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Affinity chromatography of serine proteases on the triazine dye ligand Cibacron Blue F3G-A

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Abstract

The interaction between complement component factor B and the triazine dye ligand Cibacron Blue F3G-A coupled to a cross-linked agarose matrix (Blue Sepharose) was found to involve the Bb part of the molecule, and to be inhibited by benzamidine. Human, chicken and rainbow trout factor B which had bound to Blue Sepharose could subsequently be eluted with benzamidine. Other serine proteases (C2, factor II, factor IX, trypsin, chymotrypsin, proteinase 3) also bound to Blue Sepharose but only those belonging to the trypsin family could be eluted with benzamidine. Trypsin treated with the active-site inhibitor phenylmethylsulfonyl fluoride did not bind to Blue Sepharose and pretreatment of Blue Sepharose with benzamidine did not influence binding of proteases. We conclude that trypsin-like serine proteases can be purified on Blue Sepharose and that the interaction of these serine proteases with Blue Sepharose involves the active site of the enzyme.

Keywords: Serine proteases; Factor B

1. Introduction

Since its introduction, triazine-based dye ligand chromatography has been used in the purification of numerous proteins, both on a rational ligand-mimicking basis (e.g., nucleotide-binding proteins) and on an empirical basis [1–5]. As an example of the latter, Cibacron Blue-derivatized matrices have been used to purify serum albumins [6,7]. Albumin binding is influenced by the presence of fatty acids, and the ability of caprylic acid to interfere with the binding of serum albumin to Blue Sepharose implies that the interaction of the protein with the matrix involves the fatty acid-binding sites of the protein [8]. Taking advantage of the reduced binding of albumin in the presence of caprylic acid, Williams and Sim [9] developed a procedure for isolating human complement factor B and β_2-glycoprotein I on Cibacron Blue F3G-A Sepharose (Blue Sepharose). We have explored this finding further and have observed that the interaction between factor B and Blue Sepharose involves the serine protease part of factor B, and is inhibited by the trypsin inhibitor benzamidine. This suggests that the interaction between factor B and Blue Sepharose involves the active site of the serine protease part of the molecule. This interpretation has been substantiated by investigating the interaction of other serine proteases (C2, factor II, factor IX,

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trypsin, chymotrypsin, proteinase 3) with Blue Sepharose. All the serine proteases investigated were found to bind to Blue Sepharose, but only members of the trypsin family could be eluted from the matrix by benzamidine. These results are discussed in relation to the structure of Cibacron Blue F3G-A.

2. Experimental

2.1. Chemicals

Sodium dodecylsulfate (SDS), phenylmethylsulfonyl fluoride (PMSF), tris(hydroxymethylamino)methane (Tris), *N,N,N′,N′*-tetramethylethylenediamine, glycerol, caprylic acid, mercaptoethanol, benzamidine, inulin, bovine serum albumin (BSA) and Coomassie Brilliant Blue R-250 were from Sigma (St. Louis, MO, USA). Triton X-100, Tween 20, NaCl, KCl, NaOH, NaH_2PO_4, Na_2HPO_4, $MgCl_2$, glycine, ethyleneglycol and ethylenediaminetetraacetic acid (EDTA) were from Merck (Darmstadt, Germany). Trypsin and chymotrypsin were from Boehringer Mannheim (Mannheim, Germany). Acrylamide, bisacrylamide, ammonium persulfate, bromophenol blue, and molecular mass standard proteins were from Bio-Rad (Richmond, CA, USA). Blue Sepharose CL 4 B was from Pharmacia (Uppsala, Sweden). *o*-Phenylenediamine was from Kem-En-Tek (Copenhagen, Denmark). Hydrogen peroxide was from Struers (Copenhagen, Denmark). Sulphuric acid, factor II (thrombin), factor IX, immunoaffinity-purified proteinase 3, human serum, rainbow trout serum, rabbit antisera to human factor B, rainbow trout factor B and proteinase 3, and mouse monoclonal antibodies to human factor B, factor IX and proteinase 3 were from Statens Serum Institut (Copenhagen, Denmark). Rabbit antisera to human factor B and human serum albumin, peroxidase-conjugated rabbit anti-mouse immunoglobulins (RaM^P) and peroxidase-conjugated swine anti-rabbit immunoglobulins (SaR^P) were from Dako (Copenhagen, Denmark). Maxisorp ELISA plates were from Nunc (Roskilde, Denmark). Nitrocellulose membranes were from Schleicher and Schuell (Dassel, Germany).

2.2. Enzyme-linked immunosorbent assay (ELISA)

Antigen (100 μl) was coated directly onto the surface of ELISA plates in 0.05 *M* sodium carbonate buffer, pH 9.6. Coatings were carried out overnight at 4°C or at room temperature for 2 h. After coating the plates were washed three times in 8 m*M* phosphate buffer, pH 7.2, containing 0.5 *M* NaCl, 3 m*M* KCl, 1% Triton X-100 (washing buffer). All subsequent incubations with antibodies diluted in washing buffer containing 1% BSA were carried out for 1 h at room temperature on a shaking table, and were followed by three washes with washing buffer. The final incubation was with peroxidase-conjugated rabbit anti-mouse immunoglobulins or swine anti-rabbit immunoglobulins diluted 1:1000. Staining solution was 0.066 *M* Na_2HPO_4, 0.035 *M* citric acid, pH 5.0, containing 0.4 μl 35% H_2O_2 and 0.4 mg *o*-phenylenediamine per ml. After 30 min the reaction was terminated by adding 150 μl 1 *M* H_2SO_4 and the absorbance was measured at 490 nm with background subtraction at 650 nm (Thermomax microtiter plate reader, Molecular Devices, Menlo Park, CA, USA).

For determination of factor B and factor IX in serum samples and fractions from chromatography ELISA plates were coated with 100 μl rabbit antiserum to factor B or factor IX (10 μg/ml). After washing of plates wells were incubated for 1 h with samples or control serum diluted 1:1000, followed by 1 h incubation with monoclonal antibody to factor B or factor IX diluted 1:1000. Finally, the amount of monoclonal antibody bound was quantitated as described above. Results are expressed as percent of starting material.

2.3. Rocket immunoelectrophoresis for determination of serum albumin

Serum albumin was determined by quantitative rocket immunoelectrophoresis as described [10]. The electrophoresis was performed overnight at 2 V/cm in 1% agarose gels in 20 m*M* 5,5-diethylbarbituric acid, 40 m*M* Tris, 0.5 m*M* calcium acetate, 3 m*M* sodium azide, pH 8.6, using rabbit antiserum to human serum albumin according to the manufacturer's instructions.

2.4. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE was carried out as described by Laemmli [11] using 4% stacking gels and 7% separating gels. Electrophoresis was performed overnight at 40 V, and gels were stained with 0.1% Coomassie Brilliant Blue in 10% acetic acid, 20% ethanol, and destained with 10% acetic acid.

2.5. Immunoblotting

Proteins in SDS–PAGE gels were electroblotted onto nitrocellulose membranes at 1 mA/cm^2 for 4 h. The transfer was performed with a semidry electroblotter and with 30 m*M* Tris, 0.24 *M* glycine as transfer buffer. After electrotransfer membranes were blocked with phosphate-buffered saline (PBS; 8 m*M* sodium phosphate, pH 7.2, with 0.15 *M* NaCl) containing 0.5% Tween 20. Incubation with primary antibody (rabbit anti-human factor B) diluted 1:1000 in PBS, 0.05% Tween 20 was carried out overnight at 5°C followed by 1 h incubation at room temperature. After three washes in PBS containing 0.05% Tween 20 incubation with the secondary antibody was performed at room temperature in the same buffer at a dilution of 1:1000. After three more washes in PBS containing 0.05% Tween 20, the blot was developed with diaminobenzidine (1 mg/ml) and H_2O_2 (0.03%) in 20 m*M* sodium phosphate buffer, pH 7.0.

2.6. Complement activation of serum

Inulin (100 mg/ml in PBS) was added to serum to a final concentration of 10 mg/ml and the mixture incubated for 30 min at 37°C. EDTA was added to 20 m*M* to stop the reaction.

2.7. Chromatography on Blue Sepharose

This was performed essentially as described by Williams and Sim [9] using 25 m*M* Tris, pH 7.4, containing 0.5 m*M* $MgCl_2$ and 0.5 m*M* $CaCl_2$, as buffer for all washing and elution solutions. Caprylic acid (25 m*M*), KCl (2 *M*) and benzamidine (20–100 m*M*) were included in the buffers as indicated in the experiments. All chromatographic experiments were carried out at 5°C with a flow-rate of 0.5, 1 or 2 ml/min.

2.8. PMSF treatment of trypsin

Trypsin (25 mg) was dissolved in 12.5 ml PBS and three aliquots (65 μl) of 200 m*M* PMSF in 2-propanol was added at 1 h intervals.

3. Results

During purification of factor B by chromatography on Blue Sepharose we observed that factor B did not bind to the matrix in the presence of the protease inhibitor benzamidine. This was investigated in more detail in the following experiments.

Fig. 1 shows chromatography of human and rainbow trout serum on Blue Sepharose in the presence of caprylic acid. Factor B was retained on the column and most of the immunoreactive protein could be eluted with benzamidine. A small proportion of the factor B, however, seemed to interact with the matrix in a way that was unaffected by benzamidine, but could be disrupted by 2 *M* KCl.

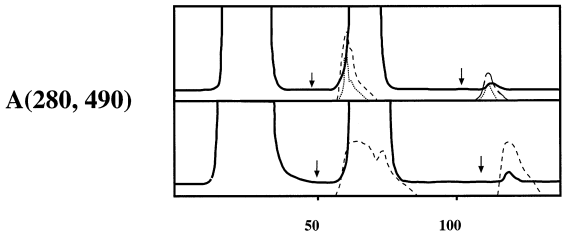

Fig. 1. Chromatography of 5 ml human serum (top) and 5 ml rainbow trout serum (bottom) on a column (5×2.5 cm) of Blue Sepharose. Caprylic acid (25 m*M*) was added to the samples and to the buffers. The samples were applied to the column which was then washed with equilibration buffer. At the first arrow the column was eluted with benzamidine (100 m*M*) and then washed. At the second arrow the column was eluted with 2 *M* KCl. Fractions were analyzed for factor B by ELISA using a polyclonal (- - -) antiserum against human or rainbow trout factor B or using a monoclonal antibody to human factor B (· · ·). The flow-rate was 1 ml/min and 2 ml fractions were collected.

Identical behavior was observed for chicken factor B and for human C2, which is homologous to factor B. From the chromatogram in Fig. 1 it can also be seen that benzamidine, which is easily detected by its strong absorbance at 280 nm, does not itself bind to Blue Sepharose, since this would have given rise to a large peak of benzamidine eluted by KCl. This interpretation was confirmed by control experiments in which benzamidine alone was passed through a Blue Sepharose column, which was then eluted with KCl. Moreover, pretreatment of Blue Sepharose with benzamidine did not influence its capacity to bind serum albumin or the serine proteases (data not shown).

When inulin-activated human serum was chromatographed on a Blue Sepharose column, which was eluted by 2 *M* KCl to release all bound material, it was found that both intact factor B and the Bb fragment of factor B could bind to Blue Sepharose (Fig. 2). This finding indicated that the serine protease active site could be involved in the interaction, and the behavior of other serine proteases on Blue Sepharose was therefore studied in similar experiments. However, as an experiment with elution of factor B by a gradient of benzamidine showed that 10–20 m*M* benzamidine was sufficient to elute all bound material, subsequent experiments were carried out with 20 m*M* benzamidine for elution instead of 100 m*M*.

Table 1 summarizes the results from chromatography of human serum on Blue Sepharose with respect to serum albumin, factor B and coagulation factor IX. It can be seen that the amount of serum albumin bound was highly influenced by the presence of caprylic acid. Moreover, serum albumin was not eluted by benzamidine to any appreciable extent. However, both factor B and factor IX could be eluted by benzamidine, regardless of whether caprylic acid was present during chromatography.

Fig. 3 shows chromatography of trypsin, chymotrypsin and proteinase 3 (PR 3) on Blue Sepharose. All three enzymes bound to Blue Sepharose, but only trypsin could be eluted by benzamidine, whereas the other two enzymes required 2 *M* KCl for elution. None of the enzymes could be eluted by an equal amount of guanidine (20 m*M*), indicating that the effect of benzamidine was not due to purely electrostatic effects and that the aromatic ring of benzamidine was involved in the interaction of this inhibitor with the trypsin-like serine proteases. When partially purified human factor II was chromatographed on Blue Sepharose it could also be eluted by benzamidine. Finally, pretreatment of factor IX and trypsin with PMSF was found to prevent their binding to Blue Sepharose. The effect of aprotinin could not be evaluated as this inhibitor bound to Blue Sepharose itself.

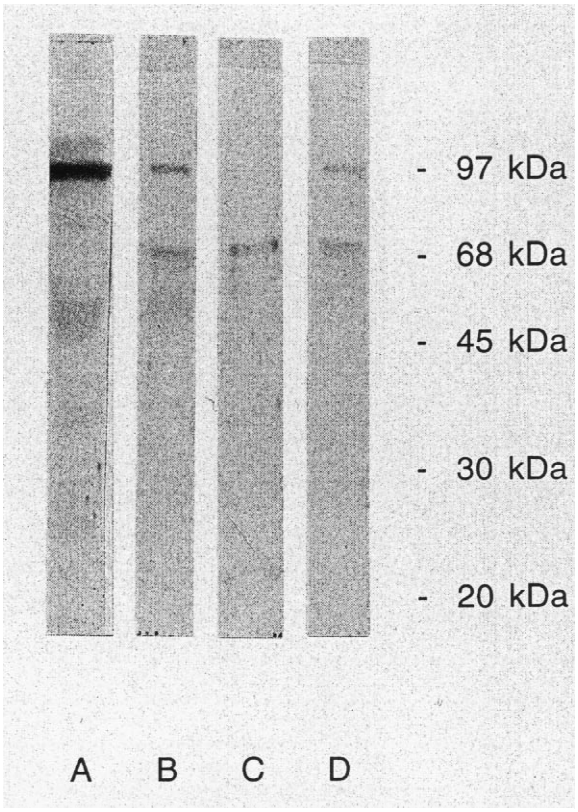

Fig. 2. Complement factor B and the Bb fragment of factor B bind to Blue Sepharose. Human serum (5 ml) was treated with inulin, then caprylic acid (25 m*M*) was added and the sample was chromatographed on a column (10×1 cm) in the presence of caprylic acid (flow-rate: 2 ml/min, fraction size: 4 ml). Lane A: human serum with EDTA. Lane B: inulin-activated human serum with EDTA. Lane C: flow through from the Blue Sepharose column. Lane D: 2 *M* KCl eluate from the Blue Sepharose column. Immunoreactive protein was detected using a rabbit serum against human factor B.

Table 1
Behaviour of serum albumin, factor B and factor IX during chromatography of human serum on Blue Sepharose (percent of starting material)

	Non-bound	Benzamidine-el	KCl-el.	Yield	Purification
Without caprylic acid					
HSA	4	8	59	71	1
Factor B	2	72	0	74	7
Factor IX	1	89	3	93	9
With caprylic acid					
HSA	65	0	1	66	1
Factor B	3	43	0	46	14
Factor IX	3	71	20	94	24

4. Discussion

The specific interaction of factor B with Blue Sepharose has previously been used by Williams and Sim [9] to obtain highly purified human factor B and β_2-glycoprotein I. When applying these procedures to the purification of human factor B and factor B from other vertebrate species we observed that the interaction of factor B with Blue Sepharose involved the C-terminal Bb part of factor B, and that the protease inhibitor benzamidine, which binds to the active site of serine proteases of the trypsin family, totally abolished binding of factor B to Blue Sepharose. Furthermore, benzamidine was highly efficient in eluting factor B and other serine proteases of the trypsin family (factor IX, trypsin, factor II, C2) from Blue Sepharose but not those outside this family (chymotrypsin, PR 3).

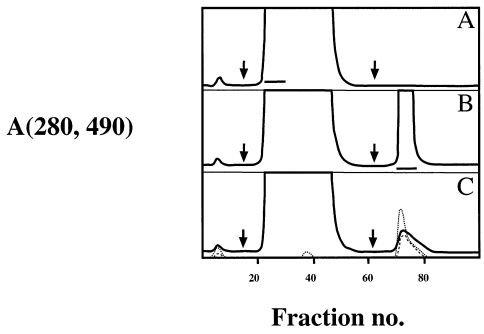

Fig. 3. Chromatography of 1.6 mg trypsin (A), 1 mg chymotrypsin (B) and 1 ml immunoaffinity-purified proteinase 3 (C) on Blue Sepharose. The purified enzymes were loaded onto a 10×1 cm column (5°C). The column was washed and eluted first with 20 m*M* benzamidine (first arrow) and then with 2 *M* KCl (second arrow). Fractions of 2 ml were collected at a flow-rate of 0.5 ml/min. In (A) and (B) the elution position of the protease was determined by SDS–PAGE and is shown by a horizontal bar. In (C) the elution of proteinase 3 was determined by ELISA using a polyclonal antiserum (· · ·) or a monoclonal antiserum (- - -).

These data suggest that the interaction of Blue Sepharose with serine proteases involves binding to the active site of the enzymes. Caprylic acid prevents serum albumin from binding, suggesting that this protein binds primarily by hydrophobic interactions. Factor B and factor IX bind in the presence of caprylic acid showing that these proteins do not primarily bind by hydrophobic interactions. Moreover, since 20 m*M* benzamidine but not 20 m*M* guanidine could elute these proteins, it can be concluded that they do not bind by electrostatic interactions, but rather by affinity (a combination of multiple interactions). The lower yield of benzamidine-eluted factor B in the presence of caprylic acid (Table 1) may suggest that factor B binds more strongly to Blue Sepharose in the presence of this fatty acid.

The fact that chymotrypsin and PR 3 also bind to Blue Sepharose but are not eluted by benzamidine, suggests that while they may well employ the active site for binding, their affinity for benzamidine is not great enough to interfere with their binding to Blue Sepharose.

The binding of serine proteases such as PR 3 to

the dye Matrex Orange A [12], and factors II, IX and X and the complement serine protease C2 to Blue Sepharose [13,14], has previously been assumed to be a general dye affinity interaction. We have shown that the binding of certain serine proteases to Blue Sepharose can be described in terms of an active site ligand affinity. The results obtained here are in complete agreement with recent results of Burton and Lowe [15], who have designed novel affinity adsorbents for trypsin-like proteases. Modeling on the basis of a dipeptide-mimicking core structure, consisting of a benzamidine-substituted triazine ring they arrived at structure **3** (Fig. 4) as a highly effective inhibitor and affinity ligand for trypsin-like proteases. A comparison of benzamidine, Cibacron Blue F3G-A and structure **3** (Fig. 4) reveals structural similarities, and this may be taken as further evidence that serine proteases bind to Blue Sepharose through the active site. This similarity in structure would imply that the ''**a**'' part of Cibacron Blue F3G-A (Fig. 4) binds to subsite S_1, flanking the active site to the left, whereas the ''**b**'' part binds to subsite S_1' flanking the active site to the right.

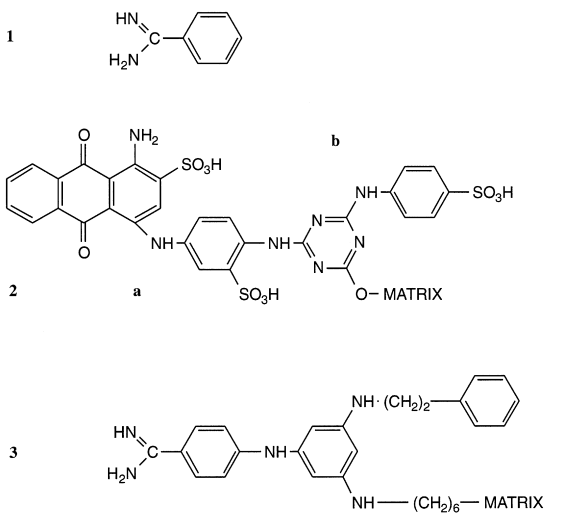

Fig. 4. Comparison of the structures of benzamidine (**1**), Cibacron Blue (**2**) and a designed affinity ligand for trypsin-like serine proteases (**3**).

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