Bioorganic Chemistry 36 (2008) 261–264

Contents lists available at ScienceDirect

Bioorganic Chemistry

journal homepage: www.elsevier.com/locate/bioorg

The putative coenzyme B_{12}-dependent methylmalonyl-CoA mutase from potatoes is a phosphatase

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ARTICLE INFO

Article history:
Received 1 February 2008
Available online 29 July 2008

Keywords:
Coenzyme B_{12} in plants?
Methylmalonyl-CoA
Phosphatase

ABSTRACT

The reported presence of a coenzyme B_{12}-dependent methylmalonyl-CoA mutase in potatoes has been reexamined. The enzyme converting methylmalonyl-CoA was purified to electrophoretic homogeneity. Examination of the reaction product by ^{1}H, ^{31}P NMR and mass spectrometry revealed that it was methylmalonyl-3′-dephospho-CoA. The phosphatase enzyme needs neither coenzyme B_{12} nor *S*-adenosylmethionine as a cofactor.

1. Introduction

During the last decades of the past century a number of publications appeared claiming the presence of coenzyme B_{12}-dependent enzymes in plants [1–5]. On the other hand, there is no evidence for the occurrence of cobalamin in these organisms. This contradiction and our continuing interest in B_{12}-dependent enzymic reactions prompted us to reinvestigate the report on the presence of the coenzyme B_{12}-dependent methylmalonyl-CoA mutase in potatoes [3].

In the latter paper it was claimed that the product of the reaction was succinyl-CoA "identified" only by the similar retention time in a certain chromatographic system. Obviously, such an identification does not meet serious standards. Though the authors also claimed that the conversion was dependent on coenzyme B_{12}, these results appeared to us rather unlikely.

In the following we describe the results we reached in our endeavors.

2. Materials and methods

The potatoes were commercially available, methylmalonyl-CoA was supplied from Sigma. Methylmalonyl-CoA mutase and oxalacetate-transcarboxylase for the spectrophotometric activity assays are isolated as described [6]. Malate dehydrogenase was supplied from Sigma.

2.1. Enzyme assay 1

The spectroscopic assay was carried out using a Cary 3 UV/VIS-spectrophotometer as described [6–9].

2.2. Enzyme assay 2

The activity of the enzyme was quantified by HPLC (Agilent 1200) chromatography (RP-18 column, 125 × 4 mm) using a method described previously [10]. Forty-five microliters of enzyme solution was added to 150 µl 0,05 M K_2HPO_4/KH_2PO_4 buffer, pH 7.5, 5 µl methylmalonyl-CoA solution (4 mM) was added to start the reaction which was stopped by 10 µl TFA after 30 min incubation at 37 °C. The protein was removed by centrifugation (5 min, 13,000 rpm). Hundred and fifty microliters of supernatant was diluted 1:1 with buffer (90% buffer A 0.2 M NaH_2PO_4, pH 4.5 and 10% buffer B (0.2 M NaH_2PO_4 and 20% acetonitrile) was applied to the RP-18 column which was equilibrated with 10% buffer B/90% buffer A. Product and substrate of the enzymatic reaction were separated with a 12 min continuous gradient run (0.75 ml/min, starting with 10% of buffer B, 63% buffer B after 5 min, 60% buffer B after 7 min, 100% buffer B after 11 min), product and substrate of the reaction are eluted as resolved peaks and can be detected at 254 nm.

2.3. Purification of the enzyme

Washed and peeled potatoes were cut into pieces and homogenized in a mixer 1:1 (w/w) with 100 mM K_2HPO_4/KH_2PO_4, pH 7.0 (4 °C). The suspension was centrifuged for 30 min at 15,000 rpm (4 °C). The supernatant was decanted, filtered through a 22 µm

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doi:10.1016/j.bioorg.2008.06.002

Table 1
Isolation of putative methylmalonyl-CoA mutase out of 450 g potatoes

Purification step	Volume (ml)	Protein (mg)	Activity (U)	Specific act. (U/mg)
Cell disruption	150	1000		
DEAE column	100	80		
Gel filtration	30	30		
Anion exchange	8	28	0.004	0.001
Chromatofocussing	7.5	10.8	0.0008	0.0006
Anion exchange II	1	0.4		

The enzyme activities were determined by the HPLC assay [10]. After the last purification step (ion exchange column II) the amount of the protein was too small for the assay and was used for SDS-electrophoresis (see Fig. 2).

sterile filter and applied to a Fractogel-DEAE (100 ml) anion exchanger purchased from Merck Chemical Company which was previously equilibrated with buffer C (20 mM K_2HPO_4/KH_2PO_4, pH 7.0). The separation was carried out at 4 °C, the column was washed with 200 ml buffer C (4–5 ml/min), then with 200 ml 20% buffer B. The active fraction was eluted with 200 ml 30% buffer B and was concentrated with centricons to a volume of 15 ml which was applied to a Superdex HiLoad (Pharmacia, Sweden) gel filtration column equilibrated with buffer (100 mM K_2HPO_4/KH_2PO_4, pH 7.2). The enzyme solution from this purification step was loaded to a Resource Q anion exchanger after equilibrating the enzyme with buffer B (BB, 50 mM K_2HPO_4/KH_2PO_4, pH 7.0). The separation was carried out at a flow rate of 3 ml/min. The elution profile starts with 15 min BB, a continuous buffer gradient ends after 75 min with 100% elution buffer (EB, 50 mM K_2HPO_4/KH_2PO_4, pH 7.0), proteins were detected at 280 nm. After this (and all following) purification step fractions were assayed and the active enzyme fractions were pooled, protein content was measured at 280 nm with a in 1 ml quartz cuvettes using a Cary 3 UV/VIS-spectrophotometer.

The active enzyme was equilibrated with buffer 1 and applied to Mono P 5/20 (4 ml) chromatofocussing column. The column was equilibrated with buffer 1 (25 mM L-histidine, 35 mM NaCl, pH 6.2) at a flow rate of 0.75 ml/min. 40 mg of protein were loaded and eluted with buffer 2 (Polybuffer 74-HCl from Pharmacia).

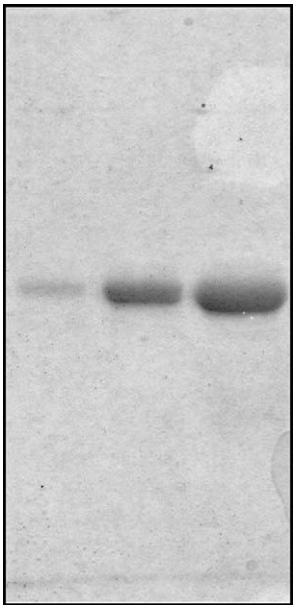

Fig. 2. SDS-electrophoresis of the active enzyme after the last purification step.

As a last purification step separation on a second Resource Q column was carried out in the same manner as described for the first column. For all protein purification steps a Pharmacia LCC-501 PLUS liquid chromatograph was used. The progress of the purification is shown in Table 1.

3. Results and discussion

3.1. Enzyme assays

A fresh extract of potatoes, prepared as previously described [3], was used as enzyme source. The putative methylmalonyl-CoA mutase was assayed by two different methods. The spectrophotometric assay starts with the substrate succinyl-CoA and is

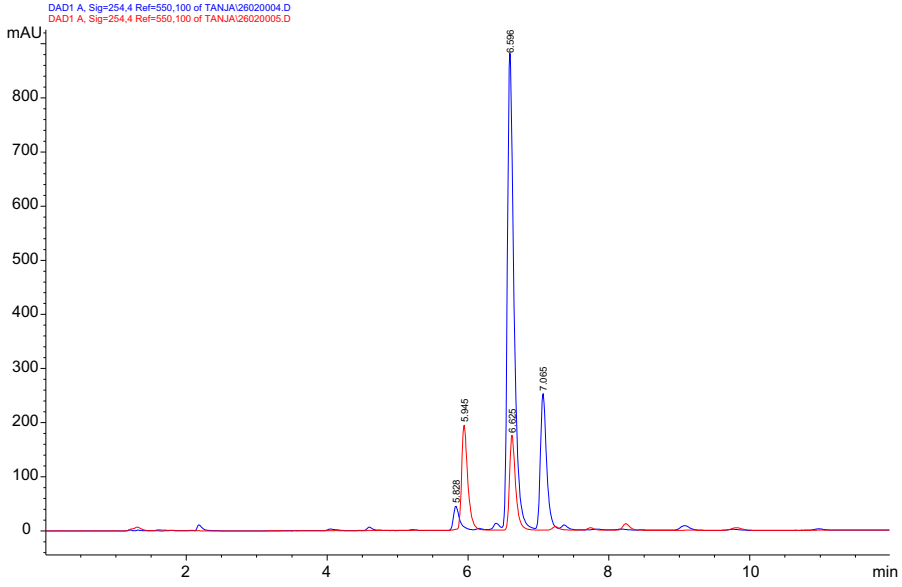

Fig. 1. Conversion of methylmalonyl-CoA with the putative mutase from potatoes and synthetic succinyl—CoA (retention time: 6.625 min), which is contaminated with some CoA (retention time: 5.945 min, red trace) and the reaction product mixture (retention times: 6.596 and 7.065 min, blue trace). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

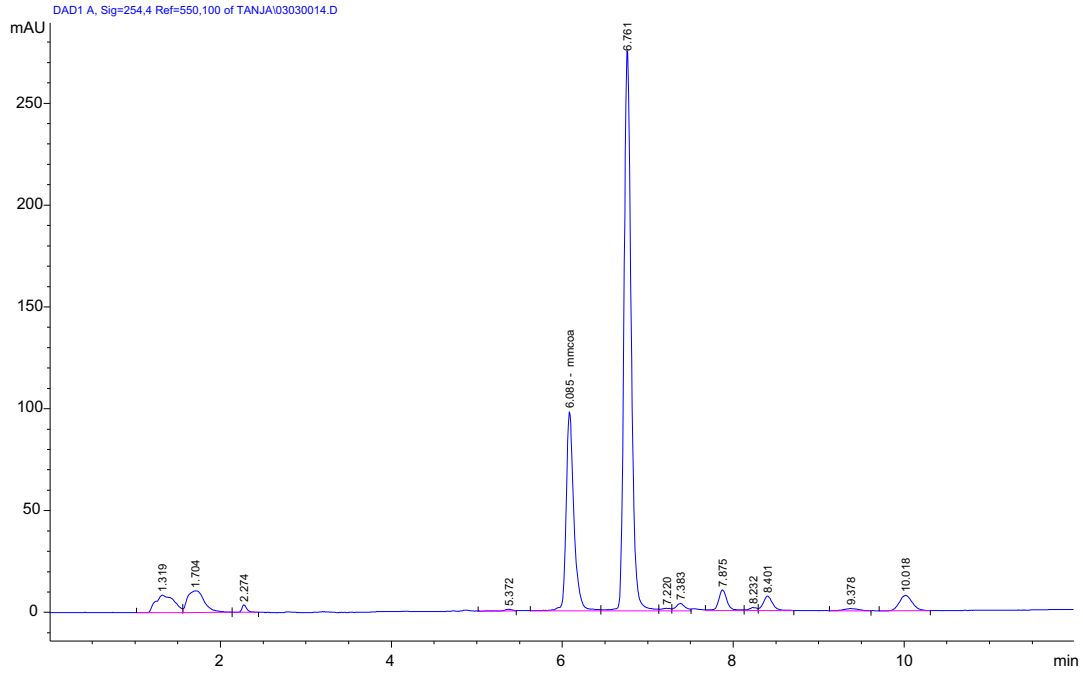

Fig. 3. Conversion of methylmalonyl-CoA. The Peak at 6.085 min retention time is methylmalonyl-CoA, the peak at 6.7 min is succinyl-CoA.

based on the indirect detection of the generated methylmalonyl-CoA by the use of the auxiliary enzymes methylmalonyl-CoA epimerase, transcarboxylase and malate dehydrogenase [7–9]. The consumption of NADH in the reaction catalyzed by the last enzyme is then monitored at 340 nm. Because of the contaminant NADH oxidase in the crude extract this assay was associated with relatively large error. After chromatographic separation no activity could be detected by the spectrophotometric assay.

The second assay of methylmalonyl-CoA mutase was based on the separation of the products by HPLC as described in the experimental section. All assays were carried out in the presence of either coenzyme B_{12} or *S*-adenosylmethionine (SAM) but none of them had an effect on the reaction.While the spectrophotometric assay gave erratic results, the HPLC-monitored assay showed a reproducible and enzyme-dependent conversion of (2 *R,S*) methylmalonyl-CoA to a product that showed almost the same retention time as succinyl-CoA (Fig. 1). Due to withdrawing samples from the reaction mixture at certain time intervals the range of error was relatively high ±50%.

3.2. Enzyme purification

The enzyme that catalyzed this reaction was purified from the crude extract by chromatography on the following columns:

Fractogel DEAE, preparative gel filtration (TSK), Resource Q anion exchanger, chromatofocussing on Mono P 5/20 and a second anion exchanger. The results of the purification procedure are summarized in Table 1. The purity of the final preparation was more than 90% (Fig. 2) and had a molecular mass (M_R) of 39 kDa. A phosphatase from potato was purified and its kinetic analysis presented already more than 40 years ago [11]. It seems to us likely that the activity detected in our work stems from the same enzyme.

The conversion of methylmalonyl-CoA was determined after each purification step by the HPLC method (Fig. 3). By incubation for 30 min with 50 μl enzyme solution at various temperatures the total conversion of methylmalonyl-CoA was also determined (Table 2). Considering that the commercial methylmalonyl-CoA contained approximately equal amounts of the (2*R*)- and (2*S*)-epimers and that methylmalonyl-CoA mutase from several sources is specific for the (2*R*)-epimer [12,13] the more than 50% conversion was unexpected.

For further characterization of the product the corresponding HPLC fraction was collected on a preparative scale and its ^{1}H, ^{31}P NMR and mass spectra were measured using a Bruker spectrometer.

The ^{1}H-NMR spectrum of the product at 600 MHz was virtually identical to that of methylmalonyl-CoA. Only the 3′-ribose signal was slightly shifted to higher field. Comparing the ^{31}P NMR spectrum recorded at 121.44 MHz for the substrate (methyl malonyl CoA): $\delta = -0.13$ (d, 1P); -10.75(d, 1P); -11.39(d, 1P); and for the isolated product (3′-dephospho methyl malonyl CoA): $\delta = -10.79$(d, 1P); -11.36 (d, 1P); the latter one contains only two phosphorous atoms while the signal for the 3′-phosphate is missing (Fig. 4). MALDI-TOF spectra were recorded using a Bruker Daltonics Biflex 3 spectrometer. Comparison of the MALDI-TOF spectra of the authentic dilithium salt of methylmalonyl-CoA with that of the enzymatic product (mol peaks at 881 and 790 Da, respectively, revealed a difference of 91 Da). This compares favorably with the difference of 92 Da calculated for the above mentioned lithium salt and methylmalonyl-dephospho-CoA.

Table 2
Enzymatic conversion of methylmalonyl-CoA at different temperatures

Temperature (°C)	Enzymatic conversion of methylmalonyl-CoA (%)
4	45
27	81
37	89
50	100

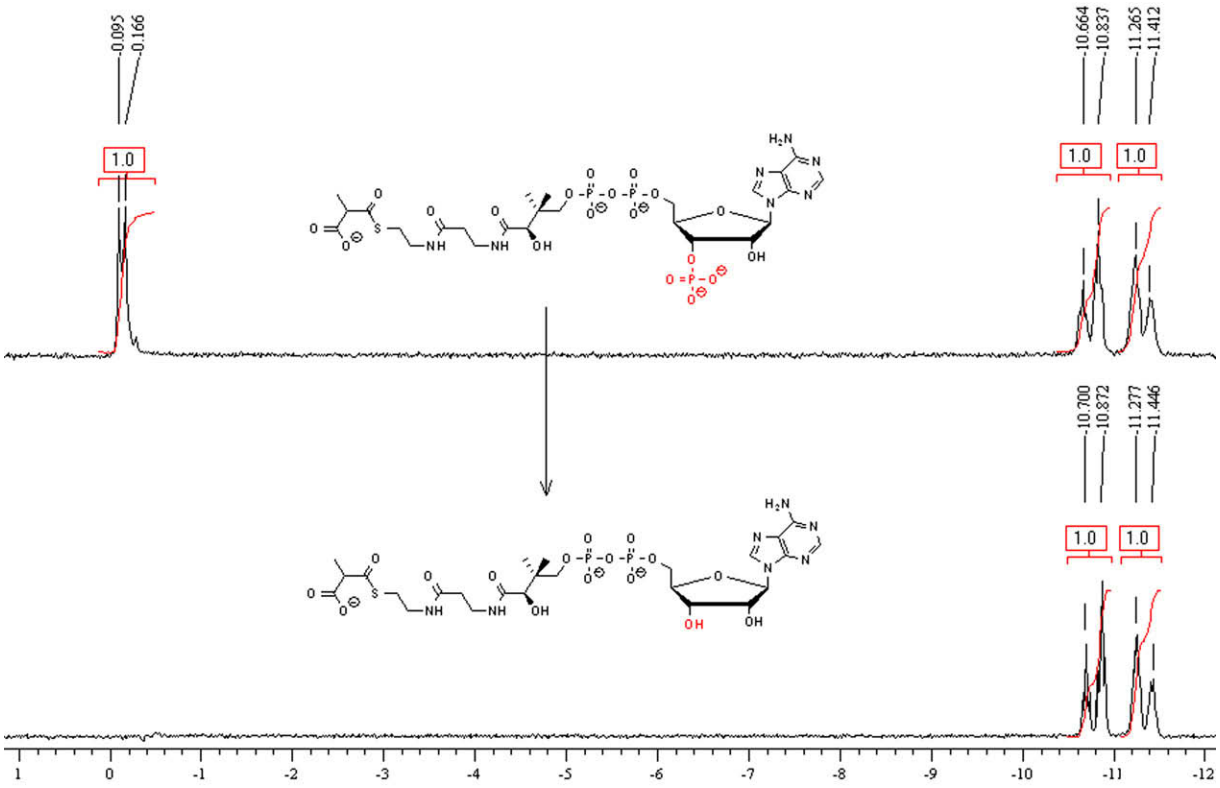

Fig. 4. The ^{31}P NMR spectrum methyl malonyl CoA and for 3′-dephospho methyl malonyl CoA.

All of these results provide evidence that the enzyme from potatoes that converts methylmalonyl-CoA is not a mutase but a phosphatase. A non-specific phosphomonoesterase from potatoes has been previously described [11]. Kinetic and inhibition experiments with various phosphatase inhibitors support the presence of the same phosphatase in our preparations (data not shown).

We looked also for leucine 2,3 aminomutase in potatoes and bean seedlings but could not reproduce the results published earlier [2] (E. Kervio & J. Rétey, unpublished).

The conclusion is therefore justified that neither vitamin B_{12} nor coenzyme B_{12}-dependent enzymes could as yet found in plants. Although their presence cannot be definitively ruled out, the fact that strict vegetarians often suffer pernecious anemia, a lethal disease caused by vitamin B_{12} deficiency, make it unlikely that plants contain B_{12}-dependent enzymes.

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