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THE APPLICATION OF DNA-CELLULOSE CHROMATOGRAPHY IN THE ISOLATION OF IMMUNOGLOBULIN M AND COMPLEMENT COMPONENT C4b-BINDING PROTEIN FROM HUMAN SERUM

MUNIR ABDULLAH, R. JEREMY H. DAVIES and J. ALAN HILL*
Biochemistry Department, Medical Biology Centre, Queen's University, Belfast BT9 7BL (U.K.)
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SUMMARY

By utilising the ability of some anionic serum proteins to bind to DNA-cellulose, at physiological pH and ionic strength, two such proteins have been purified and identified as immunoglobulin M (IgM) and complement component C4b-binding protein (C4BP). The method entails ammonium sulphate precipitation followed by QAE-Sephadex, DNA-cellulose and Sephacryl S-300 chromatographic steps. Fractionation of the anionic proteins eluting from QAE-Sephadex over a selected range of ionic strength has afforded IgM which was characterised electrophoretically and immunochemically. A mixture containing IgM complexed with C4BP has also been obtained by utilizing fractions eluted from QAE-Sephadex at higher ionic strength. C4BP can be isolated from this mixture by using immunoaffinity chromatography to remove IgM.

INTRODUCTION

DNA-cellulose chromatography has been widely used in the analysis and purification of cellular proteins with affinity for DNA[1]. The technique has also found application in the isolation of certain anionic serum proteins[2]. A physiological basis for the affinity of these serum proteins for DNA has not been established but, in general, it does not appear to be the result of a highly specific interaction as most of them also bind to other polyanionic media such as phospho-cellulose[3] and heparin-Sepharose[4]. Nonetheless, the ability of such proteins to bind to DNA can be usefully exploited in developing alternative procedures for their purification; already several DNA-binding proteins, including complement components and the proteinase inhibitor α_1-antichymotrypsin, have been isolated from human serum[5–11]. As upwards of 40 distinct polypeptide species can be detected in the human serum DNA-binding protein fraction by two-dimensional polyacrylamide gel electrophoresis[12], it is evident that many of them remain to be characterised. In this paper we describe how modifications to the chromatographic procedures originally used to purify α_1-antichymotrypsin[11] have led to the isolation of two additional DNA-binding proteins. These have been identified as immunoglobulin M (IgM) and complement com-

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ponent C4b-binding protein (C4BP) on the basis of electrophoretic and immunochemical criteria. As noted previously[13], C4BP is initially obtained as a tightly bound complex with IgM and must subsequently be separated from it by immunoaffinity chromatography[14–16].

EXPERIMENTAL

Materials

Native calf thymus DNA (Type I and V), bovine serum albumin, human transferrin, ovalbumin, soybean trypsin inhibitor and agarose (Type I) were obtained from Sigma (St. Louis, MO, U.S.A.). Human IgM was purchased from Pel-Freez Biologicals (Rogers, AR, U.S.A.) and QAE-Sephadex A-50, Sephacryl S-300 and cyanogen bromide-activated Sepharose 4B were from Pharmacia (Uppsala, Sweden). Ampholytes (pH 3.5–10) were purchased from LKB (Bromma, Sweden) and cellulose (CF 11) from Whatman (Maidstone, U.K.). Rabbit antisera specific for the following human proteins were obtained from Dako (Copenhagen, Denmark): IgM μ chains, κ-type light chains, factor B, factor H, α_2-macroglobulin and immunoglobulin A; rabbit antiserum against total human serum proteins was supplied by Behringwerke (Marburg, F.R.G). Sheep antiserum to human C4b-binding protein was purchased from Miles (Slough, U.K.). Pooled human serum was a gift from the Northern Ireland Blood Transfusion Service.

Electrophoresis and immunoelectrophoresis

Following treatment with sodium dodecyl sulphate (SDS) and 2-mercaptoethanol, protein samples were subjected to electrophoresis on 10% polyacrylamide slab gels containing 0.1% SDS according to Laemmli[17]. The electrophoresis of native unreduced protein samples was performed on rod gels[18] containing 3.5% acrylamide and 0.1% SDS as described by Villiers *et al.*[14]. Isoelectric focusing in gels containing 4% acrylamide and 2% carrier ampholytes was carried out as recommended by Wrigley[19].

Double immunodiffusion and immunoelectrophoresis were performed in 1% agarose gels according to Ouchterlony and Nillson[20] and Weeke[21].

All gels were stained with Coomassie brilliant blue.

Chromatographic procedures

All chromatographic steps except gel filtration (at room temperature) were performed at 4°C. Fractions were collected automatically with their protein content being monitored from the absorbance at 280 nm and estimated by the method of Lowry *et al.*[22] using bovine serum albumin as standard.

The scheme used is similar to that employed previously to purify α_1-antichymotrypsin[11] and has been developed from the method we used in a preliminary study to obtain a mixture of IgM and C4BP[13].

QAE-Sephadex chromatography. The 30–75% ammonium sulphate precipitate from 100 ml of pooled serum was fractionated on a QAE-Sephadex column (22 × 4.5 cm) as previously described[11]. A linear salt gradient in pH 6.5 buffer (10 m*M* KH_2PO_4–K_2HPO_4, containing 1 m*M* 2-mercaptoethanol, 1 m*M* EDTA and 0.1 m*M* phenylmethylsulphonylfluoride) was applied and the column eluate was pooled as

two fractions corresponding to eluting sodium chloride concentrations between 110 and 150 m*M* (Fraction A) and between 155 and 225 m*M* (Fraction B).

DNA-cellulose chromatography. DNA-cellulose was prepared by the method of Litman[23] and contained approximately 15 mg DNA per g cellulose when assayed according to Hoch *et al.*[24]. Fractions A and B from the QAE-Sephadex column were dialysed against pH 6.8 buffer (10 m*M* KH_2PO_4–K_2HPO_4, containing 1 m*M* 2-mercaptoethanol, 1 m*M* EDTA and 0.1 m*M* phenylmethylsulphonyl fluoride) and then separately loaded onto a column of DNA-cellulose (16.5 × 3.2 cm) equilibrated in the same buffer. After washing the column with 50 m*M* sodium chloride in the pH 6.8 buffer, the DNA-binding proteins were eluted with a linear gradient of 50-500 m*M* sodium chloride in this buffer. The required portion of the eluate was combined, dialysed against pH 6.8 buffer and lyophilised.

Gel filtration. The lyophilised material from the DNA-cellulose column was dissolved in deionised water (2.5 ml) and applied to a column of Sephacryl S-300 (84 × 3.2 cm) equilibrated in the pH 7.0 buffer (0.1 *M* KH_2PO_4–K_2HPO_4, containing 0.50 *M* sodium chloride, 1 m*M* 2-mercaptoethanol, 1 m*M* EDTA, 0.1 m*M* phenylmethylsulphonyl fluoride and 0.02% sodium azide) that was used to elute the column.

Immunoaffinity chromatography. This was performed essentially as described by Villiers *et al.*[14] to remove contaminating IgM from C4BP. The protein mixture was applied to a column (5 × 1.2 cm) containing antisera to IgM coupled to cyanogen bromide-activated Sepharose 4B conjugate and the column was left at room temperature for 2 h to allow adsorption of the IgM. The unbound C4BP was then eluted, using reversed flow, in 2 column volumes of pH 9.0 buffer (0.02 *M* NaH_2PO_4, 0.10 *M* glycine containing 0.50 *M* sodium chloride and 0.1 m*M* phenylmethylsulphonyl fluoride).

RESULTS

The anionic serum proteins bound to the QAE-Sephadex column at pH 6.5 were eluted from it with a linear salt gradient and two portions of the eluate were retained. Fraction A (eluate containing between 110 and 150 m*M* sodium chloride) and fraction B (eluate containing between 155 and 225 m*M* sodium chloride) were processed further to yield IgM and the IgM–C4BP mixture respectively. Making a cut off at 150 m*M* sodium chloride ensured that no C4BP was present in fraction A.

IgM

When the proteins in fraction A were subjected to chromatography on DNA-cellulose the eluate fractions containing between 70 and 200 m*M* sodium chloride were found to cross-react with antiserum to IgM μ chains on double immunodiffusion. These fractions were combined and, after concentration, their constituent proteins were separated on Sephacryl S-300. The elution profile of the latter column (Fig. 1) showed three peaks. Analysis of the first peak (I) by SDS–polyacrylamide gel electrophoresis (Fig. 2) revealed two polypeptide species with $M_r \approx 75\,000$ and $\approx 25\,000$; their respective mobilities and glycoprotein staining pattern[25] were the same as those of the μ and κ chains of a commercial preparation of IgM (not shown). The identity of the native protein as IgM was confirmed by the following observations.

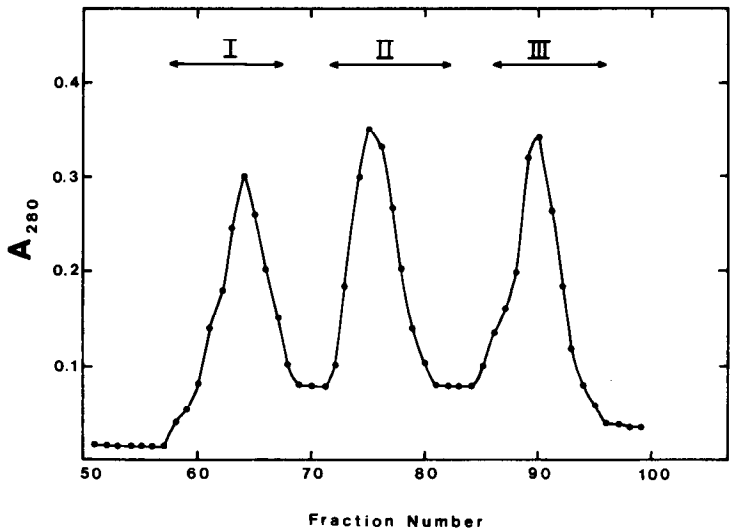

Fig. 1. Elution profile for the purification of IgM on a column (84 × 3.2 cm) of Sephacryl S-300. Fractions of 4.2 ml were collected at a flow-rate of 40 ml h^{-1}.

On gel filtration (Fig. 1) the protein emerged close to the void volume of the column as expected for IgM which has $M_r \approx 900\,000$. It gave a very heterogeneous pattern on isoelectric focusing with p*I* values extending over the range 6.0–8.0. Similar heterogeneity with a p*I* range of 5.0 to 8.2 has been reported[26] for IgM; some species with low p*I* values might have been excluded by the QAE-Sephadex separation step. Double immunodiffusion analysis of the purified protein against antiserum specific for IgM μ chains gave arcs that fused totally with the precipitates produced by commercial IgM in adjacent wells, thus demonstrating their immunochemical identity. The protein was also precipitated by antiserum specific for human κ-type light chains. On immunoelectrophoresis, it formed a single precipitin arc against monospecific antibodies to human IgM μ chains. No additional precipitin arcs were detected with polyspecific antisera to total human serum proteins thus indicating that the sample did not contain significant amounts of any serum protein other than IgM. The yield of IgM was approximately 5 mg from 100 ml of serum.

The protein composition of the second and third peaks in the Sephacryl S-300 elution profile shown in Fig. 1 was investigated by SDS–polyacrylamide gel electrophoresis and by double immunodiffusion with antisera specific for a variety of serum proteins. The predominant protein species in peaks II and III were found to be factor H and factor B respectively. Protocols for preparing both of these complement components as serum DNA-binding proteins have been published previously[8,9].

C4BP

The proteins in fraction B from the QAE-Sephadex column were further fractionated on DNA-cellulose and Sephacryl S-300 to yield a tightly bound complex of C4BP with IgM. The fractions from the DNA-cellulose column containing between 75 and 150 m*M* sodium chloride, when examined by SDS–polyacrylamide gel elec-

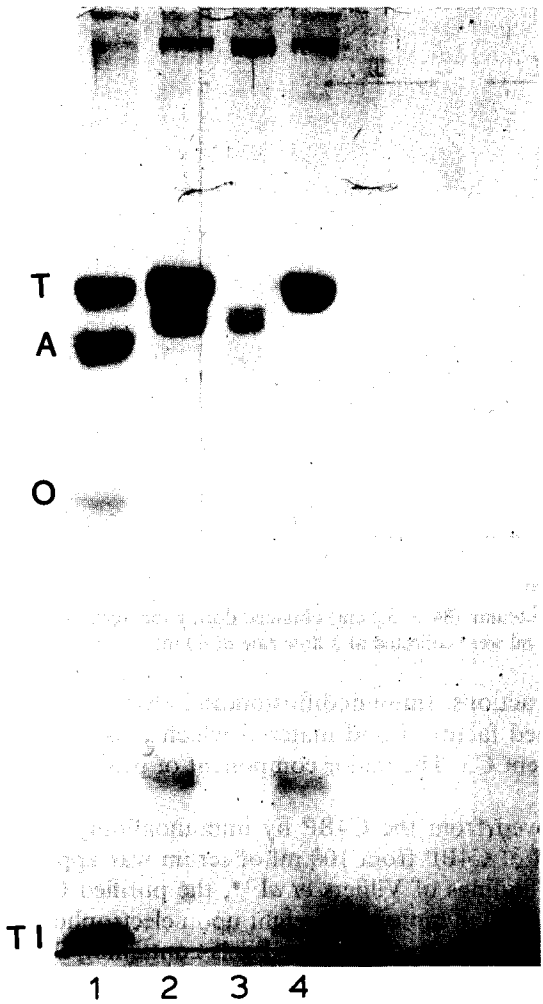

Fig. 2. SDS–polyacrylamide gel electrophoresis of IgM and C4BP as a mixture and individually. Lane 1: molecular weight markers, (T) human transferrin (76 500); (A) bovine serum albumin (67 000); (O) ovalbumin (43 000); (TI) soybean trypsin inhibitor (20 100). Lane 2: mixture of C4BP and IgM from first peak (I) of Sephacryl S-300 column (Fig. 3) before immunoaffinity chromatography. Lane 3: C4BP isolated from C4BP–IgM mixture by immunoaffinity chromatography. Lane 4: IgM purified as a DNA-binding protein, from peak I of elution profile shown in Fig. 1.

trophoresis displayed two prominent closely spaced bands with $M_r \approx 72\,000$ and 75 000 as well as a weakly staining band with $M_r \approx 25\,000$. On double immunodiffusion these fractions cross-reacted with antisera to IgM μ chains and to C4BP. The elution profile obtained after gel filtration of the material in these fractions on Sephacryl S-300 is shown in Fig. 3. The first peak (I) was eluted essentially within the void volume of the column and SDS–polyacrylamide gel electrophoresis of its component fractions indicated that they all contained a mixture of IgM and C4BP. As judged qualitatively from the protein staining intensity, the relative proportions of

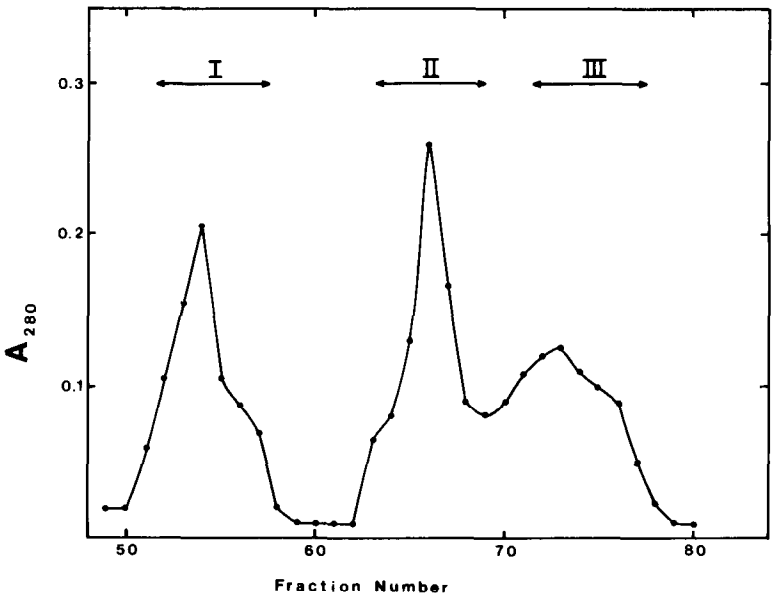

Fig. 3. Elution profile from a Sephacryl S-300 column (84 × 3.2 cm) obtained during the purification of C4BP (complexed with IgM). Fractions of 4.2 ml were collected at a flow-rate of 40 ml h^{-1}.

the two proteins were similar in all fractions. Immunodiffusion and electrophoretic analysis showed that peak II contained factor H and material which cross-reacted with antisera to complement component C3. The major component of peak III was α_1-antichymotrypsin.

Contaminating IgM was removed from the C4BP by immunoaffinity chromatography. After this step, the yield of C4BP from 100 ml of serum was approximately 1 mg. In agreement with the findings of Villiers *et al.*[14], the purified C4BP gave rise to two closely spaced bands of high molecular weight upon electrophoresis under non-reducing conditions (in 3.5% polyacrylamide rod gels containing SDS), and was also resolved into two bands, with p*I* values between 5.5 and 6.0, on isoelectric focusing. After reduction and denaturation, the protein usually migrated, on SDS–polyacrylamide gel electrophoresis, as a single polypeptide species which stained as a glycoprotein and had estimated $M_r \approx 72\,000$. This concurs with previously reported data for the C4BP subunit[14,15]. On some gels, however, the subunit appeared to be partially resolved into a doublet (see Discussion).

On immunoelectrophoresis, the purified protein formed single precipitin arcs with polyspecific antisera to total human serum proteins and with monospecific antiserum to C4BP; it did not show any cross-reactivity against antiserum specific for IgM μ chains. The purified C4BP was characterised by β mobility whereas its complex with IgM remained close to the origin and formed a precipitin arc at a position similar to that observed for free IgM.

DISCUSSION

IgM and C4BP have been purified from human serum by using an initial am-

monium sulphate precipitation step followed in turn by selective salt elution from columns of QAE-Sephadex (whose primary purpose is to remove positively charged proteins[2]) and DNA-cellulose and, finally, gel filtration. Both proteins have a high mutual affinity and all the C4BP is strongly complexed with IgM, though the precise stoichiometry of their interaction is unknown. Free IgM can, however, be separated from the IgM/C4BP complex because it is eluted from QAE-Sephadex at lower salt concentrations. To separate C4BP from IgM immunoaffinity chromatography is necessary.

Earlier protocols[27,28] for preparing IgM have been superseded by the method of Wichman and Borg[26] in which IgM is first adsorbed onto protamine-Sepharose and then subjected to two successive gel filtration steps. This gives a good yield of IgM ($\approx$ 30 mg from 100 ml of plasma) though it is contaminated with small amounts of IgA and α_2-macroglobulin. The procedure described here for purifying IgM is also quite straightforward but affords a much lower yield ($\approx$ 5 mg from 100 ml of serum). However, as judged by double immunodiffusion against appropriate antisera, the product is free from IgA and α_2-macroglobulin. Indeed, neither of these species can be detected in the DNA-binding fraction of serum proteins.

IgM is unusual in binding to both a positively charged matrix such as protamine-Sepharose and to the polyanionic DNA-cellulose at neutral pH. From isoelectric focusing results it is evident that both preparative methods lead to the isolation of IgM species bearing net negative and positive charges under the experimental conditions. It seems probable, therefore, that the binding to each type of support is mediated by different domains of opposite charge within the IgM molecule rather than by its overall net charge.

In principle, the binding of the IgM–C4BP complex to DNA-cellulose might simply reflect the affinity of its component IgM for the immobilised DNA. However, experiments with homogeneous C4BP showed that it also possesses some, albeit weaker, affinity for DNA-cellulose. At pH 6.8, 100 m*M* sodium chloride was sufficient to dissociate the protein from DNA-cellulose.

In terms of rapidity and overall yield the procedure described here does not offer any obvious advantages over existing methods[14–16,29] for preparing C4BP. As noted in these other studies, the purified C4BP migrates as two distinct species of high molecular weight on gel electrophoresis. It is well established[30] that C4BP is made up of multiple disulphide-linked glycoprotein subunits with $M_r \approx 70\,000$; the two forms of the native protein probably differ by the loss or addition of one subunit. On denaturation and reduction, the C4BP isolated after immunoaffinity chromatography usually gave a single diffuse band on SDS-gel electrophoresis with $M_r \approx$ 72 000. With lightly loaded samples this tended to resolve into a very closely spaced doublet (see Fig. 2) suggesting some heterogeneity in the subunits. This might result from differing levels of glycosylation of the individual polypeptide chains.

The protocols described here for the purification of IgM and C4BP resemble that used previously[11] to prepare α_1-antichymotrypsin as a serum DNA-binding protein. However, in each case, different sets of fractions have been processed from the salt gradients applied to the QAE-Sephadex and DNA-cellulose columns. Apart from the above proteins, fairly pure samples of factor H and factor B were obtained as distinct peaks from the final gel filtration step in the preparation of IgM. No attempt was made to purify them further because specific procedures for their isolation as

DNA-binding proteins have already been devised by Hoch and co-workers[8,9].

Although the physical basis of the affinity of some anionic serum proteins for DNA-cellulose is still unclear, this property nevertheless provides a useful variation upon which purification procedures may be based. By appropriate manipulation of elution conditions it is possible to isolate several functionally diverse serum proteins either individually or in parallel. For these proteins DNA-cellulose chromatography is a very effective method for freeing them from the great majority of serum proteins that do not bind to DNA-cellulose.

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