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Short Communication

Chromatographic profiles of cyanogen bromide fragments of unreduced human serum albumin on immobilized Cibacron Blue F3G-A

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ABSTRACT

The elution profiles of cyanogen bromide fragments A (299–585), B (1–123), C (124–298) and D (1–298) of unreduced human serum albumin (HSA) on Cibacron Blue F3G-A immobilized on Sepharose CL-6B are reported. The binding properties of fragments C and D are similar to those of HSA, whereas fragment A shows a slightly lower retention time. Fragment B, in contrast, does not interact with the dye. The different chromatographic behaviour of fragments B and C allows their fast separation by combined use of gel permeation and dye–protein affinity chromatography.

INTRODUCTION

Dye–protein affinity chromatography has gained considerable importance for purification of proteins because of the advantages of using textile dyes as "pseudo-specific" ligands in place of natural biological molecules [1]. Cibacron Blue F3G-A is most commonly used in dye–protein affinity chromatography because of its ability to bind with apparent specificity to several proteins [2]. The interaction of Cibacron Blue F3G-A with proteins containing the dinucleotide fold has been explained by assuming that the dye resembles the NAD structure. However, Cibacron Blue F3G-A is also able to bind strongly to proteins that are known not to possess the dinucleotide fold [1].

Synthetic dyes commonly possess ionic groups and apolar aromatic ring systems in the same molecule, so that they can interact with the protein by ionic, hydrophobic or charge transfer forces. Presently, it is generally accepted that any protein that possesses clusters of apolar and/or ionic groups can interact with an apparent specificity with a dye molecule having appropriately spaced apolar and ionic groups [1–5]. The specificity of Cibacron Blue F3G-A for the nucleotide binding site thus appears to be a special case of the above-mentioned requirements. Human serum albumin (HSA) interacts strongly with Cibacron Blue F3G-A [6], and this property has been largely used for isolating HSA from human plasma.

We describe here the behaviour of four large

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cyanogen bromide fragments of unreduced HSA with respect to Cibacron Blue F3G-A immobilized on Sepharose CL-6B in comparison with that of the intact HSA molecule.

EXPERIMENTAL

Materials and apparatus

Cibacron Blue F3G-A immmobilized on Sepharose CL-6B was obtained from Fluka (Buchs, Switzerland). Sephadex G-100 was provided by Pharmacia LKB (Uppsala, Sweden), HSA (Cohn Fraction V), iodoacetamide, and cyanogen bromide were purchased from Sigma (Milan, Italy). Carboxymethyl Cellulose (CM32) was obtained from Whatman (Maidstone, UK). Spectra/Por 6 dialysis membrane was obtained from Roth (Karlsruhe, Germany). All other chemicals were of the highest purity commercially available and were used without further purification. All column chromatographies were performed using Amicon glass columns. Eluates were monitored by an HP 1050 UV detector equipped with a preparative cell and coupled with an HP 3396A integrator (Hewlett-Packard). Sodium dodecyl sulphate–polyacrylamide gel electrophoresis was performed using the discontinuous method of Laemmli [7] on 12.5% (w/v) gels on a Bio-Rad instrument. Amino acid analyses were performed on a Beckman Model 119 CL amino acid analyser after acidic hydrolysis with 6 *M* hydrochloric acid for 24 h at 105°C. Circular dichroic (CD) spectra in the far-ultraviolet region of albumin solutions and isolated fragments were measured on a Jasco J-600 instrument, in the wavelength region 200–250 nm, at a protein concentration of 2.1 μM in 0.025 *M* phosphate buffer pH 7.0, using a path length of 0.5 cm.

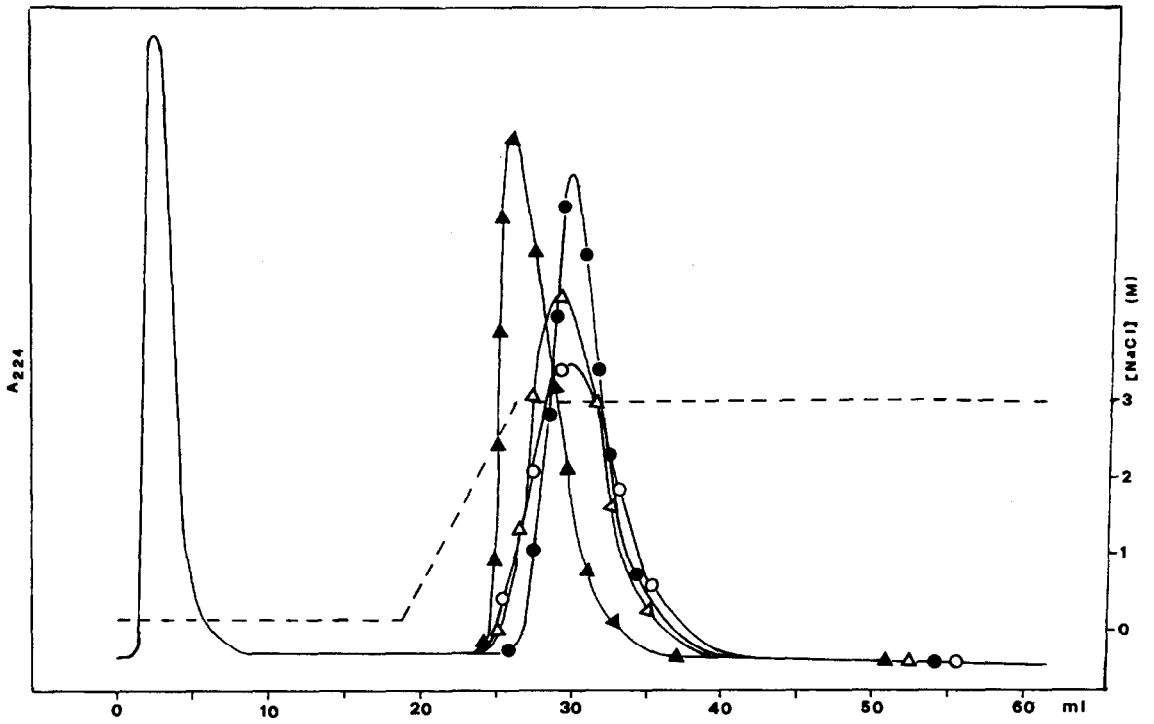

Fig. 1. Dye–protein affinity chromatography of HSA (●) and A (▲), B (—), C (△) and D (○) fragments on Cibacron Blue F3G-A–Sepharose CL-6B. The column (1.2 cm × 1 cm I.D.) was equilibrated with 0.01 *M* phosphate buffer, pH 7.5. After application of each sample the column was eluted at room temperature and at a flow-rate of 0.25 ml/min with equilibration buffer (19 ml), then with a linear gradient (----) of 0–3 *M* NaCl in phosphate buffer 0.01 *M* (8 ml) and finally with 3 *M* NaCl in phosphate buffer 0.01 *M* (35 ml).

Preparation of A, B, C and D cyanogen bromide fragments of unreduced HSA

A modification of previously described methods [8,9] was used to prepare A, B, C and D cyanogen bromide fragments of unreduced HSA. In a typical procedure the free thiol group (cysteine 34) of HSA was blocked adding 1.7 mg (six-fold molar excess) of iodoacetamide to a solution of 100 mg of HSA in 2 ml of Tris buffer 0.1 *M*, pH 8.5. The mixture was reacted for 1 h at room temperature in the dark, keeping the pH above 8 during the reaction. The solution was then dialysed against water, freeze-dried and redissolved in 1 ml of 70% formic acid. A solution of 140 mg of cyanogen bromide dissolved in 1.7 ml of 70% formic acid was added and the mixture allowed to react for 25 h at 4°C in the dark, then diluted by adding 25 ml of water, cooled in liquid nitrogen and freeze-dried. The residue, dissolved in 10 ml of 0.1 *M* NaCl in 5% propionic acid, was applied to a Sephadex G-100 column (100 cm × 3.2 cm I.D.) previously equilibrated with 0.1 *M* NaCl in 5% propionic acid. The column was eluted at room temperature using the same buffer at a flow-rate of 1 ml/min. Two fractions (peaks I and II) were collected separately and further resolved on a Whatman CM32 column. Peak I was diluted twice by water, the pH raised to 3.2, applied on a Whatman CM32 column (9 cm × 1.6 cm I.D.) and eluted at a flow-rate of 1 ml/min with a linear gradient of 0.075 *M*–0.3 *M* NaCl in phosphate buffer 0.01 *M*, pH 2.7, over 20 h. Fragments A and D were thus obtained. Peak II was fractionated in the same way, except that the starting buffer was 0.05 *M* NaCl in phosphate buffer 0.01 *M*, to give fragments B and C. Pooled peaks A, B, C and D were dialysed against water and freeze-dried. The identity of the isolated fractions was confirmed by polyacrylamide gel electrophoresis and amino acid analysis.

Column chromatography of HSA and fragments A, B, C and D on Cibacron Blue F3G-A–Sepharose CL-6B

About 3 mg of HSA and of each fragment were individually applied on the column packed with Cibacron Blue F3G-A–Sepharose CL-6B. The column was eluted according to the conditions described in Fig. 1.

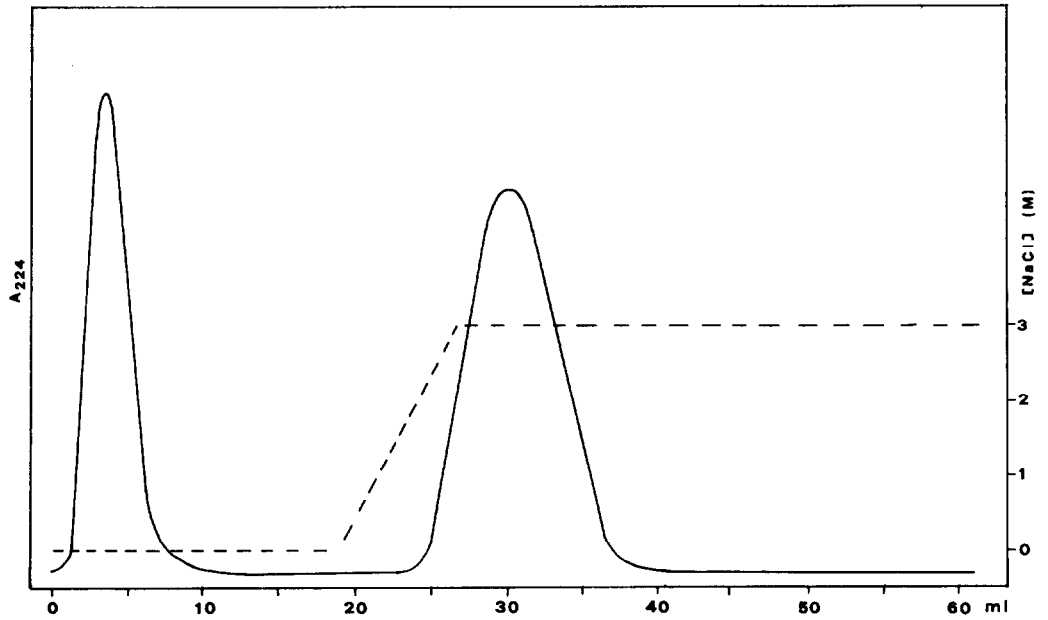

Fig. 2. Dye-protein affinity chromatography of peak II from G-100 on Cibacron Blue F3G-A–Sepharose CL-6B. Elution conditions were identical to those described in Fig. 1.

Column chromatography of peak II from Sephadex G-100 on Cibacron Blue F3G-A–Sepharose CL-6B

Peak II collected from the Sephadex G-100 column was dialysed against water and freeze-dried. About 10 mg of the sample were re-dissolved in 500 μl of 0.01 *M* phosphate buffer and applied on a column packed with Cibacron Blue F3G-A–Sepharose CL-6B. The column was eluted according to the conditions described in Fig. 2.

RESULTS AND DISCUSSION

The cleavage of unreduced HSA by cyanogen bromide gives rise to three large fragments which, according to the naming convention of McMenamy *et al.* [8], are called A, B and C (Table I).

Fragment C consists of a single polypeptide chain. Fragments A and B are composed of four and two subfragments, respectively, held together by disulphide bonds. Furthermore, incomplete cleavage at methionine 123 originates fragment D, which consists of fragments B and C linked through the uncleaved methionine [10].

To determine to what extent the secondary structure of the isolated A, B and C fragments used in this work was preserved, the CD spectra in the far-UV region were recorded. The CD

TABLE I

CYANOGEN BROMIDE FRAGMENTS OF UNREDUCED HSA

Fragment	Sequence	M_r [a]	Subfragments
A	299–585	32391	299–329 330–446 447–548 549–585
B	1–123	14040[b]	1–87 88–123
C	124–298	19990	–
D	1–298	34042	1–87 88–298

[a] Homoserine is considered to be the C-terminal amino acid.
[b] Cysteine 34 blocked with iodoacetamide.

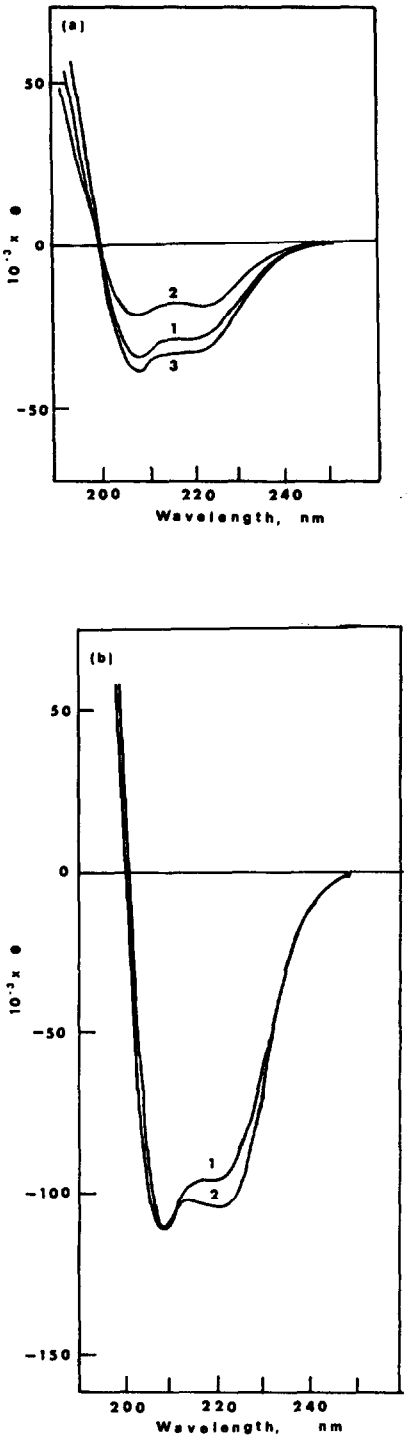

Fig. 3. Circular dichroic spectra of HSA and HSA fragments A, B and C in the far-ultraviolet region. (a) 1 = Fragment A; 2 = fragment B; 3 = fragment C; (b) 1 = equimolar mixture of A, B and C fragments; 2 = HSA.

spectra of the isolated fragments are reported in Fig. 3a. In Fig. 3b the spectra of albumin and of the equimolar mixture of the three fragments are compared. According to an earlier report [11], the spectra in Fig. 3 indicate that the secondary structure of isolated fragments is not basically impaired and that the slight decrease in the helix content observed is probably the result of disruption of bonds stabilizing the albumin tertiary structure rather than irreversible denaturation of the fragments [11].

The chromatographic pattern of HSA and A, B, C and D fragments on the Cibacron Blue F3G-A–Sepharose CL-6B column is reported in Fig. 1. Fragment B is eluted by 0.01 *M* phosphate buffer with the void column volume. The elution profile of fragments C and D is similar to that of HSA, whereas fragment A shows a slight lower retention time.

From these results it appears clearly that fragment B does not show any interaction with immobilized Cibacron Blue F3G-A, whereas fragments C and D, and to a lesser extent fragment A, interact with the dye similarly to HSA.

Since the CD spectra indicate that the secondary structure of fragments A, B and C remains essentially unchanged after cyanogen bromide cleavage, the absence of interaction for fragment B does not seem to be attributable to its denaturation. Therefore, it is very likely that the intact HSA molecule also does not present interaction sites for the immobilized dye in the sequence 1–123. The same considerations suggest, instead, that one or more interaction sites are present in each of the sequences 124–298 and 299–585 of HSA.

The different chromatographic behaviour of fragments B and C on Cibacron Blue F3G-A–Sepharose CL-6B allows their easy separation by combined use of gel permeation and dye–protein affinity chromatography.

As described in the Experimental section, peak II obtained from the Sephadex G-100 column contains unresolved fragments B and C. When peak II is applied to a Cibacron Blue F3G-A–Sepharose CL-6B column and eluted as described in Fig. 2, two peak are obtained (Fig. 2), one eluted very early by phosphate buffer and the second by 3 *M* NaCl. Gel electrophoresis and amino acid analysis identify the two peaks as highly purified fragments B and C.

As can be seen from Fig. 2, dye–protein affinity chromatography allows the separation of these two fragments in a considerably shorter time than conventional ion-exchange chromatography.

CONCLUSIONS

The three large cyanogen bromide fragments, A, C and D, interact with Cibacron Blue F3G-A immobilized on Sepharose CL-6B in a similar way to HSA, whereas fragment B does not interact with the dye. The different chromatographic behaviour of fragments B and C allows their fast separation by combined use of gel permeation and dye-protein affinity chromatography.

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