Selection and Characterization of a Mutant of the Cloned Gene for Mandelate Racemase That Confers Resistance to an Affinity Label by Greatly Enhanced Production of Enzyme†

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ABSTRACT: The plasmid pSCR1 containing the gene for mandelate racemase (EC 5.1.2.2) from *Pseudomonas putida* (ATCC 12633) allows *Pseudomonas aeruginosa* (ATCC 15692) to grow on (*R*)-mandelate as its sole carbon source [Ransom, S. C., Gerlt, J. A., Powers, V. M., & Kenyon, G. L. (1988) *Biochemistry 27*, 540]; the chromosome of the *P. aeruginosa* host apparently does not contain the gene for mandelate racemase but does contain genes for the remaining enzymes in the mandelate pathway and enables growth on (*S*)-mandelate as carbon source. However, in the presence of α-phenylglycidate, an active-site-directed irreversible inhibitor (affinity label) of mandelate racemase, *P. aeruginosa* transformed with pSCR1 can utilize (*S*)-mandelate but not (*R*)-mandelate as carbon source. This inhibition of growth on (*R*)-mandelate provides a metabolic selection for mutants that are resistant to α-phenylglycidate. When (*R*)-mandelate is used as carbon source and α-phenylglycidate is present, a few colonies of *P. aeruginosa* transformed with pSCR1 grow slowly and appear on plates after several days. The plasmid isolated from these cells confers resistance to α-phenylglycidate on newly transformed cells of *P. aeruginosa*. This resistance to the affinity label is not due to a mutation within the primary structure of the enzyme. A single base change (C → A) located 87 bp upstream of the initiation codon for the gene for mandelate racemase was detected in three independent isolates of α-phenylglycidate-resistant colonies and appears responsible for a 30-fold increase in the amount of mandelate racemase encoded by the gene contained in the plasmid. This mutation is within a sequence that is homologous to the consensus sequence observed for promoters in *Escherichia coli*; in accord with the presumed presence of a promoter sequence, nuclease S_1 mapping reveals that initiation of transcription begins 11 ± 1 bp downstream of the position of the mutation. However, nuclease S_1 mapping of mRNA isolated from *P. putida* induced for synthesis of the enzymes of mandelate pathway does not reveal significant amounts of a transcript starting at this potential promoter sequence. Thus, in *P. putida* the promoter for the mandelate racemase gene lies further upstream, suggesting that mandelate racemase is not the first gene in the putative mandelate operon. Large amounts of homogeneous mandelate racemase having the same N-terminal sequence as enzyme isolated from *P. putida* can be easily isolated from the cells of *P. aeruginosa* transformed with the plasmid containing the promoter mutation.

Mandelate racemase (EC 5.1.2.2) catalyzes the interconversion of the *R*- and *S*-enantiomers of mandelic acid (Kenyon & Hegeman, 1979); only the *S*-enantiomer of mandelate is a substrate for mandelate dehydrogenase, which initiates the oxidative catabolism of this aromatic acid by *Pseudomonas putida* (ATCC 12633) (Gunsalus et al., 1953). The genes for the enzymes in the mandelate pathway are thought to constitute an operon since the activities of all five enzymes in the operon are coordinately regulated (Hegeman, 1966b) and the genes for all five enzymes can be cotransduced (Wheelis & Stanier, 1970). However, no DNA sequence data are yet available that demonstrate that the genes for the enzymes in the pathway are arranged in an operon rather than in closely spaced, coordinately controlled regulons.

Mandelate racemase has been shown to be irreversibly inactivated by α-phenylglycidate, an oxirane analogue of mandelate. The inactivation requires Mg(II), which is also required for catalytic activity and can be prevented by the presence of either enantiomer of mandelate; thus, α-phenylglycidate is considered to be an active-site-directed affinity reagent (affinity label) for mandelate racemase (Fee et al., 1974). More recent studies have also demonstrated that the *R*-enantiomer of α-phenylglycidate is a more potent irreversible inactivator than the *S*-enantiomer (Whitman et al., 1985).

Our laboratories recently reported the cloning of the gene for mandelate racemase in *Pseudomonas aeruginosa*, its DNA sequence analysis, and its modest expression in *Escherichia coli* (Ransom et al., 1988). The screen for the cloning was based upon the absence of a gene for mandelate racemase in *P. aeruginosa* and the associated inability of this host strain to utilize (*R*)-mandelate as sole carbon source. The presence of the plasmid pSCR1 containing the gene for mandelate racemase allows growth of the transformed host on (*R*)-mandelate. In an effort to obtain more efficient expression of the gene for mandelate racemase so that crystallographic and biochemical experiments might be more easily accomplished, we have selected mutants of pSCR1 that allow the *P. aeruginosa* host to utilize (*R*)-mandelate as carbon source

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in the presence of racemic α-phenylglycidate. We have isolated a mutant that is resistant to α-phenylglycidate and have determined that its resistance can be explained by an approximate 30-fold increase in the amount of mandelate racemase encoded by the cloned gene; this increase in the level of mandelate racemase can be associated with the formation of large amounts of an mRNA transcript beginning 11 bp downstream of a C → A transversion in an apparent promoter sequence in the mutant plasmid. Large amounts of homogeneous mandelate racemase suitable for crystallization can be easily isolated from *P. aeruginosa* transformed with the plasmid containing the base change. Nuclease S_1 mapping of mRNA isolated from *P. putida* has revealed that mandelate racemase is not likely to be the first gene in the putative mandelate operon.

Materials and Methods

The plasmid pSCR1 containing the cloned and sequenced gene for mandelate racemase was described previously (Ransom et al., 1988). This plasmid was propagated in *P. aeruginosa* (ATCC 15692) and confers both streptomycin and kanamycin resistance to the transformed host.

Bacteriophages M13mp18 and M13mp19 were obtained from Bethesda Research Laboratories and were propagated in *E. coli* strain JM101. Restriction endonucleases and the M13–40 sequencing primer were obtained from New England Biolabs. Bacteriophage T_4 DNA ligase, polynucleotide kinase, and calf intestinal alkaline phosphatase were purchased from Boehringer Mannheim Biochemicals. All enzymes were used according to the supplier's instructions. DNA sequence analysis was performed by using the Sequenase kit and procedure obtained from United States Biochemicals. Other materials were as described previously (Ransom et al., 1988).

Racemic α-phenylglycidate was prepared according to the method of Fee et al. (1974).

Selection for Mutants Resistant to α-Phenylglycidate. A single colony of *P. aeruginosa* transformed with pSCR1 was grown overnight at 37 °C in 4 mL of LB[1] medium containing 1 mg/mL streptomycin. Fifty microliters of the culture was spread on a plate of minimal salts containing 0.020 M (*R*)-mandelate as sole carbon source. A glass fiber filter approximately 5 mm in diameter was placed in the middle of the plate, and 35 μL of a 54 mM solution of α-phenylglycidate was absorbed to the filter. The plates were incubated at 37 °C for two days, at which time a clear zone (halo) of no growth was observed around the filter; after incubation was continued at 37 °C for an additional two days, mutant colonies resistant to α-phenylglycidate appeared within the halo. Several of these colonies were randomly selected and purified by streaking on plates of LB containing streptomycin. Single colonies were used to inoculate 4-mL overnight cultures of LB containing streptomycin, and resistance to α-phenylglycidate was assessed by repeating the filter disk procedure. This exact procedure was repeated to obtain independently isolated mutants.

Another set of independently isolated mutants resistant to α-phenylglycidate was obtained by repeating this procedure with *P. aeruginosa* transformed with a plasmid containing a subclone of the *P. putida* DNA present in pSCR1. A *Hin*dIII fragment containing the gene for mandelate racemase as well as 1138 bp of the 5′-upstream flanking sequence was excised from pSCR1 and ligated into the *Hin*dIII site of pKT230. Transformants were selected for resistance to kanamycin so the orientation of the mandelate racemase gene in the resulting recombinant plasmid (pSCR11) is the same as that in pSCR1. *P. aeruginosa* transformed with pSCR11 is able to utilize (*R*)-mandelate as sole carbon source, and α-phenylglycidate inhibits utilization of (*R*)-mandelate.

DNA Sequence Analysis of Mutants. The DNA sequence of the mandelate racemase gene in pSCR1 as well as 1138 bp upstream of the initiation codon (S. C. Ransom and J. A. Gerlt, unpublished observations) was redetermined for the wild type and compared to that of one mutant. Subclones of appropriate restriction fragments from both wild-type and mutant plasmids were constructed in M13 and sequenced by the chain-terminating procedure using the M13–40 sequencing primer and the following (sense strand) oligonucleotide primers that begin at the designated distance upstream (negative numbers) or downstream (positive numbers) of the initiation codon for the gene for mandelate racemase: CTACTGGGCTGAACGGT (−941); CCTGCACACTGGTTACA (−742); TCTAGCTTAGAAATGCA (−539); CGCCAATGGAAGTTTTG (−328); CGGCTTCCGACGGGGTTC (−268); CGACGCACCCTTGCCCA (−122); GGAACTGTTGGCACAGC (+78); GGCAGGTTATACGGGAT (+278); and TCCGGGCGGTTAAGACC (+478).

Nuclease S_1 Mapping. Total RNA was isolated according to a published procedure (Aiba et al., 1981; Ebina & Nakazawa, 1983) from early log phase cultures of *P. putida* and *P. aeruginosa* transformed with either wild-type or mutant pSCR1 grown in (*R*)-mandelate minimal medium.

The *Hin*dIII–*Bam*HI restriction fragment from pSCR1 containing the coding region for the N-terminal portion of mandelate racemase and 1138 bp of the upstream 5′-flanking sequence (Ransom et al., 1988) was cloned in M13mp19. The primer CCGTGTCGCGGAGAACAA (approximately 4 ng) containing the sequence of the *antisense* strand starting 87 bp downstream of the initiation codon for mandelate racemase and proceeding 17 bp back toward the initiation codon was annealed to the M13 template (1 μg) containing the sense strand and elongated with Klenow fragment in the presence of [α-^{32}P]dATP (600 Ci/mmol) and unlabeled dCTP, dGTP, and dTTP. The resulting double-stranded DNA was restricted with *Sal*I to produce a 960-bp duplex in which the antisense strand is radioactively labeled. The labeled duplex was subjected to electrophoresis under denaturing conditions in an agarose gel, and the labeled DNA strand complementary to the RNA transcript was isolated by electroelution.

The labeled 960-bp DNA strand (21 000 cpm) was hybridized to samples of total cellular RNA (10 μg) under high stringency conditions. The resulting RNA–DNA duplexes were treated with nuclease S_1 (3.5 units) to remove single-stranded DNA and RNA from the labeled heteroduplexes. The amount of nuclease S_1 used in these experiments was selected to completely digest the single-stranded DNA probe; larger amounts of the enzyme were observed to partially digest the DNA probe in the presence of complementary RNA. The radioactively labeled RNA–DNA duplexes so obtained were subjected to electrophoresis on a polyacrylamide gel next to a sequence ladder generated by using the M13 containing the wild-type *Hin*dIII–*Bam*HI fragment as template and the oligonucleotide used to prepare the radioactively labeled DNA probe as primer (Davis et al., 1986).

Growth of Bacterial Strains for Comparisons of Levels of Mandelate Racemase. Parallel cultures of *P. aeruginosa* and *P. aeruginosa* transformed with either pSCR1 or pMRαPG (the plasmid conferring resistance to α-phenylglycidate) were

[1] Abbreviations: LB, Luria broth; Tris, tris(hydroxymethyl)aminomethane; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

prepared as follows. For plasmid-transformed cells, streptomycin (0.7 mg/mL) was included in all LB media and agar to ensure maintenance of the plasmid. A fresh single colony was used to inoculate 5 mL of LB medium, and after 24 h at 30 °C, 10-μL aliquots were used to inoculate separate 5-mL cultures of mandelate minimal medium (Hegeman, 1966a). After 20 h at 30 °C, 100-μL aliquots were used to inoculate separate 50-mL cultures of both mandelate minimal salts and LB media. The cells were grown at 37 °C to late log phase. Cultures grown in LB medium (OD_{590} = 2–3) were harvested after 18 h by centrifugation (5000*g* at 4 °C for 15 min), and cultures grown in mandelate minimal salts medium (OD_{590} = 1.0–1.6) were harvested after 28 h. The cells were washed with a 0.9% solution of NaCl and stored at −20 °C until assays were performed.

For measurements of enzyme activity, the frozen cells were thawed and resuspended in 4 mL of cold 50 mM Tris-HCl, pH 8.0, containing 10 mM $MgCl_2$ and 0.1 mM phenylmethanesulfonyl fluoride. The suspensions were incubated at 4 °C and 1 h with 1 mg of lysozyme and then lysed by sonication. Following centrifugation (5000*g* for 15 min), the supernatant fractions were assayed for mandelate racemase activity.

Purification of Mandelate Racemase from the Mutant Conferring Resistance to α-Phenylglycidate. A single colony of *P. aeruginosa* transformed with pMRαpg was used to inoculate 5 mL of LB medium containing 0.7 mg/mL streptomycin (LB/strep medium). After growth for 12 h at 37 °C, 1-mL aliquots were used to inoculate four 50-mL cultures of LB/strep medium. After 5 h at 37 °C, each 50-mL culture was used to inoculate 1500 mL of LB/strep medium contained in a 2800-mL Fernbach flask. After growth at 37 °C for 30 h, the cells were harvested by centrifugation (5000*g* for 15 min) and stored at 20 °C. Frozen cells (26.6 g) from 3 L of LB/strep medium were thawed and suspended in 450 mL of 50 mM Tris-HCl, pH 8.0, containing 10 mM $MgCl_2$ (Tris–Mg buffer). The suspension was disrupted by sonication (nine cycles of 3 min with a continuous duty cycle; the temperature was maintained below 15 °C with an ice/ethanol bath). Cell debris was removed by centrifugation at 12000*g* for 25 min. All further procedures were performed at 4 °C.

A solution of 530 mg of protamine sulfate dissolved in 27 mL of H_2O was added dropwise to the supernatant (470 mL) over 10 min. After an additional 15 min of stirring, the mixture was centrifuged at 12000*g* for 25 min. Solid ammonium sulfate (202 g, Sigma grade III) was added over 15 min to the supernatant (270 mL) to achieve 65% saturation, and the mixture was stirred for 75 min. The mixture was centrifuged (12000*g* for 25 min), and the pellets were resuspended in 30 mL of Tris–Mg buffer. The suspension was dialyzed overnight against 1 L of Tris–Mg buffer.

After clarification by centrifugation, the enzyme-containing solution (66.5 mL) was chromatographed on a 25 × 2.5 cm column of DEAE-Sephacel equilibrated in Tris–Mg buffer at a flow rate of 35 mL/h. The enzyme was eluted with a 1-L linear gradient from 0 to 0.5 M NaCl in Tris–Mg buffer. Fractions were collected at 12-min intervals and assayed for mandelate racemase activity. Fractions containing approximately 90% of the enzyme activity were pooled (90 mL) and concentrated by ultrafiltration (Amicon YM-30 membrane) to a volume of 3.55 mL.

After clarification by centrifugation, the enzyme-containing solution was gel filtered on a 91 × 2.5 cm column of S-200 Sephacryl equilibrated in Tris–Mg buffer at a flow rate of 13.5 mL/h. Fractions were collected at 20-min intervals and assayed for mandelate racemase activity. Fractions containing approximately 90% of the enzyme activity were pooled (26 mL) and concentrated by ultrafiltration to a volume of 3.0 mL.

After clarification by centrifugation, 200-μL aliquots of the enzyme-containing solution (approximately 8 mg each) were chromatographed by FPLC on a 10 × 1 cm column of Pharmacia Mono Q anion-exchange resin (HR 10/10) using a 20-min linear gradient of 420–560 mM ammonium bicarbonate, pH 8.5, at a flow rate of 2 mL/min. The enzyme-containing fractions were combined (140 mL) and concentrated by ultrafiltration to a volume of 1.0 mL. The ammonium bicarbonate buffer was replaced with Tris–Mg buffer by dilution of the sample to 50 mL with Tris–Mg buffer and concentration by ultrafiltration to 2 mL; this process was subsequently repeated twice. Small amounts of insoluble material (presumably aggregated mandelate racemase) were removed by centrifugation. The final volume of the solution containing electrophoretically homogeneous mandelate racemase (as judged by Coomassie Blue staining of an SDS–polyacrylamide gel) was 3.2 mL.

N-Terminal Sequence Analysis of Mandelate Racemase. One nanomole of the mandelate racemase isolated from *P. aeruginosa* transformed with pMRαpg was dried by vacuum centrifugation (Savant Speedvac) and subjected to automated N-terminal sequence analysis with an Applied Biosystems Model 470A gas-phase sequenator located in the Biomolecular Resource Center at the University of California, San Francisco.

Assay of Mandelate Racemase. Mandelate racemase activity was measured in the direction of racemization of (*R*)-mandelate at 25 °C by using the coupled assay with (*S*)-mandelate dehydrogenase described by Hegeman (1970) except that 13.26 M^{-1} cm^{-1} was used as the extinction coefficient for the change in absorbance at 550 nm. Protein concentrations were determined by the method of Lowry (Lowry et al., 1951) using bovine serum albumin as standard.

Results and Discussion

Selection for and Initial Characterization of Mutants Resistant to α-Phenylglycidate. Both *P. aeruginosa* transformed with pSCR1 and *P. putida* can utilize either enantiomer of mandelate as sole carbon source. The antibiotic activity of α-phenylglycidate in the presence of either enantiomer of mandelate was determined by the method described under Materials and Methods. In the presence of α-phenylglycidate, both *P. aeruginosa* transformed with pSCR1 and *P. putida* can utilize (*S*)-mandelate in the presence of a glass fiber filter impregnated with α-phenylglycidate, as evidenced by a continuous lawn to the edge of the disk (data not shown). However, in the presence of α-phenylglycidate, neither *P. aeruginosa* transformed with pSCR1 nor *P. putida* can utilize (*R*)-mandelate as carbon source. A pronounced halo due to growth inhibition occurs around the disk when *P. aeruginosa* transformed with pSCR1 is grown on (*R*)-mandelate as sole carbon source. In a control experiment, the hydrolysis product of α-phenylglycidate, 2-phenylglycerate (Maggio et al., 1975), inhibited the growth of neither *P. aeruginosa* transformed with pSCR1 nor *P. putida*. The simplest explanation for these observations is that in vivo α-phenylglycidate prevents racemization of (*R*)-mandelate by irreversible alkylation and inactivation of mandelate racemase. The antibiotic activity of α-phenylglycidate when (*R*)-mandelate is used as sole carbon source provides an effective screen for the isolation of mutants that are resistant to α-phenylglycidate.

Although a clear halo of growth inhibition around the filter impregnated with α-phenylglycidate is observed after two days,

further incubation for two days results in the appearance of isolated colonies within the halo. Reasoning that these colonies may have been mutants that were resistant to α-phenylglycidate either by overproduction of the enzyme or by a mutation in the primary structure of mandelate racemase, we purified colonies from three independent selections for resistance to α-phenylglycidate (as described under Materials and Methods), and these were partially but not totally resistant to the antibiotic action of α-phenylglycidate. These were subjected to further genetic and biochemical scrutiny. We note that no mutagen was used in these experiments, but we have not investigated the interesting possibility that α-phenylglycidate itself may, in fact, be a mutagen.

Isolated plasmids from each independent selection were used to transform *P. aeruginosa*, and the transformants were tested for resistance to α-phenylglycidate. In each case, when the transformation mixture was plated initially on LB plates containing streptomycin to select for transformants, replicate plating on minimal medium plates containing (*R*)-mandelate as sole carbon source as well as α-phenylglyciate and streptomycin revealed that all of the transformants were able to confer resistance to α-phenylglycidate on the transformed host. Thus, the resistance to α-phenylglycidate is associated with a mutation in the plasmid and not in the chromosome of the transformed host.

Restriction analysis of the mutant plasmids did not provide any evidence for gene amplification (Stark & Wahl, 1984) that could provide resistance to α-phenylglycidate by overproduction of mandelate racemase.

The mutation conferring resistance to α-phenylglycidate was located in either the 5′-upstream flanking region or the first 280 codons of the structural gene for mandelate racemase fragment by "swapping" pertinent restriction fragments between a mutant and the wild-type plasmid. Either the mutant 3.7-kb *Hin*dIII fragment that contains the entire coding region for mandelate racemase (1080 bp), the 1138 bp upstream of the initiation codon for mandelate racemase (Ransom et al., 1988; S. C. Ransom and J. A. Gerlt, unpublished observations) and approximately 1.5 kb of unsequenced 3′-flanking DNA, or the mutant 5.1-kb *Eco*RI–*Bam*HI fragment that contains the 5′-upstream flanking region but only the first 280 codons of the coding region for mandelate racemase was able to confer resistance to α-phenylglyciate to a transformed host when ligated to the corresponding large restriction fragment obtained from wild-type pSCR1.

DNA Sequence Analysis of Mutant Plasmids. Since the mutation responsible for the resistance to α-phenylglycidate was localized to the 1138-bp upstream 5′-flanking region of the gene or the first 280 codons of the structural gene for mandelate racemase and since we had previously sequenced both of these regions in pSCR1 (Ransom et al., 1988; S. C. Ransom and J. A. Gerlt, unpublished observations), we synthesized a series of sequencing primers that would permit rapid sequencing of these regions. The wild-type sequence in the fragment from pSCR1 was also redetermined with this series of primers. The only mutation that was detected in these regions in one of the mutant plasmids was a C → A transversion 87 bp upstream of the initiation codon for mandelate racemase in the sequence CTGACATC, where the position of the transversion is italicized. The region containing this mutation was subsequently sequenced in a total of nine plasmids from three independent selections for mutant plasmids, and in all cases the same C → A transversion was found.

We previously noted (Ransom et al., 1988) that this sequence containing the mutation appeared homologous to the −26 region of consensus sequence proposed for the promoters of *ntr*A-dependent genes in *Klebsiella* species (CTGGPyA-PyPu) (Ausubel, 1984) and to the consensus sequence for the −35 region of *E. coli* promoter (Rosenberg & Court, 1979). The promoter homology of this region containing the mutation will be further discussed in a following section describing the results of nuclease S_1 mapping that define the start of transcription initiation in pMRαpg.

Since the sequenced mutant plasmids were judged identical by virtue of occurrence of the same transversion 87 bp upstream of the initiation codon for mandelate racemase, the one plasmid in which the gene and the 5′-flanking region were totally sequenced was designated pMRαpg and subjected to further biochemical and genetic characterization.

Overproduction of Mandelate Racemase by the Mutant Plasmid. Extracts were prepared from *P. aeruginosa* and *P. aeruginosa* transformed with either pSCR1 or pMRαpg grown at 37 °C in potential inducing (minimal medium containing (*R*)-mandelate as sole carbon source) and noninducing (LB/strep medium) conditions. As expected, neither set of growth conditions produced measurable mandelate racemase activity in the untransformed *P. aeruginosa*.

In both media, *P. aeruginosa* transformed with pSCR1 produced comparable levels of enzyme activity in crude extracts as assessed by specific activity measurements of the soluble fraction (0.26 units/mg for noninducing and 0.40 units/mg for inducing medium). The similarity of these enzyme levels suggests that the uncharacterized regulatory mechanism that leads to induction by mandelate in *P. putida* (Hegeman, 1966b) does not function in *P. aeruginosa* transformed with pSCR1. The explanation for this is unclear but may be either (1) that an essential positive regulatory protein for transcription of the operon is not present or (2) that the *P. putida* DNA cloned in pSCR1 does not contain the promoter for the mandelate operon; this point will be discussed in more detail in a following section.

P. aeruginosa transformed with pMRαpg produces much higher levels of mandelate racemase whether grown on noninducing medium (7.5 units/mg) or inducing medium (16 units/mg). The increases in activity associated with the C → A transversion in pMRαpg are 30-fold in the case of noninducing medium and 40-fold in the case of inducing medium. In agreement with these measurements, SDS–PAGE of cell extracts of untransformed *P. aeruginosa* and of *P. aeruginosa* transformed with pSCR1 or pMRαpg reveals the presence of easily detectable levels of mandelate racemase only in the host transformed with pMRαpg (Figure 1). These increases in the amount of mandelate racemase demonstrate that the mutation upstream of the initiation codon for mandelate racemase causes a significant increase in the amount of enzyme produced. Therefore, the resistance of *P. aeruginosa* transformed with pMRαpg to α-phenylglycidate can be explained by elevated levels of enzyme activity within the cells.

Purification and Characterization of Mandelate Racemase Produced by P. aeruginosa Transformed with pMRαpg. The high levels of mandelate racemase present in *P. aeruginosa* transformed with pMRαpg have facilitated purification of pure enzyme. Using *P. aeruginosa* transformed with pMRαpg and grown on LB medium, the procedure described under Materials and Methods has been used to obtain 110 mg of electrophoretically homogeneous mandelate racemase from 26.6 g of cells obtained from 3 L of medium. This purification is summarized in Table I, and an SDS–PAGE gel showing the progress of the purification is shown in Figure 2. We assume that the increase in total enzymatic activity after precipitation

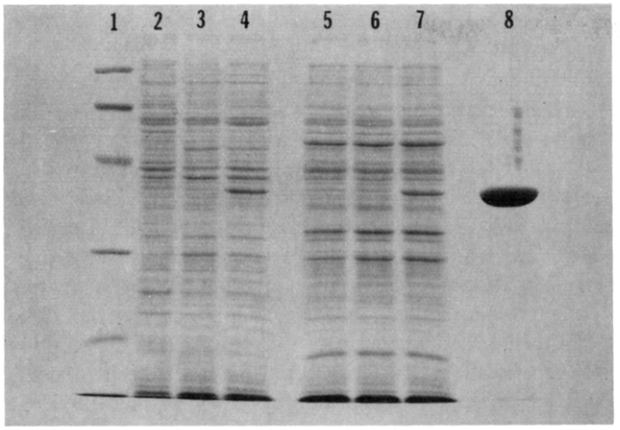

FIGURE 1: Photograph of a 12% SDS–PAGE gel showing the production of mandelate racemase by various strains of *P. aeruginosa* under different growth conditions. (Lane 1) Molecular weight standards (phosphorylase *a*, 94 000; bovine serum albumin, 67 000; ovalbumin, 43 000; carbonic anhydrase, 30 000; soybean trypsin inhibitor, 21 000; hen egg white lysozyme, 14 000); (lane 2) untransformed *P. aeruginosa* grown on LB/strep medium; (lane 3) *P. aeruginosa* transformed with pSCR1 grown on LB/strep medium; (lane 4) *P. aeruginosa* transformed with pMRαpg grown on LB/strep medium; (lane 5) untransformed *P. aeruginosa* grown on (*R*)-mandelate minimal salts medium; (lane 6) *P. aeruginosa* transformed with pSCR1 grown on (*R*)-mandelate minimal salts medium; (lane 7) *P. aeruginosa* transformed with pMRαpg grown on (*R*)-mandelate minimal salts medium; (lane 8) authentic mandelate racemase from *P. putida*.

Table I: Summary of Purification of Mandelate Racemase from *P. aeruginosa* Transformed with pMRαpg

purification step	total volume (mL)	total protein (mg)	total act. (units)	spec act. (units/mg)
crude extract	470	3200	37 300	12
protamine sulfate	470	1830	31 000	17
$(NH_4)_2SO_4$ (0–65% sat.) precipitate	66.5	1260	63 000	50
DEAE-Sephacel	3.6	390	60 000	150
Sephacryl S-200	3.0	186	58 000	310
Mono Q	3.2	110	45 000	410

with ammonium sulfate is due to the removal of a competing enzymatic activity or inhibitor. The purified enzyme was subjected to six cycles of N-terminal sequence analysis, and the sequence Ser-Glu-Val-Leu-Ile was obtained. This sequence is identical with that obtained with pure enzyme isolated from *P. putida* and reveals that the N-terminal *N*-formylmethionine is also removed by *P. aeruginosa* transformed with pMRαpg. The enzyme obtained by this procedure has been crystallized in Professor Gregory A. Petsko's laboratory at the Massachusetts Institute of Technology, and a preliminary crystallographic analysis of these crystals has already been published (Neidhart et al., 1988).

Nuclease S_1 Mapping of Transcription Initiation. The increased levels of mandelate racemase present in extracts of *P. aeruginosa* transformed with pMRαpg and the observation that a C → A transversion was present in a sequence showing strong homology to consensus sequences for promoters suggested that the overproduction of mandelate racemase was the result of increased transcription of the gene for mandelate racemase. Accordingly, total cellular RNA was isolated from *P. aeruginosa* transformed with pMRαpg, *P. aeruginosa*, and the *P. putida* from which the gene for mandelate racemase was cloned; the latter cells were grown on inducing minimal salts medium containing (*R*)-mandelate as sole carbon source. A ^{32}P-labeled DNA probe was prepared as described under Materials and Methods. Following the formation of hybrids

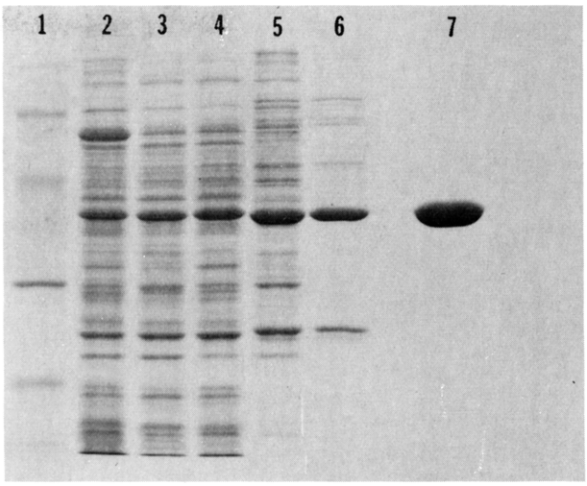

FIGURE 2: Photograph of a 13% SDS–PAGE gel showing the purification of mandelate racemase from *P. aeruginosa* transformed with pMRαpg. (Lane 1) Molecular weight standards; (lane 2) cell lysate; (lane 3) after precipitation of nucleic acids with protamine sulfate; (lane 4) after precipitation with 65% ammonium sulfate; (lane 5) after DEAE-Sephacel; (lane 6) after Sephacryl S-200; (lane 7) after Mono Q.

between this DNA and the various RNAs that were isolated, digestion with nuclease S_1 was performed to determine the point of initiation of transcription and the effect of the C → A transversion on the level of transcription. The results of this analysis are shown in Figure 3. Note that the autoradiogram shown in the figure is exposed for a long period of time so that protection of the DNA probe by RNA isolated from *P. putida* induced for the synthesis of mandelate racemase can be readily discerned; this exposure blurs the precise point of transcription initiation. Also, note that the sequence ladder in Figure 3 reads upstream from the N terminus of the gene for mandelate racemase and, therefore, is complementary to the mRNA transcript and 5′-flanking sequence.

The plasmid containing the C → A transversion within the sequence hypothesized to be a promoter sequence produces a transcript that begins 76 ± 1 bp upstream of the initiation codon for mandelate racemase and 11 ± 1 bp downstream of the mutation (lane 6 of Figure 3 and autoradiograms of the gel used to obtain Figure 3 exposed for shorter periods of time). Reading in the direction of transcription of the gene for mandelate racemase, the sequence of pMRαpg in the region containing the promoter mutation (the −35 and −10 regions that are most homologous to the *E. coli* promoter sequences are italicized and the position of the C → A transversion is indicated) and the predominant point of initiation of transcription (underlined) can be summarized:

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          -35                    -10          mRNA~~~~~~~~~~~~~~~
~~~~~CACCCTTGCCCAGATTGGATGCCCTGACATAACCTCCCTTTCTCCTGATTACCTC~~~~~
                                    ↑
                                    C  (wild type)
```

The position of initiation of transcription is neither of those anticipated on the basis of our previously discussed sequence homology with the −26 region of *ntr*A-dependent (Ausubel, 1984) and the −35 region of *E. coli* (Rosenberg & Court, 1979) promoters. Instead, the transversion mutation increases the homology of this region to the −10 region of the consensus *E. coli* promoter (CATAAC for the promoter in pMRαpg and TATAAT for the −10 consensus). With the point of transcription initiation established and the increased homology to the *E. coli* −10 consensus sequence, we now can more clearly locate an appropriately placed sequence (TTGCCC) that is partially homologous to the −35 region of the consensus pro-

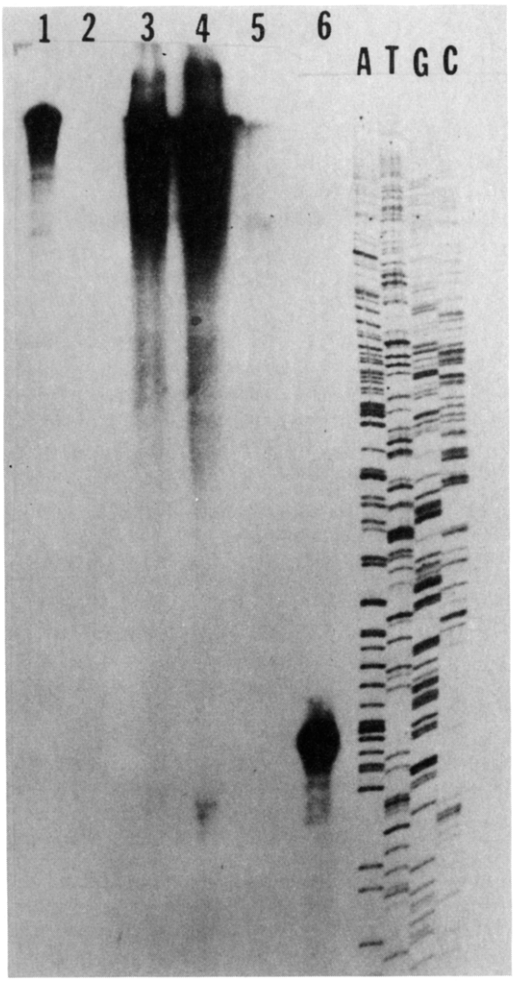

FIGURE 3: Photograph of an autoradiogram of nuclease S_1 mapping. (Lane 1) ^{32}P-labeled DNA probe (6100 cpm); (lane 2) DNA probe (21 000 cpm) in the presence of tRNA after treatment with nuclease S_1; (lane 3) DNA probe (21 000 cpm) in the presence of mRNA isolated from *P. putida* grown on (*R*)-mandelate minimal salts medium after treatment with nuclease S_1; (lane 4) DNA probe (21 000 cpm) in the presence of mRNA isolated from a mutant of *P. putida* resistant to α-phenylglycidate grown on (*R*)-mandelate minimal salts medium after treatment with nuclease S_1; (lane 5) DNA probe (21 000 cpm) in the presence of mRNA isolated from *P. aeruginosa* transformed with pSCR1 after treatment with nuclease S_1; (lane 6) DNA probe (21 000 cpm) in the presence of mRNA isolated from *P. aeruginosa* transformed with pMRαpg after treatment with nuclease S_1; (lane 7–10) A, T, G, and C, respectively, dideoxy chain-terminating sequencing lanes using the +87 oligonucleotide DNA probe primer as a sequencing primer on M13 containing the *Hin*dIII–*Bam*HI fragment cloned from pSCR1. The sequence of the promoter in pMRαpg deduced from this experiment is given in the text.

moter for *E. coli* (TTGACA); the −35 and −10 regions of the promoter in pMRαpg are separated by 16 bp in contrast to the 17-bp separation observed in the consensus *E. coli* promoter. This conclusion is in agreement with a recent compilation of known *Pseudomonas* promoters (Deretic et al., 1987) and the observation that the hybrid *E. coli tac* promoter can function in *P. putida* (Bagdasarian et al., 1983). Thus, we can now clearly recognize that the phenotype of the mutation we have isolated and characterized is the result of increasing sequence homology to a promoter known to function in pseudomonads. However, on the basis of the data shown in Figure 3 the sequence in the wild-type *P. putida* progenitor of the mutant is not an efficient promoter. The different levels of transcript that are produced by pSCR1 and pMRαpg (lanes 5 and 6, respectively) are consistent with the increased levels of mandelate racemase encoded by pMRαpg being the result of an increase in transcription of the gene for mandelate racemase.

On the basis of the homologies among the sequences approximately 90 bp upstream of the initiation codon and consensus sequences for potential promoters, we earlier postulated that the gene for mandelate racemase was the first gene in the mandelate operon (Ransom et al., 1988). However, analysis of initiation of transcription in the *P. putida* from which the gene for the racemase was cloned reveals that in *P. putida* little, if any, initiation of transcription occurs in the vicinity of the sequence that can be mutated into an efficient promoter when the gene is present in pSCR1 (lanes 3 and 4 in Figure 3). The DNA probe we constructed is protected by the RNA produced by *P. putida* under the inducing conditions of growth on (*R*)-mandelate. If the sequence that can be mutated into an efficient promoter were the actual promoter for the mandelate operon, one potential explanation for the inefficient initiation of transcription in pSCR1 and efficient initiation of transcription in pMRαpg could have been that *P. aeruginosa* lacks a regulatory protein present in *P. putida* that increases transcription efficiency at this promoter but that the C → A transversion removes the requirement for this protein. However, the fact that initiation of transcription in *P. putida* begins upstream of this sequence indicates that the gene for mandelate racemase is probably *not* the first gene in the operon after all. Consistent with this conclusion is our observation that an open reading frame 200 codons in length translated in the same direction as mandelate racemase is upstream of the sequence that acts as an efficient promoter in pMRαpg (data not shown).

Cloning and DNA sequence analysis of the remaining genes for the enzymes in mandelate pathway are presently under way, and we anticipate that these studies will reveal the actual promoter for the gene for mandelate racemase and whether the genes for the five enzymes in the pathway are present in a single operon or in multiple regulons.

REFERENCES

Aiba, H., Adhya, S., & de Crombrugghe, B. (1981) *J. Biol. Chem. 256*, 11905.

Ausubel, F. M. (1984) *Cell 37*, 5.

Bagdasarian, M. M., Amann, E., Lurz, R., Rückert, B., & Bagdasarian, M. (1983) *Gene 26*, 273.

Davis, L. G., Dibner, M. D., & Battey, J. F. (1986) *Basic Methods in Molecular Biology*, pp 276–284, Elsevier, New York.

Deretic, V., Gill, J. F., & Chakrabarty, A. M. (1987) *Biotechnology 5*, 469.

Ebina, Y., & Nakazawa, A. (1983) *J. Biol. Chem. 258*, 7072.

Fee, J. A., Hegeman, G. D., & Kenyon, G. L. (1974) *Biochemistry 13*, 2533.

Gunsalus, I. C., Gunsalus, F. C., & Stanier, R. Y. (1953) *J. Bacteriol. 66*, 538.

Hegeman, G. D. (1966a) *J. Bacteriol. 91*, 1140.

Hegeman, G. D. (1966b) *J. Bacteriol. 91*, 1161.

Hegeman, G. D. (1970) *Methods Enzymol. 17*, 670.

Kenyon, G. L., & Hegeman, G. D. (1979) *Adv. Enzymol. Relat. Areas Mol. Biol. 50*, 325.

Lin, D. T., Powers, V. M., Reynolds, L. J., Whitman, C. P., Kozarich, J. W., & Kenyon, G. L. (1988) *J. Am. Chem. Soc. 110*, 323.

Lowry, O. H., Rosenbrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265.

Maggio, E. T., Kenyon, G. L., Mildvan, A. S., & Hegeman, G. D. (1975) *Biochemistry 14*, 1131.

Neidhart, D. C., Powers, V. M., Tsou, A. Y., Ransom, S. C.,

Gerlt, J. A., Kenyon, G. L., & Petsko, G. A. (1988) *J. Biol. Chem. 263*, 9268.
Ransom, S. C., Gerlt, J. A., Powers, V. M., & Kenyon, G. L. (1988) *Biochemistry 27*, 540.
Rosenberg, M., & Court, D. (1979) *Annu. Rev. Genet. 13*, 319.
Rosenberg, S. L. (1971) *J. Bacteriol. 108*, 1257.
Rosenberg, S. L., & Hegeman, G. D. (1971) *J. Bacteriol. 108*, 1270.
Stark, G. R., & Wahl, G. M. (1984) *Annu. Rev. Biochem. 43*, 447.
Wheelis, M. L., & Stanier, R. Y. (1970) *Genetics 66*, 245.
Whitman, C. P., Hegeman, G. D., Cleland, W. W., & Kenyon, G. L. (1985) *Biochemistry 24*, 2936.

Nucleosome Reconstitution of Core-Length Poly(dG)·Poly(dC) and Poly(rG-dC)·Poly(rG-dC)†

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ABSTRACT: The double-stranded polypurine·polypyrimidines poly(dG)·poly(dC) and poly[d(A-G)]·poly[d(T-C)] and the mixed ribose–deoxyribose polynucleotide poly(rG-dC)·poly(rG-dC) have been successfully reconstituted into nucleosomes. The radioactively labeled particles comigrate in gel electrophoresis and sucrose density gradient experiments with authentic nucleosomes derived from chicken erythrocyte chromatin. These results show that nucleosomes are able to accommodate a wider variety of polynucleotides than was previously believed.

Nucleosomes are the basic organizational unit of chromatin in which 146 base pairs (bp) of DNA wrap around a core of eight histone proteins and a variable length of "linker" DNA is associated with a ninth histone, H1 (McGhee & Felsenfeld, 1980). Although the protein component of nucleosome cores is constant, the polynucleotide component is not. Since nucleosomes appear to organize almost all eukaryotic DNA, they have to accommodate a wide variety of DNA sequences. Previous experiments have shown that they have this ability. Nucleosomes cores have been reconstituted in vitro by using the synthetic polymers poly[d(A-T)·poly[d(A-T)] (Bryan et al., 1979), poly[d(G-C)]·poly[d(G-C)] (Simpson & Kunzler, 1979), and poly[d(G-m^{5}C)]·poly[d(G-m^{5}C)] (Nickol et al., 1982), as well as prokaryotic DNA (Bryan et al., 1979) and even bacteriophage T7 DNA, which contains glucosylated (hydroxymethyl)cytosine groups in place of normal cytosine residues, filling the major groove (McGhee & Felsenfeld, 1982).

There have been other reports, however, that some polynucleotides are unable to form nucleosomes. Failed in vitro attempts to reconstitute the synthetic polynucleotides poly(dG)·poly(dC) (Simpson & Kunzler, 1979; Rhodes, 1979), poly(dA)·poly(dT) (Simpson & Kunzler, 1979; Rhodes, 1979), DNA–RNA hybrids (Dunn & Griffith, 1980), plasmids substituted with ribonucleotides (Hovatter & Martinson, 1987), and the Z forms of poly[d(G-C)]·poly[d(G-C)] and poly[d(G-m^{5}C)]·poly[d(G-m^{5}C)] (Nickol et al., 1982; Ausio et al., 1987) have been published. This has usually been ascribed to the existence of the test polymer in a conformation other than the canonical B form, and thus the conclusion was drawn that nucleosomes cannot tolerate long regions of polynucleotide that deviate substantially from a normal B form.

In this paper we describe the attempted reconstitution of several polypurine·polypyrimidines and polymers containing ribonucleotides. The experiments show that nucleosomal particles can be formed by using polynucleotides that were resistant to reconstitution in other systems.

Materials and Methods

Poly[d(A-G)]·poly[d(T-C)], poly(dI), poly(rI), poly(dC), pdG_{10}, and pdC_{10} were purchased from Pharmacia. T4 polynucleotide kinase, Klenow fragment, and T4 polynucleotide ligase were obtained from U.S. Biochemicals. Micrococcal nuclease and bovine pancreatic DNase I were purchased from Bethesda Research Laboratories. Poly(rG-dC)·poly(rG-dC) was synthesized as described earlier (Wu & Behe, 1984) by using DNA polymerase I large fragment (Klenow fragment), rGTP, dCTP, and a poly[d(I-C)]·poly[d(I-C)] template in the presence of Mn^{2+}. Molecular size markers consisting of a ladder of fragments that are multiples of 123 bp were obtained from BRL.

Chicken blood was obtained in Alsever's solution from Mayer & Miles (Coopersburg, PA) and shipped on ice the same day. The time from bleeding of the animals to arrival of the blood in the laboratory was normally ~3 h.

Polymers of 150 Base Pair Length. Poly(rI)·poly(dC) and poly(rG-dC)·poly(rG-dC) [both 150 base pairs (bp) in length] were obtained by isolation of the appropriate size fragments from nuclease digests of longer starting material. Either polymer (250 μg) was digested at 37 °C with 100 units of micrococcal nuclease in 100 mL of 50 mM NaCl, 10 mM Tris·HCl, pH 8, and 2 mM $CaCl_2$. Time points were taken at 0.5, 1, 2, 3, and 4 min, the reaction was stopped by addition of EDTA to 5 mM, the solution was extracted three times with neutral phenol and chloroform, the digestion products were electrophoresed in a 6% polyacrylamide gel, and the gel was stained with ethidium bromide. For both polymers material of ~150 base pairs was excised, electroeluted with a Phar-

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