The generation of two cleavage products of human C3, termed C3o and C3p, by incubation with a C3-cleaving protease isolated from cobra venom (Naja naja siamensis) is described. The venom protease removes the C3p fragment (M, \sim 33,000) from the C3dg region of the C3 \alpha-chain. The major cleavage fragment C3o (M, \sim 140,000) contains the unaltered \beta-chain of C3 and two \alpha-chain-derived polypeptides of M, \sim 29,000 and M, \sim 38,000, respectively. Amino-terminal amino acids sequence analysis of C3p and the three chains of C3o allowed the identification of the exact location of the two \alpha-chain-derived fragments of C3o and the three cleavage sites of the venom protease. The chain structure of C3o resembles those of C3c and cobra venom factor. In contrast to C3c but like cobra venom factor (and C3b), C3o was found to support the activation of the serine protease Factor B by cleavage in the presence of Factor D and Mg\textsuperscript{2+} into Bb and Ba, and generating an enzymatically active complex that is able to cleave a fluorogenic peptide substrate for C3 convertases. Since the only stretch of amino acid residues of C3o not present in C3c is the carboxyl terminus of the M, \sim 29,000 chain of C3o, it is suggested that this region is important for the interaction with Factor B and convertase formation.

C3, the third component of complement, is activated in the classical or alternative pathway through cleavage by a C3 convertase, a protease consisting of a structural subunit and a catalytic subunit. Both cobra venom factor (CVF),\textsuperscript{1} the complement-activating protein from cobra venom, and C3b, the activated form of complement component C3, can serve as the structural subunit of an alternative pathway C3 convertase (EC 3.4.21.47) (for review, see Ref. 9). The two enzymes (CVF,Bb and C3b,Bb) are formed by the action of Factor D on Factor B when the latter is bound to CVF or C3b (10, 11). Bb serves as the catalytic subunit in both cases. Although both enzymes share the enzymatic specificity of cleaving C3 (and C5) and exhibit a similar ultrastructure in the electron microscope (12, 13), they show several important differences. The decay-dissociation at 37 °C into the two subunits occurs with a half-life of 7 h for CVF,Bb (14) and of 1.5 min for C3b,Bb (15). C3b,Bb is disassembled by Factor H (16), leading to the subsequent inactivation of C3b by Factor I (17). In contrast, CVF,Bb is resistant to these regulatory proteins (18, 19). C3b,Bb requires additional C3b to act on C5 (20, 21), whereas CVF from certain cobra species forms a CVF,Bb enzyme that can cleave C5 directly (22, 23). The two enzymes also differ in their kinetic parameters of C3 hydrolysis (14, 15). Due to its physicochemical stability and its resistance to the regulatory proteins, CVF,Bb will continuously hydrolyze C3 and C5 resulting in exhaustive complement activation. This property of CVF,Bb is frequently used to decomplement laboratory animals by injection of CVF (24-26) and has also been exploited to target an exhaustive complement activation to selected cells by covalently coupling CVF to monoclonal antibodies directed against cell surface antigens. This concept has been successful in inducing selective complement-mediated lysis of target cells such as melanoma cells (27, 28), lymphocytes and leukemia cells (29, 30), and erythrocytes (31).

As expected from the functional similarity of CVF and C3b in forming a convertase with Factor B, these two molecules exhibit several structural similarities including amino acid composition, isoelectric points, circular dichroism spectra and secondary structure, electron microscopic ultrastructure, immunological cross-reactivity in the polypeptide and carbohydrate moieties, and amino-terminal amino acid sequences (4, 32-34).\textsuperscript{2} On the other hand, structural differences must exist between the two molecules that are responsible for the different properties of the two enzymes. Furthermore, cleavage of the \alpha’-chain of C3b by Factor I abolishes the ability of the degradation products iC3b and C3c to form a convertase, although C3c structurally resembles CVF much more than C3b does (4). (For a detailed discussion of the various proteo-

\textsuperscript{2} Sequence homology of the amino terminus of the CVF \alpha-chain with the human C3 \beta-chain (4-6) and of the CVF \gamma-chain with the human C3 \alpha’-chain (4) has been reported. We recently compared the amino-terminal sequence of the CVF \beta-chain with the deduced amino acid sequence of human C3 and found homology also in the carboxy-terminal portion of the C3 \alpha-chain. The amino terminus of the CVF \beta-chain corresponds to residues 612 of the C3 \alpha-chain which is in the carboxyl-terminal portion of the C3d region, 21 residues amino-terminal from the Factor I cleavage site separating C3d and C3e. The 25 known amino-terminal amino acid residues of the CVF \beta-chain (7) correspond to residues 612-634 of the C3 \alpha-chain with 9 (39%) identical residues and 3 (13%) conservative replacements (compare Fig. 1) (M. C. O'Keefe, L. H. Caporale, and C.-W. Vogel, unpublished observation).
lytic degradation products of C3, see Refs. 8, 9, and 35.)

In an attempt to identify the structures that are required for convertase formation and that determine the properties of a convertase, we generated a human analog of CVF from human C3. Since the chain structure of CVF strongly suggests that its \( \beta \)- and \( \gamma \)-chains are derivatives of a C3-like \( \alpha \)-chain (Fig. 1) (4, 32, 34), we speculated that the molecule is made in the venom gland of the cobra by proteolytic processing of a pro-CVF with an intact C3-like \( \alpha \)-chain. Although cobra venom had been reported to contain no or only very little proteolytic activity and no protease had been described previously (36, 37), we identified a highly specific protease that cleaves human C3 into a C3c-like fragment and a C3d-like fragment, termed C3o and C3p, respectively (2, 38). We report here that C3o is a novel C3 cleavage product that is structurally similar to but not identical with C3c and CVF, and that is able to form a convertase with Factor B (C3o-Bb).

**EXPERIMENTAL PROCEDURE**

**Proteins**—Human complement proteins C3 (39), Factor B (40), and Factor D (11) were purified according to published procedures. C3o and C3c were generated from purified human C3 as described (4). Native C3 and C3(H\(_2\)O) were separated by high performance liquid chromatography using a 5 x 50-mm Mono-Q column (Pharmacia LKB Biotechnology, Inc.). Approximately 5 mg of the C3 preparation in 20 mM Tris/HCl, pH 7.6, was applied to the column. Native C3 and C3(H\(_2\)O) were eluted with a 26-ml linear gradient (20 mM Tris/HCl, pH 7.6, 900 mM sodium acetate, pH 7.6 (B), 100% A to 100% B). The Bb fragment of human Factor B was obtained by incubation of 2 mg of Factor B with 250 \( \mu \)g of C3(H\(_2\)O) and 32 \( \mu \)g of Factor D for 1 h at 37 °C in a total volume of 1.6 ml of VBS containing 2 mM MgCl\(_2\). After dialysis into 5 mM sodium phosphate, pH 7.3, the reaction mixture was applied to a 0.8 x 30-cm DEAE-cellulose column (Whatman). The Bb fragment eluted in the breakthrough. The Bb fragment and C3(H\(_2\)O) bound to the column and could be separated by elution with a 180-ml linear salt gradient (0-43 mM NaCl) in the above phosphate buffer. CVF was purified from lyophilized Naja naja siamensis venom (Serpentarium Laboratories, Salt Lake City, UT) (25). The C3-cleaving protease was purified from *N. n. siamensis* venom by sequential chromatography on Bio-Gel 0.5m (Bio-Rad), Sphero Gel TSK-5 GEX CM-3SW (Beckman), an anti-CVF immunoadsorbent column, and Mono-Q (Pharmacia LKB Biotechnology Inc.). Either highly purified protease or protease preparations containing a co-puriﬁed polypeptide of similar molecular weight that represented approximately 80% of the total protein in the protease preparations and that did not affect the enzymatic activity of the protease were used in the experiments. The puriﬁcation of the protease and its biochemical characterization have been reported in abstract form (2, 38). A full account will be published elsewhere.³

**Preparation of C3o and C3p**—In a typical preparation, 4.2 mg of human C3 was incubated for 45 min at 37 °C with approximately 0.8 mg of the highly puriﬁed (or 4.0 mg of the partially puriﬁed) C3-cleaving protease in a total volume of 3.9 ml of Veronal-buffered saline (VBS; 3.5 mM 5,5-diethylbarbituric acid, 143 mM NaCl, pH 7.4) containing 2 mM MgCl\(_2\). Subsequently, the incubation mixture was applied to a 1.5 x 119-cm Sephadex G-200 gel ﬁltration column (Pharmacia LKB Biotechnology, Inc.) equilibrated in VBS at 4 °C. The column was eluted with VBS at a flow rate of 16 ml/h. The fractions containing C3o were pooled, concentrated with an Amicon YM-10 membrane and applied to a 0.8 x 9-cm organomercurial agarose column (Affi-Gel 501; Bio-Rad) equilibrated in VBS at 4 °C to remove any contaminating C3 species with a free sulfhydryl group (C3(H\(_2\)O), C3b, C3p). The C3o, which has no free sulfhydryl group, was collected in the breakthrough. The fractions from the Sephadex G-200 column containing the C3p fragment of CVF contains the \( \gamma \)-chain-derived free sulfhydryl group, were pooled and loaded onto the regenerated organomercurial agarose column. The column was washed with VBS and the C3p was subsequently eluted with VBS containing 10 mM cysteine. The cysteine was removed by dialysis, and the C3p was concentrated by ultrafiltration on an Amicon YM-10 membrane.

**Separation of the Chains of C3o and Amino-terminal Sequence Analysis**—The chains of C3o were separated in 10% (w/v) SDS-polyacrylamide gels under reducing conditions. The separated polypeptide chains were subsequently isolated by electrophoresis from the acrylamide as described (41). The isolated proteins were stored at -20° C in 0.1 N NH\(_4\)HCO\(_3\), containing 0.92% (w/v) SDS until sequenced. Automated amino-terminal sequence analysis of the individual chains (approximately 1 nmol) was carried out in the presence of the polymeric quartenary amine Polybrene (42) by the Edman degradation method using a gas-phase sequencer (Model 470A, Applied Biosystems) (43). The phenylthiohydantoin derivatives of amino acids were identiﬁed by reversed-phase high performance liquid chromatography on a 4.6 x 250-mm C\(_18\) Ultrasphere ODS column (Beckman) (44).

**Assay for Factor B Activation**—Puriﬁed Factor B (2.66 \( \mu \)M) was incubated for 5 h at 37° C with C3o (0.97 \( \mu \)M) and Factor D (0.15 \( \mu \)M) in VBS containing 2 mM MgCl\(_2\). The samples were analyzed by electrophoresis in 7.5% (w/v) SDS-polyacrylamide gels under non-reducing conditions to monitor the appearance of the Factor B activation products Bb and Ba. Controls included the substitution of C3o with approximately equimolar amounts of CVF, C3b, or C3c and the substitution of MgCl\(_2\) with 10 mM EDTA.

**Cleavage of the Fluorogenic Peptide Substrate**—This assay was performed essentially as described (45, 46). A mixture of C3o (0.83 \( \mu \)M), Factor B (2.1 \( \mu \)M), and Factor D (0.09 \( \mu \)M) was incubated for 10 min at 37° C in a total volume of 150 \( \mu \)l of VBS containing 2 mM MgCl\(_2\). Subsequently, a ﬂuorogenic peptide derivative (t-butoxy-carbonyl-leucyl-glycyl-arginyl-aminomethylcoumarin) (Polyprotein Research Foundation, Osaka, Japan) was added to give a ﬁnal concentration of 150 \( \mu \)M. This ﬂuorogenic peptide is a substrate for C3/C5 convertases (45, 46). The ﬂuorescence at 460 nm of the liberated 7-amino-4-methylcoumarin was continuously monitored for 30 min in a Spex spectrophotofluorometer using light at 380 nm for excitation. A mixture of CVF (0.83 \( \mu \)M), Factor B, and Factor D served as positive control. Other controls included Factor B, the Bb fragment of Factor B (0.8 \( \mu \)M), Factor D, C3o, and C3c plus Factor B.

**Other Methods**—Protein determinations were performed with the Folin-Ciocalteau phenol reagent or by absorption at 280 nm for proteins with known extinction coefﬁcients (C3, CVF) (35, 39). Immunoelectrophoresis was performed in 1% (w/v) agarose gels in 43.5 mM 5,5-diethylbarbituric acid, pH 8.6, for approximately 2 h at 5 V/cm. The gels were stained with Coomassie Blue and destained with acetic acid. The gels were scanned on a Spex 3000 spectrophotometer.

**RESULTS**

**Generation and Characterization of C3c and C3p**—Fig. 2 shows a ﬂow chart describing the generation and puriﬁcation of C3o and C3p. Puriﬁed human C3 was incubated with the C3-cleaving venom protease under conditions to achieve cleavage of virtually all C3 present. The reaction mixture was chromatographed on Sephadex G-200 (Fig. 3) which separated C3o (M\(_r\) ~ 140,000) and C3p (M\(_r\) ~ 33,000) due to their size difference. C3p is derived from the region of the \( \alpha \)-chain that contains the free sulfhydryl group since it binds to organomercurial agarose-sulfhydryl afﬁnity matrix, from which it could be eluted with cysteine. This matrix was also used to remove any possible trace of remaining C3(H\(_2\)O) or C3b from C3o. Figs. 4 and 5 show SDS-polyacrylamide gels of puriﬁed C3o and C3p. C3p is a single-chain protein with an apparent molecular weight of 33,000 due to their size difference. C3p is derived from the region of the \( \alpha \)-chain that contains the free sulfhydryl group since it binds to organomercurial agarose-sulfhydryl afﬁnity matrix, from which it could be eluted with cysteine. This matrix was also used to remove any possible trace of remaining C3(H\(_2\)O) or C3b from C3o. Figs. 4 and 5 show SDS-polyacrylamide gels of puriﬁed C3o and C3p. C3p is a single-chain protein with an apparent molecular weight of 33,000 due to their size difference.

³ M. C. O'Keefe, C. W. Vogel, and L. H. Caporale, manuscript in preparation.
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**FIG. 2.** Flow chart indicating the generation and isolation of C3o and C3p. Note that any contaminating thiol-containing C3 species (C3(H2O), C3b, C3p) would be removed from C3o by the thiol-affinity column.

**FIG. 3.** Separation of C3o and C3p by gel filtration on Sephadex G-200. Shown is a typical elution profile obtained after digestion of 125I-C3 with the venom protease. The exclusion volume ($V_e$) and total volume ($V_t$) as well as the elution positions of C3o and C3p (shaded areas) are indicated.

**FIG. 4.** SDS-polyacrylamide gel electrophoresis of CVF, C3o, and C3c. Shown are gels after staining with Coomassie Blue under nonreducing (left panel) and reducing conditions (right panel). The apparent molecular weights of the individual polypeptide chains are indicated.

**FIG. 5.** SDS-polyacrylamide gel electrophoresis of purified C3p. Shown are gels after staining with Coomassie Blue under reducing and nonreducing conditions.

molecular weight of $M_r \sim 33,000$. C3o is a three-chain protein with an apparent molecular weight very similar to those of C3c and CVF ($M_r \sim 140,000$) (Fig. 4, left panel). The apparent molecular weights of the three polypeptide chains of C3o are $M_r \sim 72,000$, $M_r \sim 38,000$, and $M_r \sim 29,000$, respectively (Fig. 4, right panel). Since C3p is derived from the center portion of the C3 $\alpha$-chain, the $M_r \sim 72,000$ chain of C3o would have to be the unaltered $\beta$-chain of C3 while the $M_r \sim 38,000$ and $M_r \sim 29,000$ chains of C3o would have to be derived from the C3 $\alpha$-chain. Periodic acid Schiff staining revealed that the $M_r \sim 72,000$ and $M_r \sim 29,000$ chains of C3o are glycosylated (not shown) confirming the two known glycosylation sites of human C3 (Ref. 8, compare Fig. 1) and indicating that the $M_r \sim 29,000$ chain is derived from the amino-terminal portion of the C3 $\alpha$-chain. The chain structure of C3o therefore resembles that of C3c which consists of the unaltered $\beta$-chain and two $\alpha$-chain-derived fragments of $M_r \sim 42,000$ and $M_r \sim 29,000$, respectively (Fig. 4, right panel). However, the two $\alpha$-chain-derived polypeptide chains of C3o are different from those of C3c, indicating that C3o and C3c are two distinct molecules derived by differential proteolytic cleavage of the C3 $\alpha$-chain. It appears that the $M_r \sim 38,000$ chain of C3o is also present as a contaminant in the trypsin-generated C3c preparation (Fig. 4), suggesting that a bond at or near the cleavage site of the venom protease is partially susceptible to trypsin.

Fig. 6 demonstrates that C3o can be generated from C3b as well as C3, which contains the additional C3a domain. Although not shown in Fig. 6, both C3(H2O) and highly purified C3 after removal of C3(H2O) can serve as substrates for the venom protease. However, C3o cannot be generated from C3c. While the venom protease converts the $M_r \sim 42,000$ $\alpha$-chain fragment of C3c into the $M_r \sim 38,000$ chain of C3o, the $M_r \sim 29,000$ $\alpha$-chain fragment of C3c is cleaved into a chain that appears to be approximately 1,000 daltons smaller than the $M_r \sim 29,000$ chains of C3o (Fig. 6). Incubation of CVF with the venom protease does not cleave any of the chains of CVF as determined by SDS-polyacrylamide gel electrophoresis (not shown). Fig. 7 shows that the electrophoretic mobility of C3o resembles those of C3c and iC3b. This result is consistent with the observation that C3o can be generated from C3b and
protease. The \( \alpha \)-chain and the protease fragments of C3, C3b, and C3c before and after incubation with the venom protease.

Similarly, C3p is generated only from C3 and C3b. Note that the incubation of C3c with the venom protease results in conversion of the M₄ ~ 42,000 chain of C3c into the M₄ ~ 38,000 chain of C3o and of the M₄ ~ 29,000 chain of C3c into a chain approximately 1,000 daltons smaller than the corresponding M₄ ~ 29,000 chain of C3o.

**Fig. 6. Cleavage of C3, C3b, and C3c by the venom protease.** Shown is an SDS-polyacrylamide gel under reducing conditions of C3, C3b, and C3c before and after incubation with the venom protease. The \( \beta \)-chain and the \( M₄ \sim 38,000 \) and \( M₄ \sim 29,000 \) \( \alpha \)-chain fragments of C3o are generated by incubation with the venom protease of both C3 and C3b. Similarly, C3p is generated only from C3 and C3b. Note that the incubation of C3c with the venom protease results in conversion of the \( M₄ \sim 42,000 \) chain of C3c into the \( M₄ \sim 38,000 \) chain of C3o and of the \( M₄ \sim 29,000 \) chain of C3c into a chain approximately 1,000 daltons smaller than the corresponding \( M₄ \sim 29,000 \) chain of C3o.

**Fig. 7. Electrophoretic mobility of C3o.** Shown are agarose gels after immunoelectrophoresis and development with an antiserum to human C3 of normal human serum (NHS) (top), normal human serum after exhaustive complement activation by CVF which converts C3 into iC3b (55) (upper center), purified C3o (lower center), and purified C3c (bottom). Note the similar electrophoretic mobilities of iC3b, C3o, and C3c compared to native C3 indicating the removal of the basic C3a domain in all three cases. The anode is to the right.

indicates that C3o does not contain the highly basic C3a domain.

**Location of C3p and the Chains of C3o within the C3 Molecule**—To identify the exact location of the C3o chains in the C3 molecule, the three chains of C3o were separated by preparative SDS-polyacrylamide gel electrophoresis with subsequent electroelution (Fig. 8). The three chains of C3o and C3p were subjected to amino-terminal amino acid sequence analysis, and the obtained sequences were compared to the deduced amino acid sequence from the cDNA sequence of human C3. As shown in Fig. 9, the amino-terminal sequence of the \( M₄ \sim 72,000 \) chain of C3o is identical with the amino terminus of the human C3 \( \beta \)-chain, consistent with the above conclusion that this chain of C3o represents the unaltered \( \beta \)-chain of C3. The amino terminus of C3p could be identified as residue 319 (methionine) in the C3g region of the C3 \( \alpha \)-chain which is 35 residues carboxyl-terminal from the amino-terminal Factor I cleavage site of C3dg and 12 residues amino-terminal from the amino-terminal trypsin cleavage site of C3d. The amino termini of the \( M₄ \sim 29,000 \) and \( M₄ \sim 38,000 \) chains of C3o were found to be residues 88 (glutamic acid) and 683 (aspartic acid) of the C3 \( \alpha \)-chain, respectively. Accordingly, the \( M₄ \sim 29,000 \) chain of C3o is derived from the amino-terminal portion of the C3 \( \alpha \)-chain. The cleavage site of the venom protease that generates the amino terminus of the \( M₄ \sim 29,000 \) chain of C3o is 10 residues carboxyl-terminal from the cleavage site of the C3 convertases. The \( M₄ \sim 38,000 \) chain of C3o is derived from the carboxyl-terminal portion of the C3 \( \alpha \)-chain with its amino terminus being 33 residues carboxyl-terminal from the carboxyl-terminal Factor I cleavage site of C3f.

**Activation of Factor B by C3o and Factor D**—Fig. 10 shows that C3o supports activation of Factor B into Ba and Bb in the presence of Factor D and Mg²⁺. No Factor B activation occurs in the presence of EDTA or in the absence of Factor D. As can be seen by the decreased intensity of the Ba and Bd bands, the rate of Factor B activation by C3o is lower than that by C3b and CVF. In contrast to C3o, C3c does not support Factor B activation. To eliminate the possibility that the Factor B activation observed with C3o is due to trace contamination of the C3o preparation with C3(H₂O) or C3b not removed by the organomercurial column, the following control experiment was performed. Approximately 4 mg of C3(H₂O) was incubated under conditions as described under "Experimental Procedures" for the generation of C3o and C3p in the absence of the C3-cleaving protease. The material was subsequently passed over the Sephalex G-200 column, and the pool corresponding to the elution volume of C3o (containing the C3(H₂O)) was passed over the organomercurial column. The breakthrough was collected. The quantities of C3(H₂O) and C3b in this sample should be far greater than any quantity of these C3 species in the C3o preparation. However, when tested for its ability to support activation of Factor B, no Factor B activation was observed, indicating that the organomercurial column effectively removed all free sulphhydryl group-containing C3 species which could potentially induce Factor B activation (C3(H₂O), C3b).

**Enzymatic activity of the C3o,Bb enzyme** is demonstrated using a fluorogenic peptide substrate for C3 convertases. As shown in Fig. 11, the rate of cleavage of the peptide substrate by the mixture of C3o, Factor B, and Factor D is lower than that of the mixture containing CVF, Factor B, and Factor D, but significantly higher than those of the appropriate controls.
Fig. 9. Amino-terminal amino acid sequence of C3p and the three chains of C3o. Shown are the obtained amino-terminal sequences and the corresponding sequences deduced from the cDNA sequence of human C3 (8). The numbers refer to the amino acid sequence of the C3 α- and β-chains. X indicates residues that were not identified. Only one mismatch occurred at position 687 of the C3 α-chain where a serine was identified and a cysteine was predicted from the human cDNA sequence, which would represent a single nucleotide difference.

The highest peptide-cleaving activity in all of the control samples, on a molar basis, was observed for purified Bb, the active site-containing fragment of Factor B. However, the peptide-cleaving activity of the C3o/Factor B/Factor D mixture cannot be attributed to the enzymatic activity of the Bb fragment generated during the experiment. While the total amount of purified Bb in the control sample represents, on a molar basis, approximately 30% of the total amount of Factor B in the experimental sample, approximately only 5% of the Factor B in the experimental sample was cleaved into Ba and Bb during the fluorescence assay as determined by subsequent gel electrophoresis. These results strongly suggest that C3o not only supports activation of Factor B but that an enzymatically active C3o,Bb complex is formed. These results further demonstrate that the binding of C3o to Bb, like that of C3b or CVF, also causes enhanced enzymatic activity of the Bb fragment.

DISCUSSION

We have recently found and subsequently isolated a specific C3-cleaving protease from cobra venom (2, 38). Exposure of human C3 to the protease results in limited and specific proteolysis of the protein causing the formation of two major cleavage fragments termed C3o and C3p. The C3p fragment (M, ~33,000) is derived from the C3 α-chain and corresponds to the C3d fragment of human C3. The hydrolyzed intramolecular thioester of the C3 α-chain must be contained within C3p since it could be isolated by chromatography on thiol-reactive organomercurial agarose. The amino terminus of C3p was found to be residue 319 (methionine), which is in the C3g region of the molecule, 12 residues amino-terminal from the trypsin cleavage site that separates C3c and C3d.

The structure of the major cleavage product C3o superficially resembles the physiological C3 activation product C3c. Both C3o and C3c contain the unaltered β-chain and two α-chain-derived fragments from the carboxyl-terminal and amino-terminal region of the α’-chain, respectively. The M,
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We have not attempted in this study to identify the C3α + 10 fragment or the decapeptide. However, circumstantial evidence that these fragments are released includes the fact that the electrophoretic mobility of C3α resembles that of C3b, iC3b, and C5c, all of which lack the highly basic C3α domain (compare Fig. 7), as well as the observation that the Mγ ~ 29,000 α-chain fragment of C3c is converted by the venom protease into a chain with its apparent molecular mass being approximately 1,000 daltons smaller (compare Fig. 6).

Since C3α, the major cleavage product generated by the cobra venom protease, structurally resembles CVF, it was tested for its ability to support the activation of Factor B and to form an enzymatically active C3o,Bb enzyme analogous to CVF,Bb and C3b,Bb. We found that C3α is able, in the presence of Factor D and Mg2+, to support the activation of Factor B, however at a lower rate than that by CVF or C3b. Furthermore, a reaction mixture of C3α, Factor B, Factor D, and Mg2+ showed enzymatic activity toward a fluorogenic peptide substrate for C3 convertases, suggesting the formation of a bimolecular enzyme C3o,Bb. The C3o,Bb enzyme exhibited under our experimental conditions a lower enzymatic activity than the CVF,Bb enzyme. Further work will have to determine whether the lower activity of the C3o,Bb enzyme observed here is a consequence of a decreased rate of formation, an increased rate of decay-dissociation, different kinetic parameters, or any combination thereof. It should be noted that the affinity of Factor B for CVF, C3b, and C3(H2O) (47, 48) and the rate of decay-dissociation as well as the kinetic parameters of CVF,Bb, C3b,Bb, and C3(H2O),Bb (14, 15) differ, although the active site resides in each case in the common Bb subunit of all three enzymes.

In contrast to C3o, C3c cannot support the activation of Factor B. The rather minor structural differences between C3o and C3c may provide a clue as to the structural requirements for Factor B binding and activation. Since the only peptide portion present in C3o and not in C3c is the carboxyl-terminal end of the Mγ ~ 29,000 chain, one might suspect that this stretch of approximately 10 amino acids (residue 284 through at least residue 293) represents the core of the Factor B binding site. Consistent with this hypothesis is the fact that the carboxyl terminus of the γ-chain of CVF (Mγ ~ 32,000), which corresponds to the Mγ ~ 29,000 chain of C3o, also extends into the region homologous to residues 284–293 of the human C3 α-chain and beyond (Refs. 4, 7, compare also Fig. 4). On the other hand, this stretch of amino acid residues is also present in iC3b which cannot form a convertase with Factor B. Conformational or sterical differences of the Factor B binding site induced by the presence or absence of various polypeptide stretches in C3b, iC3b, or C3c may be responsible for the ability of a given C3 fragment to form a convertase or not, similar to the differential binding of Factor H to C3b and iC3b (49).

This study also identifies the cleavage sites of the C3-cleaving protease from cobra venom. They are all localized in the α-chain of human C3 between residues 87 and 88 (Glu-Glu), 318 and 319 (Gln-Met), and 682 and 683 (Lys-Asp). No charge or sequence pattern at the three cleavage sites is apparent except that an alanine residue precedes the amino-terminal amino acid residue at all three cleavage sites (residues 86, 317, and 381). While these alanine residues may be important, the very different chemical nature of the amino acid residues at the three cleavage sites suggests that the specificity of the C3-cleaving venom protease is not due to sequence specificity for the cleaved peptide bonds.

In addition to those three cleavage sites, our results suggest that there are at least two additional cleavage sites of the...
venom protease in the human C3 α-chain. The apparent molecular mass of C3p (M, ~ 33,000) is by at least 7,000 daltons too low to span the α-chain from residue 319 (amino terminus of C3p) to residue 683 (amino terminus of C3α). Similarly, the apparent molecular mass of the M, ~ 29,000 chain C3α is by at least 2,000 daltons too low to span the α-chain from residue 88 (amino terminus of the M, ~ 29,000 chain) to residue 319 (amino terminus of C3p).

Accordingly, one would have to postulate that two additional cleavage sites exist, generating the carboxyl termini of C3p and of the M, ~ 29,000 chain of C3o. These two additional cleavages would release a polypeptide of at least 2,000 daltons from the region between the M, ~ 29,000 chain and C3p and a polypeptide of at least 7,000 daltons from the region between C3p and the M, ~ 38,000 chain.

The physiological substrates of the venom protease remains to be determined. We propose that the venom protease participates in the posttranslational processing of a pro-CVF molecule (2, 38), similar to the processing of human C3 which is synthesized as a single-chain prepro-C3 (8). Support for this hypothesis is derived from the fact that the cleavage of C3α by the venom protease in the human C3 α-chain. The apparent molecular mass of the M, ~ 29,000 chain of CVF is by at least 7,000 daltons too low to span the a-chain from residue 88, chain c30 is by at least 2,000 daltons too low to span the α-chain from residue 319 (amino terminus of the 38,000 chain of c30). Similarly, the apparent molecular mass of the 38,000 chain of C3o is by at least 7,000 daltons too low to span the a-chain from residue 88 (amino terminus of the M, ~ 38,000 chain) to residue 319 (amino terminus of C3p).

This hypothesis is derived from the fact that the cleavage of the apparent molecular mass of the 29,000 chain of C3o is by at least 7,000 daltons too low to span the a-chain from residue 88; CVF &chain, residue 612; c30 chain, residue 278; C3p, residue 683. In this context, it would be interesting to construct a derivative of human C3 with the two a-chain fragments having their amino termini at the same relative positions as the CVF γ- and β-chains (residues 78 and 612, respectively). If such a “human CVF” molecule would exhibit functional properties in forming a stable C3 convertase similar to CVF, it would not only represent a valuable investigational tool to study the structure/function relationship of C3, but it could also replace CVF in conjugates with monoclonal anti-tumor antibodies which could be expected to exhibit a reduced or even absent immunogenicity (27, 30, 52).

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