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Purification and Some Properties of Maleylpyruvate Hydrolase and Fumarylpyruvate Hydrolase from *Pseudomonas alcaligenes*

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Hydrolysis of the gentisate ring-cleavage product, maleylpyruvate (*cis*-2,4-diketohept-5-enedioic acid), was shown to be catalyzed by an enzyme, maleylpyruvate hydrolase 11, in *Pseudomonas alcaligenes* (P25X1) after growth with 3-hydroxybenzoate. This activity was separated from fumarylpyruvate hydrolase activity during the course of its purification which accomplished an approximately 50-fold increase in specific activity. An apparent molecular weight of 77,000 was assigned on the basis of Sephadex G-200 chromatography. Despite the presence of up to three similarly migrating bands of protein on polyacrylamide-gel electrophoresis of the purified enzyme, at least two of these bands possessed maleylpyruvate hydrolase activity. Electrophoresis on sodium dodecyl sulfate-polyacrylamide before and after reduction with mercaptoethanol gave a principal band of molecular weight of 33,000 (and a minor band of molecular weight 50,000). A number of substituted maleylpyruvates also served as substrates for maleylpyruvate hydrolase 11, but maleylacetoacetate and fumarylpyruvate were not attacked. Fumarylpyruvate hydrolase was purified approximately 40-fold to give a single band on polyacrylamide gels and with an apparent molecular weight of 73,000 by Sephadex G-200 chromatography. Upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis before or after reduction with mercaptoethanol, a subunit molecular weight of 25,000 was obtained. Neither maleylpyruvate nor fumarylacetoacetate served as substrates for fumarylpyruvate hydrolase. The activities of both maleyl- and fumarylpyruvate hydrolases were stimulated by Mn^{2+} ions. Reasons are discussed for the presence of both enzyme activities, one of which appears to be redundant.

In their studies on the degradation of xylenols via the gentisate pathway, Hopper et al. (11) reported that enzymes for the hydrolysis of both maleylpyruvate and fumarylpyruvate were present in a species of *Pseudomonas* grown on *m*-cresol. The fumarylpyruvate hydrolase (FPH) had no apparent function in the pathway as the cells did not synthesize an isomerase for the conversion of maleylpyruvate to fumarylpyruvate (10). It was also reported by Hopper et al. (11) that the relative activities of the two enzymes in crude extracts varied with the aromatic compound used as the carbon source. Poh and Bayly (18) have shown that two enzymes active against maleylpyruvate are synthesized by the strain of Hopper et al. (11). One enzyme is formed constitutively during growth in the absence of aromatic compounds; the other is inducible and synthesized during growth in the presence of either *m*-cresol, 3-hydroxybenzoate, or gentisate. Poh and Bayly (18) designated these two enzymes maleylpyruvate hydrolase 1 and 11 (MPH1 and MPH11), respectively. Crawford (4) reported the presence of enzymic activity against maleylpyruvate and fumarylpyruvate in a species of *Bacillus* that utilized 3-hydroxybenzoate, and he suggested that both activities may be catalyzed by a single enzyme.

The present report shows that, in the *Pseudomonas* species used by Hopper et al. (11), the hydrolysis of maleylpyruvate and fumarylpyruvate is catalyzed by separate enzymes that are specific for their substrate. The purification and some properties of the inducible MPH (MPH11) and FPH are also reported.

MATERIALS AND METHODS

Organism and methods of cultivation. The organism used was *Pseudomonas alcaligenes* NCIB 9867 and will be referred to as P25X1. Some of its metabolic properties have been described by Hopper and Chapman (9, 11) and Poh and Bayly (18). Cultures were maintained on nutrient agar.

The basal medium was the mineral salts base de-

scribed by Crawford et al. (6) to which was added 3-hydroxybenzoate (5 mM). Cells were grown in 10-liter batches under forced aeration at 30°C and harvested in the late exponential phase. the cells were washed twice with cold 0.05 M Tris-hydrochloride buffer (pH 7.4) and stored at −20°C.

Preparation of cell extracts. Washed cells were suspended in twice their volume of cold Tris-hydrochloride buffer (pH 8.1) that contained Mg^{2+} (10^{-3} M). The suspension was kept below 5°C and stirred, and the cells were disrupted by sonic oscillation. Cell debris was removed by centrifugation at 25,000 × *g* for 30 min, and the cell extract was used on the same day.

Enzyme assays. MPH and FPH were assayed as described by Poh and Bayly (18). Maleylacetoacetate was prepared by metabolism of homogentisic acid by cell extracts of *Pseudomonas putida* (ATCc 12633) grown on tyrosine (5 mM) as sole source of carbon. Fumarylacetoacetate was prepared from maleylacetoacetate by acid-catalyzed isomerization (13).

Protein determination. The concentration of protein in cell extracts was determined by the method of Lowry et al. (16) with bovine serum albumin as standard.

Physicochemical examination of purified enzyme preparations. Molecular weights were determined by gel filtration on Sephadex G-200 (1.6 × 90 cm), as described by Andrews (1), with bovine serum albumin, γ immunoglobulin, ovalbumin, and ribonuclease as standards. Analytical disc gel electrophoresis with 7.5% polyacrylamide gels was by (i) the method of Davis (8) at pH 8.9, and (ii) the method of Williams and Reisfeld (21) at pH 7.5. Gels were stained with 0.5% amido black in 7% acetic acid for 4 to 5 h and then destained electrophoretically in 7% acetic acid. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out as described by Weber and Osborn (20) with 10% gels. The gels were stained with Coomassie brilliant blue R for 4 to 5 h and destained electrophoretically in 18% methanol-9% acetic acid. Reduced and nonreduced samples for sodium dodecyl sulfate gels were prepared as described by Tack et al. (19). Amino acid analysis of the purified enzymes was carried out by the method of Moore and Stein (17), as described previously (2).

Detection of MPH activity on polyacrylamide gels. This was performed on purified enzyme which had undergone gel electrophoresis at 2 to 4°C. Electrophoresis was carried out on gels, each of which contained 60 μg of protein. Immediately after electrophoresis, one of the gels was frozen in dry ice, and the other gel was stained with Coomassie brilliant blue R for 15 min and was destained, and the protein bands were located. The frozen gels were sliced at 0.3-mm intervals with a Mickle gel slicer (Mickle Laboratory Engineering Co., Gomshall, Great Britain), and each slice was immersed in 0.1 ml of 0.1 M Tris-hydrochloride buffer (pH 7.1) at 2 to 4°C. The extract of each slice was then assayed for MPH activity. The relative activity per slice was determined by measuring the change in absorbance at 330 nm over 1 min.

Separation of MPH and FPH activities. A 40 to 70% ammonium sulfate fraction of crude cell extract was applied to a DEAE-cellulose column (5 × 100 cm) equilibrated with 0.05 M Tris-hydrochloride buffer (pH 8.1), and protein was eluted with 2 liters of 0 to 0.7 M NaCl gradient in the same buffer. Fractions (11 ml) were collected and assayed for activity against maleylpyruvate and fumarylpyruvate. Fractions 69 to 79 were active against maleylpyruvate but not fumarylpyruvate. Fractions 86 to 110 were active against fumarylpyruvate, and low activity against maleylpyruvate was detected in fractions up to 100. Fractions 98 to 101 were pooled, concentrated to 4 ml by ultrafiltration (PM-10 Amicon membrane), and applied to a Sephadex G-150 column (2 × 50 cm), and protein was eluted with 0.1 M Tris-hydrochloride buffer (pH 8.1) which contained Mg^{2+} (10^{-3} M). Fractions (3 ml) were collected, and activity against fumarylpyruvate was detected in fractions 38 to 45. Maximum specific activity (fraction 42) was 20-fold greater than in the crude cell extract. This fraction showed activity against maleylpyruvate at less than 1% of the rate at which fumarylpyruvate was attacked. The elution profile is shown in Fig. 1.

Purification of MPH11 and FPH. All steps in the purifications were carried out at 2 to 4°C, and $MgSO_4 \cdot 7H_2O$ (10^{-3} M) was added to all buffers. The procedures used for the two enzymes were similar, and details of the separating materials, buffers, and salt gradients used and the yields obtained are given in Tables 1 and 2. When dialysis was necessary, it was carried out against the starting buffer for the column to be used, and concentration of pooled fractions was by ultrafiltration (PM-10 Amicon membrane).

At the conclusion of the purification procedures, fractions in which the elution profile for protein was coincident with enzyme activity were pooled. A portion of the pooled fractions that was to be used for amino acid analysis was dialyzed to remove salts and then lyophilized. The remainder of the pooled material was kept at 2 to 4°C, and examinations for substrate specificity, K_m values, pH optima, activation studies, molecular weight, and subunit structure were completed within 4 to 6 days.

RESULTS

Molecular weight and subunit structures. Molecular weights as determined on Sephadex G-200 were 77,000 for MPH11 and 73,000 for FPH. Polyacrylamide gel electrophoresis by the method of Davis (8) of fractions 42 to 50 from step 8 of MPH11 (Table 1) each showed bands as shown in Fig. 2 for fraction 46. Electrophoresis of fraction 46 by the method of Williams and Reisfeld (21) gave a similar pattern (Fig. 2), and examination of the same fraction on polyacrylamide gels containing urea and acetic acid, as described by Tack et al. (19), gave one major band. To determine whether enzymic activity was present in more than one of the protein bands, the preparation was subjected to electrophorsis, and MPH activity in the bands was determined as described above. A plot of enzyme activity versus gel slice number for one gel (Fig. 3) showed that two peaks of activity were resolved. Another gel showed a similar plot and

that for a third gel gave a plateau in the region of the first peak shown in Fig. 3. It was not determined which two of the three protein bands (Fig. 2) corresponded to the peaks of enzyme activity, nor were the extracts of the gel slices assayed against analogs of maleylpyruvate.

The finding that more than one protein band was present on gel electrophoresis of fractions from step 8 and that the specific activity against maleylpyruvate was not constant over the protein elution peak suggested that the preparation was not homogeneous. Polyacrylamide gel electrophoresis of fractions across the elution profile from the triethylaminoethyl-cellulose column (Fig. 4A, step 7) gave the patterns shown in Fig. 4B. This indicated that for maximum specific activity against maleylpyruvate it was necessary for the protein from more than one band to be present.

To determine whether differences in kinetic properties could be detected in different fractions from step 7, K_m values were determined for fractions 47 and 62 against the substrates shown in Table 3. No significant differences were obtained in K_m values for the same substrate with the different fractions. Sodium dodecyl sulfate-gel electrophresis of the purified preparation (Fraction 46, step 8) gave two bands: one, with a molecular weight of 33,000, was the major band; the other was a faintly staining band with a molecular weight of 50,000. Reduction of the sample before electrophoresis resulted in no change in the bands detected. The same result was obtained when the fraction with the highest specific activity from step 7 was tested by the same procedures. It is not known whether the species of molecular weight 50,000 is a contaminating protein or part of the MPH11 molecule.

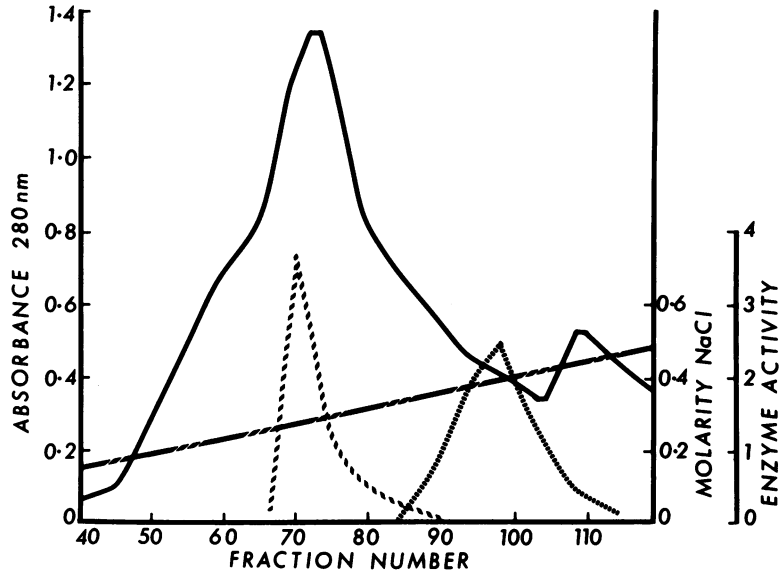


FIG. 1. *Elution from a DEAE-cellulose column of MPH and FPH from P. alcaligenes NCIB 9867 grown on 3-hydroxybenzoate. Absorbance 280 nm, ; NaCl gradient, ; MPH activity, ; FPH activity, .*

TABLE 1. *Purification steps for MPH11*

Purification step	Protein (mg)	Sp act[a]	Total activity	Purification	Recovery (%)
1. Crude extract	14,200	0.170	2,400		100
2. Ammonium sulfate (40–70%)	8,160	0.258	2,100	1.5	88
3. DEAE-cellulose; 0.15 M NaCl–0.1 M Tris-hydrochloride (pH 8.1)	2,240	0.890	1,980	5.2	82
4. DEAE-cellulose; 0–0.4 M NaCl–0.1 M Tris-hydrochloride (pH 8.1)	653	1.50	980	8.8	41
5. Sephadex G-150; 0.1 M Tris-hydrochloride (pH 8.1)	192	3.40	650	20	27
6. DEAE-Sephadex A50; 0–0.15 M NaCl–0.1 M Tris-hydrochloride (pH 8.1)	106	5.30	568	31	23
7. Triethylaminoethyl-cellulose; 0–0.2 M NaCl–0.1 M Tris-hydrochloride (pH 8.1)	42	6.60	258	39	11
8. Sephadex G-150; 0.1 M Tris-hydrochloride (pH 8.1)	7	8.80	62	52	1.7

[a] Micromoles of substrate used per minute per milligram of protein.

TABLE 2. *Purification steps for FPH*

Purification step	Protein (mg)	Sp act[a]	Total activity	Purification	Recovery (%)
1. Crude extract	45,000	0.117	5,265		100
2. Ammonium sulfate (30–50%)	14,137	0.254	3,591	2.2	68
3. DEAE-cellulose; 0.1–1.0 M NaCl–0.05 M Tris-hydrochloride (pH 8.5)	ND[b]	1.25	ND	10.7	ND
4. DEAE-cellulose; 0.1–0.7 M NaCl–0.05 M Tris-hydrochloride (pH 8.0)	ND	1.74	ND	14.8	ND
5. Sephacryl S-200; 0.05 M Tris-hydrochloride (pH 8.5)	98	7.60	746	65	14
6. DEAE-Sepharose CL-6B; 0.1–0.7 M NaCl–0.05 M Tris-hydrochloride (pH 7.6)	34	6.70	227	57	4
7. Sephadex G-200; 0.05 M Tris-hydrochloride (pH 8.5)	11	5.20	47	44	0.9

[a] Micromoles of substrate utilized per minute per milligram of protein.
[b] ND, Not determined as total volume not recorded.

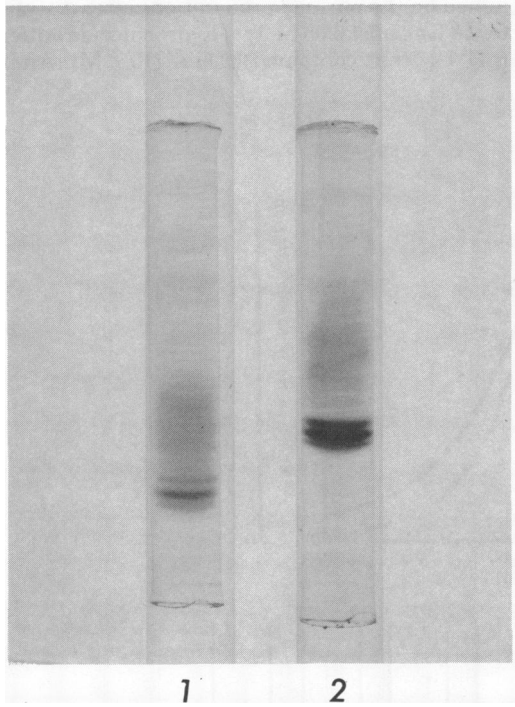


FIG. 2. *Polyacrylamide gel electrophoresis of purified MPH11. Protein (50 μg) was applied to the gels, and electrophoresis was carried out by the method of (i) Davis (8) and (ii) Williams and Reisfeld (21).*

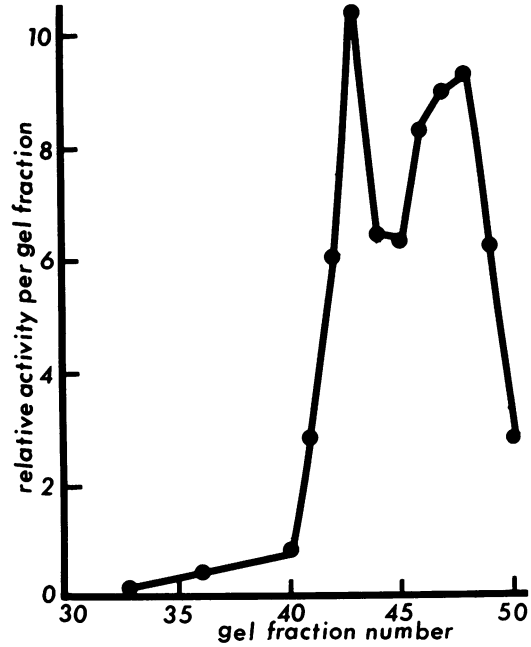


FIG. 3. *Detection of MPH activity from polyacrylamide gels of purified enzyme (Table 1, step 8).*

If the former, then MPH11 would appear to be a dimer with subunits of the same molecular weight, whereas if the latter situation was the case, it would appear to be a dimer with subunits of molecular weight 33,000 and 50,000. In both cases, the subunits would not be linked by disulfide bonds.

Gel electrophoresis of purified FPH gave only one band. Sodium dodecyl sulfate-gel electrophoresis was carried out on samples before and after reduction with mercaptoethanol, and in both samples a single band was obtained; the molecular weight of the subunits was 25,500 in each case. FPH would therefore appear to be a trimer with subunits of equal molecular weight that are not linked by disulfide bonds.

Amino acid composition. The amino acid composition of FPH is given in Table 4. The MPH11 preparation was also analyzed but since the preparation may not be homogeneous, the results are not given. In the analysis of MPH11, an unidentified compound that appeared just before histidine was detected. Analysis of the same material for carbohydrate gave a negative result and therefore the unidentified compound does not contain carbohydrate. As with FPH, a low cysteic acid value was obtained with MPH11. This is in agreement with the observation that the subunits of both enzymes are unlikely to be linked by disulfide bonds, as no

difference was found after sodium dodecyl sulfate electrophoresis of reduced and nonreduced samples.

Kinetic properties. The comparative rates of attack of purified MPH11 on maleylpyruvate and some of its alkyl- and halogen-analogs, fumarylpyruvate, maleyl- and fumarylacetoacetate are shown in Table 5 where data for crude extract from 3-hydroxybenzoate-grown cells are also given. The ratios of attack on the analogs of maleylpyruvate showed little variation between the crude and purified preparations. No activity of MPH11 against either fumarylpyruvate, maleylacetoacetate, or fumarylacetoacetate was detected.

FPH showed activity only against fumarylpyruvate; no activity was detected against either maleylpyruvate or its substituted analog, maleylacetoacetate, or fumarylacetoacetate.

The apparent K_m values calculated from Lineweaver-Burk plots were 1.4×10^{-5} M for MPH11 versus maleylpyruvate, and 7.3×10^{-6} M for FPH versus fumarylpyruvate.

Effect of pH on enzyme activity. Enzymatic activity for MPH11 and FPH against maleylpyruvate and fumarylpyruvate, respectively, was determined over the pH range 5.2 to 10.2 with phosphate and Tris-hydrochloride buffers. Maximal activity for MPH11 was between pH 7.2 to 7.7 and for FPH between pH 7.4 to 7.8, irrespective of the buffer used.

Effect of metal ions on activity. The requirement for metal ions for activity was determined on the purified preparations after dialysis for 18 h against 0.05 M Tris-hydrochloride buffer (pH 7.4) that contained EDTA (10^{-3} M). After

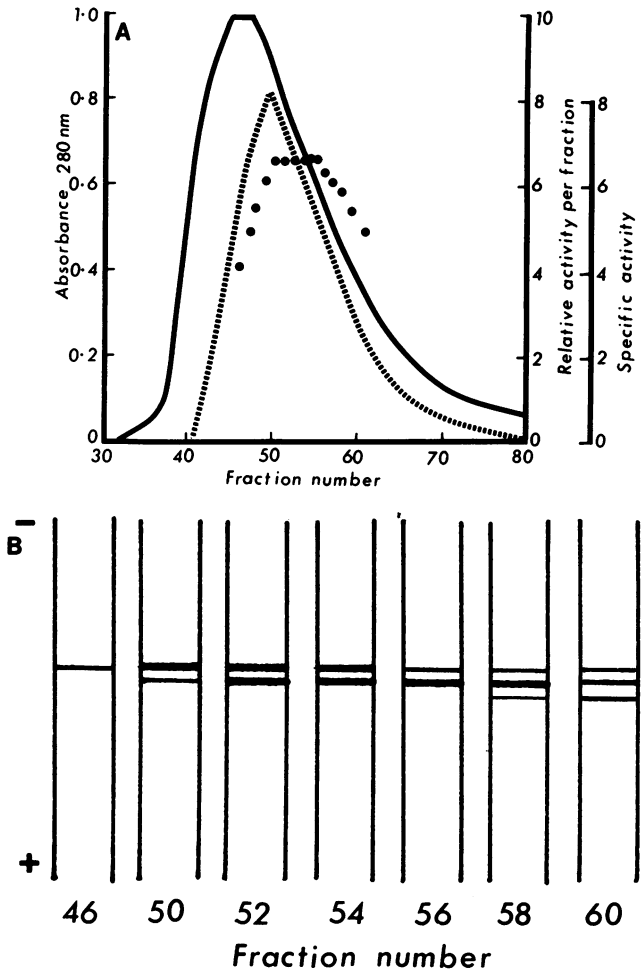


FIG. 4. *(A) Elution of MPH11 from a triethylaminoethyl-cellulose column (Table 1, step 7). Absorbance 280 nm, ▬; relative activity per fraction, ▮▮▮▮; specific activity, ●. (B) Schematic representation of protein bands in polyacrylamide gel electrophoresis of column fractions from Fig. 4A.*

dialysis, no activity was detected for either enzyme.

Addition of Mn^{2+} to MPH11 to a concentration of 5×10^{-4} M resulted in 20% of the initial activity being regained. Activity was not regained when either Mg^{2+} or Co^{2+} was used at the same concentration, but at 10^{-3} M both these ions gave slight activation. With FPH, addition of Mn^{2+} at 10^{-3} M resulted in 63% of the initial activity being regained whereas Mg^{2+} and Co^{2+} both gave 35% recovery at the same concentration. No breakdown of the substrates was detectable after addition of the same concentration of the above metal ions in the absence of the dialyzed enzyme. Addition of reduced glutathione at 5×10^{-4} M had no effect on the activity of either MPH11 or FPH against their respective substrates.

DISCUSSION

MPH11 and FPH are clearly two different proteins, as shown by their separation by chromatography. MPH11 and FPH show strict stereospecificity in that they catalyze degradation only of substrate(s) that are in either the *cis*- or *trans*-configuration, respectively.

Stereospecificity of enzymes has been reported in several degradative pathways of aromatic compounds. Collinsworth et al. (3) showed that the hydratase and aldolase for the *meta*-pathway of catechol acted on specific substrate isomers; in the *meta*-pathway of protocatechuate, the Mg^{2+}-dependent aldolase is specific for one enantiomer (19); and in the pathway studied in this report Hopper et al. (11) found that D-citramalate is formed from citraconate and that the former compound undergoes fission by an aldolase that is specific for the D-isomer.

While FPH could not be tested against any substituted derivatives of fumarylpyruvate, MPH11 was active against several alkyl- and halogen-substituted homologues of maleylpyruvate at approximately the same rate as maleylpyruvate. This differs from MPH1, synthesized constitutively by P25X1, where marked differences in activity against some of the maleylpyruvate homologues were observed (18). That the specificity of MPH11 was confined to substituted maleylpyruvate-type substrates was shown by the absence of activity against maleylacetoacetate. Similarly, FPH did not attack fumarylacetoacetate.

Both hydrolases are activated by Mn^{2+}, and in this they are similar to some enzymes of other aromatic degradative pathways where activation

TABLE 3. *K_m values of partially purified MPH11 from triethylaminoethyl-cellulose fractions*[a]

Substrate	Fraction 47	Fraction 62
Maleylpyruvate	1.4×10^{-5}	1.3×10^{-5}
6-Methylmaleylpyruvate	3.7×10^{-5}	2.0×10^{-5}
6-Ethylmaleylpyruvate	5.5×10^{-6}	5.2×10^{-6}
6-Bromomaleylpyruvate	3.1×10^{-5}	2.4×10^{-5}

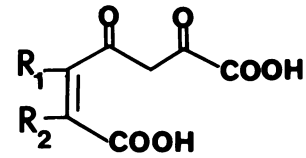
[a] See Table 1, step 7.

TABLE 4. *Amino acid composition of FPH*

Amino acid	Composition (mol fraction %)	No. of residues per 25,600 mol wt[a]
Lysine	5.4	14
Histidine	2.7	7
Arginine	4.0	10
Threonine	5.2	13
Serine	8.5	22
Glutamic acid	10.3	26
Glycine	11.7	30
Alanine	8.6	22
Cystine[b]	0.3	1
Valine	8.8	22
Methionine	1.2	3
Isoleucine	4.8	12
Tyrosine	2.4	6
Phenylalanine	3.3	8
Aspartic acid	7.1	18
Proline	6.3	16
Leucine	6.7	17

[a] To the nearest integer.
[b] Determined as cysteic acid.

TABLE 5. *Comparative rates of hydrolysis of maleylpyruvate and analogous compounds by MPH11*

Substrate		Crude extract	Purified enzyme
			
R1	R2		
H	H	1 (0.116)[a]	1 (6.7)[a]
CH_3	H	1.1	1.3
C_2H_5	H	0.6	0.5
C_3H_8	H	0.1	ND[b]
F	H	1.0	1.1
Cl	H	1.3	0.9
Br	H	1.3	1.1
H	CH_3	1.2	0.7
CH_3	CH_3	NT[c]	0.4
Fumarylpyruvate		NT	ND
Maleylacetoacetate		NT	ND
Fumarylacetoacetate		NT	ND

[a] Specific activity expressed as micromoles of substrate metabolized per milligram of protein per minute.
[b] ND, No activity detected.
[c] NT, Not tested.

by divalent metal ions has been suggested by Dagley (7) to have evolutionary significance. However, the corresponding hydrolase enzyme in the degradation of the *meta* ring-fission product of 3-methyl-catechol which acts on the vinylogous β-diketone, 2-hydroxy-6-oxo-2,4-heptadienoate (2), showed no activation by divalent metal ions.

The significance of three protein bands on gel electrophoresis of the purified MPH11 is not clear. At least two of these bands have hydrolytic activity against maleylpyruvate, but as the separate protein bands were not tested against homologues of maleylpyruvate, it is not possible to state whether the separated proteins both react as MPH11 or if one of them could react as MPH1. A further possibility is that the bands seen on gel electrophoresis are the result of disaggregation or aggregation during electrophoresis. Keat and Hopper (12) reported a similar situation in their study of *p*-cresol methylhydroxylase in *P. putida* NCIB 9869, an organism which degrades 3,5-xylenol by the same reaction sequence as that used by the strain of *P. alcaligenes* used here for study of 2,5-xylenol degradation. Pujar and Ribbons (B. J. Pujar and D. W. Ribbons, Abstr. Annu. Meet. Am. Soc. Microbiol. 1976, K51, p. 145) also reported disaggregation of a purified preparation of 4,5-dihydroxyphthalate decarboxylase into six bands on gel electrophoresis. However, the finding that maximum specific activity from chromatographic columns occurred in fractions in which at least two protein bands were detected on electrophoresis tends to suggest that disaggregation is not the explanation for the appearance of multiple bands on gels.

The reason for the synthesis of FPH by P25X1 is not clear as it has no apparent function in the gentisate pathway used by this strain (10). Poh (unpublished observations) has found that MPH11 and FPH are coincidently induced and hence under the control of the one regulatory molecule. It may be that P25X1 once possessed an isomerase that became redundant with the acquisition of broadly specific enzymes such as MPH1 or MPH11. In other systems, maleylpyruvate isomerase is unable to effect isomerization of substituted maleylpyruvates (6), suggesting that acquisition of MPH is necessary for the metabolism of 3- and 4-substituted gentisic acids. It has been reported (5) that *P. alcaligenes* may contain a low level of a glutathione-dependent activity towards maleylpyruvate. Such an activity may represent evidence that this organism once degraded gentisate via maleylpyruvate and isomerization to fumarylpyruvate as is the case for organisms selected on gentisate and its precursors (4, 5, 14, 15) and that this enzyme became redundant with the introduction of a plasmid encoding 2,5-xylenol degradation (Poh and Bayly, unpublished data). A comparison of the properties of these enzymes and their regulation with those presently reported would be of great interest.

ACKNOWLEDGMENTS

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