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Disulfide bridges in human complement component C3b

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The disulfide bridges of human complement component C3b, derived from C3 by removal of the 77-residue C3a, have been determined. The 10 bridges are Cys^{537}–Cys^{794}, Cys^{605}–Cys^{640}, Cys^{851}–Cys^{1491}, Cys^{1079}–Cys^{1136}, Cys^{1336}–Cys^{1467}, Cys^{1367}–Cys^{1436}, Cys^{1484}–Cys^{1489}, Cys^{1496}–Cys^{1568}, Cys^{1515}–Cys^{1639}, and Cys^{1615}–Cys^{1624}. Including the 3 bridges in C3a (Cys^{670}–Cys^{698}, Cys^{672}–Cys^{705}, and Cys^{685}–Cys^{706}) previously determined by high-resolution X-ray crystallography [Hoppe-Seyler's Z. Physiol. Chem. 361 (1980) 1389–1399] all disulfide bridges of C3 are localized. C3 and the strongly related C4 and C5 are members of the α_2-macroglobulin superfamily. The predicted bridge patterns of C4 and C5 are discussed and compared with that of α_2-macroglobulin.

Plasma protein; Complement C3; Disulfide bridge; Sequence homology; α_2-Macroglobulin superfamily

1. INTRODUCTION

The third component of human complement, C3, consists of a 75-kDa β-chain disulfide bridged to a 115-kDa α-chain [1]. C3 plays an important effector role in the immune response, and it is operative at a point where the classical and alternative pathways converge. It is activated by the physiological complex proteinases C4bC2a or C3bBb [2]. This activation results in the formation of fragments C3a and C3b. C3a consists of the N-terminal 77 residues of the α-chain [1,2]. The large C3b fragment binds covalently to biological targets, e.g. membrane constituents and to antibody through a transacylation reaction effected by an activated internal β-Cys-γ-Glu thiol ester located in the α-chain [3–5]. C3b can be cleaved by several proteinases in its α'-chain resulting in the major fragments C3c and C3d [6]. Different parts of the C3 structure can be recognized by specific receptors, which are important for the regulation of complement activity [7].

C3 and the related complement components C4 and C5 are members of the α_2M superfamily [8]. The amino acid sequences of human C3 [9], C4 [10], C5 [11], and α_2M [12] are known, but the complete disulfide bridge pattern is only known for α_2M [12,13]. From the high resolution X-ray structure of C3a its three disulfide bridges have been determined [14], but only limited information on the location of the remaining bridges of C3 is available from partial reduction experiments [15,16].

Here we report the location of the 10 disulfide bridges in human C3b. The putative arrangement of the disulfide bridges in the related proteins C4 and C5 is discussed, and their bridge patterns are compared with that of human α_2M.

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Abbreviations: C3(MA), C3 treated with methylamine and iodoacetamide; α_2M, α_2-macroglobulin; HPLC, high-performance liquid chromatography; RP, reverse phase; SCX, strong cation exchange; TFA, trifluoroacetic acid; PTH, phenylthiohydantoin.

2. MATERIALS AND METHODS

2.1. *Proteins*

Human C3 was prepared from the 3–10% polyethylene glycol precipitate obtained from fresh pooled plasma essentially according to ref. [1]. The final purification step consisted of chromatography on Q Sepharose FF (load 75 mg, column size 1 × 30 cm, 20 mM Na-phosphate, pH 7.4, 50–500 mM gradient of NaCl). By sodium dodecyl sulfate gel electrophoresis only bands corresponding to the α- and β-chains of C3 were seen as confirmed by sequence analysis of electroblotted samples. C3 was treated with methylamine to cleave the internal thiol ester [4] and the SH group blocked by reaction with iodoacetamide. C3b(MA) was prepared from C3(MA) by incubation with 1/100 (w/w) trypsin for 10 min at 37°C followed by chromatography on Q Sepharose FF as above. Tosyl-phenylalanine chloromethylketone-treated bovine trypsin was from Cooper Biomedicals. *Staphylococcus aureus* V8 proteinase was from Miles. Porcine pepsin was from Worthington.

2.2. *Column materials and chemicals*

Sephadex G-50 SF, Superose 12, Q and S Sepharose FF were from Pharmacia. 4 × 250 mm columns were packed with PolySULFOETHYL-A, 5 μm from PolyLC Inc, and with Nucleosil 100-5 C18 from Machery-Nagel. Standard chemicals were of analytical grade from Merck and Sigma.

2.3. *Separation of peptides by HPLC*

Pools containing peptides judged to be less than approx. 50 residues in length were separated on polySULFOETHYL-A using a phosphoric acid/NaCl/acetonitrile based SCX-HPLC system [17,18]. Selected pools were further separated by RP-HPLC on a Nucleosil C18

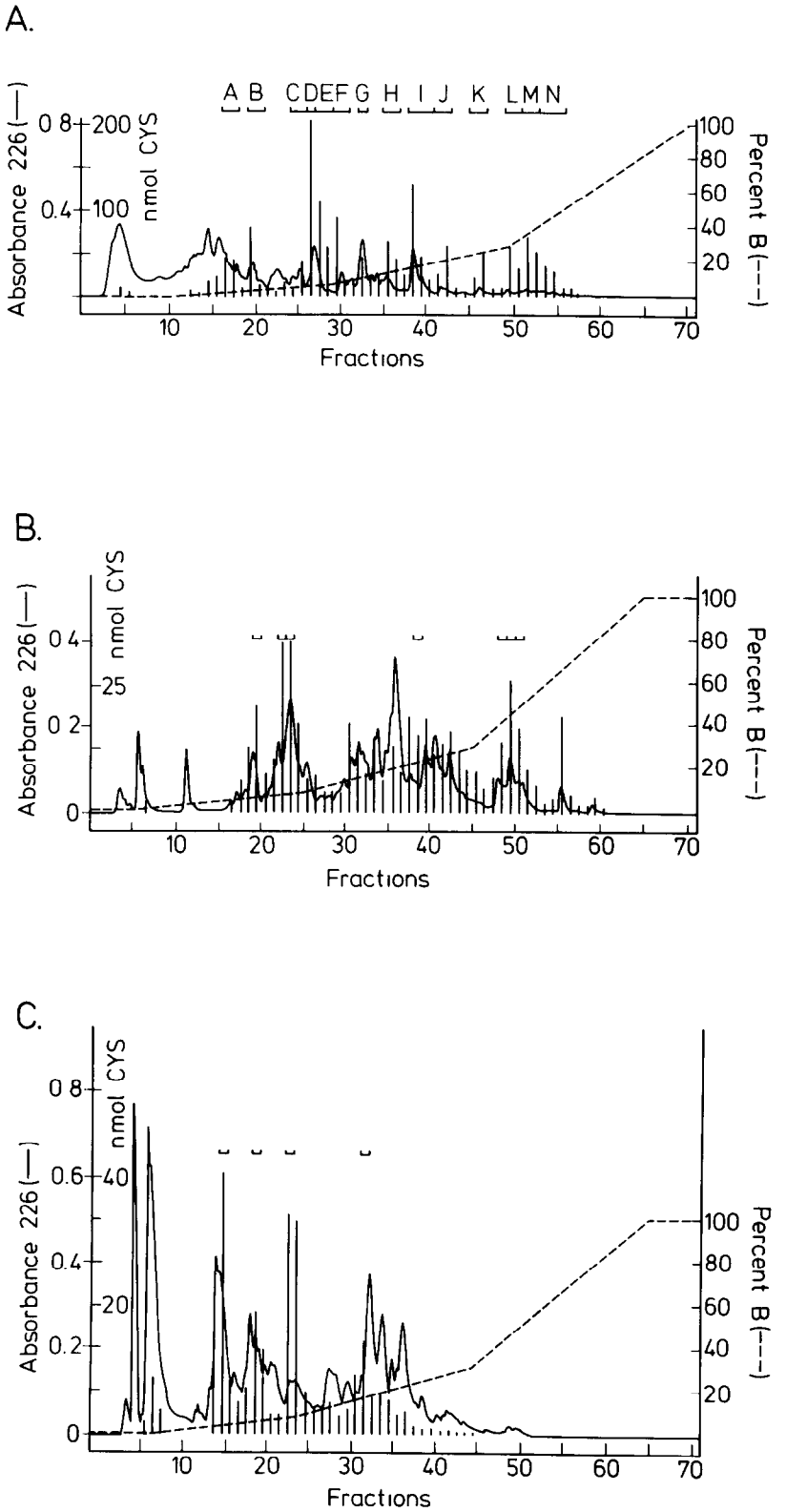

Fig. 1. SCX-HPLC separations of CNBr/tryptic peptides from C3b(MA) (panel A) and CNBr/peptic peptides from C3(MA) (panels B and C). A 4 × 250 mm column packed with polySULFOETHYL-A was equilibrated with 5 mM phosphoric acid/10 mM NaCl/25% acetonitrile and eluted at 40°C with a gradient of NaCl (----) (100% B corresponds to 1 M NaCl in the solvent). The flow rate was 1 ml/min, and the separations were monitored by recording A_{226} (—) and by determining the amount of halfcystine in each fraction (vertical bars). The separation shown in panel A represents 1/2 of the Superose 12 pool, whereas those shown in panels B and C represent 1/6 of the Sephadex G50 SF pools. The pools/fractions further separated by RP-HPLC (1/4 to 1/2 used) are marked by horizontal bars.

column eluted at a flow rate of 1 ml/min with a linear gradient formed from 0.1% TFA (A) and 90% acetonitrile containing 0.075% TFA (B) (10–60% B). The gradient was developed over 25 min, and the separations at 50°C were monitored by recording the absorbance at 226 nm. Disulfide-bridged peptides in the effluents were located by determining the content of Cya in performic acid oxidised samples or by RP-HPLC of reduced and carboxymidomethylated samples (2 mM dithiothreitol for 15 min at pH 9.0 followed by 5 mM iodoacetamide for 30 min).

2.4. *Amino acid analysis*

Peptides were hydrolyzed with 6 M HCl/1% thioglycolic acid for 20 h at 110°C in evacuated sealed tubes or in the gas phase using an evacuated desiccator. Most samples were analyzed after treatment with 1 M performic acid for 30 min at room temperature. Free amino acids were separated by SCX-HPLC on a 4 × 125 mm Mitsubishi CK10U column using a pH gradient ranging from 3.10 (Na-citrate/sulfate) to 10.10 (Na-borate) essentially as described [19].

2.5. *Sequence analysis*

Edman degradations were done for 4–10 cycles in an Applied Biosystems 477A sequenator equipped with an 120A on-line HPLC using polybrene-coated glass-filters and Normal-1 cycles. Initial yields were 100–500 pmol.

2.6. *Experimental details*

Digest 1: 58 mg C3b (MA) was degraded with CNBr in 70% formic acid and the digest separated on a 1 × 10 cm S Sepharose FF column eluted with a 0–1 M gradient of NaCl in 10 mM ammonium acetate, 6 M urea, pH 4.5. No significant fractionation occurred, and 4.5 ml portions of the material in the main irregular peak eluting at 0.43–0.78 M NaCl was loaded onto a 160 ml Superose 12 column eluted with 25% formic acid (10 runs, not shown). The material eluting at 0.32–0.50 bed vols. containing more than 90% of Cys_2 present was pooled, freeze-dried, dissolved in 0.1% TFA, made 0.1 M in ammonium bicarbonate, and digested with 120 μg trypsin for 2 h at 37°C. Aliquots of the digest were separated by SCX-HPLC, and pools A–N (Fig. 1A) were further purified by RP-HLC (not shown).

Digest 2: 59 mg C3 (MA) was dissolved in 0.1 M formic acid and digested with 1 mg pepsin for 3 h at 37°C. After freeze-drying the material was treated with CNBr in 70% formic acid and the peptides separated on a 180 ml column of Sephadex G 50 SF eluted with 1 M formic acid (not shown). More than 95% of Cys_2 present was found in effluent at 0.25–0.80 bed vols. Aliquots of two pools containing relatively small peptides eluting at 0.52–0.67 bed vols (pool 1), and 0.68–0.80 bed vols. (pool 2) were separately subjected to SCX-HPLC. Individual fractions from these separations (marked in Fig. 1B and C) were further separated by RP-HPLC (not shown).

3. RESULTS

Since the sequence of C3 is known [9], the main strategy for locating its disulfide bridges consisted of compositional analysis and partial sequence analysis of peptides having intact bridges. In a few cases the mates of bridged peptides were characterized after reduction and alkylation.

To avoid thiol–disulfide exchange reactions resulting from thiol ester cleavage C3 was treated with methylamine, forming C3 (MA), and the SH-group appearing [4] blocked with iodoacetamide. Since the bridges of the C3a portion were already known [14], C3b (MA) was used as starting material for the first digest. Degradation with CNBr followed by digestion with trypsin would be advantageous, since it could be expected that most bridged peptides would contain only 1 bridge. The CNBr fragments were subjected to cation-exchange chromatography followed by gel chromatography under denaturing conditions to possibly obtain larger sets of bridged fragments which could be further digested. However, no significant separation of such fragments was obtained. The main pool obtained was then digested with trypsin and the peptides separated by SCX-HPLC at pH 3.0 (Fig. 1A) followed by RP-HPLC.

Due to the complexity of the digest only 6 bridges could be located, and therefore a second digest was investigated. In this, C3 (MA) was degraded with pepsin followed by digestion with CNBr. The bridges not found in the first digest were planned to be present in relatively small peptides in the second digest. Therefore, the peptides were fractionated by gel chromatography on Sephadex G50 SF, and two pools containing small peptides were further separated by SCX-HPLC (Figs. 1B and C) and RP-HPLC. The peptide pools of the digest were examined until the remaining 4 disulfide bridges were found.

As summarized in Table I the minimal evidence for locating the 10 disulfide bridges in C3b was provided by 10 unique peptides or mates hereof. Most bridged peptides, in particular those derived from peptic digestion, were recovered as several cleavage variants which were also characterized (not shown).

The evidence for locating the bridges in peptides 2, 5, 6, 7 and 10 was obtained from analysis of the intact peptides. The disulfide bridges in peptides 2, 5 and 6 connected two mates, while peptides 7 and 10 each contained an internal bridge. Peptides 1, 4 and 9 were slightly impure, and the evidence for locating their bridges was obtained after separating their reduced and carboxamidomethylated mates by RP-HPLC. Peptides 3 and 8 were obtained by subdigesting a peptide set, which by compositional analysis was shown to contain the stretch C^{1491} AEENCFIQK connected to 2 other peptides, with *S. aureus* V8 proteinase followed by separation of the 2 peptide sets by RP-HPLC. Both peptides contained a bridge connecting two mates.

In the Sephadex G 50 SF gel chromatography used in digest 2 a large cluster of peptic peptides eluting at 0.25–0.51 bed vols. and containing the 3 bridges in the C3a portion was obtained (not shown). As the location of these bridges was already determined from X-ray crystallography [14] that material was not further investigated.

4. DISCUSSION

This work provides unequivocal evidence for the location of the 10 disulfide bridges in C3b. It identifies the single bridge in C3d and the bridges inferred from partial reduction experiments to connect the α- and β-chains of C3, and the N- and C-terminal pieces of the cleaved α-chain of C3c [15,16]. The recent incomplete

Table I

Summary of the evidence for the location of the 10 disulfide bridges in human C3b[a].

Peptide 1: Cys^{537}–Cys^{794} (Fig. 1C, fraction 32, 54% B, 34% B and 38% B, 1.1 nmol)[b]

Mate 1: $C_{08(1)},B_{21(2)},S_{18(2)},G_{12(1)},V_{29(3)},L_{10(1)},K_{15(1)}$[c,d]

Mate 2: $C_{08(1)},B_{21(2)},S_{08(1)},Z_{10(1)},P_{10(1)},G_{12(1)},A_{12(1)},V_{11(1)},I_{08(1)},F_{10(1)},K_{26(2)}$[c,d]

Sequence, mate 1: V^{531}DVK(DSCVGSL)[c]

Sequence, mate 2: S^{788}DKK(GICVADPFE)[c]

Peptide 2: Cys^{605}–Cys^{640} (Fig. 1A pool B, 39% B, 3.5 nmol)[b]

Intact peptide: $C_{20(2)},B_{33(3)},T_{36(4)},S_{43(5)},Z_{57(6)},P_{28(3)},G_{55(6)},A_{65(7)},V_{09(1)},I_{10(1)},L_{20(2)},Y_{09(1)},F_{19(2)},K_{10(1)},R_{10(1)}$[c,e]

Sequence: A^{601}DIGC(TPGSGKDYAGVFSDAGLTFTSSSGQQTAQR)[c,f,g]
A^{636}ELQC(PQPAA)[c,f,g]

Peptide 3: Cys^{851}–Cys^{1491} (Fig. 1A pool K, 38% B, 36% B, 1.8 nmol)[b]

Intact peptide: $C_{21(2)},B_{10(1)},T_{20(2)},S_{09(1)},Z_{19(2)},P_{09(1)},A_{27(3)},L_{30(3)},F_{10(1)},H_{12(1)},K_{11(1)}$[c,e]

Sequence: L^{844}LHNP(AFCSLATTK)[c,g]
X^{1491}AEE[h]

Peptide 4: Cys^{1079}–Cys^{1136} (Fig. 1B fraction 24, 48% B, 46% B and 44% B, 0.6 nmol)[b]

Mate 1: $C_{08(1)},B_{10(1)},S_{15(1)},Z_{12(1)},G_{14(1)},A_{21(2)},V_{19(2)},I_{18(2)},L_{11(1)},K_{08(1)}$[c,d]

Mate 2: $C_{08(1)},B_{33(2)},T_{101(1)},S_{29(3)},Z_{50(5)},P_{13(1)},G_{25(2)},A_{25(2)},V_{12(1)},I_{29(3)},L_{23(2)},K_{23(2)}$[c,d]

Sequence, mate 1: I^{1071}AIDS(QVLCGAVKW)[c]

Sequence, mate 2: I^{1125}SLQE(AKDICEEQVNSLPGSITKAG)[c]

Peptide 5: Cys^{1336}–Cys^{1467} (Fig. 1A pool I, 36% B, 5.0 nmol)[b]

Intact peptide: $C_{21(2)},B_{44(4)},T_{17(2)},S_{07(1)},Z_{29(3)},A_{19(2)},V_{09(1)},L_{30(3)},Y_{27(3)},F_{10(1)},K_{31(3)},R_{10(1)}$[c,e]

Sequence: A^{1330}KDQL(TCNKFDLK)[c,g]
V^{1457}YAYY(NLEESCTR)[c,g]

Peptide 6: Cys^{1367}–Cys^{1436} (Fig. 1A pool H, 38% B, 0.5 nmol)[b]

Intact peptide: $C_{15(2)},B_{19(2)},T_{10(1)},S_{17(2)},Z_{20(2)},A_{11(1)},V_{11(1)},I_{17(2)},L_{21(2)},F_{10(1)},H_{11(1)},K_{11(1)},R_{11(1)}$[c,e]

Sequence: I^{1363}LEIX(TR)[c,g,h]
V^{1429}SHSE(DDCLAFK)[c,g]

Peptide 7: Cys^{1484}–Cys^{1489} (Fig. 1A pool D, 36% B, 2.2 nmol)[b]

Intact peptide: $C_{20(2)},B_{10(1)},Z_{10(1)},L_{20(2)},R_{20(2)}$[c,e]

Sequence: L^{1483}XRDELCR[h,i,j]

Peptide 8: Cys^{1496}–Cys^{1568} (Fig. 1A pool K, 38% B, 16% B, 1.8 nmol)[b]

Intact peptide: $C_{21(2)},B_{12(1)},Z_{11(1)},I_{10(1)},F_{10(1)},K_{09(1)},R_{10(1)}$[c,e]

Sequence: N^{1495}CFIQK[g,k]
X^{1568}R[g,h]

Peptide 9: Cys^{1515}–Cys^{1639} (Fig. 1C fraction 23, 40% B, 28% B and 34% B, 6.7 nmol)[b]

Mate 1: $C_{10(1)},B_{21(2)},Z_{29(3)},P_{11(1)},G_{10(1)},A_{10(1)},V_{10(1)},L_{11(1)},K_{11(1)},R_{10(1)}$[c,d]

Mate 2: $C_{09(1)},B_{10(1)},P_{10(1)},G_{12(1)},V_{10(2)},F_{10(1)}$[c,d]

Sequence, mate 1: E^{1508}ERLD(KACEPGVD)[c,g]

Sequence, mate 2: V^{1635}VFGX(PN)[c,g,h]

Peptide 10: Cys^{1615}–Cys^{1624} (Fig. 1C fraction 15, 40% B, 7.5 nmol)[b]

Intact peptide: $C_{1\,8\,(2)}$,$B_{4\,0\,(4)}$,$Z_{8\,8\,(10)}$,$P_{1\,2\,(1)}$,$G_{1\,2\,(1)}$,$A_{1\,0\,(1)}$,$V_{1\,1\,(1)}$,$L_{1\,0\,(1)}$,$H_{1.0\,(1)}$,$K_{0\,9\,(1)}$[c,e]

Sequence: V^{1606}EHWP (EEDECQDEENQKQCQDLGA)[c,j]

[a] The numbering of proC3 [9] is used.
[b] The data in brackets show in succession the SCX-HPLC fraction, the percentage of solvent B required in RP-HPLC to elute the peptide with intact bridge, and the mates 1 and 2 after reduction and carboxamidomethylation or after subdigestion; the actual yield of intact peptide is listed.
[c] The numbers and residues in brackets show the expected numbers of residues and their sequences.
[d] Cys was determined as *S*-carboxymethyl-Cys.
[e] Cys was determined as Cya.
[f] *Bis*-PTH-Cys_2 eluting close to PTH-Tyr was seen in cycle 5.
[g] The appearance of 2 PTH-derivatives per cycle was consistent with the partial sequences shown.
[h] X indicates a Cys-residue. No PTH-derivative was seen in this cycle, consistent with the disulfide pairing.
[i] A small amount of *bis*-PTH-Cys_2 was seen in cycle 7.
[j] Since only 1 sequence was seen the bridge is internal in the peptide.
[k] *Bis*-PTH-Cys_2 was seen in cycle 2.

description of the bridges in C3 [23] is consistent with the results described here. Together with previous cDNA analysis [9], localization of an internal thiol ester [4], determination of 3 bridges in C3a [14], structure determination of high-mannose-type carbohydrate groups [20,21] attached to Asn^{63} and Asn^{917} [22] the primary structure determination of C3 is completed.

The disulfide bridges of C3 are shown schematically in Fig. 2. For a protein the size of C3 their number is strikingly low, and moreover, two segments, the C3a portion and the C-terminal approx. 300 residues of the α-chain contain several clustered bridges, while the β-chain only contains 1 intrachain bridge.

The complement proteins C4 and C5 are homologous with C3 [9–11] and have many halfcystine residues in common with C3. Hence, their bridge patterns are likely to be similar to that of C3 as indicated in Fig. 2. However, they differ subtly, since C4, relative to C3, is likely to have a unique bridge in the N-terminal part and also has an unpaired Cys residue at position 1103, and since C5 has an unpaired Cys residue in the C5a part, and likely a bridge in the stretch around residue 850 (Fig. 2).

Curiously, while C3, C4, and C5 are homologous with the α_2M subunit [8], and while all these proteins probably contain many domains of similar gross conformation, the bridge pattern of the α_2M subunit is not similar to that of the complement proteins (Fig. 2), except for the presence of a short bridge just before the activation cleavage regions (the α_2M bait region), a bridge spanning several hundred residues and a bridge spanning appr. 130 residues in the C-terminal part. However, the C-terminal approx. 150-residue stretches of C3, C4, and C5, which represent extensions relative to α_2M, are unique and contain a conserved and intricately bridged domain.

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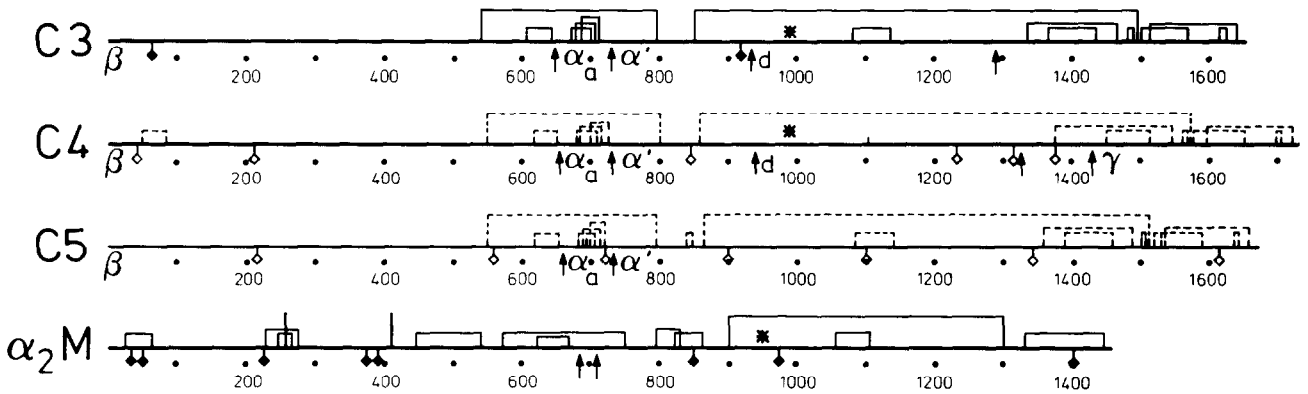

Fig. 2. Schematic representation of the structure of C3 showing the location of the disulfide bridges determined here and before (C3a portion [14]). The putative disulfide bridge patterns of C4 and C5 are shown as stippled lines. The bridges in α_2M [12,13] are shown. The 2 halfcystine residues at position 255 and 408 in α_2M form interchain bridges in the α_2M half-molecule [13]. The α-, α'-, β-, (C3, C4, C5) and γ-chains (C4) are labeled and delimited by arrows. The approx. 77-residue C3a, C4a, and C5a parts, and the approx. 400-residue C3d and C4d parts are labeled (a) and (d), respectively, and delimited by arrows. C3c and C4c (not shown) are multiple chain structures formed from C3b and C4b by excision of the C3d and C4d parts [6]. The multiple cleavage sites in the α_2M bait region [12] are indicated by 2 arrows. The thiol ester site in C3, C4, and α_2M is labeled with an asterisk. The location of carbohydrate groups in C3 [22] and α_2M [12] is shown by filled diamonds. Potential carbohydrate attachment sites in C4 [10] and C5 [11] are shown by open diamonds.

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