Biochem. J. (1980) **185**, 783–786
Printed in Great Britain

Maleylacetate Reductase from *Trichosporon cutaneum*

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(*Received 12 November 1979*)

The enzyme catalysing the reduction of maleylacetate to 3-oxoadipate was purified 150-fold from *Trichosporon cutaneum*, induced for aromatic metabolism by growth with resorcinol as a major carbon source. The enzyme separated upon electrofocusing into three species with pI values 4.6, 5.1 and 5.6. They had similar catalytic properties and the same molecular weight.

Our group has previously reported that the yeast *Trichosporon cutaneum* can be induced to metabolize phenol and resorcinol, both phenols being attacked by the same hydroxylating enzyme and the same ring-cleavage enzyme (Neujahr & Varga, 1970; Varga & Neujahr, 1970). We have since demonstrated that both phenol and resorcinol are metabolized to 3-oxoadipate (Gaal & Neujahr, 1979). The metabolism of resorcinol goes through 1,2,4-trihydroxybenzene and maleylacetate. The enzyme catalysing the reduction of maleylacetate to 3-oxoadipate was enriched from crude extracts (Neujahr, 1978; Gaal & Neujahr, 1979). The present paper describes partial purification and characterization of this enzyme. The occurrence of a similar enzyme in *Pseudomonas putida* was first observed by Chapman & Ribbons (1976). Sparnins *et al.* (1979) have since observed the occurrence of such an enzyme in crude extracts of another strain of *T. cutaneum*, induced for catabolism of L-tyrosine. An enzyme catalysing a similar reaction was recently observed in mycelial extracts of the white-rot fungus *Sporotrichum pulverulentum* grown on glucose and vanillate (Buswell & Eriksson, 1979).

Experimental

Materials and methods

Equipment, commercially available chemicals and enzymic synthesis of maleylacetate. These were as described previously (Gaal & Neujahr, 1979).

Determination of protein. Protein was determined by the biuret method (Gornall *et al.*, 1949) or was calculated from the u.v. absorbance at 280 and 260nm (Layne, 1957).

Assay of maleylacetate reductase activity. This was done by measuring the decrease in A_{340} with NADPH as co-substrate in 1.0cm cells. The final volume of 1.0ml contained 50mM-Tris/H_2SO_4 buffer, pH7.6, 0.2μmol of maleylacetate, 0.2μmol of NADPH and 50–200μg of enzyme protein. Under these conditions 1 enzyme unit corresponds to an absorbance decrease of 6.1 A_{340} units/min.

Determination of molecular weight. Molecular weight was determined by gel filtration (Andrews, 1965) on a Sephadex G-150 column (2.5cm × 85cm) equilibrated with 50mM-Tris/H_2SO_4 buffer, pH7.6. Alcohol dehydrogenase (mol.wt. 150000), lactate dehydrogenase (mol.wt. 136000), hexokinase (mol.wt. 105000) and avidin (mol.wt. 68000) were used for calibration.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. This was done in 10% polyacrylamide gels (Weber *et al.*, 1972), with serum albumin, catalase, aldolase, glyceraldehyde 3-phosphate dehydrogenase and trypsin being used for calibration.

Zymograms. After completion of disc electrophoresis (Davis, 1964), the gel slabs were cut in halves lengthwise. One half was used for protein staining with Amido Black, the other half for specific enzyme staining. The gel was first incubated for 30min with 2.0ml of 0.3mM-NADPH dissolved in 50mM-Tris/H_2SO_4 buffer, pH7.6, then with 2.0ml of 0.3mM-maleylacetate dissolved in the same buffer. After about 10min the activity appeared as a dark band against a fluorescent background under u.v. light (340nm). No such band was observed when maleylacetate was omitted.

Induction and purification of the enzyme

Cultures of *T. cutaneum* were grown on resorcinol as carbon source, harvested, stored and disrupted as described previously (Neujahr & Gaal, 1973). All buffers contained 1mM-2-mercaptoethanol and 0.1mM-EDTA. For steps 1–3 2μM-FAD was also added, as phenol hydroxylase was recovered simultaneously.

Steps 1 and 2: preparations of crude extract and protamine treatment. This was done as before

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(Neujahr & Gaal, 1973), with 300–500g of cell paste. The buffer was supplemented with 1.5mM-phenylmethanesulphonyl fluoride immediately before disintegration of the cells.

Step 3: crude separation of DEAE-Sephadex. The supernatant from step 2 was passed through a DEAE-Sephadex A-50 column (9cm × 15cm) equilibrated with 50mM-potassium phosphate buffer, pH 7.6. The column was then washed with 500ml of the same buffer containing 50mM-$(NH_4)_2SO_4$. The enzyme was eluted with a linear gradient of 50mM–0.3M-$(NH_4)_2SO_4$ in 1.5 litres of buffer. The most active fractions were pooled. The enzyme was precipitated by slowly adding $(NH_4)_2SO_4$ crystals to 75% saturation, with maintenance of pH 7.6 by addition of aq. 10% NH_3. The turbid solution was left overnight and then centrifuged at 16000*g* for 30min, and the precipitate was dissolved in 20mM-Tris/H_2SO_4 buffer, pH 7.6. This solution was desalted on a Sephadex G-50 column equilibrated with the same buffer.

Step 4: chromatography on DEAE-Sephadex A-50. This was done as described previously (Gaal & Neujahr, 1979). The most active fractions were pooled, and the enzyme was precipitated and centrifuged as in step 3. The precipitate was dissolved in 20ml of 20mM-potassium phosphate buffer, pH 7.6.

Step 5: chromatography on hydroxyapatite. The enzyme solution was applied to a hydroxyapatite column (3cm × 15cm) equilibrated with 20mM-potassium phosphate buffer, pH 7.6, and eluted with the same buffer. The most active fractions were pooled, and the enzyme was precipitated and centrifuged as in step 3. The precipitate was dissolved in 10ml of 20mM-Tris/H_2SO_4 buffer, pH 6.8. This solution was desalted on a Sephadex G-50 column equilibrated with the same buffer.

Step 6: chromatography on Blue Sepharose CL-6B. The enzyme solution was applied to a Blue Sepharose CL-6B column (2.5cm × 12cm) equilibrated with 20mM-Tris/H_2SO_4 buffer, pH 6.8, at a flow rate of 10ml/h. When all the solution had entered the column, the flow was arrested for 3h. The column was then washed with 120ml of 20mM-Tris/H_2SO_4 buffer, pH 7.3, which eluted most of the inactive protein. The enzyme was eluted by a linear gradient of Tris/H_2SO_4 buffer, pH 7.3 (20mM–0.5M; 200ml in total). The most active fractions were concentrated by ultrafiltration.

Step 7: gel filtration on Sephadex G-150. The concentrated enzyme solution was passed through a Sephadex G-150 column (2.5cm × 85cm) equilibrated with 50mM-Tris/H_2SO_4 buffer, pH 7.6. The most active fractions were concentrated by ultrafiltration and stored at −25°C.

Electrofocusing. This was performed in an LKB 8100 110ml column with a 5–50% (w/v) sucrose gradient and 1.2% Ampholine, pH 4–6. The dense electrode solution (at the anode, bottom) contained 15g of sucrose, 15ml of water and 1.5ml of H_3PO_4. NaOH (20mM) was used as the light electrode solution. The column was cooled with circulating water at 5°C. Pre-focusing was done for 15h at 1200V. The desalted enzyme solution (after the addition of sucrose to obtain the appropriate density) was applied as a narrow zone at about pH 6.0, and the electrofocusing was continued for 24h at 1200V. Fractions of volume 2.0ml were collected at a rate of 80ml/h; pH, absorbance and enzyme activity were determined in every fraction. Ampholine and sucrose were removed from the active fractions by precipitation of the enzyme with $(NH_4)_2SO_4$ crystals (to 75% saturation) after adjustment of the pH of the fractions to 7.6 with 0.1M-Tris base. The precipitate was allowed to grow for 2 days, then it was centrifuged and washed twice with 75%-saturated $(NH_4)_2SO_4$ in 0.1M-Tris/H_2SO_4 buffer, pH 7.6, and finally dissolved in 50mM-Tris/H_2SO_4 buffer, pH 7.6, and stored at −25°C until used.

Results and Discussion

Yield and homogeneity of the purified enzyme

A typical balance sheet of the purification procedure is shown in Table 1. The enzyme was purified 150-fold with an overall recovery of about 50%. A similar degree of purification was obtained in several independent runs with three different batches of cells and, in some cases, by exchanging the chromatography step on Blue Sepharose for electrofocusing.

The purified protein was eluted from the Sephadex G-150 column as a single symmetrical protein peak. So did the coincident enzyme activity. Further purification of the enzyme by use of preparative electrofocusing in a sucrose gradient did not increase its specific activity. On being electrofocused, the enzyme invariably separated into three main peaks of activity, at pH 4.6, 5.1 and 5.6, with the corresponding protein peaks slightly shifted towards higher pH (Fig. 1). The proportions of the peaks varied with cell batch (Fig. 1, Expts. A, C and E), with enzyme preparation with use of an identical isolation procedure from the same cell batch (Fig. 1, Expts. A and B), and also when the same enzyme preparation was re-focused after a long time of storage (Fig. 1, Expts. C and D). Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the preparation E (pI 5.6), both before and after electrofocusing, gave one single protein band corresponding to mol.wt. 40000.

Disc electrophoresis of the peak at pH 5.6 (Fig. 1, Expt. E) showed two equally intense protein bands, 1mm apart. Only the faster-moving band (R_F 0.34)

Table 1. *Purification of maleylacetate reductase from T. cutaneum*
Experimental details are given in the text.

Step	Volume (ml)	Protein (mg/ml)	Specific activity (units/mg)	Purification (-fold)	Yield (%)	Total activity (units)
1. Crude extract	1310	11.0	0.25	1	100	3600
2. Protamine-treated supernatant	1400	4.1	0.69	2.8	110	3960
3. Crude separation, DEAE-Sephadex A-50, eluate	980	1.3	3.02	12	107	3850
4. DEAE-Sephadex A-50, eluate	150	2.0	6.35	25	88	3175
5. Hydroxyapatite, eluate	65	3.5	11.6	46	73	2640
6. Blue Sepharose, eluate	82	0.75	33.8	135	57	2080
7. Sephadex G-150, eluate	18	2.75	34.2	152	52	1880

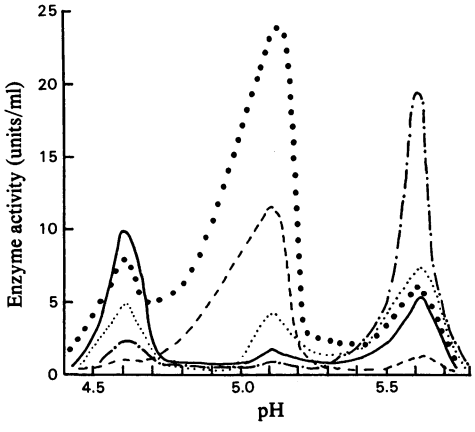

Fig. 1. *Electrofocusing of different maleylacetate reductase preparations*
The numbered purification steps refer to Table 1. See also the Experimental section. Expt. A (····), cell batch no. 1, first preparation, electrofocusing after step 4. Expt. B (· · · ·), cell batch no. 1, second preparation, electrofocusing after step 4. Expt. C (– – – –), cell batch no. 2, electrofocusing after step 7 but with step 6 omitted. Expt. D (——), re-electrofocusing of the main peak from Expt. C. Expt. E (—·—), cell batch no. 3, electrofocusing after step 7. The protein peaks (not shown) were shifted by 0.08 pH unit towards the cathode in relation to the activity peaks.

was active on zymograms. A similar phenomenon was observed with preparation C (pI 5.1), both before and after the electrofocusing step. The R_F vale of the faster-moving band was then 0.69. The small difference in the mobility of the two intense bands, only the faster one being active, correlates with the slight difference between the pI values of the activity and protein peaks (Fig. 1). Thus the slower bands may represent an inactive form of the enzyme. This is also supported by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis giving one single band.

Identification of reaction product

For identification of the reaction product, 200 units of purified enzyme were incubated with maleylacetate as described previously for crude extracts (Gaal & Neujahr, 1979). The crystalline product co-chromatographed with and had melting point (112–115°C) undepressed by authentic 3-oxoadipate.

Properties of the enzyme

The properties of maleylacetate reductase are summarized in Table 2, which compares the extensively purified enzyme with protamine-treated crude cell extracts. The only significant difference is that of molecular weight. It decreases during purification, from 109000 in the crude extract to 81000 in the purified enzyme. Affinities towards substrate and co-substrates, as well as pH-dependence, remain practically the same throughout the purification process.

From the data in Table 2, we conclude that the enzyme contains essential thiol group(s), but no easily accessible disulphide bridges of importance for activity. There is no evidence of the involvement of bivalent metal ions, iron, copper or haem. The absence of any prosthetic group is also indicated by a single absorbance peak at 278 nm, with no peaks in the visible region. The enzyme reaction seems to be irreversible, as the enzyme does not catalyse oxidation of 3-oxoadipate to maleylacetate with NAD^+ or $NADP^+$ (0.1–0.5 mM) as co-substrate. In the reaction 1 mol of NAD(P)H is oxidized by 1 mol of maleylacetate.

Crude or partially purified enzyme could be stored in 50 mM-Tris/H_2SO_4 buffer, pH 7.6, for several days at 5°C and for several months at −25°C without loss of activity.

Multiple forms of the enzyme

The three peaks of activity that were separated by electrofocusing can hardly be ascribed to complex(es) with Ampholine. The same bottle of Ampholine and identical conditions were used in, for

Table 2. *Properties of maleylacetate reductase from T. cutaneum*
Experimental details are given in the text.

	Crude enzyme (step 2 of Table 1)	Purified enzyme (step 7 of Table 1)
Absorption spectrum	—	Single peak at 278nm
Molecular weight (subunit)	109000	81000* (40000)
Co-substrate (with 0.2mM-maleylacetate)		
NADH, K_m	32μM	30μM†
NADPH, K_m	66μM	75μM†
Maleylacetate (with 0.2mM co-substrate)		
With NADH, K_m	25μM	25μM†
With NADPH, K_m	75μM	80μM†
Inhibition by maleylacetate	>300mM	>300mM
Inhibition by 3-oxoadipate, NAD^+ or $NADP^+$	None	None
Stoicheiometry		
Maleylacetate added, μmol	—	0.25, 0.10
NAD(P)H oxidized, μmol	—	0.26, 0.10
pH optimum	6.5–7.8	6.5–7.8
Effect of dithiothreitol (0.1–10mM, 10min)	None	None
Effect of $CuSO_4$ (0.1mM) or *p*-hydroxymercuribenzoate (0.1mM)	Total inhibition, reversed by thiol	Total inhibition, reversed by thiol
Effect of chelating agents‡	None	None

* Partially purified enzyme (step 4 of Table 1) gave mol.wt. 96000.
† Values differing by ±10% were obtained for each of the three peaks separated by electrofocusing.
‡ Assayed after 10min incubation with the enzyme: 10mM-EDTA, 1mM-*o*-phenanthroline, 1mM-Tiron, 1mM-diethyldithiocarbamate and 0.1mM-KCN.

example, Expts. A and B (Fig. 1), giving different proportions of the three peaks. The difference in the molecular weight between the crude and purified enzyme (Table 2) may indicate a disaggregation of some cellular complex during the purification. Proteolytic degradation is less probable, because the decrease in molecular weight occurred irrespective of the use of the proteinase inhibitor phenylmethanesulphonyl fluoride. The molecular weight (81000) and the subunit size (40000) of the purified enzyme did not undergo changes on further storage. However, the proportions of the three peaks (Fig. 1) were changing, seemingly at random (cf. Fig. 1, Expts. D and E), though their catalytic properties remained unchanged (Table 2).

The differences in the pI values of the three peaks are close to 0.5 or to 1.0 pH unit. This suggests that the multiple peaks of the purified enzyme may reflect losses of charged groups not essential for activity. The proportion of the species with higher pI decreased in some runs, but increased in others. Thus it seems that the loss of both acidic and basic components may be involved. Another possible explanation would be that the subunits of the enzyme can freely combine. If they or their dimers have different pI values, the result could be the appearance of three isoenzymes differing in pI. We did not consider further studies meaningful, as all efforts failed to obtain the enzyme in a disc-electrophoretically homogeneous form.

This research was supported by The National Swedish Board for Technical Development (Grant nos. 75-3502, 76-3648 and 77-3766 to H. Y. N.).

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