to a 2.4 × 55 cm column of phenyl-Sepharose CL-4B. The column was eluted with a 2-L linear gradient consisting of 1 L of 1 M ammonium sulfate (Chelex treated), 0.1 M Tris-HCl, 10 mM EDTA, 1 mM dithiothreitol, and 20 μM FAD and 1 L of 25 mM Tris-HCl, 1 mM EDTA, 1 mM dithiothreitol, and 20 μM FAD. The gradient was followed by elution with 1 L of the limit buffer. Fractions containing acetolactate synthase activity were collected (96–108, 283 mL, 26 400 units, 2.44 g of protein) and concentrated (60 mL) by pressure dialysis with an Amicon PM30 membrane. The concentrate was applied to a 5 × 107 cm column of Sephacryl S-200, which was preequilibrated and eluted with the limit buffer used in phenyl-Sepharose chromatography. Fractions containing enzymic activity were collected (34–46, 292 mL, 23 400 units, 1.77 g of protein) and applied to a 5 × 108 cm column of DEAE-650M Fractogel TSK. The Fractogel column was eluted with an 8-L linear gradient consisting of 4 L of 25 mM Tris-HCl, 1 mM EDTA, and 1 mM dithiothreitol and 4 L of 0.1 M Tris-HCl, 10 mM EDTA, 1 mM dithiothreitol, 0.4 M KCl (Puratronic), and 0.1 mM FAD. The column packing had been suspended in the initial buffer and the pH of the suspension adjusted to 8.1 with HCl (at room temperature) followed by equilibration of the column with the initial buffer (about 6 L) prior to sample application. Fractions containing enzymic activity (134–145, 272 mL, 23 000 units, 910 mg) were pooled and concentrated to 11.2 mL by pressure dialysis. The concentrated enzyme could be stored frozen at −80 °C indefinitely without loss of enzymic activity. Purified ALSII has an extinction at 280 nm of 0.90 ± 0.02 A mg^{-1} mL^{-1} cm^{-1}. The purification scheme is summarized in Table I.

Table I: Purification of ALSII from *E. coli* HB101/pDU9 (600 g of Cell Paste, Wet Weight)

step	protein (g)	activity (units)	sp act. (units/mg)	yield (%)
extract	56.3	36 700	0.65	"100"
heat step	45.6	30 800	0.67	84
first ammonium sulfate step	39.1	25 700	0.66	70
second ammonium sulfate step	7.3	26 100	3.57	71
phenyl-Sepharose	2.44	26 400	10.8	72
Sephacryl S-200	1.77	23 400	13.2	64
DEAE-650M Fractogel TSK	0.91	23 000	25.3	63

Stability and the Metal Requirement of ALSII. Initial attempts to purify ALSII by a procedure similar to that described above, with the exception that all cofactors (TPP, Mg^{2+}, and FAD) were included throughout the purification, resulted in severe losses of activity (2% final recovery) primarily during anion-exchange chromatography. The low recovery of enzymic activity was in contrast to a similar recovery of protein, pure by the criteria of SDS gel electrophoresis and of similar mobility to enzyme prepared by the procedure outlined above (an indication that loss of enzymic activity was not the result of proteolysis). Crude ALSII retains a full complement of FAD, TPP, and M^{2+} even after extended dialysis at 4 °C (LaRossa & Schloss, 1984). Dilution of the crude enzyme (final concentration 0.2 μM) into an assay mixture not containing cofactors (FAD, TPP, and Mg^{2+}) at 37 °C results in the reversible loss of enzymic activity with a half-time of about 10 min. After incubation for several hours in the absence of cofactors (1 mM EDTA, assay buffer, no pyruvate) at 37 °C, the enzyme exhibits an absolute dependence on TPP, FAD, and Mg^{2+}. Full enzymic activity can be regained if the cofactors are supplemented in the assay, with no perceptible lag in the assay time course (<15 s). By contrast, if Mg^{2+} and/or TPP is present during the preincubation at 37 °C, irreversible losses of enzymic activity are encountered. After incubation of ALSII (0.2 μM) in the presence

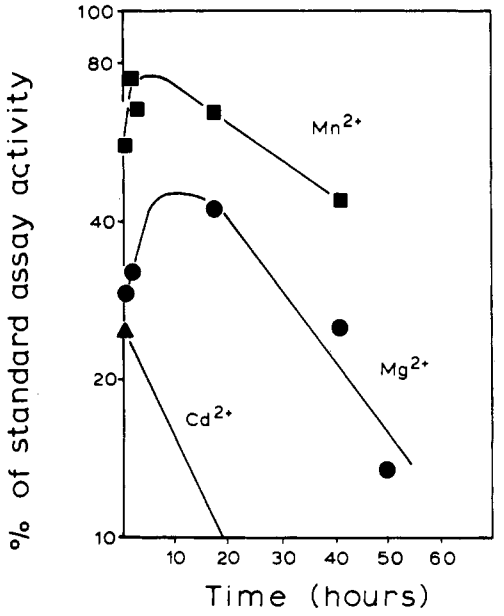

FIGURE 1: Activation of acetolactate synthase by Mn^{2+} (■), Mg^{2+} (●), or Cd^{2+} (▲). The relative enzymic activity (percent of maximal activity observed when the enzyme, not preactivated, was assayed under standard assay conditions) obtained after various times of preincubation of ALSII–FAD (at 4 °C) with thiamin pyrophosphate and metal upon dilution into an assay mixture (at 37 °C, not containing M^{2+}, thiamin pyrophosphate, or FAD, with 1 mM EDTA included) is illustrated.

of 0.1 mM TPP, 10 mM $MgCl_2$, and 0.1 mM FAD for 3 h, only 16% of the enzymic activity remained upon initiating the assay. A similar incubation in the presence of 0.1 mM FAD and 0.1 mM TPP resulted in 88% recovery; incubation in the presence of 0.1 mM FAD and 10 mM $MgCl_2$ resulted in 81% recovery; incubation in the presence of 0.1 mM TPP and 10 mM $MgCl_2$ resulted in 51% recovery.

When purified ALSII (0.2 mM) was preincubated on ice with 0.5 mM TPP and 2 mM metal and then diluted (100-fold) into an assay mixture containing no TPP, FAD, or metal (1 mM EDTA), the results illustrated in Figure 1 were obtained. After an initial activation of the enzyme, there was a slow loss of activity with a half-time of about 2 days. The relative maximal levels of activity achieved with various metals were (expressed as a percentage of the specific activity observed under standard assay conditions with Mg^{2+} as the activating metal) as follows: Mn^{2+}, 75%; Mg^{2+}, 42%; Cd^{2+}, 25%; Zn^{2+}, 4%; Ni^{2+}, 1.3%; Co^{2+}, 1.4%; Cu^{2+}, 1%; Al^{3+}, 0%; none, 0%. By contrast, if 2 mM metal was present in the assay mixture together with 0.1 mM FAD and 0.1 mM TPP (18 μM ALSII, not preactivated), the following relative activities were observed: Mn^{2+}, 136%; Mg^{2+}, "100%"; Co^{2+}, 83%; Ca^{2+}, 66%; Ni^{2+}, 38%, Cd^{2+}, 24% (66% at 0.4 mM, the enzyme precipitated with denaturation at the higher Cd^{2+} concentration); Zn^{2+}, 22%; Ba^{2+}, 19% (activation of the enzyme by Ba^{2+} proceeded with a pronounced lag, the half-time of which was about 6 min); Al^{3+}, 19%; Cu^{2+}, 9.8%; none, <0.006%. In the latter, metal-containing assays Tris buffer was substituted for Tricine due to the chelating properties of Tricine. The former set of assays, where metal was present during preincubation but not in the final assay mixture (excess EDTA present to prevent additional activation under assay conditions), will exhibit activity only if the activating metal forms a tight, slowly reversing complex with the enzyme and TPP, such that activity due to complex formation during preincubation is maintained upon dilution for the period of time required for assay (several minutes). In the latter set of assays, any metal capable of forming a productive complex with enzyme and TPP at the

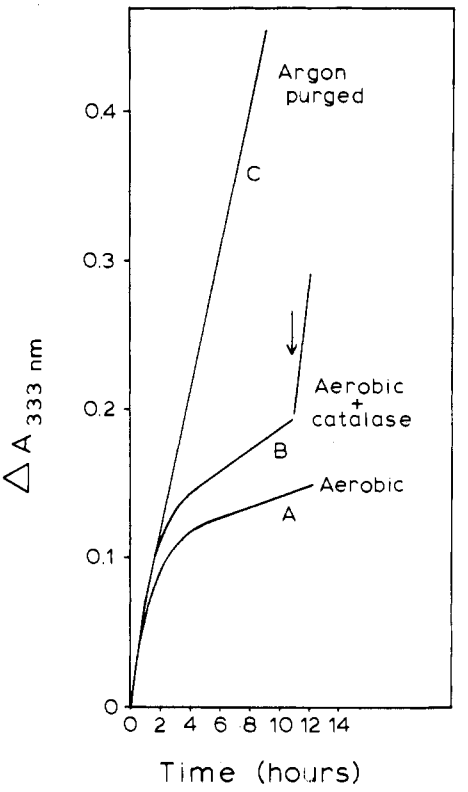

FIGURE 2: Assay progress curves (37 °C, standard assay mixture) in the absence (A) and presence (B) of catalase; also, an assay conducted under anaerobic conditions (C). The enzyme concentration in these assays was 3.3 μg/mL. A second addition of enzyme to curve B is indicated by an arrow.

concentration tested, regardless of the rate at which the complex dissociates, will exhibit activity. Thus, the difference between the two sets of assays is that the former assess the degree to which activation involves the formation of a slowly reversing complex while the latter assess only whether the metal activates.

A typical assay time course is illustrated in Figure 2. Under aerobic conditions, ALSII (48 nM) loses activity at 37 °C with a half-time of about 1.5 h. Supplementing the assay mixture with 1 mM dithiothreitol and/or 1 mg/mL bovine serum albumin had negligible effect on the time-dependent loss of activity. Addition of catalase (0.13 mg/mL, 3800 unit/mL) to the assay had a moderate effect on the stability of ALSII (Figure 2). A second addition of ALSII, after decay of the enzymic activity, resulted in an uninhibited initial rate. The lack of inhibition of fresh enzyme suggests inhibition is not due to the accumulation of an exogenous inhibitor in the assay but rather an alteration of the enzyme. When the assay mixture was purged with argon prior to assay (Figure 2), substantial protection of the enzyme during an 8-h assay resulted. In all cases, decay of the enzymic activity was not complete, but approached a new steady-state rate characteristic of the assay conditions used. Decay of enzymic activity appears to be oxygen-dependent, somewhat affected by catalase, but unaffected by thiol. Furthermore, loss of enzymic activity is dependent on the activation state of ALSII, as ALSII–FAD can be incubated for extended periods at 4 °C (at least 1 week) or 3 days at room temperature, with no loss of activity. By contrast, ALSII–FAD–TPP–M^{2+} is labile even at 4 °C.

Subunit Composition and Sequence Analysis of ALSII. ALSII was homogeneous by the criteria of gel electrophoresis (Figure 3) when carried out under native conditions (Davis, 1964). When conducted in the presence of dodecyl sulfate

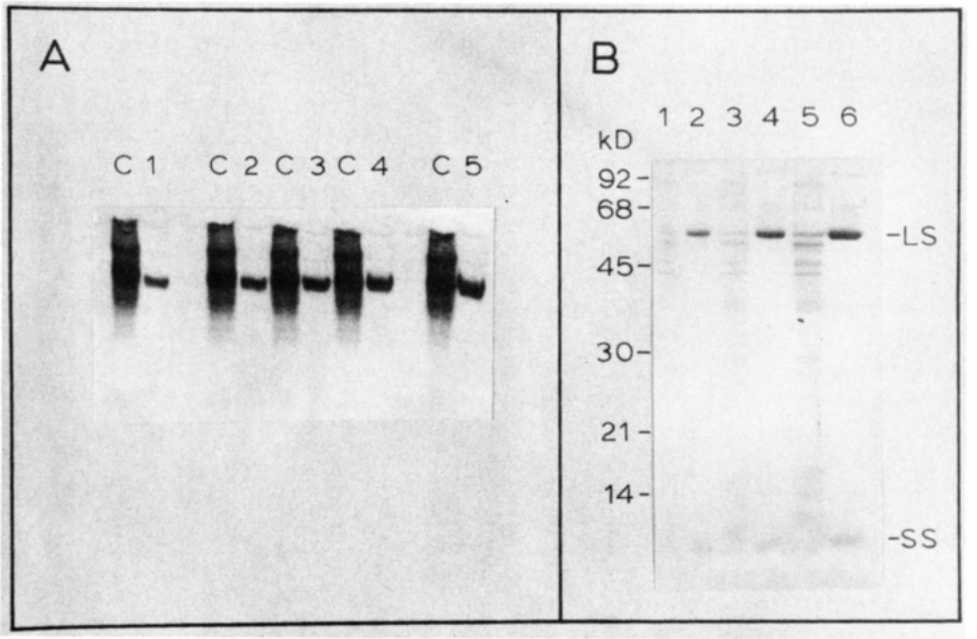

FIGURE 3: Polyacrylamide gel electrophoresis of purified ALSII under native (A) conditions and in the presence of dodecyl sulfate (B). (A) The native gel was prerun with 10 mg/100 mL FAD present in the cathode compartment. A Mini-Slab gel aparatus from Idea Scientific (Corvallis, OR) was used to run the native gels with 1–5 μg of ALSII in the lanes marked 1–5, respectively. Those lanes marked C contained about 30 μg of the initial crude extract. (B) An LKB slab-gel apparatus was used to run the lithium dodecyl sulfate–polyacrylamide gels with 5, 10, and 20 μg of ALSII in the lanes marked 2, 4, and 6. Crude extract was loaded in lanes 1, 3, and 5 (about 10, 20, and 50 μg of protein). The electrophoretic positions of several commercial molecular weights markers are designated at the left of the figure. The positions of the large and small subunits of ALSII are designated at the right of the figure by LS and SS, respectively.

(Weber & Osborn, 1969; Laemmli, 1970), gel electrophoresis resolved ALSII into two components (Figure 3). Estimates of the molecular weights of the two components of ALSII by comparison with standard molecular weight markers gave values of 59 000 and 9800. The former compares favorably to the expected molecular weight calculated from the *E. coli ilvG* gene sequence, 59 300 (Lawther et al., 1981). The latter was unexpected, especially since the isolated protein was presumed to derive from a plasmid-borne insert containing *S. typhimurium ilvG* (Blazey et al., 1981). The native molecular weight of ALSII was estimated by gel filtration with the method of Andrews (1970). A column of Sephacryl S-200 (5 × 107 cm) was calibrated with a mixture of FAD (M_r 786), horse heart cytochrome *c* (M_r 12 384; Margoliash, 1962), bovine heart malate dehydrogenase (M_r 65 000; Englard & Siegel, 1969), *Rhodospirillum rubrum* ribulosebisphosphate carboxylase (M_r 114 000; Tabita & McFadden, 1974), rabbit muscle aldolase (M_r 158 000; Kawahara & Tanford, 1966), and spinach ribulosebisphosphate carboxylase (M_r 557 000; Paulsen & Lane, 1966). ALSII eluted between *R. rubrum* ribulosebisphosphate carboxylase and aldolase; an apparent M_r of 140 000 was obtained by extrapolation.

Resolution of the large and small subunits of ALSII was initially attempted by gel filtration of the enzyme in the presence of SDS and dithiothreitol. After precipitation of 16 mg of ALSII with 2 volumes of ethanol, the precipitate was dissolved in 2 mL of 1% SDS and 0.005% dithiothreitol. This solution was applied to a 1 × 44 cm column of Sephacryl S-200, which was equilibrated and eluted (18 mL/h) with 1% SDS and 0.005% dithiothreitol. Fractions (1 mL each) 19–26, which represented the majority of the single prominent UV-absorbing (280-nm) peak eluted from the column, were pooled and subjected to SDS gel electrophoretic analysis. Both large and small subunits were present in the pool, and in similar ratio to that seen in untreated ALSII. Further, amino-terminal sequence analysis of this preparation gave a composite sequence, as though composed of two polypeptides in approximately equal molar ratio. Through cycle 35, one of the two amino acids liberated by the sequencer matched the amino acid sequence encoded by the corresponding gene sequence from *E. coli* (Lawther et al., 1981) beginning at position bp 271 (relative to the point of transcription initiation), with the exception of position 17 in which a lysine was observed rather than the expected asparagine. The remaining amino acid sequence matched that encoded by the region beginning immediately prior (bp 1914) to the termination of *ilvG*.

Resolution of the large and small subunits of ALSII was achieved by gel filtration of the carboxymethylated enzyme. The carboxymethylated, dialyzed ALSII described under Materials and Methods was brought into solution by the addition of 25 mg of SDS. A portion of this solution (2.3 mL, 3.63×10^5 cpm, 80% of total) was applied to a 2.4 × 59 cm column of Sephadex G-100, which was equilibrated and eluted with 1% SDS and 0.1% thiodiglycol. The distribution of radioactivity in the eluant of this column is illustrated in Figure 4. Two major peaks of radioactivity representing 3.12×10^5 cpm and 6.95×10^4 cpm were obtained, which on the basis of SDS gel electrophoretic analysis represent the large and small subunits of ALSII, respectively. Fractions containing the resolved large (27–33, 21.8 mL) and small (54–60, 23.2 mL) subunits were separately pooled and portions subjected to total acid hydrolysis. Amino acid analyses of the hydrolysates of the unresolved ALSII, large subunit, and small subunit revealed the presence of 11 cysteinyl residues per large–small subunit pair (69 kDa), nine (carboxymethyl)-cysteinyl residues per large subunit, and two (carboxymethyl)cysteinyl residues per small subunit, respectively (Table II). These are the predicted number of cysteinyl residues from the *E. coli* gene sequence (Lawther et al., 1981) and suggest conservation of these residues between *E. coli* and *S. typhimurium*. Overall, there is good correspondence between the observed amino acid compositions of the *S. typhimurium* subunits and those predicted for the large and small subunits from the *E. coli* gene sequence. Most notably, tyrosine and

Table II: Amino Acid Analysis of the Separated Large and Small Subunits of Acetolactate Synthase

	unfractionated (not carboxymethylated)		large subunit, M_r 59 300		small subunit, M_r 9700	
	found[a]	expected[b]	found[c]	expected[b]	found[c]	expected[b]
Asp	56 (51)	58	44	49	7	9
Thr	34 (33)	35	30	30	3	5
Ser	24 (27)	30	21	22	6	8
Glu	78 (67)	70	57	60	10	10
Pro	38 (32)	33	30	31	2	2
Gly	53 (47)	48	45	47	2	1
Ala	74 (70)	72	60	63	10	9
Cys[d]	11 (11)	11	9	9	2	2
Val	52 (54)	54	44	43	10	11
Met	21 (19)	24	16	20	3	4
Ile	24 (26)	25	22	21	4	4
Leu	67 (67)	67	60	60	7	7
Tyr	15 (12)	13	12	13	0	0
Phe	22 (20)	21	17	18	3	3
His	16 (13)	22	11	18	2	4
Lys	25 (20)	18	19	17	1	1
Arg	28 (25)	27	20	20	5	7
Trp	(4)	7	4	7	0	0

[a] Single analysis, sum of large and small subunit values in parentheses. [b] Expected amino acid composition for the corresponding *E. coli* enzyme (Lawther et al., 1981). [c] Average of three analyses. [d] As cysteic acid or as (carboxymethyl)cysteine.

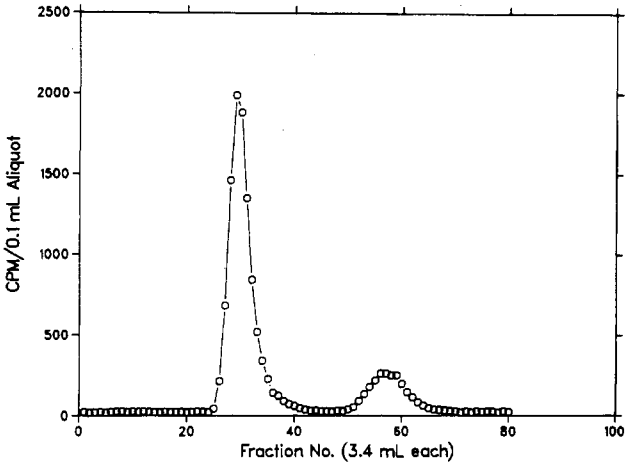

FIGURE 4: Distribution of radioactivity from [^{14}C]carboxymethylated ALSII obtained by gel filtration.

tryptophan, which were expected to be absent in the small subunit on the basis of the gene sequence, were not present. On the basis of the number of (carboxymethyl)cysteinyl residues present in the large and small subunits (nine and two, respectively), the ratio of radioactivity present in these two subunits would be 4.5 (9/2) if they were present in equal molar ratio. The observed ratio of radioactivity is 4.49 ($3.12 \times 10^5/6.95 \times 10^4$), demonstrating that the large and small subunits are equimolar. When aliquots of the isolated large subunit were subjected to Edman degradation, the following amino-terminal sequence was obtained: MNGAQWVVHALRAQGV*K*TVFGYPGGAIMPVYDALY. This sequence is in perfect correspondence with that encoded by the corresponding *E. coli* gene sequence, except for the previously observed substitution of a lysyl residue (italicized) for an asparaginyl residue at position 17. Amino-terminal analyses of the small subunit gave the following sequence: MMQHQVNVSARFNPETLERVLRVVR. This corresponds to the *E. coli* sequence through position 25.

Cofactors and Kinetics. As isolated, ALSII contains bound FAD. After exhaustive dialysis of 32 mg/mL enzyme (0.46 mM protomer) against 10 μM FAD and 0.1 M Tris-HCl, the enzyme retained 0.88 mol of FAD/mol of protomer (69 000 g) on the basis of the enzyme's absorbance at 450 nm (using the solution extinction of FAD). Since the absorption spectrum of FAD is slightly red shifted (3–9 nm) upon binding to ALSII (Schloss, 1984), this value should slightly underestimate the amount of FAD bound by the enzyme. Analyses for enzyme-bound organic phosphate by the method of Ames & Dubin (1960) gave 1.89 ± 0.06 mol of phosphate/mol of protomer, indicating 0.94 mol of FAD/mol of protomer. Extensive dialysis (6 days) of ALSII at room temperature against 2 M KCl, 0.1 M Tris-HCl, 10 mM EDTA, and 1 mM dithiothreitol removed most of the FAD from the enzyme (96% based on the absorbance at 450 nm). In the absence of FAD, the enzyme exhibited about 1.5% of its initial activity. When assayed under standard assay conditions (0.1 mM FAD), the enzyme had a specific activity of 23 units/mg, 92% of the specific activity prior to dialysis. Dilution of the FAD-free enzyme into 0.1 M Tris-HCl (1000-fold) prior to assay resulted in the loss of 86% of its enzymic activity. Inclusion of 1 mM dithiothreitol and 1 mg/mL bovine serum albumin in the dilution buffer afforded marginal protection against loss of activity (73% loss). However, inclusion of 2 M KCl in the dilution buffer dramatically improved the recovery of activity (20% loss). Similarly, extended dialysis of ALSII against 25 mM Tris-HCl, 1 mM EDTA, and 1 mM dithiothreitol without KCl, while resulting in removal of FAD from the enzyme, resulted in a poorer recovery of activity (final sp act. 17 units/mg, 68% of initial). FAD could be removed more rapidly from ALSII by treatment of the enzyme with charcoal. Incubation of 0.2 mL of 35 mg/mL ALSII (final purification buffer) with 0.2 mL of 4 M KCl and 40 μL of a 50% (w/w) charcoal slurry in water for 2 h at 37 °C resulted in the removal of >98% of the enzyme-bound FAD. The specific activity of ALSII after charcoal treatment was 20 units/mg, when assayed under standard conditions (0.1 mM FAD). ALSII that had been stripped of FAD had a somewhat lower extinction at 280 nm of 0.78 ± 0.01 A mg^{-1} mL^{-1} cm^{-1}.

Of a variety of cofactors examined, only FAD would reactivate the FAD-free ALSII. The following compounds exhibited no activity when tested at 0.1 mM in place of FAD in the assay with FAD-free ALSII: riboflavin 5′-phosphate, AMP, riboflavin 5′-phosphate and AMP, adenosine 5′-(diphosphoribose), NAD, NADH, NADP, NADPH, ATP, adenosine, adenosine and riboflavin 5′-phosphate, 3-acetylpyridine adenine dinucleotide (oxidized or reduced), pyro-

phosphate, adenosine and pyrophosphate, and adenosine, pyrophosphate, and riboflavin 5′-phosphate. Saturation of the apoenzyme by FAD, TPP, Mg^{2+}, or pyruvate appeared hyperbolic. The Michaelis constants obtained for these cofactors or substrates, with the nonvaried cofactor or substrate at saturating levels (0.1, 0.1, 2, and 50 mM, respectively; the enzyme concentration used in these assays was 14 nM), were 0.8 ± 0.1 μM, 1.5 ± 0.2 μM, 22 ± 4 μM, and 10.6 ± 0.7 mM, respectively. When the kinetic data for pyruvate were fit to eq 2 (Materials and Methods), fitted values of 8.8 ± 0.9 mM and 5.0 ± 2.2 mM were obtained for K_1 and K_2, respectively. These values result in a curve that is nearly hyperbolic over the concentration range of pyruvate examined (0.3–50 mM) and do not result in a better fit than that from eq 1. The most likely interpretation of the hyperbolic saturation by pyruvate is that the decarboxylation step (k_3, Materials and Methods) is kinetically irreversible.

Discussion

Previous attempts to purify acetolactate synthase have been hampered by the extreme lability of the enzyme (Grimminger & Umbarger, 1979). This problem is particularly acute for the isozymes of acetolactate synthase, which bind FAD more avidly, isozymes II and III. The lability of isozyme II is complex and depends upon which of the tightly bound cofactors remains bound to the enzyme. In the presence of all three cofactors (FAD, thiamin pyrophosphate, and M^{2+}), ALSII loses activity in an oxygen-dependent process. Loss of enzymic activity under these conditions does not seem to be due to oxidation of enzymic thiols as dithiothreitol does not protect the enzyme from inactivation and does not reverse the inactivation once it has occurred. Since the enzyme that has lost activity under these conditions can be substantially reactivated (at least 10-fold) by extended dialysis against high concentrations of EDTA, it seems likely that inhibition could be due to oxidation of enzyme-bound thiamin pyrophosphate to the thiazolone. Thiamin–thiazolone–pyrophosphate, a potential reaction-intermediate analogue, is an exceptionally potent, virtually irreversible inhibitor of a number of thiamin pyrophosphate dependent enzymes (Gutowski & Lienhard, 1976). Furthermore, binding of thiamin pyrophosphate, and tight binding of its analogue tetrahydrothiamin pyrophosphate (Lowe et al., 1983), to ALSII is metal dependent (L. M. Ciskanik and J. V. Schloss, unpublished observation). As such, if the oxygen-dependent loss of activity observed in the presence of thiamin pyrophosphate and metal is due to the formation of a tight-binding derivative of thiamin pyrophosphate, then the reversal of inhibition by dialysis against a metal chelator is readily explained. In the absence of thiamin pyrophosphate and metal, the enzyme–FAD complex is quite stable. This form of the enzyme can survive several days incubation at room temperature with no loss of activity. By contrast, the enzyme stripped of FAD is unstable, especially at low protein concentrations. The instability of this form of the enzyme is different from that observed with its thiamin pyrophosphate–FAD–Mg^{2+} complex in that high salt prevents loss of activity. Stabilization of the FAD-free enzyme by high salt is similar to the properties of ALSI (Grimminger & Umbarger, 1979). Although ALSII and ALSI exhibit similar activation constants for FAD (0.8 and 0.2 μM, respectively), the latter isozyme loses this cofactor far more readily (Grimminger & Umbarger, 1979). Losses of activity at low concentrations of FAD-free ALSII are likely to be due at least in part to surface adsorption. Intermediate dilution of the enzyme into a vessel at low ionic strength, followed by dilution into an assay mixture, results in greater losses than dilution of the enzyme into an FAD-free assay mixture followed by initiating the assay with FAD.

As acetolactate synthase was isolated from an organism containing the plasmid-borne *ilvG* gene from *S. typhimurium* (Blazey et al., 1981), a single type of enzyme subunit was anticipated. However, purified ALSII was composed of two large (59 kDa) and two small (9.7 kDa) subunits. Amino-terminal sequence analysis of these two polypeptides identified the position for initiation of translation of the larger, *ilvG*-encoded, subunit as bp 271 and the smaller subunit as bp 1914, relative to the point of transcription initiation (Lawther & Hatfield, 1980). The position at which translation of *ilvG* is initiated had previously been identified as bp 352 (Lawther et al., 1981), although identified earlier as bp 271 (Lawther & Hatfield, 1980), primarily due to the absence of a region complementary to the 3′ terminus of 16S ribosomal RNA prior to the latter DNA sequence. The calculated molecular weights of the large and small subunits of ALSII on the basis of the *E. coli* gene sequence (Lawther et al., 1981) are 59 303 and 9703, respectively, values that agree rather well with those estimated by SDS gel electrophoresis (59 000 and 9800).

On the basis of DNA sequence homology between the region encoding the first, amino-terminal half of *ilvH* and the region encoding the small subunit of ALSII, Squires et al. (1983) had proposed that this region (between *ilvG* and *ilvE*) may code for a small subunit of the enzyme. They had tentatively refered to this region of DNA as the "K region". However, since *ilvK* has already been used to refer to another isozyme of acetolactate synthase (Madu & Jackson, 1983), we designate the gene that encodes the small subunit of ALSII as *ilvM*. As pointed out by Squires et al. (1983), *ilvM* has a region immediately prior to the gene (bp 1902–1905) that could serve as a ribosome binding site (GGAG). The small subunit appears to have been observed by Lawther et al. (1981) as a plasmid- (pBR322, pRL5, and pAH29) encoded protein using the "maxi-cell" technique. However, they identified the plasmid-encoded protein of about M_r 11 000 as a truncated *ilvE′* gene product.

Interestingly, the genes for the large and small subunits of ALSII have overlapping start and stop codons. In the DNA sequence ATGATG (bp 1914–1919), TGA is the stop codon for *ilvG*, while the preceeding A and the TG of the *ilvG* stop codon codes for the first methionyl residue of *ilvM*. There are several examples of similar overlapping start–stop codons. The genes that code for the β and α subunits of tryptophan synthase in *E. coli* (*trpB* and *trpA*), those coding for component I and component II of anthranilate synthase (*trpE* and *trpD*) (Yanofsky et al., 1981), and the genes for the β and ε subunits of the maize chloroplast photosynthetic coupling factor CF_1 (Krebbers et al., 1982) have overlapping start and stop codons. One feature these three enzymes share in common with ALSII is that the two components encoded by the overlapping genes are in equal molar ratio.

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