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# Complement Component C2, Inhibiting a Latent Serine Protease in the Classical Pathway of Complement Activation†

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ABSTRACT: The innate immune response to infection or injury involves an antigen−antibody triggered classical pathway (CP) of complement activation, in which soluble and cell surface plasma proteins cooperatively effect elimination of foreign organisms and damaged host cells. However, protracted or dysfunctional complement activation can lead to inflammatory diseases. Complement component 2 bound to C4b is cleaved by classical (C1s) or lectin (MASP2) proteases to produce C4bC2a, a very short-lived C3 convertase ($t_{1/2}$ 2 min) that in turn cleaves C3 to C3a and C3b, leading ultimately to formation of Membrane Attack Complex (MAC) and lysis of bacteria and damaged cells. C2 has the same serine protease domain as C4bC2a but in an inactive zymogen-like conformation, requiring cofactor-induced conformational change for activity. Here, we show that C2 has catalytic protease activity in its own right above pH 7, in the absence of cofactor, processing C3 and C3-derived chromogenic peptide fragments. In contrast to the instability of C3 convertase ($t_{1/2}$ 2 min, pH 7), the C2 enzyme is indefinitely stable under alkaline conditions, facilitating studies of its catalytic properties and development of small molecule inhibitors. We characterize the catalytic activity of C2 against C3 and short paranitroanilide peptide substrates, and identify potent small molecule inhibitors of C2 that also inhibit classical pathway C3 convertase, MAC formation, and hemolysis of sensitized sheep erythrocytes. These results provide a new avenue and valuable new insights to inhibiting CP complement activation relevant to inflammatory diseases.

Human complement was originally defined as the heat-labile component of plasma that "complemented" the humoral system and aided antibody-dependent killing of bacteria (*1*). Complement is now known to be a tightly regulated proteolytic network of >30 proteins circulating in blood or attached to membrane surfaces that play crucial roles in mammalian innate immunity (*1*, *2*). Complement proteins are produced by many cell types and have diverse cooperative functions ranging from regulating antibody responses to foreign antigens, recruitment of immune cells (e.g., PMNs, macrophages) to sites of infection, opsonization of pathogens for phagocytosis, and facilitating elimination of pathogens as well as host cells damaged by injury or modified and recognized as 'nonself' by infection, necrosis, apoptosis, and inflammatory insults (*1*). Some activation fragments participate via immune cells in the adaptive immune response to infectious agents. Complement activation is tightly controlled in normal physiology, but in some circumstances (e.g., genetic mutation, metabolic disorders, chronic infection, cancer) (*19*−*21*, *23*), it becomes dysfunctional or protracted and the same properties (proinflammatory, anaphylaxis, phagocytosis, oxidation) that make complement proteins important in immune defense instead trigger tissue destruction and initiate or exacerbate disease. Despite extensive studies over many decades, the finer molecular details of complement-mediated immune defense and dysfunction remain to be fully elucidated.

Complement is activated by at least 3 routes (Figure 1); the classical (CP), mannan-binding lectin (MBL) and alternative (AP) pathways. These converge at complement component C3 which is cleaved by two distinct C3 convertase enzymes (C4b2a via CP or MBL; C3bBb via AP) to anaphylatoxin C3a and fragment C3b that amplifies the complement cascade through a feedback loop via the AP. C4b covalently binds pathogen surfaces (opsonization) before interacting with C2. The end result of the complement cascade is formation of a terminal lytic pathway, through assembly of the membrane attack complex (MAC) that facilitates phagocytic destruction of foreign organisms and damaged cells. The pivotal role of C3 in the complement cascade suggests that C3 convertase inhibitors might be able to retard complement activation. However, the complication of two convertase enzymes that cleave C3 by different pathways, the multicomponent complexity and extremely short lifetimes of each convertase, the possibility of pathway bypasses (*3*) including possible salvage paths in which other serine proteases (e.g., thrombin, trypsin, kallikrein) (*4*−*7*) might cleave complement proteins, has prevented or deterred efforts to inhibit C3 convertase. The instability of CP C3 convertase, a $Mg^{2+}$-dependent enzyme which irreversibly dissociates (with active participation by other proteins) in minutes, causes substantial difficulties in measuring activity and inhibition. Substrate-based analogues have so far failed to inhibit C3 processing by C3 convertase (*7*−*9*).

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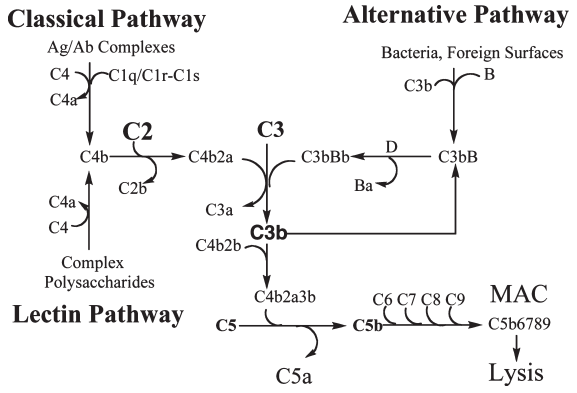


FIGURE 1: Complement activation. Activating classical or lectin pathways leads to CP C3 convertase (C4b2a). Activating the alternative path leads to AP C3 convertase (C3bBb). Both cleave C3 to C3a and C3b. C3b, which also amplifies the AP by binding factor B, enables continuation of the cascade leading to fragments that assemble into Membrane Attack Complex (MAC) and lyse bacteria and damaged host cells.

We have focused instead on better understanding C2, thought to be an inactive zymogen that possesses the same catalytic machinery as C3 convertase (C4bC2a) of the CP pathway. C2 has some structural homology with factor B (*10*, *11*), each with N-terminal triplet repeat complement control protein modules (CCP 1−3); a von-Willebrand Factor type A (vWFA) domain containing an integrin-like metal ion-dependent adhesion site (MIDAS) that may be a binding site for C4b, consistent with the requirement for $Mg^{2+}$ to form catalytically active C4bC2a; and a C-terminal serine-protease (SP) domain. However, there is only 39% sequence homology between C2 and fB (*11*, *12*), with an 8-residue conserved sequence near the SP termini in both proteins not seen in other serine proteases and that might confer similar substrate selectivity (*13*). C2 has negligible proteolytic activity on C3 at physiological pH. However, $Mg^{2+}$-dependent binding of C2 to C4b, and subsequent cleavage of C2 by C1s (CP) or MASP2 (MBL), results in C4bC2a (C3 convertase) which is a specific serine protease that cleaves C3 ($K_m$ 8.9 ± 3.9 $\mu$M, $k_{cat}$ 0.022 ± 0.005 $s^{-1}$) (*14*). The dissociated C2a has negligible protease activity (*15*). Despite an inactive zymogen-like oxyanion conformation, C2 and C2a exhibit weak esterolytic activity on activated (thiobenzyl, SBz) esters Z-Gly/Leu-Leu-Ala-Arg-SBzl. Similarly, fB and Bb cleave Z-Lys-Arg-SBzl and Z-Gly-Leu-Ala-Arg-SBzl, respectively (*16*). C2 cleaves the ester Ac-Gly-Lys-OMe ($K_m$ 1.8 × $10^{-2}$ M) (*17*). The fluorescent Boc-LeuGlyArg-AMC (AMC, amino methyl coumarin) was used to analyze kinetics of CP and AP C3 convertases ($K_m$ ~$\mu$M), and was cleaved by Factor Xa with similar $K_m$ (*18*).

Complement disorders have been associated with many inflammatory conditions (e.g., rheumatoid arthritis, lupus, ischemia-reperfusion injury, acute respiratory distress syndrome, shock, arteriosclerosis, fibrosis, inflammatory bowel diseases, psoriasis, atherosclerosis, multiple sclerosis, asthma, Alzheimer's disease, burns), but precise roles remain to be elucidated. C2 polymorphisms are linked to age-related macular degeneration and blindness (*19*), while C2 deficiency has been associated with rheumatic and autoimmune disorders and increased risk to bacterial (and viral) infections, arthritis, systemic lupus erythematosus, ischemia-reperfusion injury, atherosclerosis and schizophrenia (*20*−*23*). Recently, a $C2^{-/-}$/$fB^{-/-}$ double knockout mouse infected with *Candida albicans* showed the highest mortality rate, due to lack of complement activation (*24*). No inhibitors of C2 or C2a are known, and few modulators are known for any complement proteins. Most are based on natural complement regulators, such as recombinant factor H (inactivates AP C3 convertase), soluble CR1 (inactivates CP and AP), antibodies to components B, D, C5a and proteins that form MAC (*25*). Among small molecule inhibitors are C5a antagonists (*26*, *27*) that bind to immune cell surfaces ($IC_{50}$ 20 nM), C1s inhibitors (*28*), compstatin which binds to C3 to prevent cleavage ($IC_{50}$ 12 $\mu$M) (*29*), and FUT-175 which covalently inhibits C3 convertase ($IC_{50}$ 4 $\mu$M) and other serine proteases (*7*, *30*). Small molecule inhibitors of C2 and CP C3 convertase could be valuable physiological probes for the classical pathway *in vivo*, and for evaluating CP enzymes as therapeutic targets.

We report that C2, containing the same catalytic machinery as CP C3 convertase, has appreciable enzymatic activity and stability alone under nonphysiological alkaline conditions. This is significant because CP C3 convertase is an extremely unstable and short-lived enzyme (*15*) and difficult to study and to develop inhibitors. We characterized the catalytic activity of C2 against short peptide substrates (modified as paranitroanilides, pNA) and C3 itself, created substrate-based peptide inhibitors that block cleavage of C3 and short peptide substrates by both C2 and CP C3 convertase, and established that such inhibitors also block both formation of MAC in an immobilized ELISA format and hemolysis of isolated sheep erythrocytes, all via the classical pathway of complement activation. This work is an important advance in understanding how to develop small molecule inhibitors for the classical pathway of complement activation, something that has eluded researchers to date.

## MATERIALS AND METHODS

*Materials*. C2 and C4b and activated C1s were obtained from Calbiochem. Protein molecular weight markers for gels were purchased from Sigma-Aldrich. Fmoc protected amino acids were from Novabiochem. Gel ingredients, casting apparatus, and resolving chamber (Mini-PROTEAN Tetra Cell) were from Bio-Rad Laboratories. Proteins were visualized using colloidal Coomassie brilliant blue staining solution. Buffers were made using deionized $H_2O$, with 100 mM Tris-HCl (pH 7.5−8.5), 100 mM glycine-NaOH (pH 9−10.5), and 100 mM piperidine-HCl (pH 11−11.5). All buffers were made with 154 mM NaCl, corresponding to physiological levels of NaCl. All other chemical reagents were analytical grade from Sigma-Aldrich.

*Peptide Synthesis*. Paranitroanilide substrates (pNA) (*31*) and aldehyde inhibitors (*32*) were synthesized and characterized by standard methods (*33*), including analytical high-performance liquid chromatography, mass and NMR spectroscopy.

*Enzyme Assays*. C2 enzyme activity was assayed against short peptide substrates based on the native substrate sequence, C3, but with a chromogenic pNA group (*33*) at the P1′ position at the C-terminus. Cleavage of pNA from the synthetic peptide sequence by C2 produces a yellow color, which can be monitored at the absorbance wavelength of 405 nm. Assays were conducted in 96-well plates, using the semioptimized conditions of 100 mM glycine-NaOH buffer pH 9.5 unless otherwise stated, with 154 mM NaCl. Inhibitors were dissolved in PBS with 1% DMSO and tested at the indicated concentrations. Enzyme was suspended initially in PBS, and aliquoted to 10 $\mu$L/well, with final concentrations of 50, 75, and 100 nM. After preincubation in separate wells for 5 min at 37 °C, the reaction was initiated by mixing enzyme (and inhibitor if present) and substrate together, and the

reaction was monitored using a Fluostar Optima (BMG Labtech) microplate reader, at absorbance 405 nm. The initial velocity $V_0$ ($\mu$M/s) of the reactions was calculated as the amount of free pNA released from the average change in millioptical density per second (mOD/s). Kinetic parameters were calculated using GraphPad Prism 4 software. The kinetic parameters $k_{cat}$, $K_m$, and $k_{cat}/K_m$ were calculated assuming Michaelis−Menten equilibrium kinetics, and $v = (\text{Vmax}[S])/([S] + K_m)$. Duplicate measurements were taken for each data point, and means ± SEM are reported from duplicate or triplicate experiments as specified. Results from concentration−response curves of inhibitors are presented as % residual activity of enzyme. $IC_{50}$ is the concentration at half-maximal efficacy of the inhibitor.

*SDS-PAGE Gels*. Complement components were incubated for 12 or 24 h at 37 °C at the specified pH. After incubation, samples were denatured at 100 °C for 5 min, then loaded onto either a 7% or 12% SDS-PAGE gel. Gels were run on a Mini-Protean TETRA cell (Bio-Rad Laboratories) at 100 V for 1 h, and stained with Coomasie-blue dye. For inhibition of the CP C3 convertase, 12 $\mu$g of C4b and 6 $\mu$g of C2 were incubated with/without inhibitor for 5 min in PBS with 1 mM $Mg^{2+}$ to allow for the formation of the C3 convertase. C4b.C2 was then added to 10 $\mu$g of C3, with 0.1 $\mu$g of C1s added last to initiate the reaction, which was allowed to proceed for 30 min after which the samples were denatured and analyzed on SDS-PAGE.

*MAC Formation*. Activation of complement and measurement of terminal MAC formation via the classical pathway was assessed using Weislab Complete Complement Screen (Euro-Diagnostica, Sweden) (*34*, *35*), an ELISA-based method for detecting formation of MAC using antibodies specific for complement components involved in MAC formation. IgM coating of wells allows for the specific activation of complement via the CP, while monoclonal antibodies directed against C1q, C4, C3, and C5b−C9 detect the successive steps of the cascade and formation of terminal MAC (*34*). The assay was performed as per manufacturer's instructions, but with slight modifications to better optimize assay conditions. Briefly, human serum (Sigma-Aldrich) was diluted 1:50 in the provided assay diluent, and incubated in the presence of inhibitor over a range of concentrations. Determination of $IC_{50}$ was performed by plotting concentration of inhibitor against formation of MAC, by measuring absorbance at 405 nm. The experiment was performed three times in duplicates per plate. Results are presented as the mean ± SEM.

*Erythrocyte Hemolysis*. Red blood cell (RBC) lysis was performed using freshly isolated sheep erythrocytes (Animal Research Institute, Yeerongpilly, Queensland) in veronal buffered saline (GVB, containing 2 mM sodium barbitone, 5 mM barbituric acid, 145 mM NaCl, and 0.01% (w/v) gelatin) with 10 mM EDTA, at pH 7.4. Sheep RBCs were sensitized with rabbit anti-sheep IgG antibody (Sigma-Aldrich), to specifically activate the CP of complement, then washed in GVB containing 0.15 mM $CaCl_2$ and 1 mM $MgCl_2$ ($GVB^{2+}$). Agglutination assay was performed for each batch of sheep RBCs to determine the optimal dilution of antibody required to sensitize RBCs. Human serum (Sigma-Aldrich) with inhibitor was added to RBC solution in $GVB^{2+}$, and incubated for 30 min at 37 °C. Samples were centrifuged at 2000*g* and supernatants were collected. Lysis was monitored at 541 nm using an Ultrospec II UV/vis spectrophotometer (Biochrom, U.K.) and normalized to 100% control (no inhibitor). Data was collected from two independent experiments and are represented as mean ± SEM.

## RESULTS

*C2 Cleaves C3 at Alkaline pH*. C2 circulates in serum as an inactive zymogen at pH 7.4 and must bind to C4b before it is cleaved to C2a by C1s to form the classical pathway C3 convertase. It has also been suggested that C2 may be able to be cleaved by C1s without binding to C4b, albeit at a much slower rate (*36*). Here, we show that C2 cleaves C3 at alkaline pH in the absence of C4b and C1s. When C2 was incubated directly with C3 for 12 h at variable pH between 7.5 and 10.2, the cleavage products C3a and C3b increased in a pH dependent manner by SDS-PAGE analysis (Figure 2).

*C2 Cleaves Peptide Substrates at High pH*. C2 cleaved the chromogenic heptapeptide substrate Ac-SHLGLAR-pNA, corresponding to the nonprime side of the cleavage site in C3, in a pH dependent manner with maximal hydrolysis at pH 10 (Figure 3). Above pH 10, the rate declined, probably due to denaturation of C2. This pNA substrate did not undergo spontaneous hydrolysis under the conditions. Other factors that could influence peptide hydrolysis by C2 were briefly examined. C2 contains an integrin-like MIDAS domain which can bind $Mg^{2+}$. However, $Mg^{2+}$ (10 mM) added to the buffer at pH 9.5 caused no change in the rate of processing of heptapeptide substrate by C2. NaCl (0−250 mM), metal scavengers EDTA and EGTA (10 mM), and glycerol (0−50%) also had no effect at pH 9.5 (data not shown).

*C2 Prefers Substrates of 7−8 Residues*. The proteolytic activity of C2 was examined against synthetic peptide substrates

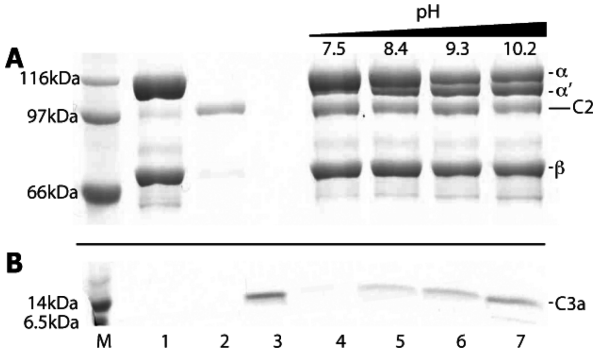


FIGURE 2: C2 cleaves C3 at alkaline pH. C3 (10 $\mu$g) was incubated with C2 (6 $\mu$g) at 37 °C in various buffers (pH 7.5, Tris; 8.4, Tris; 9.3, glycine; 10.2, glycine) for 12 h. Protein was analyzed by SDS-PAGE on (A) 7% gel and (B) 12% gel. Lane M, marker; lane 1, C3 (α-chain 115 kDa and $\beta$-chain 75 kDa); lane 2, C2 (93 kDa); lane 3, C3a (9 kDa); lanes 4−7, pH dependent cleavage of C3 to C3a (9 kDa) and C3b (α′-chain, 106 kDa; $\beta$-chain 75 kDa) by C2.

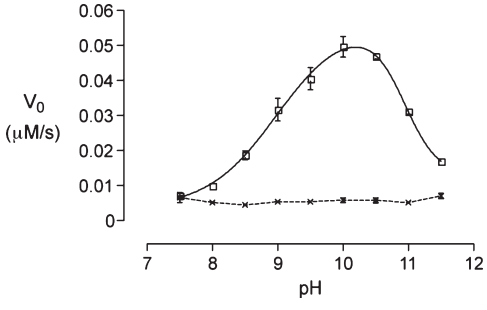


FIGURE 3: pH-dependent hydrolysis of substrate Ac-SHLGLAR-pNA by C2. C2 (50nM, □) or PBS (×) was incubated with 1 mM substrate at buffered pH with 154 mM NaCl. Initial velocity $V_0$ ($\mu$M/s) was calculated (amount free pNA released) from increase in millioptical density per second at 405 nm. Data (mean ± SEM) from $n = 3$ experiments.

(chromogenic paranitroanilides) of varying length corresponding to the native C3 sequence, ranging from the capped dipeptide Z-KR-pNA to the 10-mer Ac-ARASHLGLAR-pNA. Z-KR-SBzl, with a thio-benzyl substituent at position P1′, is reportedly a substrate for factor B (*16*) but not hydrolyzed by C2 or C2a (*16*). However, Figure 4 shows that C2 (50 nM) does slowly process Z-KR-pNA, as well as peptides of 4−6 and 9−10 residues at comparable rates, but processes the 7-mer Ac-SHLGLAR-pNA (**1**) and 8-mer Ac-ASHLGLAR-pNA (**2**) up to 3- to 4-fold faster ($V_0$ 0.03−0.04 $\mu M \cdot s^{-1}$, 50 nM C2, pH 9.5, [S] = 1 mM).

*Enzyme Kinetics for Substrate Processing*. $K_m$ and $k_{cat}$ values were measured (Table 1) for hydrolysis by C2 of different concentrations of heptapeptide Ac-SHLGLAR-pNA (**1**) and octapeptide Ac-ASHLGLAR-pNA (**2**) (Figure 5), with classic Michaelis−Menten kinetics exhibited. Structure−activity relationship studies (not shown) subsequently led us to a shorter substrate sequence of six residues, namely, Ac-RLLLAR-pNA (**3**). Relative to **1**, substrate **3** had a lower $k_{cat}$, $K_m$ improved 4-fold (0.93 mM), and the catalytic efficiency ($k_{cat}/K_m$) for substrate **3** was improved 2.5-fold (2093 $M^{-1}$ $s^{-1}$).

*Substrate-Based Inhibitors of C2*. The aldehyde Ac-SHLGLAR-H **4**, derived from heptapeptide substrate **1**, was an inhibitor (Figure 6) of C2 ($IC_{50}$ 4.2 ± 0.6 $\mu$M) under alkaline conditions (pH 9.5). The smaller hexapeptide aldehyde, Ac-RLLLAR-H **5**, derived from the substrate **3** with the lowest $K_m$ (Table 1), was an even more potent, competitive, and reversible inhibitor of C2 ($IC_{50}$ 0.33 ± 0.06 $\mu$M, Figures 6 and 7). In addition, we also show that C2 processing of its native substrate C3 can be inhibited by compound **5** (Figure 8) with an estimated $IC_{50}$ of 0.5−1.0 $\mu$M, indicating that **5** is also competitive with the protein C3.

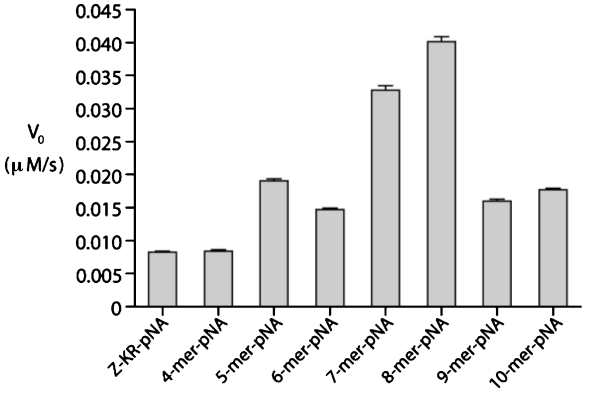


FIGURE 4: Effect of substrate length on catalysis by C2. C2 (50 nM) was incubated in 100 mM glycine-NaOH buffer pH 9.5 (154 mM NaCl, 37 °C) with substrates (1 mM): (Z-KR-pNA (2-mer), Ac-GLAR-pNA (4-mer), Ac-LGLAR-pNA (5-mer), Ac-HLGLAR-pNA (6-mer), Ac-SHLGLAR-pNA (7-mer), Ac-ASHLGLAR-pNA (8-mer), Ac-RASHLGLAR-pNA (9-mer), Ac-ARASHLGLAR-pNA (10-mer). Initial velocity $V_0$ ($\mu$M/s) was calculated (amount free pNA released) from increase in millioptical density per second at 405 nm. Data from $n$ = 3 experiments (mean ± SEM).

*Inhibition of CP C3 Convertase*. The more potent inhibitor of C2, compound **5**, was next assessed by SDS-PAGE (Figure 9) for inhibition of CP C3 convertase, assembled from recombinant C4b and C2 proteins and incubated with substrate C3. This enzyme has a very short half-life of 2 min (*15*). Compound **5** inhibited CP C3 convertase at micromolar ($\mu$M) concentrations, shown by formation of cleavage products, C3 α′ chain (Figure 9A) and C3a (Figure 9B). The inhibitor is competitive and reversible and, thus, directed at the substrate-binding active site of the enzyme.

*Inhibition of MAC Formation*. The ability of compounds **4** and **5** to inhibit C3 convertase specifically formed via the classical pathway was further analyzed using a commercially available ELISA plate with precoated IgM in the wells to specifically activate the CP. Monoclonal antibodies directed against C1q, C4, C3, and C5b−C9 allow detection of MAC produced solely via the CP, thus, enabling measurement of

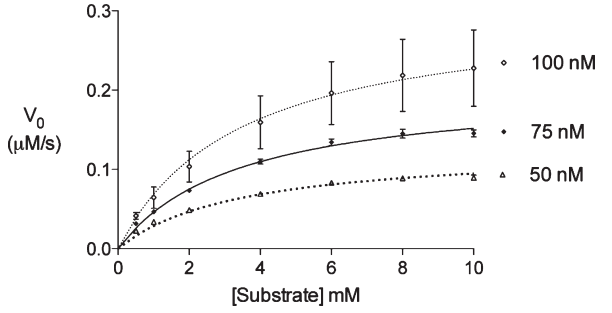


FIGURE 5: Hydrolysis of heptapeptide substrate Ac-SHLGLAR-pNA by C2. Varying concentrations of substrate were incubated with 50 nM (△) 75 nM (▲) and 100 nM (◇) C2, in 100 mM glycine-NaOH buffer pH 9.5, with 154 mM NaCl, at 37 °C. Initial velocities $V_0$ ($\mu$M/s) were calculated as amount free pNA released by increase in millioptical density per second at 405 nm. Data is from $n$ = 3 experiments, except for 100 nM ($n$ = 2;) mean ± SD.

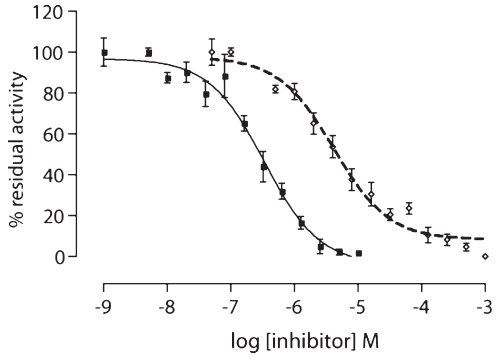


FIGURE 6: Concentration-dependent inhibition of C2 by compound **4**, Ac-SHLGLAR-H (○) and compound **5**, Ac-RLLLAR-H (■). C2 (75 nM) was incubated with inhibitor over a concentration range, in 100 mM glycine-NaOH buffer, pH 9.5, at 37 °C, with 0.5 mM substrate **1**.

Table 1: Kinetic Parameters for Processing of Substrates by C2 at pH 9.5, 37 °C[a]

| substrate[b] | $k_{cat}$ ($s^{-1}$) | $K_m$ (mM) | $k_{cat}/K_m$ ($M^{-1}$ $s^{-1}$) |
|---|---|---|---|
| (**1**) Ac-SHLGLAR-pNA (7 mer) | 2.80 ± 0.29 | 3.41 ± 0.52 | 838 ± 47 |
| (**2**) Ac-ASHLGLAR-pNA (8 mer) | 4.28 ± 0.12 | 5.24 ± 0.06 | 817 ± 14 |
| (**3**) Ac-RLLLAR-pNA (6 mer) | 1.95 ± 0.19 | 0.93 ± 0.20 | 2093.4 ± 251.7 |

[a]Data from $n$ = 3 experiments is presented as mean ± SEM. [b]Concentration of C2 was 50nM.

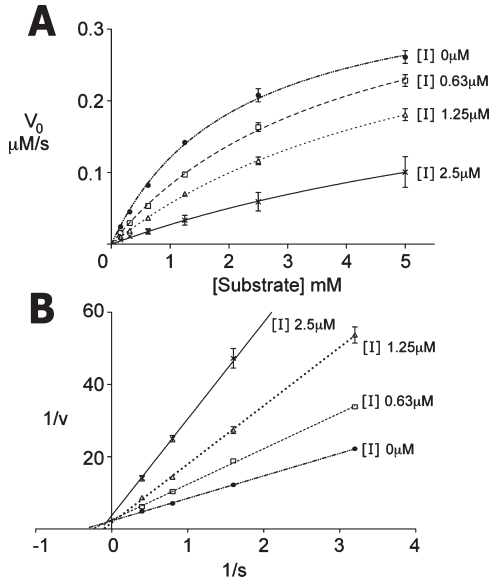


FIGURE 7: Reversible competitive inhibition of C2 by compound **5**, Ac-RLLLAR-H. C2 (75 nM) was incubated in 100 mM glycine-NaOH buffer, pH 9.5, with various concentrations of substrate **1** and the following concentrations of inhibitor: (●) 0 $\mu$M; (□) 0.63 $\mu$M; (△) 1.25 $\mu$M; and (×) 2.5 $\mu$M. (A) Michaelis−Menten kinetics and (B) double reciprocal Lineweaver−Burk plot showing a *y*-intersect indicative of competitive inhibition. Data from $n = 3$ independent experiments presented as mean ± SEM.

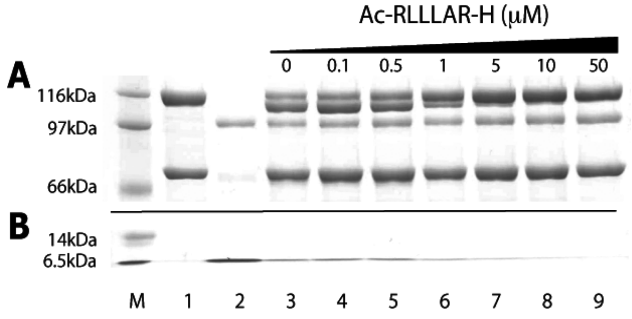


FIGURE 8: Processing of C3 by C2 is inhibited by compound **5** (Ac-RLLLAR-H). C2 (6 $\mu$g) was incubated with C3 (10 $\mu$g) along with varying concentrations of inhibitor **5**, at pH 10.2 in glycine-NaOH buffer. The reaction was allowed to proceed for 24 h at 37 °C to obtain maximal cleavage of C3. Proteins were analyzed by SDS-PAGE on either 7% (A) or 12% (B) gels. Lane M, marker; lane 1, C3 with α-chain (115 kDa) and $\beta$-chain (75 kDa); lane 2, C2 (93 kDa) in panel A and C3a (9 kDa) in panel B; lanes 3−9 show that formation of cleavage products C3a (9 kDa) and C3b (α′-chain, 106 kDa; $\beta$-chain, 75 kDa) is inhibited in a dose-dependent manner.

inhibition of terminal MAC formation (*34*, *35*). Compounds **4** and **5** were found to inhibit formation of MAC via the CP ($IC_{50}$ 13.3 and 7.3 $\mu$M, Figure 10).

*Inhibition of Erythrocyte Hemolysis*. Red blood cell hemolysis is a normal end point for monitoring the terminal stage of complement activation. Antibody-sensitized sheep erythrocytes have been shown to be highly susceptible to complement-mediated lysis, due to a more efficient utilization of C2 bound on the cell surface (*37*). Inhibitors **4** and **5** were assessed for their capacity to block hemolysis. Consistent with results above, compound **5** ($IC_{50}$ 28.9 ± 6.6 $\mu$M) was a more potent inhibitor of sheep RBC hemolysis than compound **4** ($IC_{50}$ 141 ± 45 $\mu$M), supporting inhibition of the CP C3 convertase mediated pathway (Figure 11).

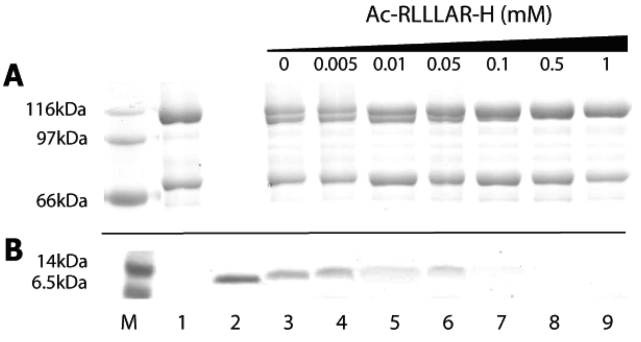


FIGURE 9: CP C3 convertase is inhibited by compound **5** (Ac-RLLLAR-H). C3 convertase was assembled in the presence of increasing concentrations of inhibitor **5** Ac-RLLLAR-H. C3 convertase was added to substrate C3, and incubated for 30 min at 37 °C in PBS with 1 mM $MgCl_2$ at pH 7.4. Proteins were analyzed by SDS-PAGE on either 7% (A) or 12% (B) gels. Lane M, marker; lane 1, C3 with α-chain (115 kDa) and $\beta$-chain (75 kDa); lane 2, C3a (9 kDa); lanes 3−9 show that formation of cleavage products C3a (9 kDa) and C3b (α′-chain, 106 kDa; $\beta$-chain, 75 kDa) is inhibited in a dose-dependent manner.

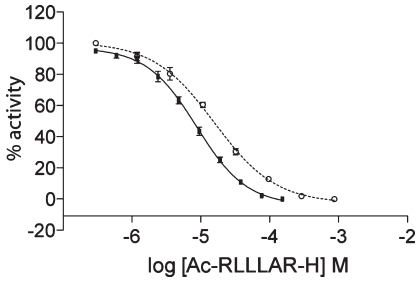


FIGURE 10: Inhibition of CP-mediated MAC formation by compound **4** Ac-SHLGLAR-H (○) and compound **5** Ac-RLLLAR-H (■) using a commercial ELISA, at pH 7.4. Measurements were taken at absorbance of 405 nm.

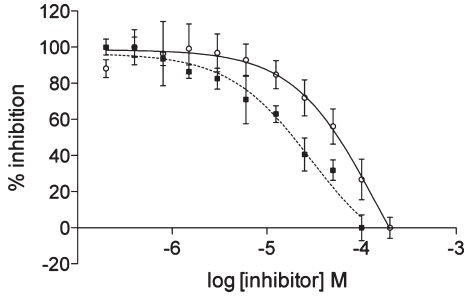


FIGURE 11: Inhibition of sheep red blood cell lysis by compounds **4** (Ac-SHLGLAR-H) and **5** (Ac-RLLLAR-H). Antibody-sensitized sheep red blood cells were incubated with various concentrations of compounds **4** (○) or **5** (■) with human serum for 30 min in $GVB^{2+}$ buffer, pH 7.4 (see Materials and Methods) at 37 °C. The degree of hemolysis measured at A541 nm is expressed as % of control (no inhibitor). Data from two independent experiments in duplicate, presented as mean ± SD.

## DISCUSSION

The classical pathway (CP, Figure 1) of complement activation is usually initiated by binding of foreign antigens to complementarity determining loops of immunoglobulins IgG and IgM, and the Fc site of these antibodies then binds to C1q, one of 3 components of the C1 complex (C1q, C1r, C1s). Following activation of C1r, then C1s, the resulting activated C1 cleaves C4 to C4a and C4b (Figure 1). C4b deposits on the membrane of

the pathogen, where it binds C2 that is subsequently cleaved by C1s to produce CP C3 convertase, C4bC2a. Subsequently, this cleaves C3 to C3a and C3b, the latter binding to C4bC2a on the membrane to produce C5 convertase (C4bC2aC3b) leading to the terminal lytic stage of complement activation (Figure 1). Complement proteins are further regulated by proteins that inhibit or degrade them, such as C1-inhibitor, complement receptor-1 (CR-1), and C4b binding protein (C4BP) (*26*).

In this study, we have found that C2 can process C3 and synthetic peptide substrates (under nonphysiological, alkaline conditions) without the need for either C4b or activating SPs from the classical (C1s) or lectin (MASP2) pathways. C2 contains the catalytic SP subunit for the CP C3 convertase. This protease is an inactive zymogen at pH 7.4, but becomes proteolytically active when attached to a specific cofactor C4b to form the functional but very short-lived C3 convertase of the CP. We suggest that, under alkaline conditions, C2 likely undergoes a pH-dependent structural change to an active conformation that can directly recognize and cleave C3. The recent crystal structures for the catalytic SP subunits C2a (and Bb of the AP) bound to $Mg^{2+}$ used crystals obtained at pH 7 (*39*−*41*), where the enzymes are catalytically inactive without cofactor. The structure for C2a shows a chymotrypsin-like fold, a catalytic triad with Asp, His and Ser in appropriate positions for catalysis to ensue, but with loops adjacent to the oxyanion hole which are shorter or missing. This resulting oxyanion hole conformation is abnormal for serine proteases, where the key oxyanion hole-forming residues are directed into a $3_{10}$-helix instead of the $\beta$-turn usually present in SPs. We surmise that this is the reason for lack of catalytic activity of C2 at physiological pH, where cofactor binding is needed to induce catalysis, probably by altering the conformation of the enzyme perhaps in the oxyanion hole region. A comparison of structures of other trypsin-like serine proteases has indicated the importance of a stabilizing water molecule that interacts with oxyanion hole residues (*42*). Enzymes with a productive oxyanion hole conformation in the active state involved either a water molecule or an oxygen atom from the bound inhibitor/substrate to stabilize the protein complex, while those with an inactive oxyanion hole conformation lacked this stabilizing effect, and in the case of Factor B, the catalytic serine residue may prevent binding of a hydrogen-binding molecule.

Here, we have been able to generate semioptimal catalysis conditions using a chromogenic assay to profile the enzymatic activity of C2 on its own using a limited set of peptide substrates. We found that C2 optimally processed synthetic peptide substrates (e.g., Ac-SHLGLAR-pNA, **1**) as well as native C3 at pH 10 (Figures 2 and 3) and that, among putative short peptide substrates examined, hepta- or octapeptides were optimal for cleavage (**1** and **2**, Figure 4). Interestingly, we found (Table 1) a 6-fold increase in $K_m$ for C2 from octa- to hexapeptide substrates (**2**, Ac-ASHLGLAR-pNA, 5.24 mM; **1**, Ac-SHLGLAR-pNA, 3.41 mM; **3**, Ac-RLLLAR-pNA, 0.93 mM), indicating the possibility at the S6 position of negative or polar enzyme residues that accommodate the Arg side chain at the P6 position of the substrate.

This difference in $K_m$ encouraged us to modify C2 substrates **1** and **3**, replacing their C-terminal pNA substituents with aldehyde, for conversion to the first small molecule C2 inhibitors, Ac-SHLGLAR-H (**4**, $IC_{50}$ 4.2 $\mu$M) and Ac-RLLLAR-H (**5**, $IC_{50}$ 0.33 $\mu$M) (Figures 6 and 7). Compound **5** also inhibited processing of C3 by C2 at pH 10 (Figure 8) and by the CP C3 convertase (Figure 9). Consistent with these results, inhibitors **4** and **5** were found to inhibit MAC formation by CP complement components immobilized on a solid support (Figure 10), as well as inhibiting human CP-mediated lysis of sheep erythrocytes (Figure 11). Together, these results unambiguously establish that the inhibitors of C2 protease activity at pH 10 also inhibit the classical pathway of complement activation at pH 7.4 leading all the way to lysis. Little is known about the conformation C2 adopts when bound to its cofactor to form the convertase, and structural changes which occur may contribute to the loss of potency of the compounds as seen across the different assays used. However, compound **5** was consistently more potent than **4**, showing at least some optimization of our lead.

In conclusion, the results show that a chromogenic assay using small peptide substrates can be used to study catalysis by two serine proteases of the classical pathway of complement activation. We have also described small molecule inhibitors derived for C2 and CP C3 convertase. These are important advances in the field for several reasons. First, the measurement of activity or inhibition of CP C3 convertase is very difficult because of the very short lifetime of the enzyme. On the other hand, C2 at nonphysiological pH is quite stable and catalytically active. Second, no C2-specific assay has been reported before and so no potent small molecule inhibitors are known. Third, the few substrate based analogues previously examined reportedly failed to inhibit processing of C3 by C3 convertase (*7*−*9*). The availability of reversible substrate-based competitive inhibitors that could stabilize the active conformation of C2, in conjunction with a pH-promoted higher processing activity, suggests a new approach to obtain crystal structures of C2 and CP C3 convertase in active conformations. Fourth, only a few inhibitors of C3 convertases are known, but there are no reports at all of small molecule inhibitors of C3 convertase of the classical pathway. This work presents new clues and a new avenue to pursue development of small molecule inhibitors that could be valuable for probing physiological and pathophysiological roles of C2 and C3 convertase *in vivo*, and for evaluating these enzymes as possible therapeutic targets for new complement-based drugs.

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