Evidence for Induced Synthesis of an Active Transport Factor for Mandelate in *Pseudomonas putida*

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1. At low concentrations of mandelate there is a lag before induction of the mandelate regulon begins at a sub-maximum rate. Cells preinduced with a saturating concentration of inducer do not exhibit this lag when they are transferred to sub-maximum inducer concentrations and are able to maintain a high rate of induction under these conditions.
2. Chloramphenicol was used to show that a protein is synthesized during the lag period that is probably responsible for the maintenance effects observed in batch and continuous cultures.
3. Direct measurements appear to show that induced cells possess an active transport factor for mandelate, sensitive to dinitrophenol, and this is presumably responsible for the maintenance effect. Mandelate is accumulated unchanged against a concentration gradient from media containing low concentrations. At higher external concentrations no accumulation occurs and only a rapid equilibration is noted. Uninduced cells merely equilibrate mandelate.

Although specific transport systems for various substrates are known in *Pseudomonas* (Kogut & Podoski, 1953; Shilo & Stanier, 1957; Rosenfeld & Fiegelson, 1969; Gryder & Adams, 1969), an investigation of the uptake of mandelate by *P. putida* failed to demonstrate the existence of an active concentrating permease for this substance (Hegeman, 1966b). Radioactive mandelate entered both induced and uninduced cells very rapidly and the internal pool equilibrated with the external medium.

However, while studying the induction of the mandelate regulon at different concentrations of inducer, we found (Higgins & Mandelstam, 1972) that the kinetics of induced enzyme synthesis were reminiscent of those encountered, for example, in the lactose system of *Escherichia coli*, in which the β -galactoside permease causes a lag and a non-linear differential rate of enzyme synthesis when low inducer concentrations are used (Monod, 1956; Cohen & Monod, 1957; Herzenberg, 1959). In this paper we describe the kinetics of induction of the mandelate regulon, which also shows maintenance effects, and we present some evidence that induction may involve the synthesis of an active-transport factor for mandelate.

Methods

Organism

A mutant (*md*⁻) of *Pseudomonas putida*, biotype A, strain 90 (Stanier *et al.*, 1966), A.T.C.C. 12633, was

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used that was unable to metabolize mandelate and possessed a negligible amount of L-(+)-mandelate dehydrogenase. It was obtained from the parent organism by mutagenesis (Mandelstam & Jacoby, 1965) and was maintained as described in the preceding paper (Higgins & Mandelstam, 1972).

Growth and induction

Cultures growing exponentially in mineral salts medium (Mandelstam & Jacoby, 1965) were obtained as described by Higgins & Mandelstam (1972). Glucose (0.5%, w/v), which is a relatively weak repressor of the mandelate regulon (Mandelstam & Jacoby, 1965; Higgins, 1971), was the carbon source in all the experiments, which were conducted at 30°C. Cells fully induced for the mandelate regulon were obtained by growing them for at least five generations in glucose medium containing mandelate (5 or 10 mM).

Procedures for culture sampling, cell harvesting, estimations of enzyme activities, measurement of growth and continuous culture of the organism were as described by Higgins & Mandelstam (1972).

Determination of extracellular space in cell pellets

Two substances generally considered to be excluded from cells were used. They were Blue Dextran 2000 (mol.wt. 2×10^6), in which the blue dye has an absorption maximum of 610 nm, and ¹³¹I-labelled serum albumin (mol.wt. 6.1×10^4). The procedures used followed those of Roberts *et al.* (1955) and Black & Gerhardt (1961).

Washed cells (10–20 mg dry wt./ml) were incubated with shaking for 10 min in mineral-salts medium containing dextran (25 mg/ml) or ^{131}I -labelled serum albumin (0.5 mg/ml, 0.3 $\mu\text{Ci}/\text{ml}$). In each case the cell suspension (2.5 ml) was centrifuged (20000g for 5 min) and the supernatant carefully removed. The walls of the centrifuge tube were wiped and the tube was then rinsed with minimal medium (5 ml) without disturbing the pellet and inverted to drain for 1–2 min. The cells were resuspended in minimal medium (1.0 ml) and centrifuged again. This second supernatant was removed and the concentrations of albumin or dextran were determined in both supernatants. Finally, the cell pellet was dried to constant weight. Since from separate experiments it was known that the total water content of cell pellets obtained by centrifuging was four times the dry weight, the ratio of the extracellular and intracellular fluid could be calculated. Both the dextran and the albumin methods gave similar results and showed that approx. 16% of the total water of the pellet was extracellular. This value is similar to that obtained with other types of bacteria (Roberts *et al.*, 1955).

Measurement of uptake of mandelate

Cells, either glucose-grown or fully induced for the mandelate regulon, were washed twice in mandelate-free medium and resuspended (10–20 mg dry wt./ml) in fresh glucose-containing minimal medium with chloramphenicol (50 $\mu\text{g}/\text{ml}$). Portions (10 ml) of these cell suspensions were incubated with shaking and, after 10 min for equilibration, DL-[carboxy- ^{14}C]-mandelate at different external concentrations (0.05–10 mM) was added. The radioactive label was present at 1×10^5 – 2×10^5 c.p.m./ml. At intervals, samples (2.5 ml) were centrifuged as described above. The pellet was dispersed in trichloroacetic acid (10%, w/v; 1.0 ml) at 0°C to release the intracellular mandelate. The cells were centrifuged again in the cold, the supernatant was removed and the wet cell pellet dried to constant weight at 105°C.

The radioactivities in the supernatants from the original incubation and from the trichloroacetic acid-extracted cells were determined by scintillation counting. To ensure comparable counting efficiencies in the two types of sample, an equal volume of 10% trichloroacetic acid was added to the first supernatant. Samples (0.5–1.0 ml) were dried in glass vials under vacuum for 1 h at 70°C with a disc of glass-fibre paper (Whatman GFA) in each to prevent splashing of the contents while drying. These conditions effectively dried the samples and removed the trichloroacetic acid without destroying the mandelate. When the vials were cool, scintillant (10 ml) was added and the vials were counted for radioactivity (Higgins & Mandelstam, 1972). Since the total water in the pellet was four times the dry weight and 16% of this was

extracellular, the intracellular mandelate concentration could be easily calculated. Because we were using a method involving centrifugation, uptake of mandelate in periods less than the first 5 min could not be measured.

Reagents

^{131}I -labelled serum albumin (human) was supplied by The Radiochemical Centre, Amersham, Bucks., U.K., as a sterile solution containing 20 mg and 1.0 mCi in 1 ml of 0.7% NaCl. Pharmacia (Uppsala, Sweden) supplied the Blue Dextran 2000. The sources of all other reagents and the synthesis of DL-[carboxy- ^{14}C]-mandelate are described by Higgins & Mandelstam (1972).

Results

Effect of 'loading' cells with mandelate on induction at low mandelate concentrations

When *P. putida* was induced with concentrations of mandelate below 2.0 mM, there was a lag before the enzymes of the mandelate regulon were synthesized (Higgins & Mandelstam, 1972). This lag could have been due to slow diffusion of the inducer into uninduced cells until eventually sufficient had entered to support the synthesis of the mandelate enzymes. If this were so, cells already 'loaded' with mandelate should show no lag in the induction of the regulon at these low concentrations.

To test this possibility, mandelate was used at 0.25 mM. At this concentration, induced synthesis of the mandelate enzymes, indicated by benzoylformate decarboxylase, started 70–80 min (about one generation) after the addition of the inducer to the culture (Fig. 1). Uninduced cells were therefore 'loaded' with mandelate at this concentration by incubating them for 70 min in glucose-containing minimal medium with mandelate (0.25 mM). Chloramphenicol (50 $\mu\text{g}/\text{ml}$) was used to prevent induced enzyme synthesis and was removed at the end of the incubation by washing the cells twice in glucose-containing minimal medium (30°C) with mandelate (0.25 mM) to maintain their internal mandelate pool. The washed cells were finally resuspended at their original density (50 μg dry wt./ml) in fresh glucose-containing minimal medium (30°C) and grown in the presence of mandelate (0.25 mM). A lag of about one generation was still observed before synthesis of benzoylformate decarboxylase occurred (Fig. 1).

On the other hand, no lag was observed in the induction if the cells had been taken through the same procedure except that chloramphenicol was omitted from the first incubation so that protein synthesis could proceed unhindered (Fig. 1). It did not matter whether mandelate (0.25 mM) was present or absent

Fig. 1. Induction of the mandelate regulon at 0.25 mM-mandelate: effect of 'loading' the cells with mandelate

An exponentially growing culture (50 μg dry wt./ml) was divided and the portions were treated as follows. (1) Mandelate (0.25 mM) was added and the synthesis of benzoylformate decarboxylase followed as a measure of the induction of the regulon (●). (2) Mandelate (0.25 mM) and chloramphenicol (50 $\mu\text{g}/\text{ml}$) were added and the culture was incubated for 70 min. The antibiotic was removed by washing the cells twice in warm medium containing mandelate (0.25 mM). The washed cells, resuspended to their original density, were induced with mandelate (0.25 mM) and samples were taken at intervals and assayed for benzoylformate decarboxylase (○). (3) Procedure (2) above was followed except that chloramphenicol was omitted. The cells were washed in the presence (▲) or absence (△) of mandelate. (4) A saturating concentration of mandelate (5 mM) was added and the synthesis of benzoylformate decarboxylase was followed until the culture density had doubled (indicated by A). Chloramphenicol was then added and the incubation continued for 70 min. The antibiotic was removed by washing the cells (see above), which were then resuspended at the same density as before the antibiotic treatment. Mandelate (5.0 mM) was added and the rate of synthesis of the enzymes over the next generation was compared with that before exposure to chloramphenicol (■).

during the washing. In a control flask, chloramphenicol was added, after one generation, to cells that had been induced with a saturating concentration of mandelate (5.0 mM). The antibiotic remained in contact with the cells for 70 min and was removed as described above. Reinduction with mandelate (5.0 mM) resulted in the resumption of benzoylformate

decarboxylase synthesis at the rate observed before the treatment with chloramphenicol (Fig. 1). This indicated that the antibiotic had been effectively removed and that the cells were undamaged.

Effect of preinduction at a high mandelate concentration on subsequent induction at a low concentration

The results of the previous experiment do not support the hypothesis that slow inducer diffusion is responsible for the lag at low mandelate concentrations. Instead, it seems that a protein is synthesized during the lag period and that until this synthesis has taken place the mandelate enzymes cannot be induced at low concentrations of inducer. If this notion is correct, it is reasonable to suppose that cells already possessing this protein should continue to synthesize the mandelate enzymes without a lag when transferred to medium containing a low concentration of mandelate, i.e. there should be a maintenance effect. An experiment to test this was carried out as follows.

Cells (50 μg dry wt./ml) were induced for one generation with mandelate at a saturating concentration (5 mM). They were then washed twice in mandelate-free medium at 30°C and resuspended (100 μg dry wt./ml) in fresh glucose-containing minimal medium (again at 30°C) with mandelate (0.25 or 5.0 mM). The synthesis of benzoylformate decarboxylase was followed after resuspension for a further two generations. Cells resuspended in 0.25 mM-mandelate recommenced synthesis of the enzyme almost immediately, in contrast with uninduced cells, which showed the expected lag (about one generation) at this mandelate concentration (Fig. 2). Further, the rate of synthesis of benzoylformate decarboxylase at this low inducer concentration was three times higher in the preinduced cells. In fact, the rate was identical with that for cells that were being induced at the maximum rate (5.0 mM-mandelate). The fact that preinduced cells resuspended without inducer synthesized no more enzyme showed that the mandelate must have been effectively washed out and that the preinduction effect could not have been due to retention of mandelate in the cells (Fig. 2).

Maintenance effect in the chemostat

The previous experiments appeared to show that cells partially induced at saturating concentrations of mandelate were able to maintain the maximum rate of induction indefinitely at sub-optimum inducer concentrations. This is true in batch culture but is better illustrated in the chemostat, so experiments of the following type were done.

The cells were grown in a nitrogen-limited chemostat with a doubling time of 90 min with glucose as sole carbon source. Mandelate was added in increasing concentrations (up to 10 mM), and after each increment the culture was allowed to reach a steady state

Fig. 2. Maintenance effect, i.e. effect of preinduction of the mandelate regulon at 5.0 mM on the subsequent induction at 0.25 mM-mandelate

Cells (50 μ g dry wt./ml) were induced with 5.0 mM-mandelate for one generation, washed twice in warm mandelate-free medium and resuspended (100 μ g dry wt./ml) in fresh medium containing mandelate at 0.25 (●) or 5.0 mM (○). The rates of synthesis of the mandelate regulon, as indicated by benzoylformate decarboxylase, were then determined. The effect of 0.25 mM-mandelate on the synthesis of the enzyme by cells not previously induced was also determined (▲), as well as that of mandelate-free medium on the pre-induced cells (Δ).

and the specific activity of benzoylformate decarboxylase was measured. The specific enzyme activity increased to a maximum at about 5 mM (Fig. 3). The curve had the form of an adsorption isotherm with an apparent K_{ind} of 0.7 mM (see also Higgins & Mandelstam, 1972). When the experiment was done in reverse and these fully induced cells were grown in the presence of decreasing concentrations of mandelate, they were able to maintain the maximum rate of induction at inducer concentrations as low as 0.3 mM (Fig. 3). At this concentration, uninduced cells would only have been about 33% induced. The curve now gave an apparent K_{ind} value of about 0.01 mM.

Uptake of radioactive mandelate by md^- cells

The maintenance effects exhibited by preinduced cells were compatible with the assumption that these cells had developed some sort of mandelate transport system. It was therefore decided to measure the internal mandelate concentration in induced and uninduced cells under different conditions.

However, when we used the well-known membrane filter technique (Kepes, 1960; Winkler & Wilson, 1966), no evidence for internal accumulation of mandelate could be obtained. This agrees with the findings of Hegeman (1966b) and it seemed that accumulation, if it occurred at all, was either very slight or that washing the cells on the filter caused the

Fig. 3. Maintenance effect in the chemostat

Cells, growing with a doubling time of 90 min (dilution rate = 0.45 vol./h) on glucose in a nitrogen-limited chemostat, received increasing concentrations of mandelate (0–10 mM). The specific activity of benzoylformate decarboxylase was determined at steady state after each increase in the mandelate concentration (●). When steady state had been reached on 10 mM-mandelate, the inducer concentration was decreased in stages and the enzyme was redetermined at each steady state (○).

loss of any mandelate that might have accumulated. Extending the sensitivity of this method would have involved using either larger volumes of culture (which clogged the filter and decreased the efficiency and speed of washing) or much higher specific radioactivities (which gave high background counts owing to adsorption to the filters). Accordingly, centrifugation had to be used to separate the cells from the surrounding medium. Although this is slower than filtration, it eliminates the need to wash the cells and is capable of detecting lower internal concentrations.

Washed cells, either glucose-grown or fully induced for the mandelate regulon, were incubated in glucose-containing medium with labelled mandelate at 0.05, 0.25, 2.5 and 10 mM. The intracellular and extracellular mandelate concentrations were determined at intervals after the addition of the mandelate (see the Methods section). At all these external concentrations mandelate had equilibrated across the membrane of induced cells by the time the first sample had been treated, i.e. at 5 min (Fig. 4a). Thereafter, there was a gradual accumulation of mandelate within induced cells at the lower external concentrations (0.05 and 0.25 mM). After 45 min, the mandelate in cells in 0.05 mM solution had been concentrated 4.5 times whereas in 0.25 mM solution it was concentrated only 1.75 times. In both suspensions, accumu-

Fig. 4. Distribution of mandelate between cells and medium

Cells at a density of 10–20 mg dry wt./ml were incubated at 30°C in glucose-containing minimal medium with chloramphenicol (50 µg/ml). After 10 min, radioactive mandelate (1×10^5 – 2×10^5 c.p.m./ml) was added at 0.05 (●), 0.25 (○), 2.5 (▲) and 10 mM (△). At intervals samples (2.5 ml) were taken and the intracellular and extracellular mandelate concentrations determined. (a) Uptake by induced cells; (b) uptake by uninduced cells.

lation seemed to be still continuing. In contrast, induced cells in media containing 2.5 and 10 mM-mandelate had not accumulated mandelate even after 40–50 min.

Uninduced cells were unable to accumulate mandelate at any of the concentrations tested, but were clearly readily permeable to mandelate, since equilibration had occurred within 5 min (Fig. 4b).

Nature of the radioactivity accumulated by induced cells

We then had to consider the possibility that the radioactive material accumulated by induced cells in the presence of 0.05 mM-mandelate might not in fact be mandelate, but another compound produced from mandelate by a process occurring in induced but not in uninduced cells. The nature of the accumulated radioactive label was therefore investigated.

Induced and uninduced cells were incubated for

45 min in glucose-containing medium with [*carboxy*- 14 C]mandelate (0.05 mM; 2×10^5 c.p.m./ml). Samples (2.5 ml) were taken and the intracellular concentration of radioactive label was determined as described above. Induced cells had accumulated the label sixfold whereas uninduced cells had equilibrated the label (internal/external concentration ratio = 0.95). Further samples (10 ml) of the cells were centrifuged and the cells extracted twice with ethanol (3 ml) and centrifuged. The ethanol supernatants were pooled and dried in a stream of warm air (30°C). The residue was dissolved in potassium phosphate buffer (0.1 M, pH 6; 0.2 ml). Portions (60 µl) of the solutions were resolved by paper chromatography (Whatman 3MM) in a downward direction for 25–30 cm. The three solvent systems used were ethanol–acetic acid–water (8:1:1, by vol.) (Hegeman, 1966a), butan-1-ol–pyridine–dioxan–water (14:4:1:1, by vol.) (Armstrong *et al.*, 1956) and butan-1-ol–ethanol–water (7:1:2, by vol.) (Smith, 1969). The chromatograms were dried and the radioactive spots detected by radioautography (Kodak, Kodirex X-ray film; 1 and 5 days). In all three solvent systems each extract gave a single spot with an R_f identical with authentic DL-[*carboxy*- 14 C]mandelate run at the same time. There was no detectable difference between the extracts from induced or uninduced cells, indicating that no conversion of mandelate into another compound had occurred in the induced cells.

Effect of dinitrophenol on the accumulation of mandelate by induced cells

Presumably the accumulation of mandelate by induced cells is energy-dependent. If this is so, 2,4-dinitrophenol should prevent this accumulation just as it stops the active concentration of β -galactosides in *E. coli* (Winkler & Wilson, 1966). Its effect on the uptake of mandelate was investigated as part of the experiment just described. A portion of the induced cells was incubated for 45 min in glucose medium containing radioactive mandelate (0.05 mM) with the addition of 2,4-dinitrophenol (5.0 mM). In separate experiments this was found sufficient to stop immediately the growth of *P. putida*. The dinitrophenol prevented the sixfold accumulation that occurred when it was omitted. Nevertheless, equilibration with the label still occurred (internal/external concentration ratio = 0.86). This sensitivity of the accumulation system is analogous to that exhibited by the galactoside permease and other active transport systems.

Discussion

At low inducer concentrations, not only was the rate of synthesis of the mandelate enzymes sub-maximal, but there was also a lag between the addition of the inducer and the start of enzyme synthesis.

The experiment with chloramphenicol seems to show that during the lag a protein is synthesized and that until this has occurred the mandelate enzymes themselves cannot be synthesized. At high concentrations there is no lag and the rate of enzyme synthesis is maximal from the start.

The protein could conceivably have been an inducible enzyme converting mandelate into an active inducer in the way that the basal β -galactosidase of *E. coli* converts lactose into an active inducer (Burststein *et al.*, 1965). However, the *md*⁻ strain used in this study was unable to metabolize mandelate by the normal catabolic route and radiochemical experiments failed to show measurable loss of label from [¹⁴C]mandelate (Higgins, 1971).

Maintenance effects are more often the result of the induction and action of an inducible transport system. For instance, β -galactosides at low concentrations enter uninduced cells of *E. coli* only with difficulty, but after a while the galactoside permease becomes induced. Accordingly at low inducer concentrations there is a lag followed by an accelerating rate of synthesis of the *lac* operon (Rickenberg *et al.*, 1956; Monod, 1956; Cohen & Monod, 1957). The existence of the *lac* permease also produces a maintenance effect in the chemostat analogous to that shown in Fig. 3 (Novick & Weiner, 1957).

Our results are very similar to these and are therefore consistent with the induction of a concentrating mechanism for mandelate. Uninduced cells are freely permeable to mandelate and, when the external concentration of mandelate is low, induced cells do in fact concentrate mandelate against a gradient by an active, dinitrophenol-sensitive route. Unlike the uptake of galactosides by *E. coli*, the active uptake of mandelate occurs slowly but is probably sufficient to account for the maintenance effects observed in batch and continuous cultures. Although they still

rapidly equilibrate with mandelate, induced cells are not able to accumulate mandelate when the external concentration is high (2.5–10 mM). It may be that under these conditions the permease is inhibited.

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References

- Armstrong, M. D., Shaw, K. N. F. & Wall, P. E. (1956) *J. Biol. Chem.* **218**, 293
- Black, S. H. & Gerhardt, P. (1961) *J. Bacteriol.* **82**, 743
- Burststein, C., Cohn, M., Kepes, A. & Monod, J. (1965) *Biochim. Biophys. Acta* **95**, 634
- Cohen, G. N. & Monod, J. (1957) *Bacteriol. Rev.* **21**, 169
- Gryder, R. M. & Adams, E. (1969) *J. Bacteriol.* **97**, 292
- Hegeman, G. D. (1966a) *J. Bacteriol.* **91**, 1140
- Hegeman, G. D. (1966b) *J. Bacteriol.* **91**, 1155
- Herzenberg, L. A. (1959) *Biochim. Biophys. Acta* **31**, 525
- Higgins, S. J. (1971) D.Phil. Thesis, University of Oxford
- Higgins, S. J. & Mandelstam, J. (1972) *Biochem. J.* **126**, 901
- Kepes, A. (1960) *Biochim. Biophys. Acta* **40**, 70
- Kogut, M. & Podoski, E. P. (1953) *Biochem. J.* **55**, 800
- Mandelstam, J. & Jacoby, G. A. (1965) *Biochem. J.* **94**, 569
- Monod, J. (1956) *Enzymes: Units Biol. Struct. Funct., Henry Ford Hosp. Int. Symp.* **1**
- Novick, A. & Weiner, M. (1957) *Proc. Nat. Acad. Sci. U.S.A.* **43**, 553
- Rickenberg, H. V., Cohen, G. N., Buttin, G. & Monod, J. (1956) *Ann. Inst. Pasteur Paris* **91**, 829
- Roberts, R. B., Cowie, D. B., Abelson, P. H., Bolton, E. T. & Britten, R. J. (1955) *Carnegie Inst. Wash. Publ.* **607**
- Rosenfeld, H. & Fiegelson, P. (1969) *J. Bacteriol.* **97**, 697
- Shilo, M. & Stanier, R. Y. (1957) *J. Gen. Microbiol.* **16**, 482
- Smith, I. (1969) *Chromatographic and Electrophoretic Techniques*, vol. 1, p. 364, Heineman Medical Books, London
- Stanier, R. Y., Palleroni, N. J. & Doudoroff, M. (1966) *J. Gen. Microbiol.* **43**, 159
- Winkler, H. H. & Wilson, T. H. (1966) *J. Biol. Chem.* **241**, 2200