HYDROPHOBIC CHROMATOGRAPHY IN THE RESOLUTION OF THE INTERCONVERTIBLE FORMS OF GLYCOGEN PHOSPHORYLASE

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1. Introduction

Homologous series of hydrocarbon-coated agaroses were recently shown to provide a powerful chromatographic tool for the purification of proteins [1–3]. This new technique was named hydrophobic chromatography, as it achieves resolution mainly through differences in the size and distribution of available hydrophobic pockets in the various proteins. Work done in our laboratory and elsewhere [1–10] has already demonstrated the wide applicability of this method. One of its advantages lies in the fact that it provides a variety of column families, each constituting a homologous series in which every member has hydrocarbon side chains one-carbon-atom longer than the preceding one. Using exploratory kits of columns [10] it becomes possible to choose rapidly the most suitable column for a desired purification and to establish optimal conditions for effective retention and subsequent elution of a given protein. This paper illustrates the resolution power of alkyl agaroses (Seph-C_n*) in the separation of the two metabolically interconvertible forms of rabbit muscle glycogen phosphorylase. These two forms are identical in their amino acid sequence, except for a unique serine residue in each of the enzyme protomers (M.W. 100 000 [11]) which becomes *O*-phosphorylated upon conversion of the *b* form (a dimer) to the *a* form (a tetramer) [12–14]. Coating agarose with methyl groups results in a column (Seph-C_1) capable of retaining the *a* form of the enzyme, while under the same conditions hydrocarbon side chains 4-carbon-atoms long are necessary to retain the *b* form of the enzyme.

2. Materials and methods

Rabbit muscle glycogen phosphorylase *b* (EC 2.4.1.1) was prepared by the method of Fischer et al. [15] and recrystallized three times before use. The enzyme was freed from AMP by passage through a charcoal–cellulose column [16] and assayed by the method of Hedrick and Fischer [17]. Enzyme concentrations were determined spectrophotometrically using an absorbance index of $A^{1\%}_{280\ nm} = 13.1$ [18]. Nonactivated phosphorylase kinase was partially purified by the method of Krebs et al. [19]. The supernatant fraction obtained after centrifugation at 30 000 rpm was used as a source of kinase activity in the preparation of phosphorylase *a* [15]. Phosphorylase *a* was recrystallized twice and passed before use through the charcoal–cellulose column mentioned above. Seph-C_n columns (n = 1–4) were prepared as described previously [1]. The elution pattern of the columns was established by monitoring the protein fluorescence (excitation at 280 nm, emission at 325 nm) and the phosphorylase activity in the presence of AMP (for the *b* form) and in the absence of the nucleotide (for the *a* form). Fluorescence was measured with a Turner 210 Spectro, and absorption measurements were carried out with a Cary spectrophotometer model 15.

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* *Abbreviation*: Seph-C_n, Sepharose 4B activated with CNBr and reacted with an α-alkylamine n-carbon-atoms long.

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3. Results and discussion

The possibility of using hydrophobic chromatography for the resolution of the *a* and *b* forms of glycogen phosphorylase was studied with a mixture of the two purified forms of the enzyme. This mixture was subjected to chromatography on a series of Seph-C_n columns (fig. 1). In each case, the non-adsorbed protein was allowed to emerge from the column and then it was attempted to elute additional protein by applying successively two gradients (both at pH 7): (A) a NaCl gradient up to a concentration of 0.5 M and (B) on imidazole-citrate gradient up to 0.8 M**, which was previously shown to act as a 'deformer' [20] on glycogen phosphorylase. As seen in fig. 1, glycogen phosphorylase *b* was excluded from Seph-C_1 and Seph-C_2, retarded on Seph-C_3 and retained by Seph-C_4, as we have already shown before [1]. In contrast, the *a* form of the enzyme was already adsorbed on Seph-C_1 and also on higher members of the Seph-C_n series. The affinity of phosphorylase *a* to the various columns increases with increasing length of the hydrocarbon side chains, as judged by the conditions required for its elution which become more drastic with increasing *n* (fig. 1). From Seph-C_1, phosphorylase *a* is released by gradient (A), the peak being eluted when the conductivity of the buffer reaches 12 mmho. From Seph-C_2 a higher salt concentration is required (peak at a conductivity of 16 mmho). From Seph-C_3, the elution is only partial even at a conductivity of 23 mmho. In the case of Seph-C_4, phosphorylase *a* is eluted only by gradient (B).

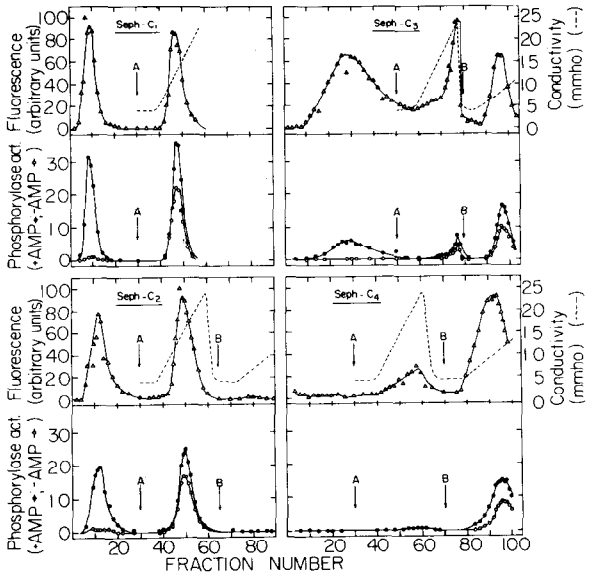

Fig. 1. Resolution of the interconvertible forms of glycogen phosphorylase by chromatography on Seph-C_n ($n = 1-4$). A mixture of the two purified enzymes (9 mg phosphorylase *a* and 5.7 mg phosphorylase *b*) were dissolved in 0.9 ml of a buffer composed of sodium β-glycerophosphate (50 mM), mercaptoacetic acid (30 mM) and EDTA (1 mM), adjusted to pH 7.0 with NaOH. Such a sample was applied on each of four alkyl agarose columns (all 8 × 1 cm) which were equilibrated at 22°C with the same buffer. Unadsorbed protein was washed off and then elution was attempted by changing the composition of the solvent as follows: (A) applying a NaCl gradient (total volume 40 ml, in the β-glycerophosphate buffer, pH 7.0) up to a concentration of 0.5 M, then washing with the β-glycerophosphate buffer until the conductivity dropped to its original value; (B) applying an imidazole–citrate gradient (40 ml), up to 0.8 M, keeping the pH at 7.0. Fractions of 25 drops (1.6 ml) were collected and their fluorescence (△--△--△), conductivity (- - - - -) and enzymatic activity (with AMP (●--●--●) and without AMP (○--○--○) were monitored.

The above results show that with such mixtures it is possible to fish out the *a* form of phosphorylase by passage through Seph-C_1 or Seph-C_2, then to extract the *b* form on Seph-C_4. As seen in fig. 2, it is also possible to extract both forms of the enzyme on Seph-C_4 (at pH 7), lower the pH of the buffer to 5.8 (with glacial CH_3COOH) to elute phosphorylase *b* and then apply the deforming buffer to elute phosphorylase *a*. Alternatively, the mixture of the two enzyme forms can be applied on a Seph-C_4 column at pH 5.8 from which phosphorylase *b* is excluded (not illustrated). Under these conditions phosphorylase *a* is still retained on the column and it can then be eluted with a gradient of imidazole–citrate (up to 0.7 M) at pH 7.0 (the enzyme is excluded at ~ 0.38 M).

In a previous paper [1] we have shown that rabbit muscle glycogen phosphorylase can be purified in the *b* form by chromatography on Seph-C_4. This paper suggests procedures for the purification of the enzyme in its fully phosphorylate *a* form. It should be

** Imidazole-citrate buffers are composed of imidazole (the given concentration), mercaptoacetic acid (30 mM) and citric acid (at the concentration necessary to adjust the pH to 7.0).

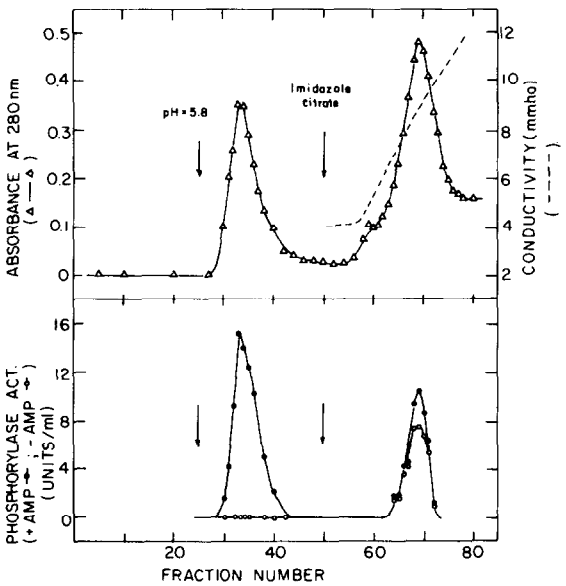

Fig. 2. Separation of the *a* from the *b* form of glycogen phosphorylase on Seph-C_4. A mixture of phosphorylase *a* (3.5 mg) and phosphorylase *b* (4.6 mg) were dissolved in 0.75 ml of the β-glycerphosphate buffer described in the legend to fig. 1 and applied on a Seph-C_4 column (8 × 1 cm) equilibrated at 22°C with the same buffer. Both enzymes were retained by the column as indicated by the fact that no protein was excluded in the first 25 fractions (compare with the experiments depicted in fig. 1 performed with columns of the same dimensions and in which fractions of the same volume were collected). Elution of the enzymes was attempted by: (A) lowering the pH of the β-glycerophosphate buffer to 5.8 with glacial CH_3COOH; (B) applying an imidazole-citrate gradient (up to 0.61 M in imidazole) while keeping the pH at 5.8. The column was monitored by following the absorption of the fractions (1.6 ml) at 280 nm (△—△—△), their conductivity (– – – –), and their enzymatic activity in the presence of AMP (—●—) and in the absence of the nucleotide (—○—).

emphasized, however, that if attempts are made to separate the *a* from the *b* form of the enzyme in crude extracts, the elution patterns may vary depending on the activity state of other enzymes in the extract (e.g. phosphorylase kinase and phosphorylase phosphatase) as well as on the concentration of certain metabolites (e.g. glucose-1-phosphate and glucose-6-phosphate). This is due to the formation (during the interconversion of phosphorylase) of partially phosphorylated species of the enzyme, whose enzymatic and physicochemical properties are neither those of phosphorylase *b* nor those of phosphorylase *a* [21, 22]. Preliminary experiments in our laboratory indicate that resolution of such 'phospho–dephospho' hybrids may be achieved with hydrophobic chromatography columns.

Several pieces of evidence summarized in a recent review [10] strongly suggest that the retention power of alkyl agaroses is derived mainly from lipophilic interactions between hydrophobic pockets or regions in the proteins and appropriately-sized side-chains on the agarose. However, purified agaroses were reported to contain negative charges [23] and positive charges may be introduced as a result of activation with CNBr [24] and subsequent coupling with alkylamines. Also, increasing the ionic strength or altering the pH of the buffer brings about elution of some proteins. Therefore it is not possible to exclude some influence of ionic interactions on the elution patterns of these columns. Still, mere activation of the beads with CNBr and subsequent reaction with ammonia (to simulate the functional groups linking the hydrocarbon chains) does not suffice for retaining phosphorylase *b*. Furthermore, the above mentioned charges should be equally present in all the members of the homologous series, and therefore cannot account for the finding that no adsorption of phosphorylase *b* occurs when the side chains are less than 4-carbon-atoms long.

The elution caused by changes in ionic strength and pH need not be attributed to ionic interactions between the column beads and the protein. When dealing with protein molecules, whose conformation and aggregation state are strongly dependent on ionic interactions, it is quite conceivable that the availability and size of hydrophobic pockets may depend on ionic strength, pH and even on the nature of the ions present. In fact, the influence of these parameters on the structure of a hydrophobic pocket in phosphorylase has been amply documented [20, 25–30].

The fact that alkyl agaroses discriminate between the *a* and *b* forms of phosphorylase further illustrates their high resolution power, which may make them useful also as probes for the detection of structural changes in regulatory proteins. In any case, the rapid and effective method for resolution of phosphorylase *a* and *b* seems to be a handy tool in physiological and biochemical studies on glycogen metabolism and its regulation, where the interconversion of phosphorylase plays a key role [31–33].

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