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α-ISOPROPYLMALATE SYNTHASE FROM *SALMONELLA TYPHIMURIUM*—AMINO ACID COMPOSITION, NH_2-TERMINAL ANALYSIS, AND FINGERPRINT ANALYSIS

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SUMMARY

α-Isopropylmalate synthase was purified from *Salmonella typhimurium* and shown to be homogeneous by gel filtration, polyacrylamide gel electrophoresis, and a method involving ^{14}C-labeled amino acids. The amino acid composition of the enzyme was determined. The number of free sulfhydryl groups estimated by KOHLHAW (*Biochim. Biophys. Acta*, 212 (1970) 58) is equal to the number of half-cystine residues, suggesting that the enzyme has no disulfide bridges. Treatment of the enzyme with potassium cyanate released 1.25 moles of amino-terminal serine per 47 500 g of protein. The number of spots on a fingerprint chromatogram prepared from trypsin-digested enzyme was similar to the number of basic amino acids in 47 500 g of protein. These data, together with genetic and electrophoretic evidence, indicate that α-isopropylmalate synthase is composed of only a single type of polypeptide chain.

INTRODUCTION

The three enzymatic reactions unique to leucine biosynthesis have been at least partially characterized[1-3]. The first reaction in the pathway is the aldol condensation of acetyl CoA and α-ketoisovalerate to form α-isopropylmalate and is catalyzed by α-isopropylmalate synthase. α-isopropylmalate synthase, like many other "branch point" enzymes[4], is inhibited by the end product of the pathway, in this case leucine.

KOHLHAW *et al.*[1] have described the purification of α-isopropylmalate synthase from *Salmonella typhimurium*. They have shown by ultracentrifugation and disc-gel electrophoresis that the molecular weight of the enzyme is dependent on both leucine and protein concentration[5]. Their evidence suggests that the enzyme exists in a monomer-tetramer relationship at minimum and maximum association of the subunits, respectively.

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This report describes the composition, NH_2-terminal amino acid analysis and tryptic fingerprint analysis of α-isopropylmalate synthase from *S. typhimurium*. These data indicate that α-isopropylmalate synthase is composed of identical polypeptide chains.

MATERIALS AND METHODS

Strains and culture techniques

The bacterial strains used in this study were derivatives of *Salmonella typhimurium ara-9 gal-205*. Strain CV123 (*ara-9 gal-205 flr-123*) was grown in a minimal salts solution containing per liter of distilled water: K_2HPO_4, 10.5 g; KH_2PO_4, 4.5 g; $(NH_4)_2SO_4$, 1.0 g; sodium citrate dihydrate, 0.97 g; $MgSO_4$, 0.05 g; glucose, 2.0 g.

Assay of α-isopropylmate synthase

The enzyme was assayed by a fluorimetric procedure developed by CALVO *et al.*[6]. A typical assay tube contained the following in a total volume of 1 ml: Tris, pH 7.5, 250 μmoles; α-ketoisovalerate, 6.25 μmoles; acetyl-CoA, 1.0 μmole; and enzyme. A standard curve prepared from α-isopropylmalate was linear to 0.5 μmole of α-isopropylmalate.

Protein determination

Protein was generally determined by the method of LOWRY *et al.*[7] using bovine serum albumin (10 mg protein nitrogen per ml) as a standard. Column effluents were monitored by light absorption at 280 nm; however, pooled fractions were analyzed by the Lowry technique.

Purification

Frozen cells were suspended in 0.03 M potassium phosphate buffer, pH 6.8 (1 g wet weight of cells per ml buffer) and sonicated for a total of 4 min at tap 8 of a Branson Sonifier Model S110. The temperature was monitored throughout the sonication and maintained below 8° with an ice bath. In all subsequent steps of the purification the temperature of the preparation was maintained at about 4°. After sonication, cell debris was removed by centrifugation at 10 000 × *g* for 10 min. The supernatant containing the enzyme was removed and the pellet was resuspended in 0.03 M phosphate buffer, pH 6.8 (one-half the volume added to the unbroken cells) and sonicated for 2 min as described above. The cell debris was removed by centrifugation and the supernatant combined with that from the first sonication. 20% of the total activity released was obtained from the second sonication. At this point, the enzyme was stable to freezing, losing less than 1% of its activity after 24 h in the frozen state. However, at 4° the enzyme lost about 30% of its activity after 24 h.

Streptomycin sulfate (Mann Research Labs) was added to the crude extract (3 g/100 g wet weight of original cells) and the mixture was stirred for 30 min. The precipitate was removed by centrifugation at 10 000 × *g* for 10 min and washed with 0.03 M phosphate buffer, pH 6.8. The supernatant and wash from the streptomycin sulfate precipitation step were combined and brought to 40% saturation with $(NH_4)_2SO_4$ by adding the appropriate volume of 3.9 M $(NH_4)_2SO_4$ (a saturated so-

lution of 0°). After centrifugation, the supernatant was made 10^{-4} M in dithiothreitol (Calbiochem) and placed in a Amicon Model 400 ultrafiltration cell (Amicon Corp.) equipped with an XM-100 membrane. The material was concentrated to about 200 ml and washed with 0.03 M phosphate buffer, pH 6.8, containing 10^{-4} M dithiothreitol until the conductivity of the filtrate was less than 10 mmho.

The concentrate was mixed with a suspension of hydroxylapatite HTP (Bio-Rad, 40 mg protein per ml bed volume) suspended in 0.03 M phosphate buffer, pH 6.8, containing 10^{-4} M dithiothreitol and poured into a column equipped with a bed of fine glass wool. It was important that the sample be applied rapidly because upon removal of $(NH_2)_4SO_4$, the enzyme was unstable, losing 70% of its activity after 24 h at 4°. When the liquid had drained to the top of the settled adsorbant, the column was washed with 0.16 M phosphate buffer, pH 6.8, containing 10^{-4} M dithiothreitol (conductivity, about 11.0 mmho) until the absorbancy at 280 nm had dropped to 0.10 or less. The enzyme was then eluted with 0.30 M phosphate buffer, pH 6.8, containing 10^{-4} M dithiothreitol.

The latter fraction was concentrated to 100 ml or less by ultrafiltration using an XM-50 filter and brought to 65% saturation with 3.9 M $(NH_4)_2SO_4$. The precipitate was harvested by centrifugation at 10 000 × *g* for 20 min and resuspended in 10 ml of 0.03 M phosphate buffer, pH 6.8 containing 10^{-3} M L-leucine and 10^{-4} M dithiothreitol. The solution was incubated for 1 h at 4° and applied to a 2.5 cm × 90 cm column of Sephadex G-100 (Pharmacia Fine Chemicals, Inc.) equilibrated with 0.03 M phosphate buffer, pH 6.8, containing 10^{-3} M L-leucine and 10^{-4} M dithiothreitol. The protein was eluted with equilibrating buffer and the fractions containing α-isopropylmalate synthase activity were pooled, dialyzed exhaustively against water, and lyophilized.

Isotopic labeling of strain CV5057

Minimal salts solution (20 ml) containing 60 mg of L-leucine and 2.0 g of glucose per l, and 20 μC of a ^{14}C-labeled reconstituted protein hydrolysate (Schwartz BioResearch) was inoculated with 0.1 ml of a culture of strain CV5057 (*ara-9 gal-205 leuAB5057*) grown to stationary phase in minimal medium containing leucine. After incubation with aeration for 24 h at 37°, the cells were centrifuged at 17 000 × *g* for 10 min and washed once with 10 ml of minimal salts solution.

Radioactivity measurements

A known quantity of protein was precipitated with 8% trichloroacetic acid, pH 1.0 and filtered on membrane filters (0.45 μm; Matheson Higgins Co., Inc.). The filters were washed with four successive 5 ml volumes of 8% trichloroacetic acid, dried for 2 h at 60° and placed in counting vials containing 10 ml of 0.4% 2,5-bis-[2-(5-*tert.*-butylbenzoxazolyl)] thiophene (BBOT) (Scintillation Grade; Packard Inst., Inc.) in toluene. Radioactivity was measured in a Packard Model 3003 TriCarb Scintillation Spectrometer.

Polyacrylamide gel electrophoresis

The procedures of DAVIS[8] were followed, except for the following modifications. Instead of incorporating the sample into a gel, it was made 10% in sucrose and layered directly on the top of the spacer gel. Electrophoresis in the presence of urea

was carried out with the running gel, spacer gel, and buffer all equilibrated with 7 M urea. Protein was stained with Coomassie Blue by the procedure of CHRAMBACH *et al.*[9].

Amino acid composition

Samples of α-isopropylmalate synthase dissolved in 2 ml of twice-distilled 6 M HCl were hydrolyzed *in vacuo* by heating to 108° for 24 h in an aluminum block heater. The samples were taken to dryness by flash evaporation at 40°, dissolved in 2 ml of 0.2 M sodium citrate buffer, pH 2,2. and analyzed with a Beckman 120 C amino acid analyzer (Beckman Inst., Inc.). To correct for destruction of serine and threonine caused by the hydrolysis procedure, samples of the enzyme were hydrolyzed for 24, 48, and 72 h. The amount of serine and threonine was plotted as a function of hydrolysis time and the zero time extrapolate was taken as the correct value. Total half-cystine was determined either as cysteic acid after performic acid oxidation of the protein[10] or as *S*-carboxymethylcysteine following carboxymethylation with iodoacetic acid[11]. Tryptophan was determined by the spectrophotometric method of BENCZE AND SCHMID[12] using a Cary model 15 recording spectrophotometer.

NH_2-terminal amino acid analysis

The analysis for the NH_2-terminal amino acid of α-isopropylmalate synthase was carried out using the cyanate procedure described by STARK AND SMYTH[13].

Trypsin digestion

Oxidized α-isopropylmalate synthase[10] was suspended in 2 M urea and the pH adjusted to 8.3 with NaOH. Digestion was started by adding trypsin dissolved in 0.001 M HCl (0.01 mg/mg α-isopropylmalate synthase) and continued at 25° in a pH stat until the release of hydrogen ion had ceased. At this point another sample of trypsin solution (0.01 mg/mg α-isopropylmalate synthase) was added and digestion continued. The reaction mixture was heated to 100° for 3 min and desalted by adjusting the pH to 2.0 and placing it on a Dowex 50W-X2 column equilibrated with water (10 ml bed volume per 5 mg α-isopropylmalate synthase). The column was washed with 100 ml of water and the peptides were eluted with 40 ml of 4 M NH_4OH, and dried by lyophilization.

Fingerprint technique

Paper chromatography and electrophoresis were carried out on Whatman No 3 MM paper (18.25 inch × 22.5 inch) using the procedure of BENNETT[14]. Chromatograms were developed by descending chromatograph for 21 h in *n*-butanol–pyridine–glacial acetic acid–water (90:60:18:72, by vol.). After drying for 2 h, electrophoresis in the second direction was carried out at pH 3.5 in a 0.5% pyridine, 5.0% acetic acid buffer. 30 V/cm were applied for 2 h in a Gilson Medical Electronics apparatus. After drying, the peptides were revealed by spraying the paper with ninhydrin and heating for 30 min at 80°.

RESULTS

Purification of α-isopropylmalate synthase

Strain CV123 was chosen as the source of α-isopropylmalate synthase because it has constitutive levels of leucine biosynthetic enzymes which are 10-fold higher than wild type. High amounts of α-isopropylmalate synthase were harvested from this strain even when it was grown in a medium rich in leucine. The mutation in strain CV123 affecting the production of leucine biosynthetic enzymes is located near *gal* on the chromosome (MIKULKA AND CALVO[23]) and was not expected to alter the primary structure of the enzyme.

The purification procedure of KOHLHAW *et al.*[1] was modified to obtain enough material to carry out structural studies. It was necessary to divide the ammonium sulfate fractionation into two steps because precipitation of the enzyme in the early stages of the purification procedure led to a sizeable loss of activity (50% in some instances). However, in the later stages of the procedure (*e.g.* after the hydroxyla-

TABLE I

PURIFICATION OF α-ISOPROPYLMALATE SYNTHASE FROM STRAIN CV123

Treatment	*Vol. (ml)*	*Units*/ml*	*Total units**	*Protein*		*Specific activity*	*Yield (%)*	*Purification (-fold)*
				mg/ml	*Total*			
Crude extract	606	1.90	1150	64.0	38 800	0.03	100	–
Streptomycin sulfate	603	2.17	1310	34.0	13 100	0.06	114	2.0
Ammonium sulfate	1172	0.95	1115	9.1	10 690	0.10	97	3.3
Ultrafiltration	539	2.08	1120	9.5	5 110	0.22	98	7.3
Hydroxylapatite	296	2.47	734	1.2	356	2.06	64	68.7
Sephadex G-100	64	6.30	403	1.9	122	3.32	35	110.5

* One unit catalyzes the production of 1 μmole α-isopropylmalate per min. Specific activity equals units per mg of protein.

patite step) only about 15% of the activity was lost. The loss of activity at both stages of purification was not eliminated by the addition of 10^{-3} M L-leucine or 10^{-4} M dithiothreitol. By separating the fractionation into a 40% cut following streptomycin sulfate precipitation and a 65% cut following hydroxylapatite chromatography, no more than 15% of the activity was lost.

The purity of the enzyme obtained using this modified procedure was evaluated by gel filtration, polyacrylamide gel electrophoresis, and a method using ^{14}C-labeled amino acids. All of the enzymatic activity from the final gel filtration step was evenly distributed throughout one of the two $A_{280\ nm}$ peaks (Fig. 1A). The presence of contaminating material was investigated by taking advantage of a unique property of α-isopropylmalate synthase reported by KOHLHAW *et al.*[1] namely, that in the absence of leucine the enzyme eluted more rapidly from Sephadex G-100 than in the presence of leucine. The material obtained from gel filtration carried out in the presence of leucine was precipitated with $(NH_4)_2SO_4$ to concentrate the enzyme and remove leucine. It was then rechromatographed on the same Sephadex G-100 column which

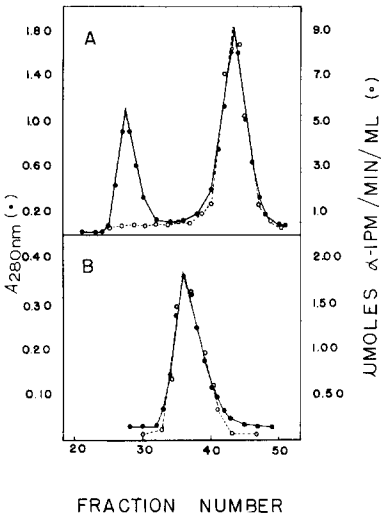

Fig. 1. Elution of α-isopropylmalate synthase from Sephadex G-100 in the presence (A) and absence (B) of 10^{-3} M L-leucine. α-IPM, α-isopropylmalate.

was equilibrated with 0.03 M phosphate buffer, pH 6.8, containing 10^{-4} M dithiothreitol. Both the activity and the $A_{280\ nm}$ profiles show significant trailing; however, the ratio of activity to $A_{280\ nm}$ was constant throughout the peak (Fig. 1B). There was no indication of 280-nm absorbing material at the position at which α-isopropylmalate synthase eluted in the presence of leucine.

When material obtained from the final step in the purification procedure was subjected to gel electrophoresis on gels of different polyacrylamide content, 2 bands of Coomassie Blue staining material were detected for all gel concentrations. A plot of the logarithm of mobility of each of the two bands versus the gel concentration is

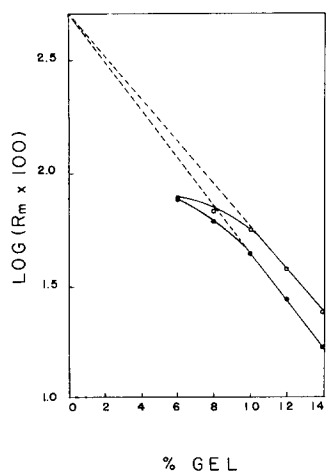

Fig. 2. Variation in relative electrophoretic mobility (R_m) of α-isopropylmalate synthase with % gel concentration.

shown in Fig. 2. If the linear portions of the curves are extrapolated, they intersect at 0% gel concentration which, according to the criteria of HEDRICK AND SMITH[15], indicates that the two bands represent material having the same charge but differing in molecular weight. Using the relationship of slope versus molecular weight published by HEDRICK AND SMITH[15], the two components correspond to molecular weights of 191 000 and 126 000. These two components probably represent different states of aggregation of one polypeptide since when a sample of α-isopropylmalate synthase was oxidized with performic acid[10] and subjected to electrophoresis on 7% gels containing 7 M urea only one band was detected even when the gel was overloaded.

The degree of purity of the enzyme was also measured by a modification of a technique described by BURNS AND ZARLENGO[16]. A strain of Salmonella (CV5057) which produces no α-isopropylmalate synthase because of a multisite mutation covering the cistron coding for the enzyme[17] was labeled with ^{14}C-containing amino acids and mixed with 30 g of unlabeled cells of strain CV123. The amount of contaminating protein in the mixture was estimated from the amount of radioactivity in the preparation. For example, a preparation that was 50% α-isopropylmalate synthase and 50% contaminating protein would have a specific radioactivity (counts/min per mg of protein) one-half that of the contaminating protein. This method indicated that the material obtained after the final Sephadex G-100 step corresponded to 94% pure α-isopropylmalate synthase.

TABLE II

AMINO ACID COMPOSITION OF α-ISOPROPYLMALATE SYNTHASE

Amino acid	*Number of residues per 47500 g protein**
Lysine	28.1 ± 2
Histidine	12.6 ± 1
Arginine	24.8 ± 1
Aspartic acid	49.0 ± 2
Threonine	23.1**
Serine	23.8**
Glutamic acid	49.7 ± 3
Proline	14.3 ± 2
Glycine	30.4 ± 2
Alanine	38.5 ± 4
Valine	30.8 ± 2
Methionine	9.4 ± 1
Isoleucine	24.6 ± 2
Leucine	27.8 ± 2
Tyrosine	10.7 ± 1
Phenylalanine	13.3 ± 1
Half-cystine	4.7 ± 0.3***
Tryptophan†	11.1
Total	427 ± 26

* Based on a minimum molecular weight of 47 500[5]. Average of four different hydrolysis experiments using three separate preparations of enzyme.

** Determined by an extrapolation to zero time of hydrolysis.

*** Analyzed as cysteic acid[10].

† Analyzed spectrophotometrically as described by BENCZE AND SCHMID[12].

Amino acid composition

Table II gives the amino acid composition of α-isopropylmalate synthase isolated from strain CV123. The serine and threonine values are corrected for destruction during hydrolysis by extrapolation to zero time hydrolysis. Throughout this time-course experiment no other amino acid appeared to vary significantly. Half-cystine was determined by the performic acid oxidation procedure of HIRS[10], and as *S*-carboxymethylcysteine following carboxymethylation of the reduced enzyme. Both methods gave approximately the same value. The ratio of tyrosine to tryptophan in the enzyme was determined from the spectrum of α-isopropylmalate synthase in 0.1 M NaOH[12]. The content of tryptophan was then calculated from the known tyrosine content.

NH_2-terminal amino acid analysis

The NH_2-terminal amino acid analysis of α-isopropylmalate synthase was performed using the cyanate procedure described by STARK AND SMYTH[13]. To test the method both ribonuclease and hemoglobin were carried through the procedure. In agreement with the published values[18,19], the analysis indicated 0.81 moles of lysine per mole of ribonuclease and 3.76 moles of valine per mole of hemoglobin. In addition, sizeable background levels of aspartic acid, serine, glutamic acid, glycine and alanine were detected in the analysis of both proteins. α-Isopropylmalate synthase carried through the same procedure gave 1.25 moles of serine per 47 500 g of

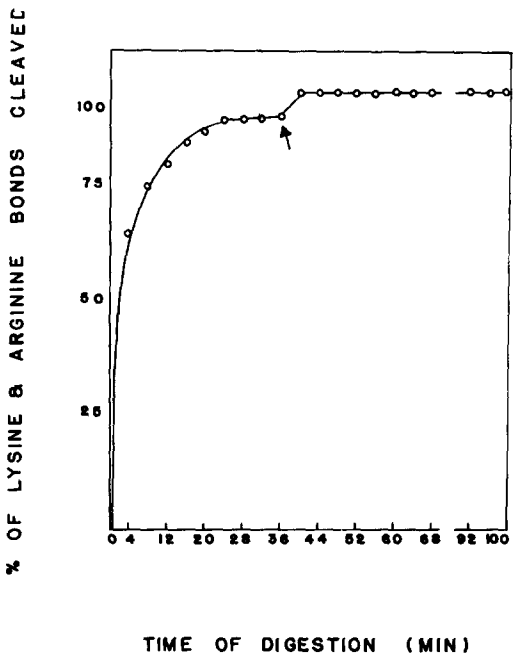

Fig. 3. Digestion of α-isopropylmalate synthase with trypsin. The ordinate was calculated by dividing the number of μmoles of H^+ liberated as determined in the pH stat by the number of μmoles of lysine and arginine in the sample as determined by acid hydrolysis followed by amino acid analysis. At the time indicated by the arrow, a fresh sample of trypsin was added to the reaction mixture.

enzyme. Aspartic acid, glutamic acid, glycine, and alanine all were present in the analysis of α-isopropylmalate synthase; however, since these same amino acids arose during the analysis of the two test proteins and in approximately the same concentrations, they were discounted as arising from the procedure.

Trypsin digestion

When 5 mg of oxidized α-isopropylmalate synthase[10] were hydrolyzed with trypsin in a pH stat the data in Fig. 3 were obtained. Hydrolysis was very rapid, being essentially completed in 30 min. After hydrolysis, the tryptic peptides were desalted and subjected to fingerprint analysis. Not counting the material at the

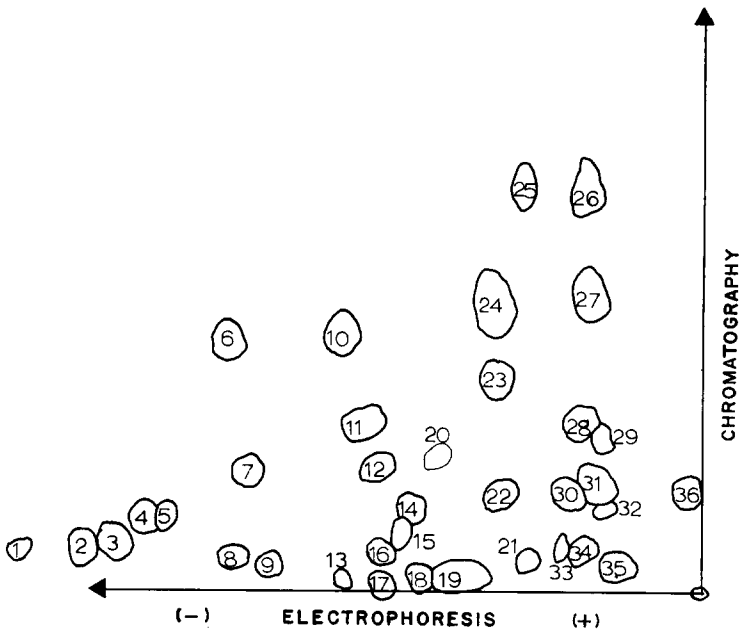

Fig. 4. Fingerprint of α-isopropylmalate synthase.

origin, 35 ninhydrin-positive spots were detected (Fig. 4). The material at the origin was eluted and subjected to acid hydrolysis and amino acid analysis. It contained 2 lysine and 2 arginine residues per residue of cysteic acid, accounting for a minimum of 4 more tryptic peptides or their equivalents. Since α-isopropylmalate synthase contains approx. 28 lysine residues and approx. 24 arginine residues per 47 500 g, the fingerprint analysis indicates that α-isopropylmalate synthase is composed of one type of polypeptide [(28 + 24 + 1)/39 = 1.36].

DISCUSSION

Strain CV123 has high, constitutive levels of leucine biosynthetic enzymes. From the data in Table I it was calculated that about 0.9% of the protein in the crude extract of strain CV123 was α-isopropylmalate synthase. A similar calculation

by KOHLHAW *et al.*[1] gave an estimate of about 1.25% α-isopropylmalate synthase in crude extracts of strain CV19. However, the specific activity of α-isopropylmalate synthase in the crude extract of strain CV19 was about 4-fold higher than reported here for the enzyme from CV123. This difference is probably due to the different assay procedures used in the two laboratories because the enzyme is derepressed to about the same extent in both strains and because the estimates of the proportion of the enzyme in crude extracts were similar.

The amino acid composition of α-isopropylmalate synthase (Table II) is not unusual in any respect. KOHLHAW[20] has titrated α-isopropylmalate synthase with 5,5′-dithio bis-(2-nitrobenzoic acid) and shown that the enzyme contains 4.7 free SH groups per 47 500 molecular weight polypeptide. Using two different procedures, reduction followed by carboxymethylation and oxidation to cysteic acid, we found that the enzyme had 4.7 half-cysteine residues per 47 500 g. The close similarity of these two values suggests that there are no disulfide bonds in the enzyme.

LEARY AND KOHLHAW[5] have shown that α-isopropylmalate synthase exists in an association–dissociation equilibrium which is dependent on both leucine and protein concentration. They estimated the minimum molecular weight of the enzyme to be about 47 500 from ultracentrifugation and gel electrophoresis in the presence of sodium dodecyl sulfate. The following points bear on the question of the number of unique polypeptide chains in α-isopropylmalate synthase: (a) About 1 mole of NH_2-terminal serine is found per 47 500 g of enzyme. (b) Carboxypeptidase releases approximately 1 mole of valine per 47 500 g of enzyme[21]. (c) Only a single band of material is observed upon gel electrophoresis in the presence of sodium dodecyl sulfate[5] or urea. (d) The number of spots on a fingerprint chromatogram prepared from trypsin-digested enzyme is similar to the number of basic amino acids in 47 500 g of enzyme. (e) Mutants lacking α-isopropylmalate synthase activity fall into a single genetic complementation group[17,22]. These data taken together clearly indicate that α-isopropylmalate synthase is composed of only a single type of polypeptide chain.

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