BBA 66435

α-ISOPROPYLMALATE SYNTHASE FROM *SALMONELLA TYPHIMURIUM*

CARBOXYPEPTIDASE DIGESTION STUDIES OF PARENT AND FEEDBACK-INSENSITIVE ENZYMES

JAMES C. BARTHOLOMEW* AND JOSEPH M. CALVO

Section of Biochemistry and Molecular Biology, Division of Biological Sciences, Cornell University, Ithaca, N.Y. 14850 (U.S.A.)

(Received June 14th, 1971)

SUMMARY

α-Isopropylmalate synthase was purified from *Salmonella typhimurium* strains CV123 and CV241. The enzyme from the latter strain is relatively insensitive to feedback inhibition by leucine due to a mutation at the extreme operator-distal end of *leuA*. The amino acid composition of the enzymes from both strains were very similar. Carboxypeptidase A treatment of the wild-type enzyme from strain CV123 released 1.21 moles of valine per 47 500 g of protein. Digestion with a mixture of carboxypeptidase A and carboxypeptidase B established –Ala–Arg–(Lys,Arg)–Val–COOH as the sequence of amino acids at the carboxyl-terminal end of the enzyme. Similar studies established the sequence –Ala–Arg–Lys–COOH for the feedback-insensitive enzyme. The simplest interpretation of these studies is that the feedback-insensitive enzyme differs from the parent enzyme in lacking the penultimate arginine and COOH terminal valine residues.

INTRODUCTION

α-Isopropylmalate synthase is the first enzyme of the leucine biosynthetic pathway in *Salmonella typhimurium*[1]. Leucine has two known effects on this enzyme. First, it controls the activity of the enzyme by feedback inhibition[2], and second, it causes a dissociation of the enzyme into monomeric subunits of 47 500 molecular weight[3]. This paper describes experiments with a strain of Salmonella containing a mutation, *leuA2010*, resulting in the production of a feedback-insensitive α-isopropylmalate synthase. The mutation has been mapped by deletion mapping studies and the amino acid alteration in the mutated enzyme established.

* Present address: The Salk Institute for Biological Research, P.O. Box 1809, San Diego, Calif. 92112, U.S.A.

MATERIALS AND METHODS

Strains and culture techniques

All the bacterial strains used in this study were derivatives of *S. typhimurium* LT2. Strain CV123(*ara-9 gal-205 flr-123*) was grown as described previously[4]. Strain CV241(*leuA2010 gal-205 flr-19*) was grown in nutrient broth containing 40 g of tryptone and 30 g of yeast extract per l. The cells were harvested at early stationary phase by centrifugation in a Sorvall RC-2 refrigerated centrifuge at 5000 × *g* for 10 min. Approx. 16 g of CV241 per l of medium were harvested. The cells were stored frozen prior to use.

Transduction techniques

Transduction was mediated by PLT22 phage. The media and methods used were those reported by MARGOLIN[5]. Transduction mixtures were diluted 3-fold in nutrient broth and 0.1-ml samples were plated on minimal agar plates[5] enriched with 0.1% nutrient broth. After incubation for 48 h at 37°, colonies were scored for leucine excretion by an auxanographic test[6].

Purification

α-Isopropylmalate synthase was assayed by a sensitive fluorometric procedure described previously[4,7]. Protein was determined by the method of LOWRY *et al.*[8] using bovine serum albumin (10 mg protein nitrogen per ml; Armor Pharmaceutical Company, Chicago, Ill.) as a standard. The purification procedure was as described elsewhere[4] except that in the case of the feedback-insensitive enzyme from strain CV241, dialysis was used to remove $(NH_4)_2SO_4$ instead of ultrafiltration. This modification was taken to expedite the procedure because the feedback-insensitive enzyme was not as stable as the enzyme from strain CV123 (Table II).

Amino acid composition

The procedures used for determining the amino acid composition of both enzymes were described elsewhere[4].

Carboxypeptidase digestion

Carboxypeptidase A (DFP treated, Worthington Biochemical Corp., Freehold, N.J.) was washed free of contaminating amino acids by suspending 1 mg in 1 ml of water and centrifuging at 17 000 × *g* for 10 min. The supernatant was discarded and the pellet of carboxypeptidase A was solubilized by adding 1 ml of 10% (w/v) LiCl. Carboxypeptidase B (DFP treated, Worthington Biochemical Corp., Freehold, N.J.) was dissolved in 1 M NaCl to give a concentration of 0.5 mg/ml. Digestion of α-isopropylmalate synthase was carried out in 0.2 M *N*-ethylmorpholine–acetate buffer (pH 8.5) by adding carboxypeptidase A (0.01 mg per mg α-isopropylmalate synthase) or carboxypeptidase B (0.001 mg per mg α-isopropylmalate synthase). The progress of the reaction was monitored by withdrawing aliquots and precipitating the protein with 12.5% trichloroacetic acid. The precipitate was removed by centrifugation at 3000 × *g* for 10 min and the supernatant analyzed for amino acids on a Beckman 120 C amino acid analyzer (Beckman Inst., Inc. Fullerton, Calif.).

RESULTS

Map location of leuA2010

Strain CV191(*leuA2010 ara-9 gal-205*), isolated as a mutant resistant to trifluoroleucine, overproduces and excretes leucine as a consequence of having an α-isopropylmalate synthase insensitive to feedback inhibition[6]. The mutation site responsible for these phenotypes was shown to lie close to or within the operator-proximal position of the leucine operon. To locate more precisely the position of this mutation, phage grown on strain CV191 were used to transduce strains having different deletions within the leucine operon (Fig. 1). Prototrophic recombinants were scored for leucine

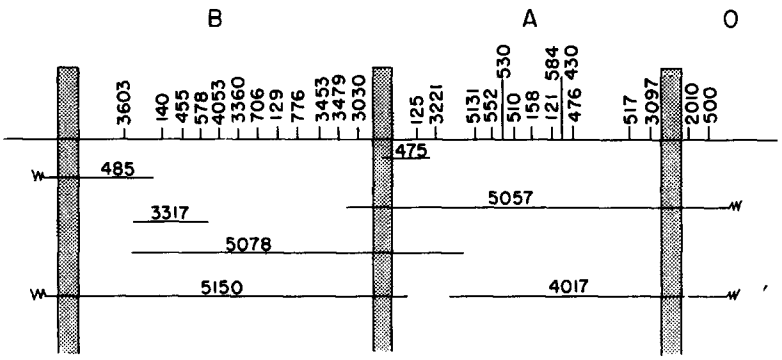

Fig. 1. Deletion map of a portion of the leucine operon of *S. typhimurium*[9].

excretion by an auxanographic technique[6]. The data indicate (Table I) that *leuA2010* is located to the left of *leuOA4017* and is either covered by or close to *leuA475*. Of 256 mutations mapping in the A cistron, only 52 are covered by *leuA475* and of those none are covered by *leuABCD5150* (ref. 9). The fact that *leuA2010* did not recombine with *leuABCD5150* (Table I) suggests that *leuA2010* is at the extreme left end of cistron A.

Purification of α-isopropylmalate synthase

α-Isopropylmalate synthase was isolated from strain CV123(*ara-9 gal-205 flr-123*) and strain CV241(*leuA2010 gal-205 flr-19*). Both strains contain mutations

TABLE I

MAP LOCATION OF *leuA2010* AS DETERMINED BY A TRANSDUCTIONAL ANALYSIS

Cross			
Recipient	*Donor phage grown on*		
leuOAB5057	CV191 (*leuA2010*)	1112	0
leuAB5078		1000	0
leuABCD5150		3940	0
leuA475		3063	1
leuBCD485		200	9
leuB3317		515	35
leuOA4017		415	12

TABLE II

PURIFICATION OF α-ISOPROPYLMALATE SYNTHASE FROM STRAIN CV241

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

Treatment	*Vol. (ml)*	*Units /ml*	*Total units*	*Protein*		*Specific activity*	*Yield (%)*	*Purification (-fold)*
				mg/ml	*Total*			
Crude extract	410	1.80	730	48.0	19670	0.04	100	—
Streptomycin sulfate	420	1.10	462	29.4	12320	0.04	63	—
Ammonium sulfate	652	0.65	425	11.1	7240	0.06	58	1.50
Dialyzed ammonium sulfate	895	0.33	295	5.0	4470	0.07	41	1.75
Hydroxylapatite	395	0.36	143	0.9	355	0.40	20	10.00
G-100 Sephadex	55	1.34	71	0.8	44	1.67	10	41.9

resulting in high, constitutive levels of leucine biosynthetic enzymes. These secondary mutations (*flr*) are located near *gal* on the chromosome[10] and were not expected to alter the primary structure of α-isopropylmalate synthase. The procedure used for purifying the enzymes has been described previously[4] and is a modification of a method reported by KOHLHAW *et al.*[11]. The feedback-insensitive enzyme was less

TABLE III

AMINO ACID COMPOSITION OF α-ISOPROPYLMALATE SYNTHASE

Molecular weight of α-isopropylmalate synthase polypeptide chain assumed to be 47 500 (ref. 3).

Amino acid	*Number of residues per 47 500 g of enzyme from*	
	Strain CV123	*Strain CV241*
Lysine	28.1	25.7
Histidine	12.6	11.9
Arginine	24.8	21.2
Aspartic acid	49.0	47.8
Threonine	23.0*	21.1*
Serine	23.8*	21.7*
Glutamic acid	49.7	50.9
Proline	14.3	15.8
Glycine	30.4	31.4
Alanine	38.5	37.3
Valine	30.8	28.3
Methionine	9.4	10.7
Isoleucine	24.6	22.2
Leucine	27.8	30.4
Tyrosine	10.7	9.0
Phenylalanine	13.3	12.8
Half-cystine	4.7**	5.1**
Total	416	413

* Corrected for destruction during hydrolysis.
** Analyzed as cysteic acid[12].

stable than the normal enzyme, losing 90% of its activity during purification (Table II) *versus* only a 65% loss for the enzyme from strain CV123 (ref. 4). In the crude extract, the specific activity was approximately the same as that for the normal enzyme, however, the final specific activity of the purified material was approx. one-half that of the normal enzyme.

Fig. 2 compares the elution of the feedback-insensitive enzyme from Sephadex G-100 in the presence of leucine with that of the normal enzyme both in the absence and presence of leucine (arrow A and B, respectively). As observed by Dorsey and Kohlhaw (cited in ref. 11), the feedback-insensitive enzyme eluted at the high molecular weight position even in the presence of leucine.

Amino acid composition

Table III gives the amino acid composition of α-isopropylmalate synthase isolated from strains CV123 and CV241. 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[12].

Carboxypeptidase digestion of α-isopropylmalate synthase from strain CV123

Carboxypeptidases A and B differ in their substrate specificity, carboxypeptidase B releasing lysine and arginine most rapidly, and carboxypeptidase A releasing all amino acids except proline, arginine, and hydroxyproline[13]. Initial experiments indicated that carboxypeptidases A and B did not release amino acids from the native enzyme nor from *S*-carboxymethylated enzyme. However, performic acid-oxidized α-isopropylmalate synthase[12] served as an excellent substrate. From the rate of release of amino acids by a mixture of carboxypeptidases A and B (Fig. 3) it appeared that valine was the carboxyl-terminal amino acid and that arginine was penultimate. Table IV lists the amino acids released by the combined action of carboxypeptidases A and B after 24 h of digestion. From this table and Fig. 3, it

TABLE IV

AMINO ACIDS RELEASED FROM α-ISOPROPYLMALATE SYNTHASE AFTER DIGESTION FOR 24 h WITH A MIXTURE OF CARBOXYPEPTIDASES A AND B

Molecular weight of α-isopropylmalate synthase polypeptide assumed to be 47 500 (ref. 3).

Amino acid	*Moles of amino acid per 47 500 g of α-isopropylmalate synthase from*	
	Strain CV123	*Strain CV241*
Lysine	1.06	1.23
Arginine	2.83	1.26
Serine	0.11	0.20
Alanine	0.50	0.63
Valine	1.43	0.92
Isoleucine	0.22	0.32
Leucine	0.42	0.69
Tyrosine	0.23	0.33

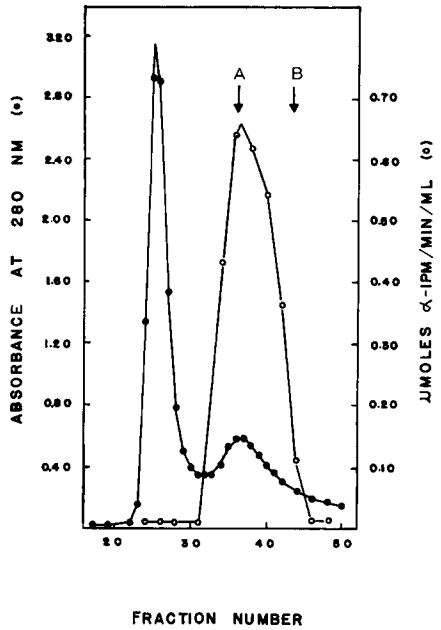

Fig. 2. Elution of feedback-insensitive enzyme from Sephadex G-100. A and B refer to the elution position of the normal enzyme in the absence and presence of leucine, respectively. α-IPM, α-isopropylmalate.

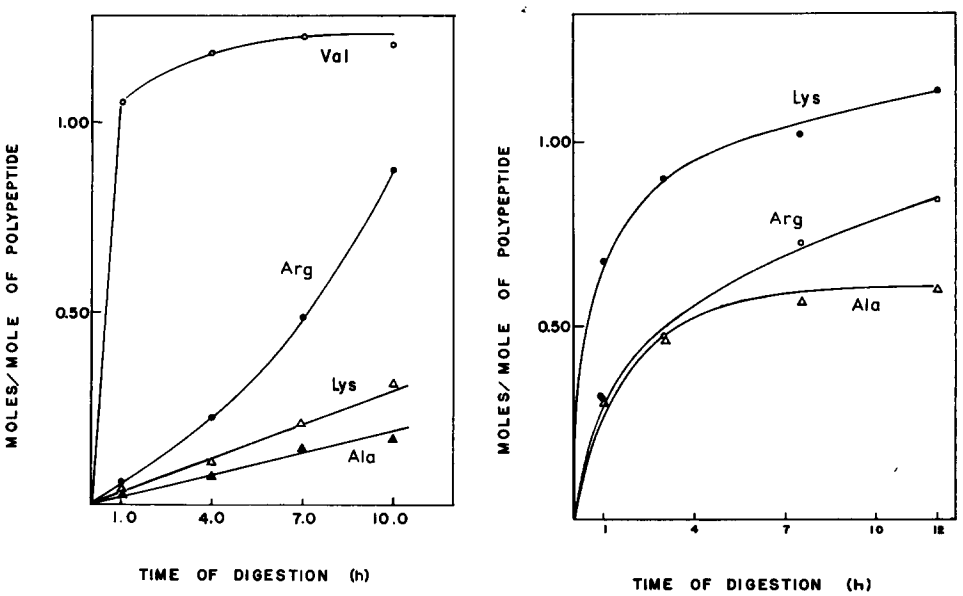

Fig. 3. Release of amino acids by carboxypeptidases A and B from oxidized α-isopropylmalate synthase isolated from strain CV123. Amino acids released at a rate slower than alanine are not shown.

Fig. 4. Release of amino acids by carboxypeptidases A and B from oxidized α-isopropylmalate synthase isolated from strain CV241. Amino acids released at a rate slower than alanine are not shown.

appears that the sequence of amino acids at the COOH-terminus of the CV123 enzyme is –Ala(Arg, Lys)Arg,Val–COOH.

When the enzyme from CV123 was digested with carboxypeptidase A alone, only valine was released. Since carboxypeptidase A is capable of releasing lysine but not arginine[13], this confirms the sequence –Arg–Val–COOH, rather than –Lys–Val–COOH. No amino acids were released when α-isopropylmalate synthase was incubated with carboxypeptidase B alone.

In order to obtain more information about the sequence of amino acids adjoining the –(Arg, Lys)Arg,Val–COOH sequence, the enzyme was treated sequentially with carboxypeptidase A, carboxypeptidase B, and carboxypeptidase A. 10 mg of α-isopropylmalate synthase were digested with carboxypeptidase A for 4 h to remove valine and the digestion was stopped by precipitating the protein with 12.5% trichloroacetic acid. After dialysis to remove the trichloroacetic acid followed by lyophilization, the protein was digested with carboxypeptidase B for 24 h to remove the basic residues. At the end of 24 h a fresh sample of carboxypeptidase A was added and samples were removed at various times and analyzed for free amino acids. The fact that alanine was released fastest further supports the sequence –Ala(Arg, Lys)-Arg,Val–COOH.

Carboxypeptidase digestion of α-isopropylmalate synthase from strain CV241

Fig. 4 shows the release of amino acids from the feedback-insensitive enzyme following addition of a mixture of carboxypeptidases A and B. Lysine was released most rapidly followed by arginine and alanine. When the release of amino acids from the feedback-insensitive enzyme is compared with the release from the normal enzyme (Fig. 4 and Fig. 3, respectively), it is apparent that the COOH-terminal sequence of the two enzymes are quite different. The normal enzyme has a sequence –Ala(Arg,Lys)Arg–Val–COOH whereas the feedback-insensitive enzyme has a sequence –(Ala,Arg)Lys–COOH. The absence of valine as the COOH-terminal amino acid of the feedback-insensitive enzyme was confirmed by treating the enzyme with carboxypeptidase B alone. If valine were COOH-terminal, no amino acids would have been released by carboxypeptidase B. After 24 h of digestion with carboxypeptidase B, 1.17 residues of lysine and 1.20 residues of arginine per 47 500 g of α-isopropylmalate synthase were released. Furthermore, because no alanine was released by carboxypeptidase B, the sequence –Ala–Arg–Lys–COOH is established. Table IV shows the release of amino acids from the feedback-insensitive enzyme after 24 h of digestion with a mixture of carboxypeptidases A and B. Other than the absence of a valine and an arginine residue, the amino acid composition of the COOH-terminal end of the CV241 enzyme is quite similar to that of the CV123 enzyme.

DISCUSSION

The behavior of the feedback-insensitive α-isopropylmalate synthase from strain CV241 was similar to the normal enzyme through all the purification steps except the final gel filtration step. In contrast to the normal enzyme, the gel filtration properties of the feedback-insensitive enzyme were not affected by the presence of leucine in the elution buffer. Thus, leucine not only fails to inhibit the enzymatic activity of the

CV241 enzyme, but it also fails to alter the enzyme's molecular weight. Possibly, dissociation of the enzyme is a necessary step in leucine-mediated inhibition.

Because α-isopropylmalate synthase is composed of identical polypeptide chains[3,4], carboxypeptidase digestion data can be analyzed in terms of a single sequence of amino acids. The most probable sequence of amino acids at the COOH-terminal end of the enzyme from strain CV123 is –Ala–(Arg,Lys)Arg–Val–COOH and from strain CV241 is –Ala–Arg–Lys–COOH. There remains the question as to how the amino acid sequences of the two enzymes are related. There is probably not a large difference in their amino acid sequences because they behave in the same fashion during purification and they have nearly identical amino acid compositions. The fact that the *leuA2010* mutation in Strain CV241 is located at the extreme operator-distal end of cistron A suggests that any alteration in α-isopropylmalate synthase from this strain would be located at the COOH-terminal end of the molecule. In this respect, three possibilities, two of which are shown below, should be considered.

A. CV123 NH_2 – – – – – – – – – – – – – Ala–(Arg, Lys)–Arg–Val–COOH
   CV241 NH_2 – – – – – – – – – – – – – Ala–Arg–Lys–COOH
B. CV123 NH_2 – – – Ala, Arg, Lys – – – Ala–(Arg, Lys)–Arg–Val–COOH
   CV241 NH_2 – – – Ala–Arg–Lys–COOH

In the first possibility, the CV241 enzyme differs from the parent in lacking the COOH-terminal valine and penultimate arginine. If true, the sequence for the CV123 enzyme is established as –Ala–Arg–Lys–Arg–Val–COOH. In the second possibility, the Ala,Arg,Lys sequence of the CV241 enzyme corresponds to some internal Ala,Arg, Lys sequence of the parent enzyme. Finally, it is conceivable that *leuA2010* is a frameshift mutation which generated a nucleotide sequence coding for Ala,Arg,Lys just before creating a nonsense codon. The conclusion from the data in Table IV, namely, that the COOH-terminal ends of α-isopropylmalate synthase from CV123 and CV241 have nearly identical compositions, is more easily reconciled with the first possibility. Also, the last two possibilities seem unlikely simply on the basis of chance. The simplest interpretation of the data, then, is that the CV241 enzyme differs from the parent in lacking two amino acids at the COOH-terminal end of the molecule. Apparently, these two amino acids are not required for catalytic activity but are necessary for feedback inhibition.

Although *leuA2010* was isolated after treatment with 2-aminopurine[6], it probably arose spontaneously because 2-aminopurine causes primarily A–T → G–C transitions[14] and the loss of Arg–Val–COOH cannot be explained by such a transition. *leuA2010* is probably either a frameshift mutation (which creates a nonsense codon in place of an arginine codon), a small deletion, or a nonsense mutation caused by a transversion [AGA(Arg) → UGA(umber)].

Taken together, the genetic mapping and carboxypeptidase digestion studies demonstrate that the COOH-terminal end of α-isopropylmalate synthase is coded for by the operator-distal end of the A cistron. This relationship, first demonstrated for tryptophan synthetase by YANOFSKY[15], is consistent with current notions of the direction of transcription and translation.

ACKNOWLEDGEMENTS

The transduction experiments were done by T. Mikulka. We thank J. Jones,

A. Tannahill and R. Rittenhouse for excellent technical assistance in the preparation of enzymes. Also, we express appreciation to G. Kohlhaw and D. Wilson for helpfull discussions. This investigation was supported by Grant GB-7636 from the National Science Foundation. One of us (J.C.B.) was supported through a training grant (5 T01 GM 824-09) from the National Institutes of Health.

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