AFFINITY CHROMATOGRAPHY OF N^5-METHYLTETRAHYDROFOLATE-HOMOCYSTEINE METHYLTRANSFERASE ON A COBALAMIN–SEPHAROSE

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Received 8 October 1977

1. Introduction

For the purification of enzymes or proteins, affinity chromatography has been widely used as one of the most attractive methods [1]. The enzymes which require coenzyme or cofactor are purified by the use of specific interaction between the enzyme and the coenzyme immobilized in the insoluble support [1].

There are several reports for the purification of B_{12}-dependent enzymes or B_{12}-binding proteins by the vitamin B_{12} affinity adsorbents. In the successful approach cobalamin was attached to agarose through a hydrocarbon spacer connected to the corrin nucleus [1–4]. The attachment of the cobalamin to the matrices through the axial ligand could be undesirable, because the K_m value of such modified cobalamin decreases drastically in adenosyl-B_{12}-dependent enzyme systems [2]. However, there is a possibility that this type of affinity adsorbent might work satisfactorily in the purification of B_{12}-dependent methionine synthetase. The apoenzyme of methionine synthetase bound to the adsorbent could be obtained as the holoenzyme by elution after the cleavage of the carbon–cobalt bond by light irradiation as follows:

In this communication we report the synthesis of a cobalamin–Sepharose by the reaction sequence are shown in fig.1, and its application to the purification of N^5-methyltetrahydrofolate–homocysteine cobalamin methyltransferase from *Escherichia coli*.

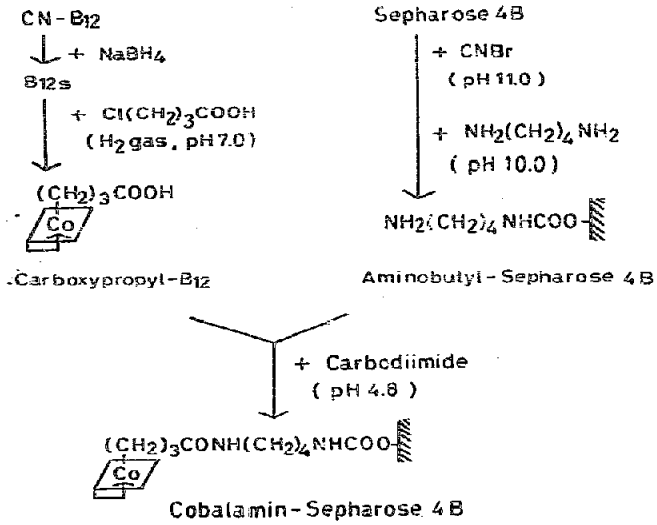
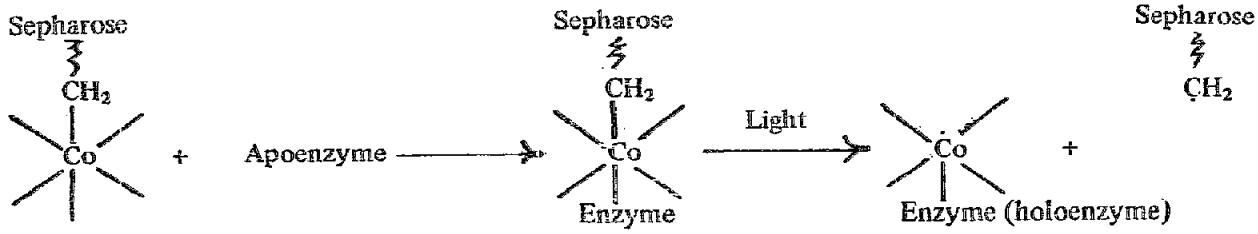


Fig.1. Scheme for the synthesis of cobalamin–Sepharose 4B.



2. Materials and methods

N^5-[^{14}C]methyltetrahydrofolate and [^{14}C]methyl iodide were purchased from Japan Radioisotope Ass. and *S*-adenosylmethionine from Kyowa Hakko Kogyo Co. 4-Chlorobutanoic acid and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide were obtained from Nakarai Chemicals. $FMNH_2$ was prepared each day by the catalytic hydrogenation of FMN with PtO_2 [5]. Unlabeled N^5-methyltetrahydrofolate was synthesized according to the method [6]. [^{14}C]Methyl B_{12} and carboxypropyl-B_{12} were synthesized in the dark by the procedure of [7].

Aminobutyl–Sepharose was prepared by the overnight reaction of cyanogen bromide-activated Sepharose 4B with 1,4-diaminobutane (2 mmol/ml Sepharose) at 4°C, according to the method [8]. Carboxypropyl-B_{12} was coupled to this aminobutyl–Sepharose in the presence of water-soluble carbodiimide to form cobalamin–Sepharose [8]. In order to determine the content of B_{12}, 0.15 ml cobalamin–Sepharose was exposed to a 300 W tungsten lamp for 2 min at a distance of 30 cm on ice and packed in a column. The B_{12} compound photolyzed was eluted with 3 ml distilled water. The amount of B_{12} in the effluent was determined by A_{351} and that of B_{12} bound to Sepharose was calculated as 40 nmol/ml Sepharose.

Escherichia coli B (wild type) was grown aerobically at 37°C in a medium containing (in g/liter): KH_2PO_4, 3.0; K_2HPO_4, 7.0; $MgSO_4 \cdot 7H_2O$, 0.1; $(NH_4)_2SO_4$, 1.0; NaCl, 0.5; sodium citrate, 0.5; yeast extract, 5.0; glucose 10.0. The cells were harvested in late logarithmic phase of growth by centrifugation at 3500 × *g* for 20 min. Cell-free extracts were then prepared by grinding the cells with 2 g alumina/g wet wt cells by using a mortar and pestle on ice [9].

This dialysate was used as crude extract for enzyme assay. For further purification of the enzyme, the supernatant obtained as above was treated with solid ammonium sulfate to get a fraction precipitating at between 35–50% ammonium sulfate saturation. The precipitate was dissolved in 0.05 M potassium phosphate buffer (5 ml/g wet wt disrupted cells) and dialyzed against 1.0 liter 0.01 M potassium phosphate buffer (pH 7.5) for 2.5 h. Dialysate, 30 ml (protein 103 mg) was incubated with DTT (dithiothreitol), 6 μmol; 1.0 M potassium phosphate buffer (pH 7.5) 6 ml and cobalamin–Sepharose 15 ml (wet wt) in a 200 ml beaker with gentle stirring for 20 min at 30°C in the dark. The subsequent work was carried out below 4°C under a dim light. The incubation mixture was packed in a column of 2.7 cm diameter and impure protein was eluted with buffer as described in the legend of fig.2. Afterwards the beads were transferred into a Petri-dish (diam. 8.5 cm) with about 20 ml of 0.05 M potassium phosphate buffer (pH 7.5) and exposed to a 300 W tungsten lamp for 2 min at a distance of 30 cm, on ice, in order to cleave the carbon–cobalt bond of cobalamin–Sepharose. Then, the sample exposed to light was repacked in a column of the same size as above. The column was eluted with 100 ml 0.5 M KCl in 0.05 M potassium phosphate buffer (pH 7.5) and the effluent was collected in 10 ml fractions.

Protein was determined by the method [10]. N^5-Methyltetrahydrofolate–homocysteine cobalamin methyltransferase was assayed essentially by a Dowex 1 (Cl^-) column method [11,12]. The complete system of the assay mixture contained: N^5-[^{14}C]-methyltetrahydrofolate 50 nmol; homocysteine 500 nmol; SAM, 25 nmol; $FMNH_2$, 200 nmol; DTT, 500 nmol; potassium phosphate buffer (pH 7.5), 25 μmol and apoenzyme (protein 1.4 mg) in total vol. 0.425 ml. For the assay of crude extract and ammonium sulfate fraction 5 nmol methyl-B_{12} was added, because enzyme exists as apoenzyme. But no B_{12} was added to the fractions after the light irradiation in the affinity chromatography, where enzyme exists as holoenzyme. The reaction mixtures were incubated in the dark for 15 min at 30°C under an atmosphere of hydrogen gas. One unit of the activity is defined as the amount of enzyme catalyzing the formation of 1 nmol methionine/15 min, at 30°C. Methyl-B_{12}-homocysteine methyltransferase was assayed by measuring the formation of [^{14}C]methionine according to the method [12] with the following modifications:

(i) The reaction mixture contained: [^{14}C]methyl-B_{12} 10 nmol; homocysteine 2 μmol; potassium phosphate buffer 100 μmol; enzyme in total vol. 0.45 ml and was aerobically incubated in the dark for 30 min at 30°C.

(ii) The product, [^{14}C]methionine, was eluted with 2.0 ml 1 M dibasic potassium phosphate from the

Table 1
Purification of B_{12}-dependent methionine synthetase from *Escherichia coli*

	Volume (ml)	Total protein (mg)	Total act. (units)	Spec. act. (units/mg)	Recovery (%)	Purification (-fold)
Crude extract	50	495	1110	2.2	100	1
Ammonium sulfate (35–50%)	30	103	880	8.5	79	3.8
Affinity chromatography						
Fraction No. 43	10	0.18	480	2670	43	1220
Fraction No. 44	10	0.10	380	3800	34	1750

column of Dowex 50 (H^+) (0.6 × 3.0 cm) and collected in a counting vial.

3. Results and discussion

Vitamin B_{12}-dependent methionine synthetase has been purified to homogeneity from *E. coli* [13,14]. However, it requires a multistep procedure to obtain a purified enzyme. In order to simplify the method we used affinity chromatography and purified apomethionine synthetase, which was converted into holoenzyme in a stage of the affinity chromatography.

A summary of the purification is given in table 1. The recovery of the activity (fraction No. 43,44) was about 77% and enzyme was purified to over 1000-fold. The specific activity is at least comparable to the one reported [5]. The purity was estimated to be about 70% from the polyacrylamide disc-gel electrophoresis.

The typical elution pattern of the chromatography is shown in fig.2. Almost all of contaminating proteins were eluted in the washing steps by 0.05 M potassium phosphate buffer and 1 M KCl–0.05 M potassium phosphate buffer. The enzyme was eluted with 0.5 M KCl–0.05 M potassium phosphate buffer after the photolysis of enzyme-bound cobalamin–Sepharose. This enzyme also catalyzes the transfer of methyl group of methyl-B_{12} to homocysteine [13]. We examined this in another experiment. Figure 3 shows the elution profile after photolysis. The peak position of the activity of methyl-B_{12}-homocysteine methyltransferase completely coincides with that of N^5-methyltetrahydrofolate–homocysteine methyltransferase. The similar result was obtained in the affinity chromatography of apomethionine synthetase from *Protaminobacter ruber*, a B_{12}-producing bacterium [9], cultivated in a cobalt deficient medium supplemented with succinate and serine (data are not shown). These results support the finding [13], that N^5-methyltetrahydrofolate–homocysteine transmethylation and methyl-B_{12}–homocysteine transmethylation occur at separate sites on the B_{12} protein.

In this study the carbon–cobalt bond in cobala-

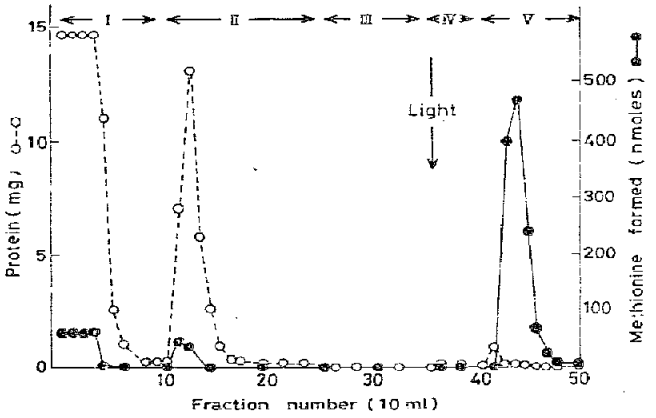


Fig.2. Affinity chromatography of N^5-methyltetrahydrofolate–homocysteine methyltransferase. The cobalamin–Sepharose binding enzyme was washed with [I] 100 ml 0.05 M potassium phosphate buffer, [II] 150 ml 1 M KCl–0.05 M potassium phosphate buffer and [III] 100 ml 0.05 M potassium phosphate buffer. After exposure to visible light, the column was washed with [IV] 50 ml 0.05 M potassium phosphate buffer and finally enzyme was eluted with [V] 100 ml 0.5 M KCl–0.05 M potassium phosphate buffer.

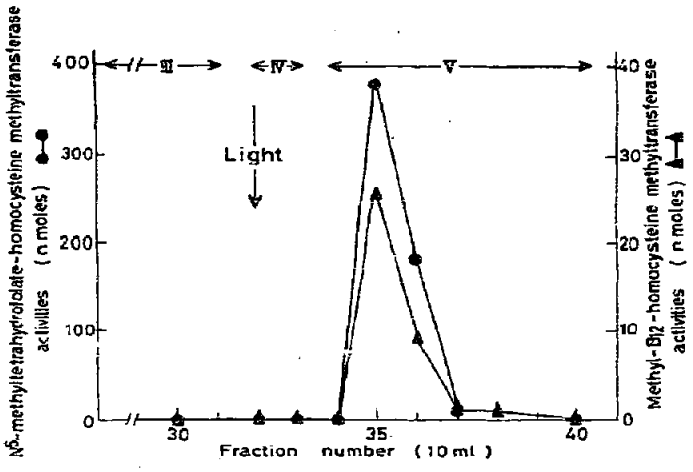


Fig.3. Elution pattern of N^5-methyltetrahydrofolate—homocysteine methyltransferase and methyl-B_{12}-homocysteine methyltransferase from a cobalamin—Sepharose column. Elution was performed as in fig.2, except that this experiment was carried out on a smaller scale.

min—Sepharose was cleaved by the photolysis and thus, the affinity adsorbent is not reusable. We are now investigating the possibility of eluting the protein without photolysis so that the adsorbent would be reusable. It might open the way for the wide application of this type of the adsorbent in the purification of B_{12}-dependent enzymes.

Acknowledgements

The authors are grateful to Drs Thomas H. Finlay and Neil Nathanson for their contribution in the preparation of the affinity adsorbent.

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