JOURNAL OF BACTERIOLOGY, Apr. 1969, p. 147-151
Vol. 98, No. 1

Oxidation of D-Malic and β-Alkylmalic Acids by Wild-Type and Mutant Strains of *Salmonella typhimurium* and by *Aerobacter aerogenes*

JOSEPH R. STERN[1] AND R. W. O'BRIEN

Department of Pharmacology, School of Medicine, Case Western Reserve University, Cleveland, Ohio 44106

Received for publication 16 December 1968

A mutant strain of *Salmonella typhimurium* (SL 1634 *dml-51*) capable of growth on D-malate as sole carbon source was shown to produce D-malic enzyme. This enzyme was absent in the parent wild-type strain which was unable to grow on D-malate. Growth of the mutant on D-malate also resulted in a greatly increased level of β-isopropylmalic enzyme compared with its level in the wild-type strain grown on citrate or L-malate. The D-malic and β-isopropylmalic enzymes, both of which catalyze a nicotinamide adenine dinucleotide- and Mg^{++}-dependent oxidative decarboxylation of their respective substrates, were shown to be distinct enzymes by selective inhibition with *erythro*-DL-β-hydroxyaspartate and by other methods. Cell extracts of the mutant strain also oxidized DL-β-methyl-, DL-β-ethyl-, DL-β-propyl- and DL-ββ-dimethylmalates, in order of decreasing activity. DL-β-Methylmalate was shown to be oxidized by both the D-malic and the β-isopropylmalic enzymes, whereas the oxidation of the other β-alkylmalates appeared to be effected exclusively by the β-isopropylmalic enzyme. β-Isopropylmalic enzyme activity was induced by D-malate but not by L-malate, showing that it behaved as a D-malic-type enzyme. Growth of *Aerobacter aerogenes* on D-malate, which caused induction of D malic enzyme, resulted in only a small increase in the activity of β-isopropylmalic enzyme.

In recent years, several investigators (2, 5-7) have reported the occurrence of three separate D-malic enzymes in a variety of bacteria. These enzymes catalyze the oxidative decarboxylation of D-malate or a D-β-alkylmalate and require nicotinamide adenine dinucleotide (NAD) as coenzyme, as well as both monovalent and divalent cations. Except for their stereospecificity with respect to the D isomer, the reaction which they catalyze is exactly analogous to that catalyzed by the equally stereospecific L-malic enzymes.

Thus, Stern and Hegre (7) have shown that extracts of *Escherichia coli* which were grown on, or in the presence of, D-malate possessed an inducible D-malic enzyme which catalyzed the oxidative decarboxylation of D-malate to pyruvate and CO_2 in the presence of NAD. Also *threo*-D_s-α-hydroxy-β-carboxyisocaproate is an intermediate in leucine biosynthesis in *Salmonella typhimurium* (2), and is oxidatively decarboxylated to α-ketoisocaproate and CO_2 by an NAD-dependent β-isopropylmalic enzyme which does not attack D- or L-malate. Extracts of a *Pseudomonas* species grown on pantothenate as sole carbon source have been shown by Magee and Snell (5) to cause oxidative decarboxylation of DL-ββ-dimethylmalate and D-malate in the presence of NAD. More recently, Rabin et al. (6) reported that extracts of *P. aeruginosa*, grown on butyrate, oxidize *erythro*-DL-β-ethylmalate to α-ketovalerate; they have suggested that this oxidation reflects a broad specificity of the β-isopropylmalic enzyme present in these extracts.

This paper demonstrates that a mutant of *S. typhimurium* (SL 1634, *dml-51*), capable of growth on D-malate as sole carbon source, produces a large amount of D-malic enzyme, whereas this enzyme is lacking in extracts of the parent strain grown on citrate or L-malate. The *dml* mutant also possessed a higher level of β-isopropylmalic enzyme and oxidized various β-alkylmalates at rates considerably higher than the parent strain. By contrast, growth of *Aerobacter*

[1] Recipient of a Special Fellowship from the National Institute of General Medical Sciences, 1967-1968, during which part of this work was done.

aerogenes on D-malate caused induction of the D-malic enzyme with only a small increase in the activity of β-isopropylmalic enzyme.

MATERIALS AND METHODS

Chemicals. The *threo*-D_s-isomer of α-hydroxy-β-carboxyisocaproic acid was kindly provided by H. E. Umbarger. [The intermediate in leucine biosynthesis was shown to have the absolute configurational assignment of *threo*-D_s-α-hydroxy-β-carboxyisocaproic acid by Calvo et al. (3). Later, this group of investigators (1) adopted the β-isopropylmalic terminology. Rabin et al. (6) have pointed out that, when viewed as a substituted malic acid rather than as a substituted isocaproic acid, the intermediate would have the configuration *erythro*-Lg-β-isopropylmalic acid. We shall refer to the intermediate as β-isopropylmalic acid.] DL-ββ-Dimethylmalic acid was supplied by E. E. Snell. DL-β-Methylmalic, DL-β-ethylmalic, and DL-β-propylmalic acids were obtained from Cyclo Chemicals Corp.; D-(+)-malic. L-(−)-malic and *erythro*- and *threo*-DL-β-hydroxyaspartic acids were purchased from Calbiochem, Los Angeles, Calif.

Organisms. The organisms used in this study were (i) a wild-type prototrophic strain of *S. typhimurium* (SL 934), obtained from B. A. D. Stocker, which was unable to utilize D-malate for growth; (ii) a spontaneous mutant of SL 934 selected by growth on D-malate as sole carbon source and designated strain SL 1634 *dml-51* (*dml* refers to the gene which determines the character of D-malate utilization); and (iii) *A. aerogenes* NCTC 418.

Media and growth conditions. *S. typhimurium* strain SL 934 was grown on the following medium (g/liter): citric acid, 12; KH_2PO_4, 2.0; $MgSO_4 \cdot 7H_2O$, 0.4; $(NH_4)_2SO_4$, 1.0; and Na_2SO_4, 0.71. The medium was neutralized to *p*H 7.0 with KOH. This strain was also grown on L-malate by substituting L-malic acid (15 g/liter) for citric acid in the above medium. Strain SL 1634 was grown on the following medium (g/liter): D-malic acid, 2.0 (or DL-malic acid, 4.0); KH_2PO_4, 2.0; $MgSO_4 \cdot 7H_2O$, 0.4; and $(NH_4)_2SO_4$, 1.0. The medium was neutralized to *p*H 7.0 with NaOH. *Aerobacter* was grown on the above citrate and D-malate media.

Each medium was inoculated with a 1% inoculum from a growing culture and was shaken on a gyratory shaker for 18 to 20 hr at 35 C. *Salmonella* cells were harvested in a Sorvall centrifuge at 8,000 × *g* for 30 min at 0 C, and *Aerobacter* cells were harvested in a refrigerated Sharples Super-centrifuge at 23,000 rev/min. The cells were stored at −20 C prior to use.

Preparation of cell extracts. Cell extracts were prepared by sonic treatment of 1.6 g of wet cells in 6 ml of 0.05 M tris(hydroxymethyl)aminomethane hydrochloride buffer (*p*H 7.5) for 2 min at 5 C with a Branson Sonifier. Cell debris was settled by centrifugation at 27,000 × *g* for 20 min at 0 C in a Sorvall centrifuge. The clear supernatant liquid contained approximately 20 mg of protein per ml.

Assays. The oxidation of D-malate and β-alkylmalates was measured by determining the rate of NAD reduction at 340 nm. The reaction mixture contained: 2-amino-2-methyl-1,3-propanediol (Diol)-chloride buffer, *p*H 9.0, 100 μmoles; $MgCl_2$, 5 μmoles; KCl, 10 μmoles; NAD, 1 μmole; and cell extract. The amount of protein present in the reaction mixture varied according to the substrate being tested, but was in the range of 0.2 to 1.0 mg. The reaction was started by adding 25 μmoles of potassium D-malate or 10 μmoles of β-alkylmalate. In the inhibition studies (*see* Tables 2 and 3), 20 μmoles of DL-β-hydroxyaspartate was added when D-malate was the substrate and 10 μmoles when β-alkylmalate was the substrate. The final volume was 1.50 ml, the light path was 0.5 cm, and the temperature was 22 C. One unit of enzyme activity is the amount causing an increase in absorbancy at 340 nm of 0.1 per minute, equivalent to 0.0048 μmole of NAD reduced. Specific activity is expressed in units per milligram of protein. Protein was determined by the biuret method (4).

RESULTS

Table 1 shows the rate of oxidation of D-malate and of various β-alkylmalates, in the presence of NAD, by cell-free extracts of *S. typhimurium* and *A. aerogenes*. Extracts of the parent *Salmonella* strain grown on citrate were devoid of D-malic enzyme and also failed to oxidize DL-β-propylmalate and DL-ββ-dimethylmalate. They did have slight activity with DL-β-methyl, DL-β-ethyl, and β-isopropyl derivatives of malate. Extracts of the *dml* mutant grown on D-malate or DL-malate had high levels of D-malic enzyme, and they oxidized β-isopropylmalate and all other β-substituted malates at greatly increased rates. The levels of L-malic enzyme were not significantly different in parent and *dml* mutant strains. Also, nicotinamide adenine dinucleotide phosphate did not substitute for NAD in the oxidation of D-malate or β-alkylmalates, demonstrating that the L-malic enzyme had no significant activity on these compounds.

With *A. aerogenes*, growth on D-malate, in contrast to that on citrate, caused inducton of D-malic enzyme, a high level of oxidation of DL-β-methylmalate, and increased rates of oxidation of the DL-β-ethyl and β-isopropyl derivatives of malate. Comparing the relative rates of oxidation of D-malate and its congeners by extracts of D-malate-grown *A. aerogenes* and by highly purified *E. coli* D-malic enzyme (C. S. Hegre and J. R. Stern, Federation Proc. **24**:351, 1965), it was evident that, except for DL-β-propylmalate and β-isopropylmalate, the order of reactivities was the same. The lack of oxidation of the former compound was more apparent than real, since the expected specific activity (13.4 × 0.035 = 0.47) was below the limits of detection. β-Isopropylmalate, on the other hand, was oxidized by its specific enzyme, which was partly induced by D-malate and which may contribute

TABLE 1. *Oxidation of D-malate and β-alkylmalates by extracts of S. typhimurium and A. aerogenes*

Substrate	*Salmonella*			*Aerobacter* on		*E. coli* D-malic enzyme
	SL 1634 on		SL 934 on citrate	D-Malate	Citrate	
	D-Malate	DL-Malate				
D-Malate	17.3[a]	16.3	0	13.4	0	100[b]
DL-β-Methylmalate	76.5 (124)	63.0 (100)	4.2 (111)	13.7 (102)	0	85.0
DL-β-Ethylmalate	41.6 (68)	34.7 (55)	1.9 (50)	3.3 (25)	0.7	11.0
DL-β-Propylmalate	6.1 (9.9)	3.9 (6.2)	0	0	0	3.5
β-Isopropylmalate	61.8 (100)	63.0 (100)	3.8 (100)	2.6 (19)	0.8	0.8
DL-ββ-Dimethylmalate	5.5	3.5	0	1.6 (12)	1.2	18.0
L-Malate + nicotinamide adenine dinucleotide phosphate	23.8	42.5	36.0	46.3	12.6	—

[a] All values are specific activities. Those in parentheses are activities relative to that of β-isopropylmalate (*Salmonella*) or D-malate (*Aerobacter*). Values less than 0.7 would not be measurable and are designated 0.

[b] Activities of β-alkylmalates relative to D-malate with highly purified *E. coli* D-malic enzyme (C. S. Hegre and J. R. Stern, *unpublished data*).

partly to the oxidation of the other β-alkylmalates (*see below*).

On comparing the rate of oxidation of β-alkylmalates with that of D-malate, one can see (Table 1) that, in both the parent strain of *Salmonella* (which lacked D-malic enzyme) and the *dml* mutant strain (which produced D-malic enzyme), the relative rates were quite different from those observed with the purified *E. coli* D-malic enzyme. Indeed, the relative rates of oxidation of the β-methyl and β-ethyl derivatives were more or less the same in both strains when compared with that of β-isopropylmalate. This suggested that the oxidation of β-alkylmalates was associated with the activity of the β-isopropylmalic enzyme, which does not oxidize D-malate (2), and was largely independent of the production of D-malic enzyme.

To analyze the relative contribution of these two enzymes to the oxidation of the β-alkylmalates, use was made of the observation (Hegre and Stern, *unpublished*) that the D-malic enzyme of *E. coli* was inhibited by *erythro*-DL-β-hydroxyaspartate but not by its *threo*-DL isomer. This was also true of the *Salmonella* enzyme (Table 2). With extracts of the *dml* mutant, *erythro*-DL-β-hydroxyaspartate caused a large inhibition (65 to 83%) of the oxidation of D-malate but did not affect the oxidation of β-isopropylmalate. The *threo* isomer did not inhibit the oxidation of either substrate. This was additional evidence that the D-malic enzyme was distinct from the β-isopropylmalic enzyme and did not contribute to the oxidation of β-isopropylmalate.

It was thus possible to utilize the differential inhibition by the *erythro*-DL-β-hydroxyaspartate to analyze the relative contribution by the two enzymes to the oxidation of the β-alkylmalates. Experiments showed (Table 3) that only the oxidation of DL-β-methylmalate was significantly inhibited by *erythro*-DL-β-hydroxyaspartate, whereas oxidation of the β-propyl and ββ-dimethyl derivatives was little affected, and that of β-ethylmalate was not affected at all. Thus, the β-isopropylmalic enzyme of *Salmonella* can oxidize all the β-alkylmalates tested. Again, from relative reaction rates, it was evident that the activity of the β-propyl- and ββ-dimethylmalates with this enzyme was too low to be detected in the parent strain. The D-malic enzyme also oxidized β-methylmalate at a significant rate, and the combined

TABLE 2. *Effect of threo- and erythro-DL-β-hydroxyaspartates on the oxidation of D-malate and β-isopropylmalate by extracts of S. typhimurium SL 1634 dml-51*

Additions	Carbon source	
	D-Malate	DL-Malate
D-Malate	23.0[a]	16.3
D-Malate + *threo*-DL-β-hydroxyaspartate	21.6	17.9
D-Malate + *erythro*-DL-β-hydroxyaspartate	8.1	2.8
β-Isopropylmalate	64.0	63.0
β-Isopropylmalate + *threo*-DL-β-hydroxyaspartate	60.2	64.5
β-Isopropylmalate + *erythro*-DL-β-hydroxyaspartate	65.0	63.8

[a] Specific activity.

TABLE 3. *Effect of erythro-DL-β-hydroxyaspartate on the oxidation of DL-β-alkylmalates by S. typhimurium SL 1634 dml-51 grown on DL-malate*

Additions	Specific activity	Inhibition
DL-β-Methylmalate	63.0	%
DL-β-Methylmalate + *erythro*-DL-β-hydroxyaspartate	44.0	30
DL-β-Ethylmalate	34.7	
DL-β-Ethylmalate + *erythro*-DL-β-hydoxyaspartate	34.7	0
DL-β-Propylmalate	3.9	
DL-β-Propylmalate + *erythro*-DL-β-hydroxyaspartate	3.5	10
DL-ββ-Dimethylmalate	3.5	
DL-ββ-Dimethylmalate + *erythro*-DL-β-hydroxy-aspartate	3.3	6

attack of the two enzymes on this substrate explains why it was oxidized more rapidly than β-isopropylmalate.

DISCUSSION

The results in Table 1 show that the wild-type parent strain of *Salmonella* lacked D-malic enzyme and hence could not grow on D-malate as carbon source. Being a prototroph, it possessed β-isopropylmalic enzyme as a component of the leucine biosynthetic pathway. Its failure to grow on D-malate confirmed, at the nutritional level, the biochemical observation of Burns et al. (2), that partly purified β-isopropylmalic enzyme did not oxidize D-malate. On the other hand, a mutant (*dml-51*) of this strain which could grow on D-malate as sole carbon source possessed levels of D-malic enzyme sufficient to account for this character.

Interestingly, the mutant also possessed a greatly increased activity (16- to 19-fold) of its β-isopropylmalic enzyme. This raised the possibility that the *dml* mutant possessed an altered β-isopropylmalic enzyme capable of oxidizing D-malate. However, the finding that *erythro*-DL-β-hydroxyaspartate, a known inhibitor of the D-malic enzyme of *E. coli*, inhibited the oxidation of D-malate but not that of β-isopropylmalate strongly supported the existence of two separate enzymes. Genetic analysis (J. R. Stern and B. A. D. Stocker, *unpublished data*) proved conclusively that this was the case, since the *dml* gene mapped in another segment of the *Salmonella* chromosome from the *leuB* gene which codes for the β-isopropylmalic enzyme.

Both *Salmonella* strains oxidized a variety of β-alkylmalates, and the relative rates of oxidation of methyl, ethyl, and isopropyl derivatives were essentially the same in both strains. Of the β-alkylmalates tested, only the oxidation of DL-β-methylmalate was significantly (30%) inhibited by *erythro*-DL-β-hydroxyaspartate, and it was concluded that the oxidation of β-ethyl, β-propyl and ββ-dimethyl derivatives was effected solely by the β-isopropylmalic enzyme. The β-methyl derivative was oxidized mostly by the β-isopropylmalic enzyme (approximately 70%) and in part by the D-malic enzyme (approximately 30%).

The results compiled in Table 1 for *Aerobacter* grown on D-malic acid and for the D-malic enzyme of *E. coli* show that the D-malic enzymes on these two organisms can oxidize β-methylmalate at about the same rate as D-malate. This supports the conclusion that the *erythro*-DL-β-hydroxyaspartate-sensitive portion of the DL-β-methylmalate oxidation by the *dml* mutant *Salmonella* extracts was contributed by endogenous D-malic enzyme activity. It will be noted that the *Salmonella* D-malic enzyme is much less reactive with β-ethyl-, β-propyl-, and ββ-dimethyl-substituted malates than is the analogous enzyme in either *E. coli* or *A. aerogenes*.

Growth of *A. aerogenes* on D-malate resulted in a large induction of D-malic enzyme, whereas the activity of the β-isopropylmalic enzyme was only slightly increased. The extract of D-malate-grown cells oxidized DL-β-methylmalate as rapidly as D-malate and had small but increased activities with DL-β-ethyl and DL-ββ-dimethyl derivatives, whereas activity was not detected with DL-β-propylmalate. The substrate specificity of *Aerobacter* D-malic enzyme is quite similar to that of the purified *E. coli* enzyme and becomes more so if one corrects for the expected contribution by the β-isopropylmalic enzyme to the oxidation of β-alkylmalates.

The problem arises as to how the β-isopropylmalic enzymes "views" the configuration of its substrate (Materials and Methods). Does it recognize the intermediate of leucine biosynthesis as *threo*-D_s-α-hydroxy-β-carboxyisocaproic acid or as *erythro*-L$_B$-β-isopropylmalic acid, i.e., as a D or L isomer? Stern and Hegre (7) have shown in *E. coli* that growth on D-malate induced both D- and L-malic enzymes, whereas growth on L-malate induced only the L-malic enzyme. *S. typhimurium* strain SL 1634 is also an inducible mutant, since extracts of cells grown on L-malate or glycerol contain only traces of the D-malic enzyme. The activity of β-isopropylmalic enzyme in extracts of *S. typhimurium* strain SL 1634 grown on L-malate (or glycerol) was found to be

no greater than that in extracts of the parent strain 934 grown on L-malate or citrate. Thus, the β-isopropylmalic enzyme, like the D-malic enzyme, was induced by growth on D-malate but not L-malate. By this criterion, as well as by its capacity to oxidize β-alkylmalates that are not attacked by L-malic enzyme (Hegre and Stern, Federation Proc. 23:351, 1965), β-isopropylmalic enzyme belongs to the class of D-malic enzymes, and the above result is strong, though indirect, evidence that the *Salmonella* β-isopropylmalic enzyme "views" its substrate as a *threo*-D_s isomer.

Rabin et al. (6) have presented evidence that in *P. aeruginosa* the β-isopropylmalic enzyme is responsible for the oxidation of *erythro*-DL-β-ethylmalate. Here too, the enzyme may "view" the active isomer, which they assume to be *erythro*-Lg, as *threo*-D_s-α-hydroxy-β-carboxyvalerate.

ACKNOWLEDGMENTS

We thank Harriet Richardson for skillful technical assistance. This investigation was supported by grant GB-4716 from the National Science Foundation.

LITERATURE CITED

1. Burns, R. O., J. Calvo, P. Margolin, and H. E. Umbarger. 1966. Expression of the leucine operon. J. Bacteriol. 91:1570–1576.
2. Burns, R. O., H. E. Umbarger, and S. R. Gross. 1963. The biosynthesis of leucine. III. The conversion of α-hydroxy-β carboxyisocaproate to α-ketoisocaproate. Biochemistry 2: 1053–1058.
3. Calvo, J. M., C. M. Stevens, M. G. Kalyanpur, and H. E. Umbarger. 1964. The absolute configuration of α-hydroxy-β-carboxyisocaproic acid (3-isopropylmalic acid), an intermediate in leucine biosynthesis. Biochemistry 3:2024–2027.
4. Gornall, A. G., C. J. Bardawill, and M. M. David. 1949. Determination of serum proteins by means of the biuret reaction. J. Biol. Chem. 177:751–766.
5. Magee, P. T., and E. E. Snell. 1966. The bacterial degradation of pantothenic acid. IV. Enzymatic conversion of aldopantoate to α-ketoisovalerate. Biochemistry 5:409–416.
6. Rabin, R., I. I. Salamon, A. S. Bleiweis, J. Carlin, and S. J. Al. 1968. Metabolism of ethylmalic acids by *Pseudomonas aeruginosa*. Biochemistry 7:377–388.
7. Stern, J. R., and C. S. Hegre. 1966. Inducible D-malic enzyme in *Escherichia coli*. Nature 212:1611–1612.