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PURIFICATION OF METHYLMALONYL-CoA MUTASE FROM *PROPIONIBACTERIUM SHERMANII* USING AFFINITY CHROMATOGRAPHY

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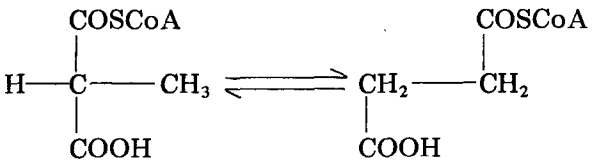
Summary

A novel procedure for the purification of methylmalonyl-CoA mutase from *Propionibacterium shermanii* has been described which employs affinity chromatography on a column of immobilized vitamin B-12 linked covalently to Sepharose. The method has the advantage of being simple and rapid, thus enabling the purification of the enzyme to near homogeneity with good yields.

The enzyme methylmalonyl-CoA mutase from *Propionibacterium shermanii* catalyzes the reversible conversion of (2*R*)-methylmalonyl-CoA to succinyl-CoA according to the following reaction:



(2*R*)-methylmalonyl-CoA Succinyl-CoA

This reaction is one of the ten distinct enzymatic reactions requiring coenzyme B-12 as a cofactor [1]. The bacterial methylmalonyl-CoA mutase has been studied extensively with particular reference to the mechanism of the enzyme catalyzed rearrangement as well as the role played by the coenzyme (for a recent review, see ref. 2). The inactive enzyme-inhibitor complex has been crystallized employing hydroxycobal(III)amine in place of the naturally active cofactor [3].

While conducting studies on the enzyme from *Propionibacterium shermanii* in our laboratory, we decided to investigate the merits of an affinity chromatographic procedure employing immobilized vitamin B-12 as the affinity ligand.

Such a procedure will have the advantage of providing a general method for the purification of coenzyme B-12-dependent enzymes to a high degree with appreciable yields, which is beyond the scope of conventional procedures described for the purification of methylmalonyl-CoA mutase [3,4]. Also, the general procedure will be a valuable tool for purifying all the known coenzyme B-12-dependent enzymes from a variety of sources, taking advantage of their common property of binding to vitamin B-12. This communication describes such a purification procedure employing affinity chromatography on a column of vitamin B-12 linked covalently to Sepharose. To our knowledge, this is the first time affinity chromatography on immobilized vitamin B-12 has been employed to purify coenzyme B-12-dependent enzymes, although cobalamin binding proteins from plasma and gastric juices have been purified using this principle [5,6].

The chemicals used were purchased from the following sources: bovine serum albumin, coenzyme B-12, ammonia free glycine and vitamin B-12 (cyanocobalamin) from Calbiochem, La Jolla, Calif., U.S.A.; DEAE cellulose, dithioerythritol, malate dehydrogenase, NADH and sodium pyruvate were obtained from Sigma Chemical Co., St. Louis, U.S.A.; DEAE Sephadex, Sephadex G-25 and Sepharose 4-B from Pharmacia, Piscataway, U.S.A.; Dowex 50 H^+ form (200—400 mesh) from Dow Chemical Company, Midland, U.S.A.; and 3,3′-diaminodipropylamine from Eastman Company, Rochester, U.S.A. All other chemicals were of reagent grade and were obtained commercially. Vitamin B-12-Sepharose was prepared according to the procedure of Allen and Majerus [7]; and all operations involving vitamin B-12 affinity chromatography were carried out in the dark at 0—5°C. A culture of *Propionibacterium shermanii* 52 W, was kindly provided by Dr. Harland G. Wood, Case Western Reserve University, Cleveland, Ohio, U.S.A. The bacteria were grown, harvested and extracted according to the procedure of Kellermeyer et al. [4] except that 1 mM dithioerythritol was used in place of cysteine. All other operations were carried out between 0—5°C. In a typical procedure, the cell-free extract (10 ml) was absorbed on DEAE-cellulose and the enzyme fraction recovered by batch elution with 0.3 M phosphate buffer (pH 6.8) essentially by the method of Zagalak and Retey [3], and passed through a Sephadex G-25 column previously equilibrated with 0.1 M phosphate buffer (pH 7.0). A column of vitamin B-12-Sepharose (1 × 6 cm) was washed extensively with several volumes of 0.1 M phosphate buffer (pH 7.0) prior to the application of the enzyme fraction from the previous desalting step. After all the sample (120 ml) had passed through the affinity column, the column was washed with 10 more vols. of 0.1 M phosphate buffer (pH 7.0). The non-specifically absorbed proteins were removed by washing the column with 20 vols. of 0.1 M glycine buffer (pH 10.0) containing 0.5 M NaCl. By this procedure, less than 2% of the absorbed enzyme protein was eluted in this step. The methylamalonyl-CoA mutase activity was selectively eluted by washing the column with 0.1 M phosphate buffer (pH 7.0) containing increasing concentrations of coenzyme B-12. Fractions of 1 ml were collected and those fractions containing the highest specific activities of the enzyme were pooled (5 ml). An effective concentration of 0.02 M coenzyme B-12 was necessary to remove all the enzyme bound to the affinity column. In subsequent trials,

the washing of the column with glycine buffer prior to eluting the enzyme was replaced by washing with 0.5 M phosphate buffer (pH 7.0) containing 0.5 M NaCl. This procedure proved to be more gentle and did not adversely affect the recovery of the enzyme. The enzyme activity in the different fractions were assayed according to the procedures outlined by Stjernholm and Wood [8] or by the method of Zagalak and Retey [3]. The protein concentration in the different fractions were determined by a microbiuret method [9]. A unit of the enzyme is defined as the amount of mutase which catalyzes the conversion of 1 μmol of succinyl-CoA per min under standard conditions of the assay procedure.

Preliminary experiments in the Beckman model E ultracentrifuge indicate that the enzyme fraction at this stage of purification obtained after affinity chromatography is homogeneous. The results of a typical purification procedure are summarized in Table I. Starting with 10 g of packed cells, a 500-fold purification was achieved after Step 3. A total of 70% of the original activity was recovered at this stage, with a final specific activity of about 24 units/mg protein. The procedure described here is simple, gentle and quite rapid. The entire procedure may be completed within 48 h. The washing procedure for the affinity column in Step 3 may be carried out employing the buffer with a salt gradient (0—0.5 M NaCl) and may be completed in 18 h, overnight. The final elution is carried out during the day to enable the assay of the active enzyme fractions to be completed without undue delay. In a typical purification trial, starting with the crude extract containing 964 mg protein and 38.9 units of the enzyme we were able to recover 26.9 units of the pure enzyme equivalent to 1.13 mg protein.

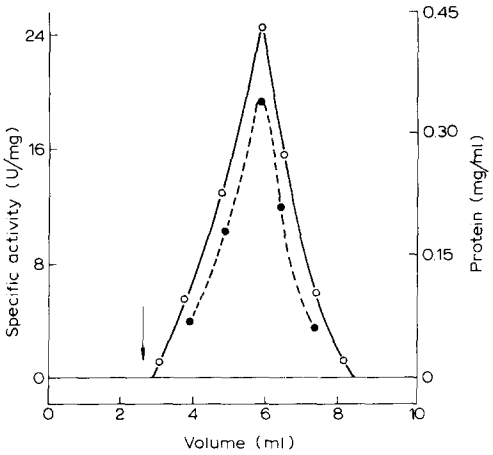
The purified mutase could be stored at $-10°C$ for at least 3 months without any loss of activity. The enzyme is active over a pH range between 6.5 and 8.0. In this investigation, the enzyme fraction 2 was routinely assayed with added coenzyme B-12 although it did possess enzyme activity without the addition of the coenzyme. The enzyme activity was linear for at least 5 min under the assay conditions used. The enzyme activity was proportional to the enzyme protein concentration and was linear between protein concentra tions of 1—15 μg in a total volume of 1 ml incubation mixture. The results of a typical affinity chromatography separation are summarized in Fig. 1.

TABLE I

PURIFICATION OF METHYLMALONYL-CoA MUTASE FROM *PROPIONIBACTERIUM SHERMANII*

The enzyme activity was derived from 10 g packed cells grown in 10 l medium for 5 days. See text for details.

Fraction number	Step	Total recovered activity		Specific activity (units/mg protein)
		%	Units	
1	Cell-free extract (10 ml)	100	38.90	0.04—0.06
2	Absorption and batch elution with 0.3 M phosphate buffer (pH 6.8) from DEAE-cellulose (120 ml)	100	38.85	0.07—0.08
3	Combined affinity chromatography fraction (5 ml)	65—70	26.91	23.8—24.0



Affinity chromatography of methylmalonyl-CoA mutase on vitamin B-12-Sepharose. After the enzyme fraction from Step 2 was applied to the vitamin B-12-Sepharose affinity column, the column was washed successively with 20 vols. each of 0.5 M phosphate buffer (pH 7.0), and 0.5 M phosphate buffer (pH 7.0) containing 0.5 M NaCl. The mutase activity was eluted from the column with phosphate buffer containing 0.02 M coenzyme B-12, as indicated by the arrow. Aliquots of active enzyme fractions were assayed for both enzyme activity and protein content. Protein was assayed after extensive dialysis of each fraction against 50 mM phosphate buffer (pH 7.0), to remove the excess coenzyme present. ○——○, specific activity; ●-----●, protein concentration.

The enzyme prepared by our procedure has properties very much similar to those described by Kellermeyer and Wood [4] and by Zagalak and Retey [3]; however, the final specific activity of our enzyme preparation is about twice as high as that reported by these researchers for the purest enzyme preparations. It is possible that, in our procedure, the coenzyme B-12 associated with the purified fractions imparts the enzyme its higher stability; thereby the losses in activity due to inactivation are kept to a minimum. Additionally, the covalently bound vitamin B-12 affords better stability to the enzyme during the affinity chromatography step. This may account for the higher specific activity of the enzyme preparations described in this report.

Methylmalonyl-CoA mutase is one of the two coenzyme B-12-dependent enzymes functional in man. A congenital deficiency or a defect in this enzyme is expressed as a genetic disorder, methylmalonic aciduria, which is refractory to vitamin B-12 therapy [10]. Our procedure may be conveniently adapted to purify the enzyme from human liver specimens, obtained during biopsy or after surgery. In view of the fact that the mutase activity in human livers is very stable in lyophilized mitochondria [11], this purification procedure could be used to advantage in obtaining enzyme preparations with high specific activity from human liver.

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References

1 Barker, H.A. (1972) Ann. Rev. Biochem. 41, 55—78
2 Babior, B.M. (1975) Acc. Chem. Res. 8, 376—384
3 Zagalak, B. and Retey, J. (1974) Eur. J. Biochem. 44, 529—535
4 Kellermeyer, R.W., Allen, S.H.G., Sterjnholm, R. and Wood, H.G. (1964) J. Biol. Chem., 239, 2562—2569
5 Allen, R.H., Burger, R.L., Mehlman, S.C. and Majerus, P.W. (1974) Methods Enzymol. 34, 305—314
6 Nexo, E. (1975) Biochim. Biophys. Acta 379, 189—192
7 Allen, R.H. and Majerus, P.W. (1972) J. Biol. Chem. 247, 7695—7702
8 Stjernholm, R.and Wood, H.G. (1971) Proc. Natl. Acad. Sci. U.S. 47, 303—313
9 Itzhaki, R.F. and Gill, D.M. (1964) Anal. Biochem. 9, 401—409
10 Mahoney, M.J. and Rosenbery, L.E. (1971) Biochem. Biophys. Res. Commun. 44, 375—379
11 Whitaker, G. (1976) Fed. Proc. 35, 1429