THE EFFECT OF TRYPSIN CLEAVAGE ON THE STRUCTURE AND FUNCTION OF HUMAN C4

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1. Introduction

The fourth component of human complement is a glycoprotein of $M_r \sim 190\,000$ [1–3]. C4 consists of three polypeptide chains (α,β,γ) linked together by non-covalent forces and disulfide bridges [4].

When activated by $\overline{C1}$s, C4 is cleaved, releasing a small peptide, C4a, of $M_r \sim 10\,000$ [5,6]. This peptide is spasmogenic for guinea pig ileum and tachyphylactic for human C3a anaphylatoxin [7]. The rest of the molecule after $\overline{C1}$s cleavage, i.e., C4b, can bind to the cell surface via a labile binding site. C4b, on the cell surface or in solution, is easily cleaved by the C4b inactivator (C4bINA) [8] in the presence of C4-binding protein (C4bp) [9,10]. Two fragments are produced, namely C4d, a fragment of the α-chain, and C4c, which consists of the residual α-chain and the intact β- and γ-chains.

One of the biological functions of C4 in the complement cascade is to form a complex with C2. This complex, stabilized by $\overline{C1}$s [11], generates proteolytic activity against C3, which is split and activated.

We have shown that methylamine-treated C4 is hemolytically inactive but combines with C2 to elicit convertase activity against C3. Here trypsin was used for degradation of highly purified human C4. The composition of released fragments was analysed with the aim of elucidating the biological functions of C4.

Abbreviations: DTT, dithiothreitol; SBTI, soybean trypsin inhibitor; EDTA, ethylene diamine tetraacetic acid; SDS, sodium dodecyl sulphate; PBS, phosphate-buffered saline (pH 7.4); GVB, veronal buffer containing 0.1% gelatin; GVBE, gelatin veronal (barbitone) buffer containing 0.04 M EDTA; M_r, relative molecular mass

Nomenclature: Complement components follow the recommendations by the World Health Organization (1968). A bar above the complement component (e.g., $\overline{C1}$s) signifies an enzymatically active form

2. Materials and methods

Fresh frozen ACD-plasma from healthy adult, purchased from the University Hospital (Uppsala) served as the origin for preparation of complement components. Sheep erythrocytes and rabbit antisheep hemolysin were from the National Bacteriological Lab. (Stockholm). D,L-Dithiothreitol (DTT) was purchased from Sigma (St Louis MO). L-(Tosylamide-2-phenyl-ethyl-chloromethyl-ketone)-treated trypsin with spec. act. 224 units/mg and soybean trypsin inhibitor (SBTI) were purchased from Worthington Biochemical Corp. (Freehold NJ).

2.1. *Human complement components*

Highly purified C2 and C4 and partially purified $\overline{C1}$ were isolated as in [12], [3] and [13,14], respectively. Treatment of C4 with [^{14}C]methylamine at slightly alkaline pH was performed as in [15]. [^{14}C]-Methylamine is incorporated into the α-chain of C4 at a ratio of 1 mol methylamine/mol C4. Here, [^{14}C]-methylamine was used as a marker of the α-chain to follow the breakdown by autoradiography.

2.2. *Hemolytic titration of complement components*

Effective molecules (EM) of C2 [16] and C4 [13] were determined according to established methods.

2.3. *Immunoelectrophoresis*

Experiments were carried out on glass plates containing 1% agarose dissolved in 0.01 M Tris–HAc buffer (pH 8.6) containing 0.002 M EDTA for 2 h at 50 V.

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2.4. *Antisera*

Monospecific rabbit antisera against human C4 were purchased from Behringwerke AG (Marburg). Antisera against C4, trypsinized for 1.5 h at 37°C with a substrate:enzyme ratio of 50:1, were raised in rabbits. The antiserum against trypsinized C4 also contains antibodies against the trypsin/SBTI complex.

2.5. *Polyacrylamide gel electrophoresis*

Sodium dodecyl sulphate (SDS) gel electrophoresis was carried out on 11% slab gels, using a discontinuous buffer system [17]. Sample proteins (20 μg), diluted in an equal volume of 8 M urea, containing 2% SDS with or without 50 mM DTT, were incubated at 37°C for 60 min before being loaded on the gel. A drop of tracking dye, 0.1% bromophenol blue, in water, was added and electrophoresis was carried out at room temperature for 4 h at 200 mA. The gel was stained with 0.25% Coomassie brilliant blue for 16 h and destained in methanol:acetic acid:water (25:7.5:67.5).

Slab gel with reference proteins of known M_r was run in the same way. The negative logarithms of the relative mobilities of these proteins were plotted against M_r. The curve obtained was used for calculating the M_r values of fragments of C4. After staining, the gel was cut to give parallel strips about 10 mM wide and these strips were analyzed in a Gilford spectrophotometer, model 2541, at 541 nm. Autoradiography was carried out by exposing the stained and dried slab gel to an X-ray film (Kodak X-Omat R5) at room temperature for 1–2 weeks.

2.6. *Cleavage of C4 by trypsin*

Highly purified C4 at 0.8 mg/ml and with 7.0×10^{12} effective molecules/ml was incubated at 37°C with trypsin (2.5 mg/ml in PBS) at a substrate:enzyme ratio of 50:1 (w/w). At known time intervals 150 μl of the mixture were withdrawn and the trypsin activity was neutralized by adding a 4-fold weight excess of SBTI (5 mg SBTI/ml PBS) over trypsin. Reaction mixture (25 μl) was subjected to SDS–polyacrylamide gel electrophoresis and 10 μl samples were analyzed by immunoelectrophoresis; 16 μl were diluted with GVB to 200 μl and used for testing the interaction with C2. C4 hemolytic activity was tested on samples serially diluted from 1:500 in a total volume of 0.4 ml.

2.7. *Formation of a C4–C2 complex*

Trypsinized C4 (16 μg) in 200 μl GVB were incubated with an equal volume of oxidized C2 (1×10^9 EM/ml) for 15 min at 37°C. After incubation, remaining ^{oxy}C2 hemolytic activity was assayed by adding 2.4 ml guinea pig serum diluted 1:100 in GVBE. Binding of trypsinized C4 to ^{oxy}C2 lowers the ^{oxy}C2 hemolytic activity and is expressed in the following as percent inhibition of ^{oxy}C2-dependent hemolysis. Native C4 was incubated with ^{oxy}C2 as a positive control for total inhibition of ^{oxy}C2 hemolytic activity; as a negative control ^{oxy}C2 was incubated with buffer. The effect of trypsin, SBTI and trypsin/SBTI complex on ^{oxy}C2 hemolytic activity was also tested.

3. Results

The cleavage of C4 by trypsin as a function of time was analyzed by immunoelectrophoresis, by polyacrylamide gel electrophoresis, by the ability to interact with C2, and by C4 hemolytic titrations.

3.1. *Immunoelectrophoretic analyses*

Proteolysis of C4 was accompanied by changes in its immunoelectrophoretic pattern as illustrated by the use of monospecific rabbit antihuman C4 serum (fig.1A). After 1 min incubation with trypsin, a faster mobility of C4 towards the anode is seen. The material might have further increased its anodicity after degradation for 2 h.

The troughs in fig.1B contain antiserum against C4 degradation products produced as in section 2. C4 trypsinized for 1 min shows a faster β-mobility arc than intact C4, and 5 min trypsin treatment gives rise to a new arc of α-mobility. This arc increases up to 20–30 min of trypsin digestion and then remains unchanged up to 120 min of this treatment.

3.2. *SDS–polyacrylamide gel analyses*

C4 migrated on SDS–polyacrylamide slab gel as a single protein band of M_r 189 000. In the presence of a reducing agent, C4 was split into 3 chains: α-chain (M_r 88 000), β-chain (M_r 72 000) and γ-chain (M_r 32 000). Incubation of C4 with trypsin resulted in progressive degradation of C4. As illustrated in fig.2, incubation of C4 for 1 min at 37°C with 2% (w/w) trypsin resulted primarily in cleavage of the α-chain into a residual α′-chain of reduced molecular size (M_r 81 500). Prolonged degradation affects the

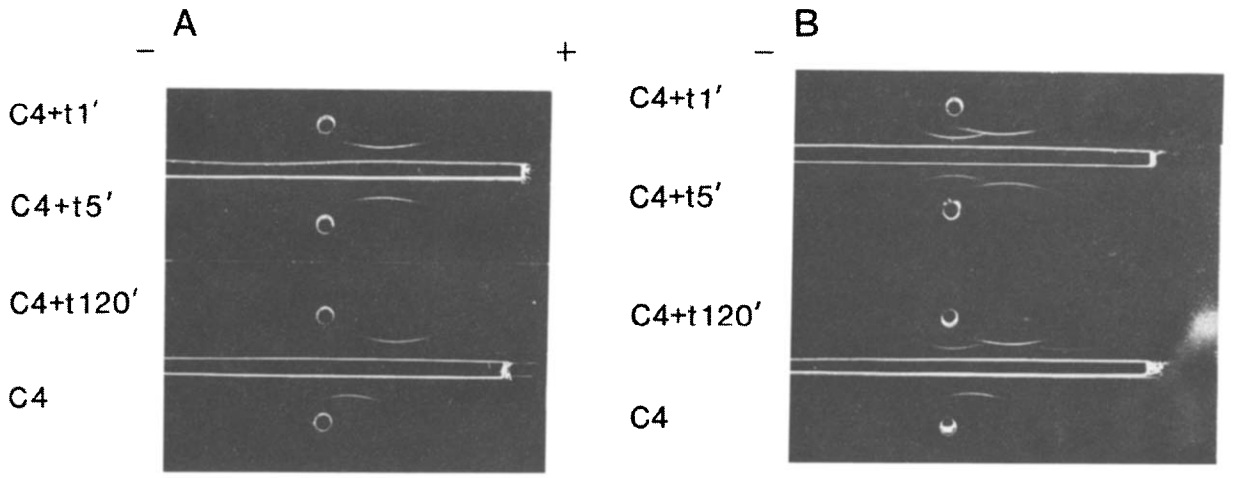

Fig.1. Immunoelectrophoretic analysis of the cleavage of human C4 by trypsin. C4 (0.8 mg/ml) was digested with trypsin at a substrate:enzyme ratio of 50:1. The reaction was terminated by adding SBTI at known time intervals and 10 μl of the mixtures was analyzed. The troughs in (A) contain monospecific rabbit anti-human C4 and in (B) rabbit antiserum against C4 digested with trypsin for 1.5 h.

α'-, β- and γ-chains. After ~10–20 min these are completely fragmented. The principal fragments formed during a digestion period of up to 2 h, which are stainable with Coomassie brilliant blue, have M_r-values of 60 500, 28 000, 24 000, 20 000 and <10 000.

To further estimate the breakdown pattern, [^{14}C]-methylamine-treated C4 was trypsinized. Methylamine is incorporated into the α-chain of C4 and the degradation of [^{14}C]methylamine-labelled α-chain of C4 was recorded by autoradiography of the stained and dried slab gel (fig.3). During the digestion period the ^{14}C-label of the α-chain is progressively found in fragments with M_r of 81 500, 60 500, 41 000 and 28 000 (fig.3). At least the last fragment showed

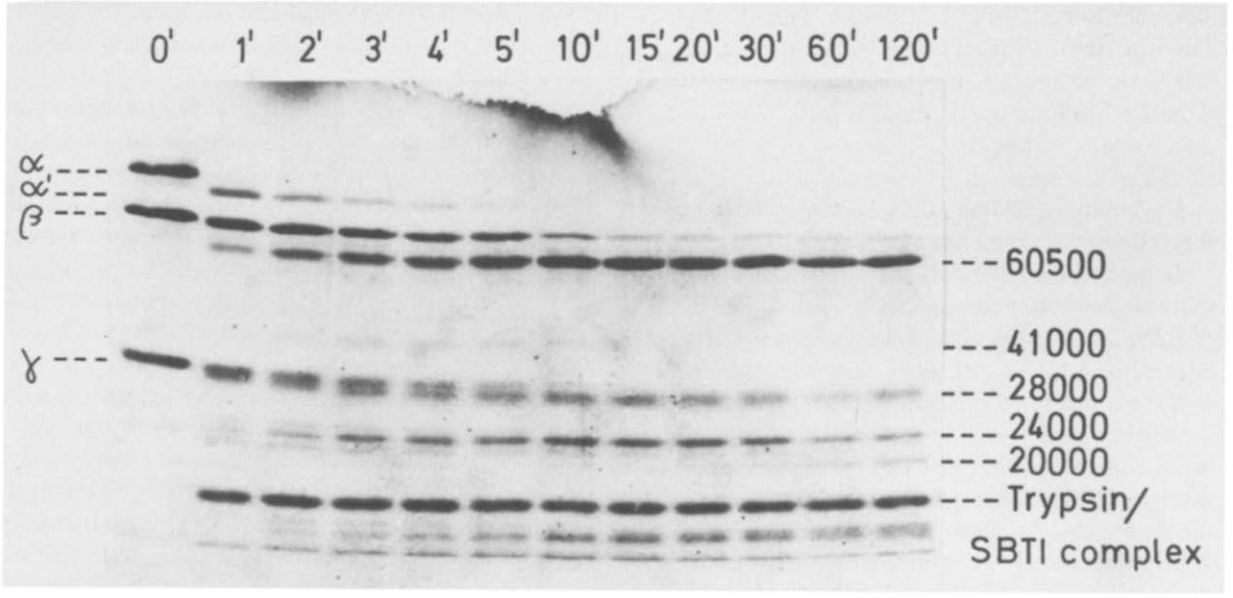

Fig.2. SDS–Polyacrylamide slab gel electrophoresis of C4, treated with trypsin at 37°C in a substrate:enzyme ratio of 50:1. Digestion was terminated at known intervals of time by a 4-fold molar excess of SBTI over trypsin. Samples were reduced with 25 mM DTT in 4 M urea and 1% SDS for 1 h at 37°C before being loaded on the 11% polyacrylamide slab gel.

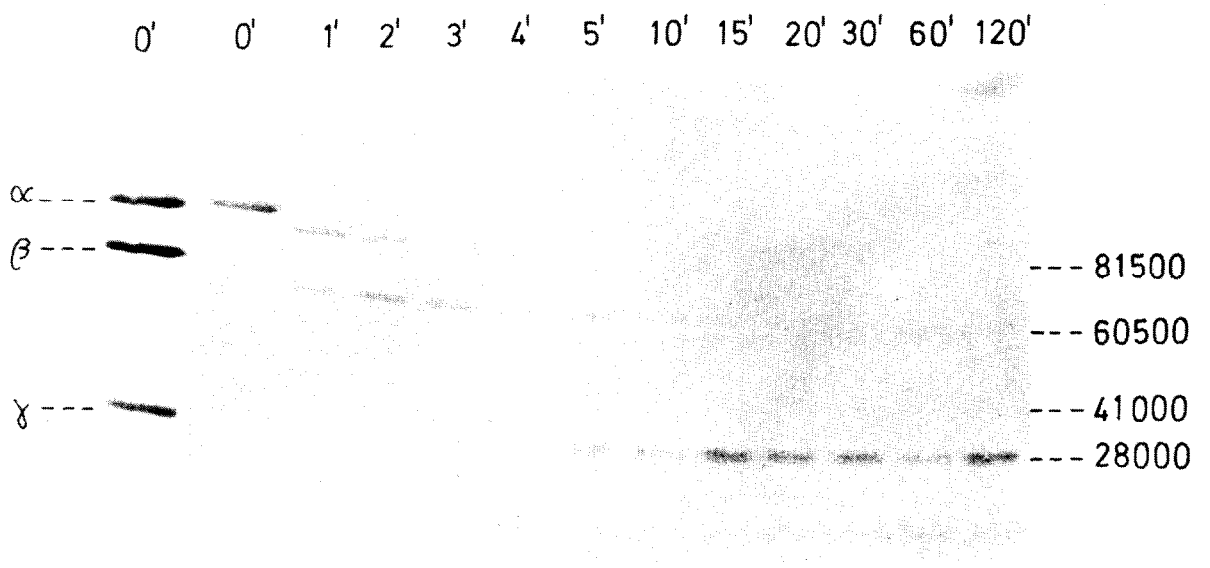

Fig.3. Autoradiography of [^{14}C]methylaminated and trypsinized C4. The material was digested at 37°C with 2% trypsin, and after reduction with 25 mM DTT in 4 M urea and 1% SDS was subjected to electrophoresis on a polyacrylamide slab gel. After staining and drying, the gel was exposed to an X-ray film at room temperature for 2 weeks. Indicated on the left side of the developed X-ray film is part of the stained gel at zero time of digestion.

microheterogeneity. These fragments correspond to fragments found in the gel stained by Coomassie brilliant blue.

The fragments on polyacrylamide slab gel produced after 2 h digestion were tested by immunodiffusion against anti-β-chain and anti-γ-chain serum. The fragment with M_r of 60 500 precipitated the anti-β-chain serum. No precipitation was obtained by the anti-γ-chain serum.

3.3. *Biological activities of degraded C4*

The ability of C4 and its degradation products to convert EAC1 to hemolytically active EAC14 intermediates was examined. For this purpose degraded C4 was added to EAC1 cells, the cells were washed and ^{oxy}C2 and guinea pig EDTA-serum was added. Trypsin treatment of C4 for 1 min reduced the hemolytic activity of this system to <1% of that of native C4.

The interaction between C4 and C2 in solution was studied by incubating C4 or its degradation products with C2 and subsequently measuring the remaining C2 hemolytic activity. The inhibitory activity of C4, degraded at 37°C for different periods of time, is illustrated in fig.4. Undegraded C4 showed even lower inhibitory activity than C4 digested for 1–3 min. The interaction with material digested for 5–10 min rapidly declines.

To further investigate the breakdown products, stained gel strips were scanned in a Gilford spectrophotometer model 2541. The cleavage of the β-chain seemed to correlate fairly well with the loss of C2-complexing activity of C4. Trypsin treatment for 5 min left ~56% of the β-chain.

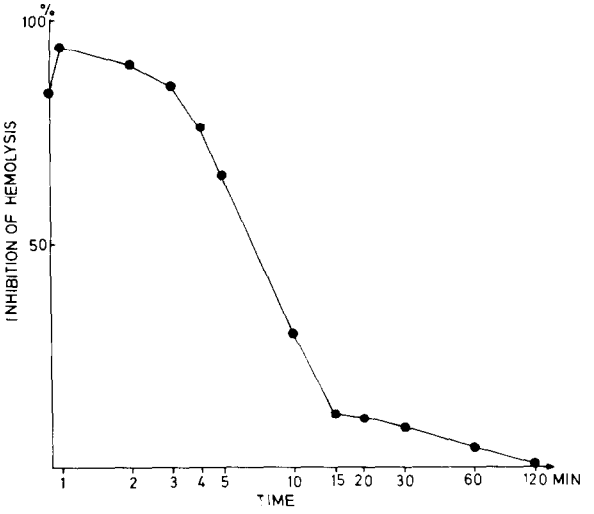

Fig.4. The ability of C4, trypsinized for a given time, to form a complex with ^{oxy}C2, expressed as inhibition of ^{oxy}C2 hemolytic activity.

4. Discussion

Earlier reports on the degradation of C4 have mostly concerned the natural enzymes $C\bar{1}s$, which splits C4 into C4a and C4b, and C4bINA, which splits the molecule into C4c and C4d. Treatment of C4 with 1% trypsin (w/w) for 1 min at 20°C totally abrogated the C4 hemolytic activity in [6]. This finding was confirmed in our study. The first tryptic attack is directed at the α-polypeptide chain, as is also evident from the polyacrylamide gel at a site responsible for binding of C4 onto the cell surface. Moreover, the mobility of trypsinized C4 on immunoelectrophoresis has been reported [6] to be slower than that of intact C4, an observation which is not in accordance with our result that trypsin-treated C4 had a faster β-mobility. A cathodal shift of the β-mobility arc has been observed, however, after cleavage of C4b by C4bINA [18] together with the generation of a new arc of α-mobility. The α-mobility arc appeared here (fig.1B) after 5 min trypsin treatment and persisted for up to 120 min degradation when an antiserum against C4 degradation products was used.

The cleavage of C4b by trypsin differs at least partly from the reported cleavage by C4bINA and C4bp, which are directed only against the α′-polypeptide chain. Trypsin also cleaves both the β- and γ-chains. Cleavage of the β-chain by crude preparations of C4bIBA, released a fragment from the β-chain of $M_r < 20\,000$ [8]. This attack can be accounted for by proteases, e.g., trypsin or plasmin, as it is quite in accordance with our results obtained in a purified system of C4 and trypsin. The remaining product of the β-chain has a calculated M_r of 61 500.

The cleavage of C4bINA and C4bp has been stated to give rise to 2 [8] or 3 [9] fragments from the α-chain of C4b with different M_r-values. In [10] the α′-chain was found to be first split by C4bINA and C4bp to a small fragment of M_r 16 000 and a large fragment the M_r of which (73 000) was very close to that of the β-chain. Secondary cleavage of the large fragment yielded an M_r 45 000 fragment (C4d), and an M_r 27 000 fragment of the α′-chain which together with intact β- and γ-chains constitutes C4c in addition to the M_r 16 000 fragment. These data are partly in agreement with ours. The initial attack by trypsin was directed at the α-polypeptide chain, resulting in an α-chain which rendered the molecule hemolytically inactive but still able to form a complex with C2. Further trypsin treatment gave an α″-chain (M_r 60 500), as evaluated by autoradiography of [^{14}C]methylaminated C4. A very low concentration of trypsin released an intermediate fragment of the α-chain of $M_r \sim 66\,000$. Cleavage of the β-chain occurred only when the α-chain was almost totally degraded. This parallelled the lost ability of C4 to complex with C2, as shown by scanning of the stained gel strips.

Degradation for 2 h gave a radiolabelled fragment of M_r 28 000, a fragment not linked by disulphide bridges to the β- and γ-chains [15]. This fragment elicited by trypsin resists further degradation by proteolytic enzymes, is micro-heterogeneous, has an α-mobility on immunoelectrophoresis and contains a thiol ester [20]. Similar data have been reported for C4d [9], and probably the C4d fragment of M_r 45 000 is rapidly split by trypsin to a lower M_r of 28 000.

Trypsin cleavage of human C4 has been described in [21]. The breakdown pattern as evaluated by polyacrylamide gel electrophoresis [21] is in accordance with that observed here in that the α-chain was readily cleaved (at room temperature), while the β-chain was not totally degraded after 1 h. The calculated M_r of β′, the main breakdown product of the β-chain, was 66 000 and compares to our value of 60 500. In [21] the γ-chain was reported to remain intact after 60 min trypsin treatment, which is contrary to our result. A possible explanation for the discrepancy is that the band seen after 60 min of degradation is not an intact γ-chain but an α-chain fragment of M_r 28 000, i.e., very close to the M_r of the γ-chain.

Native C4 and C2 tend to interact in free solution to form a reversible protein–protein complex [11] which is converted into a stable complex by the action of C1 esterase. These molecular complexes between C4 and C2 are precursors of an enzyme which is generated through the action of C1 esterase. Our studies were undertaken to determine whether C4, when partly broken down by trypsin, was still able to form a complex with C2. We found that neither an intact α- nor an intact α′-chain was a prerequisite for complex formation with C2 but that a non-digested β-chain was required. As soon as the β-chain starts to proteolyse, the complex formation between C4 and C2 decreases. It is an open question [10] whether a nicked form of C4b (C4b′) is able to bind C2. It is quite obvious, however, that the tryptic fragment of M_r 28 000 is not responsible for the binding of C2. This fragment is present after 2 h trypsinization, when no C2 binding takes place. The fact that a partly degraded C4 is still able to form a com-

plex with C2 and is perhaps able to generate C3 convertase may be of physiological importance. We have shown that methylamine-treated C4, although hemolytically inactive, can form a fluid phase convertase with C2 and C1, and most probably the same is true for partially degraded C4.

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