Purification and Properties of 2-Methylcitrate Dehydratase from *Yarrowia lipolytica*

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2-Methylcitrate dehydratase (2-methylcitrate hydro-lyase) functioning at the methylcitric acid cycle of propionyl-CoA oxidation was purified from a cell-free extract of *Yarrowia (Saccharomycopsis) lipolytica*. Disc gel electrophoresis proved that the enzyme preparation was homogeneous. The molecular weight was about 79,000 in determinations by gel filtration and SDS-disc electrophoresis. The enzyme was composed of 685 residues of amino acid per molecule. The enzyme showed an isoelectric point of 3.9. No cofactor was required for full enzyme activity. The enzyme was inhibited by sulfhydryl reagents such as *p*-chloromercuribenzoate, but not by any chelating reagents. The enzyme competitively inhibited by citrate ($K_i=4.5 \times 10^{-4}$ M), *threo*-D_s-isocitrate ($K_i=1.2 \times 10^{-2}$ M), *threo*-D_s-2-methylisocitrate ($K_i=1.1 \times 10^{-3}$ M), tricarballoylate ($K_i=3.7 \times 10^{-2}$ M), and DL-fluorocitrate ($K_i=9.5 \times 10^{-4}$ M).

In a previous paper,¹⁾ we have presented the evidence for the presence of 2-methylcitrate dehydratase (2-methylcitrate hydro-lyase), a new enzyme functioning at the methylcitric acid cycle²⁾ of propionate metabolism in yeasts. The enzyme of *Yarrowia (Saccharomycopsis) lipolytica* catalyzes only the reversible reaction between 2-methylcitrate (2-hydroxy-1,2,3-butanetricarboxylate) and 2-methyl-*cis*-aconitate (*cis*-2-butene-1,2,3-tricarboxylate), but cannot catalyze the other reversible reaction between 2-methyl-*cis*-aconitate and *threo*-D_s-2-methylisocitrate in the cycle. This paper described the purification and further characterization of this enzyme.

MATERIALS AND METHODS

Chemicals. 2-Methylcitrate used as a substrate was chemically synthesized by the method of Beach *et al.*³⁾ *threo*-D_s-Isocitrate⁴⁾ and *threo*-D_s-2-methylisocitrate⁵⁾ were the products isolated from culture broths of *Y. lipolytica*. Molecular weight markers for gel filtration and SDS-disc electrophoresis were obtained from Boehringer Mannheim GmbH and Daiichi Pure Chemicals Co., respectively. All other chemicals were obtained from the usual commercial sources.

Microorganism and cultivation. *Saccharomycopsis lipolytica* IFO 1659 was cultured by the same method described in the previous paper.¹⁾ According to the recent proposal of van der Walt and von Arx,⁶⁾ *Saccharomycopsis lipolytica* is called *Yarrowia lipolytica* (Wickerham *et al.*) Comb. Nov.

Enzyme assay. The activity of 2-methylcitrate dehydratase was assayed with synthetic 2-methylcitrate as the substrate by the same method described in the previous paper.¹⁾ The activity of aconitate hydratase [citrate (isocitrate) hydro-lyase EC 4.2.1.3] and the protein concentration were also assayed by the same method described in the previous paper.¹⁾ One unit of activity was expressed in nanomoles per min.

Disc gel electrophoresis. Electrophoresis in a 7.5% polyacrylamide gel was carried out by the method of Davis⁷⁾ at a current of 2 mA for 2 hr at 4°C. Proteins were stained with Coomassie brilliant blue R 250.

SDS-disc electrophoresis. SDS-disc electrophoresis was carried out by the method of Weber and Osborn⁸⁾ with a 3.3% polyacrylamide gel containing 0.1% SDS at a current of 8 mA for 4 hr at 25°C both with and without marker proteins. Proteins were stained with Coomassie brilliant blue R 250.

Molecular weight estimation by gel filtration. Gel filtration was performed with Sephadex G-150 and G-200 columns (2.4 × 100 cm) previously equilibrated with 0.05 M phosphate buffer (pH 6.5). Proteins were eluted with the

same buffer at a flow rate of 10 ml per hr and fractions (5 ml) were collected.

Ultraviolet absorption. The ultraviolet absorption spectrum of the enzyme preparation was measured with a Hitachi, Model 100-50, automatic spectrophotometer.

Isoelectric focusing. Electrofocusing was carried out on a column (about 110 ml) containing 0.5% carrier ampholyte with pH ranges from 3.5~10 and from 4~6 (LKB) at 600 V for 36 hr at 4°C.

Amino acid composition. The purified enzyme was hydrolyzed with 6N HCl in a sealed, evacuated glass tube at 110°C for 20, 40 and 70 hr. The hydrolysates were analyzed with a Durrum D-502 amino acid analyzer. Tryptophan was determined by the spectrophotometrical method of Goodwin and Morton.⁹⁾

Purification of the enzyme. All steps were carried out at 0 to 5°C.

Step 1. Cell-free extract. Frozen cells (1 kg) were suspended in 2 liters of 0.05 M potassium phosphate buffer (pH 6.5) containing 10 μ M phenylmethylsulfonyl fluoride (PMSF), and disrupted three times with a French press. After centrifugation at 10,000 $\times g$ for 30 min, the supernatant was used as a cell-free extract.

Step 2. Ammonium sulfate fractionation. Solid ammonium sulfate was added to 2 liters of the cell-free extract to 40% saturation and the mixture was allowed to stand in a refrigerator overnight. After centrifugation at 5000 $\times g$ for 20 min, solid ammonium sulfate was further added to the supernatant to 70% saturation and the mixture was allowed to stand overnight. The resulting precipitate was collected by centrifugation at 5000 $\times g$ for 20 min and dissolved in 0.05 M potassium phosphate buffer (pH 6.5) containing 10 μ M PMSF. The solution (120 ml) was dialyzed overnight against 5 liter of the buffer described above.

Step 3. First DEAE-Sephadex A-50 chromatography. The dialyzed protein solution (150 ml) was passed through a DEAE-Sephadex A-50 column (6 $\times$ 50 cm) containing

10 μ M of PMSF. The column was washed with 1 liter of the same buffer, and then eluted stepwise with each 1 liter of 0.125, 0.15, 0.175 and 0.2 M potassium phosphate buffers (pH 6.5). Fractions of 20 ml were collected. The active fractions (Nos. 140~155) of 2-methylcitrate dehydratase were combined and concentrated with a collodion bag to a final volume of 20 ml.

Step 4. First Sephadex G-200 gel filtration. The sample from step 3 was placed on a Sephadex G-200 column (3 $\times$ 100 cm) previously equilibrated with 0.05 M potassium phosphate buffer (pH 6.5), and the enzyme was eluted with the same buffer at a flow rate of 20 ml per hr and the fractions (Nos. 30~37) were combined and concentrated with the collodion bag to a final volume of 20 ml.

Step 5. Second DEAE-Sephadex A-50 chromatography. The sample from step 4 was placed on a DEAE-Sephadex A-50 column (3 $\times$ 30 cm) equilibrated with 0.1 M potassium phosphate buffer (pH 6.5). The column was washed with 200 ml of the buffer and then eluted with a gradient between 1 liter of 0.1 M potassium phosphate buffer (pH 6.5) and 1 liter of 0.2 M phosphate buffer at a flow rate of 20 ml per hr. Fractions of 8 ml were collected. The active fractions (Nos. 41~50) were combined and concentrated with the collodion bag to a final volume of 5 ml.

Step 6. Second Sephadex G-200 gel filtration. The sample from step 5 was placed on a Sephadex G-200 column (1.8 $\times$ 100 cm) equilibrated with 0.05 M potassium phosphate buffer (pH 6.5) and eluted with the same buffer at a flow rate of 10 ml per hr (Fig. 1). Active fractions were combined and concentrated to a final volume of 10 ml. The concentrated enzyme preparation was divided into 1 ml portions containing about 15 mg per ml of the enzyme and stored in liquid nitrogen. The purified enzyme was stable for over half a year under these conditions.

RESULTS

Purification

Table I shows the results of a purification run. The enzyme was purified about 200-fold

TABLE I. SUMMARY OF PURIFICATION

Step	Total protein (mg)	Specific activity ^a	Recovery (%)
Cell-free extract	140,000	4.8	100
(NH ₄) ₂ SO ₄ (40~70%)	27,500	20.8	84
First DEAE-Sephadex A-50	6,800	60.6	61
First Sephadex G-200	1,300	210	40
Second DEAE-Sephadex A-50	160	1,050	25
Second Sephadex G-200	120	1,080	19

^a Expressed as nanomoles per min per mg of protein.

FIG. 1. Second Sephadex G-200 Column Chromatography.

FIG. 2. Disc Gel Electrophoresis (A) and SDS-disc Electrophoresis (B) of the Purified Preparation.

from the extract with a recovery of about 20% of the original activity. In the first DEAE-Sephadex A-50 chromatography of the purification run, the peak activity of aconitate hydratase was observed at the 0.125 M phosphate buffer and the peak activity of 2-methylcitrate dehydratase was at the 0.15 M phosphate buffer, respectively.

Homogeneity

As Fig. 1 shows, the chromatogram of Step

FIG. 3. Estimation of Molecular Weight by Gel Filtration on Sephadex G-150 (I) and G-200 (II).

●, 2-methylcitrate dehydratase; Δ , cytochrome *c*; ○, chymotrypsinogen A; ●, ovalbumin; □, bovine serum albumin; ●, aldolase.

FIG. 4. Estimation of Molecular Weight by Electrophoresis on SDS-disc Acrylamide Gel.

●, 2-methylcitrate dehydratase; ○, Molecular Weight Markers-H (BDH, 53,000~265,000).

6 of the purification run showed only one protein peak. The enzyme preparation gave a single protein band both in polyacrylamide gel electrophoresis and in SDS-polyacrylamide gel

TABLE II. AMINO ACID COMPOSITION OF 2-METHYLCITRATE DEHYDRATASE

Molecular weight calculated from the total integral number of residues is 78,500.

Amino acid	Residues in 78,500	Residues to nearest integer
Lysine	39.6	40
Histidine	12.0	12
Arginine	22.6	23
Aspartate	67.2	67
Threonine	36.1 ^a	36
Serine	37.4 ^a	37
Glutamate	58.7	59
Proline	26.2	26
Glycine	57.3	57
Alanine	57.3	57
Half-cystine	31.8 ^a	32
Valine	44.6 ^b	45
Methionine	2.1	2
Isoleucine	39.6 ^b	40
Leucine	50.2	50
Tyrosine	17.0 ^a	17
Phenylalanine	24.0	24
Tryptophan	60.8 ^c	61
Amide-NH ₃	13.0	
Total		685

^a Values extrapolated to zero time of hydrolysis.

^b Values from 70 hr hydrolysis.

^c Determined by the method of Goodwin and Morton.⁸⁾

All other values are averages of hydrolysates at 20, 40 and 70 hr.

electrophoresis (Fig. 2).

Molecular weight

The molecular weight of 2-methylcitrate dehydratase was estimated to be 78,000 by gel filtration on Sephadex G-200, 79,000 by that on Sephadex G-150 (Fig. 3) and 79,000 by SDS-disc electrophoresis (Fig. 4). This suggests that 2-methylcitrate dehydratase has no subunit structure.

Amino acid composition

Table II shows the amino acid composition of the enzyme. The values are expressed as the number of residues per molecule assuming a molecular weight of 78,500. The enzyme was found to consist of 685 residues of amino acid per molecule.

FIG. 5. Determination of Isoelectric Point of the Enzyme.

○—○, 2-methylcitrate dehydratase activity; ●—●, pH at 4°C; ----, absorbance at 280 nm.

FIG. 6. Ultraviolet Absorption Spectrum.

The purified enzyme was dissolved at a concentration of 0.22 mg per ml of 0.05 M potassium phosphate buffer (pH 7.0).

Isoelectric focusing

Isoelectric focusing of the enzyme preparation in an ampholyte electrofocusing column yielded a single protein peak (Fig. 5). The peak of the enzyme activity coincided with that of the protein, and the isoelectric point (pI) of the

TABLE III. EFFECT OF IONS ON ENZYME ACTIVITY

Compound added	(mM)	Relative activity (%)
None	—	100
NaCl	10	100
K ₂ SO ₄	10	99
(NH ₄) ₂ SO ₄	10	97
MgCl ₂	10	93
CaCl ₂	10	97
MnSO ₄	2	100
FeSO ₄	0.5	97
FeCl ₃	0.5	98
CoCl ₂	0.5	100
NiCl ₂	0.5	30
CuSO ₄	0.5	65
ZnCl ₂	0.5	96
Hg(NO ₃) ₂	0.5	38
PbCl ₂	0.5	40

TABLE IV. EFFECTS OF VARIOUS REAGENTS ON ENZYME ACTIVITY

Compound added	(mM)	Relative activity (%)
None	—	100
Phenylmethylsulfonyl fluoride	0.1	99
Dithiothreitol	20	99
2-Mercaptoethanol	20	98
Cysteine	20	100
<i>p</i> -Chloromercuribenzoate	1	9
Iodoacetate	1	13
EDTA	10	100
<i>o</i> -Phenanthroline	10	99
2,2'-Dipyridyl	10	100

enzyme was estimated to be 3.9.

Ultraviolet absorption spectrum

Figure 6 shows the ultraviolet absorption spectrum of the enzyme in 0.05 M potassium phosphate buffer (pH 7.0). A peak was observed at 275 nm. No visible absorption was observed. The extinction coefficient ($E_{1\text{ cm}}^{1\%}$) of this enzyme at 280 nm was calculated to be 24.3.

Effect of various ions

Table III shows the effect of various ions on enzyme activity. No stimulation of enzyme

TABLE V. EFFECTS OF METABOLITES AND ANALOGS ON ENZYME ACTIVITY

Compound added	(mM)	Relative activity (%)
None	—	100
Citrate	2.5	62
DL-Fluorocitrate	5.0	68
<i>threo</i> -D ₅ -Isocitrate	2.5	91
<i>threo</i> -DL-Isocitrate	5.0	92
<i>cis</i> -Aconitate	2.5	60
<i>trans</i> -Aconitate	2.5	98
<i>threo</i> -D ₅ -2-Methylisocitrate	2.5	78
Tricarallylate	2.5	93
Pyruvate	2.5	97
Oxaloacetate	2.5	98
2-Oxoglutarate	2.5	99
Succinate	2.5	98
Fumarate	2.5	98
DL-Malate	5.0	100
Malonate	2.5	99
Adipate	2.5	100
Glutarate	2.5	98
L-Glutamate	2.5	99
L-Aspartate	2.5	98

activity by any ion was observed under the experimental conditions, although several bivalent metal ions, such as nickel, copper, zinc, mercury, and lead, inhibited the activity to some extent.

Effects of various reagents and metabolites

The effects of various reagents and structural analogs on enzyme activity were examined. For measuring inhibition or stimulation, the enzyme was first incubated for 10 min at 25°C with the test compound in the assay mixture minus the substrate. The reaction was then started by adding the substrate and enzyme activity was assayed.

As Table IV shows, phenylmethylsulfonyl fluoride, an inhibitor of protease, had no effect on enzyme activity and so it was added in the buffer used in the early steps of purification. Sulfhydryl compounds such as dithiothreitol or mercaptoethanol had no observable effect on enzyme activity. The enzyme was strongly inhibited by sulfhydryl binding reagents such as *p*-chloromercuribenzoate and iodoacetate,

FIG. 7. Competitive Inhibition of 2-Methylcitrate Dehydratase by Citrate.

The concentration of citrate added: ○, none; ⊙, 0.1 mM; ⊕, 0.5 mM; ⊕, 1.0 mM; ●, 2.5 mM.

as well as by the ions of copper, silver, mercury, and lead, as already shown in Table III. However, no chelating reagents showed any effect on enzyme activity.

Table V shows the effect of metabolites and structural analogs on enzyme activity. The enzyme was inhibited by several structural analogs such as citrate, *cis*-aconitate, isocitrate, 2-methylisocitrate, tricarballoylate and fluorocitrate, but not by *trans*-aconitate¹⁰⁾ or adipate¹¹⁾ known as the inhibitors of aconitate hydratase.

Effects of adenylates and coenzymes

For the evaluation of the regulatory function of this enzyme, the effects of certain adenylate compounds and coenzymes on enzyme activity were examined. The following compounds tested at a concentration of 1 mM had no effect on enzyme activity: AMP, ADP, ATP or NAD(P)(H). Pyruvate, the end product of the cycle, also had no effect on enzyme activity (Table V).

Kinetics

The rate of substrate conversion by the enzyme followed the standard Michaelis-

Menten kinetics curve. The apparent K_m value for the synthetic 2-methylcitrate consisting of the two racemic pairs of diastereoisomers was calculated to 1.9×10^{-4} M from Lineweaver-Burk plots.

As Fig. 7 shows, citrate competitively inhibited enzyme activity ($K_i = 4.5 \times 10^{-4}$ M). Competitive inhibition was also observed with the other structural analogs: *threo*-D_s-isocitrate ($K_i = 1.1 \times 10^{-2}$ M), *threo*-D_s-2-methylisocitrate ($K_i = 1.2 \times 10^{-3}$ M), DL-fluorocitrate ($K_i = 9.5 \times 10^{-4}$ M) and tricarballoylate ($K_i = 3.7 \times 10^{-2}$ M).

DISCUSSION

We have previously shown that 2-methylcitrate dehydratase is highly specific for substrates and has no activity on *threo*-D_s-2-methylisocitrate or the substrate of aconitate hydratase, namely, citrate, *cis*-aconitate or *threo*-D_s-isocitrate.¹⁾ The present experiments show that *threo*-D_s-2-methylisocitrate, citrate and *threo*-D_s-isocitrate are competitive inhibitors of this enzyme.

Differing from aconitate hydratase, 2-methylcitrate dehydratase was not stimulated by ferrous ions or by sulfhydryl compounds and was not inhibited by *trans*-aconitate or any of chelating reagents. The enzyme showed a *pI* value (3.9) close to that (4.2) of the aconitate hydratase of *Y. lipolytica*,¹²⁾ but did no visible absorption. 2-Methylcitrate dehydratase appears to be monomeric, and its molecular weight (78,500) is larger than that (68,500) of the aconitate hydratase of the same strain of *Y. lipolytica*.¹²⁾ The amino acid composition of the enzyme clearly differed from that of the aconitate hydratase described by Suzuki *et al.*¹³⁾ These results also show that 2-methylcitrate dehydratase differs from the apoenzyme of aconitate hydratase.¹⁴⁾

We have shown in a previous study that the methylcitric acid cycle enzymes are constitutive and the degree of glucose repression in the enzymes is similar to glucose repression in several enzymes of the citric acid cycle in *Y. lipolytica*.^{1,15)} 2-Methylcitrate synthase func-

tioning at the start of the cycle has been shown to be inhibited by NAD(P)H and ATP.¹⁶⁾ 2-Methylisocitrate lyase yielding pyruvate, the end product of the cycle, has been shown to be also inhibited by NAD(P)H but activated by NAD.¹⁷⁾ However, the present experimental results show that 2-methylcitrate dehydratase was not affected by any of the adenylate compounds or coenzymes. This suggests that 2-methylcitrate dehydratase does not have the mechanism of fine control in enzyme activity.

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