Memoirs of the Faculty of Science, Kyoto University, Series of Physics, Astrophysics, Geophysics and Chemistry, Vol. XXXVII, No. 1, Article 2, 1986

Characterization of Two Enzymes with Fumarase Activity in *Pseudomonas arvilla* and Their Physiological Roles

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(*Received March 15, 1985*)

Summary

Two enzymes with fumarase activity (named enzymes I and II) in cell-free extracts of *Pseudomonas arvilla* were separated from one another by DEAE-Sephadex column chromatography. Enzyme I, having a high substrate specificity, catalyzed only the dehydration of L-malate and hydration of fumarate and did not require ferrous ions for its activity. In contrast, enzyme II catalyzed the dehydration of L-citramalate and hydration of mesaconate as well as the above two reactions and required ferrous ions and some kinds of SH-compounds such as 2-mercaptoethanol. It was concluded that enzymes I and II were fumarase (EC 4·2·1·2) and mesaconase (EC 4·2·1·34), respectively. Based on the facts that enzyme II was rapidly inactivated under the conditions of low ionic strength and it had different degrees of activities against L-malate and L-citramalate, a method for the differential determination of both enzymes in crude extracts of cells was devised. Enzyme I showed a high level in the L-malate-, glucose- and L-glutamete-media not containing ferrous salt, but it was strongly repressed by the addition of the salt to each medium. In contrast, enzyme II showed a low level in the media not containing ferrous salt, but it was strongly induced by the addition of the salt. No marked difference depending on the kinds of crabon source was observed in each enzyme level among the cells grown in these media. This bacterium was found unable to be adapted to itaconate, since the addition of itaconate to the glucose-medium affected neither the growth of the bacterium nor the levels of itaconate-activating activities in the cell-free extracts. *Pseudomonas fluorescens*, which was able to be adapted to itaconate, showed about the same extent of enzyme II level in the cells grown on the itaconate- and glucose- media. From these results, it was concluded that enzyme II (mesaconase) is a constitutive enzyme, not an inducible one for the metabolism of C_5-branched-chain dicarboxylic acids. A possibility was proposed that mesaconase as well as fumarase is involved in the operation of the tricarboxylic acid cycle.

Introduction

Fumarase (EC 2·4·1·2), catalyzing reversibly the hydration of fumarate to form L-malate, is an enzyme which is involved in the operation of the tricarboxylic acid cycle. There is another enzyme called mesaconase (EC 4·2·1·34) which can catalyze the same reaction. As for the physiological role of mesaconase, whose intrinsic

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activity is to catalyze the interconversion between mesaconate and L-citramalate, the following observations were reported. According to Barker *et al.*, mesaconase was reported to be involved in the metabolism of mesaconate, one of the C_5-branched-chain dicarboxylates, which is formed in the catabolic metabolism of L-glutamate by *Clostridium tetanomorphum* (1,2). Katsuki *et al.* reported that the resting cells of *Pseudomonas fluorescens* grown in a glucose-medium could oxidize the dicarboxylates such as itaconate and measaconate after some lag time but the cells grown in a glucose-itaconate medium easily oxidized them without lag (3). As other possibilities of physiological roles of mesaconase in aerobic bacteria, the enzyme was reported to be involved in the L-glutamate biosynthesis (4), L-isoleucine biosynthesis (5, 6), catabolic metabolism of mesaconate (or *threo*-3-methylmalate) (7, 8) and in the operation of the tricarboxylic acid cycle (9). The diversity of conclusion of these reports indicates that the physiological role of mesaconase is not yet established.

Recently two genes for fumarase (*fum* A and *fum* B) have been reported to be present in *Escherichia coli* (10). No enzymatic study has been made on the product of the *fum* B gene, though that of the *fum* A gene was demonstrated to be fumarase (10). In this regard, Suzuki *et al.* reported that an aerobic soil bacterium had mesaconase but not fumarase (9). They proposed the speculation that mesaconase is involved in the operation of the tricarboxylic acid cycle, catalyzing the interconversion of fumarate into L-malate (9). Suzuki *et al.* also obtained evidence suggesting the existence of two enzymes with fumarase activity in *Pseudomonas arvilla* (11) grown in a glucose medium, but they did not separate them from one another.

The present paper deals with the separation, characterization and physiological roles of the two enzymes in this organism.

Materials and Methods

Chemicals—DL-Citramalic acid was synthesized by the method of Barker (12). A mixture of DL-*erythro*- and DL-*threo*-3-methylmalic acids was synthesized according to the method of Nakano *et al.* (13), and they were separated from one another by the method of Sasaki *et al.* (14). Sodium salts of D- and L-citramalic acids, D-malic acid, Tris, DEAE-Sephadex A-50 and Sephadex G-50 (fine) were purchased from Sigma Chemical Co. 4-(2-Hydroxyethyl)-1-piperazineethane-sulfonic acid (HEPES) and 3-[tris(hydroxymethyl)-methylamino]-1-propanesulfonic acid (TAPS) were obtained from Research Organics Inc. and Dojin Chemicals, respectively. Fumaric, mesaconic and L-malic acids were purchased from Nakarai Chemicals. Itaconic acid, a special grade producet from Nakarai Chemicals, was used after recrystallization twice. Other chemicals from Nakarai Chemicals and Wako Chemicals used were of special grade. The dicarboxylic acids described above were used after neutralization with Tris, NaOH or KOH as will be indicated.

Organisms—The following medium was used for the cultivation of *Pseudomonas arvilla* (ATCC23974) and *Pseudomonas fluorescens* (IFO3081) according to Suzuki *et al.* (8): the growth medium contained 7 g of K_2HPO_4, 3 g of KH_2PO_4, 1.5 g of

$(NH_4)_2SO_4$, 100 mg of $MgSO_4 \cdot 7H_2O$, 50 mg of $FeSO_4 \cdot 7H_2O$ (unless otherwise indicated) and 5 g of compound as a carbon source in 1 l of distilled water, and was neutralized (pH 6.8) with sodium hydroxide. When glucose or L-glutamic acid was to be used as a carbon source, it was dissolved into small volume of water, and the solution was sterilized in an autoclave separately from the other inorganic constituents. In the case of itaconic acid, its solution, after neutralized with sodium hydroxide, was sterilized by ultrafiltration. Then, each solution was mixed with other inorganic constituents to prepare each growth medium.

Adaptation Treatment to Itaconate—*Ps. arvilla* was grown with shaking in a medium containing 1 g of glucose and 4 g of itaconic acid as a carbon source according to Katsuki *et al.* (15) at 30°C for 24 hr.

Besides the above medium, the one containing only itaconate as a carbon source was used for *Ps. fluorescens* since the strain used was capable of growing in this medium.

The Crude Enzyme Preparation for Separation of Two Enzymes with Fumarase Activity—*Ps. arvilla* was grown in a medium containing and not containing ferrous salt and harvested at late logarithmic growth phase. Both samples of cells were stored at −20°C until use. The cells (5 g in wet weight) were suspended in 10 ml of 50 mM Tris-HCl buffer (pH 8.0) and sonicated at 20 kHz for 10 min under the atomosphere of nitrogen. After distruption of the cells, 15 ml of the same buffer was added to the mixture, stirred and centrifuged for 20 min at 10,000 xg. To the obtained supernatant was added 14 ml of 3% streptomycin sulfate solution, and the resulting precipitate was removed by centrifugation. To the supernatant was added ammonium sulfate (0.472 g/ml) to obtain 70% saturation, and the solution was stirred for 1 hr. The precipitate was dissolved in 10 ml of 50 mM Tris-HCl buffer (pH 8.0) ("The crude enzyme solution"). This preparation was stable enough for more than one month if stored at 0°C. Both samples of the cells grown in the media containing and not containing ferrous salt gave rise to the crude enzyme solutions with 1015 U (547 mg of protein) and 1440 U (480 mg of protein) of fumarase activity, respectively. Portions of these crude enzyme solutions (105 and 73 U, respectively) were mixed together, and the mixture was used as the crude enzyme preparation for separation of the two enzymes.

DEAE-Sephadex Column Chromatography—All the procedures which will be described in this section were performed under nitrogen atmosphere. The crude enzyme preparation obtained was passed through a Sephadex G-50 column (2.3×6.5 cm) using 50 mM Tris-HCl buffer (pH 9.0) containing 0.25 M KCl to remove substances with low molecular weight. The obtained solution was then applied to a DEAE-Sephadex A-50 column (1.3×55 cm) which had been equilibrated with the same buffer. Elution was carried out with a linear gradient of KCl concentration from 0.25 to 0.6 M in 50 mM Tris-HCl buffer (pH 9.0) (total volume, 400 ml). Elution rate was 12 ml/hr. To every 21-ml fraction collected was added 50 mM Tris-HCl buffer containing 0.5 mM ferrous ammonium sulfate and 50 mM 2-mercaptoethanol in final concentrations, and the mixture was stored at 0°C.

Reactivation of Enzyme—To the enzyme sample (250 μl) was added in final concentrations 50 mM 2-mercaptoethanol, 0.5 mM ferrous ammonium sulfate and 0.4 M

KCl according to the method of Blair and Barker (16), and the mixed solution (500 μl) was incubated for 2 hr at 30°C. Aliquot (10 μl) of the solution was used for determination of the enzyme activity.

Determination of Enzyme Activity—The enzyme activity was determined at 30°C under nitrogen atmosphere according to Suzuki *et al.* (9). The assay mixture contained, in 1 ml of total volume, 20 μmol of L-malate or DL-citramalate (neutralized with Tris, pH 8.0) and the enzyme. Reaction was initiated by the addition of the enzyme, and the increase of abosrption in ultraviolet region due to the formed fumarate (ε_{240}=2530) or mesaconate (ε_{250}=2260) was followed. One unit (U) of the enzyme activity was defined as the activity producing 1 μmol of the reaction product in 1 min under the assay conditions. For this spectrophotometric measurement, a Hitachi 214 spectrophotometer was used.

Differential Determination of Enzymes I and II—As will be described, two enzymes with fumarase activity (named enzymes I and II) were separated from one another in the crude enzyme preparation. The levels of the two enzymes in the cells grown under various conditions were determined by the method which will be described. The following procedures were performed under nitrogen atmosphere unless otherwise indicated. The cells of *Ps. arvilla* or *Ps. fluorescens* harvested at late logarithmic growth phase were suspended in 50 mM Tris-HCl buffer (pH 8.0) containing 0.4 M KCl, sonicated and centrifuged at 10,000 xg for 20 min. The resulting supernatant was subjected to the reactivation treatment. The solution was divided into two parts. One part was passed through a Sephadex G-50 column (1.0×5.0 cm) using 50 mM Tris-HCl buffer (pH 8.0) containing 0.4 M KCl, 50 mM 2-mercaptoethanol and 0.5 mM ferrous ammonium sulfate (named buffer A, in which enzyme II is stable). The other part was similarly gel-filtered using 50 mM Tris-HCl buffer (pH 8.0) containing 50 mM 2-mercaptoethanol (named buffer B, in which enzyme II is inactivated). With the enzyme preparation obtained by the gel-filtration using buffer A, the enzyme activity against L-malate (F_a) and that against DL-citramalte (M) were determined. With the enzyme preparation obtained by the gel-filtration using buffer B, the activity (F_b) was determined. When the concentration of the enzyme preparation was too high and the activity against DL-citramalate remained, the preparation was diluted with buffer B and was left to stand for about 2 hr or more (in air in the case of *Ps. fluorescens*) to inactivate measconase activity.
The activities of enzymes I and II were obtained as followes;

i) When F_b was higher than (F_a-F_b),
the activity of enzyme I=F_b, and
the activity of enzyme II=M/r,
where r represents the ratio of the activity of enzyme II
against DL-citramalate to that of enzyme II against L-malate.
(r=0.61 for *Ps. arvilla*, r=0.67 for *Ps. fluorescens*)

ii) When F_b was less than (F_a-F_b),
the activity of enzyme I=F_b, and
that of enzyme II=F_a-F_b.

Assay of Itaconate-Activating Enzyme—The activity of itaconate-activating enzyme was assayed by the hydroxamate method (17).

Other Methods—The amount of protein was determined by the method of Lowry *et al.* (18) using bovine serum albumin as a standard.

Results

Separation of Two Enzymes with Fumarase Activity in Ps. arvilla—Suzuki *et al.* reported that the mesaconase and fumarase predominated in the cells grown in the media containing and not containing ferrous salt, respectively, but they did not separate the two enzymes from one another (11). In order to separate them clearly, the crude enzyme solutions obtained from both cultures (containing 3.0 U and 1.9 U of fumarase activity per mg of protein, respectively) were mixed together, and the mixture was applied to a DEAE-Sephadex column. Elution was performed with increasing concentrations of KCl, and the enzyme activities against L-malate and DL-citramalate were determined with every fraction eluted.

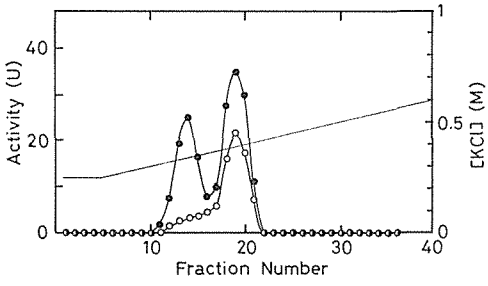

Fig. 1. Separation of two enzymes with fumarase activity by DEAE-Sephadex column chromatography. The crude enzyme preparation described in "MATERIALS AND METHODS" was used as a sample for the chromatography. The conditions for the chromatography are described in "MATERIALS AND METHODS". The enzyme activities against L-malate (-●-) and DL-citramalate (-○-) were determined.

As shown in Fig. 1, two peaks of the activity appeared when L-malate was used as a substrate, and one peak of the activity when DL-citramalate was used. This suggested that *Ps. arvilla* has two enzymes with fumarase activity. The enzyme eluted first was named enzyme I and the one eluted next enzyme II. In the subsequent study, the enzyme preparations in fractions 14 and 19 were used as enzymes I and II, respectively. Since the enzyme preparation in fraction 14 contained small amounts of enzyme II, the preparation was subjected to an inactivation treatment of enzyme II which will be described subsequently.

Stabilities of Enzymes I and II in Ps. arvilla at Low Concentrations of Salt—When the solution eluted from the DEAE-Sephadex column, which contained high concentrations (about 0.4 M) of KCl, was passed through a Sephadex G-50 column, enzyme II was found to be rapidly inactivated (preliminary experiment). This led us to examine the stabilities of both enzymes at low concentrations of salt.

KCl was removed from the enzyme preparation by passing through a Sephadex G-50 column using 50 mM Tris-HCl buffer (pH 8.0) containing ferrous ammonium sulfate and 2-mercaptoethanol. The obtained enzyme solution was left to stand at 20°C, and variations of both enzyme activities were determined (Fig. 2).

No loss of the activity was observed with enzyme I even after 190 min, whereas

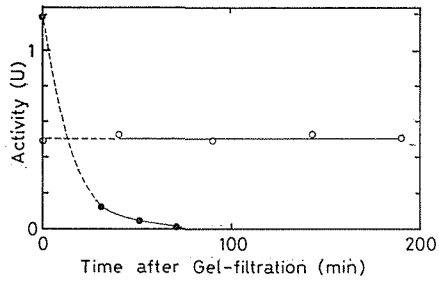

Fig. 2. Stabiliteis of enzymes I and II at low concentrations of KCl. Fraction 14 (0.49 U, 64 ng of protein) and fraction 19 (1.34 U, 70 ug of protein) obtained in the chromatography shown in Fig. 1 were used as samples of enzymes I and II, respectively. They were gel-filtered through a Sephadex G-50 column (1.0×5.0 cm) using 50 mM Tris-HCl buffer (pH 8.0) containing 50 mM 2-mercaptoethanol and 0.5 mM ferrous ammonium sulfate to obtain the enzyme preparations to be tested, respectively. They were left to stand at 20°C, and aliquots were withdrawn at definite time intervals to determine the enzyme activity against L-malate. (-●-) and (-○-) indicate the activities of enzymes I and II, respectively.

the activity of enzyme II completely disappeared within 100 min notwithstanding the presence of ferrous ammonium sulfate and 2-mercaptoethanol. However, the acticvity of enzyme II with residual activity of about 1% after the treatment was partially restored by the addition of KCl (0.4 M in a final concentration)—restoration of 10% after 70 min and 16% after 25 hr.

Stabilization of Enzyme II by Various Kinds of Alkali Salts—The observation that enzyme II was stabilized by 0.4 M KCl led us to examine the effect of other alkali salts in the stabilization. The enzyme II preparation was gel-filtered similarly as described in Fig. 2 using other alkali salts than KCl. From the obtained solution, aliquots (10 μl) were withdrawn and the enzyme activity was determined.

As shown in Table 1, LiCl, NaCl and K_2SO_4 as well as KCl stabilizde enzyme II.

Effects of Various Salts on the Activities of Enzymes I and II—Various salts were added to the assay mixture to a final concentration of 0.1 M except for $CaCl_2$ added for enzyme II assay, and the enzyme activities were determined.

Table 1. Stabilization of enzyme II by various alkali salts. The enzyme solution (fraction 19, 70 μg of protein) (Fig. 1) was gel-filtered through a Sephadex G-50 column (1.1×3.0 cm) using 50 mM Tris-HCl buffer (pH 8.0) containing 0.5 mM ferrous ammonium sulfate, 50 mM 2-mercaptoethanol and 0.4 M indicated alkali salt. The obtained enzyme preparation (23 μg of protein/ml) was left to stand until the activity of the control run (None") disappeared (about 2 hr), and aliquots (10 μl) were withdrawn for the determination of enzyme activity against L-malate and DL-citramaltae.

Addition	Enzyme activity (U)	
	L-Malate	DL-Citramalate
None	0.01	0.00
LiCl	1.29	0.71
NaCl	1.27	0.73
KCl	1.27	0.69
K_2SO_4	1.33	0.83

Table 2. Effects of various salts on the activities of enzymes I and II. The reaction mixture (pH 8.0) contained 20 mM substrate (neutralized with Tris) and indicated salt in a total volume of 1 ml. The concentration of salt added was 100 mM except for $CaCl_2$ (50 mM) for enzyme II. The amounts of enzymes I and II used for the assay against L-malate were 4.8 and 8.1 mU, respectively.

Addition	Activity (mU)		
	Enzyme I	Enzyme II	
	L-Malate[a)]	L-Malate[a)]	L-Citramalate[a)]
None	4.8	8.1	4.9
LiCl	4.8	4.7	2.3
NaCl	4.9	4.3	2.1
KCl	5.0	4.5	2.0
K_2SO_4	7.4	9.5	2.4
$KHCO_3$	4.0	2.6	0.0
KOAc	5.8	9.3	4.1
K_2HPO_4	5.7	9.1	3.5
$(NH_4)_2SO_4$	6.8	8.3	1.9
Tris-HCl	4.1	4.0	1.8
Tris-AcOH	4.8	9.5	4.2
Tris-H_2SO_4	5.5	8.5	1.9
$MgSO_4$	6.2	7.7	1.2
$CaCl_2$	3.5	4.2[b)]	1.4[b)]

a) substrate, b) 50 mM

Table 2 shows a part of the results. LiCl, NaCl and KCl showed no effect on enzyme I. $CaCl_2$, Tris-HCl and $KHCO_3$ as well as $MgCl_2$ and $BaCl_2$ (data not shown) showed a weak inhibition. K_2SO_4, K_2HPO_4, CH_3CO_2K, $MgSO_4$, $(NH_4)_2SO_4$ and Tris-H_2SO_4 showed a weak activation. The enzyme II activity against L-malate was inhibited by LiCl, NaCl, KCl, $NaHCO_3$, Tris-HCl, $MgSO_4$ and $CaCl_2$ and was activated by K_2SO_4, CH_3CO_2K, K_2HPO_4, $(NH_4)_2SO_4$, Tris-CH_3CO_2H and Tris-H_2SO_4. The enzyme II activity against DL-citramalate was inhibited by all the salts tested.

Substrate Specificities of Enzymes I and II—Table 3 shows substrate specificities of both enzymes. L-Malate and fumarate served as a substrate against enzyme I. L-Citramalate and mesaconate as well as L-malate and fumarate served as a substrate against enzyme II.

D-malate, D-citramalate, DL-*erythro*-3-methylmalate, DL-*threo*-3-methylmalate, maleate, citraconate and itaconate did not serve as a substrate against both enzymes.

Activation of Enzymes I and II—Although the activity of enzyme I was considerably stable, the omission of 2-mercaptoethanol from the mixture for the enzyme stock gave rise to decrease in the activity (Table 4).

The addition of 2-mercaptoethanol to the mixture restored the activity in spite of the presence or absence of ferrous ions. Enzyme II was inactivated by the omission

Table 3. Substrate specificities of enzymes I and II. The assay mixture (pH 8.0) for the dehydration reaction contained in a total volume of 1.0 ml, 20 μmol of substrate (neutralized with Tris) and that (pH 8.0) for the hydration contained 1 μmol of substrate and 50 μmol of Tris-HCl buffer. The amounts of enzymes I and II were 1.6 and 1.6 μg of protein, respectively. Reaction was performed at 30°C.

Substrate	Activity (mU)	
	Enzyme I	Enzyme II
Dehydration (20 mM)		
L-Malate	4.8	19.8
D-Malate	0	0
L-Citramalate	0	11.9
D-Citramalate	0	0
DL-*erythro*-3-methylmalate	0	0
DL-*threo*-3-methylmalate	0	0
Hydration (1.0 mM)		
Fumarate	3.8	27.3
Malaete	0	0
Mesaconate	0	5.5
Citraconate	0	0
Itaconate	0	0

Table 4. Reactivation of enzymes I and II. The mixture for the reactivation contained, in a total voluem of 260 μl, 0.5 mM ferrous ammonium sulfate, 50 mM 2-mercaptoethanol, 50 mM Tris-HCl buffer (pH 8.0) and the enzyme. After the reaction was performed at 30°C for 2 hr for reactivation, aliquots (10 μl) were withdrawn for the determination of enzyme activity against L-malate. Since it was favorable to use the enzyme preparations whose activity was decreased (enzyme I) or inactivated (enzyme II) for this experiment, the enzymes were gel-filtered through a Sephadex G-50 column (1.0×5.0 cm) using 50 mM Tris-HCl buffer (pH 8.0) containing 0.4 M KCl. The amounts of enzymes I and II used were 19.2 and 8.1 μg of protein, respectively.

Conditions	Activity (mU)	
	Enzyme I	Enzyme II
Before Treatment	71	0
Complete	113	40
—2-ME	21	0
—Fe^{2+}	111	0
—2-ME, —Fe^{2+}	38	0

of ferrous ions and 2-mercaptoethanol, indicating that both of them were necessary for the reactivation. The minimum concentration of ferrous ions necessary for 50% reactivation of enzyme II was 0.8 μM.

Examination was carried out whether or not divalent metal ions other than ferrous ion are effective for the reactivation of enzyme II.

As shown in Table 5, Ca^{2+}, Co^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Pd^{2+} and Zn^{2+} were ineffecitve.

Besides 2-mercaptoethanol, 3-mercaptopropionate, DL-homocysteine and glutathione were effective for the reactivation of enzyme II, but 2-mercaptoethylamine, D- and L-cysteine were ineffective.

Table 5. Effects of various divalent cations on the reactivation of enzyme II. The reaction mixture for the reactivation contained, in a total volume 250 μl, 50 mM Tris-HCl buffer (pH 8.0), 50 mM 2-mercaptoethanol, 0.5 mM indicated divalent metal salt and the enzyme (9.8 μg of protein). After the incubation at 30°C for 2 hr, aliquots (10 μl) were withdrawn for the determination of enzyme activity against L-malate.

Addition	Activity (mU)
None	9
$CaCl_2$	11
$CoCl_2$	10
$CuCl_2$	6
$FeCl_2$	68
$MgCl_2$	8
$MnCl_2$	10
$NCli_2$	7
$PdCl_2$	10
$ZnCl_2$	10

Table 6. Effects of various SH-compounds on the reactivation of enzyme II. The reaction mixture for the reactivation contained 50 mM Tris-HCl buffer (pH 8.0), 50 mM SH-compounds, 0.5 mM ferrous ammonium sulfate and the enzyme (9.4 μg of protein) in a total volume of 300 μl. After the incubation at 30°C for 2 hr, aliquots (10 μl) were withdrawn for the determination of the enzyme activity against L-malate.

SH-compound	Activity (mU)
None	0
2-Mercaptoethylamine	0
D-Cysteine	0
L-Cysteine	0
2-Mercaptoethanol	45
3-Mercaptopropionate	34
DL-Homocysteine	38
Glutathione	24

Kinetic Properties of Enzymes I and II—Kinetic properties of enzyme I and enzyme II were determined. Table 7 summarizes *K*m value, its relative value ($V_{max\ rel}$) and optimal pH value for each enzyme.

*K*m values of enzyme I against L-malate and fumarate were similar to those of enzyme II against L-malate and fumarate, respectively. Moreover, *K*m and optimal pH of enzyme II against L-citramalate and mesaconate showed similar values to those of the same enzyme against L-malate and fumarate, respectively. The ratios of V_{max} of both enzymes against L-malate and fumarate at their respective optimal pH's were 1:0.65 with enzyme I and 1:1.8 with enzyme II, respectively.

Variations of Levels of Enzymes I and II of Ps. arvilla Grown in Growth Media Containing Various Compounds as Carbon Source—In order to obtain clue to physiological roles of both enzymes, the levels of both enzymes of *Ps. arvilla* grown in the media containing various compounds as a carbon source were investigated by the method as described in "MATERIALS AND METHODS." The carbon source were L-malate, glucose, L-glutamate and itaconate-glucose (4:1).

Enzyme I was found to be repressed by the presence of ferrous salt in the growth

Table 7. Kinetic properties of enzymes I and II. The activities of enzymes I and II against indicated compounds as a substrate were determined at various pH's using 50 mM HEPES-KOH buffer (pH 6.5–8.5) or TAPS-KOH buffer (pH 7.5–9.5). Optimal pH's were determined from the plots of reaction velocities versus pH values. For the determination of the *K*m and V_{max} values of enzymes I and II against indicated substrate from Lineweaver-Burk plots, reactions were carried out at their respective optimal pH's. The reaction mixture contained 50 mM TAPS-KOH or HEPES-KOH buffer for the respective optimal pH and substrate as indicated in total volume of 1.0 ml.

Substrate	Km (mM)	Relative V_{max}	pH optimum
	Enzyme I		
L-Malate	1.2	1.00	8.7
Fumarate	0.13	0.65	7.6
	Enzyme II		
L-Malate	2.0	1.00	9.8
L-Citramalate	2.5	0.63	8.5
Fumarate	0.48	1.8	8.5
Mesaconate	0.24	0.63	8.3

Table 8. Levels of enzymes I and II of *Ps. arvilla* grown under various nutritional conditions. *Ps. arvilla* was grown in media containing indicated compounds as a carbon source in the presence (50 mg in 1 l medium) or absence of ferrous sulfate, and was harvested at late logarithmic growth phase. The cell-free extracts obtained from the cells as described in "MATERIALS AND METHODS" were used for the determination of specific activities of enzymes I and II against L-malate.

Carbon source	Fe salt	Enzyme I (U/mg protein)	Enzyme II (U/mg protein)
L-Malate	—	1.38	0.04
	+	0.05	3.3
Glucose	—	0.55	0.16
	+	0.03	2.9
Glutamate	—	1.76	0.03
	+	0.13	4.0
Itaconate-Glucose (4:1)	—	0.70	0.14
	+	0.06	3.4

medium (Table 8). The enzyme level was relatively high in the cells grown in L-malate and L-glutamate media and low in those in glucose medium. This seems to suggest that enzyme I undergoes a weak nutritional repression by glucoes and do not undergo a nutritional induction by L-malate. In contrast, the presence of

ferrous salt in the growth medium seems to be necessary for the synthesis or stabilization of enzyme II protein, suggesting a strong induction of the enzyme by the salt.

When ferrous salt was present in growth media, the enzyme level was considerably high in the glucose-grown cells. No information was obtained suggesting that the adaptaiton to itaconate is necessary for the synthesis of enzyme II protein. These results rather did not give information showing whether or not the bacterium was adapted to itaconate.

Examination Whether or not Ps. arvilla Is Able to Be Adapted to Itaconate—Katsuki *et al.* reported that the enzymes involved in the metabolism of C_5-branched-chain dicarboxylic acid such as itaconate and mesaconate were induced in *Ps. fluorescens* when the bacterium was grown in a itaconate-glucose medium for the adaptation to itaconate (7). With *Ps. arvilla,* however, the level of enzyme II in the itaconate-glucose-grown cells was about the same as that in the glucose-grown cells. Moreover, the attempt to grow the bacterium which had been grown in a itaconate-glucose medium in a itaconate medium was not successful. These obsrevations caused apprehensions that *Ps. arvilla* is unable to be adapted to itaconate. Thus, the levels of itaconate-activating enzyme in glucose- and itaconate-glucose-grown cells were determined by a modification (17) of the method of Lipmann and Tuttle (19). Itaconate and succinate were used as a substrate in the assay reaction, since itaconate is known to be activated by succinyl-CoA synthetase (EC 6·2·1·5) as well as its proper itaconate-activating enzyme (17).

As shown in Table 9, the levels of the activating enzyme against itaconate and succinate in the glucose-grown cells were almost equal to the corresponding levels in the itaconate-glucose-grown cells. The presence of ferrous salt did not affect both levels. For further confirmation, the bacterium was grown in media containing various concentrations of glucose and definite amounts of itaconate, and the effect of itaconate on the growth amounts of the bacterium was investigated. But, itaconate did not affect the growth of bacterium at all. From these results, it was conlcuded that *Ps. arvilla* is unable to be adapted to itaconate.

Since *Ps. arvilla* was found unable to be adapted to itaconate, variation of levels

Table 9. Variation of levels of itaconate-activating enzymes after the adaptation treatment to itaconate. *Ps. arvilla* was grown in glucose- and itaconate-glucose (4:1 in weight)-media. In the presence (50 mg in 1 l medium) and absence of ferrous sulfate, the activities of itaconate-activating enzymes against itaconate and succinate as a substrate were determined with the cell-free extracts obtained from the cells.

Carbon source	Fe salt	Hydroxamate formed (A_{520}/mg protein)		Ratio (SA/IA)
		Succinate (SA)	Itaconate (IA)	
Glucose	−	0.326	0.226	1.44
	+	0.303	0.211	1.44
Itaconate-Glucose (4:1)	−	0.347	0.239	1.46
	+	0.311	0.219	1.42

Table 10. Levels of enzymes I and II in *Ps. fluorescens* grown under various nutritional conditions. *Ps. fluorescens* was grown in indicated media as described in Table 8. The levels of enzymes I and II were determined by measurement of the activities against L-malate with the cell-free extracts as described in Table 8.

Carbon source	Fe salt	Enzyme I (U/mg protein)	Enzyme II (U/mg protein)
Glucose	−	0.24	0.02
	+	0.06	0.49
Itaconate	−	0.17	0.06
	+	0.10	0.57

of both enzymes was investigated with *Ps. fluorescens* which is known able to be adapted to itaconate (3). Preliminary experiment showed that the strain of *Ps. fluorescens* used can grow in a medium containing only itaconate as a carbon source without a significant lag unlike the one used by Katsuki *et al.* (3). Accordingly, the levels of both enzymes I and II of *Ps. fluorescens* were determined with both of the glucose- and itaconate-grown cells.

As shown in Table 10, the level of enzyme II in the itaconate-grown cells was almost the same as that in the glucose-grown cells. Ferrous salt showed similar effect to that on *Ps. arvilla,* but itaconate nearly completely released the repression of enzyme I by ferrous salt. The effect of itaconate seems to be noteworthy, though the mechanism is ambiguous.

Discussion

The present investigation showed the presence of two enzymes with fumarase activity in *Ps. arvilla*. Examniation of substrate specificity and ferrous-ion requirement with them revealed that enzyme I was fumarase and enzyme II was mesaconase.

The optimal pH's, *K*m values and ratio of V_{max} values using L-malate and L-citramalate as a substrate with enzyme II resembled the corresponding values of mesaconase of *Cl. tetanomorphum* (20) and a kind of soil bacterium isolated by Nakano *et al.* (9). The examination of structure specificity of SH compounds in the reactivation of *Ps. arvilla* mesaconase showed that the enzyme, different from the soil bacterium (Nakano strain) enzyme, had a considerable high specificity as in the case of the enzyme of *Cl. tetanomorphum.* (21).

From the results of coarse control experiments of enzymes I and II (Tables 8 and 10), the addition of ferrous salt to the growth media gave rise to the repression of enzyme I and the induction of enzyme II. The nutritional induction of enzyme II by ferrous salt seems to be reasonable in view of reactivation of the enzyme activity by ferous ions. However, the nutritional repression of enzyme I by ferrous salt—a marked phenomenon which presumably has not been reported before—may be difficult to be explained. As one possibility, the phenomenon may be explained

in terms of non-specific toxic action of considerably high concentrations, if so, of ferrous salt. However, such a possibility could be excluded, if one considers that the concentration of ferrous salt was not so high as to inhibit the growth of bacterium and that the levels of activating enzymes of itaconate and succinate were not lowered by the addition of ferrous salt. It is also a mraked phenomenon that the repression of enzyme I by ferrous salt in *Ps. fluorescens*—an itaconate-adaptable strain—was mostly released in the itaconate medium. The release of repression by itaconate may due to the metabolism of itaconate. Examination whether or not this repression is released by other C_5-branched-chain dicarboxylates than itaconate remains to be solved.

Comparison of levels of enzymes I and II in the cells grown on various carbon sources showed that no marked variation was found exept for repression of enzyme I by glucose to some extent. Such type of glucose repression has been very often found on enzymes involved in the tricarboxylic acid cycle.

From the following two reasons, it is evident that enzyme II was not induced in relation to the metabolism of C_5-branched-chain dicarboxylates. (i) A high level of enzyme II was found in *Ps. arvilla* which is unable to be adapted to itaconate. (ii) No increase in the level of enzyme II was found in itaconate-grown *Ps. fluorescens* which is able to be adapted to itaconate. As for the physiological role of enzyme II in these bacteria, one possibility was raised: enzyme II may play a role—at least partly—in the operation of the tricarboxylic acid cycle through catalyzing the interconversion of fumarate to L-malate—in cooperation with fumarase. The results in Table 8 show that the level of enzyme I fell down one sixtieth by the addition of ferrous salt to the medium. If the interconversion activity of fumarate to L-malate at that time was rate-limiting in the operation of the tricarboxylic acid cycle, only mesaconase (enzyme II) should have been the candidate. If mesaconase is considered to be always involved in the operation of the cycle in cooperation with fumarase, the finding of enzyme II even in glucose-grown *Ps. arvilla* cells and the non-induction of enzyme II by itaconate in *Ps. fluorescens* cells are easily understandable.

Acknowledgement

The authors wish to thank Dr. Katsura Izui for his kind discussion.

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