Importance of the Prime Subsites of the C1s Protease of the Classical Complement Pathway for Recognition of Substrates†

Grace O'Brien,‡,§,|| Noelene S. Quinsey,‡,|| James C. Whisstock,‡,||,⊥ and Robert N. Pike*,‡,||,⊥

Department of Biochemistry and Molecular Biology and Victorian Bioinformatics Consortium, Monash University, Clayton, Victoria 3800, Australia, Bioplasma Division, CSL Limited, Broadmeadows, Victoria 3047, Australia, and Cooperative Research Centre for Vaccine Technology, Brisbane, Queensland 4000, Australia

Received August 21, 2003; Revised Manuscript Received October 5, 2003

ABSTRACT: The classical complement pathway, which plays a vital role in preventing infection, is initiated by the action of the serine proteases C1r and C1s. We have examined the hydrolysis of substrates representing cleavage sequences in the physiological substrates for C1s, C2 and C4. These studies showed that the $P_1'-P_4'$ substrate residues of C2 and C4 conferred greater affinity of substrate for enzyme and also induced a sigmoidal dependence of enzyme velocity on substrate concentration. This indicates that the substrate gave rise to homotropic positive cooperative behavior in the enzyme. When C1s was in complex with C1q and C1r, as would occur under physiological conditions, the same behavior was observed, indicating that this mechanism is relevant in the complement pathway in vivo. We further investigated the requirements of C1s for prime side amino acids by examining a substrate library in which each of the $P_1'-P_4'$ positions had been substituted by different classes of amino acids. This revealed that the P_1' position was a major determinant of the selectivity of the enzyme, while certain substitutions at the $P_1'-P_4'$ positions abolished the allosteric behavior, indicating that contact residues at these positions in the C1s enzyme must mediate the cooperativity. The studies reported here highlight the importance of prime subsites in C1s for interaction with its cognate substrates in the complement pathway and therefore yield greater understanding of the mechanism of interaction between this vital protease and its physiological substrates.

The complement system plays an essential role in host defense mechanisms against infectious agents and in the inflammatory process. The activation of the complement system is achieved through the classical pathway, the alternative pathway, or the mannose binding lectin (MBL) pathway. All three pathways share a common terminal reaction sequence that leads to the generation of a membrane attack complex (MAC).

Normal activation of the classical pathway is mediated through the binding of specific antibodies to non-self-determinants. Initiation of the cascade occurs when the C1 complex binds to the Fc region of the antibody, triggering a number of events. C1q, the first complement component, undergoes a conformational change upon binding to the antibody. This conformational change induces the autoactivation of C1r, which is then able to cleave C1s (*1*, *2*). Activated C1s cleaves complement components C4 and C2 to generate the C3 convertase (C4b2a) (*3*, *4*). C3 convertase acts to cleave C3 to form C3b, which in turn attaches to C3 convertase to form the trimolecular complex C5 convertase (C4bC3bC2a). The cleavage and activation of C5 by C5 convertase initiates assembly of the MAC, which, upon formation, attaches itself to the membrane of the initiating antigenic determinant, resulting in osmotic cell lysis (*5*).

Here we report the use of substrates based on the cleavage sequences in the physiological substrates of human C1s, C2 and C4, to investigate the kinetic parameters of the interaction of these substrates with the active site of the enzyme. These studies showed that the prime side residues in these substrates mediated a large increase in affinity for C1s and also induced positive cooperativity in the enzyme. A substrate library based on the C4 P_4-P_4' [1] sequence was therefore synthesized to investigate the role of the prime side residues with respect to affinity and the observed cooperative behavior.

EXPERIMENTAL PROCEDURES

Materials. Fluorescent quenched substrates [C2 P_4-P_4' substrate (2Abz-Ser-Leu-Gly-Arg-Lys-Ile-Gln-Ile-Lys(Dnp)-NH_2) and C4 P_4-P_4' substrate (2Abz-Gly-Leu-Gln-Arg-Ala-Leu-Glu-Ile-Lys(Dnp)-NH_2)] and coumarin substrates [C2 P_4-P_1 substrate (Z-Ser-Leu-Gly-Arg-NHMec and C4 P_4-P_1 substrate (Z-Gly-Leu-Gln-Arg-AMC)] were synthe-

† This work was supported by the CRC for Vaccine Technology. J.C.W. is an NHMRC Senior Research Fellow and Monash University Logan Fellow.

* To whom correspondence should be addressed. Phone: +61 3 9905 3923. Fax: +61 3 9905 4699. E-mail: rob.pike@med.monash.edu.au.

‡ Department of Biochemistry and Molecular Biology, Monash University.

§ CSL Limited.

|| Cooperative Research Centre for Vaccine Technology.

⊥ Victorian Bioinformatics Consortium, Monash University.

[1] Abbreviations: *h*, Hill cooperation coefficient; NHMec, aminomethylcoumarin group; Abz, 2-aminobenzoyl; Lys(Dnp), lysine dinitrophenyl. The nomenclature for residues within the reactive site loop is based on that outlined by Schecter and Berger (1976) (*22*) for the substrates of proteases. The residues are numbered from the cleaved bond (P_1-P_1') as follows: $P_n-...-P_4-P_3-P_2-P_1-P_1'-P_2'-P_3'-P_4'-...-P_n'$.

10.1021/bi035507b CCC: $25.00
Published on Web 11/22/2003

sized at greater than 80% purity by Auspep (Melbourne, Victoria, Australia). Substrate library peptides were designed by substituting the P_1', P_2', P_3', or P_4' amino acid of the C4 P_4-P_4' sequence with Ala, Thr, Leu, Pro, His, Lys, Gln, Phe, Glu, or Met residues. The substrate library peptides were synthesized at greater than 70% purity by Auspep. Human C1s (activated two-chain) was purchased from Calbiochem (San Diego, CA) and made to 1 mg/mL using sterile laboratory-grade water. Human C1, human C1r (activated two chain), and human C1s (activated two-chain) were purchased from Calbiochem. Human C1 inhibitor and plasma kallikrein were purchased from Sigma (Sydney, New South Wales, Australia).

Determination of Enzyme Active Site Concentration. Human plasma kallikrein, which had previously been active site titrated, was used to determine the active concentration of C1 inhibitor. Once titrated, the C1 inhibitor was used to determine the active concentration of C1s.

Preparation of Substrate Stock Solutions. C4 P_4-P_1 substrate (5 mg) was resolubilized in 50 μL of dimethyl sulfoxide (DMSO) and made up to a final concentration of 10 mM using sterile laboratory-grade water. C2 and C4 substrates (2 mg) were resolubilized in 50 μL of dimethylformamide (DMF) and made up to a final concentration of 2 mM using sterile laboratory-grade water. Substrate library peptides were also resolubilized in 50 μL of DMF and made up to a final concentration of 2 mM using sterile laboratory-grade water. The concentration of the fluorescent quenched substrate stocks were determined by using an extinction coefficient $A_{360nm}(1\%) = 10000$.

Coumarin Substrate Assay. All assays were conducted at 37 °C. A range of substrate (Z-GLQR-AMC) dilutions, performed in triplicate, were prepared in fluorescent assay buffer (FAB; 50 mM Tris, 150 mM NaCl, 0.2% PEG 8000, 0.02% NaAzide, pH 7.4), and 100 μL was added to the wells of an assay plate. The plate was incubated at 37 °C for several minutes. C1s (1 mg/mL) was diluted to 10 μg/mL in FAB and incubated at 37 °C for several minutes. A 100 μL sample of enzyme was then added to each substrate dilution and the rate of increase in fluorescence measured immediately on a BMG Technologies FluoStar Galaxy fluorescent plate reader using an excitation wavelength of 370 nm and an emission wavelength of 460 nm.

Fluorescent Quenched Substrate Assays. All assays were conducted at 37 °C. A range of substrate dilutions, performed in triplicate, were prepared in FAB, and 100 μL was added to the wells of an assay plate. The plate was incubated at 37 °C for several minutes. C1s (1 mg/mL) was diluted to 10 μg/mL in FAB, unless otherwise indicated, and incubated at 37 °C for several minutes. A 100 μL sample of enzyme was then added to each substrate dilution and the rate of increase in fluorescence measured immediately on a BMG Technologies FluoStar Galaxy fluorescent plate reader using an excitation wavelength of 320 nm and an emission wavelength of 420 nm. To determine whether C1s in complex with the other complex components behaved the same as C1s alone, the C1 complex (1 mg/mL) was also used at a concentration of 71.8 nM as a source of C1s enzyme in this assay. The reaction using C1 was performed in the presence of 150 μM $CaCl_2$. The ability of C1r to cleave this substrate was also analyzed as a control.

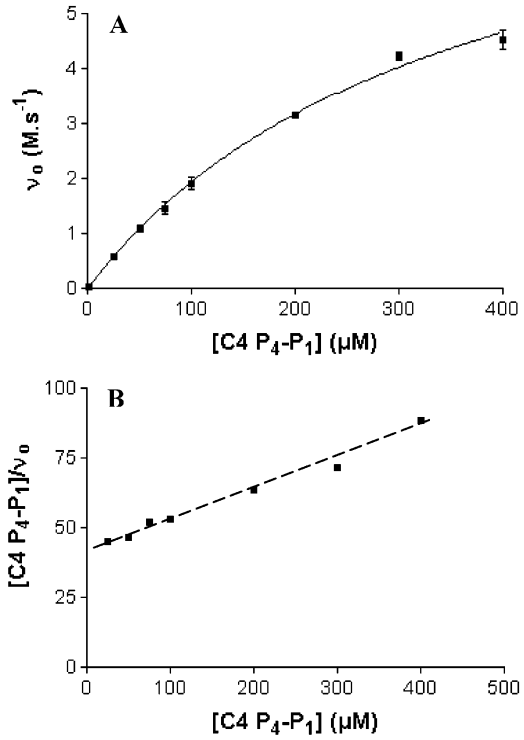

FIGURE 1: Cleavage of C4 P_4-P_1 substrate by human C1s. Kinetic analysis was performed as described in the Experimental Procedures. C4 P_4-P_1 substrate (1.0−400 μM) was incubated in the presence of active C1s (9.5 nM) for 40 min at 37 °C, during which time the increase in fluorescence was measured. The curves show (A) a Michaelis−Menten curve where initial velocity (v_o) is plotted against substrate concentration ([C4 P_4-P_1]) and (B) a Hanes plot where substrate concentration/initial velocity ([C4 P_4-P_1]/v_o) is plotted against substrate concentration ([C4 P_4-P_1]).

Determination of Steady-State Reaction Constants. The initial reaction rate was estimated at a single concentration of enzyme from triplicate measurements over a range of substrate concentrations. To determine steady-state reaction constants (K_m, k_{cat}, k_{cat}/K_m, V_{max}, $K_{0.5}$, h [Hill coefficient], and $k_{cat}/K_{0.5}$), the experimental results were fitted, using the GraphPad Prism Version 3.0 computer program, to the Michaelis−Menten single site binding equation ($V = V_{max}\{[S]/([S] + K_m)\}$, which explains the relationship between reaction rate and substrate concentration, or an equation describing positive cooperativity ($V = V_{max}[S]^h/[S]^h + [K_{0.5}]^h$), which defines the relationship between reaction rate and substrate concentration when more than one binding site applies. Hanes plots ([S]/V versus [S]) were also drawn to confirm the kinetic analyses. Plots of residual values for fits to the Michaelis−Menten equation or the equation for positive cooperativity were carried out to further resolve the best fitting of the data.

Activation of the C1 Complex. Spontaneous activation was performed according to the method of Illy et al. (*6*) by incubating the complex at 37 °C for 30 min.

RESULTS

Activity of C1s against P_4-P_1 Substrate. The kinetic parameters for cleavage of the C4 and C2 P_4-P_1 substrates by C1s were determined. The reactions followed classic Michaelis−Menten kinetics and thus demonstrated normal rectangular hyperbolic curves (see Figure 1A for results with the C4 substrate). The resultant Hanes plots were found to

Table 1: Kinetic Constants for the Proteolytic Activity of C1s on Four Synthetic Substrates Based on Cleavage Sequences in the Complement Proteins C2 and C4[a]

	Michaelis−Menten kinetics			allosteric enzyme kinetics			
substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM/s)	$K_{0.5}$ (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM/s)	h
C4 (P_4-P_1)	358.10	0.38	1.06				
C4 (P_4-P_4')				10.8	2.1×10^{-4}	0.019	1.96
C2 (P_4-P_1)	136.9	12.89	94.2				
C2 (P_4-P_4')				15.60	1.39×10^{-3}	0.089	1.40

[a] All values had errors of less than 10%.

be linear and therefore confirmed the operation of a single binding site (Figure 1B). The values for the Michaelis−Menten constant (K_m), turnover number (k_{cat}), and specificity (k_{cat}/K_m) for hydrolysis of the C4 and C2 P_4-P_1 substrates catalyzed by C1s are presented in Table 1. C1s demonstrated a K_m value for the C4 P_4-P_1 substrate of 358.1 μM, while the K_m value for the C2 P_4-P_1 substrate was somewhat lower at 136.9 μM.

Activity of C1s against P_4-P_4' Substrates. The kinetic parameters for C2 and C4 P_4-P_4' substrate cleavage by active C1s were investigated. In the example of the C4 substrate, the kinetics did not conform to Michaelis−Menten kinetics, as judged by the poor fit of the nonlinear regression line specified by the Michaelis−Menten equation (Figure 2A) and the nonrandom residuals (Figure 2B). Instead, the data were best fitted by an equation describing a sigmoidal relationship between initial velocity and substrate concentration, indicative of cooperative behavior in the interaction between enzymes and substrates (Figures 2A,C). Further, the Hanes plot for the data described an upward facing curve instead of a linear one (Figure 2D). The curve highlights a digression from normal Michaelis−Menten kinetics and suggests that C1s exhibits positive cooperativity. Similar results were obtained for the C2 P_4-P_4' substrate.

The values for the half-saturation constant ($K_{0.5}$), k_{cat}, $k_{cat}/K_{0.5}$, and Hill coefficient (h) for hydrolysis of the C4 and C2 P_4-P_4' substrates catalyzed by C1s were calculated from the fit to the equation for cooperativity and are presented in Table 1. In both cases, C1s demonstrated a much lower $K_{0.5}$ value for interaction with the P_4-P_4' substrate compared to the P_4-P_1 substrate. Both enzyme−substrate reactions displayed Hill coefficients greater than 1, confirming the positive cooperative behavior.

Proteolytic Activities of C1-Complex-Associated C1s. The kinetic parameters for C4 P_4-P_4' substrate cleavage by C1-complex-associated C1s were investigated. Analysis of the data fits (not shown) once again showed that C1-complex-associated C1s displayed positive cooperativity in the cleavage of the C4 P_4-P_4' substrate and displayed an h value greater than 1. The values of $K_{0.5}$, k_{cat}, $k_{cat}/K_{0.5}$, and h for hydrolysis of the C4 P_4-P_4' substrate catalyzed by complex-associated C1s are presented in Table 2. Association of the C1 complex occurs in a calcium-dependent manner; thus, C1s alone was analyzed under the same conditions. The presence of calcium increased the $K_{0.5}$ value somewhat and also increased the k_{cat} value. For the C1 complex, the $K_{0.5}$ value was decreased and the k_{cat} substantially increased; thus, C1s in the C1 complex was more efficient at cleaving this substrate. As expected, C1r did not cleave this substrate (data not shown), and therefore did not contribute to the reaction catalyzed by the C1 complex.

Analysis of Positive Cooperativity Using Truncated C4 P_4-P_4' Substrates. We attempted to delineate which prime side position caused the positive cooperativity and higher affinity seen for C1s by synthesizing a set of three substrates which had been truncated such that they ended at P_1', P_2', and P_3' and compared the kinetics of cleavage with that for the C4 P_4-P_4' substrate (Table 3). The substrate with an Ala residue at P_1' displayed strong positive cooperativity and increased affinity compared to the P_4-P_1 substrate, the latter very similar to that found for the P_4-P_4' substrate, indicating that the P_1' residue must be mediating most of the effects seen overall for the substrate containing all of the prime side residues. Substrates with further residues at P_2' and P_3' did not display positive cooperativity, and the affinity constant increased. One drawback of these substrates is that the bulky Lys(Dnp) quenching residue would be expected to sit at positions consistent with P_2', P_3', and P_4' in the P_1', P_2', and P_3' truncated substrates, respectively. This substrate set therefore indicated that the P_1' position may be very important, but it is also likely that the positioning of the Lys(Dnp) group might have negatively affected the overall interaction of the enzyme with these substrates.

Substrate Library Studies. We further investigated the importance of each prime side position by examining the cleavage of a library of substrates with substitutions at each position from P_1' to P_4'. All results were uniformly analyzed using nonlinear regression fitting to a sigmoidal plot. In cases where h is close to 1.0, the reaction is likely to proceed under normal Michaelis−Menten kinetics. Prior to substitution of the Lys residues in the prime side positions, we showed that a substrate in which the P_1 Arg residue was substituted for a Lys residue was not cleaved even at high substrate and enzyme concentrations, indicating that we would not be introducing secondary cleavage positions by substituting Lys residues in the prime side positions.

Substitutions of amino acids of the different classes at the P_1' position gave rise to quite small changes in $K_{0.5}$ (Table 4), although it is notable that a change from the Ala residue to a His residue abolished the positive cooperativity and increased the $K_{0.5}$ affinity constant 7.6-fold. The substitution of a Pro residue at P_1' yielded a substrate which could not be cleaved even at high enzyme or substrate concentrations. Substitutions at P_1' did give rise to large changes in k_{cat} values, particularly with the substitution of a Thr residue, which gave rise to an 8.9-fold increase in k_{cat} compared to the base C4 substrate. This yielded a substrate which had the second highest $k_{cat}/K_{0.5}$ value of any substrate in the library (Tables 4 and 5). The lowest k_{cat} in the P_1' library was for the substitution to the negatively charged Glu residue. Overall, on the basis of the $k_{cat}/K_{0.5}$ value, the selectivity for the P_1' position was 107, which was the highest of any position tested.

At the P_2' position, selectivity (factor of 50.6) was lower than at the P_1' position (Table 4). The biggest effect on $K_{0.5}$ was registered by the change to an Ala residue, with the substitutions to a Pro residue also having a measurable effect. Changes to Glu or Gln had surprisingly little effect on $K_{0.5}$, although the change to a Glu residue did significantly decrease k_{cat}, as did the substitution with a Pro residue.

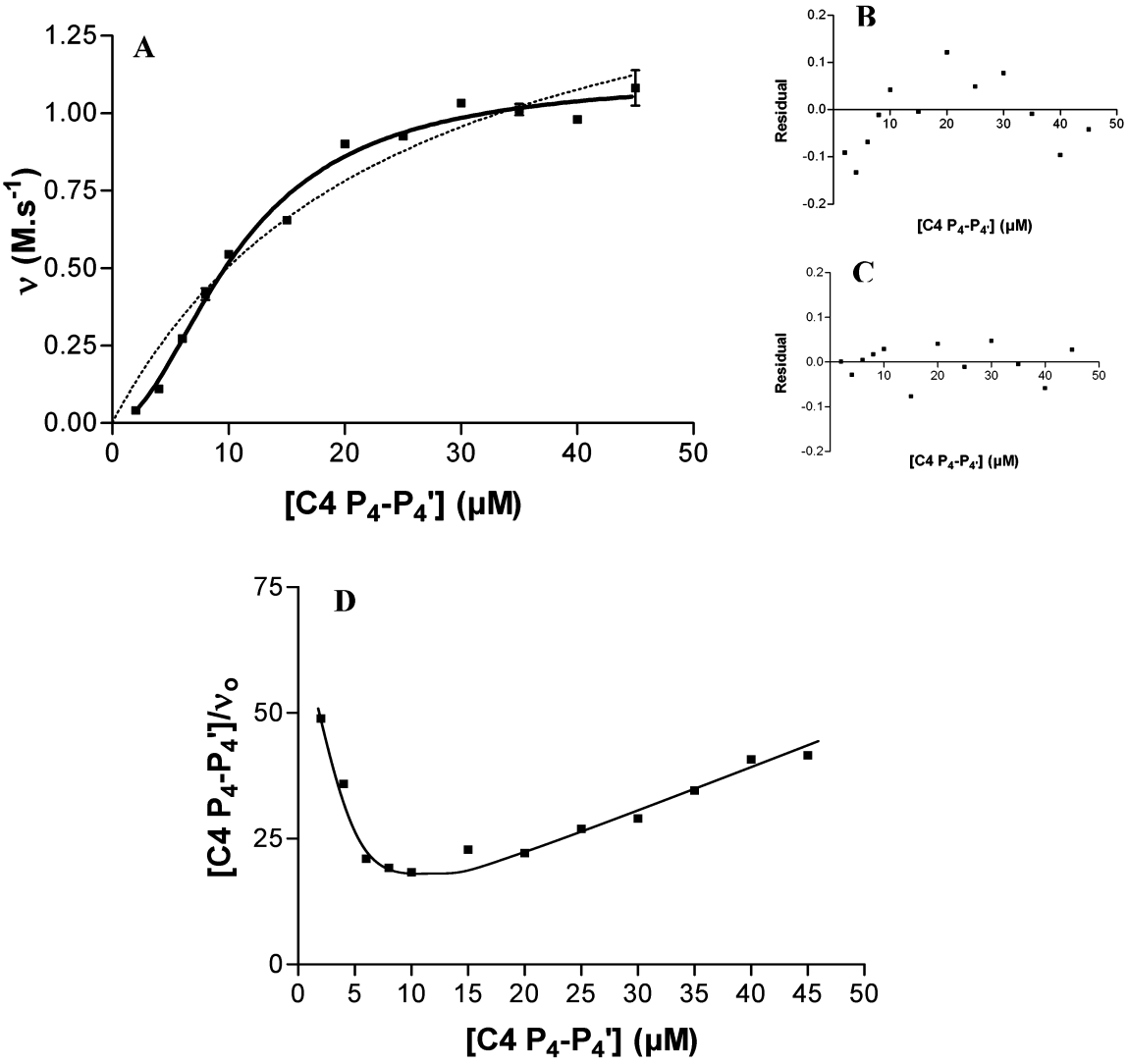

FIGURE 2: Cleavage of C4 P_4-P_4' substrate by human C1s. C4 P_4-P_4' substrate was incubated in the presence of human C1s (14.4 nM) for 40 min at 37 °C, during which time the increase in fluorescence was measured. The curves show (A) a comparison of fits for Michaelis−Menten (dotted line) and sigmoidal (solid line) curves, where initial velocity (v_0) is plotted against substrate concentration ([C4 P_4-P_4']), (B) a plot of the residuals for the Michaelis−Menten fit against ([C4 P_4-P_4']), (C) a plot of the residuals of the sigmoidal fit against substrate concentration ([C4 P_4-P_4']), and (D) a Hanes plot where substrate concentration/initial velocity ([C4 P_4-P_4']/v_o) is plotted against substrate concentration ([C4 P_4-P_4']) for 0.5−20.0 μM substrate.

Table 2: Kinetic Constants for the Proteolytic Activity of C1s Alone and as Part of the C1 Complex on a Synthetic Substrate Based on the P_4-P_4' Amino Acids of Complement Protein C4[a]

	allosteric enzyme kinetics			
enzyme preparation	$K_{0.5}$ (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM/s)	h
C1s	10.8	2.1×10^{-4}	0.019	1.96
C1s (+0.15 mM $CaCl_2$)	16.6	8.31×10^{-4}	0.050	1.70
C1 complex (+0.15 mM $CaCl_2$)	4.80	1.78×10^{-3}	0.371	1.90

[a] All values had errors of less than 10%.

Table 3: Kinetics of Cleavage of C4 P_4-P4' Substrate Truncated at P1′, P2′, and P3′ by C1s[a]

peptide sequence	$K_{0.5}$ (μM)	k_{cat} (s^{-1})	$k_{cat}/K_{0.5}$ (mM/s)	Hill slope
GLQR-ALEI	10.81	2.05×10^{-4}	0.019	1.96
GLQR-**A**	10.69	3.94×10^{-5}	3.69×10^{-3}	2.12
GLQR-**AL**	28.05	6.83×10^{-4}	0.024	0.67[b]
GLQR-**ALE**	22.13	3.19×10^{-4}	0.014	0.92[b]

[a] All values had errors of less than 10%. [b] The reaction follows normal Michaelis−Menten kinetics.

Substitution with a Lys residue yielded a substrate which was not cleaved even at high substrate and enzyme concentrations. Substitution of the Leu with a His residue once again abolished the positive cooperativity, reducing the Hill constant to a value of 1.0. The best $k_{cat}/K_{0.5}$ at this position was for the Phe residue substitution, suggesting that the enzyme prefers a hydrophobic residue at the P_2' position.

Substitutions of the P_3' Glu uniformly gave rise to large increases in the k_{cat} value, the largest of which was for the substitution with a His residue (the largest k_{cat} of any substrate in the $P_1'-P_4'$ library) (Table 5). This was accompanied by a 14-fold increase in $K_{0.5}$, however, and once again an abolition of the positive cooperativity. Reduction of the Hill constant to unity was also seen with a substitution to a Met or a Gln residue. Selectivity at the P_3' position was 24, with the substitution of a Phe residue at this position rather surprisingly yielding the highest $k_{cat}/K_{0.5}$ value. Substitution with a Lys residue at P_3' yielded the lowest $k_{cat}/K_{0.5}$ value.

Table 4: Kinetic Constants for the Proteolytic Activity of C1s on Synthetic Substrate Library Peptides[a]

position of substitution	substrate library peptide	$K_{0.5}$ (μM)	k_{cat} (s^{-1})	$k_{cat}/K_{0.5}$ (mM/s)	h
control	GLQR-ALEI	10.8	2.1×10^{-4}	0.019	1.96
P_1'	GLQR-**T**LEI	9.1	1.55×10^{-3}	0.170	1.49
	GLQR-**L**LEI	5.2	7.02×10^{-5}	0.014	2.0
	GLQR-**P**LEI	no cleavage			
	GLQR-**H**LEI	48.6	4.15×10^{-4}	8.54×10^{-3}	0.90
	GLQR-**K**LEI	28.6	7.62×10^{-5}	2.66×10^{-3}	1.38
	GLQR-**Q**LEI	4.8	7.02×10^{-5}	0.015	2.0
	GLQR-**E**LEI	12.9	2.05×10^{-5}	1.59×10^{-3}	1.5
	GLQR-**F**LEI	2.5	1.55×10^{-4}	0.062	2.2
	GLQR-**M**LEI	5.9	2.39×10^{-4}	0.041	1.6
P_2'	GLQR-A**A**EI	40.9	1.69×10^{-4}	4.32×10^{-3}	2.1
	GLQR-A**T**EI	11.67	1.47×10^{-4}	0.013	1.7
	GLQR-A**P**EI	20.8	1.89×10^{-5}	9.09×10^{-4}	1.4
	GLQR-A**H**EI	11.2	3.23×10^{-4}	0.029	1.0
	GLQR-A**K**EI	no cleavage			
	GLQR-A**Q**EI	7.6	1.83×10^{-4}	0.024	1.5
	GLQR-A**E**EI	6.5	4.21×10^{-5}	6.48×10^{-3}	2.8
	GLQR-A**F**EI	7.2	3.30×10^{-4}	0.046	1.9
	GLQR-A**M**EI	7.1	1.19×10^{-4}	0.017	1.3

[a] Substitutions for P_1' and P_2' amino acids are shown in bold text. All values had errors of less than 10%.

Table 5: Kinetic Constants for the Proteolytic Activity of C1s on Synthetic Substrate Library Peptides[a]

position of substitution	substrate library peptide	$K_{0.5}$ (μM)	k_{cat} (s^{-1})	$k_{cat}/K_{0.5}$ (mM/s)	h
control	GLQR-ALEI	10.8	2.1×10^{-4}	0.019	1.96
P_3'	GLQR-AL**A**I	16.3	2.74×10^{-3}	0.168	1.3
	GLQR-AL**T**I	23.94	2.79×10^{-3}	0.117	1.9
	GLQR-AL**L**I	32.61	2.96×10^{-3}	0.091	2.2
	GLQR-AL**P**I	18.08	2.44×10^{-3}	0.135	1.6
	GLQR-AL**H**I	88.53	8.21×10^{-3}	0.093	0.9
	GLQR-AL**K**I	16.3	1.36×10^{-4}	8.34×10^{-3}	1.45
	GLQR-AL**Q**I	35.45	2.32×10^{-3}	0.065	1.1
	GLQR-AL**F**I	14.20	2.88×10^{-3}	0.203	1.8
	GLQR-AL**M**I	32.74	2.27×10^{-3}	0.069	1.1
P_4'	GLQR-ALE**A**	15.62	3.92×10^{-4}	0.025	1.07
	GLQR-ALE**T**	27.99	4.40×10^{-4}	0.016	0.89
	GLQR-ALE**L**	10.53	9.78×10^{-5}	9.31×10^{-3}	1.50
	GLQR-ALE**P**	16.67	8.07×10^{-4}	0.048	1.20
	GLQR-ALE**H**	22.10	2.44×10^{-4}	0.011	1.36
	GLQR-ALE**K**	12.0	8.67×10^{-5}	7.35×10^{-3}	1.7
	GLQR-ALE**Q**	9.63	9.78×10^{-5}	0.010	1.85
	GLQR-ALE**E**	6.70	9.78×10^{-5}	0.015	1.56
	GLQR-ALE**F**	5.89	2.20×10^{-4}	0.037	1.47
	GLQR-ALE**M**	5.45	1.71×10^{-4}	0.031	1.16

[a] Substitutions for P_3' and P_4' amino acids are shown in bold text.

Overall, any substitution at the P_3' position gave rise to an increase in the $K_{0.5}$ values. Substitutions at the P_4' position generally had smaller effects on $K_{0.5}$ and k_{cat} values, although substitutions with Ala or Thr decreased the Hill constant to unity (Table 5). The selectivity factor at P_4' was only 6.5, indicating that this position had the least overall effect on C1s specificity of any of the prime sites investigated.

DISCUSSION

The classical complement pathway plays a vital role in host defense against infection. The pathway is set off after initial binding events mediated through C1q, following which there is a cascade of proteolytic events. The action of complement proteases is highly specific; thus, one would expect these enzymes to be highly specific for their cognate substrates in the pathway. Complement is also associated with detrimental events, such as the activation of the pathway by prion aggregates (*7*) or Alzheimer's tangles (*8*, *9*), which leads to an augmentation of these diseases. Detailed study of the specificity of these enzymes is a precursor not only to further understanding of the detailed mechanism of interaction with their cognate physiological substrates and inhibitors, but also to the rational design of effective inhibitors against the proteases. In this study, we have shown that the prime site binding residues of the complement proteases are vital in the recognition of amino acids at the cleavage site of substrates, such as the C4 complement protein.

C1s is highly restricted in terms of its cleavage of protein substrates, with C2 and C4 the only protein substrates cleaved by this enzyme. Initially, the activity of C1s against the P_4-P_1 sequences found at the cleavage points in C4 and C2 was examined. The cleavage of the C4 and C2 P_4-P_1 substrates by C1s followed Michaelis−Menten kinetics and showed moderately good kinetics, with the C2 sequence having a significantly better $k_{cat}/K_{0.5}$ value. However, the K_m values were ultimately too high to allow optimum substrate concentrations to be used in assays to study the active site characteristics of the enzyme. It is interesting to note that the Z-GLQR sequence has previously been used to produce an efficient substrate for C1s ($K_m = 41$ μM), but in this case the substrates were synthesized with a thioester leaving group (*10*). It is possible that the NHMec group in the P_1' position had a negative effect on binding, which is concordant with later results showing that this position is important in recognition of substrates by C1s (see below).

We then examined the cleavage of substrates on the basis of the full cleavage sequence in C2 and C4, incorporating the P_4-P_4' residues from these substrates. This was done in the form of a fluorescence quenched peptide, a strategy which has proved useful in the evaluation of cleavage of physiological sequences in proteins in the past (*11*, *12*). When we initially attempted to analyze results from cleavage of these substrates by C1s using Michaelis−Menten kinetics, we had difficulty due to the poor fit of the curves. Attempting to linearize the data by means of the Hanes plot showed that we were dealing with positive cooperativity in the system. Thus, subsequent analysis of the data was performed using nonlinear regression fitting to a sigmoidal plot (Figure 2). In doing so, we found that C1s had a much higher affinity for the C4 and C2 P_4-P_4' sequences, in addition to the positive cooperativity displayed. This implies that the prime site residues in the substrate must be inducing the cooperativity, since this was not seen with the P_4-P_1 substrate.

The positive cooperativity was also found for the C1 complex, which represents the physiologically relevant form for this pathway. Proper assembly and function of the C1 complex is thought to depend on the presence of calcium ions; therefore, assays with the complex were carried out in the presence of an appropriate concentration of calcium. We also carried out assays with C1s alone in the presence of calcium, which resulted in a 1.5-fold increase in the $K_{0.5}$ value, indicating that calcium was affecting the active site of the enzyme. Of further interest is the finding that this effect was negated in the C1 complex, where the $K_{0.5}$ value

was reduced 3.5-fold compared to that of C1s in the presence of calcium. There was also a concomitant substantial increase in the k_{cat} value. The overall $k_{cat}/K_{0.5}$ value of the C1 complex therefore increased 7.4-fold compared to that of C1s in the presence of calcium. This indicates that the C1 complex cleaves the representative C4 cleavage site sequence with much greater efficiency than C1s alone.

Initially, we considered that the positive cooperativity seen in cleavage of the C2 and C4 substrates might be due to interaction between active sites in a C1s dimer, analogous to the dimer seen in the crystal structure of C1r (*13*). However, C1s dimers are calcium-dependent (*14*), and we were able to demonstrate positive cooperativity even in the presence of EDTA (results not shown), indicating that dimerization of C1s is unlikely to be important for the effect. At present, therefore, the localization of the binding site on C1s mediating cooperativity with the active site of the enzyme is unknown. The finding that the enzyme is capable of undergoing conformational transitions in its structure is interesting in light of the possibility of designing molecules to interact with the second binding site on the enzyme to either positively or negatively affect the binding of substrates at the active site and thus modulate the activity of this crucial enzyme.

Overall, these results indicate that the prime binding sites of the complement proteases are highly important for efficient cleavage of substrates. We therefore screened a limited library of substrates for their kinetics of cleavage by C1s to further investigate the residues required for both the higher affinity seen and the positive cooperativity induced. The amino acid substitutions made at each of the four positions in the library were selected to incorporate changes to at least each of the major classes of amino acids found.

We were able to show that the P_1' position is the most selective site of these four positions, yielding a selectivity factor of 107 for cleavage of the best compared to the worst substrate at this position. The introduction of a Thr residue at this position gave rise to a very good substrate for C1s, which is interesting given that this is the residue found at P_1' in its physiological inhibitor, C1-inhibitor (*15*). It must be noted that substitution to a Pro residue at this position rendered the substrate apparently noncleavable, which is similar to the results seen for other serine proteases such as thrombin and factor Xa (*16*, *17*). Substitution of a His residue at any of the $P_1'-P_3'$ positions gave rise to a loss of the positive cooperativity seen, as well as large decreases in affinity for the substrate. At the P_4' position, substitution of Ala or Thr residues gave rise to the same effect, although at this position the loss in affinity was smaller. The screening of the substrate library for C1s has allowed greater insight into the contribution of the prime side binding sites of the enzyme to the positive cooperativity seen with the enzyme and also to the large increase in affinity seen with substrates that incorporate $P_1'-P_4'$ residues.

Some of the substrate specificity data were able to be related to the X-ray crystal structure of C1s (pdb identifier 1ELV; *18*). The S_1' pocket is shallow and relatively hydrophobic and thus would be predicted to accommodate a Thr side chain well in contrast to a bulky charged residue such as Glu or His. The S_2' pocket is also relatively shallow and contains an Arg residue (R563) at its base. This would presumably be an explanation for the noncleavage of the peptide substrate with a Lys residue substituted at the P_2' position. Interestingly, C1s had the greatest affinity for substrates containing a P_2' Glu: these data may reflect an ionic interaction between P_2' Glu and R563. Despite enhanced affinity for a P_2' Glu containing peptide, turnover of this substrate was poor, perhaps reflecting impaired product release. The S_3' and S_4' binding pockets were less defined in the structure of C1s, and thus, it was not possible to make a meaningful interpretation of the data for these positions.

What is the physiological relevance of these findings? Most usually, the induction of positive cooperativity in enzymes may be seen to allow reactions to occur in the physiological milieu in which they operate. It is interesting to note that the C4 protein is found at a concentration of approximately 1.95 μM in plasma (given a molecular weight of 205000 (*19*) and a plasma concentration of 400 μg/mL (*20*)). Thus, if one examined the kinetics of cleavage displayed by C1s for cleavage of the P_4-P_1 sequence of this protein, the low affinity displayed by this enzyme for this substrate would imply that the reaction would be unlikely to occur in physiological conditions. Taking into account the full sequence, however, the affinity is brought much closer to the physiological concentration of the substrate. The influence of the prime binding site residues in the substrate would therefore be expected to markedly enhance the activation of the complement pathway. It must be noted that others have postulated that additional parts of the complement protease structure, such as the CCP modules (*21*), are required to efficiently interact with protein substrates, and this may indeed be the case. The findings of this study indicate both that the interaction with the prime side residues at the cleavage point in C1s enhances the affinity of the enzyme for C2 and C4 substrates and also that these residues mediate positive cooperativity in the cleavage of the substrate. The present and future studies in our laboratory aim to establish whether similar principles govern the cleavage of substrates by other complement proteases and the localization of sites of interaction mediating the positive cooperativity.

ACKNOWLEDGMENT

We thank Dr. Steve Bottomley and Dr. Robyn Minchinton for useful discussions regarding the data in the manuscript.

REFERENCES

1. Dodds, A. W., Sim, R. B., Porter, R. R., and Kerr, M. A. (1978) Activation of the first component of human complement (C1) by antibody−antigen aggregates, *Biochem. J. 175*, 383−390.
2. Arlaud, G. J., Gaboriaud, C., Thielens, N. M., Budayova-Spano, M., Rossi, V., and Fontecilla-Camps, J. C. (2002) Structural biology of the C1 complex of complement unveils the mechanisms of its activation and proteolytic activity, *Mol. Immunol. 39*, 383−394.
3. Schumaker, V. N., Zavodszky, R., and Poon, P. H. (1987) Activation of the first component of complement, *Annu. Rev. Immunol. 5*, 21−42.
4. Arlaud, G. J., Colomb, M. G., and Gagnon, J. A. (1987) A functional model of the human C1 complex: Emergence of a functional model, *Immunol. Today 8*, 106−111.
5. Meri, S., and Jarva, H. (1998) Complement regulation, *Vox Sanguinis 74*, 291−302.
6. Illy, C., Thielens, N. M., and Arlaud, G. J. (1993) Chemical characterization and location of ionic interactions involved in the assembly of the C1 complex of human complement, *J. Protein Chem. 12*, 771−781.

7. Klein, M. A., Kaeser, P. S., Schwarz, P., Weyd, H., Xenarios, I., Zinkernagel, R. M., Carroll, M. C., Verbeek, J. S., Botto, M., Walport, M. J., Molina, H., Kalinke, U., Acha-Orbea, H., and Aguzzi, A. (2001) Complement facilitates early prion pathogenesis, *Nat. Med. 7*, 488−492.
8. Rogers, J., Cooper, N. R., Webster, S., Schultz, J., McGeer, P. L., Styren, S. D., Civin, W. H., Brachova, L., Bradt, B., Ward, P., and Lieberburg, I. (1992) Complement activation by beta-amyloid in Alzheimer disease, *Proc. Natl. Acad. Sci. U.S.A. 89*, 10016−10020.
9. Tacnet-Delorme, P., Chevallier, S., and Arlaud, G. J. (2001) Beta-amyloid fibrils activate the C1 complex of complement under physiological conditions: evidence for a binding site for A beta on the C1q globular regions, *J. Immunol. 167*, 6374−6381.
10. McRae, B. J., Lin, T.-Y., and Powers, J. C. (1981) Mapping the substrate binding site of human C1r and C1s with peptide thioesters. Development of new sensitive substrates, *J. Biol. Chem. 256*, 12362−12366.
11. Lourbakos, A., Chinni, C., Thompson, P., Potempa, J., Travis, J., Mackie, E. J., and Pike, R. N. (1998) Cleavage and activation of proteinase-activated receptor-2 on human neutrophils by gingipain-R from *Porphyromonas gingivalis*, *FEBS Lett. 435*, 45−48.
12. Smith, R., Jenkins, A., Lourbakos, A., Thompson, P., Ramakrishnan, V., Tomlinson, J., Deshpande, U., Johnson, D. A., Jones, R., Mackie, E. J., and Pike, R. N. (2000) Acrosin activates proteinase-activated receptor-2: expression of the receptor on oocytes, *FEBS Lett. 484*, 285−290.
13. Budayova-Spano, M., Lacroix, M. B., Thielens, N. M., Arlaud, G. J., Fontecilla-Camps, J. C., and Gaboriaud, C. (2002) The crystal structure of the zymogen catalytic domain of complement protease C1 reveals that a disruptive mechanical stress is required to trigger activation of the C1 complex, *EMBO J. 21*, 231−239.
14. Rivas, G., Ingham, K. C., and Minton, A. P. (1992) Calcium-linked self-association of human complement C1s, *Biochemistry 31*, 11707−11712.
15. Eldering, E., Huijbregts, C. C. M., Lubbers, Y. T. P., Longstaff, C., and Hack, C. E. (1992) Characterization of recombinant C1 inhibitor P1 variants, *J. Biol. Chem. 267*, 7013−7020.
16. Bianchini, E. P., Louvain, V. B., Marque, P. E., Juliano, M. A., Juliano, L., and Le Bonniec, B. F. (2002) Mapping of the catalytic groove preferences of factor Xa reveals an inadequate selectivity for its macromolecule substrates, *J. Biol. Chem. 277*, 20527−20534.
17. Ludemann, J. P., Pike, R. N., Bromfield, K. M., Duggan, P. J., Cianci, J., Le Bonniec, B. F., Whisstock, J. C., and Bottomley, S. P. (2003) Determination of the P_1', P_2' and P_3' subsite-specificity of factor Xa, *Int. J. Biochem. Cell. Biol. 35*, 221−225.
18. Gaboriaud, C., Rossi, V., Bally, I., Arlaud, G. J., and Fontecilla-Camps, J. C. (2000) Crystal structure of the catalytic domain of human complement c1s: a serine protease with a handle, *EMBO J. 19*, 1755−1765.
19. Budzko, D. B., and Müller-Eberhard, H. J. (1970) Cleavage of the fourth component of human complement (C4) by C1 esterase: isolation and characteristics of the low molecular weight product, *Immunochemistry 7*, 227−234.
20. Hammer, C. H., Wirtz, G. H., Renfer, L., Gresham, H. D., and Tack, B. F. (1981) Large scale isolation of functionally active components of the human complement system, *J. Biol. Chem. 256*, 3995−4006.
21. Rossi, V., Bally, I., Thielens, N. M., Esser, A. F., and Arlaud, G. J. (1998) Baculovirus-mediated expression of truncated modular fragments from the catalytic region of human complement serine protease C1s. Evidence for the involvement of both complement control protein modules in the recognition of the C4 protein substrate, *J. Biol. Chem. 273*, 1232−1239.
22. Schechter, I., and Berger, A. C. (1967) On the size of the active site in proteases. I. Papain, *Biochem. Biophys. Res. Commun. 27*, 157−162.

BI035507B