ENZYMIC HYDRATION OF MESACONATE BY *PSEUDOMONAS FLUORESCENS*

H. KATSUKI, N. ARIGA, F. KATSUKI, J. NAGAI, S. EGASHIRA AND S. TANAKA
Department of Chemistry, Faculty of Science, Kyoto University, Kyoto (Japan)

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SUMMARY

Sonicated extract of *Pseudomonas fluorescens* could, in the presence of Fe^{2+}, reversibly hydrate mesaconate to produce *d*-citramalate. This enzyme was partially purified by protamine treatment, heating and ammonium sulfate fractionation. In the purified preparation, the reaction proceeded stoichiometrically and reached the equilibrium state in which the proportion of *d*-citramalate to mesaconate was about 6:1. K_m of this enzyme for *d*-citramalate was $5 \cdot 10^{-4} M$ and its optimum pH was 8.2. It was inhibited by various metal ions, iodoacetate and cysteine.

INTRODUCTION

The occurrence of several kinds of organic acids having a methyl or methylene side-chain has been reported and attracted the author's interests in the mutual conversion of these acids in the biological systems.

Several years ago, WACHSMAN[1] and BARKER *et al.*[2] reported that the extract of *Clostridium tetanomorphum* converted glutamate *via* methylaspartate and mesaconate to citramalate. But they did not investigate precisely the step of the hydrating reaction of mesaconate. On the other hand, ADLER *et al.*[3] studied the metabolism of itaconate by animal mitochondria and proved that itaconyl-CoA was converted by way of citramalyl-CoA to mesaconyl-CoA (see ref. 4).

The present paper deals with the purification and some properties of the mesaconate hydrating enzyme of *Pseudomonas fluorescens*.

MATERIALS AND METHODS

Cultivation of the cells

Pseudomonas fluorescens No. 3081* was grown in a medium of the following composition (in per cent): KH_2PO_4, 0.25; $MgSO_4 \cdot 7H_2O$, 0.02; Oriental yeast extract, 0.1; peptone, 0.8; glucose, 0.5; disodium itaconate, 0.25 and distilled water. Of the nutrients, disodium itaconate was previously dissolved in a small amount of water and filtered through ultrafilter aseptically. The filtrate was combined with the auto-

Abbreviations: PCA, perchloric acid; EDA, ethylenediamine.

* We were kindly supplied with this strain from the Institute for Fermentation.

claved medium containing the other components. After being inoculated directly from the stock slant agar, cultivation was carried out by shaking for 20–24 h at 28°.

Preparation of substrates

Mesaconic acid was synthesized by the hydrolysis of citraconic anhydride which was obtained by distillation of itaconic anhydride[5]. *dl*-Citramalic acid was synthesized by the method of MICHAEL AND TISSOT[6].

Enzyme assay

The reaction mixture contained Tris buffer (pH 8.2), 125 μmoles; $Fe(NH_4)_2(SO_4)_2$, $3 \cdot 10^{-2}$ μmoles; *dl*-citramalate, 10 μmoles, and 0.1 ml of the enzyme in a final volume of 3.2 ml. The reaction was carried out in the cell (1.0 cm) of a Beckmann Spectrophotometer model DU at 30°. The increase in absorbancy at 230 mμ caused by the appearance of mesaconate, was measured at 30 sec intervals during the period from 15 to 195 sec after the addition of substrate. The concentration of the enzyme was so adjusted that the absorbancy, due to the protein at 230 mμ, did not exceed 0.150.

The reaction rate was constant during 5 min and was directly proportional to the enzyme concentration in the presence of Fe^{2+}.

The unit of the enzyme is defined as the amount forming 1 μmole of mesaconate in 1 min under the conditions described.

The reverse reaction was assayed by adding 1 μmole of sodium mesaconate instead of *dl*-citramalate. In this case the activity was represented as the decrease in absorbancy at 230 mμ/min.

Determination of mesaconic acid and citramalic acid

Mesaconic acid was determined by measuring the absorbancy at 230 mμ. A 1 m*M* solution of mesaconate in 0.05 *M* phosphate buffer (pH 7.0) had an absorbancy of 3.27 at 230 mμ; 2.33 at 240 mμ; and in 0.04 *M* piperazine buffer (pH 5.0) 3.55 at 230 mμ. These values were different from those reported by MUNCH-PETERSEN AND BARKER[7].

dl-Citramalic acid was determined spectrophotometrically by the method of one of the authors[8] which was based upon the reaction between *cis*-dichlorobis(ethylendiamine)chromium(III) chloride and organic acids. The sample solution was deproteinized with PCA which was then removed as potassium perchlorate by the addition of potassium hydroxide. To 1 ml of this deproteinized sample solution was added 0.5 ml of 3% chromium complex reagent and 0.5 ml of 0.5 *M* EDA-hydrobromide buffer (pH 7.5) and the mixture was heated on a boiling-water bath for 5 min. Then 0.5 ml of 20% ammonium thiocyanate was added and the mixture was heated for another 5 min, then cooled with running water and allowed to stand for more than 15 min. The mixture was extracted twice with 3 ml each of a butanol–ethyl acetate mixture (2:1). After removing the upper layer, 0.5 ml of 0.6 *M* tartaric acid and 1 ml of 1.5% EDTA disodium salt were added to the lower layer and heated on a boiling-water bath for 10 min. After cooling, the mixture was diluted with water to 10 ml and the intensity of the developed colour was measured at 543 mμ. The value obtained with water instead of the sample solution, was substracted as the control value. The value due to 10 μmoles of citramalic acid was 0.611 and that of

mesaconic acid 0.229. The amount of citramalate in the mixture was derived by subtracting the value due to mesaconate, which was obtained by measuring the absorbancy at 230 mμ, from the total value.

The concentration of enzyme was determined by measuring the amount of protein[9] or tentatively by measuring the absorbancy at 230 mμ.

Ion exchange chromatography

A 200–400 mesh of Dowex-1 resin (Br-form) was slurried into a column. A sample solution, deproteinized with PCA and then freed from the PCA by adding potassium hydroxide, was passed through the column and then eluted with 0.04 *M* piperazine–hydrobromic acid buffer solution (pH 5.0) at a rate of one drop/7 sec*. The effluent of 5 ml each was collected by a fraction collector.

RESULTS

Extraction and purification

Harvested cells were washed twice with 0.9% sodium chloride by centrifugation and suspended in 35 ml of 1/15 *M* phosphate buffer (pH 7.2) per cells, obtained from 1 l of culture. 20 ml each of the cell suspension was exposed to sonic vibration for 6 min at 5° in a Kubota's 10 kc Sonic Oscillator and centrifuged for 20 min at 12000 × *g*. The precipitant was discarded and the supernatant was stocked in a refrigerator. This extract was kept active for about 1 week by occasional additions of small amounts of ferrous sulfate.

To 40 ml of the crude extract (12.3 units/ml containing 41.4 mg protein) 15 ml of a 2% protamine sulfate solution, was added dropwise whilst stirring the mixture at 0°. After stirring for a further 30 min, the solution was centrifuged at 12000 × *g* for 20 min. The precipitate was discarded.

To the protamine-treated extract powdered ammonium sulfate was slowly added whilst stirring to give a 30% saturation, and stirring was continued for a further 10 min. The mixture was centrifuged and the precipitate was discarded. To this supernatant powdered ammonium sulfate was added carefully to give a 70% saturation. The precipitate obtained by centrifugation was dissolved in 40 ml of 0.05 *M* Tris buffer (pH 8.2).

The solution obtained above was heated by stirring on a bath to 50° and kept for 2 min. The solution was then cooled rapidly in an ice bath and allowed to stand for 30 min after which the precipitate was centrifuged off. The heat-treated supernatant was dialysed by stirring for 6 h against 0.05 *M* Tris buffer, pH 8.2, at 0°.

To 1 ml of the dialysed solution was added a hydroxyapatite suspension in 0.001 *M* phosphate buffer (pH 6.8) (see ref. 11). After stirring for 2 min and being allowed to stand for 20 min at 0°, the gel was centrifuged down and discarded.

The supernatant obtained by hydroxyapatite treatment was fractionated by adding ammonium sulfate and the fraction between 60% and 70% saturation was collected and dissolved in 0.05 *M* Tris buffer (pH 8.2) in the same way as shown in the first ammonium sulfate treatment. This enzyme preparation lost about a half of its activity in two days even when refrigerated in the presence of ferrous ion. The specific activity and yield of the enzyme during the purification process described above, are summarized in Table I.

* Later, the Dowex-1 (Cl-form)–0.2 *M* NaCl system was found to give better results[10].

TABLE I

PURIFICATION OF ENZYME

Purification step	*ml*	*mg protein*	*Units*	*Per cent*	*Units/mg*
Crude extract	40	1656	491.7	100	0.297
Protamine sulfate supernatant	50	1426	493.7	100	0.346
1st ammonium sulfate fractionation (30–70%)	20	564	403.7	82	0.716
Heat-treated supernatant	18	432	360	73	0.833
Hydroxyapatite supernatant	20	360	321	65	0.892
2nd ammonium sulfate fractionation (60–70%)	20	69	162	33	2.35

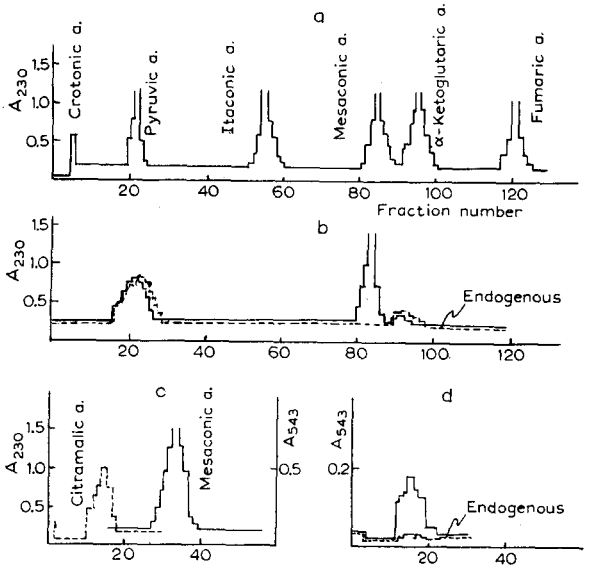

Fig. 1. Identification of reaction products by column chromatography. In 1a and b, a column of 1 cm^2 × 24 cm was used and in Fig. 1c and d, a smaller one of 1 cm^2 × 12 cm. Enzyme, purified by the first ammonium sulfate fractionation, was used. The amounts of unsaturated acids and keto acids are expressed in the absorbancy at 230 mμ and citramalate in the absorbancy at 543 mμ developed by chromium complex reagent. Fig. 1a shows the chromatogram of authentic acids. Fig. 1b shows the enzymic formation of mesaconate from citramalate. The reaction was carried out in 1/15 *M* phosphate buffer (pH 8.2) on a large scale. Fig. 1c shows the chromatogram of authentic mesaconate and citramalate and Fig. 1d, the enzymic formation of citramalate from mesaconate. The reaction was carried out in 0.05 *M* Tris buffer, pH 8.2 on a large scale.

TABLE II

STOICHIOMETRY OF THE REACTION

The reaction mixtures contained 1 ml of the purified enzyme, 5 ml of 0.05 *M* Tris buffer (pH 8.2), Fe^{2+} and 153 μmoles of substrate. Final volume, 10 ml. Determinations after incubation for 90 min at 30°.

μmoles decreased	*μmoles formed*	*Per cent*
Citramalate 12.8	Mesaconate 13.6	106
Mesaconate 126	Citramalate 128	101

dl-Citramalate or mesaconate was incubated for 3 h at 30° in a reaction mixture containing the purified enzyme. The reaction was stopped by adding PCA and the precipitate was removed by centrifugation. As a control the reaction mixture from which the substrate was omitted, was incubated in the same conditions and the substrate was added after deproteinization. After the PCA in the supernatants was removed as the potassium salt, each reaction mixture was passed through a Dowex-1 (Br-form) column as described in MATERIALS AND METHODS. The chromatograms as shown in Fig. 1, demonstrate the formation of mesaconate from citramalate and *vice-versa*.

The formation of these acids could also be shown by paper chromatography.

TABLE III

INHIBITIVE EFFECTS OF VARIOUS SUBSTANCES

The reaction conditions were the same as those described in the enzyme assay, except that the addition of ferrous ion was omitted. The enzyme used was the one purified 5-fold and stocked in the presence of Fe^{2+}. Just before the experiment it was precipitated by 80% saturation with ammonium sulfate and dissolved again in Tris buffer.

Substances	*M*	*Inhibition (%)*
Mg^{2+}	10^{-3}	9
Ca^{2+}	10^{-3}	9
Mn^{2+}	10^{-3}	36
Cu^{2+}	$5 \cdot 10^{-5}$	13
Hg^{2+}	10^{-4}	97
Na_3AsO_4	10^{-3}	0
KCN	10^{-3}	100
NH_2OH	10^{-3}	23
NaN_3	10^{-4}	15
Ascorbic acid	10^{-4}	33
Sodium iodoacetate	10^{-4}	13
Glutathione	10^{-4}	21
Cysteine	10^{-4}	100

Stoichiometry of the reaction

The stoichiometry of the reaction was shown by an experiment (see Table II) in which 153 μmoles of the substrate were incubated with 1 ml of the purified enzyme preparation in 0.05 *M* Tris buffer (pH 8.2) containing Fe^{2+}, for 90 min at 30°.

Properties of the enzyme

As shown in Table III, several substances inhibit the reaction, in particular Hg^{2+}, CN^- and cysteine. The inhibition by Hg^{2+}, Cu^{2+} and iodoacetate would suggest that the enzyme is an –SH enzyme. But the strong inhibition by cysteine, contrary to the result of BARKER *et al.* could not be explained.

The conversion of *d*-citramalate follows Michaelis-Menten kinetics. From the Lineweaver-Burk plots obtained, the constants $K_m = 5 \cdot 10^{-4}\,M$ and $pK_m = 3.3$ were derived.

Experiments, in which the enzyme was incubated for 50 min at 14.5°, demonstrated that the optimum of stability was at pH 8.2. The pH-optimum of the activity was found at 8.2–8.4, both in phosphate and in Tris buffer.

In accordance with the findings of BARKER *et al.*[2], the citramalate formed from mesaconate was found in the *d*-form*. Presuming that *l*-citramalate is inactive in the reaction mixtures in experiments with *dl*-citramalate and mesaconate at equilibrium, the ratio *d*-citramalate to mesaconate is 6:1 (see Fig. 2).

The purified enzyme preparation also showed fumarase activity but the complete separation of both enzymes was unsuccessful. According to MASSEY[12], crystalline

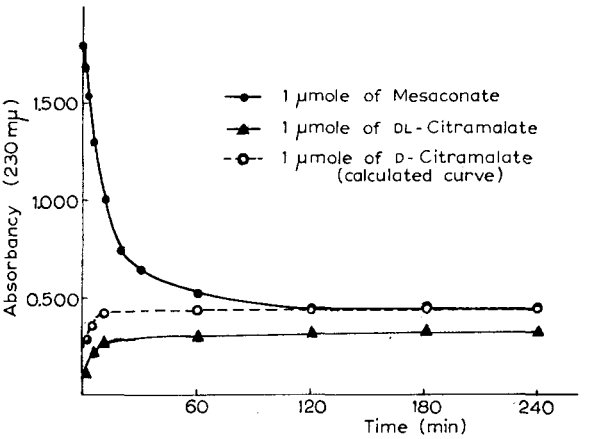

Fig. 2. Equilibrium of the reaction. The enzyme purified 6.8-fold was used. The experimental conditions were described in the text.

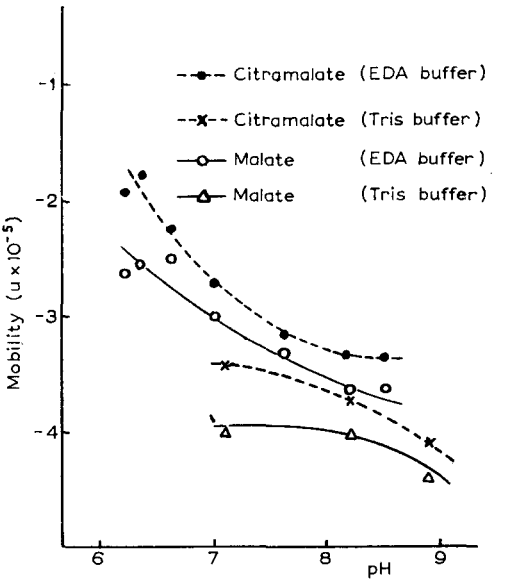

Fig. 3. Mobilities of the mesaconate hydrating enzyme and fumarase. An enzyme which was purified from 3 to 6 fold by first ammonium sulfate fractionation and charcoal treatment, was used. The experimental conditions were the same as in the enzyme assay except that the buffer and pH were changed as indicated. The fumarase activity was measured in the same way as in the enzyme assay except that malate was used as substrate instead of citramalate.

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fumarase of animal source has no activity hydrating mesaconate. In the present case, it was proved that the two enzymes differ. Firstly, any activating effect by Fe^{2+} was not seen with malate as a substrate. Secondly, in the course of the enzyme purification, the specific fumarase activity did not increase in proportion to the specific mesaconase activity. Thirdly, in electrophoresis at pH 6–9, the mobility of the malate dehydrating enzyme was always greater than that of the citramalate dehydrating enzyme (see Fig. 3).

The enzyme is also different from aconitase as the purified preparations had only insignificant activity toward citrate or *cis*-aconitate.

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