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Purification and Characterization of Maleate Hydratase from *Pseudomonas pseudoalcaligenes*

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Maleate hydratase (malease) from *Pseudomonas pseudoalcaligenes* has been purified. The purified enzyme (98% pure) catalyzes the stereospecific addition of water to maleate and citraconate (2-methylmaleate), forming D-(+)-malate and D-(+)-citramalate, respectively. 2,3-Dimethylmaleate was also a substrate for malease. The stability of the enzyme was dependent on the protein concentration and the addition of dicarboxylic acids. The purified enzyme (89 kDa) consisted of two subunits (57 and 24 kDa). No cofactor was required for full activity of this colorless enzyme. Maximum enzyme activity was measured at pH 8 and 45°C. The K_m for maleate was 0.35 mM, and that for citraconate was 0.20 mM. Thiol reagents, such as *p*-chloromercuribenzoate and iodoacetamide, and sodium dodecyl sulfate completely inhibited malease activity. Malease activity was competitively inhibited by D-malate (K_i = 0.63 mM) and D-citramalate (K_i = 0.083 mM) and by the substrate analog 2,2-dimethylsuccinate (K_i = 0.025 mM). The apparent equilibrium constants for the maleate, citraconate, and 2,3-dimethylmaleate hydration reactions were 2,050, 104, and 11.2, respectively.

Hydratases are commercially interesting enzymes since they can hydrate unsaturated compounds, forming optically pure alcohols (100% yield and 100% enantiomeric excess are theoretically possible). An example is the industrial production of L-malic acid from fumarate with immobilized cells of *Brevibacterium flavum* (9).

Since hydration does not involve a net oxidation or reduction, no expensive cofactors should be required or have to be regenerated. Not all hydratases are cofactor independent, however. In fact, most of the hydratases described so far require one or more cofactors.

Cofactors reported for hydratases are pyridoxal phosphate (for amino acid dehydratases [26]), coenzyme A (e.g., for crotonase [1]), metal ions (e.g., for enolase [6]), enzyme-bound pyridine nucleotide (NAD^+) (e.g., for CDP-glucose-4,6-dehydratase [20]), iron-sulfur clusters (e.g., for aconitase [2]), pyrroloquinoline quinone (for nitrile hydratase [19]), and adenosylcobalamin (coenzyme B_{12}) (e.g., for propanediol dehydratase [27]). These cofactors have a function in substrate binding (including water) (e.g., iron-sulfur clusters, pyrroloquinoline quinone, and metal ions), in polarization of the substrate (e.g., coenzyme A and metal ions), in temporary binding of H^+ or OH^- during acid-base catalysis (e.g., iron-sulfur clusters), in stabilization of carbocation intermediates (e.g., pyridoxal phosphate), in converting the substrate after it is bound to the enzyme into a substrate which can be dehydrated (enzyme-bound NAD^+), or as a producer of radicals (coenzyme B_{12}).

Maleate hydratase (malease; EC 4.2.1.31) purified from rabbit kidney (10) catalyzes the hydration of maleate to D-(*R*)-malate. It contains an iron-sulfur cluster and is dependent on a divalent metal ion and a sulfur compound for activity.

Malease is also present in microorganisms (25), and D-malate production from maleate was studied in more detail in *Pseudomonas pseudoalcaligenes* NCIMB 9867. This strain produced D-malate with an enantiomeric purity of more than 99.9%. To further assess the potential of this malease as an industrial biocatalyst, characterization of the microbial enzyme with respect to cofactor requirements, substrate specificity, and stability was required.

In this report we describe the purification and characterization of maleate hydratase from *P. pseudoalcaligenes*.

MATERIALS AND METHODS

Organism and growth conditions. *P. pseudoalcaligenes* NCIMB 9867 was grown in a 30-liter fermentor with 20 liters of mineral salts medium (13) containing 1 g of 3-hydroxybenzoate per liter at 30°C and pH 7. When all the 3-hydroxybenzoate was consumed, as judged by the drop in the oxygen consumption rate, an additional amount of 3-hydroxybenzoate (20 g) was added. This two-stage addition of substrate was used to prevent toxicity problems. Cells were harvested immediately after all the 3-hydroxybenzoate was consumed (as judged by the decrease in the oxygen consumption rate). The broth was cooled to 10°C, and cells were concentrated with a Pellicon cross-flow filtration unit (Millipore) at 0°C to a volume of 2 liters. The cells were then collected by centrifugation (4°C, 10 min at 16,000 × *g*) and washed with 50 mM phosphate buffer (pH 7.0). The pellet was resuspended in 100 ml of the same buffer and stored at −20°C in 7-ml aliquots.

Purification of malease. All purification steps were performed at 4°C, and the pH of the buffers used was 7.0 unless specified otherwise.

(i) Preparation of cell extract. Three 7-ml aliquots of the frozen cell suspension were thawed and disrupted by sonication (6 min; 30% duty cycle; output control, 2.3) with a Branson Sonifier 250. Cell debris was removed by centrifugation at 20,000 × *g* for 20 min. The supernatant was used as the cell extract (≈ 420 mg of protein).

(ii) Anion-exchange chromatography. The cell extract was applied to a DEAE–Sepharose CL-6B column (5.5 by 30 cm) equilibrated with 10 mM phosphate buffer containing 20 mM

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DL-malate. The column was washed with 400 ml of the same buffer (flow rate, 2.3 ml/min; collected fraction volume, 23 ml), and subsequently the enzyme was eluted with a 0 to 500 mM linear gradient of NaCl in the same buffer (total volume, 2 liters). Malease eluted at an NaCl concentration of 40 mM. Active fractions were pooled. Protein was precipitated by bringing the pooled fractions to 80% ammonium sulfate saturation. After 15 min at 0°C, the precipitate was collected by centrifugation (15 min at 27,000 × *g*). The pellet was resuspended in 4 ml of 50 mM phosphate buffer.

(iii) Gel filtration. The solubilized precipitate was applied to a Sephacryl S300 column (3 by 65 cm) equilibrated with 25 mM phosphate buffer containing 50 mM DL-malate and eluted with the same buffer (flow rate, 0.4 ml/min; collected fraction volume, 4 ml). Fractions containing malease were pooled and concentrated by ultrafiltration with an Amicon ultrafiltration unit, using a membrane with a cutoff of 30.000 under helium at a pressure of 4×10^5 Pa, to a volume of approximately 4 ml.

(iv) Hydroxyapatite. The concentrate from the gel filtration step was applied to a hydroxyapatite column (1.2 by 15 cm) equilibrated with 10 mM phosphate buffer containing 10 mM DL-malate and eluted with the same buffer (flow rate, 0.2 ml/min; collected fraction volume, 2.5 ml). The fractions containing malease were pooled and concentrated by ultrafiltration under helium at a pressure of 4×10^5 Pa to a volume of about 4 ml.

(v) Mono Q. Samples of 1 ml from the hydroxyapatite step were applied to a Mono Q column (0.6 by 6 cm) operated with a fast protein liquid chromatography system (Pharmacia) at room temperature. The column was equilibrated with 10 mM Tris-HCl buffer (pH 8.0) containing 20 mM DL-malate. The enzyme was eluted with a 0 to 50 mM linear gradient of NaCl in the same buffer (flow rate, 1 ml/min; gradient in 11 min; collected fraction volume, 1 ml). Malease eluted at an NaCl concentration of 25 mM. Fractions exhibiting malease activity were pooled.

Assay of malease and citraconase activities. Malease and citraconase activities were determined spectrophotometrically by monitoring the decrease in the absorbance due to the disappearance of substrate. The absorption of the hydroxyacid formed is negligible compared with the absorption of the unsaturated acid. Activity in the fractions was routinely measured as follows. Fifty microliters of enzyme, 100 μl of 10 mM maleate or citraconate, and 1.85 ml of 50 mM Tris-HCl (pH 7.5) were incubated at 30°C, and the decrease in A_{240} was monitored for at least 3 min (for extinction coefficients, see Table 1). Specific activities were determined at 270 nm by using initial substrate concentrations of 5 mM in the activity assay and are expressed in units (micromoles per minute) per milligram of protein. The effects of inhibitors and ions were studied by adding 200 μl of a 10 mM solution to the activity assay mixture (the total volume was held at 2 ml by adding less buffer).

Protein determination. Protein was determined by the method of Bradford (5) with bovine serum albumin (BSA) as the standard.

Stability of malease. Cell extract was diluted 1:1 with 50 mM phosphate buffer (pH 7) containing the additive. These mixtures were stored at 4°C, and malease activity was measured each day.

Determination of molecular weight. The molecular weight of the native protein was determined by gel filtration on a Sephacryl S300 column as described above ("Purification procedure, step 3"). The column was calibrated by using a Pharmacia low-molecular-weight calibration kit containing phosphorylase *b* (molecular weight, 94,000), BSA (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), and α-lactalbumin (14,400).

TABLE 1. Extinction coefficients of maleate and citraconate at different wavelengths

Wavelength (nm)	ϵ^a	
	Maleate	Citraconate
230	3,300	5,400
235	2,600	4,100
240	1,900	2,900
245	1,400	1,900
250	900	1,100
255	560	650
260	350	350
265	210	190
270	120	100
275	70	55
280	40	30

[a] In liters · mole^{-1} · centimeter^{-1}.

The molecular weights of the subunits were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). A 12.5% (wt/vol) separation slab gel was prepared by the method of Laemmli (15). The reference proteins were the same as used for the determination of the molecular weight of the native protein. Proteins were stained with Coomassie brilliant blue G.

Determination of amino acid composition. Purified malease was hydrolyzed in 6 N HCl for 16 h at 105°C. HCl was removed by evaporation with a rotary evaporator at 50°C, and the resulting residue was used for analysis on a Biotronic LC 6000E amino acid analyzer equipped with a separation program for physiological solutions. No modifications for cysteine or methionine were performed, and tryptophan was destroyed by the method used, so these amino acids could not be determined.

Determination of kinetic constants. Specific activities were determined spectrophotometrically at initial substrate concentrations of 0.25, 0.5, 1, 2.5, 5, and 10 mM. Depending on the substrate concentration, the wavelength was adjusted to obtain an absorbance of between 0.8 and 1.2 at the start of the activity assay. The decrease in absorption was monitored at 30°C.

The extinction coefficients of maleate and citraconate between 220 and 280 nm were determined in 50 mM phosphate buffer at pH 7 and are given in Table 1. The extinction coefficients did not vary significantly between pHs 5.5 and 9.

The initial activities at the different substrate concentrations were set out in Lineweaver-Burk plots, and the best line through the points was obtained by linear regression. K_m and V_{max} for maleate and citraconate were calculated from these Lineweaver-Burk plots.

The inhibition constants were determined by measuring the initial activities at the above-mentioned maleate concentrations in the presence of inhibitor at a concentration that inhibited the malease activity at 0.5 mM maleate around 30 and 60%. The inhibition constants were calculated from the change in the slope of the Lineweaver-Burk plot.

Substrate specificity. The activity of purified malease towards other compounds was determined spectrophotometrically (240 nm, 30°C), either by monitoring a possible increase in absorbance for the hydroxy acids, the amino acid, and the mercapto acid or by monitoring a decrease in absorbance for the unsaturated acids. The substrate concen-

TABLE 2. Purification of malease from *P. pseudoalcaligenes*

Purification step	Total protein (mg)	Sp act (U/mg of protein)	Ratio of malease to citraconase activity	Purification (fold)	Yield (%)
Cell extract	423	2.0	1.78	1	100
Anion exchange	33.7	27.7	1.72	13.6	108
Gel filtration	14.8	38.3	1.71	18.8	66.0
Hydroxyapatite	7.2	72.2	1.83	35.5	60.3
Mono Q	2.4	108	1.81	53.2	30.2

trations of the unsaturated compounds were chosen to give absorbance values of between 0.5 and 1.0 at the beginning of the incubation. The amount of malease used in determining the substrate specificity was varied to allow the detection of activities as low as 1% of the malease activity with maleate. With the tartrates and acetylene dicarboxylate as potential substrates, L-malate dehydrogenase was added to the assay to determine if oxaloacetate was formed. The activity assay contained 100 μl of malease (≈ 3 U), 100 μl of 100 mM substrate, 100 μl of 5 mM NADH, 5 μl of L-malate dehydrogenase (30 U), and 1.7 ml of 50 mM Tris-HCl (pH 7.5). A possible decrease in absorption was monitored at 340 nm and 30°C.

Apparent equilibrium constant. To 1.7 ml of 25 mM potassium phosphate buffer (pH 7) containing 40 mM maleate, D-malate, citraconate, D-citramalate, or 2,3-dimethylmaleate in a 30-ml serum bottle, 300 μl of malease (≈ 10 U) was added. The serum bottles were incubated in a water bath at 25°C. The maleate-malate, citraconate-citramalate, and 2,3-dimethylmaleate concentrations in the mixtures were monitored by high-performance liquid chromatography (HPLC) until equilibrium was reached.

HPLC analysis. Malate, citramalate, maleate, citraconate, and 2,3-dimethylmaleate were analyzed by HPLC as described before (25). The dicarboxylic acids were separated on a C_{18} column with 2 mM octylamine and 25 mM potassium phosphate buffer (pH 7.0) as the mobile phase.

Optical rotation. The optical rotation of the malate and citramalate produced was assayed as described previously (25).

Chemicals. L-Malate dehydrogenase (for analytical purposes, solution in glycerol) and NADH were from Boehringer. Octylamine, D-citramalate, and 2,3-dimethylmaleic anhydride were from Aldrich. Maleate, DL-malate, and D-malate were from Janssen Chimica. DEAE–Sepharose CL-6B, Sephacryl S300, and Mono Q were obtained from Pharmacia, and hydroxyapatite was from Bio-Rad.

2,3-Dimethylmaleate was prepared from 2,3-dimethylmaleic anhydride by adding sodium hydroxide. Isopropylmaleate was isolated from the culture medium of *Candida maltosa* G587, a kind gift of R. Bode, according to the method described by Bode et al. (4). All other chemicals were of analytical grade (at least 98% pure).

RESULTS

Malease stability. Because considerable losses in malease activity were observed during initial attempts to purify malease, the stability of this enzyme was studied first. Addition of DL-malate (50 mM) to the phosphate buffer (pH 7) stabilized malease. This stabilization was concentration dependent; higher DL-malate concentrations resulted in a better stabilization of malease activity, and D- and L-malate were equally effective. Succinate also stabilized malease, although it was less effective than DL-malate.

Therefore, phosphate buffer (pH 7) containing DL-malate was used during the different purification steps. Because DL-malate has an ionic strength which is three times higher than that of NaCl, the concentrations of buffer and DL-malate were varied depending on the purification step.

The stability of purified malease was also dependent on the protein concentration. In the absence of DL-malate, a diluted malease preparation (0.008 mg/ml) lost 80% of its activity after 30 min at 30°C, while the activity of the undiluted malease preparation (0.32 mg/ml) remained constant. Addition of 3 mg of BSA per ml to the diluted malease preparation still resulted in a 60% loss of malease activity after 30 min at 30°C.

Purification of malease. The purification scheme for the malease of *P. pseudoalcaligenes* is presented in Table 2. Malease was purified ≈53-fold, with an overall yield of ≈30%. SDS-PAGE revealed two distinct bands, corresponding to proteins with molecular weights of 24,000 and 57,000, and showed that the enzyme was ≈98% pure (Fig. 1). The molecular weight of the native protein was 89,000 as determined by gel filtration. The enzyme consists of two subunits in an αβ composition.

The absorption spectrum of this colorless protein did not give any indications of the presence of prosthetic groups.

From Table 2 it can be calculated that malease represents ≈2% of the total soluble cellular protein. By using the molecular weight determined with SDS-PAGE (81,000), a turnover number of 150 molecules of maleate per molecule of malease per s could be calculated for the purified enzyme.

Amino acid composition. The amino acid composition of malease from *P. pseudoalcaligenes* is shown in Table 3. The amino acid compositions of malease from rabbit kidney (10) and of the cofactor-independent fumarase from *Escherichia coli* (28) are also shown. Surprisingly, malease from *P. pseudoalcaligenes* did not appear to contain proline (Table 3).

Temperature optimum and pH optimum. Malease activity was maximal at approximately 45°C (Fig. 2). At temperatures above 45°C, inactivation of malease was observed during the time of the activity assay (3 min).

Maleate hydratase has a quite broad pH optimum around 7.5 (Fig. 3). Succinate and phosphate buffer (50 mM) inhibited malease activity (Fig. 3; see also Table 5). At pH values above 9, inactivation of malease occurred during the time of the spectrophotometric assay.

Substrate specificity. The substrate analogs citraconate and 2,3-dimethylmaleate were also substrates for malease from *P. pseudoalcaligenes*. Citraconate and 2,3-dimethylmaleate (1 mM) were hydrated at, respectively, 54 and 0.8% of the rate of maleate hydration. The ratio of malease and citraconase activities remained constant during the purification procedure (Table 2), indicating that no additional citraconase is present in the cell extract.

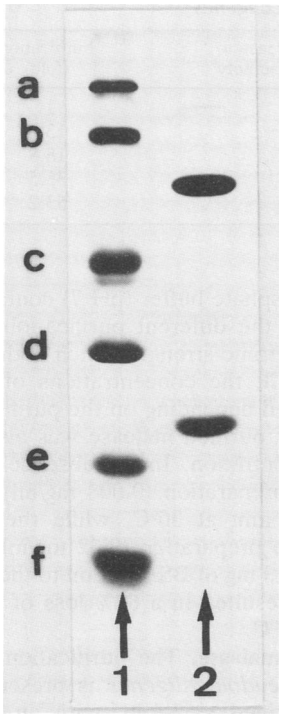

FIG. 1. SDS-PAGE of malease from *P. pseudoalcaligenes*. Lane 1, molecular weight markers: a, phosphorylase *b* (94,000); b, BSA (67,000); c, ovalbumin (43,000); d, carbonic anhydrase (30,000); e, soybean trypsin inhibitor (20,100); f, α-lactalbumin (14,400). Lane 2, 20 μg of malease.

The product of malease activity with maleate is D-malate (25), and the product of citraconate had the same optical configuration as commercially available D-citramalate (analyzed by optical rotation).

Malease showed less than 1% of the maleate-hydrating activity with the following compounds: fumarate, mesaconate, itaconate, dihydroxymaleate, maleatedimethylester, acetylenedicarboxylate, acrylate, methacrylate, crotonate, *cis*-3-chloroacrylate, *trans*-3-chloroacrylate, 2-chloroacrylate, 2-pentenoic acid, 3,3-dimethylacrylate, tiglic acid, glutaconate, *cis*-aconitate, *trans*-aconitate, 2-isopropylmaleate, L-malate, L-citramalate, D-tartrate, L-tartrate, *meso*-tartrate, DL-aspartate, and DL-mercaptosuccinate.

TABLE 3. Amino acid composition of malease

Amino acid	Amt[a] in:		
	P. pseudoalcaligenes malease	Rabbit kidney malease (10)	*E. coli* fumarase (*fumC*) (28)
Glutamate + glutamine	100	100	100
Aspartate + asparagine	110	173	88
Threonine	71	76	49
Serine	58	73	55
Proline	0	173	37
Glycine	134	116	67
Alanine	140	100	112
Valine	129	78	57
Isoleucine	84	57	43
Leucine	145	86	102
Tyrosine	23	149	8
Phenylalanine	62	70	22
Lysine	53	73	47
Histidine	33	30	29
Arginine	105	81	41

[a] The amino acid compositions of the purified enzymes are shown as a percentage of the glutamate-plus-glutamine content.

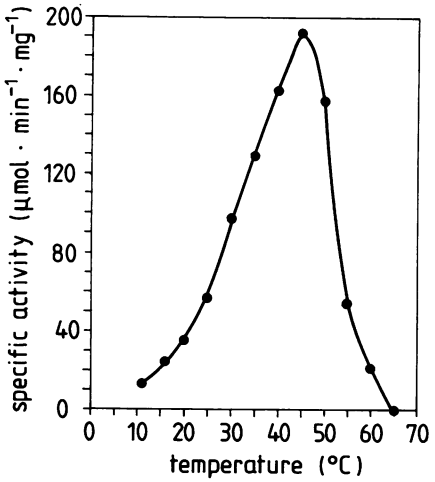

FIG. 2. Specific activity of malease as a function of temperature (50 mM Tris-HCl, pH 7).

Kinetics. The K_m for maleate was determined at different pH values. Lineweaver-Burk plots and Eadie-Hofstee plots resulted in comparable values for K_m and V_{max}. The apparent K_m for maleate depended strongly on the pH (Fig. 4). Recalculation of the K_m values on the basis of the concentrations of the dianionic form of maleate (pK_{a_2} = 6.23 [23]) gave values which varied much less with the pH (Fig. 4), indicating that only maleic acid present in the dianionic form is a substrate for malease.

When the K_m of malease for maleate was determined in 50 mM phosphate buffer (pH 7.0) instead of in imidazole or Tris-HCl buffer, a much higher K_m (1.9 mM) was found, while the V_{max} was two times lower.

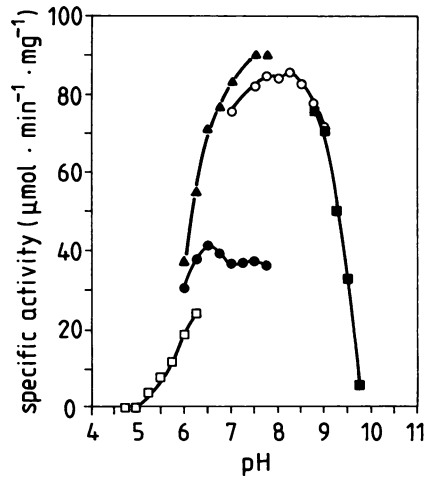

FIG. 3. Specific activity of malease as a function of pH (30°C). □, 50 mM succinate buffer; ●, 50 mM phosphate buffer; ▲, 50 mM imidazole buffer; ○, 50 mM Tris-HCl buffer; ■, 50 mM glycine buffer.

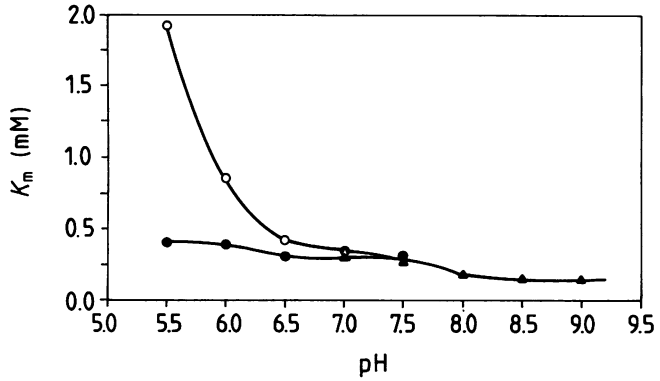

FIG. 4. Michaelis-Menten constant (K_m) as a function of pH (30°C). Open symbols, K_ms based on the total maleate concentration; closed symbols, K_ms based on the concentration of dianionic maleate. Circles, 50 mM imidazole buffer; triangles, 50 mM Tris-HCl buffer.

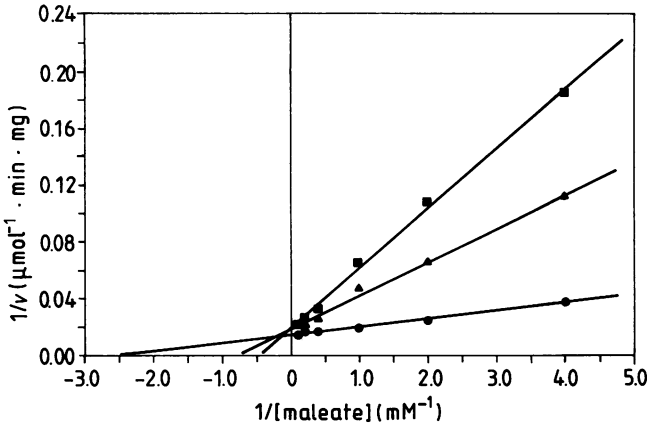

FIG. 5. Competitive inhibition of malease by D-malate (50 mM Tris-HCl, pH 7, 30°C). ●, no D-malate; ▲, 2 mM D-malate; ■, 4 mM D-malate.

The K_m of malease for citraconate was 0.20 mM in Tris-HCl buffer (pH 7.5). Correction for the amount of citraconate present in the dianionic form (pK_{a_2} = 6.15 [23]) did not significantly change this value.

Effects of ions. The effects of several ions on malease activity were tested (Table 4). No stimulation of malease activity was observed with any of the salts tested. $NiCl_2$, $CuSO_4$, and $Fe_2(SO_4)_3$ inhibited malease activity to some extent, while $HgCl_2$ and $ZnSO_4$ completely inhibited malease activity.

The monovalent ions KCl, LiCl, CsCl, NH_4Cl, and NaCl (all at 10 mM concentration) did not have any significant influence on malease activity. NaCl at concentrations higher than 10 mM inhibited malease activity; 50, 100, and 200 mM NaCl inhibited malease activity by 8, 21, and 38%, respectively.

Inhibitors. A variety of enzyme inhibitors were tested for their ability to inhibit malease activity. The thiol reagents iodoacetamide and *p*-chloromercuribenzoate (at, respectively, 1 and 0.1 mM) completely inhibited malease activity, while iodoacetate (1 mM) inhibited malease activity only slightly (4%). The carbonyl reagents hydroxylamine, phenylhydrazine, and semicarbazide (all at 1 mM) did not inhibit malease activity. The chelating agents EDTA, *o*-phenanthroline, nitrilotriacetate, and α,α'-dipyridyl (all at 1 mM) also did not inhibit malease. The reducing agents dithiothreitol, cysteine, 2-mercaptoethanol, and glutathione (all at 1 mM) did not affect malease activity. When malease was preincubated with one of these reducing agents for 30 min at 30°C in the presence of 1 mM $FeSO_4$ (which is the activation mixture as reported for the related iron-sulfur cluster-containing carbon-2-substituted maleate hydratases [10]), a decrease of malease activity was observed. SDS (1 mM) completely inhibited malease activity, while KCN (1 mM) did not cause any inhibition.

Inhibitory effect of various substrate analogs and substrates. D-Malate (Fig. 5), D-citramalate, and also some product and substrate analogs were tested for their abilities to inhibit malease activity with maleate as the substrate. The inhibition constants (K_i), representing the dissociation of the enzyme-product complex in the case of the products D-malate and D-citramalate or the dissociation of the enzyme-inhibitor complex in the case of product and substrate analogs, are given in Table 5. Dicarboxylic acids (C_4) with two substituents at C-2 (e.g., 2,2-dimethylsuccinate and D-citramalate) were the most effective competitive inhibitors of malease (Table 5). The L enantiomers of malate and citramalate were also competitive inhibitors of malease activity (Table 5).

Equilibrium constant. The equilibrium constant of the maleate hydration reaction was determined. Under standard

TABLE 4. Effect of ions on malease activity

Salt[a]	Relative activity (%)
None	100
$HgCl_2$	0
$CaSO_4$	97
$ZnSO_4$	0
$CoSO_4$	98[b]
$BaCl_2$	99
$NiCl_2$	85
$MnSO_4$	96
$CuSO_4$	40[b]
$MgSO_4$	98
$FeSO_4$	97[b]
$Fe_2(SO_4)_3$	33[b]

[a] All ions were at 1 mM final concentration.
[b] Determined by HPLC because of complex formation between maleate and the cation, which disturbed the spectrophotometric assay.

TABLE 5. Competitive inhibitors of malease

Inhibitor	K_i (mM)
Succinate[a]	6.3
2-Methylsuccinate[a]	0.76
2,2-Dimethylsuccinate[a]	0.025
L-Malate[a]	8.2
D-Malate[b]	0.63
L-Citramalate[a]	0.65
D-Citramalate[b]	0.083
L-Tartrate[a]	0.70
D-Tartrate[a]	10
Itaconate[a]	0.74
DL-Mercaptosuccinate[a]	0.88

[a] K_i is for dissociation of the enzyme-inhibitor complex.
[b] K_i is for dissociation of the enzyme-product complex.

biochemical conditions (25°C, pH 7.0, and an ionic strength of 0.1 M [18]), the apparent equilibrium constant (K_{app} = [hydroxy acid]$_{total}$/[unsaturated acid]$_{total}$) was 2,050 ± 100, starting either from maleate or from D-malate.

The apparent equilibrium constants for hydrating citraconate (starting either from citraconate or from D-citramalate) and 2,3-dimethylmaleate were 104 ± 6 and 11.2 ± 0.5, respectively.

DISCUSSION

This report describes the purification and characterization of maleate hydratase (malease) from *P. pseudoalcaligenes*. This strain was previously selected for the production of D-malate (25), which can be used as a synthon (starting compound for chemical synthesis) in the fine-chemical industry. Special attention was paid to the substrate specificity and possible cofactor requirements of malease and to the apparent equilibrium constant of the hydration reaction, since these factors are of prime importance for the commercial use of such enzymes.

Malease from *P. pseudoalcaligenes* does not require the addition of cofactors for full enzyme activity. The enzyme, unlike maleate hydratase from rabbit kidney (10) and unlike aconitate hydratase, homoaconitate hydratase, and isopropylmaleate hydratase (11), does not contain an iron-sulfur cluster. Oxidation of the iron-sulfur cluster in these enzymes results in the loss of one of the irons and in loss of activity (2). Subsequent incubation with iron and a sulfur compound results in reincorporation of iron in the iron-sulfur cluster and restoration of activity. These brownish enzymes are completely inhibited by metal-complexing agents at concentrations higher than the metal ion concentration present in the activity assay. Although citraconase (2-methylmaleate hydratase) (22) and 2,3-dimethylmaleate hydratase (14) were only partially purified, they had the same characteristics as the other reported iron-sulfur hydratases (reactivation by a metal ion and a sulfur compound and complete inhibition by metal-complexing agents), suggesting that these enzymes also contain an iron-sulfur cluster.

Malease from *P. pseudoalcaligenes*, however, is not activated (it is even inactivated) by incubation with iron and a sulfur compound, and it is not strongly inhibited by chelating agents (and thiol reagents), suggesting that it is another type of enzyme different from malease from rabbit kidney.

A similar situation with two different lyases catalyzing the same reaction exists with fumarases. These enzymes catalyze the hydration of the *trans* isomer of maleate. One type is cofactor independent, while the other type contains an iron-sulfur cluster (24).

Malease from *P. pseudoalcaligenes* also differs from the enzyme from rabbit kidney because it is dimeric rather than monomeric. Also, its amino acid composition is quite different from that of the rabbit enzyme (Table 3), and high NaCl concentrations inhibit rather than stimulate (7) the enzyme. The bacterial enzyme has a much higher affinity for maleate than the rabbit enzyme (K_m = 0.35 versus 10 mM) and, in contrast to the rabbit enzyme, also catalyzes the hydration of citraconate and 2,3-dimethylmaleate.

Malease from *P. pseudoalcaligenes* is also quite different from the cofactor-independent fumarase from *E. coli* (24), which has four identical subunits and a completely different amino acid composition (Table 3).

The enzyme apparently catalyzes the hydration only of substrates containing two carboxyl groups in the *cis* position (maleate, citraconate, and 2,3-dimethylmaleate). Substrates having the *cis* configuration but containing a methyl or a chloro group instead of a second carboxyl group were not hydrated. The fact that two carboxyl groups are necessary for activity suggests that these two groups are bound in the reaction center of the enzyme by bases and that when only one acid group is present, the substrate cannot be bound in the right configuration for hydration.

Because the enzyme is very unstable at pH values below 5 and above 9, the K_m could not be determined in these pH regions. Therefore, we are unable to say anything about the nature of the active groups in the reaction center, except that histidine is probably not involved as is the case with fumarase (16). With fumarase, large (10-fold) changes in the K_m are observed between pHs 6 and 8 (16).

The equilibrium constants for the maleate hydration (K_{app} = 2,050) and the citraconate hydration (K_{app} = 104) reactions are much higher than the equilibrium constants for the hydration of the *trans* analogs (for the fumarate hydration reaction, K_{app} = 4.4 [3], and for the mesaconate hydration reaction, K_{app} = 5.7 [8]). This is in contrast with the group contribution theory, which does not correct for *cis* or *trans* configuration at the double bound (17), thus giving the same equilibrium constants for the hydration of the *cis* and the *trans* compounds. However, the K_{app} for the hydration of *cis*-crotonyl coenzyme A (K_{app} = 5.5) is also higher than the K_{app} for the *trans* isomer (K_{app} = 3.5) (21). The K_{app} for the 2,3-dimethylmaleate hydration reaction was 11.2 (25°C). Previously the K_{app} for this reaction was reported to be 2.3 at 35°C (14).

Unfortunately, both potential commercial products of the hydration reaction, D-malate and D-citramalate, inhibit malease from *P. pseudoalcaligenes* (Table 5). For application of this enzyme on a commercial scale, it may therefore be necessary to (continuously) remove the product of the reaction, for instance, by precipitation with Ca^{2+} (12).

Subsequent work will focus on the thermodynamics of the maleate and citraconate hydration reactions. Also, the optimization of malease induction in *P. pseudoalcaligenes* and further optimization of the reaction conditions for the production of D-malate and D-citramalate will be studied.

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REFERENCES

1. **Bahnson, B. J., and V. E. Anderson.** 1991. Crotonase-catalyzed β-elimination is concerted: a double isotope effect study. Biochemistry **30:**5894–5906.
2. **Beinert, H., and M. C. Kennedy.** 1989. Engineering of protein bound iron-sulfur clusters. A tool for the study of protein and cluster chemistry and mechanism of iron-sulfur enzymes. Eur. J. Biochem. **186:**5–15.
3. **Bock, R. M., and R. A. Alberty.** 1953. Studies of the enzyme fumarase. I. Kinetics and equilibrium. J. Am. Chem. Soc. **75:**1921–1925.
4. **Bode, R., I. A. Samsonova, and D. Birnbaum.** 1991. Production of α- and β-isopropylmalate by a mutant from *Candida maltosa*. Zentralbl. Mikrobiol. **146:**35–39.
5. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
6. **Brewer, J. M.** 1981. Yeast enolase: mechanism of activation by metal ions. Crit. Rev. Biochem. **11:**209–254.
7. **Britten, J. S., H. Morell, and J. V. Taggart.** 1969. Anion activation of maleate hydratase. Biochim. Biophys. Acta **185:** 220–227.
8. **Buckel, W., and S. L. Miller.** 1987. Equilibrium constants of

several reactions involved in the fermentation of glutamate. Eur. J. Biochem. **164**:565–569.
9. **Chibata, I., T. Tosa, and I. Takata.** 1983. Continuous production of L-malic acid by immobilized cells. Trends Biotechnol. **1**:9–11.
10. **Dreyer, J.-L.** 1985. Isolation and biochemical characterization of maleic-acid hydratase, an iron-requiring hydro-lyase. Eur. J. Biochem. **150**:145–154.
11. **Emptage, M. H.** 1988. Active-site similarities among aconitase, isopropylmalate isomerase, and homoaconitase. Biochemistry **27**:3104.
12. **Furui, M., N. Sakata, O. Otsuki, and T. Tosa.** 1988. A bioreactor-crystallizer for L-malic acid production. Biocatalysis **2**:69–77.
13. **Hartmans, S., J. P. Smits, M. J. van der Werf, F. Volkering, and J. A. M. de Bont.** 1989. Metabolism of styrene oxide and 2-phenylethanol in the styrene-degrading *Xanthobacter* strain 124X. Appl. Environ. Microbiol. **55**:2850–2855.
14. **Kollmann-Koch, A., and H. Eggerer.** 1984. Nicotinic acid metabolism. Dimethylmaleate hydratase. Hoppe-Seyler's Z. Physiol. Chem. **365**:847–857.
15. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
16. **Massey, V.** 1953. Studies on fumarase. 4. The effects of inhibitors on fumarase activity. Biochem. J. **55**:172–177.
17. **Mavrovouniotis, M. L.** 1991. Estimation of standard Gibbs energy changes of biotransformations. J. Biol. Chem. **266**: 14440–14445.
18. **Miller, S. L., and D. Smith-Magowan.** 1990. The thermodynamics of the Krebs cycle and related compounds. J. Phys. Chem. Ref. Data **19**:1049–1073.
19. **Nagasawa, T., and H. Yamada.** 1990. Large-scale bioconversion of nitriles into useful amides and acids, p. 277–318. *In* D. A. Abramowicz (ed.), Biocatalysis. Van Nostrand Reinhold, New York.
20. **Russell, R. N., and H.-W. Liu.** 1991. Stereochemical and mechanistic studies of CDP-D-glucose oxidoreductase isolated from *Yersinia pseudotuberculosis*. J. Am. Chem. Soc. **113**:7777–7778.
21. **Stern, J. R., and A. del Campillo.** 1956. Enzymes of fatty acid metabolism. II. Properties of crystalline crotonase. J. Biol. Chem. **218**:985–1002.
22. **Subramanian, S. S., and M. R. Raghavendra Rao.** 1968. Purification and properties of citraconase. J. Biol. Chem. **243**:2367–2372.
23. **Tu An, T., K. Tóth, and E. Pungor.** 1974. Electrochemical investigations in non-aqueous media. I. Investigation of the dissociation of dibasic acids. Anal. Chim. Acta **71**:151–156.
24. **Ueda, Y., N. Yumoto, M. Tokushige, K. Fukui, and H. Ohya-Nishiguchi.** 1991. Purification and characterization of two types of fumarase from *Escherichia coli*. J. Biochem. **109**:728–733.
25. **van der Werf, M. J., W. J. J. van den Tweel, and S. Hartmans.** 1992. Screening for microorganisms producing D-malate from maleate. Appl. Environ. Microbiol. **58**:2854–2860.
26. **Vederas, J. C., and H. G. Floss.** 1980. Stereochemistry of pyridoxal phosphate catalyzed enzyme reactions. Acc. Chem. Res. **13**:455–463.
27. **Wang, Y., and R. G. Finke.** 1989. Coenzyme B_{12} dependent diol dehydratase model studies: chemical evidence against Co(B_{12}) participation in the rearrangement step. Inorg. Chem. **28**:983–986.
28. **Woods, S. A., J. S. Miles, R. E. Roberts, and J. R. Guest.** 1986. Structural and functional relationships between fumarase and aspartase. Nucleotide sequences of the fumarase (*fumC*) and aspartase (*aspA*) genes of *Escherichia coli* K12. Biochem. J. **237**:547–557.