Is there a mandelate enzyme complex in *Acinetobacter calcoaceticus* or *Pseudomonas putida*?

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Several methods were used in a vain search for a possible specific, isolable complex of the enzymes involved in the conversion of mandelate into benzoate in *Acinetobacter calcoaceticus* and *Pseudomonas putida*. Previous *in vivo* cross-linking experiments done by other workers with *P. putida* were repeated and slightly extended. Extracts prepared from bacteria that had been treated with dimethylsuberimidate were analysed by ultracentrifugation and gel-filtration chromatography. In confirmation of the original observations, some of the mandelate enzymes were found to be linked to high- M_r material. However, several control enzymes behaved in the same way and only a small proportion of the 'soluble' mandelate enzymes were found in exactly the same fraction as the membrane-bound L(+)-mandelate dehydrogenase. Similarly, no specific cross-linking was observed in experiments with *A. calcoaceticus*, even when the bacteria were supplied with substrates and cofactors for the mandelate enzymes. There was no evidence for co-fractionation of the mandelate enzymes from extracts that had been prepared in various ways. Treatment of homogenates with antiserum that had been raised against phenylglyoxylate decarboxylase failed to co-precipitate the other mandelate enzymes. Incubation of homogenates with anti-D(–)-mandelate dehydrogenase did lead to the removal of some phenylglyoxylate decarboxylase from the supernatant fraction, but this is ascribed to the interaction of phenylglyoxylate decarboxylase with a membrane component other than D(–)-mandelate dehydrogenase. Control and cross-linked extracts were analysed by SDS-PAGE followed by immunoblotting with the three antisera. Although there was evidence for the formation of high- M_r species, there was no sign of specific cross-linking of any of the three enzymes to each other. Experiments involving cross-linking with other dimethylimidates and with phenylene dimaleimide all failed to provide evidence for a mandelate complex.

Introduction

Halpin *et al.* (1981) reported evidence consistent with the idea of physical association of the enzymes of the mandelate pathway in *Pseudomonas putida*. The experiments involved the cross-linking reagent DMSI. When extracts from control and DMSI-treated *P. putida* were subjected to differential centrifugation, there was a 9- to 25-fold increase in the 'soluble' enzyme activities recovered in the pellet fraction [where most of the membrane-bound L(+)-mandelate dehydrogenase activity was already located] after DMSI treatment. In addition, when extracts from bacteria which had been treated with DMSI *in vivo* were subjected to gel-filtration chromatography, an extra peak of enzyme activities appeared in the void volume, which in control extracts

had only L(+)-mandelate dehydrogenase activity. This was taken to indicate the possible presence of a high- M_r aggregate of the mandelate enzymes. The cross-linked complex was also studied by ^{13}C -NMR spectroscopy (Halpin *et al.*, 1981). On oxygenation of the isolated complex, DL- $[\alpha\text{-}^{13}\text{C}]$ mandelate was converted into $[\alpha\text{-}^{13}\text{C}]$ benzoate; $[\alpha\text{-}^{13}\text{C}]$ phenylglyoxylate and $[\alpha\text{-}^{13}\text{C}]$ benzaldehyde were also detected. This confirmed that all the enzymes necessary for the metabolism of mandelate to benzoate were present in the high- M_r material. However, it was also reported that in one experiment, ^{13}C bicarbonate was detected after oxygenation of the isolated complex suggesting that the two enzymes that convert benzoate into catechol were also present in the high M_r material. Some glutamine synthetase was also present in the cross-linked sample.

Acinetobacter calcoaceticus, like *P. putida*, can use mandelate as the sole source of carbon and energy for growth (Fewson, 1988). In *A. calcoaceticus*, L-mandelate

Abbreviations: DMPI, dimethylpimelimidate; DMSI, dimethylsuberimidate; TPP, thiamin pyrophosphate.

is oxidized to phenylglyoxylate by L(+)-mandelate dehydrogenase in the wild-type strain NCIB 8250 and some mutants also have a D(-)-mandelate dehydrogenase which allows growth on D-mandelate. L(+)-Mandelate dehydrogenase and D(-)-mandelate dehydrogenase, which are both membrane-bound flavoproteins, as well as phenylglyoxylate decarboxylase and benzaldehyde dehydrogenase I, are coordinately synthesized and are referred to as the 'mandelate enzymes' (Fewson, 1988). Unlike the enzymes of mandelate metabolism in *P. putida*, the mandelate enzymes of *A. calcoaceticus* have all been purified, partly characterized and antibodies have been raised against them (Allison *et al.*, 1985a, b; Barrowman & Fewson, 1985; Hoey *et al.*, 1987; Chalmers & Fewson, 1988).

In the present work, some of the experiments on *P. putida* described by Halpin *et al.* (1981) have been repeated, extended and the original results largely confirmed. The possibility of some sort of interaction amongst the enzymes of the mandelate pathway in *A. calcoaceticus* was investigated using a variety of techniques. However, the results do not seem to support the notion of a 'mandelate complex' in either *A. calcoaceticus* or *P. putida*.

Methods

Organisms. *Acinetobacter calcoaceticus* mutant strains C1005 [NCIB 11339; constitutive L(+)-mandelate dehydrogenase, no D(-)-mandelate dehydrogenase], C1123 [NCIB 11456; constitutive D(-)-mandelate dehydrogenase, no L(+)-mandelate dehydrogenase], C1219 [NCIB 11457, constitutive D(-)- and L(+)-mandelate dehydrogenase activities] and C1408 [NCIB 10789, no D(-)- or L(+)-mandelate dehydrogenase activities] were derived from the wild-type strain NCIB 8250 (Allison *et al.*, 1985a). *Pseudomonas putida* wild-type strain NCIB 9494 (strain A3.12) is inducible for the mandelate enzymes (Hegeman, 1966). The wild-type strains were obtained from the National Collection of Industrial Bacteria, Aberdeen, UK.

Growth of bacteria. *A. calcoaceticus* was maintained and grown in complex medium containing mandelate (Allison *et al.*, 1985a). Inocula were prepared by aseptically transferring 0.1 ml of a stock culture into 50 ml of medium (in 250 ml conical flasks) and grown at 30 °C with shaking (120 r.p.m.) for 24 h. These cultures were used to inoculate (2.5%, v/v) 400 ml volumes of medium (in 2 litre conical flasks) which were incubated at 30 °C with shaking for 16 h. The bacteria were then harvested by centrifugation and used immediately.

P. putida was grown in minimal medium containing mandelate. This medium was adapted from that used by Hegeman (1966) and contained (g l⁻¹): DL-mandelate, 1.52 (10 mM); (NH₄)₂SO₄, 1; KH₂PO₄, 3.4; Na₂HPO₄, 3.55; and 2 ml of trace metals solution l⁻¹. The trace metals were prepared as described by Beggs & Fewson (1977). The solution containing mandelate was adjusted to pH 6.9 with NaOH, made to the correct volume with distilled water and autoclaved. Immediately before inoculation, sterile 2% (w/v) MgSO₄·7H₂O (20 ml l⁻¹) was added. A stock nutrient broth culture of *P. putida* was inoculated (0.2%) into 50 ml of minimal medium containing mandelate and grown for 24 h at 30 °C with shaking. This culture was used as the inoculum (2.5%, v/v) for 400 ml volumes of medium (in 2 litre flasks) which were

incubated at 30 °C with shaking. These cultures were harvested when the OD₅₀₀ reached 0.8 (after about 5 h).

Disruption of bacteria. For ultrasonic disruption, bacteria were resuspended in the appropriate buffer (usually 50 mM-potassium phosphate buffer, pH 7.5) to a final concentration of 0.25 g wet wt ml⁻¹. Portions (5 to 7 ml) in 10 ml glass vials were placed in a chilled brass holder. This was screwed onto the horn of a 13 mm probe of a Dawe Soniprobe (type 7532A) and lowered into an ice/water slurry. *A. calcoaceticus* was disrupted at 80 W for 5 × 30 s periods alternated with cooling periods of 30 s. *P. putida* was also disrupted at a current of 80 W but for 3 × 30 s periods alternated with cooling periods of 30 s. The homogenates were centrifuged at 15000 g for 20 min at 4 °C in an MSE 18 Highspeed centrifuge to remove intact bacteria and debris.

For disruption in the French press, bacteria were resuspended in an appropriate buffer, usually to a final concentration of 0.5 g wet wt ml⁻¹, and disrupted by one to four passages through a French press (4-3398A; Aminco) at a pressure of 98 MPa (14300 lbf in⁻²). Intact bacteria and debris were removed by centrifuging the homogenate at 15000 g for 20 min at 4 °C in an MSE 18 Highspeed centrifuge.

In the case of lysozyme treatment, freshly harvested bacteria were washed three times in 50 mM-Tris/HCl buffer, pH 7.8, and resuspended (50 mg wet wt ml⁻¹) in about 200 ml 50 mM-Tris/HCl buffer, pH 7.8, containing 0.6 M-sucrose. The suspension was incubated with lysozyme [10 mg (g wet wt)⁻¹] for 150 min at 23 °C, after which spheroplasts were harvested by centrifuging at 15000 g for 20 min at 4 °C in an MSE 18 Highspeed centrifuge. The spheroplasts were washed once by resuspension/centrifugation in 50 mM-Tris/HCl buffer, pH 7.8, containing 0.6 M-sucrose. The spheroplast sludge was homogenized for 45 s at top speed in a glass homogenizer, then a crystal of deoxyribonuclease was added and the homogenate was incubated at 23 °C for 5 min. The homogenizing and deoxyribonuclease treatment was repeated twice (Mowbray & Moses, 1976). Finally, intact bacteria were removed by centrifuging at 15000 g for 20 min at 4 °C in an MSE 18 Highspeed centrifuge.

Cross-linking with dimethylimidates. Cross-linking methods were generally based on those developed by Professor J. R. Coggins of this Department (personal communication and Coggins, 1978), but in some experiments 50 mM-potassium phosphate buffer, pH 7.5, was used instead of TNM buffer (i.e. 50 mM-triethanolamine/HCl buffer, pH 8.0, containing 0.1 M-NaCl and 10 mM-MgCl₂). Cross-linking intact bacteria with dimethylimidates used a method based on that described by Halpin *et al.* (1981). Exponentially growing bacteria (after about 5 h growth) were harvested and washed once with TNM buffer, pH 8.0, and resuspended in the same buffer (0.4 g wet wt ml⁻¹). The cross-linker (0.1 M) was added to give a final concentration of 6 mM. The bacterial suspension was incubated for 60 min at 23 °C with gentle swirling. In experiments where potassium phosphate buffer, pH 7.5, was used instead of TNM buffer, pH 8.0, the same volume of dimethylimidate solution was added every 15 min over the 60 min incubation period.

In cross-linking experiments using homogenates prepared with the French press, freshly harvested bacteria were resuspended in TNM buffer, pH 8.0, to a final concentration of 0.5 g wet wt ml⁻¹. An appropriate volume of 0.1 M-dimethylimidate was added to the bacterial suspension to give a final concentration of 6 mM, after which the bacterial suspension was passed through the French press. The same volume of a 0.1 M solution of dimethylimidate was added to the resulting homogenate which was passed through the French press again. When all four passages through the French press had been completed, a further volume of 0.1 M-dimethylimidate was added to the homogenate which was incubated at 23 °C for 60 min with gentle swirling. In experiments where the bacteria were resuspended in 50 mM-potassium phosphate buffer, pH 7.5, the same volume of the 0.1 M-dimethylimidate solution was added every 15 min throughout the

60 min incubation period. Unbroken bacteria and debris were removed by centrifuging at 15000 g for 20 min at 4 °C in an MSE 18 Highspeed centrifuge.

Gel-filtration chromatography. (a) Extracts prepared by ultrasonication of control and DMSI-treated *P. putida* were applied to a 1.4 litre column (71 cm long $\times$ 5.2 cm diam.) of Ultrogel AcA 34 which had been equilibrated with TNM buffer, pH 8.0. Enzymes were eluted with the same buffer at a flow rate of 60 ml h⁻¹. (b) Homogenates that had been prepared by French press treatment were applied to a column (30 cm long $\times$ 0.7 cm diam.) of Ultrogel AcA 34 equilibrated with 0.1 M-potassium phosphate buffer, pH 7.5, containing 100 μ M-TPP, 100 μ M-DTT, 2 mM-MgSO₄ and 1 mM-EDTA. Material was eluted with the same buffer at a flow rate of 4 ml h⁻¹ and fractions were collected every 7.5 min.

High-speed centrifugation. (a) Extracts prepared from control and cross-linked bacteria were centrifuged at 105000 g for 1 h at 4 °C and the pellets were washed three times by resuspension and centrifugation. (b) In other cases, supernatants produced by disruption and centrifugation at low speed were centrifuged at 165000 g for 150 min and the high-speed pellets ('wall + membrane' fraction) were washed twice by resuspension and centrifugation under the same conditions.

Percoll density-gradient centrifugation. A stock Percoll (Pharmacia) solution was prepared by adding 18 ml Percoll to 2 ml 2.5 M-sucrose in 0.5 M-potassium phosphate buffer, pH 7.5. A 20% (v/v) Percoll solution was obtained by making 22.2 ml of the stock Percoll solution up to 100 ml. Sample (1 ml) was layered onto 20 ml of this 20% Percoll solution in a Ti60 polycarbonate centrifuge tube. The sample was then centrifuged for 30 min at 64000 g_{av}, after which 1 ml fractions were collected from the top of the self-generated gradient. Density-gradient marker beads (Pharmacia) were included in each run in a separate tube to calibrate the density gradient formed.

Toluene treatment of bacteria in the presence of poly(ethylene glycol). Bacteria were treated with toluene according to the method described by Beggs & Fewson (1977) except that buffers contained 8.5% (w/v) poly(ethylene glycol) (Matlib *et al.*, 1977). Bacteria (3.8 ml; 0.8 g wet wt ml⁻¹) were pipetted onto 200 μ l 4% (v/v) toluene in ethanol. The mixtures were vortexed for 30 s and incubated at 27 °C in a water-bath for 90 min.

Enzyme assays. Enzyme activities were measured spectrophotometrically at 27 °C using 3 ml reaction mixtures in cuvettes with 1 cm light-paths. Activities are expressed as enzyme units, where 1 unit is the amount of enzyme catalysing the conversion of 1 μ mol of substrate min⁻¹. For NAD(P)-linked enzymes a change in A₃₄₀ of 2.07 was taken to correspond to the conversion of 1 μ mol of substrate.

L(+)-Mandelate dehydrogenase and D(-)-mandelate dehydrogenase activities were assayed by measuring the reduction of 2,6-dichloroindophenol at 600 nm in the presence of *N*-methylphenazonium methosulphate and BSA (Allison *et al.*, 1985b). A decrease in A₆₀₀ of 7.18 units was taken to correspond to the oxidation of 1 μ mol of substrate at pH 7.5.

The method for measuring mandelate racemase activity was adapted from that described by Hegeman (1966). Mandelate dehydrogenase activity was assayed as for D-mandelate dehydrogenase but, in addition, a sufficient quantity of a 'wall + membrane' fraction from *A. calcoaceticus* mutant strain C1005 which had been grown in complex medium supplemented with DL-mandelate was added to give non-rate-limiting amounts of L-mandelate dehydrogenase activity. D-Mandelate (0.1 ml of a 12.5 mM solution) was added to initiate the reaction.

A new method was developed for measuring the activity of phenylglyoxylate decarboxylase. The formation of benzaldehyde was coupled to the oxidation of NADH by horse-liver alcohol dehydrogenase. The enzyme was assayed in reaction mixtures containing (total

volume 3.0 ml): 1 mmol (2 ml of 0.5 M) potassium phosphate buffer, pH 6.0; 0.5 μ mol (0.1 ml of 5 mM) NADH; 17 μ g of horse-liver alcohol dehydrogenase (0.1 ml of a 0.5 mg protein ml⁻¹ solution); 200 nmol TPP (0.1 ml of 2 mM stock solution in 0.25 M-potassium phosphate buffer, pH 7.0); enzyme sample (usually 50 μ l) and 15 μ mol (0.1 ml of 150 mM) phenylglyoxylate, adjusted to pH 6.0, was added to initiate the reaction. The alcohol dehydrogenase was confirmed to be present in non-rate-limiting concentrations for any given sample of extract by measuring the rate of oxidation of NADH in the presence of increasing concentrations of alcohol dehydrogenase. Minus-substrate blanks were included to compensate for any NADH oxidase activity present in the samples.

Benzaldehyde dehydrogenase I activity of *A. calcoaceticus* was measured by following the reduction of NAD at 340 nm as described by Beggs & Fewson (1977). NAD- and NADP-dependent benzaldehyde dehydrogenase activities of *P. putida* were measured by following the reduction of NAD(P) at 340 nm as described by Hegeman (1966).

Catechol 1,2-dioxygenase activity was measured by following the production of *cis,cis*-muconate at 260 nm (Hegeman, 1966). An increase in A₂₆₀ of 5.6 units corresponded to the conversion of 1 μ mol of catechol to *cis,cis*-muconate.

Isocitrate dehydrogenase activity was assayed by the method of Borthwick *et al.* (1984) by following the reduction of NADP at 340 nm.

NADH oxidase was measured by following the oxidation of NADH at 340 nm (Allison *et al.*, 1985a).

Glutamine synthetase was assayed by the coupled procedure described by Kingdon *et al.* (1968), using pyruvate kinase and lactate dehydrogenase.

Preparation of antisera. (a) The recovery of L(+)-mandelate dehydrogenase at the final stage of its purification was poor (Hoey *et al.*, 1987) and so a modified version of the method used by Mihara & Blobel (1980) was used to produce protein for injection. L(+)-Mandelate dehydrogenase was purified as described by Hoey *et al.* (1987) up to the gel-filtration stage. The pooled fractions from the gel-filtration column were treated with (NH₄)₂SO₄ to yield a 15–30% saturated fraction which was resuspended in 5 to 10 ml 20 mM-potassium phosphate buffer, pH 7.5, containing 0.05% (w/v) Triton X-100, 100 μ M-DTT and 10% (v/v) ethanediol. The (NH₄)₂SO₄ fraction was subjected to electrophoresis on a 12.5% (w/v) SDS/polyacrylamide slab gel which was then stained with Coomassie Blue and destained. The band corresponding to L(+)-mandelate dehydrogenase was cut out and the gel was washed in distilled water until the pH of the wash was 7.0. The gel was ground in liquid nitrogen and the powder was immediately transferred to the barrel of a 2 ml syringe. Distilled water (0.5 ml approx.) was added and the sample was sonicated for 3 $\times$ 30 s using the microtip of the Dawe Soniprobe (type 7332A). An equal volume of Freund's complete adjuvant (samples for boost inoculations were prepared with Freund's incomplete adjuvant) was added and the sonication was repeated. The emulsion was then loaded into 1 ml syringes. A rabbit weighing about 4 kg was injected subcutaneously at several different sites with a total of 100 μ g L(+)-mandelate dehydrogenase. After 5 weeks a further 100 μ g was injected and another booster injection was given 2 weeks later. After a further two weeks, blood was collected from the rabbit's ear vein. Blood samples were allowed to clot at room temperature and left overnight at 4 °C. The serum was pipetted off and centrifuged at 15000 g for 10 min to remove the red blood cells. Samples (1 ml) were stored at -20 °C. (b) Antisera against D-mandelate dehydrogenase were prepared as described by Fewson *et al.* (1988). (c) Antiserum was raised against phenylglyoxylate decarboxylase as described by Barrowman *et al.* (1986).

Immunoblotting. Cross-linked species were analysed by first subjecting them to SDS-PAGE (Laemmli, 1970) on slab gels containing 7.5% (w/v) polyacrylamide and then immunoblotting them using the method described by Towbin *et al.* (1979) and modified by Batteiger *et al.*

(1982). Bands were visualized with peroxidase conjugate/chloronaphthol (Fewson *et al.*, 1988).

Immunoprecipitation studies. Appropriate concentrations of extracts (100 µl) were incubated with undiluted antisera (100 µl), or with normal rabbit serum or buffer as controls, for 1 h at 23 °C. Protein A-Sepharose (100 µl) was added and the samples were incubated for a further hour at 23 °C before immunocomplexes were precipitated by centrifuging for 5 min in an Eppendorf 3200 centrifuge.

Analytical methods. Protein was estimated by the Lowry procedure and BSA was used as the standard.

Results and Discussion

Cross-linking intact *P. putida* with DMSI

P. putida was treated with DMSI in the presence of TNM buffer, pH 8.0, following the general procedures of Halpin *et al.* (1981). After disrupting the bacteria by ultrasonication, the extracts were analysed by high-speed centrifugation (Table 1). Catechol 1,2-oxygenase was used as a control 'soluble' enzyme from a related part of metabolism and isocitrate dehydrogenase and glutamine synthetase were used as control 'soluble' enzymes from unrelated parts of metabolism. Furthermore, glutamine synthetase served as a control enzyme with a high M_r (600 000) whereas isocitrate dehydrogenase was an example of an enzyme with a lower M_r (100 000). Halpin *et al.* (1981) also used glutamine synthetase as a control enzyme but they assayed *cis,cis*-muconate lactonizing enzyme instead of catechol 1,2-oxygenase.

In general, DMSI did not affect the total specific activities of the enzymes, although L(+)-mandelate

dehydrogenase (which is a membrane-bound enzyme) was slightly inhibited (Table 1). There was a 5- to 50-fold increase in the specific activities of the 'soluble' enzymes of the mandelate pathway present in the high-speed pellet after DMSI treatment. However, this was not a specific effect since the specific activities of catechol 1,2-oxygenase, isocitrate dehydrogenase and glutamine synthetase in the pellet were also increased by about the same extent (Table 1). No increases in the specific activities of the enzymes in the high-speed pellets were observed after DMSI treatment of stationary phase bacteria. The results in Table 1 are very similar to those reported by Halpin *et al.* (1981) who also found increases in the specific activities of control enzymes in the high speed pellets. Unfortunately, Halpin *et al.* (1981) did not express their results in terms of total percentage of enzyme activity nor does the information in their paper allow the percentages to be calculated. However, when the results from the present experiments are expressed in this way (Table 1) it can be seen that the proportion of the 'soluble' mandelate enzymes recovered in the pellet fraction after DMSI treatment (2 to 10%) is very small in relation to the proportion of L(+)-mandelate dehydrogenase (97%) present.

Halpin *et al.* (1981) also analysed sonic extracts from control and DMSI-treated *P. putida* by gel filtration through Ultrogel AcA 22. They suggested that there was an 'all-or-nothing' trapping of the high- M_r complex of protein since there was little evidence of dimers, trimers or tetramers of the individual enzymes and so they concluded that the cross-linking was not completely random. We did similar experiments, but used Ultrogel

Table 1. Cross-linking *P. putida* with DMSI

P. putida was grown in minimal mandelate medium, harvested in late exponential phase and treated with DMSI in the presence of TNM buffer, pH 8.0, as described in Methods. After disrupting the bacteria by ultrasonication, the extracts were centrifuged at 105 000 g_{av} for 60 min and the high-speed pellets so formed were washed three times by resuspension/centrifugation. The pellets, supernatants and washes were assayed for enzyme activity so that the results could be expressed in terms of the percentage of the total enzyme activity recovered. The 'total specific activities' were derived from the addition of the enzyme units and protein, respectively, recovered in the pellet, supernatant and washes of each sample. ND, Not detectable.

Enzyme	Total specific activity [munits (mg protein) ⁻¹]		Specific activity in pellet [munits (mg protein) ⁻¹]		Activity recovered in pellet (%)	
	Control	DMSI-treated	Control	DMSI-treated	Control	DMSI-treated
L(+)-Mandelate dehydrogenase	309	247	661	622	95	97
Mandelate racemase	433	408	2.2	100	0.2	9.4
Phenylglyoxylate decarboxylase	215	248	2.9	13.3	0.6	2.1
NAD-linked benzaldehyde dehydrogenase	79	76	ND	ND	ND	ND
NADP-linked benzaldehyde dehydrogenase	49	56	0.8	7.3	0.7	5
Catechol 1,2-oxygenase	498	499	2.7	40	0.2	3.1
Isocitrate dehydrogenase	524	412	2.9	33	0.3	3.1
Glutamine synthetase	25	33	2.3	18	4.2	22
Protein	—	—	—	—	45	38

AcA 34 because of its higher resolving power. All the 'soluble' enzymes assayed (mandelate racemase, phenylglyoxylate decarboxylase, NADP-linked benzaldehyde dehydrogenase, catechol 1,2-oxygenase, isocitrate dehydrogenase and glutamine synthetase), except NAD-linked benzaldehyde dehydrogenase, showed higher- M_r species after DMSI treatment. In each case one of the high- M_r species co-eluted with L(+)-mandelate dehydrogenase. However, with the exception of NAD-linked benzaldehyde dehydrogenase, the enzymes eluted over a broad range of M_r values after DMSI treatment. In other words, not only were there enzyme activities in the very-high- M_r fractions, but there were also intermediate- M_r species smaller than the fraction containing most of the L(+)-mandelate dehydrogenase activity but larger than the untreated enzymes.

Cross-linking intact *A. calcoaceticus*

Although the results from the experiments with *P. putida* suggested that only non-specific cross-linking had occurred, a series of experiments on *A. calcoaceticus* was done in the hope that cross-linking of a specific mandelate complex could be obtained.

A. calcoaceticus mutant strain C1005 was treated with DMSI, disrupted by ultrasonication and the homogenate analysed by high-speed centrifugation. The results obtained for *A. calcoaceticus* (Table 2) were similar to those for *P. putida* (Table 1). L(+)-Mandelate dehydrogenase (total specific activity) was again inhibited by the DMSI treatment. There was an increase of about 10-fold in the specific activity of phenylglyoxylate decarboxylase recovered in the pellet but this corresponded to only 1% of the total activity. As with *P. putida*, the specific activities of the 'soluble' control enzymes in the high-

speed pellets were also increased (Table 2). Again as for *P. putida*, the increases in activities in the pellet fractions after DMSI treatment were observed only in bacteria that had been growing exponentially.

The following experiments all failed to provide any evidence for the cross-linking of a specific mandelate complex. (a) Different strains of *A. calcoaceticus* (wild-type strain 8250, mutant strains C1219 and C1123) all gave similar results to those obtained for mutant strain C1005. (b) Different dimethylimidates [structures $R-(CH_2)_n-R$], i.e. dimethylmalonimide ($n=1$), dimethylsuccinimide ($n=2$), dimethylglutarimide ($n=3$), dimethyladipimide ($n=4$), DMPI ($n=5$) and DMSI ($n=6$), all of which cross-link lysine residues, were studied. In addition, the effect of phenylene dimaleimide which cross-links thiol groups was also investigated. No specific cross-linking was observed after treatment with any of these reagents and in all cases L(+)-mandelate dehydrogenase (total specific activity) was inhibited. Slightly more of all the 'soluble' enzymes (about 3% in the case of phenylglyoxylate decarboxylase) were recovered in the pellet after cross-linking with DMPI than with any of the other cross-linkers. (c) Slightly more cross-linking (up to 6% phenylglyoxylate decarboxylase associated with the pellet) was observed after bacteria had been made permeable with toluene in the presence of 8.5% (w/v) poly(ethylene glycol). A 3- to 20-fold increase in the specific activities of the 'soluble' enzymes in the pellet was also observed in stationary phase bacteria after toluene/poly(ethylene glycol) treatment. However, these effects were seen for the control enzymes as well as for the mandelate enzymes. (d) In some experiments bacteria were treated with toluene/poly(ethylene glycol) and then cofactors and substrates of the mandelate enzymes were added. However, there was

Table 2. Cross-linking *A. calcoaceticus* mutant strain C1005 with DMSI

A. calcoaceticus mutant strain C1005, grown in complex medium supplemented with DL-mandelate, was harvested in late exponential phase and treated with DMSI in the presence of TNM buffer, pH 8.0, as described in Methods. After disrupting the bacteria by ultrasonication, the extracts were centrifuged at 105 000 g_{av} for 60 min and the pellets were washed three times by resuspension/centrifugation. All the fractions were assayed for enzyme activity. ND, Not detectable.

Enzyme	Total specific activity [munits (mg protein) ⁻¹]		Specific activity in pellet [munits (mg protein) ⁻¹]		Activity recovered in pellet (%)	
	Control	DMSI-treated	Control	DMSI-treated	Control	DMSI-treated
L(+)-Mandelate dehydrogenase	90	63	184	200	57	63
Phenylglyoxylate decarboxylase	166	166	0.7	8.0	0.11	1.0
Benzaldehyde dehydrogenase I	86	79	ND	ND	ND	ND
Catechol 1,2-oxygenase	82	175	12	49	4.2	5.6
Isocitrate dehydrogenase	157	166	6	27	3.5	3.2
Glutamine synthetase	53	63	10	28	5.2	8.8
Protein	—	—	—	—	18	20

still no indication for specific cross-linking of a mandelate complex.

Enzyme distribution after disruption of A. calcoaceticus by different methods

Since no specific cross-linking of the mandelate enzymes was observed, we investigated alternative methods of examining enzyme interactions and first checked the effects of different methods of disruption on the distribution of enzyme activities in fractions obtained by high-speed centrifugation (165 000 g_{av} , 150 min).

(a) *Ultrasonic disruption.* When bacteria were disrupted by ultrasonication, 50 to 60% of the L(+)-mandelate dehydrogenase was recovered in the pellet fraction whereas only 4% (or less) of phenylglyoxylate decarboxylase, benzaldehyde dehydrogenase I, catechol 1,2-oxygenase and isocitrate dehydrogenase and about 30% of the glutamine synthetase were present in this fraction.

(b) *Disruption by lysozyme treatment.* After bacteria had been treated with lysozyme as described in Methods, as much as 95% of the L(+)-mandelate dehydrogenase, but only 0 to 17% of the 'soluble' enzymes phenylglyoxylate decarboxylase, benzaldehyde dehydrogenase I, catechol 1,2-oxygenase and isocitrate dehydrogenase were recovered in the pellet fraction along with 30% of glutamine synthetase.

(c) *Use of the French pressure cell.* When bacteria had been passed through the French press once or twice, 97% of the L(+)-mandelate dehydrogenase, 40 to 55% of phenylglyoxylate decarboxylase, 3 to 15% of benzaldehyde dehydrogenase I, catechol 1,2-oxygenase and isocitrate dehydrogenase and 40% of glutamine synthetase were recovered in the pellet fraction, whereas when bacteria had been passed through the French press three or four times, 95% of the L(+)-mandelate dehydrogenase but only 20% of phenylglyoxylate decarboxylase still remained in the pellet with 3 to 8% of the benzaldehyde dehydrogenase I, catechol 1,2-oxygenase and isocitrate dehydrogenase and 40% of glutamine synthetase. The presence of deoxyribonuclease or Mg^{2+} did not alter these enzyme distributions. About 30 to 40% of the glutamine synthetase sedimented, irrespective of the method of disruption and this presumably illustrates the behaviour of a high- M_r protein under the conditions of centrifugation used.

The protein concentration of French press homogenates was varied by suspending bacteria to different concentrations (0.03 to 1.0 g wet wt ml^{-1}). This had little effect on the proportions (86 to 98%) of L(+)-mandelate dehydrogenase and NADH oxidase (another 'control' enzyme, this time one that is membrane-bound) recovered in the pellet. However, there was a substantial effect on the amount of phenylglyoxylate decarboxylase recov-

ered in the pellet; at high protein concentration, 92% of the phenylglyoxylate decarboxylase was recovered in the pellet fraction, whereas at low protein concentration only 22% was recovered in this fraction. The distribution of the other enzymes also varied somewhat with the protein concentration of the homogenate (from highest to lowest protein concentration): benzaldehyde dehydrogenase I, 56 to 9%; isocitrate dehydrogenase, 58 to 34%; catechol 1,2-oxygenase 30 to 9%; glutamine synthetase 54 to 32%; protein, 50 to 30%. These results may perhaps illustrate an interaction of phenylglyoxylate decarboxylase with the membrane, and therefore possibly, but not necessarily, with L(+)-mandelate dehydrogenase, at high protein concentrations. If the amount of isocitrate dehydrogenase (58%) recovered in the pellet fraction is taken as a measure of non-specific binding, then it would be possible to argue that 34% (92% minus 58%) of the total phenylglyoxylate decarboxylase interacts (at high protein concentration) with the membrane fraction. However, it would be difficult to use these data as evidence for the existence of a mandelate complex in view of the results for catechol 1,2-oxygenase and isocitrate dehydrogenase.

Buffer conditions

Conditions commonly used to dissociate extrinsic membrane proteins (e.g. 0.2 M-NaCl, 0.5 M-KCl, 0.2 M- $MgCl_2$, 0.02 M-EDTA, 0.02 M-EGTA, buffers at pH 6.0 and 8.5) were used to try to achieve a differential release of isocitrate dehydrogenase from high-speed pellets prepared by centrifugation of homogenates produced in the French pressure cell while leaving phenylglyoxylate decarboxylase associated with the pellet. In all cases, however, where isocitrate dehydrogenase was washed off, phenylglyoxylate decarboxylase was also washed from the pellets although there was always more phenylglyoxylate decarboxylase than isocitrate dehydrogenase recovered in the membrane fraction.

Percoll-gradient centrifugation and gel-filtration chromatography

French press homogenates were analysed by Percoll density-gradient centrifugation and by gel-filtration chromatography. No co-elution of phenylglyoxylate decarboxylase with L(+)-mandelate dehydrogenase was observed even in the presence of high protein concentrations (30 mg BSA ml^{-1}) or in the presence of 20% (v/v) glycerol.

Cross-linking French press homogenates with dimethylimidates

Since such a high percentage of phenylglyoxylate decarboxylase could be associated with the membrane

fraction after disruption of the bacteria in the French pressure cell, it was thought that a putative specific complex might be stabilized by cross-linking the French press homogenate. The earlier cross-linking experiments using intact bacteria were done in the presence of TNM buffer, pH 8.0, in order to allow direct comparison with the experiments of Halpin *et al.* (1981). However, the phenylglyoxylate decarboxylase tetramer dissociates at this high pH and so a method for cross-linking with the dimethylimidates at a lower pH was developed (see Methods). Freshly harvested *A. calcoaceticus* mutant strain C1005 which had been grown in complex medium supplemented with DL-mandelate was treated with DMSI and DMPI at pH 7.5 during the preparation of French press homogenates, as described in Methods. After incubating with the cross-linker a 'wall + membrane' fraction was prepared from the homogenate. There was no increase in the proportion of phenylglyoxylate decarboxylase recovered in the pellet fraction after treatment with DMSI or DMPI. When the pellets were washed by resuspending to half the original volume of the homogenate, about 70% of the total phenylglyoxylate decarboxylase and 40% of the isocitrate dehydrogenase were recovered in the high-speed pellets of control and treated samples alike. When the pellets were washed by diluting to 2.5 times the original volume, 36% of the phenylglyoxylate decarboxylase and 6% of the isocitrate dehydrogenase remained associated with the membrane fractions of control samples, and 44% of the phenylglyoxylate decarboxylase and 6% of the isocitrate dehydrogenase were recovered in the pellet fraction of the cross-linked homogenate.

Analysis of cross-linked French press homogenates by immunoblotting

Control and cross-linked homogenates were analysed by immunoblotting with anti-L(+)-mandelate dehydrogenase, anti-D(-)-mandelate dehydrogenase and anti-phenylglyoxylate decarboxylase in order to see if there were any bands that contained two or three of these proteins. In the control (uncross-linked) samples there were bands corresponding to L(+)-mandelate dehydrogenase, D(-)-mandelate dehydrogenase or phenylglyoxylate decarboxylase whereas in the cross-linked samples there were bands with high apparent M_r values. In all cases DMSI and DMPI gave similar results. In the anti-L(+)-mandelate dehydrogenase blot there were bands with apparent M_r values of 140 000 (this band was not always detected and sometimes appeared in the control sample), 100 000 and 40 000 [equivalent to L(+)-mandelate dehydrogenase; Hoey *et al.* (1987)]. In the anti-D(-)-mandelate dehydrogenase blot, there was a

band with an apparent M_r value of 66 000 [equivalent to D(-)-mandelate dehydrogenase; Allison *et al.* (1985b)] in the control sample; however, there was no band with this M_r value in the cross-linked sample but there was one with a slightly higher M_r value (68 000) which may be due to intramolecular cross-linking. In addition there was a smear in the cross-linked samples which probably indicates that D(-)-mandelate dehydrogenase was cross-linked to proteins of various M_r values. In the anti-phenylglyoxylate decarboxylase blot bands with apparent M_r values of 135 000, 120 000, 98 000 and 56 000 (the latter being equivalent to phenylglyoxylate decarboxylase; Barrowman & Fewson, 1985) were detected. A similar blotting pattern with anti-phenylglyoxylate decarboxylase was obtained with extracts from mutant strain C1005 [L(+)-mandelate dehydrogenase and phenylglyoxylate decarboxylase activities present] and mutant strain C1123 [D(-)-mandelate dehydrogenase and phenylglyoxylate decarboxylase activities present]. Comparison of the native M_r value obtained by gel filtration and the monomeric M_r obtained by SDS-PAGE indicated that phenylglyoxylate decarboxylase is a tetramer (Barrowman & Fewson, 1985). However, there was no evidence for trimers or tetramers of phenylglyoxylate decarboxylase being formed on cross-linking although the band with an apparent M_r of 120 000 may be a dimer. Cross-linking experiments were also done using mutant strain C1408 which has no mandelate dehydrogenase and similar immunoblots were obtained with anti-phenylglyoxylate decarboxylase indicating that whatever phenylglyoxylate decarboxylase was cross-linked to, it was not L(+)-mandelate dehydrogenase.

The crucial observation from these experiments was that there was no single band that reacted with two or three antisera; in other words no material was detected that contained more than one of the enzymes.

Immunoprecipitation of French press homogenates

French press homogenates from mutant strains C1005 and C1219 were immunoprecipitated with anti-D(-)-mandelate dehydrogenase and anti-phenylglyoxylate decarboxylase to see if precipitation of one enzyme by its specific antiserum resulted in the co-precipitation of a second or third (presumably associated) enzyme. Only 27% of the D(-)-mandelate dehydrogenase and 0 to 5% of the phenylglyoxylate decarboxylase remained in the supernatant after incubating extracts with anti-D(-)-mandelate dehydrogenase and anti-phenylglyoxylate decarboxylase respectively. Anti-phenylglyoxylate decarboxylase did not precipitate either L(+)- or D(-)-mandelate dehydrogenase. However, there was a small co-precipitation (7 to 24%) of phenylglyoxylate decarboxylase [but not L(+)-mandelate dehydrogenase] by

anti-D(-)-mandelate dehydrogenase, presumably because when D(-)-mandelate dehydrogenase is precipitated it takes with it, being a membrane-bound enzyme, some of those membrane components with which phenylglyoxylate decarboxylase appears to associate.

Conclusions

In their work with the mandelate enzymes of *P. putida*, Halpin *et al.* (1981) were careful not to conclude that they had provided evidence for the existence of a specific mandelate complex. They did not rule out the possibility that the enzymes were not complexed to each but rather were individually cross-linked to cellular components of high M_r . Our results support the latter suggestion. The chief conclusions that we draw from this work are as follows. (a) Cross-linking of proteins within intact *A. calcoaceticus* and *P. putida* can be achieved, especially if adequate attention is paid to the physiological state of the cells at harvesting and to the conditions (including if necessary permeabilizing agents) used during the cross-linking procedure. (b) There is no convincing evidence that the mandelate enzymes of either *A. calcoaceticus* or *P. putida* exist as a specific, isolable complex. The present work was not intended to address the question as to whether substrate channeling or other possible consequences of enzyme interactions might occur during mandelate metabolism. (c) Phenylglyoxylate decarboxylase shows some inclination to associate with an unidentified component of the cytoplasmic membrane.

The outcome of these experiments has been largely negative but this does not exclude the possibility that other experimental approaches will unambiguously demonstrate the existence of a mandelate complex.

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