CHROM. 5970

Affinity chromatography of phosphofructokinase using Cibacron blue F3G-A

Phosphofructokinase (EC 2.7.1.11) binds to Blue Dextran 2000® with high specificity and affinity[1-4]. It was also found by HAECKEL *et al.*[5], STAAL *et al.*[6] and BLUME *et al.*[7] that this dye binds to pyruvate kinase (EC 2.7.1.40) as well. In contrast, other enzymes tested apparently do not show any affinity to Blue Dextran[1,4] under certain conditions.

The chromophoric group of Blue Dextran 2000 seems to be responsible for these interactions, since free Dextran 2000 was found to be completely inert[4]. Blue Dextran, immobilized by cross-linked polyacrylamide gel, can be used as a suitable adsorbent for phosphofructokinase in chromatographic experiments. The dye–enzyme complex can be split either by eluting with solutions of increasing ionic strength or more specifically by ATP at low concentrations. Underlining the specificity of the ATP effect, it was shown that ITP does not split the chromophore–enzyme complex. Thus, it seemed possible that a particular case of affinity chromatography could be developed by making use of the specific and reversible interactions between this type of dye fixed to an insoluble support and phosphofructokinase.

Materials and methods

Enzymes and reagents for the phosphofructokinase assay were obtained from C. F. Boehringer & Söhne GmbH, Mannheim-Waldhof; Sephadex G-200, Sepharose 4 B, Dextran 2000 and Blue Dextran 2000® from Pharmacia, Uppsala; Cellulose HL from Serva, Heidelberg, and the reactive dyes from Ciba AG, Basel. All other chemicals were p. a. grade and were purchased from VEB Berlin-Chemie and VEB Labor-Chemie, Apolda, D.D.R. Yeast phosphofructokinase was prepared according to ref. 8.

Coupling of Cibacron blue F3G-A to Sephadex G-200. A solution of 2 g of Cibacron blue F3G-A in 60 ml of water was added dropwise with vigorous stirring to a suspension of 10 g of Sephadex G-200 (or another polysaccharide) in 350 ml of water at a temperature of 60°. After stirring for 30 min, 45 g of sodium chloride were added and the stirring was continued for 1 h. After that the mixture was heated to 80°, treated with 4 g of Na_2CO_3 and kept for a further 2 h with stirring at this temperature. After cooling to room temperature the gel was filtered by suction on a Büchner funnel and washed with water until the filtrate became colourless.

Column chromatography. The gel was packed into a column kept at 4° and equilibrated with 0.05 *M* phosphate buffer, pH 7.1, containing 0.5 m*M* EDTA and 5 m*M* 2-mercaptoethanol. Before applying the enzyme it was dialyzed for about 2 h against a buffer having the same composition as that just described. The gradients in ionic strength of the eluting solution were obtained by means of an Isco Gradient Pump 180.

Results and discussion

Identification of the structure of Blue Dextran 2000. At the beginning of this study the structure of the dye, Blue Dextran, was unknown to us, therefore some chemical reactions for the identification of the chromophore were tested. Cleavage with sodium dithionite under alkaline conditions gives a compound which has been

identified as quinizarine. From the visible spectrum of undegraded Blue Dextran and its behaviour during acidic and alkaline hydrolysis we concluded that Blue Dextran is composed of a reactive dye having a 4-phenylamino-1-amino-anthraquinone structure coupled covalently to Dextran 2000 by means of cyanuric chloride. In order to test the chromophore specificity of phosphofructokinase binding, different products have been prepared by coupling Dextran 2000 with a series of Cibacron dyes. Only one of them, the coupling product with Cibacron blue F3G-A (Fig. 1A) was found to have the same high affinity for phosphofructokinase as commercial Blue Dextran 2000®. From these results it was concluded that the structure of the chromophore of Blue Dextran is very similar, if not identical, to that of Cibacron blue F3G-A* (ref. 3).

Chromatographic experiments. It has been shown in a previous paper[4] that the power of affinity of phosphofructokinase–Blue Dextran can be estimated from the ionic strength of the eluting buffer required to dissociate the enzyme from the immo-

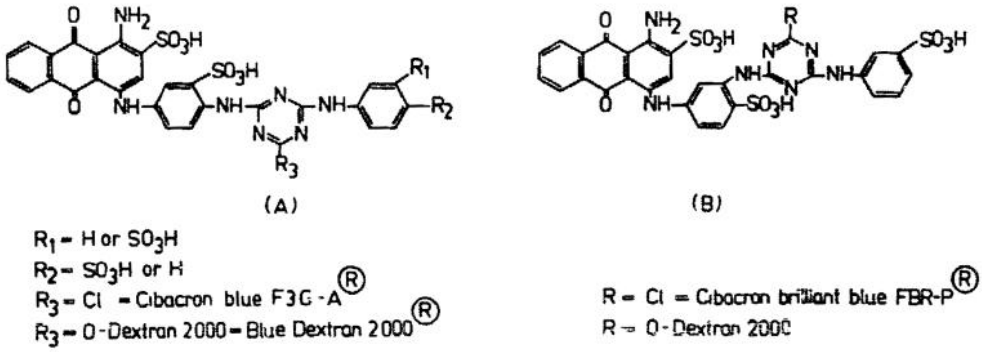

Fig. 1. Structural formulae of (A) Cibacron blue F3G-A and (B) Cibacron brilliant blue FBR-P.

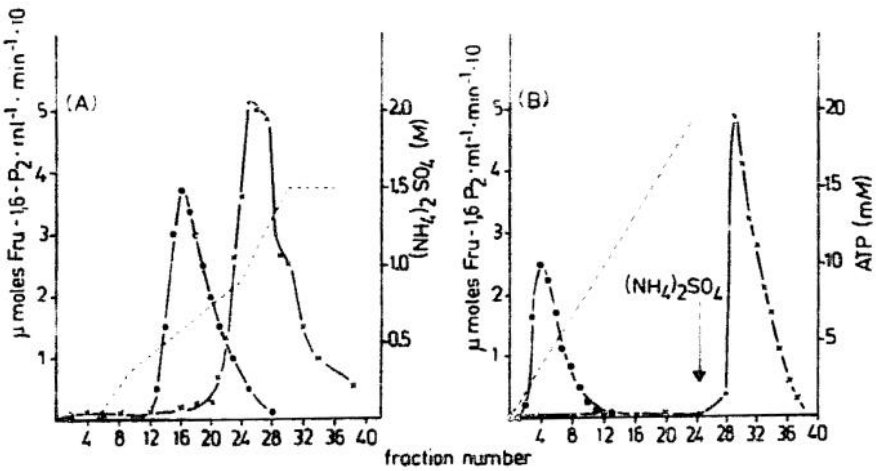

Fig. 2. Elution profiles of yeast phosphofructokinase depending on the concentration of ammonium sulphate and ATP, respectively. (A) Elution with ammonium sulphate; (B) elation with an ATP gradient followed by 1.5 *M* ammonium sulphate. ●, Elution of the enzyme using immobilized Blue Dextran in cross-linked polyacrylamide; ×, elution of the enzyme using F3G-A–Sephadex G-200 gels. Both kinds of experiments were performed under the same conditions. Column dimension, 4.0 × 1.0 cm. The programme of the gradient pump is indicated by the broken lines; duration of the programme, 6 h; flow rate 20 ml/h; temperature, 4°. The enzyme (100–150 µg) was applied to the column after dialysis for 3 h against the elution buffer without ammonium sulphate or ATP.

* The structure of Blue Dextran 2000® published by Pharmacia, Uppsala[9] while our manuscript was in preparation is indeed identical with the structure of Cibacron blue F3G-A (Fig. 1A).

bilized Blue Dextran packed into a chromatographic column. This can be carried out by eluting with a concentration gradient of ammonium sulphate. Experiments with Cibacron blue F3G-A-substituted Sephadex G-200 (abbreviated to F3G-A–Sephadex G-200) showed that this gel binds phosphofructokinase very strongly (Fig. 2A). The ionic strength of the eluting buffer necessary for dissociation of the enzyme from the F3G-A–Sephadex matrix is apparently higher than that required to split the Blue Dextran enzyme complex. Furthermore, the binding capacities of the two gels are different. F3G-A–Sephadex G-200 binds approximately fifty times more phosphofructokinase than immobilized Blue Dextran in polyacrylamide gel, with respect to their packing volume in the column. Fig. 2B shows the elution profile using an ATP concentration gradient. The Blue Dextran enzyme complex is dissociated with approximately 2 m*M* ATP (see ref. 4 for details), however the phosphofructokinase complex with F3G-A–Sephadex G-200 is not dissociated by ATP, not even with still higher concentrations (up to 20 m*M*). On the other hand, a higher ionic strength obtained by adjusting with ammonium sulphate splits the F3G-A–enzyme complex. Because there are no differences in the chromophoric component of the two matrix systems, it can be concluded that the polysaccharide carrier influences the interactions between the protein and the chromophore. This assumption is strengthened by the observation that phosphofructokinase bound to Cibacron blue F3G-A-substituted cellulose cannot be eluted by either high concentrations of ammonium sulphate or ATP.

Fig. 3 illustrates binding experiments with phosphofructokinases from different sources*. All the enzymes exhibited a remarkable affinity to the Blue Dextran polyacrylamide gel. They can be eluted with increasing concentrations of ammonium sulphate.

The binding power of the chromophore which can be deduced from the ionic

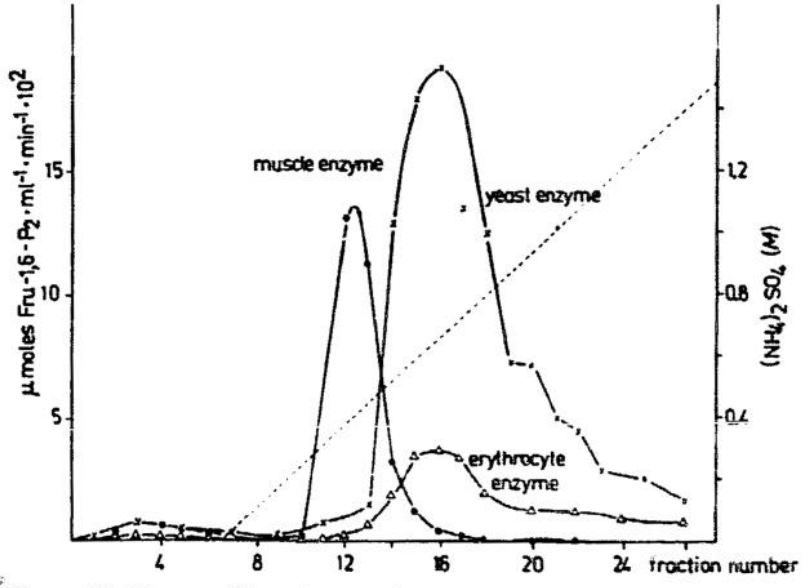

Fig. 3. Elution profiles of several species of phosphofructokinase from Blue Dextran polyacrylamide gel. The experiment was performed under the same conditions as in Fig. 2; duration of the programme, 8 h. The broken line indicates the programme of the gradient pump.

* We are grateful to Dr. K.-W. WENZEL from this Institute for the gift of purified human erythrocyte phosphofructokinase.

strength of the medium necessary for dissociation of the chromophore complex (see above) also depends on the pH value of the buffer in the range 6 to 8. The enzyme binds more strongly at acidic pH values than under alkaline conditions. This effect is evidently caused by changing the ionization of some of the amino acid residues in the protein within this range, which may be responsible for these protein–chromophore interactions.

Aspects of chromophore specificity. The chemical nature of the interactions between phosphofructokinase and the chromophore is not yet completely clear. From the following observations it may be assumed that several functional groups of the dye seem to be essential for binding the enzyme. The condensation product of Dextran 2000 with Cibacron brilliant blue FBR-P (Fig. 1B), which differs from Cibacron blue F3G-A only in the position of the sulphonic acid and amino groups in the phenylenediamine ring, and the reaction product of cyanogen bromide-activated Sepharose 4 B (ref. 10) with Blue Dextran, do not show any affinity to phosphofructokinase. Since Blue Dextran is attached to the activated Sepharose 4 B by the 1-amino group of the anthraquinone, it may be deduced from these studies that this amino group as well as the sulphonic acid residue in the 2'-position of the phenylenediamine ring are evidently responsible for the high specific interactions of phosphofructokinase with this dye. Fig. 4. shows that the steric arrangement of these two groups indeed shows some resemblance to that of the amino and γ-phosphate groups within the ATP molecule. Hence, the conclusion may be drawn that ATP and the dye are bound by the same or similar sites of the enzyme. This assumption is consistent with the following observations: (a) the substrate and allosteric inhibitor ATP specifically dissociates the phosphofructokinase–Blue Dextran 2000 complex; (b) ITP, although a substrate but not an inhibitor for phosphofructokinase, does not cause this dissociation; (c

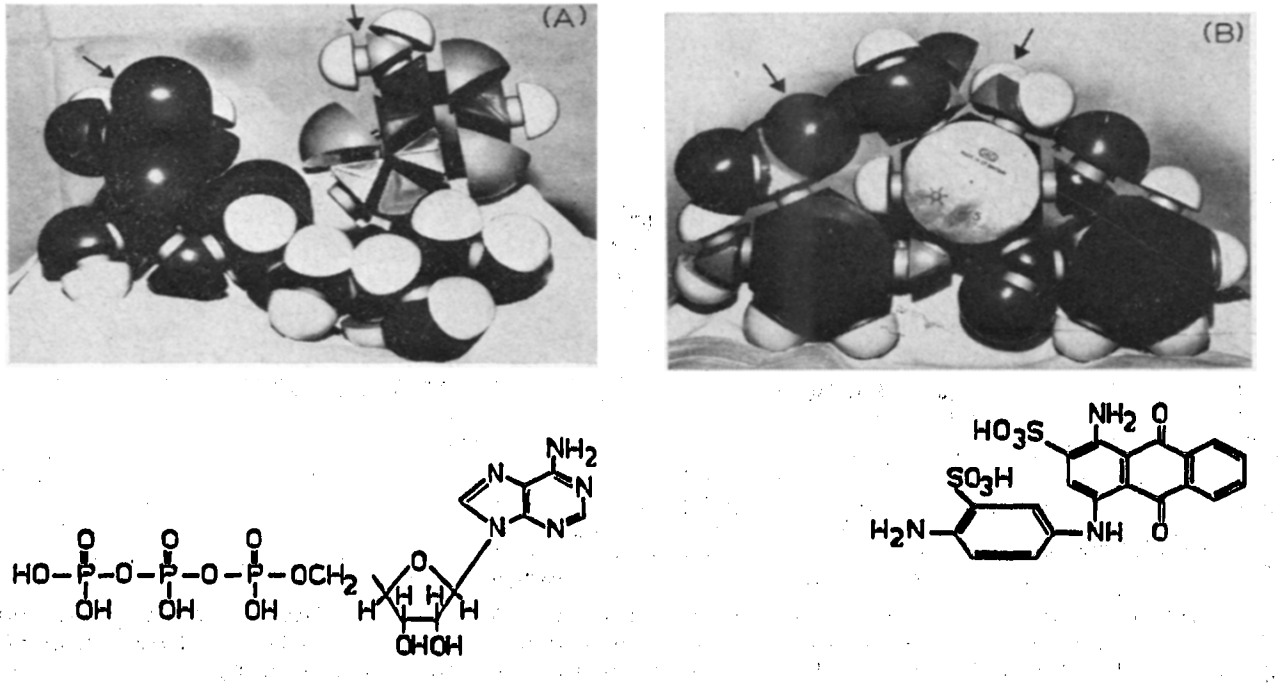

Fig. 4. (A) Calotte model of ATP. (B) Calotte model of 1-amino-4-(1'-amino-2'-sulphophenylamino) anthraquinone-2-sulphonic acid, the proposed phosphofructokinase-binding part of Blue Dextran. The arrows indicate identical regions of both molecules.

Blue Dextran-induced enzyme inhibition is competitive with ATP[4]. In addition, other ATP-dependent enzymes, such as pyruvate kinase and adenylate kinase (unpublished observations) also show a significant affinity to Blue Dextran.

Applicability to enzyme preparation. The applicability of Cibacron-substituted Sephadex gel for enzyme chromatography on a preparative scale has been investigated in a study of the isolation of yeast phosphofructokinase. After equilibration of a suitable column with phosphate buffer (see *Materials and methods*) and application of the crude enzyme solution to the column, the gel was first washed with the buffer (see above) for about 5 h, followed by elution of the enzyme with phosphate buffer containing 1.5 *M* ammonium sulphate. As shown in Table I, a remarkable increase of specific activity of the enzyme could be obtained after this chromatographic procedure. Within certain limits, the purification factor was found to be independent of the specific activity of the starting preparation.

TABLE I

PURIFICATION OF YEAST PHOSPHOFRUCTOKINASE USING CIBACRON BLUE F3G-A – SEPHADEX G-200 GEL CHROMATOGRAPHY

The specific activity is expressed as μmoles Fru-1,6-P_2 · mg^{-1} protein · min^{-1}.

Specific activity of the enzyme before affinity chromatography	*Specific activity after chromatography*	*Purification factor*	*Yield* (%)
0.3	3.8	13.0	84
2.3	12.4	5.5	85
15.0	91.0	6.0	75

This method may be of general interest for the purification of enzymes like phosphofructokinase and others. Several groups of workers have taken advantage of the affinity of proteins to Blue Dextran 2000 for the purification of a variety of enzymes[5,6,7,11–13]. Experiments in our laboratory showed that glucose-6-phosphate dehydrogenase from yeast, adenylate kinase and 3-phosphoglycerate kinase from muscle also show significant affinity to the Cibacron–Sephadex system.

It seems to us, that these interactions cannot only be applied to enzyme purification but also to the specific removal of certain enzymes from various mixtures, where they would have an undesirable effect.

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