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Purification and Properties of the Physically Associated *meta*-Cleavage Pathway Enzymes 4-Hydroxy-2-Ketovalerate Aldolase and Aldehyde Dehydrogenase (Acylating) from *Pseudomonas* sp. Strain CF600

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The final two steps in the *dmp* operon-encoded *meta*-cleavage pathway for phenol degradation in *Pseudomonas* sp. strain CF600 involve conversion of 4-hydroxy-2-ketovalerate to pyruvate and acetyl coenzyme A (acetyl-CoA) by the enzymes 4-hydroxy-2-ketovalerate aldolase and aldehyde dehydrogenase (acylating) [acetaldehyde:NAD^+ oxidoreductase (CoA acetylating), EC 1.2.1.10]. A procedure for purifying these two enzyme activities to homogeneity is reported here. The two activities were found to copurify through five different chromatography steps and ammonium sulfate fractionation, resulting in a preparation that contained approximately equal proportions of two polypeptides with molecular masses of 35 and 40 kDa. Amino-terminal sequencing revealed that the first six amino acids of each polypeptide were those deduced from the previously determined nucleotide sequences of the corresponding *dmp* operon-encoded genes. The isolated complex had a native molecular mass of 148 kDa, which is consistent with the presence of two of each polypeptide per complex. In addition to generating acetyl-CoA from acetaldehyde, CoA, and NAD^+, the dehydrogenase was shown to acylate propionaldehyde, which would be generated by action of the *meta*-cleavage pathway enzymes on the substrates 3,4-dimethylcatechol and 4-methylcatechol. 4-Hydroxy-2-ketovalerate aldolase activity was stimulated by the addition of Mn^{2+} and, surprisingly, NADH to assay mixtures. The possible significance of the close physical association between these two polypeptides in ensuring efficient metabolism of the short-chain aldehyde generated by this pathway is discussed.

One of the central metabolic routes for the bacterial degradation of aromatic compounds is the *meta*-cleavage pathway for the catabolism of catechol (1,2-dihydroxybenzene) and methyl-substituted catechols. This pathway operates in a number of different organisms, but the best-studied examples are those found in pseudomonads (see reference 1 for a review). The name for this sequence of reactions is derived from catechol 2,3-dioxygenase, which is responsible for cleavage of the aromatic ring adjacent to ("*meta*") the two hydroxyl groups of catechol. A variety of compounds, including naphthalene, phenol, toluene, and toluates, can be channeled into this pathway via conversion to a catechol by auxiliary enzymes. Upon oxygenative fission of the aromatic ring, a series of enzyme-catalyzed reactions results in the formation of pyruvate and a short-chain aldehyde.

Although studies of the *meta*-cleavage pathway are now into their fourth decade, surprisingly little is known about many of the enzymes involved, especially those of the lower pathway. Just in the past year, a new enzyme activity, aldehyde dehydrogenase (acylating), was discovered in the *dmp* operon-encoded *meta*-cleavage pathway of *Pseudomonas* sp. strain CF600. This activity is associated with the product of the *dmpF* gene (18). It has long been known that acetaldehyde and pyruvate are produced from 4-hydroxy-2-ketovalerate via an aldolase-catalyzed reaction (Fig. 1) when catechol is metabolized via the *meta*-cleavage pathway (8). Similarly, propionaldehyde is generated when 4-methylcatechol is metabolized via the same sequence of reactions (10). However, the fate of these aldehydes has never been firmly established. While it is likely that the operon-encoded aldehyde dehydrogenase (which uses NAD^+ and coenzyme A [CoA] to form acetyl-CoA from acetaldehyde) is responsible for this, the presence of competing alcohol dehydrogenase (alcohol:NAD^+ oxidoreductase, EC 1.1.1.1) is a complicating factor (8, 18). Identification and characterization of the enzymes involved in acetaldehyde metabolism is therefore necessary to establish relative rates and affinities for this substrate.

An intriguing possibility raised earlier (18) is that aldehyde dehydrogenase (acylating) is intimately associated with the preceding enzyme in the pathway, 4-hydroxy-2-ketovalerate aldolase (Fig. 1). This was suggested by the observation that aldolase activity, which is associated with the product of the *dmpG* gene, was not detectable in the absence of coexpression of the *dmpF* gene product. Although it was not possible to completely rule out low expression levels as a cause, an alternative conclusion is that the aldolase is inactive or less active when disassociated from the dehydrogenase. Complex formation between these two polypeptides would allow metabolic channelling of the toxic aldehyde formed by the action of the aldolase to the operon-encoded dehydrogenase.

This paper describes the purification and some properties of these two enzyme activities, which indeed were found to be tightly associated.

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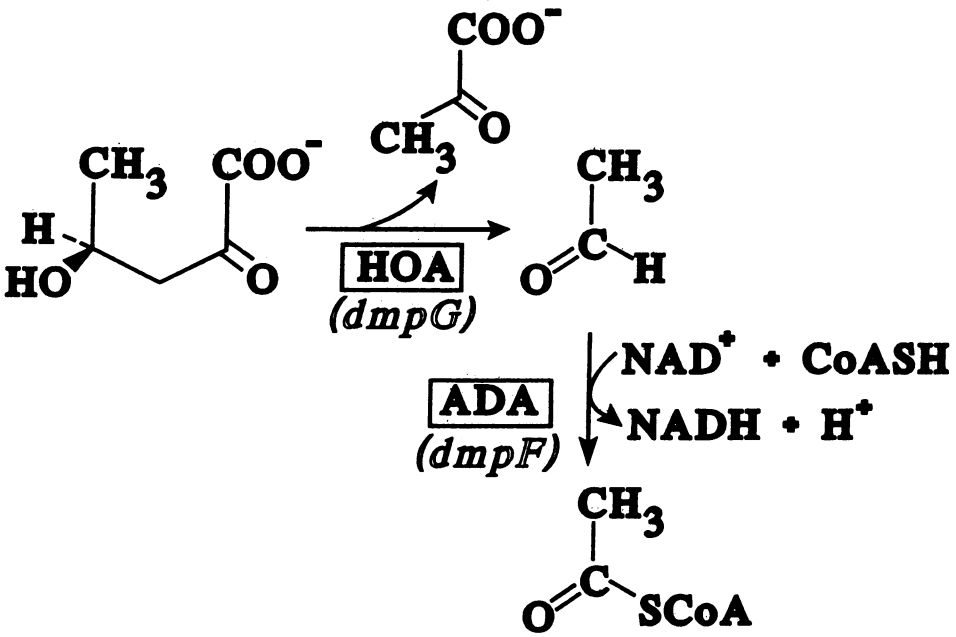

FIG. 1. Reactions catalyzed by 4-hydroxy-2-ketovalerate aldolase (HOA) and acetaldehyde dehydrogenase (acylating) (ADA). Gene names are also shown.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Pseudomonas* sp. strain CF600 was grown in Fernbach flasks shaken at 30°C with minimal medium supplemented with trace metals and phenol or acetate as described previously (15). The construction of *Escherichia coli* strains bearing plasmids pVI1311Δ and pVI1300Δ, which mediate production of the *dmpF* and *dmpQ* gene products, respectively, has been described previously (18). *E. coli* strains were grown in Luria-Bertani medium with ampicillin (100 μg/ml) selection at 37°C.

Enzyme assays. The assays for 4-hydroxy-2-ketovalerate aldolase and aldehyde dehydrogenase (acylating) were performed essentially as described previously (18 and references therein). The 4-hydroxy-2-ketovalerate used in the aldolase assay was the L-(*S*) isomer unless the racemic mixture is otherwise specified. One unit of enzyme activity is defined as the amount of enzyme required to produce 1 μmol of product per minute under the assay conditions used.

Coenzyme A-independent aldehyde dehydrogenase [aldehyde:NAD(P)$^+$ oxidoreductase, EC 1.2.1.5] was assayed as described for the CoA-dependent enzyme, but in the absence of CoA. Alcohol dehydrogenase was assayed at 25°C in 50 mM Na^+-K^+ phosphate buffer (1 ml), pH 7.5, containing ethanol (10 mM) and NAD^+ (0.29 mM). One unit of enzyme activity is defined as the amount of enzyme required to produce 1 μmol of NAD^+ per minute under these assay conditions. Assays of ATP-dependent acetyl-CoA formation at 30°C were performed essentially by following a published procedure (2) involving conversion of acetyl-CoA, formed from acetate in the presence of ATP and CoA, to acetohydroxamate.

Protein purification. All purification steps were carried out at 4°C. The buffer used was 50 mM Na^+-K^+ phosphate buffer, pH 7.5, containing 1 mM dithiothreitol (buffer A).

Step 1: preparation of crude extract. Phenol-grown *Pseudomonas* sp. strain CF600 cells (wet weight, 29 g) were suspended in buffer A (60 ml). This suspension was sonicated by using a Sonic 300 Dismembrator (Artex Systems Corp., Farmingdale, N.Y.) at 60% of full power for seven bursts of 15 s each. The temperature of the suspension was not allowed to rise above 8 to 9°C during this procedure. Cell debris was removed by centrifugation at 62,000 × *g* for 1 h, and the supernatant was used as the crude extract.

Step 2: Fast-Flow DEAE-Sepharose chromatography. Crude extract was applied to a Fast-Flow DEAE-Sepharose column (36.5 by 2.6 cm) equilibrated with buffer A. After the sample had been loaded, the column was washed with buffer A (300 ml) at a flow rate of approximately 6 ml/min. A linear gradient of 0 to 0.3 M NaCl in buffer A (1,500 ml) was then applied. Fractions eluted by the increasing NaCl concentration and containing peak aldolase activity were combined, and ammonium sulfate was added to 20% of saturation.

Step 3: phenyl-Sepharose chromatography. The preparation described above was applied to a phenyl-Sepharose column (14.5 by 1.6 cm) equilibrated with buffer A that was 20% saturated with ammonium sulfate. The column was then washed with the equilibration buffer (47 ml) at a flow rate of 1.1 ml/min and then with a linear gradient of ammonium sulfate (20% saturated to 0%) in buffer A (360 ml). After the gradient was finished, the column was washed with additional buffer A (86 ml) to completely elute the proteins of interest.

Step 4: ammonium sulfate fractionation. Fractions eluting from the phenyl-Sepharose column and containing aldolase and dehydrogenase activities were combined and brought to 30% saturation with solid ammonium sulfate. The solution was then centrifuged and, although little precipitate was visible, the supernatant was carefully decanted and brought to 55% saturation with solid ammonium sulfate. The resulting white precipitate was collected after centrifugation and dissolved in buffer A (11 ml).

Step 5: Sephacryl S-300 gel filtration chromatography. The protein was next applied to a Sephacryl S-300HR column (95 by 2.6 cm) equilibrated with buffer A, and column elution

was performed at a flow rate of 1.9 ml/min. Fractions with peak aldolase and dehydrogenase activities were combined and concentrated to 10 ml with a PM-30 ultrafiltration membrane in an Amicon stirred cell. In order to decrease the salt concentration in preparation for the next step, this sample was diluted fivefold with water containing dithiothreitol (1 mM).

Step 6: Blue-Sepharose chromatography. The preparation from the previous step was applied to a Blue-Sepharose column (14 by 1.6 cm) equilibrated with 10 mM phosphate buffer, pH 7.5, containing 1 mM dithiothreitol. Neither of the proteins of interest bound to this column, and they were eluted with the starting buffer. The ratio of aldolase activity to dehydrogenase activity was essentially the same at the beginning and the end of the activity peak. All of the active fractions were combined and concentrated to 8 ml using ultrafiltration.

Step 7: Matrex Gel Green-A chromatography. The final step involved chromatography on a Matrex Gel Green-A column (3 by 1 cm) equilibrated with 10 mM phosphate buffer, pH 7.5, containing 1 mM dithiothreitol. The proteins of interest did not bind to this column and were eluted with the equilibration buffer. All active fractions were combined, and the final preparation was stored at −80°C.

Molecular mass estimation. The native molecular mass of the purified protein was estimated by using gel filtration on a calibrated Sephacryl S-200HR column (91 by 1.6 cm). The proteins used for calibration were rabbit muscle aldolase (158 kDa), bovine serum albumin (68 kDa), egg albumin (45 kDa), chymotrypsinogen A (25 kDa), and cytochrome *c* (12.5 kDa). Chromatography was carried out using 50 mM Na^+-K^+ phosphate buffer, pH 7.5, at a flow rate of 1 ml/min.

Analytical methods. High-pressure liquid chromatography (HPLC) of CoA esters was carried out by using a Waters chromatograph equipped with an OD5 Octadecyl reversed-phase column (Burdick and Jackson, Muskegon, Mich.). Detection was performed at 254 nm. The mobile phase consisted of 0.05 M phosphate buffer (pH 5.3):acetonitrile (94:6) (7), and a flow rate of 1.5 ml/min was used. The retention times of authentic acetyl-CoA and propionyl-CoA were 5.3 and 12.7 min, respectively. These peaks were well resolved from those of NADH, NAD^+, and CoA, all of which eluted at 2 to 3 min after injection. Samples from reaction mixtures were withdrawn at various times and adjusted to pH 5 by the addition of 3 N HCl. The resulting precipitate was removed by centrifugation, and the sample was kept on ice until HPLC analysis; this procedure effectively stopped the reaction. Authentic acetyl-CoA and propionyl-CoA were used to construct standard curves for quantitation purposes.

Estimates of protein content were made by using a modification (3) of the BCA (bicinchoninic acid) assay (Pierce Chemical Co.) (60°C protocol). Published extinction coefficients were used in order to estimate the concentrations of stock solutions of CoA (20), NAD^+ (9), and CoA ester (9).

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed by using standard techniques (12) with 10 to 20% polyacrylamide gradient gels. Electroblotting of proteins from gels to polyvinylidene difluoride membranes was accomplished by using standard procedures (13). N-terminal sequencing and amino acid analyses were performed at the Sheldon Biotechnology Center, McGill University, Montreal, Quebec, Canada.

Materials. All chemicals were reagent grade or better. The L-(*S*) substrate for 4-hydroxy-2-ketovalerate aldolase was synthesized from L-(*S*)-4-methyl-2-ketobutyrolactone as described previously (4, 18). The starting lactone was judged to be chromatographically pure by using a C-18 reversed-phase HPLC column (0.01 M phosphoric acid–1% isopropanol: methanol [72.5 : 27.5]) at a flow rate of 1 ml/min and with detection at 210 and 228 nm. Alkaline hydrolysis of 4-methyl-2-ketobutyrolactone samples (8) was carried out using standardized solutions of NaOH. The concentration of 4-hydroxy-2-ketovalerate in the resulting solution was then assumed to be that of the starting lactone.

Acetaldehyde was redistilled for use in enzymatic rate determinations. Coenzymes, CoA esters, and proteins were purchased either from Boehringer Mannheim or from Sigma Chemical Co and were generally the purest available. Electrophoresis standards were obtained from Pharmacia, as were most of the chromatography column packings. Matrex Gel Green A was obtained from Amicon, and hydroxylapatite was purchased from Bio-Rad.

RESULTS

CoA ester formation from acetaldehyde and propionaldehyde is catalyzed by the *dmpF* gene product. It has previously been shown (18) that the *dmpF* gene product of the *meta*-cleavage operon of *Pseudomonas* sp. strain CF600 is associated with CoA-dependent acetaldehyde dehydrogenase activity. This activity probably serves to metabolize acetaldehyde generated from catechol by the action of the other enzymes of the *meta*-cleavage pathway. However, the product of the reaction has not previously been identified. It has also not been shown whether propionaldehyde, which is generated by metabolism of 3-methylcatechol by this pathway (10), is also a substrate for this enzyme.

In order to address these questions, the formation of acetyl-CoA and propionyl-CoA from acetaldehyde and propionaldehyde, respectively, were monitored by using HPLC (Fig. 2). Crude extracts were from *E. coli* harboring pVI1311Δ, which expresses *dmpF*, and *E. coli* harboring pVI1300Δ, which is derived from the same cloning vector and expresses a gene (*dmpQ*) encoding an unrelated protein. The results shown clearly indicate that CoA esters are formed from both acetaldehyde (Fig. 2A, solid lines) and propionaldehyde (Fig. 2B, solid lines) in the presence of the *dmpF* gene product. The control extract, which lacked the *dmpF* gene product, did not catalyze formation of these compounds in any significant quantities. The initial rate of reaction with propionaldehyde was 2.7-fold slower than that with acetaldehyde.

Similar results were obtained by using extracts from phenol-grown *Pseudomonas* sp. strain CF600, with extract from acetate-grown cells serving as a control (Figs. 2A and B, dotted lines). However, the reactions appeared to be markedly slower than those observed with the *E. coli* extracts. It is not clear whether this reflects a lower level of expression or the presence in the crude extract of other enzymes that are capable of metabolizing either the substrates or the products of the reaction. It has already been noted, for example, that the presence of alcohol dehydrogenase in extracts from phenol-grown *Pseudomonas* sp. strain CF600 interferes with the spectrophotometric assay of the enzyme (18). The initial rate obtained with extract from phenol-grown *Pseudomonas* sp. strain CF600 with propionaldehyde was 2.4-fold slower than that obtained with acetaldehyde. This ratio is essentially the same as that using extracts from the recombinant strains (see above).

In order to characterize the protein that catalyzes this reaction further, purification of the *dmpF* gene product from

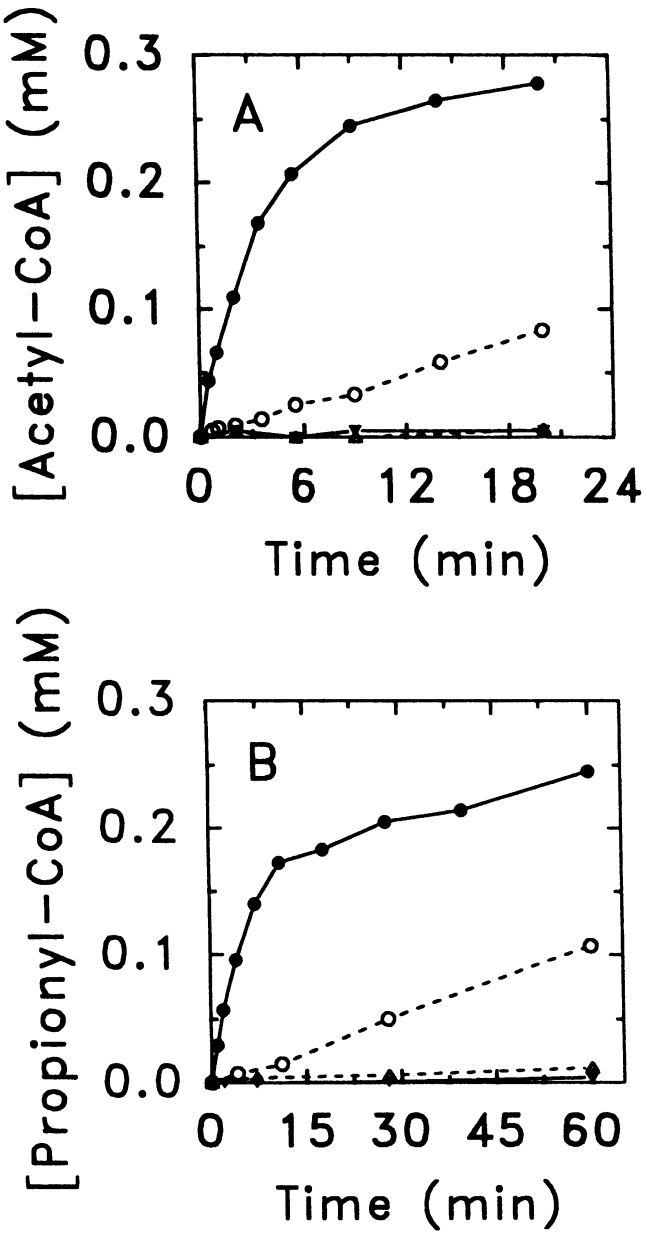

FIG. 2. Formation of CoA esters from acetaldehyde (A) or propionaldehyde (B) by dialyzed crude extract protein in the presence of CoA and NAD^+. Reactions were carried out in a total volume of 5 ml and contained phosphate buffer (pH 7.5) (230 μmol), NAD^+ (7.2 μmol), aldehyde (50 μmol), dialyzed crude extract protein (2.2 to 3.9 mg), and coenzyme A (1.6 μmol). Crude extract protein was from *E. coli* cells expressing *dmpF* (●) (3.9 mg) or *dmpQ* (▼) (3.2 mg) or from *Pseudomonas* sp. strain CF600 grown with phenol (○) (2.9 mg) or acetate (△) (2.2 mg). Product formation was quantitated by HPLC. The maximum theoretical yield of product was 0.32 mM.

phenol-grown *Pseudomonas* sp. strain CF600 was carried out. Since preliminary evidence suggested that 4-hydroxy-2-ketovalerate aldolase, which is the enzyme responsible for generation of the aldehyde in the previous step of the pathway, might be associated with the *dmpF* gene product, column fractions were also monitored for the presence of aldolase activity.

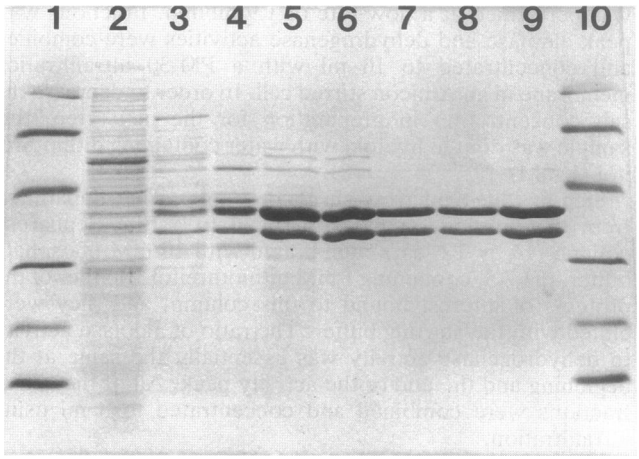

FIG. 3. SDS-polyacrylamide gel electrophoresis analysis of fractions from the purification procedure. Electrophoresis conditions are described in the text. Lanes: 1, molecular mass standards of 94, 67, 43, 30, 20.1, and 14.4 kDa; 2, crude extract protein (22.5 μg); 3, Fast-Flow DEAE-Sepharose fraction (10.8 μg); 4, phenyl-Sepharose fraction (10.2 μg); 5, ammonium sulfate fractionation (19.4 μg); 6, Sephacryl S-300HR fraction (14.1 μg); 7, Blue Sepharose fraction (7.6 μg); 8 and 9, Matrex Gel Green (5.9 and 11.8 μg); 10, molecular mass standards (same as lane 1).

Copurification of aldehyde dehydrogenase (acylating) and 4-hydroxy-2-ketovalerate aldolase from *Pseudomonas* sp. strain CF600. Aldehyde dehydrogenase (acylating) activity was inseparable from 4-hydroxy-2-ketovalerate aldolase activity during passage through six purification steps (Fig. 3, Table 1). These activities copurified on anion-exchange (Fig. 4), phenyl-Sepharose, and gel filtration chromatography columns (Table 1). Neither activity bound to Blue-Sepharose, although this step was effective in removing some contaminants from the preparation (Fig. 3). Further chromatography on a Matrex Gel Green-A column did not increase the specific activity any further, although the ratios of the two activities changed somewhat (Table 1). In a separate experiment, these activities also coeluted with increasing phosphate buffer concentration from a hydroxylapatite column to which both activities bound in the absence of phosphate (data not shown).

This purification procedure resulted in a 28% yield of aldolase activity. The yield of aldehyde dehydrogenase (acylating) activity could not be calculated for reasons discussed below. Interestingly, the activity of the aldolase appeared to increase somewhat after the DEAE-Sepharose step. The cause of this apparent increase has not been determined, although removal of an inhibitor present in the crude extract is one possibility.

Alcohol dehydrogenase activity interferes with the assay for aldehyde dehydrogenase (acylating) and, to a lesser extent, with that for 4-hydroxy-2-ketovalerate aldolase. Alcohol dehydrogenase was present in crude extract and coeluted with the enzymes of interest from the Fast-Flow DEAE-Sepharose column (Fig. 4). Therefore, no aldehyde dehydrogenase (acylating) activity figures are reported in Table 1 for these two steps. The aldolase activities reported in Table 1 for crude extract and the combined DEAE fractions are corrected for alcohol dehydrogenase activity by assuming that the presence of this enzyme results in overestimation of the activity by a factor of 2 (18). Alcohol

TABLE 1. Summary of enzyme purification[a]

Purification step	Vol (ml)	Amt of protein (mg)	HOA activity (U)	ADA activity (U)	Activity ratio (HOA/ADA)	Sp act (U of HOA/mg)	HOA yield (%)
Crude extract	71	1,650	225	ND		0.14	100
DEAE-Sepharose	157	173	284	ND		1.6	126
Phenyl-Sepharose	99	68	140	95	1.47	2.1	62
$(NH_4)_2SO_4$ (30 to 55%)	11	29	108	77	1.40	3.7	48
Sephacryl S-300HR	10	19	68	64	1.06	3.6	30
Blue Sepharose	8	17	69	62	1.11	4.1	30
Matrex Gel Green A	9.6	15	63	47	1.34	4.2	28

[a] HOA, 4-hydroxy-2-ketovalerate aldolase; ADA, aldehyde dehydrogenase (acylating); ND, not determined.

dehydrogenase activity was removed by chromatography on the phenyl-Sepharose column (data not shown), so it did not interfere with enzyme activity determinations in this and subsequent steps.

The final preparation exhibited two major bands migrating at 34.5 and 40 kDa on a 10 to 20% SDS-polyacrylamide gradient gel (Fig. 3). This result agrees reasonably well with the molecular masses of 32.5 and 37.5 kDa deduced from the nucleotide sequences of *dmpF* and *dmpG* (18). The lower band appeared to be less intense than the upper band, suggesting that the 34.5-kDa polypeptide is present in a somewhat smaller amount. However, variations in staining intensities may also account for this apparent difference.

The relative activities of 4-hydroxy-2-ketovalerate aldolase and aldehyde dehydrogenase (acylating) were similar in those fractions where both could be measured (Table 1). This result suggests that little dissociation of the two polypeptides occurred during the purification procedures used. It is also unlikely that any dissociation occurred during the anion exchange step, since only a single peak eluted from this column for both aldehyde dehydrogenase (acylating) and 4-hydroxy-2-ketovalerate dehydrogenase activities (Fig. 4). However, the possibility that dissociated polypeptides lose activity, and therefore escape detection, cannot be ruled out.

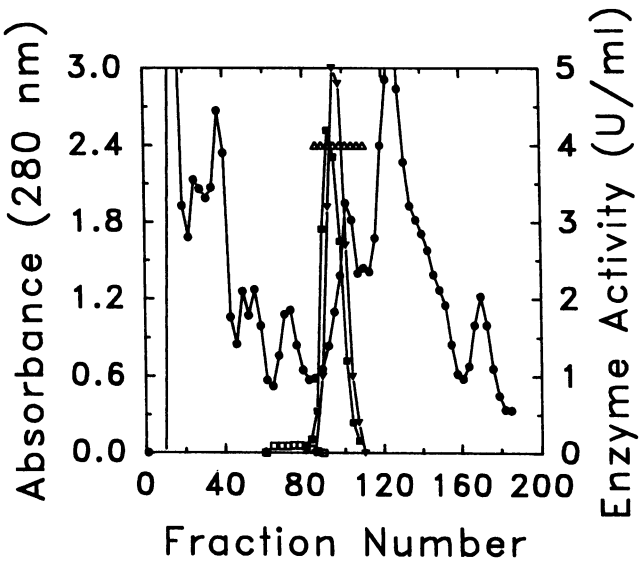

FIG. 4. Copurification of 4-hydroxy-2-ketovalerate aldolase (HOA) and aldehyde dehydrogenase (acylating) (ADA) during Fast-Flow DEAE-Sepharose chromatography. The NaCl gradient was started at fraction 40. Alcohol dehydrogenase (■), CoA-independent aldehyde dehydrogenase (□), HOA (▼), and ADA (△) activities were monitored, as was A_{280} (●). ADA activity could not be quantitated because of contaminating alcohol dehydrogenase activity, so ADA symbols (△) simply denote which fractions contained ADA activity, determined on the basis of the ability of the fraction to cause transient formation of NADH upon addition of CoA to the assay mixture (18).

Amino-terminal sequences and amino acid analysis. Amino-terminal sequencing of the purified proteins allowed unambiguous assignment of each polypeptide to a previously sequenced gene (18) from the *dmp* operon. The amino-terminal sequence of the 34.5-kDa polypeptide was determined to be M-N-Q-K-L-K from a sample electroblotted to a polyvinylidene difluoride membrane from an SDS-polyacrylamide gel. This corresponds to the amino-terminal sequence deduced from *dmpF*, which has been shown to encode aldehyde dehydrogenase (acylating) activity. The amino-terminal sequence of the other polypeptide present was then determined, from the second of two signals observed for the intact complex, to be T-F-N-P-S-K. This is in agreement with that deduced for the *dmpG* gene product after processing of the amino-terminal methionine. These two sequences were the only ones detected in the purified sample.

In order to estimate the quantities of each polypeptide present in the sample, amino acid analysis was performed on a purified sample of the native protein. A best fit, using simultaneous equations, of the observed quantities of amino acids (excluding tryptophan and cysteine) with the amounts predicted from the deduced amino acid sequences was consistent with a ratio of 1.0 mol of DmpF to 0.83 mol of DmpG in the sample (Table 2). The discrepancies between the observed and calculated quantities of serine and threonine shown in Table 2 are due to partial destruction of these amino acids during sample preparation.

Stability of enzyme activities. Purified preparations retained full dehydrogenase and aldolase activities upon storage at −80°C for at least 6 months. At 4 to 6°C, aldolase activity appeared to be stable for a period of at least 6 days. By contrast, dehydrogenase activity was unstable, dropping to 87, 56, and 47% of full activity at 2, 4, and 6 days, respectively, after storage at 4 to 6°C.

Estimation of the molecular mass of the aldolase-dehydrogenase complex. The native molecular mass of the purified protein was estimated by chromatography on a calibrated Sephacryl-S200HR column. The single peak observed eluted in a volume corresponding to a molecular mass of 148 kDa. Since the two polypeptides appear to be present in approximately equal proportions, this result is consistent with the presence of two of each of the constituent polypeptides per complex [(2 × 32.5 kDa) + (2 × 39 kDa) = 148 kDa]. No peaks that absorbed at 280 nm were detected in the elution

TABLE 2. Amino acid composition

Amino acid	Observed quantity (nmol)	No. of residues[a] in:		Calculated quantity[b] (nmol)	Observed quantity/calculated quantity (%)
		DmpF	DmpG		
Asx	12.19	24	37	11.99	101.6
Thr	5.09	16	14	6.05	84.2
Ser	5.58	17	18	7.00	79.8
Glx	11.66	28	28	11.23	103.8
Pro	5.00	15	7	4.56	109.7
Gly	10.98	25	31	11.12	99.8
Ala	18.50	50	44	18.96	97.6
Val	10.92	29	27	11.27	96.9
Met	5.41	13	14	5.40	100.4
Ile	7.66	23	18	8.31	92.1
Leu	11.44	25	33	11.48	99.6
Tyr	4.03	9	12	4.16	97.0
Phe	2.79	7	7	2.81	99.3
His	3.80	4	16	3.79	100.3
Lys	5.21	14	13	5.43	95.8
Arg	6.08	11	21	6.23	97.6

[a] Deduced from nucleotide sequences (18).
[b] Best-fit solution to simultaneous equations: DmpF, 219 pmol; DmpG, 182 pmol.

volume expected for the monomers or homodimers of these polypeptides. A small percentage of complexes lacking one subunit would not have been resolved from the intact protein with this column.

Dependence of enzyme activities on protein concentration. Since low concentrations of protein might favor dissociation, it was of interest to determine whether the two activities were maintained over a range of protein concentrations. As is evident in Figure 5, enzyme activities increased linearly over an approximately 30-fold increase in protein concentration. This result suggests either that dissociation does not occur under these conditions or that if it does, neither activity is affected.

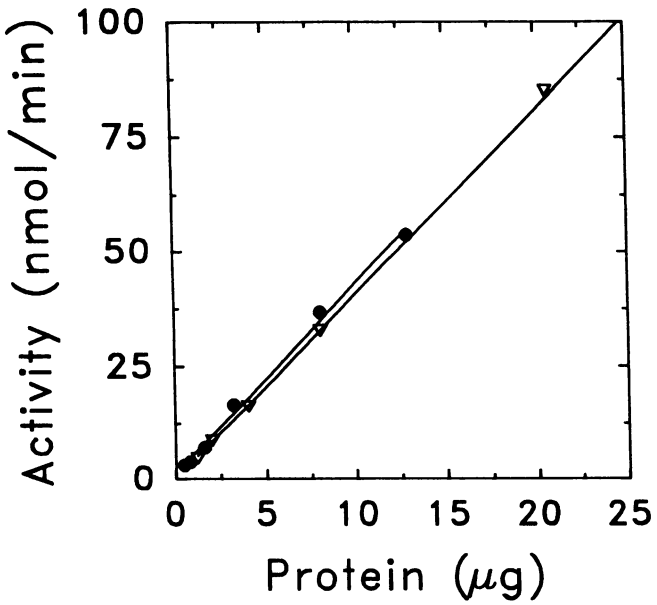

FIG. 5. Dependence of aldehyde dehydrogenase (acylating) (▽) and 4-hydroxy-2-ketovalerate aldolase (●) activities on protein concentration. Reactions were run as described in Materials and Methods, except that the NAD^+ concentration in the dehydrogenase assay was doubled; the enzyme used was a sample from the second-to-last step in the purification procedure. A point at 32 μg (128 nmol/min) (▽) has been omitted for clarity.

Dependence of enzyme activities on pH. The effects of pH on each activity were tested. Aldolase activity was examined over the pH range of 6.5 to 9.0 by using Tris (pH 8 to 9) and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 6.5 to 8.5) buffers (0.05 M). Assays were carried out with two different concentrations of lactate dehydrogenase in order to ensure that the observed rates were not limited by lactate dehydrogenase activity at different pH values. Aldolase activity was lowest at pH 6.5 and appeared to reach a maximum between pH 8.5 to 9.0; little difference in rate was noted between reactions carried out in Tris or HEPES buffer at a given pH.

Dehydrogenase activity appeared to increase gradually over the pH range from 6.5 to 8.5. However, the apparent product yield reached after this reaction was significantly different at pH 8.5 than it was at the lower pH values. At any given pH, dehydrogenase activity was highest in HEPES buffer and somewhat lower in phosphate buffer, and the rate in Tris buffer was about half the rate in HEPES buffer (data not shown).

Properties of 4-hydroxy-2-ketovalerate aldolase activity. Experiments using *Pseudomonas putida* U indicated that Mg^{2+} stimulated 4-hydroxy-2-ketovalerate activity in crude extracts from this organism (8). The availability of a purified preparation of 4-hydroxy-2-ketovalerate aldolase allowed investigation of the metal ion requirements of the *Pseudomonas* sp. strain CF600 enzyme. Mn^{2+} (1 mM) stimulated aldolase activity of the purified preparation six- to eightfold, while Zn^{2+} was strongly inhibitory and Mg^{2+} and Ca^{2+} had no effect. Aldolase activity in the absence of added Mn^{2+} was essentially unchanged by overnight dialysis against several changes of buffer containing EDTA (5 mM).

An unexpected result was the finding that NADH and, to a lesser extent, NAD^+ were also apparently able to stimulate activity of the aldolase (Fig. 6). Aldolase activity was routinely assayed by coupling the production of pyruvate by the enzyme to oxidation of NADH in the presence of excess lactate dehydrogenase. Under the conditions shown in Fig. 6, the reaction was over approximately 3 min after data collection was initiated and was unaffected by the presence of NAD^+. In order to test the possible effects of the components of the coupled reaction on aldolase activity, lactate dehydrogenase or NADH were each omitted from assays in turn. These components were added after approximately 3 min (Fig. 6), at which time the reaction was expected to be finished on the basis of the results obtained for the complete assay mixtures. When lactate dehydrogenase was added in this manner, the full amount of pyruvate expected had clearly accumulated (Fig. 6). However, when NADH was omitted and added at the time the reaction was expected to be over, very little pyruvate had accumulated; the reaction then proceeded with kinetics similar to those observed for the complete assay mixture (Fig. 6). Thus, the aldolase-catalyzed reaction is markedly slower in the absence of NADH than it is in its presence. A less-pronounced stimulation of activity was observed in the presence of NAD^+ (Fig. 6).

The results shown in Fig. 6 also demonstrate that a less-than-stoichiometric quantity of pyruvate was formed from L-(*S*)-4-hydroxy-2-ketovalerate. In our experiments, solutions of this compound, which were obtained by mild alkaline hydrolysis of the corresponding lactone (8), never

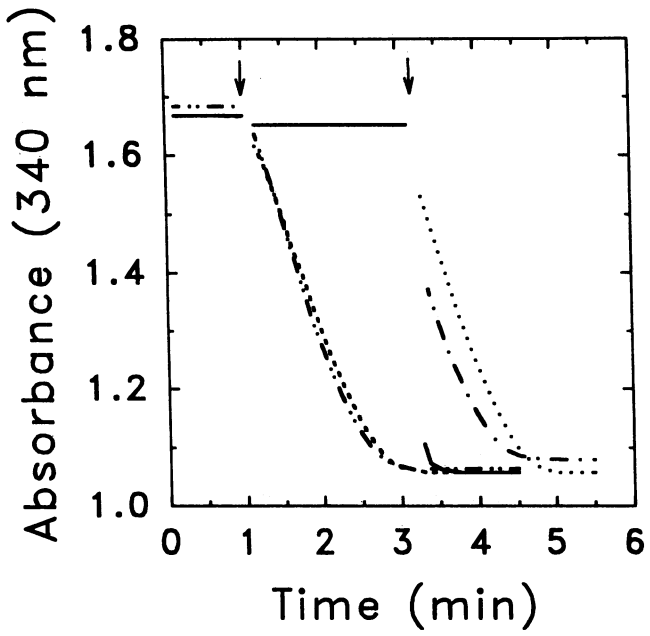

FIG. 6. Dependence of 4-hydroxy-2-ketovalerate aldolase activity on the addition of NADH to the reaction mixture. Assay mixtures (1 ml) contained HEPES buffer (pH 8.0) (48 μmol), $MnCl_2$ (1 μmol), NADH (270 nmol), lactate dehydrogenase (14 U), and purified enzyme (20 μg). All assay components except substrate were mixed, and the reaction was initiated by the addition of 4-hydroxy-2-ketovalerate at the first arrow (1 min). Two of the traces correspond to the complete assay mixture plus (— ·· — ··) or minus (– – –) NAD^+ (270 nmol), while the other three traces were obtained with identical assay mixtures in which one component was initially omitted and added only at the second arrow. Lactate dehydrogenase (——) or NADH (···) were added in this fashion to assay mixtures lacking NAD^+; NADH was omitted and added to an assay mixture that contained NAD^+ (— · — · —).

yielded more than 77% of the expected amounts of pyruvate in aldolase assays. Yields were not increased significantly by extending the incubation time shown in Fig. 6 to 10 min. These results suggest either that the substrate is impure or that equilibria involved in the assay are unfavorable for full product formation. Control assays run under identical conditions to those shown in Fig. 6 but with equimolar pyruvate and acetaldehyde substituted for 4-hydroxy-2-ketovalerate yielded more than 95% of the expected amount of NADH upon addition of lactate dehydrogenase 3 min after the other components were mixed. This result rules out unfavorable equilibria as an explanation of the low pyruvate yields. Some substrate samples that were obtained from hydrolysis mixtures that were inadvertently made slightly too alkaline gave even lower yields of pyruvate (approximately 50%). We therefore suggest that some of the 4-hydroxy-2-ketovalerate generated early during the overnight hydrolysis with equimolar NaOH is unavoidably degraded. Purification of 4-hydroxy-2-ketovalerate from hydrolysis mixtures was not attempted.

Properties of aldehyde dehydrogenase (acylating) activity. Aldehyde and nucleotide specificities of the dehydrogenase reaction were examined. Activities of the purified preparation (15 μg) with different aldehydes (10 mM) were determined in the standard assay with the substitution of 0.05 M HEPES, pH 8.0, for phosphate buffer. Measured rates (in nanomoles per minute, with standard deviations in parentheses) were as follows: acetaldehyde, 58.5 (1.0); propionaldehyde, 35.6 (1.0); butyraldehyde, 14.9 (2.0); isobutyraldehyde, 10.5 (1.9); formaldehyde, <2.0. $NADP^+$ was able to substitute for NAD^+ in the standard assay, although the rate of the reaction in the presence of $NADP^+$ was only 7% of that observed in the presence of NAD^+.

Since the closely associated aldolase activity was stimulated in the presence of Mn^{2+}, the metal dependence of the dehydrogenase reaction was also investigated. No stimulation of activity was observed when Mn^{2+}, Mg^{2+}, Ca^{2+}, Zn^{2+}, or Co^{2+} (1 mM) was added to HEPES-buffered assay mixtures.

Other acetaldehyde-metabolizing enzymes present in crude extracts. Although the CoA-dependent dehydrogenase appears to be the only *dmp* operon-encoded acetaldehyde-metabolizing enzyme, the presence of noninduced alcohol dehydrogenase in phenol-grown *Pseudomonas* sp. strain CF600 (18) (Fig. 4) suggests that other metabolic fates for acetaldehyde are possible. One obvious additional alternative is that acetaldehyde formed by the *meta*-cleavage pathway is converted to acetyl-CoA via two other steps, namely, dehydrogenation by CoA-independent aldehyde dehydrogenase to yield acetate followed by conversion of acetate to acetyl-CoA by using ATP and CoA either by acetyl-CoA synthetase [acetate:CoA ligase (AMP-forming), EC 6.2.1.1] or by acetate kinase (ATP:acetate phosphotransferase, EC 2.7.2.1) and phosphotransacetylase (acetyl-CoA:orthophosphate acetyltransferase, EC 2.3.1.8) (see reference 14). Activity of the non-CoA-dependent aldehyde dehydrogenase cannot easily be determined in crude extracts because of the presence of other NAD^+-utilizing enzymes, such as alcohol dehydrogenase. However, fractionation of crude extract on the DEAE-Sepharose column allowed detection of this activity (Fig. 4), albeit at a relatively low level. ATP-dependent acetyl-CoA-synthesizing activity was found to elute from the DEAE column shortly after the aldolase (data not shown). Thus, other fates for acetaldehyde generated by this pathway are indeed possible and must include reduction to ethanol, or oxidation to acetate, followed by conversion to acetyl-CoA via an ATP-dependent pathway.

DISCUSSION

Copurification of the enzyme activities catalyzing the final two steps of the *meta*-cleavage pathway of *Pseudomonas* sp. strain CF600 has been reported here. It was found that these two activities, 4-hydroxy-2-ketovalerate aldolase and aldehyde dehydrogenase (acylating), are tightly associated. During the purification procedures used, proteins were subjected to a wide variety of separation procedures involving both high- and low-ionic-strength solutions. Nonetheless, the two activities were maintained at a similar ratio through each step, and the final preparation contained two polypeptides, the products of the *dmpF* and *dmpG* genes, in approximately equal amounts. The results of native molecular mass estimation by gel filtration chromatography suggested that two of each polypeptide are included in the intact complex. An examination of the dependence of each activity on protein concentration showed no tendency of the activities to drop off at low protein concentrations. Together, these results suggest that separation of the two polypeptides would require relatively harsh conditions.

Although earlier experiments using genetically manipulated strains showed that the polypeptide expressed from *dmpF* was sufficient to encode aldehyde dehydrogenase (acylating) activity, it was not clear whether the *dmpG* gene

product alone possessed aldolase activity (18). These experiments showed that coexpression of *dmpF* and *dmpG* appeared to be required in order to observe aldolase activity. Expression of *dmpG* alone did not allow detection of aldolase activity, although low levels of expression were not completely ruled out as the cause. The demonstration that these polypeptides are physically associated may help to explain the difficulties in obtaining expression of *dmpG* in isolation from *dmpF*. The possible consequences of physically separating the two polypeptides include instability of the subunits in isolation, destruction of an active site made up of amino acid residues from each subunit, or abolition of allosteric regulation. While no evidence has yet been obtained regarding the first two possibilities, the third may well be involved (see below).

This is the second reported example of close physical association between two *meta*-cleavage pathway enzymes. Previously, it was found that 4-oxalocrotonate decarboxylase and 2-oxopent-4-enoate hydratase from the TOL plasmid-encoded *meta*-cleavage pathway copurified to homogeneity (11). We have made a similar observation for the *dmp*-encoded analogs of these enzymes (J. Powlowski, unpublished observation). We have also reported (18) the sequences of the *dmp*-encoded genes for the decarboxylase and hydratase enzymes, which show 37% identity at the amino acid level. This structural similarity, which could have resulted from evolution of both polypeptides from a common dimeric ancestor, could account for the close physical association of these two enzymes. However, the amino acid sequences deduced from *dmpF* and *dmpG*, the genes encoding aldehyde dehydrogenase (acylating) and 4-hydroxy-2-ketovalerate aldolase, respectively, show no sequence homologies (18).

One obvious consequence of the physical association of catabolic enzymes is the possibility of channelling metabolic intermediates. The association described here is between 4-hydroxy-2-ketovalerate aldolase, which generates an aldehyde, and aldehyde dehydrogenase (acylating), which metabolizes the aldehyde. Two advantages could be envisioned as a result of this association. One is that the toxicity of the aldehyde might be minimized by keeping it enzyme bound and efficiently metabolizing it as it is generated. A second advantage of close physical association might be to maximize the flux of the aldehyde through the *dmpF*-encoded ATP-independent dehydrogenase. Other acetaldehyde-metabolizing enzymes, such as alcohol dehydrogenase and non-CoA-dependent aldehyde dehydrogenase (leading to an ATP-dependent acetyl-CoA synthetase), are present and may compete with the CoA-dependent dehydrogenase. Thus, it would be advantageous to ensure that as much aldehyde as possible is metabolized through the ATP-independent pathway.

The purification of a *meta*-cleavage pathway aldehyde dehydrogenase (acylating) has not previously been described. Indeed, it has not yet been shown that it is present in all *meta*-cleavage operons, although its presence in the TOL plasmid-encoded pathway is probable (18). We have shown here that this enzyme is able to use either the acetaldehyde which would be generated from metabolism of catechol or 3-methylcatechol and, less efficiently, the propionaldehyde which would be generated by the degradation of 4-methyl- or 3,4-dimethylcatechol. Dehydrogenases catalyzing these reactions have been purified previously from *Clostridium kluyveri* (5) and *E. coli* (16). Unfortunately, little information on the physical properties or metabolic roles of these two proteins has been published.

The presence of 4-hydroxy-2-ketovalerate aldolase in *meta*-cleavage pathways is well known (1, 8), although only partial purifications of the enzymes from *Pseudomonas putida* U (6) and *Azotobacter* sp. (17) have previously been published. The pH optimum is approximately 8.8 for all three enzymes. Significantly, we have found that the purified protein from *Pseudomonas* sp. strain CF600 is stimulated by Mn^{2+} rather than by Mg^{2+}, as the enzyme from *P. putida* U is. This result suggests that the metal ion dependence should be carefully investigated for the aldolase in each individual strain of pseudomonad. The purified aldolase was also active in the absence of added Mn^{2+}, albeit at a lower rate. It is not clear from our results whether Mn^{2+} is simply an activator or whether it is absolutely essential, in a tightly bound form, for activity. Obviously, it will be of interest to determine this.

Unexpectedly, aldolase activity was also apparently stimulated by NADH or, much less efficiently, by NAD^+. These compounds are the product and substrate, respectively, of the closely associated acetaldehyde dehydrogenase (acylating). Activation could be the result of an allosteric site for pyridine nucleotide on the aldolase or of a pyridine nucleotide-induced conformational change in the dehydrogenase that is sensed by the associated aldolase. The latter hypothesis is more attractive, since while a known NAD^+-binding motif is present in the deduced sequence of the *dmpF*-encoded polypeptide (dehydrogenase) (18, 19), none has been detected in the deduced sequence of the aldolase. Allosteric activation may be a mechanism to ensure that acetaldehyde is generated only slowly from 4-hydroxy-2-ketovalerate unless either the coenzyme required to metabolize acetaldehyde in the next step is present or the dehydrogenase signals that it is turning over by generating an activator, NADH.

Despite the possible significance of this observation for regulation, it is also important to be aware that when attempting to purify this enzyme, aldolase activity is likely to be difficult to detect in the absence of NADH. The possibility that an NADH-induced conformational change in the dehydrogenase is required for rapid turnover of the aldolase could also explain the apparent lack of activity when *dmpG* was expressed independently of *dmpF* (18). Without activation, aldolase activity would clearly have been below the limits of detection in the earlier experiments.

Since amounts less than the expected yields of pyruvate from 4-hydroxy-2-ketovalerate were obtained with the purified aldolase, it appears that generation of this substrate by mild alkaline hydrolysis of the corresponding lactone yields an impure preparation. Other workers using partially purified aldolase preparations from *Azotobacter* sp. (17) or *P. putida* U (6) have also reported less-than-stoichiometric product yields (84% and 80 to 90%, respectively) when substrate prepared by this method was used. Although product yields approaching 100% have been reported (8), this was with crude extract, which may contain additional enzymes capable of degrading other compounds which may be generated during hydrolysis of the lactone. One possible alternate hydrolysis product is 2-oxopent-4-enoate, which is the intermediate preceding 4-hydroxy-2-ketovalerate in the *meta*-cleavage pathway. The enzyme which catalyzes this conversion would be present in crude extract but would not be present in a purified preparation, and this could explain the differing product yields. However, addition of purified 2-oxopent-4-enoate hydratase to reaction mixtures did not increase yields of pyruvate in the aldolase-catalyzed reaction (data not shown). Further studies of this interesting enzyme

will clearly require the availability of a method for preparing purer preparations of the substrate.

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