THE REGULATION OF NAPHTHALENE METABOLISM IN PSEUDOMONADS

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SUMMARY: The activities of three enzymes specifically involved in naphthalene metabolism have been measured in Pseudomonas NCIB 9816 after induction with salicylate or 2-hydroxybenzyl alcohol. The results indicate that naphthalene oxygenase, 1,2-dihydroxynaphthalene oxygenase and salicylaldehyde dehydrogenase are induced coordinately and it is suggested that all of the enzymes converting naphthalene to salicylate are regulated coordinately.

The enzyme naphthalene oxygenase which catalyses the first step in the degradation of naphthalene by certain pseudomonads is induced by salicylate, and may be induced gratuitously with 2-aminobenzoate or 2-hydroxybenzyl alcohol (1). The first identified product of naphthalene oxidation, 1,2-dihydro-1,2-dihydroxynaphthalene (2, 3), is further metabolized in a series of steps which lead to salicylaldehyde and then salicylate (4). Salicylate accumulates in high yield when Pseudomonas NCIB 9816 that has been induced gratuitously is incubated in a medium containing naphthalene (1), and it seems possible that enzymes catalyzing the first steps of naphthalene metabolism and which are specifically involved in the latter may be regulated coordinately. We have now measured the activities of enzymes catalyzing the reactions of three of the five steps leading from naphthalene to salicylate, and have shown that they may all be induced by salicylate or 2-hydroxybenzyl alcohol.

MATERIALS AND METHODS

The pseudomonad used in this work, NCIB 9816, was obtained from the National Collection of Industrial Bacteria, Aberdeen, and was maintained on

nutrient agar. Cultures were grown at 25° in shaken flasks containing a defined mineral medium and succinate as a carbon and energy source (1), and solutions of inducers, sterilized by filtration, were added during the early exponential phase of growth (apparent absorbance about 0.2 at 600 nm measured with 1 cm cuvettes in a Unicam SP 800 spectrophotometer). After the population had doubled between one and twofold, the cells were collected by centrifugation at 2°, the pellet was resuspended in ice cold 50 mM potassium dihydrogen phosphate - NaOH (pH 7.0) and sedimented again. The washing was repeated, and the cells were finally resuspended at a concentration of about 0.1 g wet wt/ml in phosphate buffer. Part of the suspension was disrupted with an ultrasonic oscillator (Model W185, Heat Systems - Ultrasonics, Inc., Plain View, New York, U.S.A.) at a nominal power of 70W. A 2 ml portion of the suspension initially at 0° was disrupted for four half minute periods. Each period of disruption was followed by 1 min during which the disrupted cells and oscillator probe were cooled in ice water. Particulate matter was then removed from the extract by centrifugation at 105 000 x g for 1 hr at 4°.

All enzymes were measured at 25° in cuvettes containing a final volume of 3.0 ml. Naphthalene oxygenase was measured by a spectrophotometric method using whole cells (1). Salicylaldehyde dehydrogenase was determined from the rate of increase of the absorbance at 340 nm caused by adding diluted cell extract (0.05 ml) to a reaction mixture containing 2.75 ml of 20 mM tetrasodium pyrophosphate - HCl (pH 8.5), 0.1 ml salicylaldehyde (3 mM aqueous solution of freshly redistilled aldehyde), and 0.1 ml NAD (150 mM). Tris at moderate concentrations reacts with salicylaldehyde and cannot be used as a buffer in this determination. The rate was calculated using an extinction coefficient of 3.84 $mM^{-1}cm^{-1}$, the difference between that of NADH (6.22 $mM^{-1}cm^{-1}$) and that of salicylaldehyde (2.38 $mM^{-1}cm^{-1}$) determined experimentally at 340 nm and pH 8.5. Salicylate hydroxylase (EC 1.14.13.1) was determined spectrophotometrically at 340 nm by measuring the rate of oxidation of NADH in a cuvette containing phosphate (pH 7.0) and cell free extract (combined volume 2.6 ml),

0.2 ml NADH (1.5 mM), and 0.2 ml sodium salicylate (1.5 mM). The reported results were corrected for the small NADH oxidation which occurred independently of added salicylate. Catechol 1,2-dioxygenase (EC 1.13.11.1) and catechol 2,3-dioxygenase (EC 1.13.11.2) were determined by published methods (5, 6).

1,2-Dihydroxynaphthalene oxygenase was determined spectrophotometrically. To a cuvette containing 50 mM acetic acid - NaOH buffer (pH 5.5) cell extract (usually 50 µl) was added to give a volume of 2.85 ml. 1,2-Dihydroxy-naphthalene (15 µl, 40 mM) was added and the initial rate of decrease of the absorbance at 285 nm was measured. The rate was corrected for the rate of the non-enzymic reaction in which the quinone is produced, and the specific activity was calculated using an extinction coefficient of 2.13 $mM^{-1}cm^{-1}$, the difference between that of 1,2-dihydroxynaphthalene (4.05) and the reaction product (1.92).

Protein was determined on the fraction of whole cells or cell extracts insoluble in 5% trichloracetic acid (7).

The stoicheiometry of the oxidation of salicylaldehyde by NAD was determined using cell extract in a reaction medium containing 5 mM 1,10-phenanthroline to inhibit salicylate hydroxylase (8). A reaction mixture containing 20.1 ml pyrophosphate buffer (pH 8.5), 2.7 ml salicylaldehyde (0.3 mM), 0.24 ml 1,10-phenanthroline (0.5 M) and cell extract (0.16 ml) was incubated at 25° for 10 min. The reaction was then started by the addition of 0.8 ml NAD (150 mM). The NADH produced was calculated from the absorbance increase at 340 nm using an extinction coefficient of 3.84 $mM^{-1}cm^{-1}$. Part of the reaction mixture (20 ml) was extracted with five 4 ml portions of ether and the extracts were discarded. The aqueous residue was acidified with 0.2 ml concentrated HCl, and the acidified solution was extracted with five 4 ml portions of ether. The combined ether extracts were evaporated on a warm water bath to low volume, and the residual solution was dissolved in phosphate buffer (pH 7.0) and diluted with the same buffer to 5.0 ml. The solution was clarified by centrifugation, and its absorption spectrum was measured. There

were present peaks corresponding to 1,10-phenanthroline (263 nm) and salicylate (295 nm), and the absorbance at these wavelengths was measured as the difference from the absorbance at 350 nm. The absorbance at 295 nm contained a contribution due to 1,10-phenanthroline, and was reduced by 11.9% of the absorbance at 263 nm to correct for this. The salicylate concentration was then calculated using the experimentally determined extinction coefficient of 3.45 $mM^{-1}cm^{-1}$. The identity of salicylate was confirmed by acidifying the buffer solution with HCl, extracting the solution with ether, and chromatographing the extract on 0.25 mM thick layers of silica gel G with benzene, ether, acetic acid ethanol (120:60:18:1 by vol.) as developing solvent.

RESULTS AND DISCUSSION

A polarographic method for the measurement of 1,2-dihydroxynaphthalene oxygenase has been proposed (9), but we have found it unsatisfactory because of the very rapid rate of oxygen uptake that is measured. With a conventional membrane-covered oxygen electrode the rates appeared to be higher than the responsiveness of the electrode itself. Consequently we examined the reaction spectrophotometrically. 1,2-Dihydroxynaphthalene has an absorption maximum in aqueous solution at 285 nm, and this absorbance decreases both in the non-enzymic oxidation to naphthalene 1,2-quinone and in the enzyme catalyzed reaction. The absorption spectrum of the product of oxidation of 1,2-dihydroxynaphthalene formed in the presence of excess extract from induced cells was very closely similar to that reported for 2-hydroxybenzalpyruvate (4). The activity of the enzyme was as high at pH 5.5 as it was at pH 6.0, whereas at this pH the non-enzymic reaction was significantly slower, and so pH 5.5 was used for enzymic measurements. At this pH the enzymic reaction product was stable in the presence of crude bacterial extracts. The chief disadvantage of the method lies in the need to measure rapid initial reaction rates. With extracts of induced cells these were usually in the range of 0.3 to 0.6 absorbance units per minute so that the rate of the non-enzymic reaction (0.05

absorbance units per minute) would be less than 17% of the enzymic rate. Probably as a consequence of this requirement the standard deviation of a set of 11 measurements made with induced cells was 11.5% of the mean. This in no way obscured the large increase in the activity of this enzyme which occurred when Pseudomonas NCIB 9816 was induced with salicylate or 2-hydroxybenzyl alcohol or was grown on salicylate or naphthalene alone (Table 1).

The presence of an NAD-dependent enzyme converting salicylaldehyde to salicylate in pseudomonads grown on naphthalene has been reported previously (4), and it now appears that it is induced in cells growing on succinate and induced with either salicylate or 2-hydroxybenzyl alcohol. The optimum pH for the reaction was 8.5, and the K_m for NAD was 0.15 mM. The K_m for salicylaldehyde was not determined, but it was very low for the rate of the reaction was independent of the concentration of salicylaldehyde at concentrations at least as low as 20 μM. NADP (5 mM) effected oxidation at 7% of the rate given by 5 mM NAD.

In a reaction mixture which contained 5 mM 1,10-phenanthroline to inhibit salicylate hydroxylase there were produced on completion of the reaction 0.88 mole NADH and 0.95 mole salicylate per mole salicylaldehyde originally present. The identity of salicylate, which was measured spectrophotometrically in the presence of 1,10-phenanthroline, was confirmed by re-extraction into ether and chromatography of the extract. A spot was observed which behaved identically with authentic salicylate (blue fluorescence under UV light, R_F 0.70) and which was separated from 1,10-phenanthroline (R_F 0.00). It was not well separated from salicylaldehyde (R_F 0.76), but was readily distinguished from the latter by its characteristic fluorescence.

The levels of some of the enzymes of naphthalene metabolism found in Pseudomonas NCIB 9816 under different conditions of growth are given in Table 1. Naphthalene oxygenase, 1,2-dihydroxynaphthalene oxygenase and salicylaldehyde dehydrogenase were induced when the organism was grown on naphthalene or salicylate, or was grown on succinate and induced with

TABLE 1

Specific Activities of Enzymes of Naphthalene Metabolism in Induced and Non-Induced Cells of Pseudomonas NCIB 9816

Initial carbon source (mM)	Inducer (mM)	Specific Activity (μmole / min / mg protein)					
		Naphthalene oxygenase	Dihydroxynaphthalene oxygenase	Salicylaldehyde dehydrogenase	Salicylate hydroxylase	Catechol 1.2-dioxygenase	Catechol 2,3-dioxygenase
Succinate (8.5)		0.005	0.05	0.06	0.001	0.003	0.040
Naphthalene (0.1%)		0.345	3.60	1.40	0.088	0.277	0.029
Salicylate (3.5)		0.300	3.95	1.36	0.118	0.281	0.021
Catechol (3.0)		0.009	0.02	0.05	0.002	0.300	0.031
Succinate (8.5)	Salicylate (2.0)	0.250	2.61	0.56	0.090	0.138	0.044
Succinate (8.5)	2-Hydroxybenzyl alcohol (3.5)	0.200	2.36	0.56	0.006	0.001	0.022
Succinate (8.5)	2-Hydroxybenzyl alcohol (3.5); exposure for nine generations	0.240	2,39	0.60	0.030	0.008	0.019
Succinate (8.5)	Catechol (3.0)	0.006	0.02	0.05	0.001	0.298	0.035

The methods of measurement are described in the text. Specific activities are computed per mg protein in whole cells (for naphthalene oxygenase) or per mg protein in extracts (other enzymes).

salicylate (which was metabolized) or 2-hydroxybenzyl alcohol (which was not metabolized) (1). These enzymes were not induced by catechol present either as a sole carbon source or added to a culture growing on succinate. Under the conditions which induced the aforementioned enzymes catechol 2,3-dioxygenase was not induced but remained at a significant constitutive level (1, 10), and catechol 1,2-dioxygenase was induced only after growth on or in the presence of naphthalene, salicylate or catechol.

It appears therefore that the three early enzymes of naphthalene metabolism examined are induced under similar conditions and, in particular, are distinguished from the other enzymes of naphthalene metabolism by their gratuitous induction by 2-hydroxybenzyl alcohol. This, together with the observation that these enzymes are induced in the presence of salicylate but not of catechol, suggests that salicylate is the inducer of the whole block of enzymes catalyzing reactions leading from naphthalene to salicylate. This coordinate induction probably does not extend as far as salicylate hydroxylase for in the presence of 2-hydroxybenzyl alcohol this enzyme shows little or no induction. This remains true when the organism has been exposed to the gratuitous inducer for as many as nine generations.

These general conclusions are probably valid in spite of uncertainties in the interpretation of some of the reaction rates presented. These arise because of the problems associated with measuring single enzymes in either whole cells or crude extracts. Naphthalene oxygenase values measured with whole cells are minimal ones (1), and this is probably also true for the levels of salicylaldehyde dehydrogenase. Both products of this reaction are consumed in the reaction catalyzed by salicylate hydroxylase, but the latter enzyme was never present at a level greater than 17% of that of salicylaldehyde dehydrogenase and at the pH used for measurement of the latter this percentage would be even less. Catechol 1,2-dioxygenase is clearly induced in the presence of naphthalene and salicylate, but it seems unlikely that the presence of this enzyme prevents the measurement of enhanced levels of catechol 2,3-di-

oxygenase for both substrates, catechol and oxygen, are present at concentrations well above the K_m values reported for either enzyme. Furthermore we have shown (1) that 2-hydroxymuconic semialdehyde, the measured reaction product of catechol 2,3-dioxygenase, is not removed under the conditions of the oxygenase determination. The results, therefore, confirm the view (10) that catechol 2,3-dioxygenase is constitutive in Pseudomonas NCIB 9816 and also that 1,2-dihydroxynaphthalene oxygenase and catechol 2,3-dioxygenase are distinct enzymes (9).

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