JOURNAL OF BACTERIOLOGY, Oct. 1984, p. 391–394
0021-9193/84/100391-04$02.00/0

Vol. 160, No. 1

ilvB-Encoded Acetolactate Synthase Is Resistant to the Herbicide Sulfometuron Methyl

ROBERT A. LAROSSA* AND DANA R. SMULSKI

Central Research and Development Department, E. I. du Pont de Nemours & Co., Wilmington, Delaware 19898

Received 26 March 1984/Accepted 15 June 1984

The herbicide sulfometuron methyl is a potent inhibitor of the branched-chain amino acid biosynthetic enzyme acetolactate synthase (ALS) isolated from bacteria, fungi, and plants. However, it did not prevent growth of wild-type *Salmonella typhimurium* LT2 or *Escherichia coli* K-12. These species each contain two acetolactate synthase isozymes. Growth of *S. typhimurium* and *E. coli* mutants lacking ALS I was prevented by the herbicide, suggesting that activity of the remaining ALS isoenzyme (II or III, respectively) was stopped by sulfometuron methyl. Synthesis of ALS I requires either an *relA* function or an elevated cyclic AMP level. A *relA* mutant of *S. typhimurium* was inhibited by sulfometuron methyl on rich carbon sources that display a basal cyclic AMP level but not on poor carbon sources where the cyclic AMP concentration is elevated. When L-valine, which allosterically inhibits ALS I activity, was added, growth retardation of the *relA*$^-$ strain by sulfometuron methyl was observed on both poor and rich carbon sources. Enzymological analyses indicated that ALS I activities derived from both species were resistant to the herbicide. In contrast, activities of *S. typhimurium* ALS II and *E. coli* ALS III were abolished by sulfometuron methyl.

The herbicide sulfometuron methyl (SM) (*N*-[(4,6-dimethylpyrimidin-2-yl)aminocarbonyl]-2-methoxycarbonylbenzene sulfonamide) is also an inhibitor of bacterial (13) and fungal growth (S. C. Falco and K. Dumas, manuscript in preparation). It disrupts branched-chain amino acid biosynthesis in organisms as diverse as peas (17), yeasts (Falco and Dumas, in preparation), and *Salmonella typhimurium* (13). Biochemical genetic analyses of *Saccharomyces cerevisiae* (Falco and Dumas, in preparation), *Nicotina tabacum* (4), and *S. typhimurium* (13) have demonstratesd that an acetolactate synthase (ALS; EC 4.1.3.18) activity of each organism is the target of the herbicide. ALS catalyzes two biosynthetic reactions: (i) 2 pyruvate → α-acetolactate + CO_2 and (ii) pyruvate + α-ketobutyrate → α-aceto-α-hydroxybutyrate + CO_2. These are parallel reactions in valine and isoleucine biosynthesis (22) with thiamine pyrophosphate as a cofactor (12).

In the enteric bacteria *S. typhimurium* and *Escherichia coli*, ALS activity is specified by isozymes I, II, and III encoded by the genes *ilvB*, *ilvG*, and *ilvHI*, respectively (8). The *ilvG* gene product, ALS II, is not produced by *E. coli* K-12; the *ilvHI* gene is cryptic in *S. typhimurium* (8). It has been suggested that the ALS I activity of *S. typhimurium* is unaffected by SM (13). This report demonstrates that the ALS I of both *E. coli* and *S. typhimurium* was insensitive to SM. Thus enterobacterial ALS I was unique; it was the only wild-type acetolactate-forming enzyme described whose catalytic activity was resistant to obstruction by SM.

MATERIALS AND METHODS

Chemicals. Chemicals were of reagent grade. SM is a product of the Du Pont Co. (Wilmington, Del.) Agricultural Chemicals Department.

Media. The rich LB medium and the minimal E and M9 media have been described (5). E medium contains citrate; therefore, M9 medium was used to study the effects of carbon source on SM inhibition. All other experiments utilized E medium as the minimal medium. Bochner selection plates, prepared with 12 μg of fusaric acid, a lipophilic chelating agent (5), per ml, were used to select tetracycline-sensitive mutants of Tn*10*-containing *S. typhimurium*.

Strains. Strains used are listed in Table 1.

Genetics. P22 transduction was performed with P22HTΔ*int-4* (supplied by B. N. Ames) as described by Davis et al. (5). Tetracycline-sensitive progeny of the *relA*::Tn*10* strain TT7542 caused by excision of Tn*10* were identified by the method of Bochner et al. (3). Cells (ca. 10^6) were plated on Bochner selection medium, which permits propagation of tetracycline-sensitive cells while retarding growth of tetracycline-resistant bacteria (3). Imprecise excision mutants of strain TT7542 unlike true revertants, retain a RelA$^-$ phenotype. That *relA* mutants are more sensitive than isogenic *relA*$^+$ strains to many antagonists of amino acid biosynthesis (19) provides the basis for their identification. Imprecise excision derivatives were thus recognized by screening for progeny displaying the RelA$^-$ phenotypes of sensitivity to the histidine antimetabolite aminotriazole (20 mM; K. Rudd and J. Roth, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, H163, p. 133) and of sensitivity to the ALS substrate α-ketobutyrate (1 mM), in the presence of 0.64 mM L-isoleucine (T. VanDyk and R. LaRossa, unpublished data).

SM inhibition. SM inhibition was quantitated by the agar diffusion method (19) as modified by LaRossa and Schloss (13) for SM.

ALS assay. Cells were grown in E medium supplemented with 0.2% glucose and required nutrients (0.17 μg of thiamine per ml, 100 μg of L-threonine per ml, 83 μg of L-leucine per ml, and 167 μg of L-proline per ml). Overnight cultures (15 ml each) were used to inoculate 2-liter Erlenmeyer flasks containing 500 ml of medium. Cultures were shaken at 37°C until densities of ~7×10^8 cells per ml were attained. The cultures were chilled on ice before harvesting by centrifugation at 4°C. Cells were suspended at 0°C in 30 ml of unsupplemented E medium. After collection by centrifugation, the cells were suspended in 3 ml of 50 mM potassium phosphate (pH 7.2)–0.2 mM dithiothreitol. Sonic extracts

* Corresponding author.

TABLE 1. Bacterial strains used

Species and strain	Genotype	Source or reference
S. typhimurium mutants		
LT2	+	B. N. Ames
TA2439	*relA2 zga*::Tn*10*	B. N. Ames
TT7542	*relA*::Tn*10*	J. Roth
TV7542.3	*relA*::IE[a]	Spontaneous Tc^s [RelA$^-$] of TT7542
TT66	*ilvG*::Tn*10*	C. Berg
CBS003	*ilvG*::Tn*10 ilvB*::Tn*5*	18
RL511	*ilvB*::Tn*5*	P22(LT2) × CBS003 → Ilv$^+$ (Tc^s Km^r Val^r)
TR3381	*relA1*	J. Roth
E. coli K-12 mutants		
MC4100	*araD139* Δ(*lacIZYA*)*U169 rpsL thi*	M. Casadaban
MI253	*ilvI614 ilvH612 thr-10 thi-1 relA1 ara-14*	6
CU200	HfrH Δ*lac* Δ(*ara-ilvHI-leu*) *thi*	H. E. Umbarger
CU847	*ilvB2101 ara thi* Δ(*pro-lac*)	H. E. Umbarger
Acinetobacter sp. CHOL	+	P. Chapman
Citrobacter freundii ATCC 8454	+	J. Romesser

[a] IE, Imprecise excision of Tn*10* from indicated gene.

were prepared by ten 15-s bursts of a microtip-equipped Heat Systems-Ultrasonics cell disrupter at maximal setting (the extracts were prepared at 0°C). The sonicates were clarified by centrifugation for 2 min at 4°C in an Eppendorf microfuge. The resulting supernatant was assayed for ALS activity. The enzyme assay was performed as previously described (2) at pH 8 with phosphate buffer.

RESULTS

Inhibition of bacterial species by SM. The ability of SM to inhibit the growth of bacterial species (listed in Table 1) on minimal medium was investigated. Wild-type *Citrobacter freundii* and *Acinetobacter* sp. CHOL were inhibited, whereas *S. typhimurium* LT2 and *E. coli* K-12 were resistant to the compound. Reversal of *Citrobacter* inhibition by the branched-chain amino acid isoleucine was demonstrated (data not shown). The effects of branched-chain amino acids upon the growth of *Salmonella typhimurium* in the presence of SM were studied. ALS I activity is inhibited by the end product L-valine; ALS II activity is insensitive to L-valine (8). Valine inclusion caused wild-type *S. typhimurium* to be inhibited by SM on glucose minimal medium. This inhibition could be reversed by isoleucine (13; data not shown). An interpretation (13) is that SM inhibits *S. typhimurium* ALS II but not ALS I; if this interpretation is correct, growth inhibition is only manifested when ALS I activity is blocked or absent. Consistent with this interpretation was the response to SM of strain RL511, which harbors an insertion of Tn*5* in the *ilvB* structural gene. Although lacking ALS I activity, the strain was prototrophic due to ALS II activity; however, it was unable to grow in the presence of SM (Table 2), suggesting that valine potentiates wild-type growth retardation via feedback inhibition of ALS I.

Strains carrying *relA* mutations are often hypersensitive to amino acid antagonists (19). Three different *relA* mutants were inhibited by SM on glucose minimal medium (Table 3). This finding is consistent with the known dependence of ALS I expression upon *relA* function (10). SM inhibition of these *relA* mutant strains was slightly reversed by L-valine. This result contrasts with the wild-type response of L-valine potentiation of herbicide inhibition. This contrast reinforces the suggestion (10, 21) that only ALS II is present at a significant concentration in *relA*$^-$ cells grown on minimal glucose medium.

cAMP and carbon source effects. The *relA* requirement for transcription of the ALS I structural gene *ilvB* can be replaced by cyclic AMP (cAMP) (10, 11, 21). In solidified M9 medium with glucose as a carbon source, the zone diameter of SM-mediated growth inhibition of the *relA*$^-$ strain TV7542.3 was decreased from 38 mm without cAMP to 28 mm with 2mM cAMP. Similar though less complete reversal was observed with sorbitol as the carbon source; cAMP inclusion caused a decrease in zone diameter from 39 to 34 mm. Growth on poor carbon sources, known to cause elevated cAMP levels (1, 15) completely abolished SM inhibition of the *relA*$^-$ strain TV7542.3 (Table 4). Inclusion of valine in the medium prevented the reversal of SM inhibition of this strain by poor carbon sources (Table 4). Since valine inhibits ALS I activity, this result was again consistent with the hypothesis that ALS I is responsible for cell growth in the presence of SM.

***S. typhimurium* ALS I activity was resistant to inhibition by SM in vitro.** In *S. typhimurium* there are two physiologically significant ALS (I and II) activities (8). An extract (prepared from strain TT66) containing ALS I, but no other ALS isozyme, was assayed for acetolactate-forming capacity. ALS I activity was uninhibited by SM at concentrations ranging from 1 to 100 μM. Conversely, ALS II activity has previously been shown to be significantly inhibited by SM; biphasic inhibition with an initial K_i of 660 nM and a final K_i of 65 nM has been reported (13).

TABLE 2. Inactivation of ALS I causes SM hypersensitivity

S. typhimurium strain	*ilvB* allele	Valine concn (μg/ml)	Zone of inhibition[a] (diam [mm])
LT2	+	0	<6
LT2	+	83	27c, 32t[b]
RL511	ΩTn*5*	0	36c, 39t

[a] Caused by a 6-mm filter paper disk impregnated with 40 μg of SM placed on a lawn of cells growing in medium E supplemented with 0.2% glucose.
[b] c and t, Clear and turbid zones, respectively.

TABLE 3. *relA* allele dependence of SM inhibition

S. typhimurium strain (allele)	Zone of inhibition (diam [mm])[a] with valine at concn (μg/ml):	
	0	83
LT2 (+)	<6	23c, 29t
TA2439 (A2)	38c	36c
TR3381 (A1)	42c	37c
TT7542 (ΩTn*10*)	40c	36c
TV7542.3 (IE[b])	46c	41c

[a] See footnotes to Table 2 for experimental details and symbols.
[b] IE, Imprecise excision of Tn*10* from the *relA* gene.

***E. coli* isozymes.** A different pair of ALS isozymes (I and III) exists in *E. coli* K-12 (9). Wild-type *E. coli* K-12 was resistant to SM (Table 5). The *E. coli* strains MI253 and CU200 (*ilvH ilvI*), containing only ALS I, were as resistant to SM growth retardation as was the wild type (Table 5). In contrast, an *ilvB* mutant (CU847) that harbors only ALS III was sensitive to SM. This indicates that, of the three enterobacterial isozymes, only ALS I is resistant to SM. Acetolactate formation catalyzed by various *E. coli* extracts in the presence and absence of SM was measured. Activity of strain MC4100, containing both ALS I and III, was partially inhibited by inclusion of 1 mM SM in the assay mix (data not shown). ALS I activity extracted from the *ilvH ilvI* strain MI253 was quite insensitive to 1 mM SM (Fig. 1A). Similar results were obtained with the *ilvH ilvI* deletion strain CU200 (data not shown). In contrast, ALS III activity present in strain CU847 was completely inhibited by this SM concentration (Fig. 1B).

DISCUSSION

Inhibition of ALS by SM is a widespread phenomenon occurring with extracts obtained from plant, fungal, and bacterial sources. Only a single wild-type ALS is known to be completely resistant to SM. This distinctive enzyme is ALS I of the enteric bacteria *S. typhimurium* and *E. coli*. The insensitivity of ALS I to SM is advantageous in many ways. It provides a first means for measuring ALS I activity in crude extracts containing ALS II, ALS III, or both. Thus, activity of this isozyme can be conveniently monitored in any strain. Since ALS I is insensitive to SM, *ilvB* is potentially useful as a dominant selectable marker for the genetic transformation of diverse organisms.

The basis for differential sensitivity of ALS isozymes to SM is unknown. The three isozymes catalyze identical reactions, albeit at differing efficiencies and under distinct allosteric controls (8). Perhaps the preference of *S. typhimurium* and *E. coli* ALS I for pyruvate as the recipient of the cofactor-bound hydroxyethyl moiety (9, 16, 18) is significant. Evidence suggests that the recipient pocket within the catalytic site of ALS I cannot efficiently accommodate the larger substrate, α-ketobutyrate (9). Furthermore, we suggest that the ability to efficiently accommodate α-ketobutyrate is required for SM interaction with the active sites. All known SM-sensitive ALS proteins (4, 13, 17; Falco and Dumas, in preparation) efficiently utilize α-ketobutyrate. Although enzymological experiments have not yet addressed these points, certain in vivo data are consistent with this

TABLE 4. SM inhibition of the *relA* strain TV7542.3 as a function of carbon source

Carbon source[a]	Zone of inhibition (diam [mm]) in medium[b]:	
	Without valine	With valine[c]
Glucose	31c	32c
Galactose	31c	31c
Mannitol	27c	29c
Sorbitol	26c	37c
Xylose	15t	37c
Glycerol	<6	33c
Citrate	<6	41c

[a] At 0.4% in M9 medium.
[b] For symbols, see footnote *b* of Table 2.
[c] Medium was supplemented with 83 μg of L-valine per ml.

TABLE 5. ALS I responsibility for resistance to SM growth inhibition[a]

Species and strain	ALS isozyme(s) present	Diam of SM-mediated inhibition zone (mm)
S. typhimurium		
LT2	I, II	<6
TV7542.3	II	13c, 24t
E. coli K-12		
MC4100	I, III	<6
MI253	I	<6
CU200	I	<6
CU847	III	13c, 17t

[a] All strains were plated on thiamine and glucose containing Vogel-Bonner minimal E medium. Auxotrophies required that this medium be supplemented with threonine, leucine, or proline for the growth of MI253, CU200, or CU847, respectively. For symbols, see footnote *b* of Table 2.

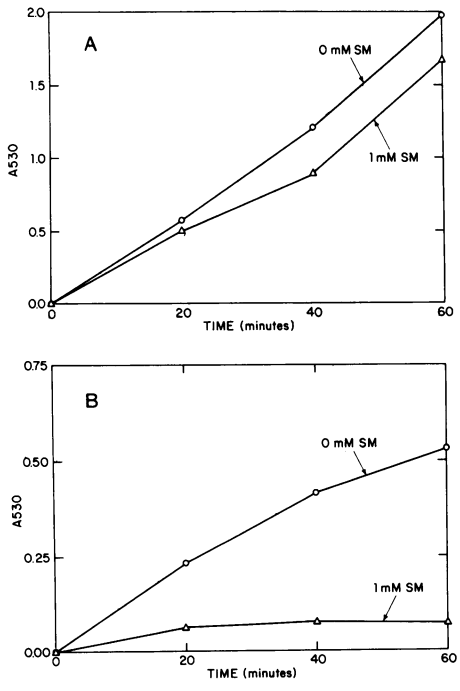

FIG. 1. Response of *E. coli* ALS I (A) and ALS III (B) to SM. SM was included in assay mixtures at 0 (○) or 1 (△) mM. ALS I and ALS III activities were derived from strains MI253 and CU847, respectively.

view. Inclusion of α-ketobutyrate and thiamine in minimal medium prevents SM growth inhibition of *S. typhimurium* mediated through interaction with ALS II (13). In contrast, the other substrate, pyruvate, in conjunction with thiamine does not reverse SM growth inhibition (unpublished data). Thus, SM may compete for that ALS II region normally occupied by α-ketobutyrate and thiamine pyrophosphate. Alternatively, *ilvB* (ALS I) may have evolved from an ancestral membrane protein gene towards an amino acid biosynthetic function. Genetic experiments have suggested ALS I involvement with the membrane proteins encoded by *cpxA* and *cpxB* (14). ALS I synthesis (10, 21), like that of many surface proteins (1, 15), is controlled by the *cya* and *crp* genes. Such control of amino acid biosynthetic genes is most unusual (21, 22); this cAMP-mediated control system does not regulate *ilvHI* and *ilvGEDA* expression (21). Catabolite repression of ALS I (21) could also suggest its resemblance to the pH 6 acetolactate-forming enzyme of *Klebsiella aerogenes* involved in 2,3-butanediol fermentation (20). Thus, ALS I may markedly differ in structure from other ALS proteins. Finally, ALS I may simply represent a variant enzyme resistant to the herbicide. Spontaneous structural gene mutations which alter sensitive ALS isozymes to resistant forms are readily obtained in bacteria (13) and yeasts (Falco and Dumas, in preparation).

It has been hypothesized that ALS I does not normally catalyze α-acetohydroxybutyrate formation in *S. typhimurium*, although it can form both this compound and acetolactate in vitro (18). We suggest that this catalysis can occur in stress situations since (i) SM does not inhibit the wild type on minimal media in the absence of valine (13; this work) and (ii) conditions causing elevated ALS I levels in *relA* strains reverse SM growth inhibition in the absence of L-valine (this work). These results extend a previous study (18) which demonstrated that ALS I can synthesize α-acetohydroxybutyrate in vivo when the culture is supplied with α-ketobutyrate exogenously. The isozymes thus appear somewhat interchangeable with respect to pathway. If the isozymes are redundant in function, other roles, perhaps regulatory, for their presence need to be considered. Multiple isozymes effecting the first common step are common features of branched amino acid biosynthetic pathways (i.e., aromatic, Ile-Leu-Val, and aspartate family) in enteric bacteria (22). In the aromatic and aspartate family pathways, each isozyme is controlled by a separate end product (22). This is not true of the ALS isozymes (8). Thus, the ability to selectively interfere with a single enzymic species may be useful in studies of the catabolic- (8) and metabolic-interlock (7) functions attributed to the ALS isozymes.

ACKNOWLEDGMENTS

We thank our colleagues who have provided us with mutant strains. T. Van Dyk constructed strain TV7542.3. Discussions with J. Schloss, T. Van Dyk, B. Mazur, and S. Falco have been most stimulating.

LITERATURE CITED

1. **Alper, M. D., and B. N. Ames.** 1978. Transport of antibiotics and metabolite analogs by systems under cyclic AMP control: positive selection of *Salmonella typhimurium cya* and *crp* mutants. J. Bacteriol. **133**:149–157.
2. **Bauerle, R. H., M. Freundlich, F. C. Stormer, and H. E. Umbarger.** 1964. Control of isoleucine, leucine and valine biosynthesis. II. End-product inhibition by valine of acetohydroxy acid synthase in *Salmonella typhimurium*. Biochim. Biophys. Acta **92**:142–149.
3. **Bochner, B. R., H.-C. Huang, G. L. Schieven, and B. N. Ames.** 1980. Positive selection for loss of tetracycline resistance. J. Bacteriol. **143**:926–933.
4. **Chaleff, R. S., and C. J. Mauvais.** 1984. Acetolactate synthase is the site of action of the herbicides chlorsulfuron and sulfometuron methyl in higher plants. Science **224**:1443–1445.
5. **Davis, R. W., D. Botstein, and J. R. Roth.** 1980. Advanced bacterial genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
6. **De Felice, M., J., Guardiola, B. Esposito, and M. Iaccarino.** 1974. Structural genes for a newly recognized acetolactate synthase in *Escherichia coli* K-12. J. Bacteriol. **120**:1068–1077.
7. **De Felice, M., J. Guardiola, W. Schreil, M. Levinthal, and M. Iaccarino.** 1977. Metabolic interlock between acetolactate synthase isozymes and lysine biosynthesis in *Escherichia coli* K-12. Mol. Gen. Genet. **156**:9–16.
8. **De Felice, M., C. T. Lago, C. H. Squires, and J. M. Calvo.** 1982. Acetohydroxy acid synthase isoenzymes of *Escherichia coli* K-12 and *Salmonella typhimurium*. Ann. Microbiol. (Paris) **133A**:251–256.
9. **De Felice, M., C. H. Squires, and M. Levinthal.** 1978. A comparative study of the acetohydroxy acid synthase isoenzyme of *Escherichia coli* K-12. Biochim. Biophys. Acta **541**:9–17.
10. **Freundlich, M.** 1977. Cyclic AMP can replace the *relA*-dependent requirement for derepression of acetohydroxy acid synthase in *E. coli* K-12. Cell **12**:1121–1126.
11. **Friden, P., T. Newman, and M. Freundlich.** 1982. Nucleotide sequence of the *ilvB* promoter-regulatory region: a biosynthetic operon controlled by attenuation and cyclic AMP. Proc. Natl. Acad. Sci. U.S.A. **79**:6156–6160.
12. **Grimminger, H., and H. E. Umbarger.** 1979. Acetohydroxy acid synthase I of *Escherichia coli*: purification and properties. J. Bacteriol. **137**:846–853.
13. **LaRossa, R. A., and J. V. Schloss.** 1984. The sulfonylurea herbicide sulfometuron methyl is an extremely potent and selective inhibitor of acetolactate synthase in *Salmonella typhimurium*. J. Biol. Chem. **259**:8753–8757.
14. **McEwen, J., and P. Silverman.** 1980. Mutations in genes *cpxA* and *cpxB* of *Escherichia coli* K-12 cause a defect in isoleucine and valine syntheses. J. Bacteriol. **144**:68–73.
15. **Pastan, I., and S. Adhya.** 1976. Cyclic adenosine 5′-monophosphate in *Escherichia coli*. Bacteriol. Rev. **40**:527–551.
16. **Primerano, D. A., and R. O. Burns.** 1982. Metabolic basis for the isoleucine, pantothenate, or methionine requirement of *ilvG* strains of *Salmonella typhimurium*. J. Bacteriol. **150**:1202–1211.
17. **Ray, T.** 1984. The site of action of chlorsulfuron: inhibition of valine and isoleucine biosynthesis in plants. Plant Physiol. **75**:827–831.
18. **Shaw, K. J., C. M. Berg, and T. J. Sobol.** 1980. *Salmonella typhimurium* mutants defective in acetohydroxy acid synthases I and II. J. Bacteriol. **141**:1258–1263.
19. **Stephens, J. C., S. W. Artz, and B. N. Ames.** 1975. Guanosine 5′-diphosphate 3′-diphosphate (ppGpp): positive effector for histidine operon transcription and general signal for amino-acid deficiency. Proc. Natl. Acad. Sci. U.S.A. **72**:4389–4393.
20. **Stormer, F. C.** 1975. 2,3-Butanediol biosynthetic system in *Klebsiella aerogenes*. Methods Enzymol. **41**:518–533.
21. **Sutton, A., and M. Freundlich.** 1980. Regulation by cyclic AMP of the *ilvB*-encoded biosynthetic acetohydroxy acid synthase in *Escherichia coli* K-12. Mol. Gen. Genet. **178**:179–183.
22. **Umbarger, H. E.** 1978. Amino acid biosynthesis and its regulation. Annu. Rev. Biochem. **47**:533–606.