

ISOLATION AND CHARACTERIZATION OF THE THIRD COMPLEMENT COMPONENT OF AXOLOTL (*AMBYSTOMA MEXICANUM*)

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Abstract—1. Using a monoclonal anti-human C3 antibody and a polyclonal anti-cobra venom factor antibody as probes, a protein homologous to the mammalian third complement component (C3) was purified from axolotl plasma and found to be axolotl C3.

2. Axolotl C3 consists of two polypeptide chains ($M_r = 110,000$ and $73,000$) linked by disulfide bonds. An internal thiolester bond in the α chain was identified by the incorporation of [14 C]methylamine and NH_2 -terminal sequence from the C3d fragment of C3.

3. Digestion of C3 by trypsin resulted in the cleavage of both the α and β chains, generating fragments with a cleavage pattern similar to that of human C3.

4. The amino acid composition of axolotl C3 and the amino acid sequences of the thiolester site (and the surrounding amino acids), the cleavage site for the C3-convertase, and one of the factor I cleavage sites are similar to C3 from other vertebrates.

5. In contrast to human C3, which has concanavalin A binding carbohydrates on both the α and β chains, only the β chain of axolotl C3 contains such carbohydrates.

INTRODUCTION

The human complement system has been studied in great detail and has been the focus of intensive studies due to its pivotal role within the complement system (Lambris, 1988; Becherer *et al.*, 1989). By contrast, very little is known about the existence and function of the different complement components in lower vertebrates. The third protein of complement (C3) is one of the most versatile and multifunctional molecules within the complement system, interacting with several serum proteins, cell surface receptors, and proteins of foreign origin (Lambris, 1988; Becherer *et al.*, 1989). Until now C3 has been purified from several species, including human (Tack and Prahl, 1976), rabbit (Horstmann and Müller-Eberhard, 1985), guinea-pig (Thomas and Tack, 1983), mouse (Gyongyossy and Assimch, 1977), rat (Daha *et al.*, 1979), quail (Kai *et al.*, 1983), cobra (Eggertsen *et al.*, 1983), frog (Sekizawa *et al.*, 1984), trout (Nonaka *et al.*, 1984b), and lamprey (Nonaka *et al.*, 1984a) (for review see Becherer *et al.*, 1989). The complete amino acid sequence of human (De Bruijn and Fey, 1985) and mouse C3 (Wetsel *et al.*, 1984; Lundwall *et al.*, 1984), as well as the partial sequence of rabbit (Kusano *et al.*, 1986) and *Xenopus* C3 (Grossberger *et al.*, 1989), have been deduced from their cDNA sequences. Limited amino acid sequence has also been obtained for the C3 from other species (Becherer *et al.*, 1989). The elucidation of the structure and functions of C3 and other complement proteins from different species provides not only evolutionary information on the complement system,

but also additional information on the structural elements involved in its multiple interactions with other ligands within the human complement system (Becherer *et al.*, 1989). In this study we report the purification and partial structural analysis of axolotl C3. Its similarities to C3 from other vertebrates, the presence of a thiolester bond and Con A binding carbohydrates, and its fragmentation by trypsin are analyzed.

MATERIALS AND METHODS

Materials

Plasma from adult axolotls (*Ambystoma mexicanum*, Amphibia, Urodela) was kindly provided by Dr James Kaufman (Basel Institute for Immunology). Trypsin was purchased from Cooper Biomedical. Concanavalin A (Con A) was from Vector Laboratories. Polyvinylidene difluoride (PVDF) membranes were from Millipore. Bovine serum albumin (BSA), 2,2'-azino-bis(3-ethylbenzothiazole-6-sulfonic acid), sodium azide and Tween-20 were purchased from Sigma Chemical Company. Polyethyleneglycol 4000 (PEG), polyvinyl pyrrolidone (PVP), thioglycolic acid and phenol were from Merck. Constant boiling 6 N HCl and 4-ethylmorpholine were from Pierce. All chemicals for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) were from Bio-Rad Laboratories. [14 C]Methylamine-HCl was purchased from Amersham. All chemicals for automated amino acid sequencing and composition were from Applied Biosystems.

Antibodies

The monoclonal anti-human C3 antibody (MoAb 013111) was obtained from Cytotech and the monoclonal anti-human C3 antibody (MoAb 133H11) was a gift from Jochem Alsenz (Basel Institute for Immunology). A polyclonal antibody against cobra venom factor was

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raised in goats as previously described (Vogel *et al.*, 1984). Affinity purified peroxidase conjugated anti-IgG antibodies were purchased from Bio-Rad Laboratories.

Purification of axolotl C3

Axolotl plasma, treated with 2 mM diisopropylfluorophosphate, was brought to 4% (w/v) PEG, and after 30 min with constant stirring at 4°C, the plasma was centrifuged (10,000 *g*, 20 min). The supernatant was brought to 16% PEG, stirred for 1 hr at 4°C, centrifuged (10,000 *g*, 20 min), and the pellet redissolved in 5 ml of 20 mM Tris-HCl, pH 7.5/2 mM diisopropylfluorophosphate. Residual PEG was removed using a PD-10 desalting column (Pharmacia) and the 4–16% PEG fraction was applied to a Mono Q HR 5/5 anion exchange column equilibrated in 20 mM Tris-HCl, pH 7.5. After washing the column with 5 ml of buffer, C3 was eluted with a linear gradient of NaCl (0–0.5 M). Fractions containing C3 were identified by their reactivity with MoAb 013III1 and anti-CVF antibodies, and by their mobility in a 9% SDS-PAGE gel (Laemmli, 1970). The fractions were pooled, concentrated by ultrafiltration using an Amicon YM30 Diaflo Membrane, then applied to a Superose 12 gel filtration column (20 × 300 mm) equilibrated in 10 mM sodium phosphate, pH 7.2/150 mM NaCl (PBS) containing 3 mM sodium azide. All chromatographic separations were performed using the Fast Protein Liquid Chromatography system (Pharmacia). Fractions containing C3 were pooled and frozen at –70°C.

Detection of axolotl C3 by an enzyme-linked immunosorbent assay (ELISA)

Microtiter plates were coated overnight at 4°C with either axolotl C3, human C3, or the 16% PEG precipitate of axolotl plasma (8 µg/ml PBS). The plates were saturated with 1% (w/v) bovine serum albumin for 30 min at 25°C. Serially diluted MoAb 013III1 or anti-CVF was added for 30 min at 25°C. Bound antibody was detected with either peroxidase conjugated antimouse IgG antibody (1/1000 dilution) or peroxidase conjugated anti-goat IgG antibody (1/1000 dilution) followed by the addition of 0.07 mM H₂O₂/5 mM 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)/10 mM sodium citrate, pH 4.5. After each step the wells were washed with 0.05% (v/v) Tween-20/PBS.

Fragmentation of axolotl C3 by trypsin

Axolotl C3 was digested with 1% (w/w) trypsin at 37°C and samples removed at time intervals of 0, 3, 15, 30, and 60 min. The reaction was stopped by the addition of 2% (v/v) 2-mercaptoethanol/2% (w/v) SDS/10% (v/v) glycerol/0.0025% (w/v) Bromophenol Blue/0.0625 M Tris-HCl, pH 6.8. The tryptic fragments were separated by SDS-PAGE on a 9% gel and visualized after staining with Coomassie Brilliant Blue R (Sigma). The molecular weights of the tryptic fragments were calculated using reference proteins (Bio-Rad Laboratories).

Identification of the internal thiolester site in axolotl C3

The presence of a thiolester site in axolotl C3 was determined by the incorporation of [¹⁴C]methylamine and amino acid sequence analysis. The incorporation of [¹⁴C]methylamine to axolotl C3 was performed as previously described (Harrison *et al.*, 1981; Tack, 1983). Briefly, axolotl C3 (100 µg) was allowed to react with [¹⁴C]methylamine (0.054 M, 0.3 mCi) for 2 hr at 37°C. Unlabeled methylamine (0.1 M) was added to the sample and incubated for 30 min at 37°C. The ¹⁴C-axolotl C3 was brought to a final volume of 100 µl, spun through a Bio-Spin 6 column (Bio-Rad Laboratories) to remove unincorporated methylamine, and digested with trypsin as described. The ¹⁴C-axolotl C3 fragments were visualized by autoradiography.

The NH₂-terminal sequence of the site was obtained by Edman degradation (see below) of the ¹⁴C-axolotl C3 fragment that is equivalent to the human C3d. This fragment

was isolated by selectively absorbing out the C3c fragment using Con A-Sepharose (Pharmacia). Trypsinized C3 (100 µg) was incubated with Con A-Sepharose in the presence of 1 mM MnCl₂/1 mM CaCl₂ for 30 min at 37°C. After centrifugation, the supernatant containing the thiolester fragment was reduced, separated on a 9% SDS-PAGE gel and the fragment containing the thiolester bond was sequenced after electroblotting to a PVDF membrane as described in this paper.

Detection of Con A binding carbohydrates on axolotl C3

Con A binding carbohydrates were detected as previously described (Becherer and Lambris, 1988). Briefly, samples from trypsinized C3 were run on a 9% SDS-PAGE gel followed by electroblotting to a nitrocellulose membrane. Nitrocellulose was saturated by incubation in 2% (w/v) PVP-40/PBS for 1 hr. ¹²⁵I-Con A, labeled by the Iodogen method (Fraker and Speck, 1978), was added to the electroblotted C3 (10⁶ cpm/ml, 7.3 × 10⁹ cpm/mg) and incubated for 1 hr at room temperature. Nitrocellulose was extensively washed with 2% (w/v) PVP-40/PBS followed by several washes with 0.05% (v/v) Tween-20/PBS. The binding of ¹²⁵I-Con A was detected by autoradiography.

Amino acid composition and NH₂-terminal sequence of axolotl C3

Gas phase hydrolysis of axolotl C3 was performed in 6 N HCl/1% (v/v) phenol at 110°C. Analysis of the amino acid composition was performed after 24, 48, and 72 hr using an Applied Biosystems 420A Derivatizer connected on-line to a 130A Analyzer and a 920A Data Analysis Module. The compositions for serine and threonine were determined after extrapolation to zero time hydrolysis.

The axolotl C3 tryptic fragments were sequenced by a modified method of Matsudaira (1987), using an Applied Biosystem 470A Protein Sequencer connected on-line to a 120A PTH-analyzer and a 900A Control/Data Analysis Module. Briefly, SDS-PAGE gels were pre-run (10 mA/gel) for 30 min before application of samples. Thioglycolic acid (0.002%) was added to the running buffer to scavenge remaining free radicals in the gel. Following electrophoresis, gels were equilibrated in 0.025 M 4-ethylmorpholine (pH 8.3) for 20 min and then electroblotted onto PVDF membranes (1 mA/cm² gel) using a semi-dry electroblot apparatus (JKA-Biotech). Bands were visualized after staining with 0.1% (w/v) Coomassie Brilliant Blue in methanol/acetic acid/water (50/10/40) for 5 min followed by destaining for 10 min. The electroblotted membranes were rinsed with tri-distilled water before drying under vacuum. Samples were stored under vacuum at 4°C until sequenced. Alternatively, the α chain of axolotl C3 was sequenced by direct application of C3 to a polybrene-coated glass fiber filter.

RESULTS

Purification of axolotl C3

In order to establish a detection assay for axolotl C3, several monoclonal and polyclonal antibodies directed against human C3 and CVF were tested for their reactivity with the 16% PEG fraction of axolotl plasma. Using an ELISA, two antibodies were found to react with the 16% PEG fraction (Fig. 1) of axolotl plasma and were further used to assay axolotl C3 during the different purification steps.

The isolation of axolotl C3 was accomplished in essentially three steps. Precipitation of axolotl plasma with 4% PEG followed by the precipitation of C3 with 16% PEG resulted in the removal of approximately 50% of the plasma proteins. Using an ELISA to assay for residual C3 in the 16% PEG supernatant

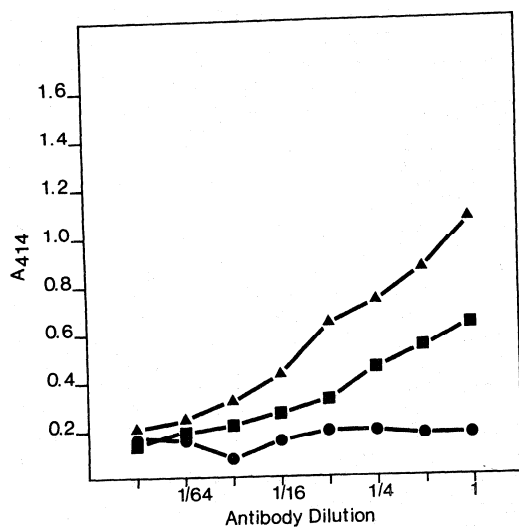


Fig. 1. Cross-reactivity of anti-CVF and MoAb 013III1 antibodies with axolotl C3. Serially diluted monoclonal antibodies 013III1 (▲), 133H11 (●), or goat anti-CVF (■) were incubated for 30 min at 25°C with a 16% PEG precipitate from axolotl plasma (20 µg protein/ml PBS) which was previously fixed to a microtiter plate. The bound antibodies were detected as described in Materials and Methods. The starting dilutions for MoAb 013III1 and MoAb 133H11 were 50 µg/ml (purified from ascites by ammonium sulfate precipitation) and for goat anti-CVF 200 µg/ml (total immunoglobulin fraction from serum after ammonium sulfate precipitation).

it was determined that the axolotl C3 was in the 4–16% PEG pellet. The pellet was resuspended into 5 ml of 20 mM Tris-HCl, pH 7.5, and then applied to a Mono Q anion-exchange column. Five major peaks were detected after elution with a gradient of 0–500 mM NaCl (Fig. 2); the third peak, identified by ELISA and SDS-PAGE, contained the axolotl C3. The concentrated C3 fractions from the Mono Q column were further purified by gel filtration on a Superose 12 column. As shown in Fig. 3, the third peak to elute from the Superose 12 column reacted with both MoAb 013III1 and anti-CVF antibodies, and contained only one major protein when analysed by SDS-PAGE. The total recovery of purified axolotl C3 from 6 ml plasma was 2.2 mg. The purified axolotl C3 has an apparent molecular weight of 200,000 as determined by gel filtration and SDS-PAGE (Fig. 3) and is comprised of two chains having M_r s of 110,000 (α chain) and 73,000 (β chain) which are linked by a disulfide bond(s).

Fragmentation of axolotl C3 by trypsin

Tryptic proteolysis of axolotl C3 resulted in the preferential cleavage of the α chain (Fig. 4A, lane 2). The initial cleavage near the NH_2 -terminus of the α chain generates a 100 kDa fragment (α' chain) and a 10 kDa fragment (C3a). Further cleavage of the α' chain generates fragments having molecular masses of 45, 38, 36, 34 and 23 kDa (Fig. 4A, lane 2). The 62 kDa fragment generated after 3 min (Fig. 4A, lane 2) represents a cleavage fragment of the β chain as determined by its ability to bind ^{125}I -Con A (Fig. 4C).

Further cleavage of the α chain fragments resulted in a band with molecular mass of 34 kDa (Fig. 4A, lane 5). This band is composed of two α chain fragments as determined by NH_2 -terminal sequencing. One fragment contains the thiolester site and the second fragment contains the factor I cleavage site (Fig. 5).

Identification of the internal thiolester site in axolotl C3

Treatment of axolotl C3 with [^{14}C]methylamine resulted in the covalent binding of [^{14}C]methylamine to the α chain (Fig. 4B, lane 1). Tryptic digestion of the α chain shows that the [^{14}C]methylamine is covalently bound to the α' chain of the C3b fragment and the three smaller fragments of 45, 38 and 34 kDa (Fig. 4B, lane 2). When the electrophoresis was run under non-reducing conditions it was found that the C3b is cleaved into two fragments of 145 (C3c) and 45 kDa (data not shown), the latter being labeled with [^{14}C]methylamine. Further cleavage of the 45 kDa fragment leads to the generation of the 38 and 34 kDa fragments (Fig. 4B, lanes 2–5). Two 34 kDa fragments were sequenced after separation on a Con A-Sepharose column and electroblotting to a PVDF membrane. One fragment containing the thiolester site (Fig. 5) shows a high amino acid sequence similarity to the region of C3 from other vertebrates containing the thiolester site (the thiolester site being completely conserved).

Detection of Con A binding carbohydrates on axolotl C3

The detection of carbohydrate moieties on the axolotl C3 was done using ^{125}I -Con A (Hirani *et al.*, 1985). Binding of ^{125}I -Con A to reduced axolotl C3

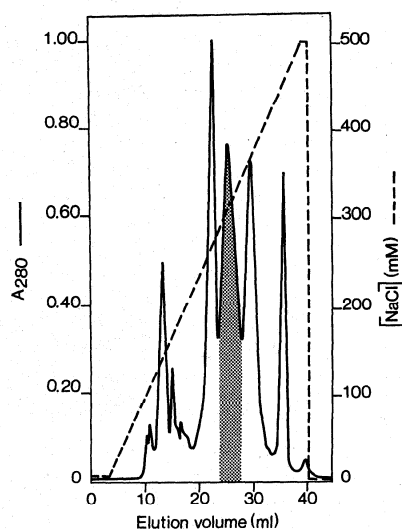


Fig. 2. Elution profile of the 4–16% PEG fraction of axolotl plasma from Mono Q column. The 4–16% PEG fraction of axolotl plasma was applied to a Mono Q column equilibrated in 20 mM Tris-HCl, pH 7.5, then eluted with a NaCl gradient (0–500 mM) in the same buffer. Protein concentration was monitored by absorbance at 280 nm. The shaded peak was reactive with both MoAb 013III1 and polyclonal anti-CVF antibodies and has an α and β chain structure with molecular weights similar to those of mammalian C3.

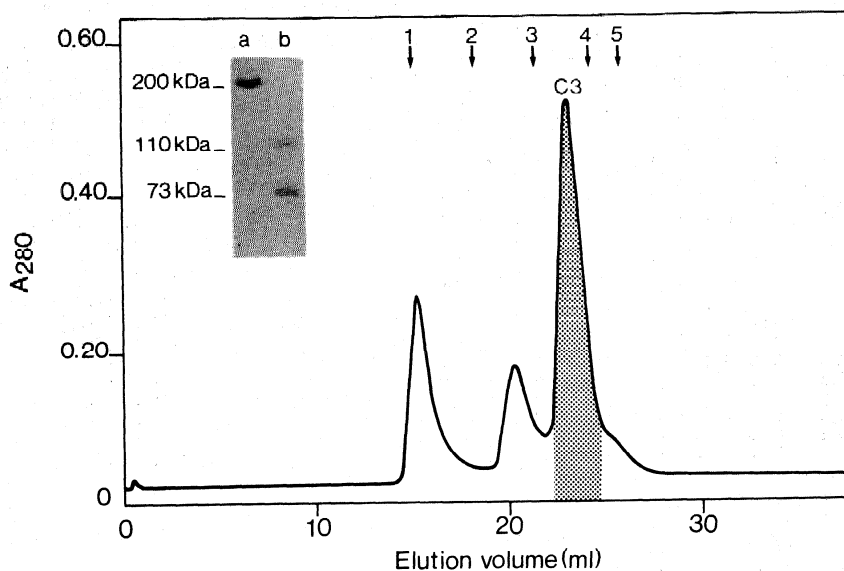


Fig. 3. Elution profile of the axolotl C3 from Superose 12 column. The axolotl C3 containing fractions from the Mono Q purification step was applied to a Superose 12 column equilibrated in 25 mM sodium phosphate, pH 7.5/150 mM NaCl/3 mM sodium azide. Axolotl C3 was eluted with the same buffer at a flow rate of 0.5 ml/min and protein elution was monitored by absorbance at 280 nm. The shaded peak represents axolotl C3 as determined by its reactivity with anti-C3 antibodies and molecular weight determination. Insert shows (a) non-reduced and (b) reduced axolotl C3. Gel filtration standards, as indicated by arrows, are (1) thyroglobulin, 669,000, (2) ferritin, 440,000, (3) catalase, 232,000, (4) aldolase, 158,000, and (5) bovine serum albumin, 66,000.

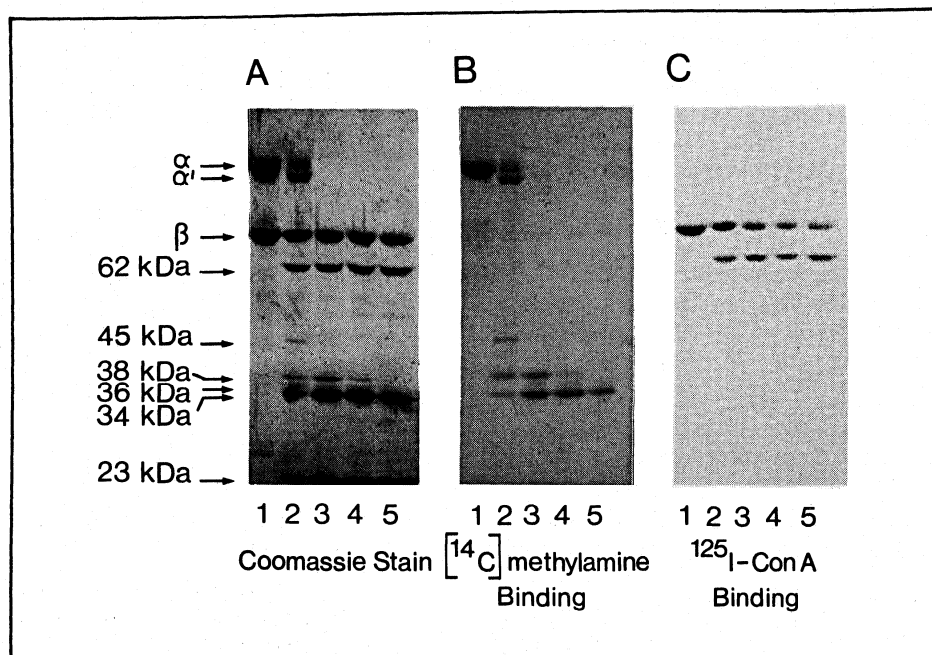


Fig. 4. Characterization of axolotl C3 by tryptic proteolysis. Axolotl C3 (80 μ g) was digested using trypsin (0.8 μ g) at 37°C. Samples were removed at 0, 3, 15, 30, and 60 min (lanes 1 through 5, respectively). The reaction was stopped by the addition of sample reducing buffer and the fragments were separated by SDS-PAGE on a 9% gel. Gels were stained with Coomassie Brilliant Blue R (A), or assayed by autoradiography for covalently bound [14 C]methylamine (B) and binding of 125 I-Con A (C). The calculated molecular masses for the fragments are shown to the left.

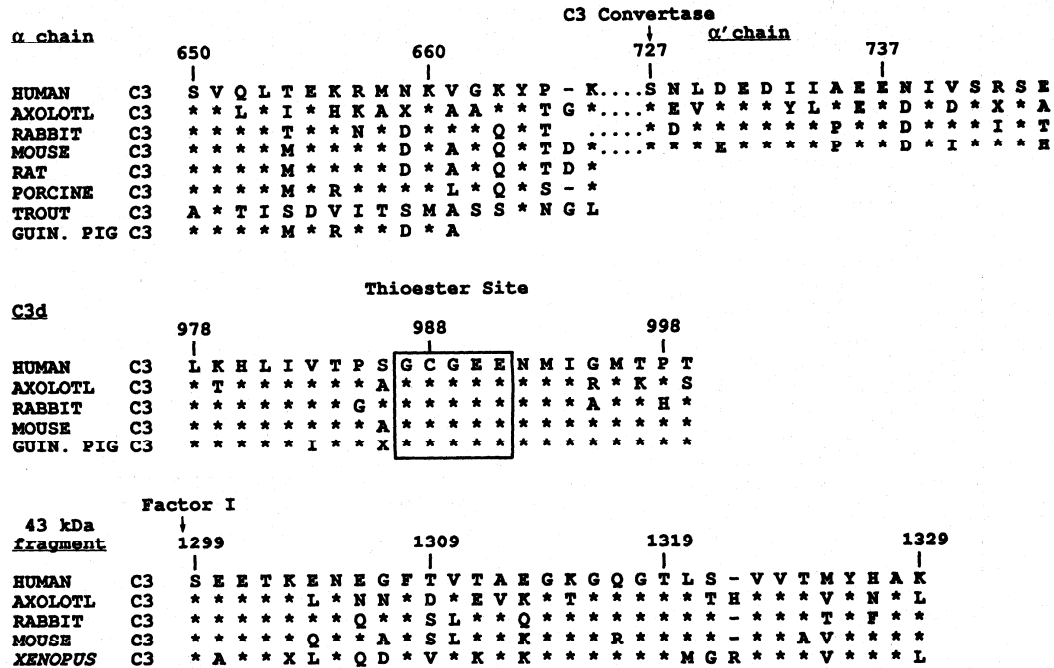


Fig. 5. Alignment of axolotl C3 sequence with known C3 sequences from other species. Identical amino acids are indicated by an asterisk. The numbering is based on the human C3 sequence (De Bruijn and Fey, 1985) after subtracting the signal peptide sequence. The amino acid sequence of rabbit (Becherer *et al.*, 1989; Kusano *et al.*, 1986), mouse (Wetsel *et al.*, 1984; Lundwall *et al.*, 1984), guinea-pig (Thomas and Tack, 1983), rat (Daha *et al.*, 1979), and trout (Nonaka *et al.*, 1984b) C3 are from the indicated references. The amino acid sequence of *Xenopus* C3 was obtained using methods similar to those described for axolotl C3 (unpublished observations). The factor I and convertase cleavage sites of human C3 (arrows) and the thiolester site (box) are indicated.

and its tryptic fragments shows that only the β chain contains Con A binding carbohydrate(s) (Fig. 4C, lane 1). This carbohydrate is retained on a 62 kDa fragment of the β chain after cleavage by trypsin (Fig. 4C, lanes 2-5). Further cleavage of the β chain and its 62 kDa fragment was not observed after 1 hr.

Amino acid composition and NH₂-terminal sequence of axolotl C3

The amino acid composition of axolotl C3 (Table 1) was compared to that of human, trout, and *Xenopus* C3. The amino acid composition similarities between the axolotl C3 and these species are shown in Table 1.

Several axolotl C3 fragments obtained by tryptic proteolysis were sequenced by Edman degradation. Alignment of the obtained sequences with the C3 sequences from other species (Fig. 5) reveals that a high similarity exists between the axolotl C3 sequence and that of C3 from other species. Similar tryptic fragments have been previously generated for the human counterpart (Eggertsen *et al.*, 1985), indicating that the trypsin cleavage sites are similar in both C3 molecules. One particular tryptic fragment, which was found to encompass the thiolester site, is 82% identical with mouse and 77% identical with human C3.

All attempts to sequence the β chain and its 62 kDa fragment were unsuccessful, leading us to believe that the NH₂-terminus is blocked. A blocked NH₂-termi-

nus was confirmed by sequencing of the intact C3 molecule, which resulted in a single sequence from the α chain and not the expected double α/β sequence.

DISCUSSION AND CONCLUSIONS

Analysis of complement in lower species not only enhances our understanding on the evolution of the

Table 1. Comparison of the amino acid composition of axolotl C3 with those from other species

Amino acid	Axolotl	Human*	Trout†	<i>Xenopus</i> ‡
Asx	11.2	9.1	10.4	11.6
Glx	11.7	12.8	11.2	11.6
Ser	6.2	6.4	7.4	5.9
Gly	7.2	5.7	6.7	6.6
His	2.1	1.7	1.7	1.4
Arg	4.1	4.9	3.5	4.3
Thr	7.6	6.3	7.1	6.5
Ala	6.8	5.7	6.8	6.4
Pro	4.8	4.7	3.8	5.1
Tyr	4.5	3.4	3.6	4.1
Val	8.9	8.8	10.0	8.2
Met	2.6	2.1	2.1	2.1
Cys	—§	0.8	2.4	—§
Ile	6.0	5.1	4.5	6.2
Leu	7.9	9.2	7.5	8.2
Phe	4.4	3.6	3.1	3.1
Trp	—§	0.7	1.2	0.9
Lys	7.7	6.8	7.3	7.7

*Taken from Tack and Prahl (1976).
 †Taken from Nonaka *et al.* (1984b).
 ‡Taken from Sekizawa *et al.* (1984).
 §Not determined.

complement system but is also a necessary element in the structural and functional analysis of the individual complement proteins. The best phylogenetically studied complement component is C3, no doubt due to its key role in complement activation. It has been purified from several vertebrates, including members of the mammals, aves, reptilia, amphibia, osteichthyes, and agnatha (for review see Becherer *et al.*, 1989). In this study we isolated and characterized C3 from the axolotl (*Ambystoma mexicanum*), a neotenic salamander. Among the interesting characteristics of the axolotl are: (a) the low production of thyroxin which accounts for its inability to achieve anatomical metamorphosis; and (b) its poor immune responses (chronic graft rejections, absence of immunoglobulin isotype switch and affinity maturation, imperceptible mixed lymphocyte reaction, etc.) (Ching and Wedgwood, 1967; Charlemagne and Tournefier, 1977).

In this study, axolotl C3 was purified from axolotl plasma using two cross-reactive antibodies as probes: a monoclonal anti-human C3 antibody and a polyclonal anti-CVF antibody. Our finding that the anti-CVF antibodies crossreact with axolotl C3 agrees with previous findings suggesting that antibodies to CVF recognize structures which are conserved in C3 from different vertebrates (Eggertsen *et al.*, 1983). We have extended this study to other species and found that the anti-CVF antibodies, in addition to reacting with CVF, cross-react with human, rabbit, mouse, chicken, cobra, and *Xenopus* C3 (unpublished observations). The procedure described for the purification of axolotl C3 is simple and has been used to purify C3 from other species such as chicken, cobra, and *Xenopus* (Alsenz *et al.*, 1989). That the isolated molecule represents axolotl C3 is based on the following properties: (a) its molecular structure is similar to mammalian C3, composed of two polypeptide chains ($M_r = 110,000$ and $73,000$) linked by disulfide bond(s); (b) it contains an internal thiolester site; (c) the amino acid composition shows a high degree of resemblance to human, trout, and *Xenopus* C3; and (d) the NH_2 -terminal sequence of the α chain and other tryptic C3 fragments show a high degree of similarity with the corresponding regions of C3 from other species. Comparisons of the limited amino acid sequences obtained from the tryptic fragments of axolotl C3 with that of C3 from other species show it to be between 55 and 61% identical (average of all fragments) to human, rabbit, mouse, and *Xenopus* C3. Its identity with mouse C3 increases to 82% in the tryptic fragment containing the thiolester bond; the thiolester site, GCGEE, is 100% conserved in all species. This conservation of the thiolester site and its surrounding hydrophobic amino acids emphasizes the functional importance of this region in maintaining, throughout evolution, the molecule's capacity to fix itself to surfaces. The region surrounding the thiolester bond in C3, C4 and α_2 -macroglobulin is relatively hydrophobic and it has been proposed that it serves to shield the thiolester from the aqueous environment and nucleophilic attack (Tack, 1983; Levine and Dodds, 1989; Zhao *et al.*, 1988; Fontaine *et al.*, 1982).

Cleavage of human C3 by C3 convertases leads to the generation of C3a and C3b, the C3b having the thiolester bond exposed. The thiolester bond is either

hydrolyzed or mediates the covalent binding reaction with cell surfaces or other receptive molecules (Tack, 1983; Law *et al.*, 1980). The fixation of C3b to the activating surfaces is important for the amplification of the complement cascade, initiation of the membrane attack complex, phagocytosis of foreign particles, and the enhancement of effector-target cell contact. Depending on the surface to which C3b is bound, it will either participate in the amplification of the complement cascade or will be degraded by factor I in the presence of cofactor molecules (Lambris, 1988). Although it is not known whether the amino acids Arg⁷²⁶ and Arg¹²⁹⁸ (human C3 numbering) are conserved in axolotl C3, the conservation of Ser⁷²⁷ and Ser¹²⁹⁹ in the α chain of axolotl C3 suggests that the axolotl C3 convertases and factor I have similar specificities as in human complement.

The human C3 fragments generated during complement activation, in surface bound or fluid phase, react with several other complement components and receptors as well as with proteins of foreign origin (Lambris, 1988). Among them are the factors, I, H, B, C5, P, CR1, CR2, CR3, CR4, etc. (for review see Lambris, 1988; Becherer *et al.*, 1989). Comparison of the ability of C3 from various species to bind the different C3 binding proteins and correlation of this information to amino acid sequence similarity has been instrumental in identifying the binding sites in C3 for these ligands (Becherer *et al.*, 1989; Daoudaki *et al.*, 1988). Since the binding sites for many of the ligands of human C3 have been localized (Lambris, 1988; Becherer *et al.*, 1989), studies on the reactivity of axolotl C3 with the ligands of human C3 and subsequent cloning of axolotl C3 will greatly enhance our understanding of the structural features in C3 involved in these interactions. Preliminary experiments comparing C3s from different vertebrates suggest that the sequence conservation of the binding sites for H, P, CR1, and CR2 is important in maintaining binding of its ligand (Alsenze *et al.*, 1989).

One discrepancy with human C3, which contains Con A binding *N*-linked high mannose carbohydrates in both chains (Hase *et al.*, 1985; Hirani *et al.*, 1986), is the absence of Con A binding carbohydrates on the α chain of axolotl C3. This does not exclude the possibility that carbohydrates not recognized by Con A are present on the α chain. Similar to axolotl C3, trout and *Xenopus* C3 do not have Con A binding carbohydrates in the α chain (Becherer *et al.*, 1989). The difference in glycosylation is of interest because the α chain carbohydrate moiety on human C3 has been found to be involved in the binding to bovine conglutinin (Hirani *et al.*, 1985), and recently the human analog to bovine conglutinin has been found in human serum (Thiel *et al.*, 1987). Even though the exact function of conglutinin-iC3b interaction is not known it will be of interest to see if the axolotl has a protein analogous to bovine or human conglutinin and, if such a protein exists, to test its interaction with human and axolotl C3.

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