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Synthesis of the Enzymes of the Mandelate Pathway by *Pseudomonas putida*

II. Isolation and Properties of Blocked Mutants

G. D. HEGEMAN

Department of Bacteriology and Immunology, University of California, Berkeley, California

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ABSTRACT

HEGEMAN, G. D. (University of California, Berkeley). Synthesis of the enzymes of the mandelate pathway by *Pseudomonas putida*. II. Isolation and properties of blocked mutants. J. Bacteriol. 91:1155–1160. 1966.—Mutants of *Pseudomonas putida* blocked in early reactions of the pathway for oxidation of D-mandelate were isolated and partially characterized. The specific genetic lesions in these mutants made normal inducer-metabolites of the pathway nonmetabolizable. Under the conditions of gratuitous enzyme synthesis so obtained, it could be shown that the D and L isomers of mandelate are equipotent inducers, and that the synthesis of the first five enzymes of the mandelate pathway is coordinate. Further experiments with the blocked mutants showed that benzoylformate, the third intermediate of the pathway, acts as an inducer without prior conversion to mandelate, and that there is no inducible, concentrating permease for mandelate.

Synthesis of the enzymes for D-mandelate oxidation by the wild-type of *Pseudomonas putida* A.3.12 may be induced by any of the first three intermediates of the pathway. Phenoxyacetate, a nonmetabolizable inducer, elicits synthesis of the first five enzymes of the pathway (the mandelate group), but of no others (4).

Two mutants, which had lost respectively the ability to synthesize the first and second enzymes of the mandelate pathway, were isolated and characterized. These blocked mutants permitted a more refined analysis of control mechanisms and induction than had been possible with the wild type.

MATERIALS AND METHODS

Organism and methods of cultivation. P. putida A.3.12, described in the first paper of this series (4), and mutants derived from it were used in this study. The media and methods of cultivation were the same as those described previously (4).

Enzymological methods and extraction procedures. The extraction and assay procedures used in this work were described in the first paper of this series (4).

Induction and selection of blocked mutants. The mutagens used to induce blocked mutants in populations of the wild type were ethyl methane sulfonate (EMS) and ultraviolet light (UV). For treatment by irradiation, an exponentially growing culture was harvested, washed in basal medium, and resuspended in the same medium. A portion of this suspension was irradiated with an unfiltered UV lamp (model V-41, Ultra-Violet Products, Inc., San Gabriel, Calif.) while being agitated gently in a shallow layer in a petri dish. When 99.9% of the cells had been killed (60 sec, 25-cm distance from lamp to dish), the treated suspension was appropriately diluted in mineral base, and 0.1-ml portions containing approximately 200 viable cells were spread on petri plates of solidified basal medium containing 0.01% asparagine and 0.2% D-mandelate as carbon and energy sources. These manipulations, and subsequent incubation of the plates, were conducted in dim light to prevent photoreactivation. After about 48 hr, the plates were examined. The smallest colonies were picked from among the more numerous large colonies, and purified by streaking. From the streaked plate of each clone, a single colony was picked and transferred to a plate, the inoculum being spread heavily over a small area to produce a patch of confluent growth. After growth had occurred, the patched plate was replicated by Lederberg's technique (7) to a series of mineral base plates containing, in order of replication, D-mandelate, L-mandelate, benzoylformate, benzoate, succinate, and to a final yeast extract plate to control the success of replication.

Strains which failed to grow on D- or L-mandelate, benzoylformate, or benzoate, but which grew on succinate, were selected for further examination. The identity of each such strain as *P. putida* was established by examining the Gram reaction, motility, microscopic morphology, and ability to produce fluorescent pigment.

For preparing mutants with EMS, the general procedures described by Loveless and Howarth (8) and Strauss (10) were used. Specifically, an exponentially growing culture was harvested and resuspended in the phosphate buffer portion of the basal medium. A portion of this suspension was mixed with an equal volume of a 4% (v/v) solution of EMS (Eastman Organic Chemicals, Rochester, N. Y.), in the same buffer. After incubation with shaking for 1 hr at 30 C, 99.5% of the cells had been killed. At this time, the suspension was diluted, first into 5% (w/v) $Na_2S_2O_3 \cdot 6H_2O$ to destroy residual EMS, and subsequently into basal medium. Plating and subsequent manipulations were carried out as described in connection with the isolation of blocked mutants after UV treatment.

Primary selection on the basis of colony size greatly reduced the effort required to collect a reasonable number of mutants with appropriate lesions, since 1 to 5% of the small colonies were mutants with specific blocks in mandelate metabolism. The remainder proved to be leaky or cross-fed auxotrophs, leaky mandelate pathway mutants, and mutants bearing undetermined growth-attenuating lesions.

RESULTS AND DISCUSSION

Characterization of blocked mutants. Mutants unable to use D-mandelate as sole carbon and energy source fell into three broad categories as defined by their ability to grow on three aromatic acids (Table 1). Only members of group I, two of which were chosen for intensive study, will be discussed here. The first of these, designated *rac*$^-$, failed to grow with D-mandelate, but grew normally with L-mandelate as sole carbon source. This mutant was isolated after EMS treatment. Racemase activity was undetectable by spectrophotometric methods in extracts of *rac*$^-$ prepared from cells grown in the presence of DL-mandelate. The second mutant, designated *md*$^-$, was isolated after UV treatment. It was unable to grow at the expense of either isomer of mandelate, but grew normally with benzoylformate. Examination of extracts of induced *md*$^-$ showed it to be devoid of detectable L(+)-mandelate dehydrogenase activity by spectrophotometric assay. Both mutants can synthesize the genetically unaffected enzymes of the mandelate pathway at normal rates.

The genetic lesion in the *rac*$^-$ mutant makes the D isomer of mandelate nonmetabolizable; the genetic lesion in the *md*$^-$ mutant makes both isomers of mandelate nonmetabolizable, although they remain interconvertable by virtue of the retention of racemase function. In effect, therefore, these two mutants permit a determination of the role of both the D(−) and L(+) isomers of mandelate as possible inducers of later enzymes in the mandelate pathway.

Leakiness and reversion. It was important for the interpretation of later experiments to establish that each of these genetic lesions did indeed involve essentially complete loss of enzyme function, and that the contribution of spontaneous revertants was negligible even during growth in the presence of mandelate. To this end, extracts were prepared from cells of the *rac*$^-$ and *md*$^-$ grown in asparagine-basal medium supplemented with 0.01 M DL-mandelate. These extracts were then examined for the respective mutationally deleted activities by radiochemical assay, the sensitivity of which is limited only by efficiency of counting and the specific activity of the substrate. It is evident from the results (Table 2) that reversion is negligible and that the blocks are substantially complete. The activities found correspond to those obtained by dilution of extracts prepared from fully-induced wild-type cells with at least 10^7 parts of inactive protein.

Response of blocked mutants to D(−) and DL-mandelate. Two cultures each of the *rac*$^-$ and *md*$^-$

TABLE 1. *Growth patterns among blocked mutants*

Group	Carbon and energy sources			Position of block
	D(−)-Mandelate	Benzoate	*p*-Hydroxybenzoate	
I	−	+	+	Mandelate group
II	−	−	+	Benzoate oxidase or catechol group before β-ketoadipate enol-lactone
III	−	−	−	Between β-ketoadipate enol-lactone and the tricarboxylic acid cycle

TABLE 2. *Radiochemical assay of genetically deleted mandelate group enzymes performed on rac*$^-$ *and md*$^-$ *extracts prepared from cells grown in the presence of 0.01 M DL-mandelate**

Mutant	Specific activity†		Specific activity as per cent of that of fully induced wild type‡
	Mandelate racemase	L(+)-mandelate dehydrogenase	
rac$^-$	0.002	—	6×10^{-6}
md$^-$	—	0.0015	2×10^{-6}

* The average of determinations on two of each type of extract.

† Expressed as millimicromoles of substrate converted per minute per milligram of extract protein.

‡ The specific activities as a percentage of the mean values of those for extracts of DL-mandelate-grown cells (4).

mutants were established in asparagine-basal medium. One *rac*$^-$ culture was furnished with 0.01 M D(−)-mandelate, the other with 0.01 M L(+)-mandelate; one *md*$^-$ culture was furnished with 0.01 M DL-mandelate, and the other with 0.01 M benzoylformate. When the cultures had grown, they were harvested, extracted, and assays for the enzymes of the mandelate pathway were performed on the extracts. The results are reported in Table 3. For the *rac*$^-$ mutant, the nonmetabolizable D(−) isomer of mandelic acid is as effective as the metabolizable L(+) isomer as an inducer of the four later enzymes of the mandelate group [L(+)-mandelate dehydrogenase, benzoylformate decarboxylase, nicotinamide adenine dinucleotide (NAD)-benzaldehyde dehydrogenase and NAD phosphate (NADP)-benzaldehyde dehydrogenase]. There is, however, a striking difference in the inductive properties of the two isomers with respect to pyrocatechase and lactonizing enzyme, whose synthesis is elicited only by the metabolizable L(+) isomer of mandelate. These data provide unambiguous evidence that the first inducer-substrate of the mandelate pathway is capable of inducing all the enzymes concerned in the conversion of D(−)-mandelate to benzoate.

The inductive responses of the *md*$^-$ mutant (Table 3) were entirely congruent: in this case, a mixture of the two isomers of mandelate, neither of which can be converted to later members of the pathway, fully induces benzoylformate decarboxylase and the two benzaldehyde dehydrogenases, in addition to mandelate racemase. Again, there was no induction of two enzymes of the catechol group: pyrocatechase and lactonizing enzyme. The inductive response of the *md*$^-$ mutant to benzoylformate is also noteworthy. Benzoylformate elicits full induction of mandelate racemase, even though this mutant is devoid of L(+)-mandelate dehydrogenase. Hence, this specific back-induction cannot be attributed to an endogenous formation of mandelate, serving secondarily as an inducer of racemase; benzoylformate must, therefore, directly induce the synthesis of the racemase. In their totality, these experiments demonstrate that all five enzymes of the mandelate group can be induced to full levels of activity by any one of the first three intermediates of the mandelate pathway.

Coordinateness of the mandelate group and affinity of apparent inducers for the inducer-sensitive system. The blocked mutants were also used to determine the dependence of the rate of enzyme synthesis on inducer concentration under conditions of gratuity. These experiments additionally provide a more powerful test of the degree of coordinacy of synthesis within the mandelate group. Flasks of liquid asparagine-basal medium were supplemented with DL-mandelate at concentrations ranging from 10^{-2} to 10^{-6} M, and provided with an inoculum of uninduced cells of the *md*$^-$ mutant. When the cultures had grown for approximately four generations, the cells were harvested and extracted, and assays for the mandelate pathway enzymes were performed. The specific activities at each inducer concentration are given in Table 4. The enzyme levels of the four nondeleted enzymes of the mandelate group increase coordinately with DL-mandelate concentration. These data, when plotted by the method of Ames and Garry (1) give straight lines running through the origin for each of the six possible pairs of four enzymes. A similar experiment carried out with the *rac*$^-$ mutant, in which D(−)-mandelate served as the gratuitous inducer, gave completely consistent results. The specific activities obtained at each inducer concentration in this latter experiment are presented in Table 5.

With both mutants and their respective non-

TABLE 3. *Levels* of mandelate pathway enzymes found in extracts of cells of the rac*$^-$ *and md*$^-$ *mutant grown in asparagine-basal medium supplemented with 0.01 M inducer*

Enzyme	*rac*$^-$		*md*$^-$	
	D(−)-Mandelate†	L(+)-Mandelate	DL-Mandelate†	Benzoylformate
Mandelate racemase	—‡	—‡	265	231
L(+)-Mandelate dehydrogenase	285	325	—‡	—‡
Benzoylformate decarboxylase	420	344	377	320
NAD-benzaldehyde dehydrogenase	305	225	252	223
NADP-benzaldehyde dehydrogenase	162	130	177	153
Pyrocatechase	≦0.05	294	≦0.05	270
Lactonizing enzyme	≦0.01	434	≦0.01	415

* Expressed as millimicromoles of substrate converted per minute per milligram of extract protein.
† Nonmetabolizable inducer.
‡ This enzyme is absent in the mutant in question.

TABLE 4. *Specific activities* of mandelate pathway enzymes in extracts of md⁻ prepared from cells grown in asparagine-basal medium supplemented with several concentrations of* DL-*mandelate*

Enzyme	DL-Mandelate concn†				
	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Mandelate racemase	300	265	110	0.2	0.1
L(+)-Mandelate dehydrogenase‡	≦0.03	—‡	—	—	—
Benzoylformate decarboxylase	580	528	212	≦1.0	≦0.5
NAD-benzaldehyde dehydrogenase	310	272	124	≦1.0	≦0.2
NADP-benzaldehyde dehydrogenase	147	132	66	≦1.0	≦0.5
Pyrocatechase	≦0.05	—	—	—	—
Lactonizing enzyme	≦0.01	—	—	—	—

* Expressed as millimicromoles of substrate converted per minute per milligram of extract protein.
† Moles per liter.
‡ This enzyme is not present in the *md⁻* mutant.

TABLE 5. *Specific activities* of four mandelate group enzymes in extracts of the rac⁻ mutant prepared from cells grown in asparagine-basal medium supplemented with* D(−)-*mandelate*

Enzyme	D(−)-Mandelate concn†						
	10^{-3}	3×10^{-4}	10^{-4}	3×10^{-5}	10^{-5}	3×10^{-6}	10^{-6}
Mandelate racemase	415	358	208	140	8	≦0.05	≦0.03
Benzoylformate decarboxylase	630	580	360	226	10	≦0.5	≦0.5
NAD-benzaldehyde dehydrogenase	375	326	194	96	4	≦0.8	≦0.4
NADP-benzaldehyde dehydrogenase	174	175	103	56	9	≦0.2	≦0.2

* Expressed as millimicromoles of substrate converted per minute per milligram of protein.
† Moles per liter.

metabolizable inducers, all four nondeleted enzymes of the mandelate group responded to varied inducer concentration by coordinated adjustment of rates of synthesis, as reflected by relative specific activities. A concentration of about 2×10^{-4} M D(−) or DL-mandelate evoked half the specific levels evoked by 10^{-2} M inducer. Both stereoisomers of mandelate are equally effective inducers of the mandelate group enzymes.

Search for an inducible concentrating permease for mandelate. The entry of material into cells of bacteria is often mediated by transport systems. These systems, often termed "permeases" (2), are known to function in the metabolism of di- and tricarboxylic acids by *Pseudomonas* sp. (6, 9). Permeases are specific, may concentrate components of the medium within the cell, and are often inducible. The existence of an inducible concentrating permease for mandelate in strain A.3.12 would vitiate the conclusions drawn above concerning both the specificity of induction and the dependence of induction upon inducer concentration, since the specificity and concentrating effect of the permease would mask the response of the regulatory apparatus to the inducer.

Two kinds of experiments were undertaken in an attempt to demonstrate an inducible concentrating permease. The first approach depended on the observation that an induced population of cells responds to the inducer in a different way than a noninduced population if a concentrating permease mediates the entry of the inducer into the cells (3). Cells of mutant *md⁻* growing exponentially in asparagine-basal medium in the presence of 0.01 M DL-mandelate were harvested, washed, and transferred to unsupplemented asparagine-basal medium. After four doublings of turbidity in the noninducing medium, this culture was used to inoculate a series of cultures containing graded concentrations of DL-mandelate, as in the previous experiment on the de-

TABLE 6. *Specific activities[a] of mandelate group enzymes in extracts of mutant md⁻ grown in the presence of graded concentrations of DL-mandelate in asparagine-basal medium[b]*

Enzyme	DL-Mandelate concn[c]				
	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Mandelate racemase	440	410	102	34	32
L(+)-Mandelate dehydrogenase[d]	≦0.03	—	—	—	—
Benzoylformate decarboxylase	400	326	130	51	41
NAD-benzaldehyde dehydrogenase	326	290	76	29	24
NADP-benzaldehyde dehydrogenase	—	128	97	20	16

[a] Expressed as millimicromoles of substrate converted per minute per milligram of extract protein.
[b] The inoculum was preinduced by prior growth in the presence of DL-mandelate.
[c] Moles per liter.
[d] Genetically deleted in the *md⁻* mutant.

pendence of the rate of enzyme synthesis on inducer concentration. The several cultures were harvested when the appropriate amount of growth had occurred, and the cells were extracted. Assays for members of the mandelate group of enzymes were performed on the extracts. Allowing for the carry-over of 6.25% of the fully induced enzyme level due to the dilution of the induced inoculum, the specific activities at each inducer concentration (Table 6) were virtually identical with those obtained when a noninduced inoculum was used (Table 4). Despite previous exposure of the cells to inducer, nonsaturating quantities of inducer evoked enzyme levels that were not measurably higher than those obtained in a parallel experiment for which the inoculum had been grown on noninducing medium for many transfers. The absence of preinduction effects on the dependence of rate of enzyme synthesis on inducer concentration indicates that the entry of mandelate into the cell is not mediated by a concentrating permease.

A direct attempt to demonstrate a permease was undertaken by use of the membrane-filter technique of Cohen and Monod (2). DL-Mandelate labeled in the carboxyl group with C^{14} at a specific activity of 4.92 mc/mmole was used in these experiments. Twice-washed cells of mutant *md⁻* grown (i) in asparagine-basal medium and (ii) the same medium supplemented with 0.01 M DL-mandelate, were compared with regard to the ability to concentrate 10^{-3}, 10^{-4}, and 10^{-5} M labeled DL-mandelate. The effects of 0.01 M KCN and 0.4% asparagine on the concentration process were also studied.

Radioactivity rapidly entered the cells in all experiments, the process of equilibration having a half-time of less than 5 sec at 30 C. Assuming a cell-water space of about 50%, the activity was equilibrated with the external medium without concentration. The state of induction of the cells and the presence of asparagine or KCN affected neither the rate of attainment nor final value of this equilibrium.

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