Amino acid sequence of the trypsin-generated C3d fragment from human complement factor C3

Ulf HELLMAN,† Gösta EGGERTSEN,* Åke ENGSTRÖM† and John SJÖQUIST*

*Department of Medical and Physiological Chemistry, Box 575, and †Department of Immunology, Box 582, Biomedical Center, University of Uppsala, 751 23 Uppsala, Sweden

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Human C3d (try-C3d), prepared from trypsin-digested C3, was fragmented by cleavage with CNBr. Eight peptides were defined and separated by h.p.l.c. on reversed-phase columns. By automatic Edman degradation the complete sequences of five peptides and partial sequences of three peptides were determined. To obtain overlapping peptides the latter three fragments were digested with trypsin, chymotrypsin or *Staphylococcus aureus* V8 proteinase, after which the fragments were separated on reversed-phase columns. Two of the CNBr-cleavage peptides were completely sequenced, and 70% of the sequence of the remaining CNBr-cleavage peptide was determined. The non-sequenced part represents a very hydrophobic segment of try-C3d. The sequence data obtained represent 90% of the primary structure of try-C3d. Alignment of the CNBr-cleavage fragments was made easier by comparison with the cDNA sequence of mouse pro-C3 [Wetsel, Lundwall, Davidson, Gibson, Tack & Fey (1984) *J. Biol. Chem.* **259**, 13857–13862]. Comparison of try-C3d with the equivalent part of human C4B revealed an extensive sequence homology in the *N*-terminal half of the molecules.

Complement factor C3 is a key factor of the complement cascade, forming a link between the classical pathway and the alternative pathway. C3 consists of two disulphide-linked polypeptide chains, α and β , with M_r values of 125000 and 75000 respectively (Bokisch *et al.*, 1975; Nilsson *et al.*, 1975). C3 is activated by proteolytic liberation of a peptide of 77 amino acids (C3a) from the *N*-terminal end of the α -chain. In the activated state (C3b) it is able to bind covalently to various structures via a labile thioester group in the α -chain, formed between a cysteine and a glutamic acid residue (for review see Lachmann & Peters, 1983). A similar labile binding site is also found in complement factor C4 and in α_2 -macroglobulin, and the amino acid sequence around the thioester group is highly conserved in all three proteins (Swenson & Howard, 1979; Tack *et al.*, 1980; Campbell *et al.*, 1981). Limited proteolysis of C3 with, for example, trypsin or elastase generates the fragments C3c (M_r approx. 140000) and C3d (M_r

25000–34000), of which the latter contains the thioester group (Bokisch *et al.*, 1975; Taylor *et al.*, 1977; Eggertsen *et al.*, 1985). C3d can also attach itself to cell receptors for complement (for review see Fearon & Wong, 1983), and has also been claimed to contain the Factor H-binding site (Burger *et al.*, 1982). The *N*-terminal sequence of human C3d prepared from elastase-digested C3 (Thomas *et al.*, 1982), and from trypsin-digested C3 (Eggertsen *et al.*, 1985) has been determined, but no further information on the sequence has been reported. The present study was undertaken to determine the primary structure of human C3d, prepared from trypsin-digested C3.

Materials and methods

Materials

The following materials were purchased: Sephadex G-75 (superfine grade) and Sephadex G-50 superfine grade (Pharmacia Fine Chemicals, Uppsala, Sweden); L-1-chloro-4-phenyl-3-tosylamido-butan-2-one (TPCK-) treated trypsin (Worthington Biochemical Corp., Freehold, NJ, U.S.A.); *Staphylococcus aureus* V8 proteinase (Miles Laboratories, Stoke Poges, Berks., U.K.); chymotrypsin

† To whom correspondence should be sent, at present address: Pharmacia AB, Division of Biotechnology, Research and System Development, S-751 82 Uppsala, Sweden.

from bovine pancreas and soya-bean trypsin inhibitor (Sigma Chemical Co., St. Louis, MO, U.S.A.); di-isothiocyanate (LKB, Bromma, Sweden); controlled-pore glass (CPG 10; pore size 7.5 nm (Serva Feinbiochemica, Heidelberg, Germany)); iodo[^{14}C]acetamide (13.2 mCi/mmol) (New England Nuclear, Boston, MA, U.S.A.). Chemicals for solid-phase and liquid-phase sequencing (Rathburn Chemicals, Walkerburn, Peeblesshire, Scotland, U.K.); chemicals for gas-phase sequencing (Applied Biosystems, Foster City, CA, U.S.A.).

Human C3 and C3d

Human C3 was prepared from fresh-frozen plasma as described by Lundwall *et al.* (1984). Tryptic C3d (try-C3d) was purified from methylaminated and trypsin-digested C3 (Eggertsen *et al.*, 1985). Briefly, C3 was methylaminated as described by Lundwall *et al.* (1982) and subjected to limited proteolysis by trypsin for 3 h at 37°C, after which the digest was applied to a DEAE-Sephrose column and try-C3d was eluted with a linear salt gradient.

Preparation of CNBr-cleavage fragments from try-C3d

Reduction and carboxymethylation with iodo[^{14}C]acetamide of try-C3d (15–20 mg) was performed as described previously (Eggertsen *et al.*, 1981). After dialysis against 1.0 M-acetic acid, the material was freeze-dried and CNBr-cleavage fragments were prepared by the procedure of Gross (1967) with 75-fold molar excess of CNBr over methionine residues in 70% (v/v) formic acid at room temperature for 24 h. The sample was diluted with 20 vol. of distilled water and freeze-dried. The material was dissolved in 6 M-guanidinium chloride to a concentration of 10 mg/ml and separated by h.p.l.c. on a ProRPC HR5/10 column (see below). The M_r values of CNBr-cleavage fragments were determined by gel filtration on a Sephadex G-75 (superfine grade) column (1 cm $\times$ 135 cm) in 1.0 M-acetic acid.

Proteolytic digestion of CNBr-cleavage fragments

The specimens were dissolved in 60 μl of 8 M-urea in 0.1 M- NH_4HCO_3 , and then diluted with 180 μl of 0.1 M- NH_4HCO_3 containing trypsin to give a final enzyme concentration of 1 unit/6–10 nmol of CNBr-cleavage peptide. Digestion was carried out at 37°C for 3–6 h, and stopped by the addition of a double-weight amount of soya-bean trypsin inhibitor over trypsin. Insoluble material was removed by centrifugation. Digestion of CNBr-cleavage fragments with chymotrypsin and *Staphylococcus aureus* V8 proteinase was performed essentially as for trypsin. However, to increase solubility the specimens for chymotryptic

digestion were dissolved in a small amount of 100% trifluoroacetic acid which was then evaporated over KOH. Chymotrypsin was added in an enzyme/substrate ratio of 1/100 (w/w) and *Staphylococcus aureus* V8 proteinase in a ratio of 1/50 (w/w).

Separation of peptides by h.p.l.c.

Peptide fractionation was carried out on reversed-phase columns, either a ProRPC HR5/10 column (5 mm $\times$ 100 mm) (Pharmacia Fine Chemicals, Uppsala, Sweden) or a C18 column (8 mm $\times$ 100 mm) (Waters Radial Compression Separation System). Fractionations were performed in aq. (v/v) 0.16% trifluoroacetic acid, with a linear 0–60% (v/v) gradient of acetonitrile containing 0.10% (v/v) trifluoroacetic acid, pH 2.1 (designated solvent B). Incompletely separated peptides were subjected to chromatography on the same columns in 25 mM-ammonium acetate buffer, pH 6.5, with a linear 0–50% (v/v) gradient of acetonitrile. The columns were operated with a flow rate of 1.0 ml/min at room temperature, and the column effluent was monitored at 220 nm. Amino acid analysis and, when needed, automatic Edman degradation were performed to identify the various fragments.

Amino acid analysis and sequence determination

Amino acid analysis was performed on a Beckman 121M amino acid analyser as described by Spackman *et al.* (1958). Automatic Edman degradation on solid phase was performed on an LKB 4020 solid-phase peptide sequencer in accordance with the manufacturer's instructions except that 25 mM-triethylamine in 100% methanol replaced the recommended buffer. CNBr-cleavage peptides with a C-terminal homoserine residue were immobilized on aminopropyl-glass by the method described by Horn & Laursen (1973). Peptides without C-terminal homoserine were either coupled to di-isothiocyanate-glass (Laursen *et al.*, 1972) or immobilized on aminopropyl-glass by carbodi-imide as described by Salnikow *et al.* (1981). For extended runs automatic Edman degradation in liquid phase with Polybrene (Tarr *et al.*, 1978) was utilized, performed in a Beckman 890C liquid-phase sequencer equipped with a cold trap, with the use of a programme described by Thomsen *et al.* (1976). Certain runs were performed on a gas-phase sequencer (Applied Biosystems) with the use of the PROTFA programme with minor modifications. The amino acid phenylthiohydantoin derivatives were identified by h.p.l.c. on a 5 μm -particle-size Nucleosil ODS column (4 mm $\times$ 300 mm) in 6 mM-sodium acetate buffer, pH 4.9, with a gradient of acetonitrile at 37°C at a flow rate of 0.9 ml/min.

Results

Isolation of CNBr-cleavage peptides of Try-C3d

The chromatogram of CNBr-degraded try-C3d on the ProRPC HR5/10 column is shown in Fig. 1. Appropriate fractions were subjected to amino acid analysis and *N*-terminal sequence determination. In this way seven fragments were defined, designated CN2, CN4, CN6, CN7, CN8x, CN8y and CN8z. The amino acid compositions and yields of the peptides are shown in Table 1. Peptide CN7 lacked homoserine and was considered to represent the C-terminal peptide. Peptides CN2, CN6 and CN8x were radioactively labelled, and amino acid analysis showed that these fragments contained one cysteine residue each. The CNBr-cleavage fragments could also be prepared by gel filtration on Sephadex G-75 (superfine grade) in 1.0M-acetic acid (Fig. 1 inset). The M_r values were assessed to be 12700 (peptide CN2), 6800 (peptide CN4), 4000 (peptide CN6), 2900 (peptide CN7) and 1800 (peptides CN8x, CN8y and CN8z). However, the recovery of peptides CN2, CN4 and CN6 decreased markedly when more than 50nmol of digested try-C3d was applied,

since precipitation of these fragments occurred on the column. Peptides CNBx, CN8y and CN8z were eluted together, but could be separated by h.p.l.c. on C18 or ProRPC columns (results not shown).

Sequence analysis of CNBr-cleavage peptides of try-C3d

Peptides CN7, CN8x, CN8y and CN8z. These peptides could be completely sequenced without fragmentation (Fig. 3). The sequence of peptide CN8x corresponded to the *N*-terminal sequence of try-C3d as reported by Eggertsen *et al.* (1985), and the thioester-forming residues were located at positions 9 and 12.

Peptide CN6. The *N*-terminal 27 amino acid residues of peptide CN6 were determined by solid-phase sequence analysis (Fig. 3, residues 141–167). Peptide CN6 was subjected to trypsin digestion, and the fragments were separated on the C18 column (Fig. 2a). Three fragments (T1₆–T3₆) were identified by amino acid analysis, and the complete sequences of fragments T1₆ and T2₆ were determined. Fragment T3₆ (14 residues) represent-

Fig. 1. Separation of CNBr-cleavage peptides from try-C3d

The CNBr digest of try-C3d (29nmol) was dissolved in 6M-guanidinium chloride and applied to a ProRPC HR5/10 column (5mm × 100mm), equilibrated with aq. 0.16% (v/v) trifluoroacetic acid. Solvent B was 100% acetonitrile containing 0.1% (v/v) trifluoroacetic acid. The flow rate was 1.0ml/min, and 1.0ml fractions were collected. The straight line represents the concentration of solvent B. —, A_{220} . The inset shows the fractionation of CNBr-cleavage peptides from try-C3d (170nmol) by gel filtration on a Sephadex G-75 column (1cm × 135cm) in 1.0M-acetic acid. The first peak represents the void volume, which contains incompletely digested material. The last peak indicates the total volume. Pooling was as indicated. —, A_{280} ; ·····, radioactivity.

Table 1. *Amino acid composition of CNBr-cleavage fragments of try-C3d and the non-sequenced segment of peptide CN2 (X₂)*
 Values for threonine and serine were corrected by using the recovery factors 0.96 and 0.89 respectively, except in peptide CN2, where extrapolation was performed from 24 h and 72 h hydrolysis. Tryptophan was determined after hydrolysis in 3M-toluene-*p*-sulphonic acid. Values within parentheses represent numbers of residues found in the sequence determination. Abbreviations: Cm, carboxymethyl; N.D., not determined.

Amino acid	Peptide ...	Amino acid composition (mol of residue/mol of peptide)							
		CN2	CN4	CN6	CN7	CN8x	CN8y	CN8z	X ₂
Trp		2.6	1.8 (2)	N.D.	N.D.	N.D.	N.D.	N.D.	1
Lys		8.3	5.8 (6)	2.1 (2)	1.1 (1)	—	1.0 (1)	0.3	0–1
His		1.9	0.2	—	0.9 (1)	1.0 (1)	—	0.2	—
Arg		3.2	3.7 (4)	0.4	1.3 (1)	—	1.0 (1)	0.9 (1)	1
Cm-Cys		1.1	—	0.9 (1)	—	0.7 (1)	—	—	—
Asx		6.3	8.0 (8)	4.0 (4)	3.7 (4)	1.0 (1)	4.0 (4)	1.0 (1)	3
Thr		6.1	5.0 (5)	2.1 (2)	0.5	1.0 (1)	—	0.9 (1)	2
Ser		5.3	2.3 (2)	3.2 (3)	2.2 (2)	1.1 (1)	—	1.0 (1)	3
Hse		0.6	0.4 (1)	0.3 (1)	—	0.3 (1)	0.4 (1)	0.3 (1)	—
Glx		17.7	7.7 (7)	5.6 (6)	5.6 (6)	1.8 (2)	1.1 (1)	2.3 (2)	2
Pro		5.0	3.7 (4)	1.2 (1)	1.8 (2)	0.8 (1)	—	0.4	1
Gly		6.7	6.2 (7)	2.4 (2)	0.7	2.0 (2)	2.1 (2)	0.9 (1)	0–1
Ala		12.0	5.4 (5)	3.8 (5)	3.2 (3)	—	—	3.2 (4)	4
Val		10.5	4.2 (4)	2.0 (2)	2.0 (2)	0.7 (1)	—	1.0 (1)	5
Ile		5.7	0.6	2.2 (3)	0.3	0.7 (1)	0.9 (1)	0.9 (1)	2
Leu		11.8	11.8 (11)	5.0 (5)	5.0 (5)	1.0 (1)	1.1 (1)	2.0 (2)	5
Tyr		3.1	5.0 (5)	1.2 (1)	1.4 (1)	—	—	2.0 (2)	1
Phe		5.8	4.0 (4)	1.7 (2)	0.8 (1)	—	—	—	1
Total no of residues:			(75)	(40)	(29)	(14)	(12)	(18)	30–33
Yield of peptide (%)		71	100	44	70	64	75	45	

ed the *N*-terminal end of peptide CN6, fragment T₂₆ (16 residues) was located immediately *C*-terminal to fragment T₃₆, and fragment T₁₆ (ten residues) contained homoserine, and consequently represented the *C*-terminal. The alignment T₃₆–T₂₆–T₁₆ was established by digestion of peptide CN6 with *Staphylococcus aureus* V8 proteinase, whereby an overlapping fragment (S₂₆, residues 153–176; Fig. 3) was obtained on the C18 column (results not shown). The total number of residues in peptide CN6 equalled 40.

Peptide CN4. Liquid-phase automatic Edman degradation was performed for 66 cycles (Fig. 3, residues 199–264), but residues 259, 261 and 263 could not be determined unambiguously. Trypsin digestion of peptide CN4 with subsequent fractionation of the digest on C18 yielded seven fragments, designated T₁₄–T₇₄ (Fig. 2b). Fragments T₃₄ and T₄₄ were eluted together, but were completely separated by rechromatography on the same column at pH 6.5. (results not shown). Homoserine was only found in fragment T₄₄, indicating that this fragment represented the *C*-terminal. The complete sequence of fragments T₆₄ and T₄₄ were determined. The first (Tyr), third (Gly) and fifth (Gly) residues in T₄₄ corresponded to residues 260 (Fig. 3), 262 and 264 respectively in the 66-cycle sequence of peptide CN4. The tryptic-digest peptide T₃₄ was found to be located within

positions 254–259 of peptide CN4. The localization of the other tryptic-digest fragments within peptide CN4 was estimated from their amino acid compositions. Alignment of the tryptic-digest fragments of peptide CN4 thus results in the following order:

Gly–Arg–T₅₄–T₂₄–Asp–Lys–Asn–Arg–T₁₄–T₇₄–T₆₄–T₃₄–T₄₄

The *N*-terminal dipeptide of peptide CN4 and the tetrapeptide located between fragments T₂₄ and T₁₄ (residues 215–218) were not recovered from the tryptic digest. Altogether peptide CN4 consisted of 75 amino acid residues.

Peptide CN2. A double sequence was found when peptide CN2 was subjected to gas-phase automatic Edman degradation, starting with Ile/Thr–Gly/Pro–Hse/Thr-. The sequence Thr–Pro–Thr- accounted for 60–70% of the total amount of the material, as estimated by comparing the peak heights of recovered amino acid phenylthiohydantoin derivatives. Comparison with the *N*-terminal sequence of try-C3d (Eggertsen *et al.*, 1985) and of a tryptic-digest α -chain fragment of human C3 (Thomas *et al.*, 1982) shows that the sequence Ile–Gly–Met–Thr–Pro–Thr- follows immediately after peptide CN8x. This suggests an incomplete CNBr cleavage of the peptide bond between the methionine and threonine residues, as

Fig. 2. Separation of peptides from tryptic digests of CNBr-cleavage fragments of try-C3d (a) Tryptic digest of peptide CN6 (35 nmol). (b) Tryptic digest of peptide CN4 (20 nmol). (c) Tryptic digest of peptide CN2 (16 nmol). The digests were directly loaded on a C18 column (8 mm $\times$ 100 mm) equilibrated in aq. 0.16% (v/v) trifluoroacetic acid, pH 2. Solvent B was 100% acetonitrile containing 0.1% (v/v) trifluoroacetic acid. The flow rate was 1.0 ml/min and 1.0 ml fractions were collected. All the peaks obtained in (a) with $A_{220} > 0.1$ were screened by amino acid analysis, but only those designated T1, T2 and T3 contained protein material. The straight line represents the concentration of solvent B. —, A_{220} .

is known to occur (Schroeder *et al.*, 1969). The released Ile-Gly-Hse fragment (designated CN9) was not recovered. The repetitive yield of the phenylthiohydantoin derivatives of the amino acids of the Ile-Gly-Met-CN2 fragment decreased

markedly after a few cycles, thereby improving the analysis of the sequence of peptide CN2, which was determined for 44 steps (Fig. 3, residues 18–61).

Peptide CN2 was more hydrophobic than the

Fig. 3. Amino acid sequence and alignment of CN-cleavage (CN), tryptic-digest (T) and chymotryptic-digest (Ch) fragments of try-C3d

Subscripts on designations for proteolytic fragments refer to the original CNBr-cleavage fragment. The boxed sequence is taken from De Bruijn & Fey (1985) and represents the non-determined part of the CN2 fragment. — denotes positive sequence determination performed; |—| denotes identification of fragments by amino acid analysis. The — link indicates the position of the thioester group. Repetitive yields of amino acid phenylthiohydantoin derivatives were about 90% for solid-phase sequencing and 96–98% for liquid-phase and gas-phase sequencing.

other CNBr-cleavage fragments, and did not dissolve completely even in the presence of urea. During trypsin digestion a precipitate was generated, and this was collected by centrifugation. Most of the radioactivity was found in the pellet. The chromatogram from the C18 column of the supernatant is shown in Fig. 2(c). Fragments T2₂ and T3₂ were eluted in one peak, as was the case also with fragments T5₂ and T6₂. These fragments were completely separated by rechromatography on C18 at pH6.5 (results not shown). Fragment T7₂ constituted the *N*-terminal fragment of peptide CN2. The amino acid compositions of fragments T7₂ and T8₂ were identical except that fragment T8₂ also contained one residue each of isoleucine, glycine and methionine, suggesting that fragment T8₂ originates from the CN9–CN2 fragment. Thus seven tryptic-digest fragments of peptide CN2 were defined, none of which was radioactively labelled. Five of the fragments could be aligned with the help of the obtained *N*-terminal sequence of intact peptide CN2 (44 residues), as follows (see Fig. 3):

Fragment T4₂ contained homoserine, but failed to give any amino acid phenylthiohydantoin

derivatives by solid-phase sequencing except for trace amounts of that of glutamic acid in the first step. This suggested that an *N*-terminal glutamine residue had cyclized to pyroglutamate during the homoserine coupling process. To avoid this, the peptide was incubated for 5 min instead of the recommended 1 h with 100% trifluoroacetic acid to convert it into its homoserine lactone form, after which coupling proceeded as described (Horn & Laursen, 1973). By this modification the complete sequence of fragment T4₂ (18 residues) was obtained. Fragment T5₂ (six residues), which contained one tryptophan residue, would be located somewhere between fragments T6₂ and T4₂. Since the estimated *M_r* for peptide CN2 was 12700, the tryptic-digest fragments (77 residues) obtained accounted for approx. 70% of the total sequence.

To obtain overlapping fragments, peptide CN2 was digested with chymotrypsin, and the material was separated on ProRPC or C18. This procedure generated a large number of fragments, many of which were difficult to purify (results not shown). A homoserine-containing peptide was found (Ch1₂), which included the fragment T4₂, and another five residues in its *N*-terminal end (Leu-Ile-Leu-Glu-Lys). This sequence was also recog-

nized in fragment T5₂, where it is preceded by an *N*-terminal tryptophan residue. Another, radioactively labelled, chymotryptic peptide (Ch2₂) yielded the sequence Cys-Gly-Ala-Val-Lys-Trp. Provided that this tryptophan residue corresponded to the first residue of fragment T5₂, the following alignment is proposed:

Cys-Gly-Ala-Val-Lys-T5₂-T4₂

By subtracting the amino acid composition of the sequenced parts of peptide CN2 from the analysis of intact peptide CN2, a composition was deduced for the non-sequenced part of peptide CN2 (X₂; Table 1).

In summary, eight CNBr-cleavage fragments from try-C3d have been defined, and seven of them completely sequenced. The alignment

CN8x-CN9-CN2-(CN4,CN6,CN8y,CN8z)-CN7

was made possible by analysis of pre-existing sequence data of human C3 fragments (Thomas *et al.*, 1982; Eggertsen *et al.*, 1985). Alignment of the other fragments was brought about by comparing their sequences with the amino acid sequence of mouse pro-C3, deduced from the cDNA sequence (Wetsel *et al.*, 1984). Because of the high degree of homology between the mouse pro-C3 and human

C3 (about 90%), the alignment was performed without any serious obstacles, and is as follows:

CN8x-CN9-CN2-CN8y-CN6-CN8z-CN4-CN7

For details see Fig. 3. The location of the chymotryptic-digest peptide Ch2₂ within peptide CN2 could be confirmed at the same time.

The amino acid sequence of try-C3d was compared with that of the equivalent segment of human C4B (residues 983-1317) (Belt *et al.*, 1984). The primary structure for the non-sequenced part of human try-C3d (residues 71-99) was made accessible to us before publication by De Bruijn & Fey (1985), who have deduced it from the cDNA sequence. The computer program used in this analysis was DIAGON (Staden, 1982). The comparison matrix of try-C3d with the C4B part (Fig. 4) shows a strong homology at the *N*-terminal half of both segments and also in the *C*-terminal region.

Discussion

This study describes 90% of the primary structure of try-C3d. Cleavage of the intact molecule with CNBr generated eight fragments, which is in accordance with the presence of seven methionine residues in try-C3d (Eggertsen *et al.*, 1985). The

Fig. 4. Comparison matrix of the sequence of try-C3d with the equivalent part of the α -chain of C4B (residues 983-1317; numbering of residues in accordance with Belt *et al.*, 1984)

A span of 21 and a proportional score of 230 were used in the calculation.

Comparison between the primary sequences of try-C3d and human C4d originating from the C4B variant revealed an extensive homology in the N-terminal half of the molecules, emphasizing the ancestral relationship between C3 and C4. Within this part of C4 (C4d) a clustered exchange of four amino acids occurs between C4A and C4B (Hellman *et al.*, 1984; Belt *et al.*, 1984). Two of these in C4B are identical with two amino acids in the corresponding segment of try-C3d, whereas no such identity is found between C4A and Try-C3d, suggesting a closer relationship between C4B and C3 (Fig. 5). Whether this might have any functional significance is difficult to say, but it has been shown that the thioester groups of C4B and C3 preferably react with hydroxy groups, whereas that of C4A interacts with amino groups (Isenman & Young, 1984; Law *et al.*, 1984a, b). Polymorphism in the sequenced parts of try-C3d was not detected, although the molecule displays a heterogeneity on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, similarly to try-C4d (Eggertsen *et al.*, 1985). C3 is synthesized at one gene locus on chromosome 19 (Whitehead *et al.*, 1982), and, even if several allelic variants of C3 occur, the structural basis for its genetic variation is presently not known.

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