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Purification of the coenzyme B_{12}-containing 2-methyleneglutarate mutase from *Clostridium barkeri* by high-performance liquid chromatography

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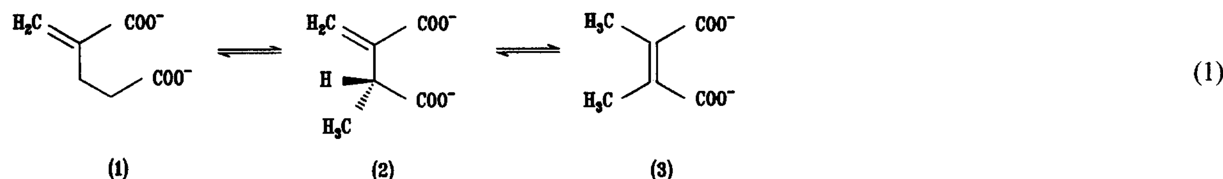
ABSTRACT

Two methods are described by which the enzymes 2-methyleneglutarate mutase and 3-methylitaconate Δ-isomerase from *Clostridium barkeri* have been separated by high-performance liquid chromatography on a much larger scale than reported previously. First, the mutase eluted before the Δ-isomerase after incubation with the mild detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate (CHAPS) followed by high-performance anion-exchange chromatography on Mono Q in the presence of the same detergent. Second, an even better separation, although with a lower yield of mutase, was obtained by hydrophobic interaction chromatography on phenyl-Sepharose HiLoad, whereby the enzymes were eluted in the reverse order. Final high-performance anion-exchange chromatography of the latter preparation on Mono Q at pH 8 gave highly purified 2-methyleneglutarate mutase (>95% purity) which had a pink-orange colour (λ_{max} 280, 375, 470 and 532 nm). The enzyme was active in the absence of coenzyme B_{12} (adenosylcobalamin) and contained 2.1 mol of this coenzyme per homotetramer (molecular mass, m = 300 kilodalton).

INTRODUCTION

2-Methyleneglutarate mutase (E.C. 5.4.99.4) and 3-methylitaconate Δ-isomerase (E.C. 5.3.3.6) are involved in two consecutive steps in the fermentation of nicotinate to ammonia, propionate, acetate and carbon dioxide by *Clostridium barkeri* [1,2]; for a review of the pathway, see ref. 3. The former enzyme catalyses the reversible, coenzyme B_{12}-dependent rearrangement of 2-methyleneglutarate (**1**, eqn. 1) to (*R*)-3-methylitaconate (**2**) with inversion of configuration at the methylene carbon (C-4 of **1**), which becomes the methine carbon of **2** [4]. The second enzyme, 3-methylitaconate Δ-isomerase, catalyses the reversible shift of the *exo* double bond of (*R*)-3-methylitaconate to 2,3-dimethylmaleate (**3**).

In order to study the mechanism of the unusual rearrangement of 2-methyleneglutarate to (*R*)-3-methylitaconate, a pure mutase free of Δ-isomerase is required. In addition, the Δ-isomerase serves as an auxiliary enzyme for the spectrophotometric assay [5]. However, purification of both cytosolic enzymes was impeded by the fact that they could not



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be separated by conventional techniques or by fast protein liquid chromatography (FPLC) on the anion exchanger Mono Q and on the molecular sieve Superose 6. Although the enzymes were recently resolved by non-denaturing polyacrylamide gel electrophoresis (PAGE), the scale was very small [5]. This paper describes two methods by which 2-methyleneglutarate mutase free of Δ-isomerase is obtained in larger amounts using FPLC. A short account of one method was published elsewhere [6].

EXPERIMENTAL

Materials

All chemicals were of analytical-reagent grade or higher quality and were purchased from Merck (Darmstadt, Germany), Sigma (Deisenhofen, Germany) and Biomol (Hamburg, Germany) if not stated otherwise. The syntheses of 2-methyleneglutarate and (*R*,*S*)-3-methylitaconate and the growth of *C. barkeri* were described previously [4,5].

Spectrophotometric enzyme assays

The activities of 2-methyleneglutarate mutase and 3-methylitaconate Δ-isomerase were determined in a continuous spectrophotometric assay at 256 nm under aerobic conditions as described recently [5] and modified according to ref. 6. The assay is based on the higher absorption of dimethyl maleate (**3**, eqn. 1) compared with 2-methyleneglutarate (**1**) and 3-methylitaconate (**2**), $\Delta\varepsilon = 0.66$ l $mmol^{-1}$ cm^{-1}.

Protein determination

Protein concentrations were determined by the bicinchonic acid method [7] with bovine serum albumin as a standard. The reagent kit was purchased from Pierce (Rockford, IL, USA).

Gel electrophoresis

PAGE in the presence of sodium dodecyl sulphate (SDS) was performed in 12% gels by the method of Laemmli [8] using a Mini Protean II equipment from Bio-Rad Labs. (Munich, Germany). Protein bands were stained with Coomassie Brilliant Blue R-250 from Serva (Heidelberg, Germany).

Fast protein liquid chromatography (FPLC)

All chromatographic procedures were performed with an FPLC system on the following chromatographic media or prepacked columns (Pharmacia, Freiburg, Germany). Anion-exchange chromatography was carried out on Q-Sepharose fast flow or on a Mono Q HR 10/10 column. For hydrophobic interaction chromatography and gel filtration a phenyl-Sepharose HiLoad 26/10 column and a Superdex 200 HiLoad 26/60 column, respectively, were used. All buffers were degassed and filtered through 0.45-μm membrane filters (Sartorius, Göttingen, Germany). Prior to loading the columns by a super loop, samples were passed through 0.2-μm sterile filters (Schleicher & Schüll, Dassel, Germany).

Enzyme separation and purification

The purification procedures were carried out in a cold room (4°C) under red light. Potassium phosphate buffer (20 m*M*, pH 7.4) containing 2 m*M* dithiothreitol (buffer A) was used in all purification steps if not stated otherwise. According to the desired amount and quality of the enzyme preparations, two different purification methods were applied.

Fast separation protocol. Frozen *C. barkeri* cells (15 g) were suspended in 25 ml of 50 m*M* potassium phosphate (pH 7.4)–5 m*M* magnesium chloride–5 m*M* dithiothreitol–deoxyribonuclease I (10 μg ml^{-1}, Boehringer, Mannheim, Germany). The cells were sonicated in three 10-min intervals at 0°C with a Branson sonifier (Branson Ultrasonics, Danbury, CT, USA) at a power of 80 W. Cell debris and membranes were removed by centrifugation at 130 000 *g* for 90 min.

The cell-free extract was diluted with two volumes of buffer A and pumped on a Q-Sepharose column (60-ml bed volume) which had previously been equilibrated with buffer A at a flow-rate of 2 ml min^{-1}. After washing with 100 ml of buffer A a linear NaCl gradient (0–1 *M* NaCl in 500 ml of buffer A) was applied, whereby both enzymes were eluted in one peak between 350 and 380 m*M* NaCl. The active fractions were combined (35 ml) and desalted by repeated ultrafiltration through a YM 100 membrane (Amicon, Witten, Germany) with buffer A.

Solid CHAPS was added to this solution to a concentration of 6 m*M* and incubated for 30 min.

This mixture was applied to a Mono Q column equilibrated with buffer A containing 2 mM CHAPS at a flow-rate of 2 ml min^{-1}. After washing with 50 ml of the same buffer, the enzymes were eluted by a linear NaCl gradient (see Fig. 1B). Fractions containing either mutase or isomerase were pooled, concentrated by ultrafiltration and stored at −80°C.

Large-scale purification protocol. The same procedure as in the fast separation protocol was carried out with 45 g of frozen cells in 80 ml of buffer yielding 105 ml crude extract. Solid ammonium sulphate (313 mg ml^{-1}) was added to the cell-free extract while stirring at 0°C to achieve 50% saturation. After 30 min the suspension was centrifuged at 25 000 g for 30 min and the precipitate was dissolved in 70 ml of buffer A. This solution was dialysed overnight against buffer A. Anion-exchange chromatograhpy on Q-Sepharose was performed, applying the same conditions as described in the fast separation protocol.

The mutase pool from Q-Sepharose was concentrated to 14 ml by ultrafiltration (YM 100 membrane) and applied to the Superdex 200 column which had been pre-equilibrated with buffer A containing 0.1 M NaCl at a flow rate of 3 ml min^{-1}. Mutase and Δ-isomerase appeared together in an elution volume from 160 to 180 ml. These fractions were concentrated to 7 ml by ultrafiltration.

Solid ammonium sulphate was added to the active Superdex fractions to a final concentration of 0.85 M. After incubation for 30 min this solution was pumped on a phenyl-Sepharose HiLoad column which had already been equilibrated with 0.85 M ammonium sulphate in buffer A at a flow-rate of 3 ml min^{-1}. After washing with 50 ml of this buffer the enzymes were eluted by a linear descending ammonium sulphate gradient from 0.85 to 0 M (see Fig. 3). The peaks containing mutase and Δ-isomerase were each concentrated by ultrafiltration in Centricon 30 microconcentrators (Amicon). The Δ-isomerase was not further purified and was stored at −80°C. The mutase was dialysed overnight against 20 mM potassium phosphate (pH 8.0).

Unlike the Mono Q step in the fast separation protocol, this run was performed at pH 8.0 in the absence of CHAPS. The Mono Q column was equilibrated with 20 mM potassium phosphate (pH 8.0) containing 2 mM dithiothreitol. The running conditions and the gradient were the same as in the above-described Mono Q step (Fig. 1). The mutase, eluting at 360 mM NaCl, was concentrated in a Centricon 30 microconcentrator and stored at −80°C.

Determination of the corrinoid content

Pure 2-methyleneglutarate mutase was incubated in 1 M KCN (pH 12) for 15 h at ambient temperature. During this time dicyanocobalamin was formed, which was indicated by its typical UV–VIS spectrum. The amount of the corrinoid was calculated from its absorbance at 369 nm (ε = 30.4 l mmol^{-1} cm^{-1}) and 543 nm (ε = 8.6 l mmol^{-1}cm^{-1}) [9].

Determination of the N-terminal amino acid sequence

2-Methyleneglutarate mutase samples (ten lanes, 10 μg of protein each) for N-terminal sequence determination were obtained from SDS-PAGE (12% acrylamide). The protein was blotted onto siliconized glass-fibre membranes (Glassybond; Biometra, Heidelberg, Germany) using the procedure supplied by the manufacturer. Electrotransfer was performed with a constant current of 6 mA cm^{-2} for 10 h at 4°C in a Mini Trans-Blot Cell (Bio-Rad Labs.). The transferred protein was stained with 0.1% Coomassie Brilliant Blue dissolved in 10% methanol for 3 min.

Destaining was performed in an aqueous solution of 45% methanol in 10% acetic acid for 20 min with two changes of the solution. The membrane was thoroughly washed overnight in distilled water. The stained bands were then excised, dried under air and subjected to amino acid sequence analysis [10]. The bands were placed on top of a Biobrene Plus pretreated glass-fibre disc (Applied Biosystems, Weiterstadt, Germany) and mounted in the cartridge of a Model 477A Protein/Peptide Sequenator (Applied Biosystems). Sequence determination was performed by the Edman procedure using the standard protocol given by the manufacturer (normal-1). The identification of the phenylthiohydantion (PTH) amino acid derivatives was performed with a Model 120A on-line PTH analyser (Applied Biosystems).

RESULTS AND DISCUSSION

Fig. 1A shows that 2-methyleneglutarate mutase and 3-methylitaconate isomerase eluted almost together by FPLC on the anion exchanger Mono Q at pH 7.4. The enzymes were also not separated by running the same chromatography at pH 8.0 (data not shown). However, when the enzymes were preincubated with 6 m*M* CHAPS and the FPLC was repeated in the presence of 2 m*M* of the same non-denaturating detergent at pH 7.4, complete separation was achieved (Fig. 1B). CHAPS influenced neither the activity nor the stability of 2-methyleneglutarate mutase. This separation was apparently specific, as the general elution profile of the protein as measured at 280 nm was not changed much by CHAPS. Notably, 2-methyleneglutarate mutase and 3-methylitaconate isomerase are both soluble cytosolic proteins, whereas CHAPS is usually applied for solubilization of membrane proteins [11]. At this stage the mutase was not pure (about 27% purity), as revealed by its specific activity (Tables I and II) and by SDS-PAGE (Fig. 2), whereby this enzyme represented the lower of the double band at 67 000 dalton. However, this preparation could be used for studies on the sensitivity of the mutase towards light and oxygen [6]. The whole purification protocol is summarized in Table I. Further but not complete purification was achieved by FPLC on Phenyl-Superose HR 10/10 (data not shown). By the same procedure (Table I) 3-methylitaconate Δ-isomerase was purified 24-fold up to a specific activity on 108 U mg^{-1} and 46% yield, but the preparation was also not homogeneous (about 10% purity).

In order to obtain pure 2-methyleneglutarate mutase, another protocol involving additional steps was developed (Table II). Starting from a larger amount of cell-free extract, precipitation with ammonium sulphate was introduced in order to remove much of the inactive protein. Thus, the same Q-Sepharose column as in the former method could be used. Subsequently the enzyme was subjected to FPLC on the molecular sieve Superdex 200. By each of the two chromatographic steps the mutase was purified threefold with recoveries of 82% and 95%, respectively. The separation of the mutase from the Δ-isomerase was achieved on a phenyl-Sepharose column in the absence of CHAPS (Fig. 3). Although this step resulted in a loss of 85% of the mutase, it could not be replaced by a milder

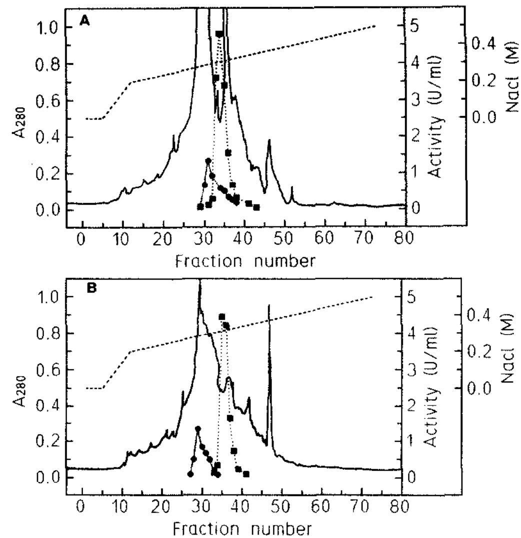

Fig. 1. FPLC on the anion exchanger Mono Q 10/10 (fast separation protocol). Solid line, absorbance at 280 nm; dashed line, NaCl gradient; dotted lines, enzymatic activity: ● = 2-methyleneglutarate mutase; ■ = 3-methylitaconate isomerase (the activity of the isomerase is 15 times higher than indicated on the scale "activity"). (A) In the absence of CHAPS; (B) in the presence of CHAPS.

TABLE I

FAST SEPARATION PROTOCOL FOR 2-METHYLENEGLUTARATE MUTASE

Step	Volume (ml)	Protein (mg)	Activity (U)	Specific activity (U/mg)	Yield (%)
Cell-free extract	19	917	64	0.07	100
Q-Sepharose	9	146	52	0.36	81
Mono Q/CHAPS	6	25	37	1.47	58

TABLE II

LARGE-SCALE PURIFICATION PROTOCOL FOR 2-METHYLENEGLUTARATE MUTASE

Step	Volume (ml)	Protein (mg)	Activity (U)	Specific activity (U/mg)	Yield (%)
Cell-free extract	105	4935	444	0.09	100
Ammonium sulphate	70	2450	337	0.14	76
Q-Sepharose	14	616	276	0.45	62
Superdex 200	7	172	262	1.53	59
Phenyl-Sepharose	5	10	38	3.8	9
Mono Q at pH 8.0	6	5	27	5.4	6

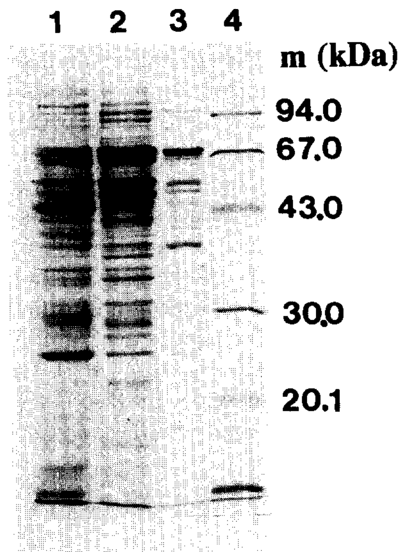

Fig. 2. SDS-PAGE of the steps of the fast separation protocol of 2-methyleneglutarate mutase. Lanes: 1 = cell-free extract (19 μg); 2 = Q-Sepharose (6.5 μg); 3 = Mono Q (4 μg); 4 = marker proteins (m = molecular mass; kDa = kilodalton).

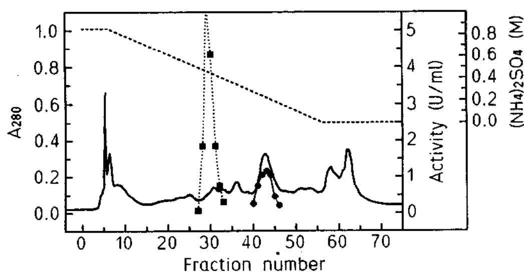

Fig. 3. FPLC on phenyl-Sepharose HiLoad (large-scale protocol). Solid line, absorbance at 280 nm; dashed line, ammonium sulphate gradient; dotted lines, enzymatic activity: ● = 2-methyleneglutarate mutase; ■ = 3-methylitaconate isomerase (the activity of the isomerase is 15 times higher than indicated on the scale "activity").

procedure. On a smaller scale, phenyl-Superose HR 10/10 instead of phenyl-Sepharose was used with almost identical results. Nearly homogeneous mutase was obtained by the final purification on Mono Q at pH 8 (Fig. 4, lane 6). When ten times as much mutase was applied to the gel, an impurity (67 000 dalton) of less then 5% became visible. The specific activity of the highly purified enzyme (5.4 U mg^{-1}, Table 2) was 2.4 times higher than that obtained earlier by preparative PAGE (2.4 U mg^{-1}) [5]. The 3-methylitaconate Δ-isomerase preparation which

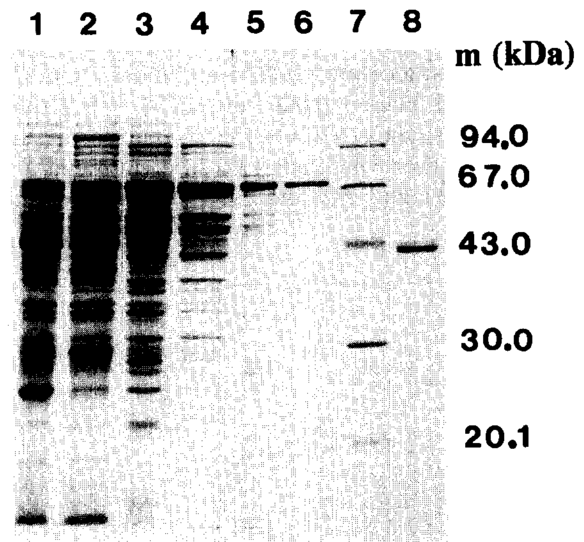

Fig. 4. SDS-PAGE of the steps of the large-scale purification protocol of 2-methyleneglutarate mutase. Lanes: 1 = cell-free extract (19 μg); 2 = ammonium sulphate fraction (21 μg); 3 = Q-Sepharose (18 μg); 4 = Superdex 200 (12 μg); 5 = phenyl-Sepharose HiLoad (2 μg); 6 = Mono Q (1 μg); 7 = marker proteins; 8 = phenyl-Sepharose fraction of the 3-methylitaconate isomerase (2 μg).

was obtained from the phenyl-Sepharose was not as active (44 U mg^{-1}) as that obtained with the CHAPS procedure (108 U mg^{-1}). The enzyme probably represented the less intensively stained upper band of the SDS-PAGE (Fig. 4, lane 8).

The successful separation of 2-methyleneglutarate mutase and 3-methylitaconate isomerase by a detergent or by chromatography on phenyl-Sepharose suggested a possible association of both enzymes by hydrophobic interactions. In addition to solubilization of membrane proteins, the application of mild detergents should be considered in related separation problems in order to improve the resolution of chromatographic steps. Hydrophobic interaction chromatography was also very efficient in purifying other enzymes, *e.g.*, 2-hydroxyglutaryl-CoA dehydratase from *Fusobacterium nucleatum* [12].

If the purification of 2-methyleneglutarate mutase was performed in the dark, the enzyme was active without added coenzyme B_{12} (adenosylcobalamin). The pink-orange colour of the mutase and the UV–VIS spectrum resembling that of adenosylcobalamin (Fig. 5) indicated that the coenzyme remained bound to the enzyme during purification. Using the molar absorption coefficient of adenosylcobalamin (ε_{522} = 8.0 l $mmol^{-1}$ cm^{-1}), the content of this coenzyme was calculated as 2.15 mol mol^{-1} homotetramer (mol. mass 300 000 dalton [5]). When the coenzyme was converted into the dicyano form [9], similar values were obtained (two different preparations gave 2.11 and 2.08 mol mol^{-1}). The protein

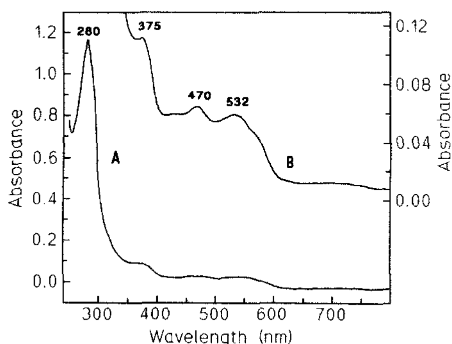

Fig. 5. UV–VIS spectrum of highly purified 2-methyleneglutarate mutase (0.8 mg ml^{-1}, purified through the Mono Q step, Table II) in 20 m*M* potassium phosphate (pH 7.4). Line A, left-hand scale; line B, right-hand scale.

1				5					10
Met	Gln	Glu	Lys	Thr	Lys	Arg	Ile	Ile	Lys
11				15					20
Glu	Asp	Ile	Glu	Ala	Val	Arg	Ala	Tyr	Ser
21									
Asp									

Fig. 6. N-Terminus of 2-methyleneglutarate mutase.

content of a sample was thereby taken from the average of the values estimated by three different methods [7,13,14]. Notably, the mutase preparation obtained earlier by non-denaturing preparative PAGE without protection from light was also pink but its enzymatic activity was completely dependent on added adenosylcobalamin [5].

The purity of the 2-methyleneglutarate mutase was also checked by the determination of the N-terminal amino acid sequence of the polypeptide. Therefore, an SDS-PAGE of the mutase was blotted on glass-fibre membranes and the protein band was subjected to Edman degradation by a gas-phase sequenator. The sequence obtained (Fig. 6) demonstrated the homogeneity of the enzyme but had no similarity to those of the N-termini of other adenosylcobalamin-dependent enzymes, *e.g.*, glutamate mutase from *Clostridium cochlearium* (E.C. 5.4.99.1) [15] or methylmalonyl-CoA mutase (E.C. 5.4.99.2) from mice [16] or *Propionibacterium shermanii* [17].

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