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Two Benzaldehyde Dehydrogenases in Bacterium N.C.I.B. 8250

DISTINGUISHING PROPERTIES AND REGULATION

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Evidence is presented for the existence in bacterium N.C.I.B. 8250 of two inducible NAD^+-linked benzaldehyde dehydrogenases. They may be distinguished in crude extracts by their different thermal stabilities at high pH values, benzaldehyde dehydrogenase I being much more heat-stable than benzaldehyde dehydrogenase II. Only benzaldehyde dehydrogenase I is activated by K^+ and certain other univalent cations. Gel-filtration experiments indicate that both enzymes have molecular weights of about 180000. Both enzymes are induced by growth on L-mandelate or phenylglyoxylate; only benzaldehyde dehydrogenase I is gratuitously induced by thiophenoxyacetate and only benzaldehyde dehydrogenase II is induced by benzyl alcohol, by benzaldehyde, and by a number of heterocyclic compounds which do not support growth. Mutants have been isolated that lack either benzaldehyde dehydrogenase II or benzyl alcohol dehydrogenase, or both of the enzymes. Results obtained in induction experiments with the wild-type bacterium N.C.I.B. 8250 and with the mutants show that benzaldehyde dehydrogenase II and benzyl alcohol dehydrogenase are co-ordinately regulated. Overall, the results suggest that benzaldehyde dehydrogenase I is associated with the metabolism of L-mandelate whereas benzaldehyde dehydrogenase II is associated with the metabolism of benzyl alcohol.

In bacterium N.C.I.B. 8250 (Fewson, 1967*a*), benzaldehyde may be derived from benzyl alcohol or from L-mandelate and is oxidized to benzoate (Kennedy & Fewson, 1968*a*). Each of these compounds supports the growth of this organism when present as the sole source of carbon and energy. Experiments on heat inactivation of benzaldehyde dehydrogenase activity in cell extracts indicated that two isofunctional NAD^+-linked enzymes might be involved in benzaldehyde oxidation (Kennedy & Fewson, 1968*b*). In the present paper we give results that confirm these observations and we give information on the properties and regulation of the two enzymes. We have designated the more heat-stable enzyme 'benzaldehyde dehydrogenase I' and the more heat-labile enzyme 'benzaldehyde dehydrogenase II.'

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Materials and Methods

Organisms

Bacterium N.C.I.B. 8250 was obtained from the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, U.K. This organism was the parent of the various mutant strains isolated in this work. Stock cultures, subcultures and the inocula used in the growth experiments were prepared as described by Kennedy & Fewson (1968*b*).

Media

The 'basal medium' used throughout this work is described by Fewson (1967*b*). 'Salts medium' was prepared by the aseptic addition of 20ml of sterile 2% (w/v) $MgSO_4,7H_2O$/l of sterile basal medium. Glutamic acid was usually dissolved in the basal medium before adjustment of the pH, and then sterilized by autoclaving at 109°C for appropriate

lengths of time. Other compounds were dissolved in glass-distilled water, adjusted to pH7.0 and sterilized by filtration through 0.22μm-pore-size filters [Sartorius–Membranfilter G.m.b.H., Göttingen, Germany or Millipore (U.K.) Ltd., Abbey Road, London N.W.10, U.K.] before their aseptic addition to sterile basal medium.

Batch cultures

Cells were grown on a 2–5-litre scale in salts medium containing 5mM-L-mandelate, 5mM-phenylglyoxylate (benzoylformate), 5mM-benzyl alcohol, 1mM-benzaldehyde or 5mM-L-glutamate+1mM-thiophenoxyacetate as described by Kennedy & Fewson (1968*b*).

Growth of bacterium N.C.I.B. 8250 for measurement of kinetics of enzyme induction

Aseptic techniques were used in the procedures described in this section. An inoculum of 10ml of a 24h nutrient broth culture was added to 4 litres of 10mM-L-glutamate–salts medium in a 10-litre flat-bottomed flask containing a 45mm stirring bar coated with polypropylene. The culture was grown for 12–15h at 30°C with rapid stirring on the apparatus described by Harvey *et al.* (1968). An additional quantity of L-glutamate equal to the amount present at inoculation, and in a volume of 200ml, was added to the culture. About 30min later the flask was removed from the growth apparatus and surrounded by ice. The cells were harvested as soon as possible by batch centrifugation at 15000*g* for 20min at 4°C. The supernatant medium was decanted and the pellet was washed by resuspension in ice-cold sterile basal medium and centrifugation at 12000*g* for 25min at 4°C. The cells were then weighed and resuspended in basal medium at 30°C to a concentration of 75mg wet wt./ml and 35ml of this suspension was then added to 1690ml of L-glutamate–salts medium in a 2-litre flask fitted with a side arm to facilitate sampling. (The final glutamate concentration when all the additions had been made was 10mM.) The culture was grown at 30°C with rapid stirring and aeration (200ml/min) in the apparatus of Harvey *et al.* (1968). Growth was followed by taking samples for turbidity measurements (E_{500}) in 10mm light-path cuvettes by using a Unicam SP.800 Spectrophotometer (Pye Unicam Instruments Ltd., Cambridge, U.K.) connected to a Servoscribe Chart Recorder (Smiths Industries Ltd., Kelvin House, Wembley, Middx., U.K.). The protein concentration of each sample was determined by reference to a calibration curve in which E_{500} was plotted against cell protein as determined by digestion of cell suspensions in 0.66M-NaOH for 24h and subsequent determination of protein content (Kennedy & Fewson, 1968*b*). After approximately a generation, the inducer was added in a volume of 75ml, whereupon samples were taken for E_{500} measurement every 5min. Samples (100ml) for enzyme estimation were withdrawn from the growth flask at suitable time-intervals on to 40g of crushed ice prepared from distilled water. This procedure was as effective at stopping enzyme synthesis as the addition of chloramphenicol to a final concentration of 20μM. The cells were harvested as described above and the pellets washed by resuspension in ice-cold water and centrifugation at 12000*g* for 25min at 4°C. The cells were weighed and then stored at −60°C until required for enzyme assay.

Growth of wild-type and mutant strains of bacterium N.C.I.B. 8250 for measurement of enzyme activities

An inoculum of 5ml of a 24h nutrient broth culture was added to 500ml of sterile 10mM-L-glutamate–salts medium in a 2-litre flask. The culture was grown for 12 to 14h at 30°C on the apparatus of Harvey *et al.* (1968) and 20ml amounts were then added aseptically to 60ml quantities of sterile 13.3mM-L-glutamate–salts medium containing appropriate concentrations of inducers, in 250ml Erlenmeyer flasks. The cultures were grown for 2.5h at 30°C on a rotary shaker (Mk V; L. H. Engineering Co., Bells Hill, Stoke Poges, Bucks., U.K.) moving at about 180 cycles/min. The cells were harvested by batch centrifugation at 15000*g* for 20min at 4°C and the pellets were washed as described above. The cells were weighed and stored at −60°C.

Isolation of mutants

Cells of bacterium N.C.I.B. 8250 grown in 10mM-glutamate– or 10mM-succinate–salts medium were washed and resuspended either in basal medium adjusted to pH6.0 (in Reading) or in 10mM-sodium citrate buffer, pH6.0 (in Glasgow). They were treated with *N*-methyl-*N*-nitroso-*N'*-nitroguanidine (Adelberg *et al.*, 1965) at a final concentration of 0.1mg/ml for 20min (basal medium) or 30min (citrate buffer) at 30°C and then thoroughly washed. Mutant strains unable to grow on benzyl alcohol but able to grow on L-mandelate or benzoate were isolated by two methods: (*a*) growth of the mutagen-treated cells on a 'limited nutrient' medium [5mM-benzyl alcohol+1mM-L-glutamate–salts medium solidified with 1.3% (w/v) Oxoid Ionagar no. 2] and selection of small colonies, and (*b*) replica plating (Lederberg & Lederberg, 1952) on to plates containing, in order of replication, 5mM-L-mandelate, 5mM-benzyl alcohol, 2mM-benzoate and finally on to nutrient agar, to monitor the success of replication. The mutants isolated were shown to be identical with the parent strain of bacterium N.C.I.B. 8250 (Fewson, 1967*a*)

except that they were unable to grow on benzyl alcohol.

Ultrasonic disruption

Cells were resuspended to 50mg wet wt./ml in a suitable buffer, usually 0.08M-sodium pyrophosphate, pH9.5; occasionally other buffers were used as described below. A Trident container (Johnson & Jorgensen Ltd., London, U.K.) containing 5ml of this cell suspension was positioned in a chilled brass holder (Holms & Bennett, 1971) screwed on to the horn of a 13mm probe of the Dawe Soniprobe (type 1130A; Dawe Instruments Ltd., London, U.K.) and lowered into an ice–water slurry. During the disruption the current (2.5A) was switched off for every alternate half-minute to aid cooling, the power to the probe being on for a total time of 3.5min. The homogenate was centrifuged at 12000*g* for 25min at 4°C to remove whole cells and debris, and the supernatant solution ('extract') used for assays of enzyme activity and protein concentration.

In a few experiments cells were disrupted in a Rosett (1965) cell by using an MSE 100W Ultrasonic Disintegrator (M.S.E. Ltd., London, U.K.) at 20kHz at a reading of approximately 6.2μm peak-to-peak. In these cases the homogenate was centrifuged at 35000*g* for 30min at 4°C to prepare the extract.

Enzyme assays

Spectrophotometric assays were done in 10mm path-length silica cuvettes. Measurements of extinction were made with a Unicam SP.800 Spectrophotometer connected to a Servoscribe Chart Recorder. Reactions were done at 27°C in Glasgow or at room temperature (approx. 20°C) in Reading. Reactions were initiated by addition of substrate. Enzyme units are defined as μmol of substrate converted/min; specific activities are given as milliunits (munits) of enzyme/mg of protein. All activities were derived from initial rates under conditions where the amount of product was proportional to the concentration of enzyme and time of incubation.

The activities of both benzaldehyde dehydrogenases were measured by a modification of the method of Gunsalus *et al.* (1953); this measures the benzaldehyde-dependent reduction of NAD^+ at 340nm. The assay mixture contained, in a total volume of 3.0ml: 200μmol of sodium pyrophosphate buffer, pH9.0; 150μmol of K_2HPO_4; 1.5μmol of NAD^+; 300nmol of benzaldehyde; extract. The extract was heated at 37°C and samples taken at intervals of approx. 5min for assay in this system. The activities of the two enzymes were then calculated by extrapolation of the heat inactivation curve, as described in the Results section for Fig. 1, although in that example the calculation is complicated by the initial activation of benzaldehyde dehydrogenase I in tris buffer; this activation did not occur in pyrophosphate buffer and the calculation was therefore more straightforward.

In some cases, especially in the initial experiments, the cells were suspended for ultrasonication in 0.04M-tris buffer, pH10.3. The benzaldehyde dehydrogenase activity was measured in a reaction mixture containing, in a total volume of 3.0ml: 200μmol of tris buffer, pH9.5; 150μmol of K_2HPO_4; 1.5μmol of NAD^+; 300nmol of benzaldehyde; extract. The activity was also measured after heating a portion of the extract at 37°C for 120min. The residual activity was taken as a measure of benzaldehyde dehydrogenase I, since the amount of this enzyme inactivated in 120min at 37°C approximately equals the increase in activity over the first few minutes (Fig. 1) and since the activity of benzaldehyde dehydrogenase II is decreased to a negligible proportion (about 6%) of its original value under these conditions. The difference between this activity and the total benzaldehyde dehydrogenase activity before heating was taken as a measure of benzaldehyde dehydrogenase II. This method with extraction in tris buffer gives estimates of enzyme activity that are less accurate than when pyrophosphate buffer is used. In practice, however, the errors introduced are small and there is the considerable advantage that the other enzymes of the mandelate pathway may be assayed in the tris extract (Livingstone & Fewson, 1972).

In a few of the later experiments done in Reading, benzaldehyde dehydrogenase was assayed in a modified reaction mixture containing, in a total volume of 3.0ml, 55μmol of glycine buffer, pH9.5, 1.5μmol of NAD^+, 900nmol of benzaldehyde, extract and, in the case of benzaldehyde dehydrogenase I, 30μmol of KCl.

Benzyl alcohol dehydrogenase was measured in a reaction mixture modified from that of Kennedy & Fewson (1968*b*) containing, in a total volume of 3.0ml: 200μmol of sodium pyrophosphate buffer, pH9.0; 1.5μmol of NAD^+; 300nmol of benzyl alcohol; extract.

Molecular weight determination

Washed cells were resuspended to 500mg wet wt./ml before ultrasonic treatment with the MSE Ultrasonic Disintegrator (see above). A portion (5ml) of the resulting extract was applied to a column (72cm×2.3cm) of Sephadex G-200 (Pharmacia Fine Chemicals, Uppsala, Sweden) previously equilibrated with 0.1M-tris–maleate buffer, pH7.1, containing 0.1M-NaCl. Ferritin [5.0mg, mol.wt. 560000 (Rothen, 1944)], lactate dehydrogenase [50 units, mol.wt. 135000 (Markert, 1963)], malate dehydrogenase [50 units, mol.wt. 50000 (Cánovas & Stanier, 1967)] and cytochrome *c* (5.0mg, mol.wt. taken as 22000;

see Ornston, 1966) were added as standard markers. The proteins were eluted with 0.1M-tris–maleate buffer, pH7.1, containing 0.1M-NaCl at a flow rate of 10–15ml/h. Samples of the eluate were assayed for the standard markers as follows: lactate dehydrogenase by the method of Meister (1950); malate dehydrogenase by the method of Ochoa (1955); ferritin and cytochrome *c* by measurement of E_{310} and E_{406} respectively. The elution volume of each of the proteins was determined to the nearest 0.1ml (Whitaker, 1963) and the molecular weights of the benzaldehyde dehydrogenases were determined from the position of their elution volume on the plot of log (mol.wt.) against elution volume obtained by using the standard markers (Andrews, 1965).

Analytical methods

Wet weights of bacteria and protein concentrations were determined as outlined by Kennedy & Fewson (1968*b*).

Glassware

All glassware used for the growth of bacteria was washed for 15min in boiling 10% (v/v) nitric acid and then thoroughly rinsed in tap-water and demineralized or distilled water.

Materials

2-Mercaptoethanol and 2-, 3- and 4-pyridylcarbinol were obtained from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K. D-Thiophenemethanol and 2- and 3-pyridine-carboxaldehyde were purchased from Ralph N. Emanuel Ltd., Wembley, Middx., U.K. Phenylglyoxylic acid (puriss), L-mandelic acid (puriss) and 2-pyridylethyleneglycol were from Fluka A.-G., Buchs, Switzerland. Sources of other compounds were: 2,3,4,5,6-pentafluorobenzyl alcohol (Aldrich Chemical Co. Inc., Milwaukee, Wis., U.S.A.); ferritin and dithiothreitol (Calbiochem, Los Angeles, Calif., U.S.A.); α-monothioglycerol, thiophenoxyacetic acid and styrene glycol (K & K Laboratories, Plainview, N.Y., U.S.A.); cysteine and L-glutamic acid–HCl (T. J. Sas and Son Ltd., London W.C.1, U.K.); rabbit muscle lactate dehydrogenase and pig heart malate dehydrogenase (C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany); horse heart cytochrome *c* (type VI), *p*-chloromercuribenzoate and *N*-ethylmaleimide [Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.]; tris (enzyme grade) (Mann Research Laboratories, New York, N.Y., U.S.A.). *N*-Methyl-*N*-nitroso-*N'*-nitroguanidine was purchased from both Sigma (London) Chemical Co. Ltd., and Koch–Light Laboratories Ltd. The sources of some of the other reagents are listed by Kennedy & Fewson (1968*a*,*b*) and the remainder were obtained from British Drug Houses Ltd., Poole, Dorset, U.K., generally as AnalaR grade.

Benzaldehyde was redistilled and stored under nitrogen in the dark. Benzyl alcohol was also redistilled before use.

All solutions were prepared in glass-distilled water.

Results

Kinetics of heat inactivation of the benzaldehyde dehydrogenases in cell extracts

Cells containing only benzaldehyde dehydrogenase I were prepared by growth on glutamate+thiophenoxyacetate; these cells also contained L-mandelate dehydrogenase and phenylglyoxylate carboxylyase but not benzyl alcohol dehydrogenase (Livingstone & Fewson, 1972). Cells containing only benzaldehyde dehydrogenase II were prepared by using benzyl alcohol as inducer. The benzaldehyde dehydrogenase activity in extracts of both types of cells was measured periodically over 3 to 4h during their incubation at 37°C; the results are given in Fig. 1. In this experiment the buffer used for extraction and assay was 0.04M-tris, pH10.3, but because of the buffering capacity of the cell extract, the actual pH was 9.18. In a number of experiments of this type the observed half-lives of benzaldehyde dehydrogenases I and II were 520 and 30min respectively. The activity of benzaldehyde dehydrogenase I rose by about 35% during the first 10min incubation at 37°C and then fell exponentially. When 0.08M-pyrophosphate buffer, pH9.5 (pH of extract 9.16) was used for extraction and assay, as in the experiments of Kennedy & Fewson (1968*b*), similar results were obtained except that there was no activation of benzaldehyde dehydrogenase I during the first few minutes at 37°C. The pH dependence of the rate of inactivation of benzaldehyde dehydrogenase II was very marked, with a half-life rising to about 113min when the buffer used for extraction and heating was 0.04M-tris, pH8.5. At still lower pH values (7 to 7.5) both enzymes were equally heat-stable.

The presence of both enzymes in cells grown on L-mandelate (Kennedy & Fewson, 1968*b*) is confirmed by the other results plotted in Fig. 1. Extrapolation to zero time of the experimental points for the samples heated for the period from 120 to 240min shows that before heating the extract contained about 60munits of benzaldehyde dehydrogenase I/mg of protein; this value allows for the initial activation and for the subsequent exponential decrease. It can be seen that there were originally about 180munits of total enzyme activity/mg of protein and there must therefore have been 120munits of benzaldehyde de-

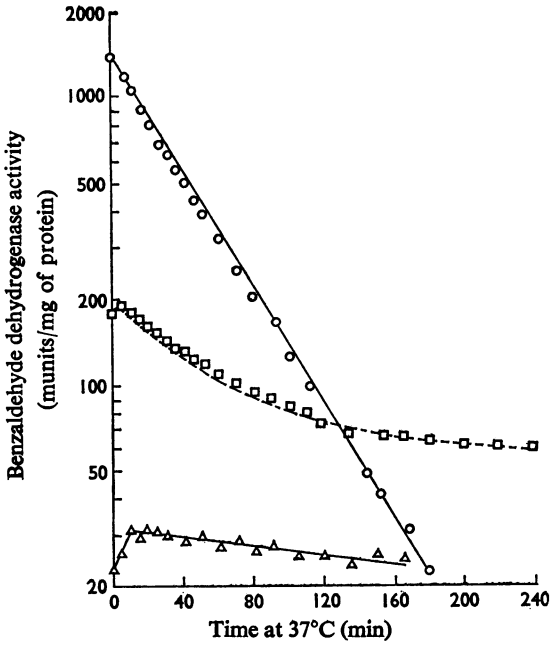

Fig. 1. *Kinetics of heat inactivation of benzaldehyde dehydrogenases*

Bacterium N.C.I.B. 8250 was grown on 5mM-benzyl alcohol or on 5mM-L-glutamate+1mM-thiophenoxyacetate before being harvested, washed and resuspended in 0.04M-tris buffer, pH10.3. The cells were disrupted and centrifuged as described in the Materials and Methods section. The extracts prepared from cells induced with benzyl alcohol, o, or with thiophenoxyacetate, Δ, were heated at 37°C and samples withdrawn at intervals for determination of benzaldehyde dehydrogenase activity. An extract of cells grown on 5mM-L-mandelate, □, was prepared and tested in the same way. Extrapolation of the experimental points indicated that the original extract contained two enzymes of half-lives 520 and 30min and specific activities of about 60 and 120munits/mg of protein respectively; --- represents the theoretical curve for such a mixture of enzymes, assuming that the relatively heat-stable enzyme showed an initial activation of 35%, as described in the text.

hydrogenase II/mg of protein present at zero time. Calculations were then made assuming that the original extract contained two enzymes having half-lives of 30 and 520min and specific activities of 120 and 60munits/mg of protein respectively, the latter rising to 80munits/mg of protein because of the stimulation over the first 10min at 37°C. The heat inactivation curve for this hypothetical mixture was drawn, and it can be seen in Fig. 1 that this theoretical curve agrees well with the experimental data, supporting the earlier conclusion that the two enzymes are present in cells grown on L-mandelate. Reconstitution experiments in which extracts prepared from cells grown on benzyl alcohol or on glutamate+thiophenoxyacetate were mixed confirmed that the activities of the two enzymes were additive.

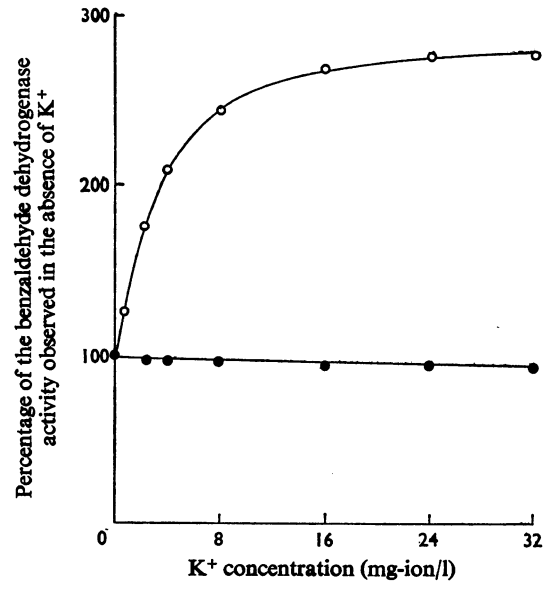

Fig. 2. *Effect of K^+ on the activity of benzaldehyde dehydrogenase*

Bacterium N.C.I.B. 8250 was grown on 5mM-benzyl alcohol or on 5mM-L-glutamate+1mM-thiophenoxyacetate before being harvested, washed and resuspended in 0.08M-sodium pyrophosphate buffer, pH9.5. Samples were disrupted and centrifuged as described in the Materials and Methods section. Extracts from cells induced by benzyl alcohol, ●, or by thiophenoxyacetate, o, were assayed in a reaction mixture containing pyrophosphate buffer (see the Materials and Methods section) and various concentrations of K_2HPO_4.

Activation by cations

Benzaldehyde dehydrogenase I, but not benzaldehyde dehydrogenase II, was activated by K^+ ions (Fig. 2) and also by certain other univalent cations ($K^+ > Rb^+ > NH_4^+ > Cs^+ > Na^+ > Li^+$). Neither enzyme was activated by Mg^{2+}, Ca^{2+}, Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Al^{3+} or Fe^{3+} when tested over the range 3μg-ion/l to 33mg-ion/l. The presence

of 1mM-EDTA, 0.5mM-KCN or 0.5mM-8-hydroxyquinoline had no significant effect on the activity of either enzyme.

Involvement of thiols in enzyme activity

Stachow *et al.* (1967) assayed the $NADP^+$-specific benzaldehyde dehydrogenase from *Pseudomonas putida* in the presence of dithiothreitol to provide protection against inhibitory thiol-binding agents. However, none of the following compounds activated either of the two benzaldehyde dehydrogenases from bacterium N.C.I.B. 8250: dithiothreitol, cysteine, reduced glutathione, 2-mercaptoethanol, α-monothioglycerol (all at 3μM to 10mM) or thioglycollate (10mM). Both enzymes were completely inhibited by 0.5mM-*p*-chloromercuribenzoate or 0.5mM-*N*-ethylmaleimide.

Effect of pH on enzyme activity

High activities of both enzymes were extracted at pH9.0–9.5 although there appeared to be somewhat lower enzyme recovery when tris buffer rather than pyrophosphate, glycine or carbonate buffer was used for the ultrasonication. This high pH of extraction lowered interference by benzyl alcohol dehydrogenase and NADH oxidase in the subsequent assays for benzaldehyde dehydrogenase. Both enzymes displayed optimum activity when assayed at pH9.0–9.5. The activity of benzaldehyde dehydrogenase II was independent of the nature of the assay buffer (pyrophosphate, glycine, tris or carbonate) whereas benzaldehyde dehydrogenase I was more active when assayed in glycine buffer.

Molecular weight

Gel filtration of extracts through Sephadex G-200 indicated molecular weights of about 180000 for both enzymes.

Enzyme induction

Experiments in which differential rates of enzyme synthesis (Monod *et al.*, 1952) were measured under a variety of conditions showed that benzaldehyde dehydrogenase II (correlation coefficient = 0.97), but not benzaldehyde dehydrogenase I (correlation coefficient = −0.33), was synthesized co-ordinately with benzyl alcohol dehydrogenase (Fig. 3). The same results showed that the two benzaldehyde dehydrogenases were synthesized independently (correlation coefficient for the differential rates of synthesis = −0.28). Benzaldehyde dehydrogenase I was synthesized co-ordinately with L-mandelate dehydrogenase

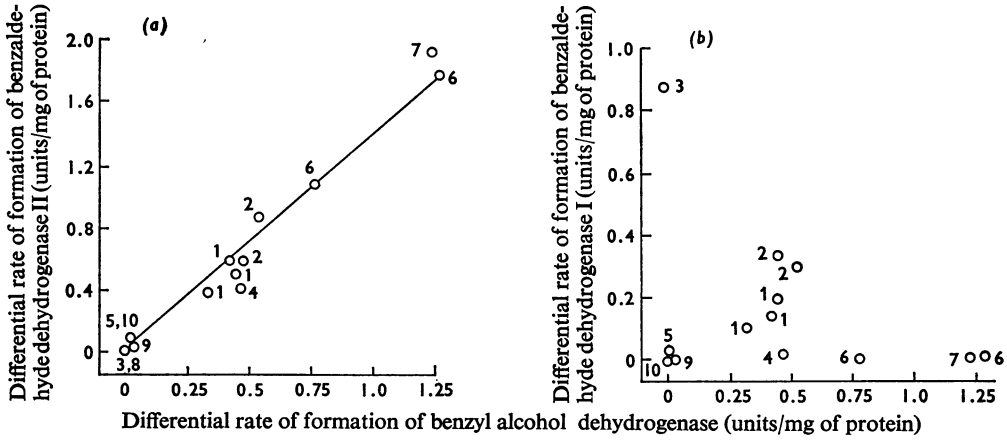

Fig. 3. *Co-ordinacy of induction of benzaldehyde dehydrogenase activity*

The kinetics of induction of the two benzaldehyde dehydrogenases and of benzyl alcohol dehydrogenase by a variety of inducers was measured as described in the Materials and Methods section. Differential plots (Monod *et al.*, 1952) were constructed for each enzyme and each inducer, the slopes giving the differential rates of enzyme synthesis. For each inducer the differential rate of synthesis of benzaldehyde dehydrogenase II (*a*) or benzaldehyde dehydrogenase I (*b*) is plotted against the differential rate of synthesis of benzyl alcohol dehydrogenase. The inducers were: 1, 5mM-L-mandelate; 2, 5mM-phenylglyoxylate; 3, 1mM-thiophenoxyacetate; 4, 5mM-L-mandelate+5mM-benzyl alcohol; 5, 5mM-L-mandelate+5mM-acetate+5mM-succinate; 6, 5mM-benzyl alcohol; 7, 1mM-benzaldehyde; 8, 5mM-benzyl alcohol+5mM-acetate+5mM-succinate; 9, 1mM-benzoate; 10, 1mM-catechol. Different points for the same inducer represent completely independent experiments.

Table 1. *Activities of benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II in wild-type and mutant strains of bacterium N.C.I.B. 8250 grown in the presence of various inducers*

Bacterium N.C.I.B. 8250 and various mutant strains derived from it were grown for 2.5h in L-glutamate–salts medium and in this medium supplemented with inducer (5mM-benzyl alcohol or 1mM-benzaldehyde) as described in the Materials and Methods section. The cells were harvested, washed and disrupted, and the activities of the two enzymes measured in the cell-free extracts.

Strain	Inducer	Enzyme activity (munits/mg of protein)	
		Benzyl alcohol dehydrogenase	Benzaldehyde dehydrogenase II
Wild-type	None	7	2
Wild-type	Benzyl alcohol	233	324
Wild-type	Benzaldehyde	143	126
Class 1 mutant			
AL 37	None	6	2
AL 37	Benzyl alcohol	10	6
AL 37	Benzaldehyde	10	6
Class 2 mutant			
CF 16	Benzyl alcohol	2	325
CF 16	Benzaldehyde	2	94
Class 3 mutant			
SK 3	Benzyl alcohol*	389	<1

* Grown for 16h on 2mM-benzyl alcohol+2mM-benzoate.

and phenylglyoxylate carboxy-lyase (Livingstone & Fewson, 1972). Benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II, but not benzaldehyde dehydrogenase I, were synthesized in cells grown on glutamate with 10mM-D-thiophenemethanol, 10mM-2-pyridylcarbinol, 2mM-3-pyridylcarbinol, 2.5mM-4-pyridylcarbinol, 0.4mM-2-pyridinecarboxyaldehyde or 2mM-3-pyridinecarboxaldehyde as inducer. It is doubtful whether any of these is a genuine gratuitous inducer since although they do not support growth, they are all oxidized to some extent by bacterium N.C.I.B. 8250 (C. A. Fewson, unpublished work). The following compounds did not induce significant amounts of either benzaldehyde dehydrogenase: cinnamyl alcohol (0.2 or 2mM), DL-1-phenylethanol (0.2 or 2mM), 2-phenylethanol (0.2 or 10mM), 2-pyridylethyleneglycol (0.4 or 10mM), styrene glycol (0.4 or 10mM) or 2,3,4,5,6-pentafluorobenzyl alcohol (0.4 or 5mM).

Mutant strains of bacterium N.C.I.B. 8250

Three classes of mutant were obtained that were unable to utilize benzyl alcohol as carbon source for growth, but were able to grow on L-mandelate or benzoate. Table 1 gives the results of induction experiments with one representative of each of the three classes of mutant. The class 1 mutant AL 37 lacks both benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II; the class 2 mutant CF 16 lacks benzyl alcohol dehydrogenase; and the class 3 mutant SK 3 lacks benzaldehyde dehydrogenase II. The class 2 mutants lacking benzyl alcohol dehydrogenase must nonetheless have been able to oxidize benzyl alcohol to some extent since low but detectable amounts of catechol 1,2-oxygenase were formed when these strains were grown in the presence of benzyl alcohol (A. Livingstone, unpublished work). In none of these mutants was the ability to synthesize benzaldehyde dehydrogenase I altered when the cells were grown in the presence of L-mandelate or phenylglyoxylate.

Mutants of class 1, which may be regulatory mutants, all failed to grow, except in some cases after very long lag-times, on 2-hydroxybenzyl alcohol, 4-hydroxybenzyl alcohol or 4-hydroxy-3-methoxybenzyl alcohol, confirming the idea that these compounds are all oxidized by the same relatively nonspecific enzymes (Kennedy & Fewson, 1968*a*,*b*). The ability of all these mutants to grow on L-mandelate or phenylglyoxylate confirms the view that benzyl alcohol is not an obligatory intermediate in mandelate oxidation (Stanier, 1948; Kennedy & Fewson, 1968*a*).

Discussion

The marked difference in sensitivity to thermal inactivation, the different response to univalent cations, the different specificities of induction, and

the properties of the mutant strains, all provide good evidence supporting the conclusion that bacterium N.C.I.B. 8250 can synthesize two NAD^+-linked benzaldehyde dehydrogenases. The present paper also provides evidence that the more heat-labile enzyme, benzaldehyde dehydrogenase II, is associated with the regulon controlling the oxidation of benzyl alcohol and the results of Livingstone & Fewson (1972) show that benzaldehyde dehydrogenase I is controlled by the same regulatory system as L-mandelate dehydrogenase and phenylglyoxylate carboxy-lyase.

The two isofunctional enzymes show no evidence for co-ordinancy of induction and are presumably products of different structural genes; the latter could be quite unrelated both in structure and in location on the genome as well as in their regulation. However, in a similar situation in a related organism *Acinetobacter calcoaceticus* A.T.C.C. 23393 that apparently possesses two isofunctional enzymes (enol-lactone hydrolases) mediating the first reaction common to the catechol and protocatechuate pathways (Cánovas & Stanier, 1967), Katagiri & Wheelis (1971) have pointed out that it is difficult to rule out models of enzyme biosynthesis in which the product of a single structural gene is modified after synthesis. It is also possible that isofunctional enzymes might be the products of structurally related, but nonetheless different, structural genes, separately regulated, arising by some process such as gene duplication (Lewis, 1951; see review by Hegeman & Rosenberg, 1970). The molecular weights, as determined by gel filtration, of the benzaldehyde dehydrogenases are similar, if not identical, as is the case with the enol-lactone hydrolases of *A. calcoaceticus*, and this is not inconsistent with either of these concepts. Also, the differences between the pairs of enzymes based on thermal inactivation depend on the pH in the case of benzaldehyde dehydrogenases (see the Results section) and on the degree of purity in the case of the enol-lactone hydrolases (Katagiri & Wheelis, 1971). Help in resolving these problems will come from a study of the purified benzaldehyde dehydrogenases (S. I. T. Kennedy & L. J. Zatman, unpublished work).

Benzaldehyde dehydrogenase II and benzyl alcohol dehydrogenase are regulated co-ordinately in bacterium N.C.I.B. 8250 as shown by the kinetics of enzyme induction (Fig. 3) and by their induction by compounds such as the substituted pyridines. The behaviour of the mutants also supports this conclusion, particularly the induction of benzaldehyde dehydrogenase II by benzyl alcohol (and by benzaldehyde) in mutant strain CF 16 which lacks benzyl alcohol dehydrogenase. The ability of both benzyl alcohol and benzaldehyde to induce this system contrasts with the specificity of phenylglyoxylate as the inducer of the enzymes of L-mandelate oxidation (Livingstone & Fewson, 1972). Although aliphatic compounds cannot induce the enzymes for benzyl alcohol oxidation (Fewson, 1966), the specificity of induction is wide, as the inducer can be a substituted benzene, a substituted pyridine or a thiophene compound. We have not eliminated the possibility that benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II are not separate enzymes but instead reflect different activities of the same protein (see, e.g., Abeles & Lee, 1960). The existence of class 2 and class 3 mutants that lack one or other enzyme activity (Table 1), however, makes this unlikely. Results reported in a paper by Suhara *et al.* (1969) imply that at least in *Pseudomonas putida* T-2, benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase are separate enzymes.

Clearly, different bacteria capable of oxidizing benzaldehyde have evolved quite different enzyme systems for doing so. The results in the present paper and elsewhere (Kennedy & Fewson, 1968*a*,*b*) indicate that bacterium N.C.I.B. 8250 has only one benzaldehyde dehydrogenase associated with the regulon controlling mandelate oxidation and a different enzyme, benzaldehyde dehydrogenase II, associated with the regulon controlling benzyl alcohol oxidation. Mandelate oxidation by *P. putida* involves both an NAD^+- and an $NADP^+$-linked enzyme, although the significance of this is not at all clear (Ornston, 1971). *P. putida* can form a third benzaldehyde dehydrogenase, which is induced by benzaldehyde (Stevenson & Mandelstam, 1965). In *Pseudomonas aeruginosa* an $NADP^+$-linked benzaldehyde dehydrogenase is involved in mandelate oxidation; this organism may contain a second $NADP^+$-linked benzaldehyde dehydrogenase with very similar properties to the first enzyme but induced by β-oxoadipate (Rosenberg, 1971). A comprehensive comparative study of bacterial benzaldehyde dehydrogenases is obviously needed.

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