HUMAN C4-BINDING PROTEIN: N-TERMINAL AMINO ACID SEQUENCE ANALYSIS AND LIMITED PROTEOLYSIS BY TRYPSIN

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1. Introduction

Initiation of the classical pathway of complement involves the interaction and activation of the C1 complex with an activator such as immune aggregates [1]. Component C4, which is composed of 3 chains (α, β and γ of M_r 93 000, 75 000 and 33 000, respectively [2]) is then activated by the $\overline{C1}$ complex by the cleavage of the C4a peptide ($M_r \sim 8000$) from the N-terminus of the α-chain to yield C4b. The freshly activated C4b can bind covalently, via a reactive glutamyl group in its α'-chain, to a variety of surfaces by the formation of an ester, or perhaps amide, band [3–5] in a similar manner to that described for C3b binding [6–8]. The C4b can be utilized in the formation of the C3 converting enzyme, the complex $\overline{C142}$. The control of this complex is partly mediated by the C4-binding protein (C4bp) which interacts with both the surface bound, and soluble, forms of C4b, acting as a cofactor in the rapid cleavage of the α'-chain of C4b, at 2 positions, by the enzyme C3b/C4b inactivator [9–12].

Human C4bp has been reported to give 2 closely spaced bands of $M_r \sim 590\,000$ and $\sim 540\,000$ on SDS–PAGE using non-reducing conditions, and a single band of $M_r \sim 75\,000$ upon reduction [13]. The 2 bands, seen under non-reducing conditions, can be separated by ion-exchange chromatography and both have cofactor activity [14]. The γ-carboxyglutamic acid-containing, vitamin K-dependent, protein S appears to form a strong complex with a major portion of the M_r 590 000 band of C4bp found in serum [15]. It was also found that C4bp isolated from a supernatant, which lacked protein S, was composed of one band, under non-reducing conditions, which appeared equivalent to the M_r 540 000 band in [13].

Here, the N-terminal sequence of 36 residues of a preparation of C4bp, which gave both high M_r bands on SDS–PAGE under non-reducing conditions, is compared with the sequence of 13 residues obtained for C4bp from a study of the C4bp–protein S complex [15]. The limited proteolysis of C4bp by trypsin, which generates discrete fragments of M_r 39 000–36 000 is also described. This procedure should be useful in future structural studies and also in the study of the interaction between C4bp and C4b since the trypsin-treated C4bp retains its cofactor activity.

Abbreviations: SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; EDTA, ethylene diamine tetracetic acid; DFP, diisopropylphosphorofluoridate; SBTI, soybean trypsin inhibitor; IgG, immunoglobulin G; IgM, immunoglobulin M; PTH, phenylthiohydantoin; Fluram, fluorescamine

Nomenclature: The nomenclature of complement components is that recommended by the World Health Organisation (1968); a bar over a component indicates that the component is activated

2. Materials and methods

Human C4bp was prepared from outdated plasma by polyethylene glycol precipitation, salt gradient elution from Bio-Rex-70, DEAE–Sephadex A-50, heparin–Sepharose and DEAE–Sephacel and finally gel-filtration on Sephacryl S-300. The polyethylene glycol precipitation and heparin–Sepharose chromatography were performed as in [16] and the DEAE–Sephadex A-50 and Bio-Rex-70 steps were performed as in [13]. Ion-exchange chromatography on DEAE–Sephacel was performed as follows: C4bp from heparin–Sepharose was concentrated to 15 ml by ultrafiltration and dialysed against 70 mM NaCl, 20 mM Tris–HCl, 2 mM EDTA (pH 7.4). The dialysed

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sample (15 ml A_{280} = 1.05) was applied to a column (1.3 X 14 cm) of DEAE–Sephacel equilibrated with 70 mM NaCl, 20 mM Tris–HCl, 2 mM EDTA (pH 7.4) at a flow rate of 30 ml/h. After washing with 80 ml of the same buffer, the protein bound to the column was eluted with a linear gradient (chamber 1, 200 ml equilibrating buffer; chamber 2, 200 ml equilibrating buffer made 0.4 M with respect to NaCl) (fig.1a). Gel-filtration was performed by application of a concentrated sample (7 ml A_{280} = 1.86) of C4bp, from DEAE–Sephacel, to a column (3 X 90 cm) of Sephacryl S-300 eqilibrated with 140 mM NaCl, 20 mM Tris–HCl, 2 mM EDTA (pH 7.4) at a flow rate of 20 ml/h. To remove traces of IgM a Sepharose anti-IgM column was prepared (using the IgG fraction of a rabbit antiserum raised against a human myeloma IgM) and run as described in [17]. The ability of C4bp fractions, and the trypsin-digested C4bp, to act as a cofactor in the splitting of C4b by C3b/C4b inactivator was assessed by SDS–PAGE using weight ratios of C4bp:C4b:C3b/C4b inactivator of 0.6:1.0:0.01. The preparations of C4b and C3b/C4b inactivator were supplied by Dr R. D. Campbell (Oxford) and Dr L.-M. Hsiung (Oxford).

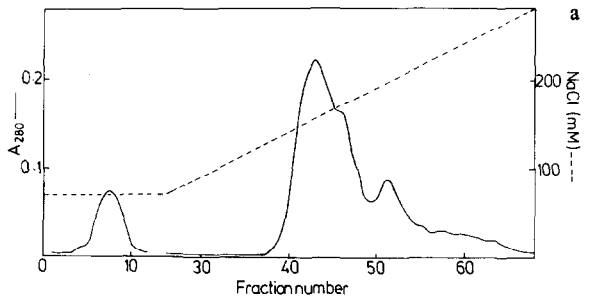
Samples and marker proteins for SDS–PAGE were prepared and run as in [18]. Amino acid analysis and hexosamine analysis was performed as in [18]. Human C4bp was reduced and alkylated (with iodo[2-^{14}C]-acetic acid) and subjected to automated Edman degradation in a Beckman 890c sequencer using the procedures given in [18]. At step 13 in the sequencer run the sample was reacted with Fluram as in [19].

Human C4bp was treated with trypsin at a variety of substrate-to-enzyme ratios (from 30/1–1000/1, w/w) in 0.2 M NH_4HCO_3 (pH 8.0) at 25°C for 16 h. The digestion was stopped by the addition of DFP in the preparative procedures, or SBTI in the analytical procedures.

3. Results

3.1. *Purification*

The yield of C4bp was ~12 mg/l of plasma and traces of IgM could be removed from the preparation by passage down a Sepharose anti-IgM column. The purified C4bp behaved as a single symmetrical peak on Sephacryl S-300 (being eluted between 190–120 ml, in a similar position to that of human IgM) but appeared heterogeneous on ion-exchange chromatography on DEAE–Sephacel (fig.1a). There were



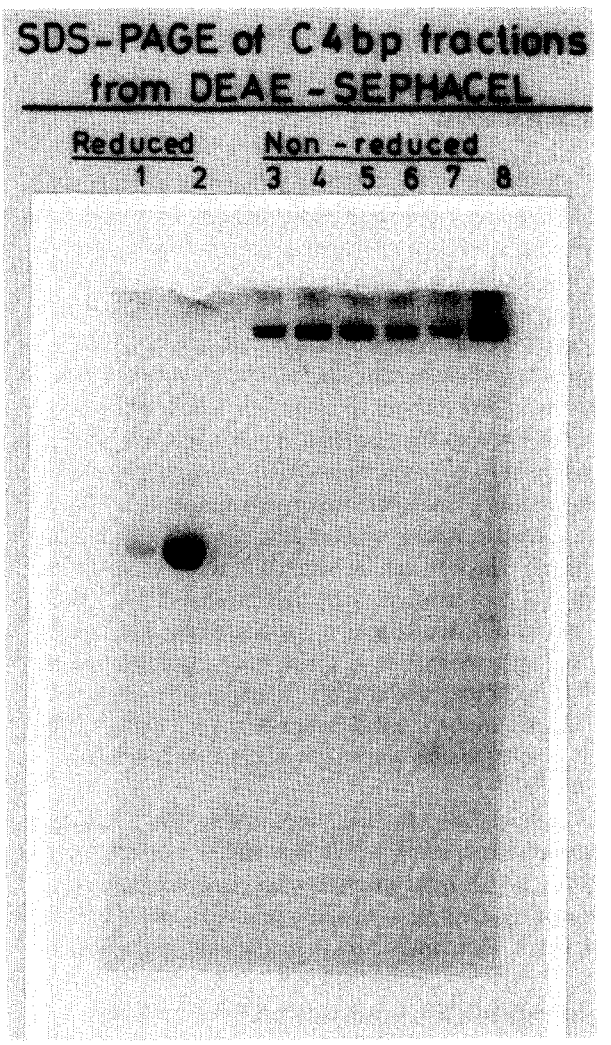


Fig.1. (a) Ion-exchange chromatography of C4bp (15 ml A_{280} = 1.05), from heparin–Sepharose, on a column (1.3 X 14 cm) of DEAE–Sephacel. The protein bound to the column was eluted with a linear salt gradient as described in the text: 4.3 ml fractions were collected. (b) SDS–PAGE, on a 7% slab gel, of non-reduced samples from fractions 41, 43, 46, 48 and 52 are shown in tracks 3, 4, 5, 6 and 7, respectively. All these samples gave a single band, on reduction, as shown in track 2. A non-reduced sample of the material applied to the column is shown in track 8.

at least 3 peaks of protein as judged by the A_{280} but each peak had the same composition as judged by SDS–PAGE (fig.1b) in non-reducing, and reducing, conditions. The non-reduced samples gave 2 closely spaced bands, which stained with about the same intensity, in the M_r 540 000–590 000 region, while all the reduced samples gave a single band of apparent $M_r \sim 75\,000$ (fig.1b). Each peak from the DEAE–Sephacel showed C4bp activity as judged by its ability to act as a cofactor in the splitting of C4b by C3b/C4b inactivator.

All the fractions from DEAE–Sephacel containing C4bp, as judged by cofactor activity measurements and SDS–PAGE, were pooled and this material, after gel-filtration on S-300, was used for amino acid analysis, hexosamine analysis, N-terminal sequence determination and limited proteolysis by trypsin.

3.2. *Amino acid and hexosamine compositions*

The amino acid composition found for human C4bp was very similar to that reported in [16] except for a higher half-cystine value (6.17% *vs* 4.89%) and a lower glutamic acid value (10.99% *vs* 13.65%) (table 1). The C4bp was found to contain 3.5% (w/w) glucosamine and no galactosamine.

Table 1
Amino acid composition of human C4bp[a]

Residue	Residues/100 residues
Asx	7.87
Thr	6.38
Ser	7.96
Glx	10.99
Pro	7.62
Gly	7.05
Ala	3.39
1/2 Cys[b]	6.17
Val	5.97
Met	0.87
Ile	4.38
Leu	6.11
Tyr	4.35
Phe	3.08
His	2.81
Lys	5.71
Arg	5.23
Trp	3.95

[a] Samples were hydrolysed in 5.7 N HCl for 24, 48 and 72 h at 110°C under vacuum and the serine and threonine values were estimated by extrapolation to zero time

[b] Half-cystine was estimated as *S*-carboxymethylcysteine

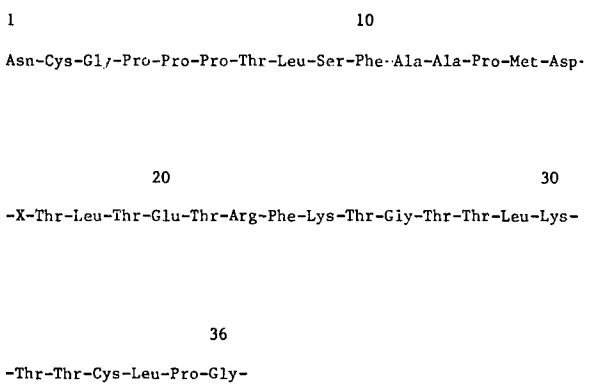


Fig.2. N-Terminal amino acid sequence of human C4bp.

3.3. *N-Terminal amino acid sequence*

Automated N-terminal amino acid sequence analysis of 45 nmol reduced and alkylated C4bp gave a single unambiguous sequence for 36 residues (fig.2). The N-terminal amino acid found was asparagine, and the yield of PTH-asparagine on the first cycle was ~60%, of that expected. A repetitive yield of 97% was obtained during the run based on the yield of leucine at positions 8, 18, 29 and 34. There was very little background, or 'carry-over', of PTH-amino acids during the run, however the identification of PTH-amino acids beyond cycle 13 was probably enhanced by the treatment of the sample with Fluram at cycle 13.

3.4. *Limited proteolysis of C4bp by trypsin*

Treatment of C4bp with trypsin (50/1, w/w) in 0.2 M NH_4HCO_3 (pH 8.0) at 25°C for 16 h yielded a molecule which behaved in an identical fashion to the untreated molecule on gel-filtration on Sephacryl S-300 and with respect to its mobility on SDS–PAGE in non-reducing conditions (fig.3). On reduction of the trypsin-digested C4bp, and examination on SDS–PAGE, it could be seen that all the intact (M_r 75 000) chain had been converted to 2 major fragments with app. M_r-values of 37 500 and 36 000 (fig.3) plus some low M_r material. Using milder conditions of trypsin digestion (fig.4) the 37 500 M_r fragment could be seen to be generated via a fragment of app. M_r 39 000 The M_r 37 500 and 36 000 fragments were separated from low M_r peptide material, after reduction and alkylation of the trypsin digested C4bp, by gel filtration on Sephadex G-50 (superfine) in 0.2 M NH_4HCO_3 (pH 8.0). N-Terminal sequence analysis of a mixture

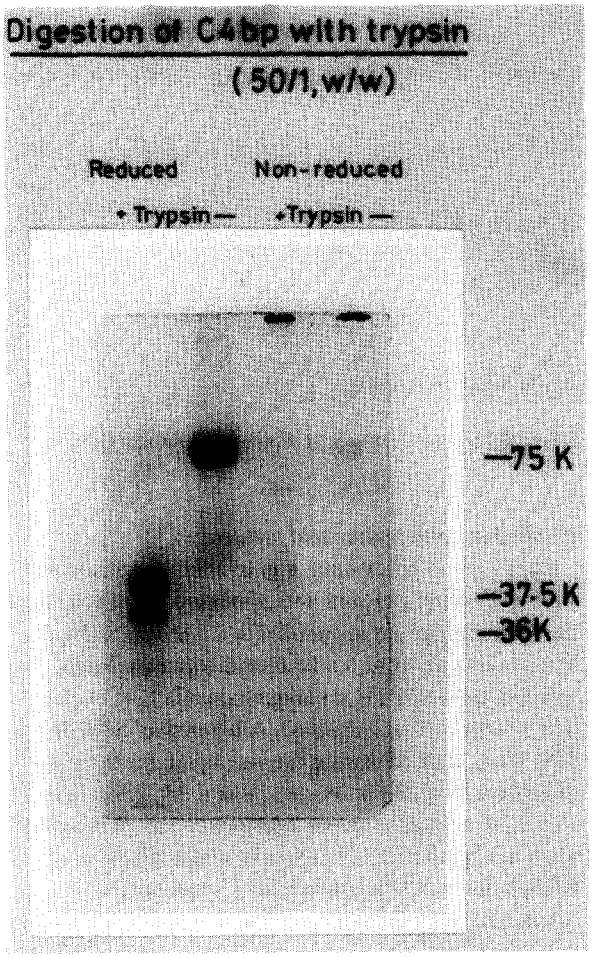


Fig.3. SDS–PAGE, on a 10% slab gel, of C4bp incubated at 25°C for 16 h in 0.2 M NH_4HCO_3 with (+) and without (–) trypsin at 50/1 (w/w) substrate/enzyme. DFP was added at 16 h to inhibit further digestion.

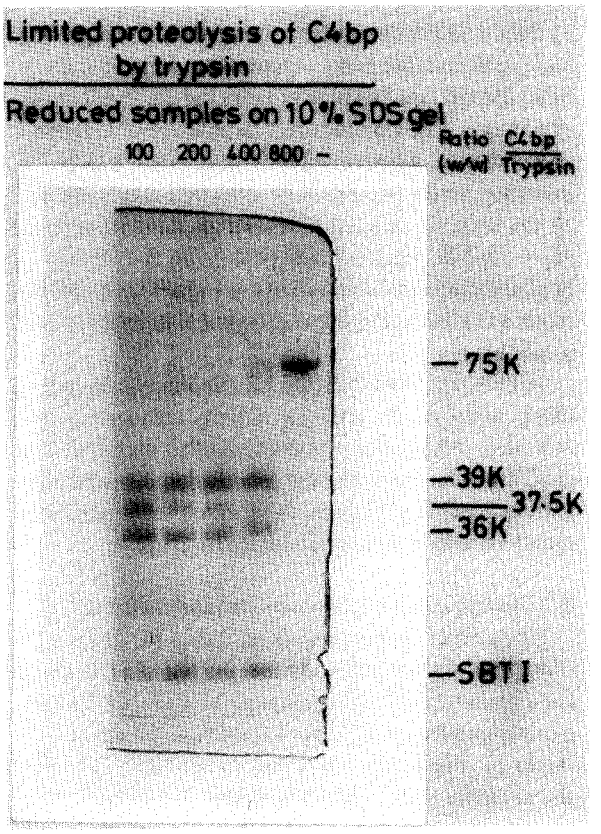


Fig.4. SDS–PAGE, on a 10% slab gel, of C4bp treated with trypsin at 25°C for 16 h in 0.2 M NH_4HCO_3. Ratios of 100/1, 200/1, 400/1 and 800/1 (w/w) C4bp/trypsin are shown in the first 4 tracks and a control sample (–) incubated without trypsin is shown in track 5. All the samples are reduced. The equivalent non-reduced samples all showed the same gel pattern as undigested C4bp. SBTI was added at 16 h to inhibit further digestion.

of the M_r 37 500 and 36 000 fragments indicated that at least the N-terminal 40 residues of the intact chain of C4bp are cleaved off during limited proteolysis by trypsin.

The trypsin-digested C4bp, which showed no intact M_r 75 000 chain under reducing conditions (fig.3), still retained its ability to behave as a cofactor for the breakdown of C4b by C3b/C4b inactivator.

4. Discussion

The C4bp preparation used in this study appeared to be composed of about equal amounts of the M_r 540 000 and 590 000 bands [13] when examined on SDS–PAGE in non-reducing conditions (fig.1b). Only a very minor band could be seen in the M_r 70 000 region, in non-reducing conditions, even with a high loading of the C4bp preparation (fig.1b, fig.3). This indicated that if protein S, which has been shown to copurify with the M_r 590 000 band [15], was present then the amount was low (<5%, assuming equal intensity of staining by the M_r 75 000 chain of C4bp and protein S). The results of the amino acid sequence analysis (fig.2) confirm, and extend, the N-terminal sequence of 13 residues obtained for C4bp from the sequence of analysis of a mixture of C4bp and protein S [15]. The yield of PTH-amino acids, in first few cycles of the sequence analysis of C4bp, indicate that C4bp is composed of a single type of polypeptide

chain and also that it was unlikely that any other polypeptide chain, with a free N-terminal amino acid, was present at a level >5% of that of the C4bp chain.

The amino acid composition found for C4bp (table 1) is very similar to the only other composition published for this protein [16] and the hexosamine analysis has established that C4bp has a relatively high (3.5%) glucosamine value. The difference between the 2 forms of C4bp with app. M_r-values of 590 000 and 540 000 is unknown but could be related to the carbohydrate content, or the number of M_r 75 000 chains present. The presence of 2 forms of C4bp may account for some of the apparent heterogeneity seen on ion-exchange chromatography seen in fig.1a and reported in [13,14].

It is of interest that the trypsin-treated C4bp retained its cofactor activity and behaved in an identical fashion to the untreated molecule on gel-filtration, and on SDS–PAGE under non-reducing conditions (fig.3), when it had been split in at least 2 positions (fig.4) in each of its M_r 75 000 chains. The trypsin-treated molecule could therefore be useful in studies designed to determine which portion of the C4bp molecule interacts closely with C4b.

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