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Enzymology of the β-Ketoadipate Pathway in *Trichosporon cutaneum*

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Cell extracts were prepared from *Trichosporon cutaneum* grown with phenol or *p*-cresol, and activities were assayed for enzymes catalyzing conversion of these two carbon sources into 3-ketoadipate (β-ketoadipate) and 3-keto-4-methyladipate, respectively. When activities of each enzyme were expressed as a ratio, the rate for methyl-substituted substrate being divided by that for the unsubstituted substrate, it was apparent that *p*-cresol-grown cells elaborated pairs of enzymes for hydroxylation, dioxygenation, and delactonization. One enzyme of each pair was more active against its methyl-substituted substrate, and the other was more active against its unsubstituted substrate. Column chromatography was used to separate two hydroxylase activities and also 1,2-dioxygenase activities; the catechol 1,2-dioxygenases were further purified to electrophoretic homogeneity. Extracts of phenol-grown cells contained only those enzymes in this group that were more active against unsubstituted substrates. In contrast, whether cells were grown with phenol or *p*-cresol, only one muconate cycloisomerase (lactonizing enzyme) was elaborated which was more active against 3-methyl-*cis*,*cis*-muconate than against *cis*,*cis*-muconate; in this respect it differed from a cycloisomerase of another strain of *T. cutaneum* which has been characterized. The cycloisomerase was purified from both phenol-grown and *p*-cresol-grown cells, and some characteristics were determined.

A reaction sequence has been described for *Trichosporon cutaneum* by which *p*-cresol is degraded using *ortho*-fission of 4-methylcatechol (11). This route is precluded in most of the procaryotes investigated thus far because the β-ketoadipate pathway (13) is blocked at the lactonizing step when substrates carry a methyl-group substituent. In the present study we examined the enzymes of the eucaryote *T. cutaneum* that convert *p*-cresol into 3-keto-4-methyladipate (Fig. 1) and compared them with the enzymes used to degrade phenol to β-ketoadipate (3-ketoadipate). Beyond this point in the metabolic sequences the types of reactions used, as well as the enzymes involved, have been found to differ (11). We also report that, like procaryotes, *T. cutaneum* uses succinyl coenzyme A (succinyl-CoA) to activate β-ketoadipate, whereas ATP plus CoA is used for 3-keto-4-methyladipate (11).

MATERIALS AND METHODS

Organism, assays of enzymes, and examination by physical methods. The conditions of growth of the organism, *T. cutaneum* ATCC 58094, preparation of cell extracts, and assays of enzymes were as described previously (11). The protein content of enzyme preparations was determined spectrophotometrically (4). The procedures used for analytical disc electrophoresis, electrophoresis in the presence of sodium dodecyl sulfate, and determination of molecular weights by gel filtration have been described (14). The sources of biochemicals and materials for column chromatography were also as given previously (11, 14), and in addition, some separations were performed using Affi-Gel Blue (Bio-Rad Laboratories, Richmond, Calif.).

Purification of *cis*,*cis*-muconate cycloisomerase. It was necessary to purify muconate cycloisomerase to show that this enzyme of the β-ketoadipate pathway (13) also functions in the reaction sequence (Fig. 1) for converting 3-methyl-*cis*,*cis*-muconic acid (III) into β-methylmuconolactone (IV). The enzyme was purified from extracts of both phenol-grown and *p*-cresol-grown cells, and at each step in purification, activities against *cis*,*cis*-muconate and 3-methyl-*cis*,*cis*-muconate were measured (11). All operations were performed at 4°C in 0.05 M Tris-H_2SO_4 buffer (pH 8.3) containing 0.5 mM dithiothreitol (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) and 0.1 mM EDTA, the mixture being referred to below as the buffer mixture.

Cell-free extracts were prepared from two batches (each 150 g) of cells grown with phenol and *p*-cresol, respectively, and submitted to the same purification procedure. Extract was brought to 40% saturation with $(NH_4)_2SO_4$ by adding a neutralized saturated solution of $(NH_4)_2SO_4$. After 2.5 h the precipitate was removed by centrifugation, and the supernatant solution was brought to 60% saturation with $(NH_4)_2SO_4$. After standing for 40 min the mixture was centrifuged, and the pellet was resuspended in buffer mixture and dialyzed overnight against 2 liters of the same buffer; the preparation was then clarified by centrifugation. The enzyme solution was then applied to a column of DEAE-Sephacel (3.2 by 46.5 cm) which had been equilibrated with degassed buffer mixture. After the sample had been loaded, the column was washed with 200 ml of buffer mixture, and a linear gradient of 0 to 0.45 M KCl (725 ml/725 ml) was applied. The gradient was diluted by the addition of 14.5 ml of buffer on top of the column bed, and 85 16-ml fractions were collected. A single peak was observed for the enzyme activities from cells grown on either carbon source; in each case the maximum was at fraction 52, which corresponds to approximately 0.13 M KCl. Peak fractions (49 to 54) were combined, concentrated on a PM-10 (Amicon Corp., Lexington, Mass.) ultrafiltration membrane, and assayed.

Combined fractions from the previous step were dialyzed overnight against buffer mixture to remove KCl and then applied to an Affi-Gel Blue (Bio-Rad) column (1.5 by 15 cm) which had been equilibrated with buffer mixture. After the

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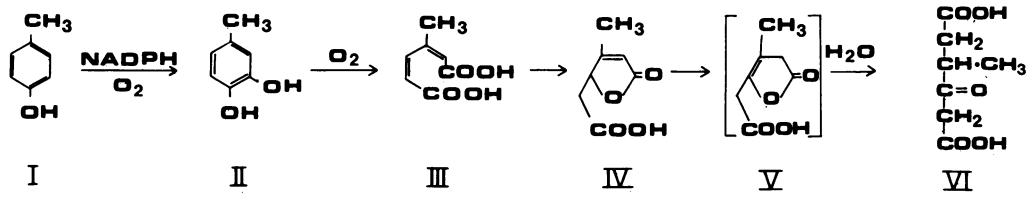

FIG. 1. Reactions used for converting *p*-cresol into 3-keto-4-methyladipic acid. Metabolites shown include *p*-cresol (I), 4-methylcatechol (II), 3-methyl-*cis*,*cis*-muconic acid (III), β-methylmuconolactone (IV), and 3-keto-4-methyladipic acid (VI).

enzyme was loaded, the column was washed with 54 ml of this buffer, and a linear gradient of 0 to 0.3 M KCl (150 ml/150 ml) was applied, diluted with 6 ml of buffer on the column bed. When this gradient was finished, 0.3 M KCl was washed through until all of the activity had been eluted from the column and 4-ml fractions were collected. For both batches of cells, two peaks of activity were eluted: a minor peak that appeared in the initial wash in both cases and a major peak that was eluted by 0.2 to 0.3 M KCl. The minor peak constituted 10% of the total activity from the column for enzyme from *p*-cresol-grown cells and 18% for enzyme from phenol-grown cells. For both of the minor peaks, the ratio of activity expressed against 3-methylmuconate to that against muconate was approximately 1.8; this was not significantly different from the corresponding ratios for the major peaks of *p*-cresol-grown and phenol-grown cells, namely, 1.70 and 1.75, respectively. The purification of this enzyme eluted in the early, minor peak was not continued further; its activity might have been due to an isoenzyme (3) of the major component eluted later. Most of the fractions of this major peak of activity were combined and concentrated for gel filtration chromatography on a column (1.5 by 92 cm) of Sephacryl S-200 (Pharmacia Fine Chemicals, Piscataway, N.J.). In this step, the enzyme was eluted with buffer mixture, and 100 1.6-ml fractions were collected. Activity was found at 82 to 99 ml with a maximum at 88 ml for the extract of *p*-cresol-grown cells and at 80 to 110 ml with a maximum at 89 ml for phenol-grown cells. This observation indicates that the proteins in both preparations were the same size.

The final step consisted of rechromatography on DEAE-Sephacel. The combined fractions in 0.05 M KCl were applied to a small column (1.5 by 22.5 cm) equilibrated with buffer mixture containing 0.05 M KCl. A linear gradient (200 ml/200 ml) of 0.05 to 0.25 M KCl was run, diluted with 5 ml of buffer on the column bed, and 5-ml fractions were collected. The enzymes eluted as single peaks: that from *p*-cresol-grown cells with maximum activity at fraction 38 and that from phenol-grown cells at fraction 36. Fractions with maximal activity were combined, concentrated, and stored at 4 or −15°C. The procedures used are summarized in Table 1.

RESULTS AND DISCUSSION

Activities of enzymes in crude cell extracts. The specific activities of enzymes present in an extract of phenol-grown cells were compared with those of an extract of *p*-cresol-grown cells (Table 2). The same spectrophotometric method was used for assays of hydroxylase activity against either *p*-cresol or phenol, whereas dioxygenase activity for either of the catechols was calculated from O_2 consumption measurements in both cases. It was therefore possible to compare directly the effect due to the presence of a methyl group upon a particular activity by taking the ratio of the specific activity for the methyl-substituted assay substrate to that for the unsubstituted substrate. When this is done with the values shown in Table 2, it can be seen that for *p*-cresol-grown cells the ratios are 1.1 and 1.8 for hydroxylase and dioxygenase specific activities, respectively, whereas the corresponding ratios for phenol-grown cells are much lower, namely, 0.1 and 0.45. Similar numerical comparisons for delactonization are less satisfactory because assays were based upon different procedures. However, as shown in Table 2, *p*-cresol-grown cells possess activity against β-methylmuconolactone approximately seven times greater

TABLE 1. Purification of muconate cycloisomerase from cells grown with phenol or *p*-cresol

Fraction	Vol (ml)	Protein (mg)	Total activity (U)[a]	Ratio[b]	Sp act (U/mg)	Purification (fold)	Yield (%)
Phenol-grown cells							
Crude extract	402	5,470	3,330	1.47	0.61	1	100
Ammonium sulfate treatment	80	1,190	2,510	1.49	2.1	3.4	75
DEAE-Sephacel chromatography	8.5	149	1,480	1.53	10.0	16.4	45
Affi-Gel Blue chromatography	8.2	25.4	670	1.75	26.4	43.3	20
Sephacryl S-200 chromatography	16.0	12.8	641	1.51	50.0	82.0	19
DEAE-Sephacel chromatography	7.0	3.2	404	1.55	126	207	12
p-Cresol-grown cells							
Crude extract	395	7,310	820	1.51	0.11	1	100
Ammonium sulfate treatment	78	2,150	750	1.65	0.35	3.2	91
DEAE-Sephacel chromatography	8.1	209	561	1.49	2.7	24.5	68
Affi-Gel Blue chromatography	9.9	18.8	384	1.50	20.4	185	47
Sephacryl S-200 chromatography	16.5	7.9	412	1.73	52.2	475	50
DEAE-Sephacel chromatography	33	2.4	240	1.68	100	909	29

[a] Expressed in micromoles of 3-methyl-*cis*,*cis*-muconate formed per minute.
[b] Ratio of micromoles of 3-methyl-*cis*,*cis*-muconate per minute to micromoles of *cis*, *cis*-muconate per minute.

than that of phenol-grown cells, whereas the preferred substrate of the latter appears to be the unsubstituted muconolactone. It may be concluded that the enzymes catalyzing hydroxylation, oxygenative ring fission, and delactonization act upon both methyl-substituted and unsubstituted substrates; but enzymes more specific for the methyl-substituted analogs appear to be elaborated, in addition, by *p*-cresol-grown cells. However, this conclusion cannot be drawn in the case of cycloisomerase activity (Table 2); here, the above-mentioned ratios are close: 1.47 for phenol-grown cells and 1.51 for *p*-cresol-grown cells. This suggests that both extracts contain only one cycloisomerase. Moreover, of all the activities listed in Table 2, this was the only one assayed in extracts of phenol-grown cells that showed a higher specific activity for the methyl-substituted analog than for the unsubstituted substrate. *T. cutaneum* owes its ability to grow at the expense of *p*-cresol to this unusual cycloisomerization, which furnishes degradable β-methylmuconolactone instead of giving the γ-isomer, an isomer that most of the other organisms investigated form but do not degrade (11).

For the remainder of this study, these conclusions were tested by partially separating the proteins that catalyze each activity discussed above except the delactonization reaction. In the case of β-methylmuconolactone this activity is monitored by using an exceptionally time-consuming colorimetric assay (11) for compound VI, which may be the product of either a single enzyme or two consecutive enzymatic reactions (Fig. 1).

Separation of two phenol hydroxylase activities. Phenol hydroxylase (phenol 2-monooxygenase, EC 1.14.13.7) has been purified from phenol-grown *T. cutaneum* (8) and characterized (9, 10); the enzyme can oxidize *p*-cresol but is more active against phenol (8, 9). In Fig. 2 we present evidence that *T. cutaneum* can elaborate a second hydroxylase for which *p*-cresol is the preferred substrate. However, unlike the phenol hydroxylase of Neujahr and Gaal (8), this enzyme lost most of its activity during the course of purification and further characterization was not attempted. Separation of the two hydroxylases was achieved by chromatography on phenyl Sepharose (Pharmacia). Crude extracts of *T. cutaneum*, grown with either *p*-cresol or phenol, were prepared in 0.05 M Na^+-K^+ phosphate buffer (pH 7.4) containing 0.1 mM EDTA, 2 μM flavin adenine dinucleotide, and 1 mM 2-mercaptoethanol. The extract was brought to 10% saturation with solid $(NH_4)_2SO_4$, and 13 ml (117 mg of extract protein) was loaded onto a phenyl Sepharose column (1.5 by 14.6 cm) equilibrated with the same buffer containing $(NH_4)_2SO_4$. The column was then washed with 100 ml of this buffer followed by 100 ml of 0.01 M Na^+-K^+ phosphate buffer (pH 7.4) containing all of the additions specified except $(NH_4)_2SO_4$. With 5 ml of buffer layered on top of the column bed, a linear gradient of 0 to 50% ethylene glycol (150 ml/150 ml) was applied; the buffer containing the ethylene glycol was adjusted to maintain constant ionic strength and concentration of stabilizing agents. The elution profile of fractions from the column (Fig. 2) exhibited two peaks of hydroxylase activity for the extract of cells grown with *p*-cresol. The first peak eluted between fractions 70 and 90 and contained a hydroxylase that was active against phenol; but only a trace of activity was shown when *p*-cresol was the assay substrate. The second peak eluted between fractions 90 and 105, and in this case the hydroxylase showed higher activity against *p*-cresol. When this experiment was repeated, using an extract of phenol-grown cells, a single broad peak was observed with maximal activity toward phenol around tube 70; there was little activity against *p*-cresol. No hydroxylase activity for either substrate was eluted in fractions 90 to 110, in contrast to the results obtained with the extract of *p*-cresol-grown cells.

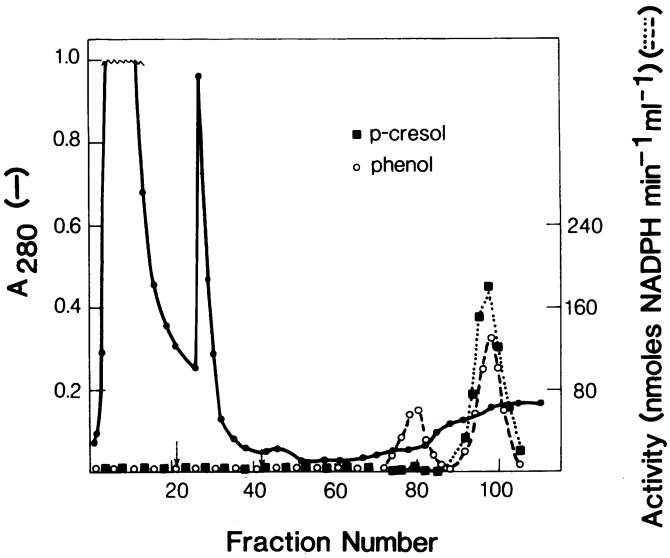

FIG. 2. Chromatograph of an extract of *p*-cresol-grown cells using a column of phenyl Sepharose. The column was first equilibrated with 0.05 M phosphate buffer; this was changed to 0.01 M phosphate buffer at the first arrow (fraction 20), and a gradient of 0 to 50% ethylene glycol was begun at the second arrow (fraction 42). Hydroxylase activities were measured separately against phenol and *p*-cresol, together with total protein (A_{280}).

TABLE 2. Enzyme activities in crude extracts of cells grown with phenol or *p*-cresol

Enzyme activity assayed	Sp act (U/mg of protein) in cells grown with:	
	p-cresol	phenol
p-Cresol hydroxylase[a]	68	28
Phenol hydroxylase[a]	62	280
4-Methylcatechol dioxygenase[b]	229	109
Catechol dioxygenase[b]	129	240
3-Methylmuconate cycloisomerase[c]	112	596
Muconate cycloisomerase[c]	73	339
Delactonization of β-methylmuconolactone[d]	82	12
Delactonization of muconolactone[c]	161	477

[a] Rate of oxidation of NADPH measured.
[b] Rate of uptake of oxygen measured.
[c] Rate of disappearance of substrate measured.
[d] Rate of formation of 3-keto-4-methyladipate measured.

Separation of two catechol 1,2-dioxygenase activities. Varga and Neujahr (16) purified catechol 1,2-dioxygenase from phenol-grown *T. cutaneum* using a procedure that included ammonium sulfate fractionation and column chromatography first with DEAE-Sephadex and then with hydroxyapatite. We used similar procedures but replaced the materials for chromatography with DEAE-Sephacel and Sephacryl S-200, respectively. An extract was prepared in 300 ml of 0.05 M Tris hydrochloride (pH 8.3) using 175 g of *p*-cresol-grown cells. Ammonium sulfate fractionation (16) was followed by dialysis for 24 h against this buffer, and the extract (80 ml) was then applied to the column (3.2 by 45 cm)

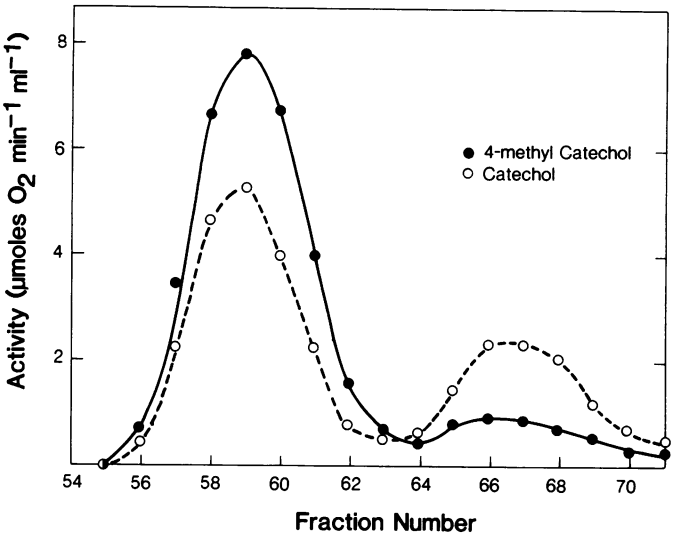

FIG. 3. Chromatography of an extract of *p*-cresol-grown cells using a column of DEAE-Sephacel. Oxygenase activities were measured separately against 4-methylcatechol and catechol as substrates.

of DEAE-Sephacel and eluted by a linear gradient of 0 to 0.5 M KCl in 0.05 M Tris hydrochloride (pH 8.3) containing 1 mM 2-mercaptoethanol and 10% acetone. Samples of eluate were collected, and dioxygenase activities were determined for each one, using first catechol and then 4-methylcatechol as assay substrates in the oxygen electrode. The elution profile (Fig. 3) showed two peaks of dioxygenase activity: the first peak (fractions 56 to 62) corresponded to enzyme activities that were greater against 4-methylcatechol than against catechol, and the second (fractions 64 to 70) corresponded to activities greater against catechol than against 4-methylcatechol. The separated enzymes, both red in color, were precipitated with ammonium sulfate, dialyzed, and chromatographed on a column of Sephacryl S-200, and when examined by analytical disc gel electrophoresis, each gave a single band. Examination by sodium dodecyl sulfate-polyacrylamide gel electrophoresis confirmed that the isolates were separate and distinct proteins. The enzyme which was more active against catechol (catechol 1,2-dioxygenase) gave two bands of about equal intensity corresponding to molecular weights 35,500 and 32,400; the second enzyme (4-methylcatechol 1,2-dioxygenase) gave two bands corresponding to molecular weights 51,300 and 23,400. Other intradiol dioxygenases that consist of two nonidentical subunits include the two *Pseudomonas* enzymes, catechol 1,2-dioxygenase (7) and protocatechuate 3,4-dioxygenase (17), and also hydroxyquinol 1,2-dioxygenase from *T. cutaneum* (14). The subunit molecular weights for the latter compound are 38,200 and 39,600, and it has been suggested that this enzyme, like bacterial catechol 1,2-dioxygenase, is a dimer (14), although close agreement is not reported with the molecular weight as measured by gel filtration, namely, 93,000. This was also the case in the present study. We obtained values of 90,000 and 104,000, respectively, for the molecular weights of catechol 1,2-dioxygenase and 4-methylcatechol 1,2-dioxygenase from gel filtration measurements using a column of Sephacryl S-200; a value of 109,000 was obtained by Varga and Neujahr (16) who used gel filtration but did not investigate subunit composition. Although we conclude that catechol 1,2-dioxygenase and 4-methylcatechol 1,2-dioxygenase from *p*-cresol-grown *T. cutaneum* are separate proteins, each composed of nonidentical subunits, the number of these subunits constituting each protein must remain an open question on the basis of present data.

When the experiment that provided the data presented in Fig. 3 was repeated for an extract of phenol-grown cells, one peak of activity was obtained; dioxygenase activity was present only in fractions beyond 62, and catechol was the preferred substrate. The properties of the catechol 1,2-dioxygenase from either batch of cells were essentially those reported by Varga and Neujahr (16). Thus, the optimum pH was around 8.2, and the rates of oxidation as measured in the assay were in the order: catechol > hydroxyquinol > 4-methylcatechol; pyrogallol was oxidized slowly and was an inhibitor of catechol oxidation. 4-Methylcatechol 1,2-dioxygenase from the present investigation shared all of these characteristics except that the order of the rates of oxidation was: 4-methylcatechol > catechol and hydroxyquinol, for which rates were approximately equal. A similar situation exists among procaryotes for a *Pseudomonas* sp. that grows with 3-chlorobenzoate and also elaborates two catechol 1,2-dioxygenases, one of which shows typical high activity toward catechol whereas the second is more active on 3- and 4-chlorocatechol than on unsubstituted catechol (2).

Evidence for a single cycloisomerase. The data presented in Table 2 suggest that our strain of *T. cutaneum* used *cis,cis*-muconate cycloisomerase to convert 3-methyl-*cis,cis*-muconate (III) into β-methylmuconolactone (IV): cells grown with either phenol or *p*-cresol appeared to possess a single enzyme for both lactonizations, in contrast to other enzymes catalyzing the reactions shown in Fig. 1. For another strain of *T. cutaneum*, Gaal and Neujahr (3) showed that muconate cycloisomerase from phenol-grown cells is resolved into two isoenzymes by isoelectric focusing. These enzymes have the same molecular weight and the same affinity for *cis,cis*-muconate and act upon 3-methyl-*cis,cis*-muconate at 25% of the rate for unsubstituted muconate. Activities in *p*-cresol-grown cells were not examined (3). We obtained further evidence for the presence of a single cycloisomerase in our strain, which used 3-methylmuconate as its preferred substrate, by purifying the enzyme from both phenol- and *p*-cresol-grown cells and measuring the ratio of activities for 3-methylmuconate to muconate at every stage in each purification. Details of the procedures are given in Materials and Methods and are summarized in Table 1, where it can be seen that the ratios of activities for the two substrates remained in the ranges 1.47 to 1.75 for phenol-grown cells and 1.50 to 1.73 for *p*-cresol-grown cells, indicating no systematic trend in either case. When the two samples of purified cycloisomerase were examined, that from phenol-grown cells was found to be essentially homogeneous as determined by polyacrylamide gel electrophoresis, although a second band appeared on gels after the enzyme had been stored at 4°C. The enzyme from *p*-cresol-grown cells showed a major band with two minor bands; the major band cochromatographed on polyacrylamide gels with the enzyme isolated from phenol-grown cells when the two preparations were run in the same gel.

The sole product formed from either sample was β-methylmuconolactone, with no trace of γ-methylmuconolactone from which it is readily separated by high-pressure liquid chromatography (11). Our enzyme, like that isolated by Gaal and Neujahr (3), did not require

divalent metal ions for activity, in contrast to the procaryotic cycloisomerase (6, 12). The dependence of activity on pH was determined for the two samples of enzyme purified from, respectively, phenol- and *p*-cresol-grown cells. A pH range from 3 to 8 was provided by the following buffers, each at 0.05 M: formic acid-sodium formate, acetic acid-sodium acetate, morpholinoethanesulfonic acid-NaOH, and Tris-H_2SO_4. Each cycloisomerase gave a broad pH optimum at pH 4 to 5.6, with activity reduced to half its maximum at pH 3.2 and 6.5. This behavior contrasts with the pH dependence of the muconate cycloisomerase of Gaal and Neujahr (3), which has a sharp pH optimum at pH 6.6, with activity reduced by one-half at pH 6.0 and 7.3. Kinetic studies were performed for each preparation in 0.05 M morpholinoethanesulfonic acid-NaOH buffer (pH 7.0) at 23°C. Since this enzyme is rather unstable, V_{max} values were not compared directly for preparations isolated at different times; instead, a specificity constant (1), V_{max}/K_m, was calculated. When this constant for 3-methylmuconate was divided by that for muconate, the ratio obtained was 2.04 for cells grown with phenol. The same procedure for cells grown with *p*-cresol gave a ratio of 1.84. For each substrate, with either preparation, the K_m was approximately 60 μM; however, the accuracy of this value is reduced by the instability of the enzyme. All of these parameters are compatible with the rest of the evidence, indicating that the two preparations contained the same cycloisomerase. Affi-Gel Blue chromatography (see Materials and Methods) revealed the existence of a minor component having a substrate activity ratio very similar to that of the purified enzyme. Therefore, the possibility remains that isoenzymes with the same molecular weight and affinity for the substrate may be present, as Gaal and Neujahr (3) found for muconate cycloisomerase purified from another strain of *T. cutaneum*. It is of interest that, of all the investigations conducted separately by Neujahr et al. and by us, the only significant difference to emerge between the two strains of *T. cutaneum* concerns the characteristics of the isomerases.

We drew attention to the fact that, whereas *Pseudomonas desmolyticum* forms γ-methylmuconolactone from 4-methylcatechol, *T. cutaneum* gives β-methylmuconolactone (11). In an analogous fashion, *P. putida* gives γ-carboxymuconolactone (6) from protocatechuate, while *Aspergillus niger* forms β-carboxymuconolactone (15). However, the analogy between fungal and bacterial catabolic sequences for carboxy- and methyl-substituted aromatic substrates ceases at this point. A carboxyl group can be released as carbon dioxide, whereas a methyl group cannot be eliminated in a similar fashion. Protocatechuate is eventually converted into β-ketoadipate by both the fungal and bacterial systems; γ-methylmuconolactone, however, is a "dead-end" bacterial metabolite, and the methyl group of β-methylmuconolactone formed by *T. cutaneum* eventually appears in pyruvate (11).

Degradation of β-ketoadipate. The degradation of β-ketoadipate by *T. cutaneum* has not been examined previously. It is evident from the results shown in Fig. 4 that the degradation of β-ketoadipate by phenol-grown *T. cutaneum*, as in *Pseudomonas* spp. (5), depends upon the presence of CoA plus succinyl-CoA. In another experiment, spectroscopic evidence was obtained for the formation of β-ketoadipyl-CoA as a reaction intermediate. An extract of phenol-grown cells was incubated with β-ketoadipate, succinyl-CoA, and CoA in Tris hydrochloride buffer (pH 8.2) containing Mg^{2+}. A species that absorbed light at 305 nm formed rapidly and then disappeared slowly; absorption was

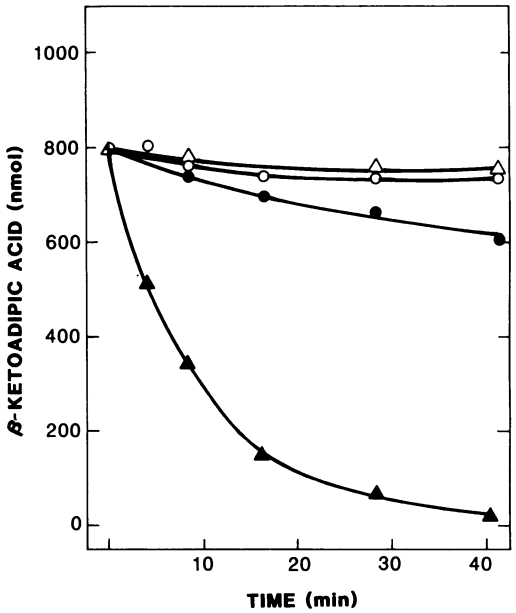

FIG. 4. Dependence of β-ketoadipic acid degradation upon CoA and succinyl-CoA. Reactions took place in 1.35 ml of 0.066 M Tris hydrochloride buffer (pH 8.2) containing 3.3. μM $MgCl_2$. All mixtures contained 0.8 μmol of β-ketoadipic acid and either 50 μmol of ATP and 2.3 μmol of CoA (●, ○) or 1.6 μmol of CoA and 1.4 μmol of succinyl-CoA (▲, △). Reactions were started by adding crude extract (0.47 mg of protein) from phenol-grown cells; extract was boiled before it was added to two of the reaction mixtures (○, △). At various intervals of time, 100 μl of each solution was withdrawn and discharged into trichloroacetic acid, the precipitate was removed by centrifugation, and the amount of β-ketoadipate remaining was determined colorimetrically as described by Powlowski and Dagley (11).

abolished upon addition of CoA. These observations are similar to those of Katagiri and Hayaishi (5) using *Pseudomonas* spp. Reaction with succinyl-CoA converts β-ketoadipic acid to its CoA ester, which absorbs at 305 nm; this is followed by thiolytic fission by CoA to give acetyl-CoA and succinyl-CoA (5). The slow disappearance of β-ketoadipate upon addition of ATP and CoA is illustrated in Fig. 4. This reaction is also observed in the *Pseudomonas* system except that the ATP-dependent activity makes a greater contribution to the total metabolism of β-ketoadipate by cell extracts than was observed here. Katagiri and Hayaishi (5) attribute this activity either to the presence in extracts of residual succinate and its activating enzyme or to the presence of a separate enzyme that activates β-ketoadipate directly, using ATP.

The succinyl-CoA-dependent enzyme for activating β-ketoadipate did not accept 3-keto-4-methyladipate as a substrate, and a different enzyme is present in *p*-cresol-grown *T. cutaneum* which converts 3-keto-4-methyladipic acid to its CoA ester and requires ATP plus CoA (11). The reason for this difference becomes evident when features of the activation processes are considered. First, each molecule of succinyl-CoA used in activation is regenerated as soon as β-ketoadipyl-CoA is cleaved; thus another enzyme is not required to provide succinyl-CoA. Second, succinyl-CoA is not formed by fission of 3-keto-4-methyladipyl-CoA and if used for activation would have to be resynthesized; further, 2-methylsuccinyl-CoA, formed in the cleavage reaction, is immediately hydrolyzed enzymically to the free acid (11).

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