BINDING BEHAVIOUR OF PYRUVATE CARBOXYLASE FROM *BACILLUS STEAROTHERMOPHILUS* ON SEPHAROSE–AVIDIN

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1. Introduction

The specific affinity of avidin for biotin [1] affords a potentially powerful facility for the affinity chromatography of biotin-containing carboxylases. The extreme tightness of this non-covalent binding has, however, presented problems with regard to the elution of these enzymes from agrose–avidin columns [2]. To our knowledge, no whole, undissociated biotin-containing carboxylase has yet been isolated in satisfactory yield from such columns. We report here the specific binding of pyruvate carboxylase (EC 6.4.1.1) from *Bacillus stearothermophilus* to Sepharose–avidin and its elution in relatively good yield in an undissociated, enzymically active state.

2. Materials and methods

The prototrophic strain of *B. stearothermophilus* var. *nondiastaticus* used in this work and conditions for its growth have been described previously [3]. Procedures for purifying the pyruvate carboxylase from this thermophile to homogeneity have been reported elsewhere [4,5].

Cyanogen bromide-activated Sepharose 4B was purchased from Pharmacia, avidin (tetramer) from Sigma (London) Chemical Company, urea (Analar grade) and guanidinium chloride (Aristar grade) from British Drug Houses Ltd. and D-[carbonyl-^{14}C] biotin of specific radioactivity 58 mCi/mmol from the Radiochemical Centre, Amersham.

The methods for the assay of pyruvate carboxylase and for the disc electrophoresis of protein in polyacrylamide have been given in earlier publications [4,6]. Protein was determined according to Lowry et al. [7].

3. Results and discussion

Sepharose–avidin was prepared as follows, essentially according to the procedure of Guchhait et al. [8]. Cyanogen bromide-activated Sepharose 4B (3.5 g) suspended in 0.1 M sodium acetate, pH 5.5, (volume of the gravity-packed gel was approximately 10 ml) was reacted with 10 mg of avidin at 4°C. The Sepharose–avidin tetramer after being washed as recommended [8] had a biotin-binding capacity of 0.58 μg of biotin per ml of packed gel as determined with [^{14}C] biotin. It was converted to Sepharose–avidin monomer by treatment with 6 M guanidinium chloride. The washed gel–avidin monomer had a biotin-binding capacity of 0.2 μg per ml, which represented approximately 65% dissociation of the subunits of the avidin tetramer, the expected theoretical dissociation being 75%. The monomer form is preferred for affinity chromatography owing to its lower affinity for biotin as compared with the tetramer [8].

A partially purified pyruvate carboxylase preparation was treated with the Sepharose–avidin monomer (volume of packed gel was 5 ml) in 50 mM potassium phosphate (pH 7) containing 0.5 M potassium chloride and 0.4 M ammonium sulphate for 1 hr at 4 °C. At this stage no pyruvate carboxylase activity could be detected in the supernatant portion, indicating that all the enzyme had been bound to the gel–avidin. The slurry was then packed in a column tube of 1 cm diameter and the gel was successively eluted with 20 ml of 50 mM potassium phosphate buffer (pH 7) con-

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Table 1
Fractionation of pyruvate carboxylase on Sepharose–avidin

Fraction	Protein (mg)	Enzyme activity (U)	Specific activity (U/mg protein)
Starting material (partially purified preparation)	30	40	1.3
Breakthrough fraction plus potassium phosphate–potassium chloride buffer eluate	29.5	0	0
Sodium glycinate buffer eluate	0	0	0
Sodium glycinate–biotin buffer eluate	0.03	1	33
Potassium phosphate–urea eluate	0.25	10	40

Experimental details are given in the text. One unit of enzyme activity corresponds to the oxidation of 1 μmol NADH/min. The potassium phosphate–urea eluate (3 ml) was dialyzed for 18 hr at room temperature against 50 mM Tris–HCl buffer (pH 7.5) containing 5 μM acetyl–coenzyme A, 0.1 mM ATP, 0.1 mM $MgCl_2$ and 0.1 μM $ZnSO_4$ before assay.

taining 0.5 M potassium chloride, 20 ml of 0.2 M sodium glycinate buffer (pH 9), 20 ml of the same buffer containing 10 mM D-biotin and 20 ml of 6 M urea in 50 mM potassium phosphate (pH 7). These operations were carried out at room temperature. As shown in table 1, over 98% of the protein in the initial sample did not bind to the Sepharose–avidin and was devoid of pyruvate carboxylase activity. Elution with the biotin-containing buffer released a little more than 2% of the enzyme from the column and the urea-buffer stripped a further 25% of the enzyme. Removal of the urea by dialysis against a suitable buffer (table 1) was essential for reconstitution of the active enzyme. Prolonged exposure to urea resulted in loss of enzyme activity. More protein could be eluted from the column with guanidinium chloride at pH 2, but no enzyme activity was regained from this fraction when the denaturant was removed. In a second, separate run a very impure sample comprising discarded fractions from several experiments and containing 200 mg of protein and 30 units of pyruvate carboxylase (the specific enzyme activity of this preparation, 0.15, was only about 60% of that generally seen in crude cell-free extracts of the thermophile) was fractionated on Sepharose–avidin with results similar to those presented in table 1: 7 units of enzyme were recovered in the urea-buffer eluate at a specific activity of 40.

Fig. 1 presents the protein profiles after polyacrylamide disc gel electrophoresis of samples before and following fractionation on Sepharose–avidin in the first experimental run. The fraction obtained after elution with urea-buffer and removal of the urea contained a single major protein component, which had the mobility expected for native pyruvate carboxylase. In the second experiment the purification factor was over 250 and in both experiments a relatively high yield (approximately 25%) of the enzyme was achieved. Our previously published purification procedures [4,5] can obviously be simplified by the incorporation of this specific affinity chromatography step.

Affinity chromatography on Sepharose–avidin has been exploited by Berger and Wood [2] to isolate the

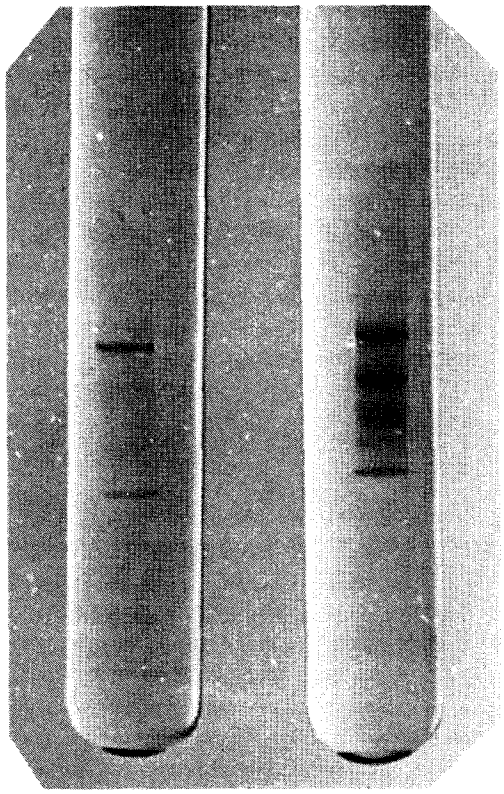

Fig.1. Protein profiles after polyacrylamide disc gel electrophoresis of samples before and after fractionation on Sepharose–avidin. Acrylamide concentration in the gels was 5%. After electrophoresis gels were stained with Amido Black and then destained in several changes of 7% (v/v) acetic acid. The profile on the right is of the starting material and that on the left is of the potassium phosphate–urea buffer eluate after removal of the urea by dialysis (see table 1). The bottom band (seen clearly in the gel on the left) is due not to protein but to the tracker dye used.

two non-biotin subunits of methyl malonyl coenzyme A pyruvate carboxytransferase. The biotin subunit of this enzyme remained firmly bound to the Sepharose–avidin and could be eluted by sodium dodecyl sulphate with only partial success. Guchhait et al. [8] used this technique to purify the relatively small biotin subunit of acetyl coenzyme A carboxylase; 25% of the subunit was eluted with biotin-containing buffer, another 15% with 6 M guanidinium chloride (pH 7) and a further 15% with 6 M guanidinium chloride (pH 2). In the present work it has been possible to release the whole, undissociated pyruvate carboxylase, which is a large protein with a molecular weight of approximately 600 000 [6], in the enzymically active form from Sepharose–avidin in fairly good yield with a relatively mild denaturant, urea. This result lends support to the conclusion from our earlier work [6] that the thermophile pyruvate carboxylase is made up of only one kind of subunit, which has a molecular weight of approximately 150 000. The situation in regard to mesophile pyruvate carboxylases seems unclear. The report of Barden and Taylor [9] suggests that they contain only one type of polypeptide, while the results of McClure et al. [10] and of Warren and Tipton [11] indicate that, like other biotin-containing carboxylases such as acetyl coenzyme A carboxylase, they may be composed of more than one kind of subunit.

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