Regulation of Expression of Novel Mandelate Dehydrogenases in Mutants of *Acinetobacter calcoaceticus*

By CELIA A. HILLS† AND CHARLES A. FEWSON*

Department of Biochemistry, University of Glasgow, Glasgow G12 8QQ, U.K.

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Wild-type strains of *Acinetobacter calcoaceticus* able to grow on only L(+) or D(–)-mandelate gave rise to mutants that could grow on the other isomer of mandelate. Each mutant contained an additional mandelate dehydrogenase which was not expressed in the parent strain. The novel enzymes were shown to be controlled co-ordinately with the pre-existing enzymes for the conversion of mandelate into benzoate when induced with phenylglyoxylate, gratuitously induced with thiophenoxyacetate, subjected to anti-induction by 2-phenylpropionate or catabolite-repressed by succinate. Mutants which had been selected on the basis of possession of a constitutive phenylglyoxylate decarboxylase also constitutively expressed both the original and the novel mandelate dehydrogenases.

INTRODUCTION

Acinetobacter calcoaceticus wild-type strain NCIB 8250 can grow on L(+)–mandelate but not on D(–)-mandelate and has only an L(+)–mandelate dehydrogenase; however, we isolated mutants that can also grow on D(–)-mandelate and possess a novel D(–)-mandelate dehydrogenase (Hills & Fewson, 1983). Conversely, wild-type strain EBF 65/65 has only a D(–)-mandelate dehydrogenase but gives rise to mutants containing an L(+)–mandelate dehydrogenase (Hills & Fewson, 1983). Both strains can also grow on benzyl alcohol. The converging metabolic pathways for the oxidation of mandelate and benzyl alcohol are shown in Fig. 1.

The properties of the novel mandelate dehydrogenases proved to be very similar to those of the original dehydrogenases (Hills & Fewson, 1983) and the question arose as to how these new enzymes are regulated. In the present paper we describe how two independent approaches indicate that the novel enzymes are regulated co-ordinately with the pre-existing enzymes that convert mandelate into benzoate. Some of these results have been presented in a preliminary form (Hills & Fewson, 1979).

METHODS

Bacteria. *Acinetobacter calcoaceticus* wild-type strain NCIB 8250 was obtained from the National Collection of Industrial Bacteria, Aberdeen, Scotland. Wild-type strain EBF 65/65 and mutant C48 were kindly provided by Dr A. Vivian, Thames Polytechnic, London.

The isolation and properties of the mutants used in this work are summarized in Fig. 2. Some of the strains have been deposited in the National Collection of Industrial Bacteria and these have been listed by Hills & Fewson (1983). Mutant C4248 was previously designated 61c (Towner, 1978).

Growth of bacteria and preparation of extracts. General methods for the maintenance of cultures and for the growth, harvesting and storage of bacteria have been summarized by Hills & Fewson (1983). 'Basal medium' contained 2 g KH_2PO_4 and 1 g $(\text{NH}_4)_2\text{SO}_4$ in 1 litre of glass-distilled water and was adjusted to pH 7.0 with NaOH. 'Salts medium' was prepared by adding 20 ml 2% (w/v) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ per litre of basal medium.

In experiments where enzyme activities were to be measured, 0.1 ml samples of stock cultures were inoculated into Oxoid CM1 nutrient broth (100 ml in 500 ml Erlenmeyer flasks) and grown at 23 °C without shaking for 48 h in the case of strain NCIB 8250 and its mutants or for 40 h in the case of EBF 65/65 and its mutants. These cultures

† Present address: Institute of Cancer Research, Clifton Avenue, Belmont, Surrey SM2 5PX, U.K.

Fig. 1. Metabolism of mandelate and benzyl alcohol by *A. calcoaceticus*. In wild-type strain NCIB 8250 L(+)-mandelate dehydrogenase (1), phenylglyoxylate (benzoylformate) decarboxylase (3; EC 4.1.1.7) and benzaldehyde dehydrogenase I (5) are co-ordinately induced by phenylglyoxylate and gratuitously induced by thiophenoxyacetate (Livingstone & Fewson, 1972). In wild-type strain EBF 65/65 D(-)-mandelate dehydrogenase (2), phenylglyoxylate decarboxylase (3) and benzaldehyde dehydrogenase I (5) are co-ordinately induced by phenylglyoxylate and thiophenoxyacetate (results in this paper and unpublished results of A. Scott and C. A. Fewson). In both wild-type strains benzyl alcohol dehydrogenase (4) and benzaldehyde dehydrogenase II (6) are co-ordinately induced by benzyl alcohol or benzaldehyde (Livingstone *et al.*, 1972; unpublished results of A. Scott and C. A. Fewson) and are repressed whenever phenylglyoxylate decarboxylase is present (Beggs & Fewson, 1977). Benzoate oxygenase (7) and cyclohexadienediolcarboxylate dehydrogenase (8) appear to form a separate regulon (Reiner, 1971), as does catechol-1,2-oxygenase (9; EC 1.13.11.1) which is induced by *cis,cis*-muconate (Stanier & Ornston, 1973).

were used as inocula (25%, v/v) for 5 mM-L-glutamate/salts media containing appropriate supplements as described in the Tables. All media were prepared so that the concentrations were correct after inoculation. The cultures (400 ml in 1 litre flasks) were grown at 23 °C with vigorous stirring and aeration (Harvey *et al.*, 1968; Beggs & Fewson, 1977). Samples were taken at intervals for measurement of turbidity and the bacteria were harvested when the A_{500} reached 0.5 to 0.7; this corresponded to approximately two generations and took between 2 and 5 h depending on the strain and the medium used. The bacteria were harvested by centrifuging at 6000 *g* for 20 min at 4 °C, washed by resuspension in ice-cold basal medium and then centrifuging at 12000 *g* for 30 min at 4 °C and finally stored at -18 °C.

Extracts were prepared by suspending bacteria in 0.1 M-KH₂PO₄/K₂HPO₄ buffer, pH 7.5, so that a 20-fold dilution had an A_{500} of 0.25. The suspensions were then disrupted by ultrasonic treatment as described by Livingstone *et al.* (1972) except that the current was 3 A. Intact bacteria and debris were removed by centrifuging at 12000 *g* for 30 min at 4 °C. The supernatant 'extracts' were then assayed immediately or stored at -18 °C.

Isolation of constitutive mutants. Mutants constitutive for phenylglyoxylate decarboxylase were isolated by a modification of the procedure of Fewson *et al.* (1978). Samples (0.1 ml) of 24 h nutrient broth cultures of the parent strains were spread on plates containing 2 mM-2-phenylpropionate + 2 mM-phenylglyoxylate/salts medium and 1.5% (w/v) Oxoid no. 1 agar. A few crystals of *N*-methyl-*N*-nitro-*N*-nitrosoguanidine were placed in the centre of some of the plates. Presumptive mutant colonies appearing during 10 d incubation at 30 °C were transferred to nutrient broth (10 ml), grown for 24 h at 30 °C and then cloned by streaking on plates of 2-phenylpropionate +

Fig. 2. Genealogy of mutants of *A. calcoaceticus*. (a) Isolation of strains with constitutive phenylglyoxylate decarboxylase activity by selection for growth on 2 mM-2-phenylpropionate (as anti-inducer) + 2 mM-phenylglyoxylate/salts medium (see Methods; Fewson *et al.*, 1978); (b) isolation of blocked mutant able to grow on phenylglyoxylate but not on mandelate (Livingstone & Fewson, 1972); (c) isolation of strains able to grow on L(+) or D(-)-mandelate (Hills & Fewson, 1983); (d) transformation and selection for ability to grow on D(-)- but not L(+)-mandelate (Ahlquist *et al.*, 1980; Hills & Fewson, 1983); (e) double auxotroph isolated by Towner & Vivian (1976). A few of the steps (*) involved mutagenesis, usually with *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine, as described in the appropriate references, but the others were all spontaneous mutants. Phenotypes: Mdl, L(+)-mandelate dehydrogenase; Mdd, D(-)-mandelate dehydrogenase; Pgc, phenylglyoxylate decarboxylase; the superscripts -, i and c denote no enzyme, inducible activity, and constitutive activity respectively.

phenylglyoxylate medium. Only about one-fifth of the colonies proved to have constitutive enzyme activities; the rest had presumably gained some other property that allowed them to grow in the presence of 2-phenylpropionate. To eliminate these unwanted strains, we made use of the observation that mutants possessing a constitutive phenylglyoxylate decarboxylase cannot grow readily on benzyl alcohol (Beggs & Fewson, 1977). We therefore made serial dilutions of the isolates and placed drops (10^{-4} and 10^{-5} dilutions in basal medium) on to plates of nutrient agar and on to plates of 5 mM-benzyl alcohol/salts agar. Strains that showed very faint or no growth after incubation for 48 h on benzyl alcohol were checked for the constitutive synthesis of phenylglyoxylate decarboxylase; inducible strains (including the wild-types, which were always included as controls) had formed large colonies by this time.

Measurements of enzyme activities. The following enzyme activities were measured using extracts: D(-)- and L(+)-mandelate dehydrogenases by the method of Hills & Fewson (1983), benzaldehyde dehydrogenase by the method of Beggs & Fewson (1977) and catechol-1,2-oxygenase by the method of Hegeman (1966). Phenylglyoxylate decarboxylase and benzyl alcohol dehydrogenase activities were measured using bacteria that had been treated with toluene, as described by Beggs & Fewson (1977) and Beggs *et al.* (1976) respectively. Toluene-treated bacteria were used because of the greater speed and convenience of this method and because smaller amounts of bacteria were required; unfortunately we have not yet developed a procedure for measuring D(-)-mandelate dehydrogenase in toluene-treated bacteria, and whilst L(+)-mandelate dehydrogenase can be measured in such cells (Beggs *et al.*, 1976), it seemed prudent to measure the activities of both mandelate dehydrogenases in the same preparation. All these assays were done in triplicate. Results are given as means and the replicates rarely showed a spread of more than 5%.

There is no convenient assay procedure for benzoate oxygenase so rates of O_2 uptake by intact bacteria were measured using Clark-type O_2 electrodes maintained at 27 °C (Hills & Fewson, 1983). The reaction mixture (final volume 3.0 ml) contained 67 mM- KH_2PO_4/K_2HPO_4 buffer, pH 7.0, and bacteria equivalent to an A_{500} of 1. Endogenous rates of respiration were measured for about 5 min and then benzoate was added (0.1 ml of a 15 mM solution adjusted to pH 7.0) and O_2 uptake followed for a further 5 min. Duplicate measurements were made in each case.

Materials and analytical methods. These were as listed by Hills & Fewson (1983).

RESULTS AND DISCUSSION

Occurrence of mutants with novel mandelate dehydrogenases

We made approximate estimates of the frequency of spontaneous occurrence of mutants able to grow on the second isomer of mandelate. Wild-type NCIB 8250 gave rise to colonies able to grow on D(–)-mandelate at a frequency of about 1 in 3×10^{10} . The frequency for strain C1408 (a mutant of NCIB 8250 lacking L(+)-mandelate dehydrogenase; Fig. 2) was very similar (approximately 1 in 2×10^{10}); its frequency of reversion to an active L(+)-mandelate dehydrogenase was rather lower (about 1 in 9×10^{10}). Strain C48 showed a considerably higher frequency of occurrence of mutants able to grow on L(+)-mandelate (about 1 in 3×10^8). Mutagenesis with *N*-methyl-*N*-nitro-*N*-nitrosoguanidine increased these frequencies. In the original experiments a trace amount of phenylglyoxylate (0.25 mM) was included in the mandelate plates used for selection in order to ensure that the mandelate enzymes were induced; however, omission of phenylglyoxylate did not seem to affect the frequency at which mutants were detected. After the discovery that D(–)-mandelate dehydrogenase is very heat-labile (Hills & Fewson, 1983), some experiments were done at 23 °C rather than at 30 °C but, again, this had little effect on the frequencies.

A few of the mutants (e.g. C1219) are rather susceptible to lysis, particularly when grown in defined media at higher temperatures (e.g. 30 °C) with vigorous agitation.

Eleven mutants (including C1041 and C4248) were examined in some detail. The D(–)-mandelate dehydrogenase appeared to have identical properties in every strain and was almost completely inhibited by 10 µM-*p*-chloromercuribenzoate and lost about 85% of its activity after incubation at 30 °C for 4 h, whereas in every strain the L(+)-mandelate dehydrogenase activity was virtually unaffected by these treatments (see also Hills & Fewson, 1983).

Regulation of the inducible novel mandelate dehydrogenases

The novel enzymes, D(–)-mandelate dehydrogenase in mutant C1041 and L(+)-mandelate dehydrogenase in C4248, were inducible. Like the original enzymes, they were synthesized in the presence of D(–)- or L(+)-mandelate or phenylglyoxylate as well as in the presence of the gratuitous inducer thiophenoxyacetate (Table 1). All the enzymes were subject to almost complete anti-induction by 2-phenylpropionate and to co-incident catabolite repression by succinate (Table 1).

In a series of experiments of the type described in Table 1, using mutants C1041 and C4248, correlation coefficients were calculated on the assumption that a linear relationship existed between the activities of the pairs of enzymes L(+)-mandelate dehydrogenase and D(–)-mandelate dehydrogenase, L(+)-mandelate dehydrogenase and phenylglyoxylate decarboxylase, and D(–)-mandelate dehydrogenase and phenylglyoxylate decarboxylase. The lowest correlation coefficient was 0.86 and most values were between 0.95 and 0.98.

Mutants with constitutive enzyme activities

The aim of these experiments was to test whether in mutants which synthesized the original mandelate enzymes constitutively the novel mandelate dehydrogenases were also constitutive or whether they were still inducible. It should be stressed that the isolation procedure was based solely on the selective advantage of having a constitutive phenylglyoxylate decarboxylase; neither isomer of mandelate was present in the selection medium.

Mutants expressing phenylglyoxylate decarboxylase constitutively were isolated on the basis of ability to grow on phenylglyoxylate in the presence of the anti-inducer 2-phenylpropionate (Fewson *et al.*, 1978). The screening procedure (see Methods) introduced to eliminate strains that were not constitutive but could grow in the presence of 2-phenylpropionate as a result of some other, unidentified, mutation proved successful. Twenty-three putative mutants (derived from strains C1041, C1040, C48 and C4248) all proved to have a constitutive phenylglyoxylate decarboxylase. Conversely, none of a random selection of 36 strains able to grow in the presence of 2-phenylpropionate but also capable of ready growth on benzyl alcohol synthesized the enzyme constitutively. This reciprocal expression of phenylglyoxylate decarboxylase and of

Table 1. *Induction and repression of D(–)- and L(+)-mandelate dehydrogenases and phenylglyoxylate decarboxylase in A. calcoaceticus mutant strains C1041 and C4248*

The mutants were grown in 5 mM-L-glutamate/salts medium containing appropriate additions, which were all at 5 mM except for thiophenoxyacetate, which was used at 2 mM in the case of strain C1041. The bacteria were harvested and then enzyme activities measured in extracts as described in Methods.

Addition to medium	Enzyme specific activities [nmol min ⁻¹ (mg protein) ⁻¹]					
	Mutant C1041			Mutant C4248		
	D(–)-Mandelate dehydrogenase	L(+)-Mandelate dehydrogenase	Phenylglyoxylate decarboxylase	D(–)-Mandelate dehydrogenase	L(+)-Mandelate dehydrogenase	Phenylglyoxylate decarboxylase
None	1	0	0	3	0	0
L(+)-Mandelate	54	57	466	59	37	367
D(–)-Mandelate	46	52	541	74	31	492
Phenylglyoxylate	46	71	595	94	57	494
Thiophenoxyacetate	14	24	109	41	44	256
Phenylglyoxylate + 2-phenylpropionate	0	0	0	2	3	0
Phenylglyoxylate + succinate	21	16	272	26	13	179

benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II extends the previous observations of Beggs & Fewson (1977) but is still unexplained.

The frequency of spontaneous occurrence of constitutive mutants in all the parental strains tested was about 1 in 10⁷. The frequency could be increased by mutagenesis (see Methods) but most of our work has been done with spontaneous mutants.

Mutants of wild-type NCIB 8250 were isolated by alternative routes (Fig. 2). In some, the ability to oxidize D(–)-mandelate was introduced first, then second-stage mutants were selected which had a constitutive phenylglyoxylate decarboxylase (e.g. strains C1123 and C1219). In others, mutants were first made constitutive for the original mandelate enzymes and then second-stage mutants were isolated that could grow on D(–)-mandelate (e.g. C1006).

Measurements of enzyme activities were made on several mutants with constitutive phenylglyoxylate decarboxylase activity: twelve (e.g. C1219) had been derived from C1041, three (e.g. C4654) from C48; five (e.g. C1123) from C1040, four (e.g. C1006) from C1005 and three (e.g. C4664) from C4248. In all cases the evolved dehydrogenases, as well as the original mandelate enzymes, were synthesized constitutively. Representative results are listed in Table 2 along with results for the parental strains. None of the constitutive strains grown on L-glutamate without inducer oxidized benzoate or possessed any detectable catechol-1,2-oxygenase activity (results not shown). The inducible strains NCIB 8250, C1408, C1041, C1040, C48 and C4248 had no detectable activities of any of the enzymes listed in Table 2 unless they had been grown in the presence of phenylglyoxylate (results not shown); after induction, they contained not only the enzymes necessary to form benzoate (Table 2) but they could also oxidize benzoate [100 to 450 nmol O₂ min⁻¹ (mg protein)⁻¹] and possessed catechol-1,2-oxygenase activity [100 to 200 nmol min⁻¹ (mg protein)⁻¹].

Conclusions

There are two types of evidence that the regulation of the enzymes converting D(–)-mandelate into benzoate in wild-type strain EBF 65/65 is extremely similar to the regulation of the enzymes converting L(+)-mandelate into benzoate in wild-type strain NCIB 8250. First, in mutants such as C4248 phenylglyoxylate, thiophenoxyacetate and 2-phenylpropionate act as inducer, gratuitous inducer and anti-inducer respectively just as they do in mutants such as C1041 or in NCIB 8250 itself (Table 1; Livingstone & Fewson, 1972; Fewson *et al.*, 1978).

Table 2. *Enzyme activities of various mutant strains of A. calcoaceticus*

The mutants were grown in 5 mM-L-glutamate/salts medium with or without 5 mM-phenylglyoxylate. The bacteria were harvested and the enzyme activities measured as described in Methods.

Strain	Addition	Enzyme specific activities [nmol min ⁻¹ (mg protein) ⁻¹]			
		D(–)-Mandelate dehydrogenase	L(+)-Mandelate dehydrogenase	Phenylglyoxylate decarboxylase	Benzaldehyde dehydrogenase
NCIB 8250	Phenylglyoxylate	0	93	614	14
C1005	None	0	81	709	13
C1408	Phenylglyoxylate	0	0	466	25
C1041	Phenylglyoxylate	79	90	597	27
C1040	Phenylglyoxylate	62	0	642	12
C1006	None	97	126	1012	10
C1123	None	107	0	901	13
C1219	None	61	71	761	22
C48	Phenylglyoxylate	103	0	814	25
C4654	None	111	0	552	36
C4248	Phenylglyoxylate	137	46	770	44
C4664	None	125	48	824	38

Secondly, mutants (e.g. C4654) can be isolated that constitutively synthesize the first three enzymes of the mandelate pathway (Table 2); in this respect they resemble the equivalent mutants of NCIB 8250 such as C1005 (Table 2; Fewson *et al.*, 1978).

The most important conclusion of the work described here is that in mutants of *A. calcoaceticus* that have gained an extra mandelate dehydrogenase, the novel enzymes are invariably regulated co-ordinately with the pre-existing mandelate enzymes (Tables 1 and 2). It was already known that all the mandelate dehydrogenases have very similar properties and in particular that the novel D(–)-mandelate dehydrogenase of NCIB 8250 is remarkably like the original D(–)-mandelate dehydrogenase of EBF 65/65 and the novel L(+)-mandelate dehydrogenase of EBF 65/65 is equally similar to the original L(+)-mandelate dehydrogenase of NCIB 8250 (Hills & Fewson, 1983). Therefore if, as we have suggested (Hills & Fewson, 1983), the new enzymes arose as a result of expression of silent genes then it would appear that these genes are intimately related to those that are expressed in the wild-type strains.

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