THE COEXISTENCE OF TWO PATHWAYS FOR THE METABOLISM OF 2-HYDROXYMUCONIC SEMIALDEHYDE IN A NAPHTHALENE-GROWN PSEUDOMONAD

F.A. CATTERALL, J.M. SALA-TREPAT AND P.A. WILLIAMS

Department of Biochemistry and Soil Science,
University College of North Wales, Bangor, Great Britain.

Received February 20, 1971

SUMMARY. Catechol is degraded by meta-cleavage in a naphthalene-grown pseudomonad to yield 2-hydroxymuconic semialdehyde. It is shown that there exist two enzymes in naphthalene-grown cells which degrade 2-hydroxymuconic semialdehyde, one NAD^+-dependent and one non NAD^+-dependent. Both are induced by growth upon naphthalene. It is suggested that the more important of these enzymic reactions in vivo is the NAD^+-dependent dehydrogenation to γ-oxalocrotonate.

Two different pathways have been reported in Pseudomonas sp. (1,2) for the metabolism of 2-hydroxymuconic semialdehyde (2-HMS), the product of meta cleavage of catechol. In the preceding communication from this laboratory (3) it has been shown that the enzymes responsible for the first metabolic steps in both these pathways are present in benzoate-grown Azotobacter cells. However, the low levels of activity of the non NAD^+-dependent hydrolase in these cells appear negligible for metabolic purposes and the NAD^+-dependent pathway seems to be the only one of physiological significance. In similar experiments carried out with a naphthalene-grown pseudomonad we found a different metabolic pattern. We wish to report here results giving evidence for the coexistence of the two meta cleavage pathways in this Pseudomonas strain. Our finding could explain the different results obtained in other laboratories (1,2,4,5) and establishes the essential role played by γ-oxalocrotonate in the metabolism of 2-HMS.

MATERIALS AND METHODS. A Pseudomonas strain (NCIB 9816) was grown at 30° in a mineral salts medium of the following composition (g/l): $(NH_4)_2\ SO_4$, 1.0; KH_2PO_4, 0.5; $MgSO_4.7H_2O$, 0.1; $FeSO_4.7H_2O$, 0.005; and stock salt solution of Bauchop and Elsden (6), 1 ml/l; the pH was adjusted to pH 7.0 with 5N NaOH. Naphthalene was ground to a fine powder before addition to the medium at a

concentration of 0.1% (w/v). 10 l cultures were harvested at the end of the logarithmic phase, washed in phosphate buffer, pH 7.0, and either used immediately or stored at -25° until required. 1 g of wet weight of cells was suspended in 7 ml of 50 mM phosphate buffer, pH 7.0, and broken by ultrasonic treatment for 5 min with a 100 W ultrasonic disintegrator. The extracts were centrifuged at 33,000 g for 60 min at 0° and the pellets discarded. The supernatant fluids were decanted and used as the crude extracts for measurement of enzyme activity. Extracts were further centrifuged at 150,000 g for 60 min to get rid of the formate dehydrogenase activity, prior to the estimation of formate.

The enzyme assays, preparation of NADase (NAD glycohydrolase EC 3.2.2.6) and other experimental methods were performed as described in the preceding communication (3), except where indicated.

RESULTS AND DISCUSSION. Previous studies on the metabolism of naphthalene by Pseudomonas NCIB 9816 showed that growth on this substrate elicited the induction of high levels of both catechol 2,3 oxygenase and the enzymes involved in the further metabolism of 2-HMS. When this strain was grown on benzoate, salicylate or catechol a catechol 1,2 oxygenase and associated enzymes of the ortho cleavage pathway (β-ketoadipate pathway) were induced (unpublished observations).

The early enzymes of the catechol meta cleavage pathway in naphthalene-grown cells were investigated by the techniques reported in the preceding paper (3). The results (Table 1) present a general pattern similar to the one found in Azotobacter species (3), except for the much higher levels of the non NAD^+-dependent activity. This enzyme, which accounts for the approximately constant levels of activity against 2-hydroxy-6-oxohepta-2,4-dienoic acid and for the residual levels against 2-HMS and 2-hydroxy-5-methylmuconic semi-aldehyde (2-hydroxy-5-MMS) after NADase treatment, represents about 20% of the total activity measured in crude cell-free extracts in the absence of added NAD^+; in Azotobacter species this activity is negligible (about 0.2% of the

Table 1. Specific activities* of 2-HMS, 2-hydroxy-5-MMS and 2-hydroxy-6-oxohepta-2,4-dienoic acid metabolizing enzymes in cell-free extracts of Pseudomonas NCIB 9816.

Assay substrate	No NAD added to the reaction mixture	NAD (0.4 µmole) added to the reaction mixture	Ratio of activities NAD/non NAD	Cell-free extracts treated with NADase**	Remaining activity after NADase treatment %
NAPHTHALENE-GROWN CELLS.					
2-HMS	12.7	63	5.0	2.4	18
2-hydroxy-5-MMS	7.8	40	5.1	1.8	23
2-hydroxy-6-oxohepta-2,4-dienoic acid	2.7	2.7	1.0	2.4	88
SUCCINATE-GROWN CELLS.					
2-HMS	0.40	0.72	1.8	0.30	75
2-hydroxy-5-MMS	0.30	0.50	1.7	0.25	83
2-hydroxy-6-oxohepta-2,4-dienoic acid	0.30	0.30	1.0	0.28	93

*Expressed as nmoles/min/mg protein.

**1 ml of cell-free extract was incubated for 2 hr at room temperature with 1 ml NADase preparation.

Table 2. Formation of γ-oxalocrotonate and formate from 2-HMS by cell-free extracts of Napthalene-grown Pseudomonas NCIB 9816.

Type of extract.	Addition to the reaction mixture	2-HMS metabolized nmoles	γ-oxalocrotonate formed nmoles	Formate formed nmoles
Crude	None	200	140	40
		150	105	28
		100	80	+
Crude	NAD (0.4 μmole)	200	192	ND
		150	136	ND
		100	91	ND
NADase treated	None	200	ND	180
		150	ND	134
		100	ND	86

+ Positive test for formate. ND Not detectable

The reaction mixtures contained in a final volume of 3 ml, 250 μmoles of phosphate buffer, pH 7.5, 50 μmoles of Na_2-EDTA, 3 mg of protein and 0.1 (0.15 or 0.20) μmole of 2-HMS. The disappearance of 2-HMS was followed spectrophotometrically at 375 nm. When all the 2-HMS was metabolized the γ-oxalocrotonate and formate formed were estimated as previously described (3).

total activity). The endogenous NAD^+ and the NADH oxidase activity present in crude extracts would be responsible for the activity not accounted for in non NAD^+-supplemented reaction mixtures. These results suggest the presence of two different enzymes, one NAD^+-dependent and the other non NAD^+-dependent, for the metabolism of 2-HMS in crude cell-free extracts of this naphthalene-grown pseudomonad.

The results shown in Table 2 for the quantitative formation of γ-oxalocrotonate and formate from 2-HMS by cell-free extracts support this conclusion. In the absence of any added NAD^+ approximately 80 nmoles of γ-oxalocrotonate and 20 nmoles of formate were formed from 100 nmoles of 2-HMS by these extracts. When the reaction mixture was supplemented with NAD^+ virtually all the 2-HMS was converted to γ-oxalocrotonate: formate was not detectable in this reaction mixture. On the other hand formate was formed from 2-HMS in almost stoicheiometric amounts when NADase-treated cell-free extracts were used and γ-oxalocrotonate could not be detected in this case. These results are in perfect agreement with the ones shown in Table 1. The NAD^+-dependent activity would correspond to a dehydrogenase (2-HMS dehydrogenase, 2-HMS:NAD oxidoreductase) converting 2-HMS into γ-oxalocrotonate and NADH (reaction 1, fig.1), in agreement with Nishizuka et al (1); the non NAD^+-dependent activity would be responsible for a hydrolytic fission of 2-HMS to formate and 2-oxopent-4-enoic acid (reaction 2, fig.1), corresponding to the one reported by Dagley and Gibson (2) and other workers (4,7).

As can be deduced from Table 1, both of the activities involved in the metabolism of 2-HMS in this strain are inducible by growth on naphthalene. Extracts prepared from succinate-grown cells contain low but detectable levels of 2-HMS dehydrogenase (estimated by subtracting the activity after NADase treatment from the activity found in NAD^+-supplemented reaction mixtures) and 2-HMS hydrolase (estimated by the activity after NADase treatment). Growth in the presence of naphthalene elicits the induction of the two enzymes, but the level of induction is much lower for the activity of 2-HMS hydrolase

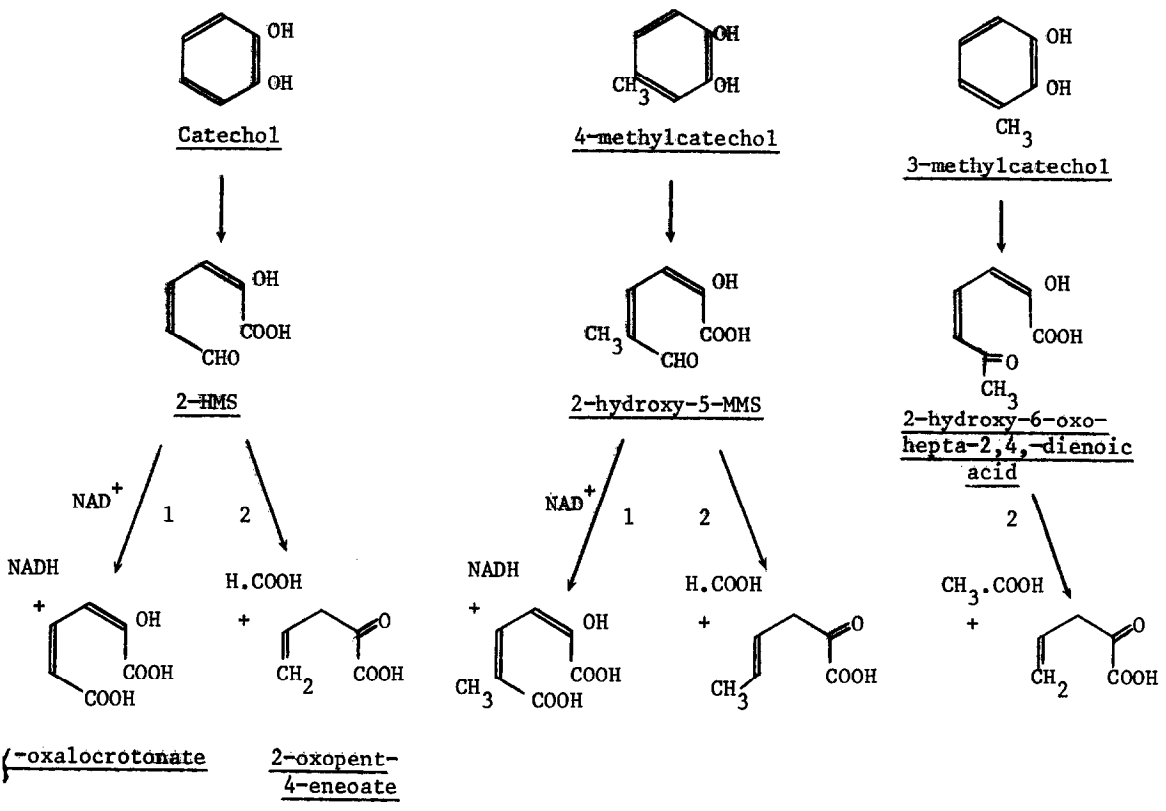

Fig.1.

(about 8-fold) than for that of 2-HMS dehydrogenase (about 150-fold increase). The induction characteristics of these two enzymes in this Pseudomonas strain are different from the ones found in Azotobacter species (3). In Azotobacter, the low levels of hydrolase activity are not inducible, but the 2-HMS dehydrogenase activity is induced at higher levels than in the naphthalene-grown pseudomonad studied.

Our results are evidence of the coexistence in this Pseudomonas strain of the two previously reported pathways for the metabolism of 2-HMS after meta cleavage of catechol (1,2) and explain the apparently contradictory results found in different laboratories (1,2,4,5). Dagley and Gibson (2) using a Pseudomonas strain (Pseudomonas U, NCIB 10105) proposed the non NAD^+-dependent pathway for the metabolism of 2-HMS. They did not, however, report

any effect of NAD^+ on the rate of conversion of 2-HMS to non-absorbing products. An increase in the rate of this conversion by added NAD^+ has been demonstrated by other workers using different Pseudomonas strains (4,5,8); Feist and Hegeman (5) reported an identical effect using the Pseudomonas U strain of Dagley. Cain and Farr (4) supported the general scheme of Dagley and Gibson for the metabolism of 2-HMS in a Pseudomonas strain grown on benzenesulphonates: although they found that γ-oxalocrotonate was formed in NAD^+-supplemented incubation mixtures, they concluded that this compound could not be regarded as a direct metabolite of catechol.

Our results which show a higher level of activity, as well as a higher level of induction, of the NAD^+-dependent enzyme indicate that γ-oxalocrotonate is an intermediate of physiological importance in the metabolism of 2-HMS. We think that the metabolic pattern we found in the strain studied could be a very general one among the Pseudomonas species.

REFERENCES.

(1) Y. Nishizuka, A. Ichiyama, S. Makamura and O. Hayaishi, J. Biol. Chem. 237, PC268 (1962).
(2) S. Dagley and D.T. Gibson, Biochem. J. 95, 466 (1965).
(3) J.M. Sala-Trepat and W.C. Evans, Biochem. Biophys. Res. Comm., this issue.
(4) R.B. Cain and D.R. Farr, Biochem. J. 106, 859 (1968).
(5) C.F. Feist and G.D. Hegeman, J. Bacteriol. 100, 869 (1969).
(6) T. Bauchop and S.R. Elsden, J. Gen. Microbiol. 23, 457 (1960).
(7) R.C. Bayly and S. Dagley, Biochem. J. 111, 303, (1969).
(8) U. Zubair, Ph.D. Thesis, University of Leeds (1963).