PURIFICATION OF GLYCOGEN PHOSPHORYLASE BY AFFINITY CHROMATOGRAPHY ON 5'-AMP SEPHAROSE

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SUMMARY

Glycogen phosphorylase from human polymorphonuclear leukocytes was purified by a factor of 2500 using differential centrifugations and affinity chromatography on 5'-AMP bound to agarose. The preparation had a specific activity of 95 units per mg protein and the recovery was 26%.

INTRODUCTION

Conventional procedures of purifying glycogen phosphorylase (EC. 2. 4. 1. 1) include several time-consuming steps (1, 2). Mosbach and Gestrelius (3) showed, that muscle phosphorylase b is strongly absorbed to N^6(6-aminohexyl) adenosine 5'-monophosphate coupled to agarose (4). Miller et al. (5) used this principle as one of the steps in preparing phosphorylase from adipose tissue, thus achieving a 12-fold purification.

The present communication describes a rapid and simple method for the purification of glycogen phosphorylase from human polymorphonuclear leukocytes by a factor of 2500 using differential centrifugation and chromatography on 5'-AMP Sepharose.

MATERIALS AND METHODS

Chemicals: 5'-AMP-Sepharose was the product of Pharmacia, Sweden, and radioactive chemicals were from The Radiochemical Centre, Amersham. All other chemicals were reagent grade and used without further purification except glycogen (Boehringer) which was passed through a mixed ion exchange resin (Amberlite MB 3) before use. Glycogen Synthase I from human polymorphonuclear leukocytes purified on concanavalin A bound to agarose (6) was a gift from dr. H. Sølling.
Assays: Phosphorylase was measured in the direction of glycogen synthesis by a method previously described (7). The reaction mixture contained 67 mM glucose-1-phosphate (spec. act. 17000 cpm/μmole), 1% rabbit liver glycogen and 1 mM AMP when required. Glycogen synthase was assayed by the method of Thomas et al. (8) in the presence of 6,7 mM glucose-6-phosphate and amylo-1, 6-glucosidase was measured by

the method described by Nelson and Larner (9). Phosphorylase kinase was assayed by the method of Lederer et al. (10) using commercial muscle phosphorylase b (Boehringer) as substrate. Phosphorylase phosphatase activity was estimated by incubating the gelfiltered (Sephadex G25 Fine equilibrated with 50 mM Tris-HCl (pH 7. 4) and 10 mM mercaptoethanol) purified phosphorylase at 30°C for 30 min; at intervals, samples were withdrawn for the assay of phosphorylase with and without AMP.
Protein was determined by the method of Lowry et al. (11) except in the eluate from the column, where the micromethod of Schaffner (12) was used. Glycogen was degraded enzymatically and determined as glucose using the filter paper method of Sølling et al. (13).

PURIFICATION OF GLYCOGEN PHOSPHORYLASE

Human polymorphonuclear leukocytes were isolated as previously described (14) and suspended in 2 volumes of cold 50 mM Tris-HCl (pH 7. 4), 50 mM NaF, 1 mM dithiothreitol buffer. The cells were sonicated in the cold and all further procedures were carried out at 2°. The homogenate was centrifuged at 17,000 x g for 30 min and the resulting supernatant was centrifuged at 65,000 x g for 90 min. The sedimented glycogen with adhering enzymes, including phosphorylase, was carefully rinsed, suspended in buffer, and slowly applied to a 5'-AMP-Sepharose column (bed volume 8 ml) equilibrated with buffer. Approximately 30 mg of protein was applied to the column in the present experiments, but considerably more protein can be applied without overloading. The column was washed with 4 bed volumes buffer to remove unbound protein and the ionic strength was then increased by adding NaCl in a final concentration of 0,5 M to the elution buffer, which removed further protein. After 2-3 bed volumes of 0,5 M NaCl in buffer, the salt was removed by washing the column with 2 bed volumes of 50 mM Tris-HCl (pH 7. 4), 1 mM dithiothreitol and phosphorylase could then be eluted by adding AMP in a final concentration of 10 mM to the buffer. The purified phosphorylase was stable in solution for about a week at 4°. The enzyme activity was unchanged for months after lyophilisation in the presence of 0,1% lactose and 10 mM AMP.

RESULTS AND DISCUSSION

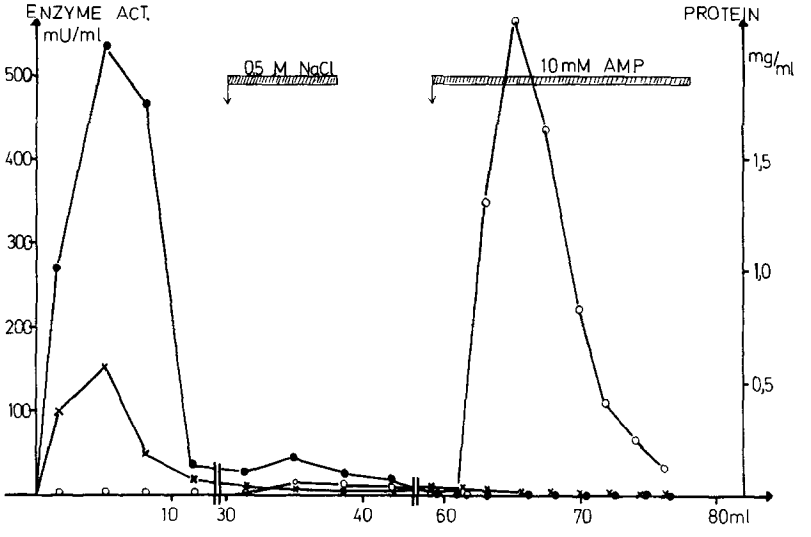
Table 1 shows the result of a typical experiment. The enzyme was purified 2500 times with a resulting specific activity of 95 units per mg protein and with a 26% recovery. The enzyme preparation was not contaminated with measurable phosphorylase kinase, phosphorylase phosphatase or amylo-1,6-glucosidase activity, whereas glycogen synthase activity was just detectable in the eluate and totalled 9

TABLE I

Purification of glycogen phosphorylase

	Volume (ml)	Phosphorylase units [1]/ml	Specific activity units/mg protein	Recovery %
Crude homogenate	3.50	14.6	0.038	-
17,000 x g supernatant	3.30	13.0	0.046	89
55,000 x g pellet (resuspended)	2.00	10.7	0.36	73
5'-AMP-Sepharose eluate	13.2	3.84	95	26

1) One unit of phosphorylase incorporates 1 μmole of glucose from glucose-1-phosphate into glycogen per minute at 30°.



Chromatography of glycogen phosphorylase from human polymorphonuclear leukocytes on 5'-AMP-Sepharose (cf. methods). Phosphorylase activity: o—o, Synthase activity x—x, Protein ●—●.

munits as compared to 3.84 units of phosphorylase. The preparation contained 178 μg glycogen or 5% of the amount present in the crude homogenate. Polyacrylamide electrophoresis in the presence of 0.1% sodium dodecyl sulphate showed a band with a molecular weight of

94.000 daltons, corresponding to the phosphorylase monomer.
The elution diagram of the chromatography on 5'-AMP-Sepharose is shown in Fig. 1. It is observed that most protein is eluted in the void volume, but that significant amounts of presumably hydrophobic bound protein is first removed when the ionic strength of the buffer is increased by the addition of NaCl. This also elutes most of the glycogen synthase bound to the column.
The residual glycogen synthase activity found in the phosphorylase preparation could be removed by prior dialysis of the resuspended 65,000 x g glycogen pellet for 3 hours at room temperature against a 100 mM phosphate buffer, pH 6.5, which degrades glycogen. We are inclined to believe that synthase is bound to the column in a 5'-AMP-phosphorylase-glycogen-synthase complex and that 0.5 M NaCl loosens the binding between phosphorylase and glycogen since 1: glycogen is eluted with 0.5 M NaCl together with synthase, 2: when the 0.5 M NaCl step is omitted, glycogen synthase is eluted with 10 mM AMP, 3: when glycogen is removed prior to chromatography, no synthase is bound to the column, and 4: purified synthase-I does not bind to the column.
The described procedure takes 4-5 hours. The column can be used again several times after extensive washing with buffer.

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