Effect of *N*-methylation on the modulation by synthetic peptides of the activity of the complement-Factor-B-derived serine proteinase CVFBb

Alla BERKOVICH,* M. Clare O'KEEFE, Preston HENSLEY† and Lynn CAPORALE‡

Department of Biochemistry, Georgetown University Medical Center, 4000 Reservoir Road N.W., Washington, DC 20016, U.S.A.

Although they share the active-site catalytic triad of less-specific enzymes such as trypsin and chymotrypsin, the serine proteinases of the complement and coagulation cascades each cleave a highly restricted set of substrates. Peptides with sequences similar to that at which C3 is cleaved by the alternative-pathway complement proteinase CVFBb were synthesized by solid-phase methodology and examined for their effects on the activity of this enzyme as measured by three different types of assays. It was found that a peptide methylated at the scissile bond was a far more effective inhibitor of the cleavage of the protein substrate C5 and of the lysis of guinea-pig erythrocytes by the alternative pathway than was the equivalent unmethylated peptide. Whereas the unmethylated peptide inhibited cleavage of the peptide substrate, the methylated peptide actually stimulated cleavage in this assay. This stimulation was found to be due to a 2.8-fold increase in k_{cat} ; the dissociation constant for the substrate was not altered significantly. One model consistent with this behaviour is that the binding of the activator peptide in the extended substrate-recognition region stabilizes a catalytically more active conformation of the active site. A small peptide substrate may have access to such an activated active site, whereas the larger substrate, C5, may be excluded from the site. These results demonstrate that the observed effect of a given compound on activity of an enzyme with an extended substrate-recognition region may depend upon the substrate.

INTRODUCTION

The proteinases of the complement system (Müller-Eberhard, 1988) are highly specific for their physiological protein substrates. As this system is of major importance in the host response to infections and in inflammation, inhibitors of key steps may hold promise in controlling inflammation. We have focused our attention on design of inhibitors of the C3/C5 convertases, enzymes that cut the complement proteins C3 and C5, as C3 cleavage is the first step in common between complement activation by the alternative and classical pathways.

Small peptides with sequences that are similar to those cleaved in C3 and C5 are recognized by the C3/C5 convertases (Caporale *et al.*, 1981; Kam *et al.*, 1987). Consistent with the view that specificity arises from the use of an extended substrate-recognition region, extending the length of peptide substrates can improve their specificity for a given enzyme (Gutierrez *et al.*, 1983).

Because *N*-methylated peptide bonds resist proteolysis (Pena *et al.*, 1974; Laufer *et al.*, 1981; Sandberg *et al.*, 1981) we wanted to examine the effect of *N*-methylation of the scissile bond on the ability of such peptides to inhibit these enzymes. The specific reason for the resistance of *N*-methylated peptides to proteolysis is unknown. If the methyl group blocks the peptide's entrance into the active site, the peptide might be expected to resist proteolysis itself but not inhibit the cleavage of other substrates. In contrast, if the peptide binds to the active site, but the methyl group interferes with a step in the action of the catalytic triad (Kraut, 1977), the peptide would block access of other substrates to the active site and thus should be an effective inhibitor of the enzyme.

Thus we prepared two peptides with sequences similar to that at which C3 is cleaved by these proteinases, Leu-Gly-Leu-Ala-Arg-Gly and Leu-Gly-Leu-Ala-Arg-Sar. The sequences of these peptides are identical except that in one case the bond analogous to the bond in the protein substrate C3 that is cleaved by CVFBb is methylated and in the other it is not. We found that the methylated peptide is an inhibitor when tested in a haemolytic assay, but that it actually activates the enzyme in an assay involving a small peptide substrate.

EXPERIMENTAL

Materials

Polystyrene-co-1% divinylbenzene resin substituted with chloromethyl groups at 1 mmol/g, *N*-methylglycine and glycine were from Vega Biochemicals (Tucson, AZ, U.S.A.). Boc-Leu-Gly-Arg-NH-Mec was from the Peptide Institute (Osaka, Japan), and *N*^ε-Boc-L-arginine hydrochloride, Boc-L-alanine, Boc-L-leucine, Boc-glycine and L-Leu-Gly were from United States Biochemical Corp., Cleveland, OH, U.S.A.). 2-(*t*-Butyloxycarbonyloxyimino)-2-phenylacetoneitrile was from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Dicyclohexylcarbodi-imide was from Pierce Chemical Co. (Rockford, IL, U.S.A.). Hydroxybenzotriazole was from Chemical Dynamics (South Plainfield, NJ, U.S.A.). Amino acid calibration standards were from Beckman (Palo Alto, CA, U.S.A.) and Pierce Chemical Co. All solvents used were spectral grade. Dichloromethane (Eastman, Rochester, NY, U.S.A.) was redistilled over Ca(OH)₂.

Abbreviations used: CVF, cobra-venom factor from *Naja naja*; CVFBb, stable analogue of alternative pathway of complement C3/C5 convertase; Boc-, *t*-butyloxycarbonyl-; -NH-Mec, 7-(4-methyl)coumarinamide; Sar (in peptide sequences), sarcosine.

* Present address: Fidia-Georgetown Institute for the Neurosciences, Medical Dental Building (Room 5E-101), 3900 Reservoir Road N.W., Washington, DC 20007, U.S.A.

† Present address: Macromolecular Sciences L940, Smith, Kline and French Research and Development Laboratories, P.O. Box 1539, King of Prussia, PA 19406-0939, U.S.A.

‡ To whom correspondence should be sent, at present address: Merck, Sharp and Dohme Research Laboratories, P.O. Box 2000, Rahway, NJ 07065-0900, U.S.A.

Methods

Analysis of amino acids and peptides. Before synthesis the purity of all amino acids and peptides was checked by t.l.c. (Analtech glass plates with silica gel type HL) in three different systems, butan-1-ol/acetic acid/water (4:1:1, by vol.), butan-1-ol/acetic acid/pyridine/water (15:3:10:12, by vol.) and butan-1-ol/acetic acid/water (2:1:1, by vol.). Sarcosine hydrochloride was recrystallized from ethanol, dried, weighed and dissolved in water; following determination by its u.v.-absorption spectrum that sarcosine was free of highly absorbing impurities, which we have observed in commercially available preparations of *N*-methylamino acids, the absorbance at 210 nm of different concentrations was measured for determination of the molar absorption coefficient.

After hydrolysis in 6 M-HCl at 130 °C for 3 h for the glycine-containing peptide and 12 h for the sarcosine-containing peptide, amino acid analyses were performed on a Beckman model 119C analyser with the manufacturer's standard one-column methodology except for samples containing sarcosine. Because *N*-methylated amino acids react only weakly with ninhydrin (Coggins & Benoiton, 1970), to increase the sensitivity of detection of sarcosine the column temperature was increased to 54 °C and the time of the reaction between the amino acids and the ninhydrin was increased (Ebata *et al.*, 1966) by using a buffer flow rate half the normal.

Synthesis. Boc-sarcosine and Boc-Leu-Gly were prepared by reaction with 2-(*t*-butoxycarbonyloxyimino)-2-phenylacetonitrile (Itoh *et al.*, 1975) in dioxan/water (1:1, v/v) at room temperature, adjusted to pH 9 by addition of triethylamine, with stirring. The standard conditions (2 h) were used for Leu-Gly; sarcosine was allowed to react for 16 h. After extraction between ethyl acetate and water, the pH of the aqueous layer was lowered to approx. 2.5 with 5% (w/v) citric acid, following which the *t*-butoxycarbonyl derivatives crystallized out of solution. The crystals were evaporated and dried over P₂O₅ *in vacuo*. Upon t.l.c. in chloroform/acetic acid (19:1, v/v) the *R_F* for Boc-sarcosine is 0.86 (compared with Boc-Gly, *R_F* 0.65). The *R_F* of Boc-Leu-Gly in chloroform/methanol/acetic acid (17:2:1, by vol.) is 0.76.

Boc-Gly resin was prepared as described previously (Gutierrez *et al.*, 1983). A 1.95 mmol portion of the caesium salt (Gisin, 1973) of Boc-sarcosine, dried over P₂O₅ by vacuum pump for 2 days, was dissolved in *N*-methylpyrrolidinone and added to 1.95 g of the resin. This suspension was stirred for 10 h at 50 °C and for 48 h at 24 °C in a screw-capped flask. This resulted in the coupling of 416 µmol of Boc-sarcosine (substitution 0.21 mmol/g determined by amino acid analysis following hydrolysis of the dried resin). Refluxing Boc-sarcosine, dissolved in *N*-methylpyrrolidinone, and resin in 12 ml of ethanol with 0.5 ml of triethylamine at 80 °C for 20 h (Merrifield, 1964) resulted in 90 µmol of Boc-sarcosine coupled at 0.25 mmol/g. The combined resins were thoroughly washed with *N*-methylpyrrolidinone, *N*-methylpyrrolidinone/water (9:1, v/v), *N*-methylpyrrolidinone and ethanol, and then dried over P₂O₅ *in vacuo*, providing 2.65 g of Boc-sarcosine plus resin.

Peptides were synthesized by the solid-phase method of Merrifield (Barany & Merrifield, 1980) on a Vega model 50 peptide synthesizer with standard 'Boc-benzyl' strategy of protection. Trace-labelling with [³H]leucine enabled us to use specific radioactivity as a rapid check of peptide concentration before assay, and to facilitate peptide detection during purification. The *t*-butoxycarbonyl group was removed with 50% (v/v) trifluoroacetic acid in dichloromethane (30 min); the substituted resin was then washed nine times for 1 min each with

dichloromethane, and then neutralized [two 5 min rinses with 5% (v/v) *NN*-di-isopropylethanolamine in dichloromethane interspersed with six washes with dichloromethane].

All amino acids were dissolved in dichloromethane; Boc-Leu was first added to a small quantity of *N*-methylpyrrolidinone to aid solubility. The coupling reagents dicyclohexylcarbodi-imide and hydroxybenzotriazole were added at a 1:1 ratio with the Boc-amino acid. The ratio of *t*-butoxycarbonyl group to resin-bound amino acid was 4:1 for Boc-arginine, 3:1 for Boc-alanine, 2:1 for Boc-leucine and 2:1 for Boc-Leu-Gly. Coupling reactions were allowed to proceed for 30 min, except for the coupling of Boc-arginine to resin-bound sarcosine, as noted below.

After each step the efficiency of coupling was determined by amino acid analysis for the sarcosine-containing peptide. Resin samples were hydrolysed with propionic acid/12 M-HCl (1:1, v/v) (Westall & Hesser, 1974), and by examining the unmodified amino group by the Kaiser test (Kaiser *et al.*, 1970) for the glycine-containing peptide; couplings were repeated as necessary.

The synthetic step that was expected to be the most difficult was the coupling of arginine (protected only by protonation) to the sarcosine resin. Arginine was used without covalent protection of the guanidino group (Gish & Carpenter, 1953; Coy & Branyas, 1979; Inman, 1981). As longer coupling times are recommended to obtain complete coupling to *N*-methylated amino acids, and we were concerned about side reactions involving the guanidino group (Photaki Yiotakis, 1976; Inman, 1981), the reaction was repeated three times for 2 h each, with the addition of fresh arginine and coupling reagents, instead of increasing the time of the reaction. Resin containing 510 µmol of sarcosine was deprotected and neutralized as described above and mixed with 2 mmol of Boc-arginine hydrochloride (a 4:1 ratio) dissolved in dichloromethane for 15 min, following which the coupling reagents were added equimolar to the arginine. The ratio of resin-bound arginine to sarcosine was 1:1 as determined by amino acid analysis after hydrolysis of the resin.

Synthetic peptides were cleaved from the resin by reaction with 10 equivalents (5 mmol) of trifluoromethanesulphonic acid (density 1.70 g/ml; 0.3 ml), with 3 equivalents (1.5 mmol, 0.163 ml) of anisole (to scavenge cations) (Chaudhuri & Najjar, 1979; Yajima *et al.*, 1974), and enough trifluoroacetic acid/dichloromethane (1:1, v/v) to cover the resin; this mixture was stirred at 40 °C for 1 h. The resin was filtered, and washed with trifluoroacetic acid, dichloromethane, diethyl ether and 50% (v/v) acetic acid. Although 80% of the radioactivity was in the trifluoroacetic acid washes, all subsequent eluates contained significant radioactivity, and thus were combined and evaporated on the rotary evaporator.

Purification of peptides. The extract was passed over a Bio-Gel P-2 column, monitored by t.l.c. of the radioactive fractions, followed by h.p.l.c. with a gradient between 0.05% (v/v) trifluoroacetic acid in double-distilled deionized water and 0.05% (v/v) trifluoroacetic acid in acetonitrile on a 4.6 mm × 25 mm reverse-phase (ODS) column (Beckman) with a flow rate of 1 ml/min (Beckman model 324). The eluate was monitored for absorbance at 210 nm and for radioactivity. Shoulder fractions were re-run. The glycine-containing peptide was eluted by 16% (v/v) acetonitrile, and the sarcosine-containing peptide by 10% (v/v) acetonitrile. Purity was determined by t.l.c. and by amino acid analysis. After h.p.l.c., peptides were freeze-dried, washed twice with water and freeze-dried again. The amino acid compositions of the synthetic peptides were sarcosine/glycine/alanine/leucine/arginine (1.10:1.03:1.00:2.05:0.99) and glycine/alanine/leucine/arginine (2.12:1.09:2.07:1.00). In order to determine whether the conditions used for the coupling of arginine to sarcosine resulted in significant racemization, the

chiral purity of the arginine in the purified peptide was demonstrated by the use of a chiral mobile phase containing *NN*-di-*n*-propyl-L-alanine and cupric acetate on a reverse-phase column by the method of Hare and co-workers (Weinstein *et al.*, 1982). Dr. Hare generously performed this test in his laboratory, and found no detectable D-arginine.

Immediately before the fluorescence assay the synthetic peptides were dissolved in phosphate-buffered saline (0.14 M-NaCl/10 mM-sodium phosphate buffer, pH 7.2); their concentration was checked by specific radioactivity and amino acid analysis.

Protein purification. CVF was purified by the method of Vogel & Müller-Eberhard (1984). C5, isolated by the method of Hammer *et al.* (1981), was generously given by Dr. Carl Hammer. Factor B and Factor D were purified starting from a plasma pool by a method developed in this laboratory (M. C. O'Keefe & L. Caporale, unpublished work). Factor B was purified from plasma by sequential chromatography on DEAE-Sephadex at pH 7.2, DEAE-cellulose at pH 7.6, Sephadex G-100, Bio-Rex 70 at pH 7.0 and CM-cellulose at pH 6.0. Factor D was purified from plasma by sequential chromatography on DEAE-Sephadex at pH 7.4, CM-cellulose at pH 6.0 and Sephadex G-75.

Enzyme preparation. The conditions chosen for formation of CVFBb (EC 3.4.21.47; a stable analogue of the alternative-pathway C3-cleaving enzyme C3bBb) from CVF, Factor B and Factor D were those in which Factor B is in excess so that the enzyme concentration is equal to the concentration of input CVF (Vogel & Müller-Eberhard, 1982). CVF (36 μ g) was incubated at 37 °C with a 2-fold molar excess of Factor B and 0.6 μ g of Factor D in a total volume of 300 μ l of Veronal-buffered saline (143 mM-NaCl/3.5 mM-5,5'-diethylbarbituric acid buffer, pH 7.2) containing 2 mM-MgCl₂. The reaction was stopped after 60 min by the addition of an equal volume of phosphate-buffered saline containing 20 mM-EDTA. The enzyme was diluted with phosphate-buffered saline to the desired concentration before the fluorescence assays. The CVFBb used in the haemolytic assays and C5-cleavage experiments was purified on a Sephadex G-200 column equilibrated in Veronal-buffered saline containing 2 mM-MgCl₂.

Fluorescence assay. CVFBb was assayed with the fluorogenic substrate Boc-Leu-Gly-Arg-NH-Mec as described previously (Caporale *et al.*, 1981). The concentration of the substrate stock solution was checked spectroscopically. The solution containing substrate and test peptide was prewarmed to 37 °C in the cuvette for 5 min; enzyme solution, also prewarmed in a water bath at 37 °C, was then added to a final concentration of 70 nM.

Boc-Leu-Gly-Arg-NH-Mec was incubated with CVFBb in 0.14 M-NaCl/10 mM-sodium/potassium phosphate buffer, pH 7.4, in a cuvette holder maintained at 37 °C in a Spex Fluorolog fluorimeter in the presence and in the absence of the test peptides. Release of free 7-amino-4-methylcoumarin was monitored by measuring fluorescence at an excitation wavelength of 380 nm and an emission wavelength of 460 nm (Morita *et al.*, 1977; Zimmerman *et al.*, 1978). The data were converted from fluorescence into mol of substrate cleaved by reference to a standard curve prepared daily with 7-amino-4-methylcoumarin on the same instrument with the same settings for slit widths.

Data analysis. The kinetic data for the hydrolysis of the small peptide substrate Boc-Leu-Gly-Arg-NH-Mec, in the presence and in the absence of the effector molecule Leu-Gly-Leu-Ala-Arg-Sar, were analysed by non-linear least-squares methods with multiple independent variables (Johnson *et al.*, 1976, 1981). Here

the dependent variable was the rate of hydrolysis and the independent variables were the concentration of the fluorogenic substrate Boc-Leu-Gly-Arg-NH-Mec and the concentration of the effector molecule Leu-Gly-Leu-Ala-Arg-Sar. As shown in the Results section, the addition of the effector molecule caused the rate of hydrolysis of the fluorogenic substrate to increase. A model, in the literature (Segel, 1981; Frieden, 1964), that describes this activation, is given in Scheme 1. This rapid-equilibrium model has been proposed to describe the non-essential activation of an enzyme by an activator molecule A. In the model shown in Scheme 1, K_s is the dissociation constant for the substrate S from the enzyme, K_A is the dissociation constant for effector from the enzyme, k_p is the rate constant for the production of product, α is the fractional change in K_A in the presence of the substrate S and also the fractional change in K_s in the presence of the activator A, and β is the fractional change in k_p in the presence of the activator A. The velocity equation that results from this model is:

$$v = \frac{V_{\max} [S] / K_s + \beta V_{\max} [A] [S] / \alpha K_A K_s}{1 + [S] / K_s + [A] / K_A + [A] [S] / \alpha K_A K_s} \quad (1)$$

Hence the data were analysed simultaneously in the presence of changing concentrations of the activator A and the substrate S, and the values of $V_{\max}$, K_s , K_A , α and β were determined.

K_i , the dissociation constant for the binding of the inhibitor Leu-Gly-Leu-Ala-Arg-Gly to the enzyme, was calculated from a Dixon plot. In this plot of $1/v$ versus $[I]$ all lines pass through $1/V_{\max}$ at $-K_i$, consistent with competitive inhibition (Segel, 1975).

Haemolytic assays. The peptides were tested for their effect on the complement-mediated lysis of guinea-pig erythrocytes. The assay is based on the cleavage of C5 by CVFBb with subsequent lysis of bystander erythrocytes. Briefly, 10⁷ guinea-pig erythrocytes were incubated with 20 μ l of guinea-pig serum and 412 ng of CVF in the presence and in the absence of the peptides in a total volume of 60 μ l of Veronal-buffered saline containing 0.5 mM-MgCl₂, 0.15 mM-CaCl₂ and 0.1% (w/v) gelatin for 20 min at 37 °C. The reaction was stopped by addition of 1 μ l of cold Veronal-buffered saline, vortex-mixing and centrifugation. The released haemoglobin was determined spectrophotometrically at 412 nm (Vogel & Müller-Eberhard, 1984).

C5 cleavage. The purified peptides were dissolved in Veronal-buffered saline containing 2 mM-MgCl₂. Highly purified C5 (1.7 μ M) was cleaved by CVFBb (380 μ M) in Veronal-buffered saline containing 2 mM-MgCl₂ at 37 °C for 105 min. This cleavage assay was performed in the presence and in the absence of 1.7 mM-Boc-Leu-Gly-Leu-Ala-Arg-Sar. After incubation at 37 °C, the samples were reduced and separated on SDS/7.5%-PAGE to determine the extent of cleavage of the C5 α -chain to the C5b α' -chain. SDS/PAGE was performed by the method of Laemmli (1970).

Scheme 1.

RESULTS

Chemical properties of amino acids and peptides

The R_F values of the synthetic peptides are reported in Table 1. The molar absorption coefficient of sarcosine at 210 nm was found to be $46 \text{ M}^{-1} \cdot \text{cm}^{-1}$, compared with the literature value of $54 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for glycine at 205 nm (Goldfarb *et al.*, 1951) and values for other carboxylic acids at 210 nm of between 10 and $40 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Ham & Platt, 1951). As reported by Coggins & Benoiton (1970), sarcosine was eluted ahead of glycine upon amino acid analysis, in a symmetrical peak with a retention time of 60 min. By the use of their method we observed an 18-fold increase in the colour factor for sarcosine over that obtained by standard methodology.

Fluorescence assays

The addition of increasing concentrations of Leu-Gly-Leu-Ala-Arg-Gly led to decreasing rates of liberation of 7-amino-4-methylcoumarin from the peptide substrate (Fig. 1). A K_i of $250 \mu\text{M}$ and V_{max} for the peptide substrate of 0.52 mol of 7-amino-4-methylcoumarin/min per mol of CVFBb were obtained from a Dixon plot (Fig. 2). The lines intersect in the upper left-hand quadrant, indicating that, as expected, the peptide is a competitive (or partially competitive) inhibitor of CVFBb (Segel, 1975).

In contrast, the methylated peptide Leu-Gly-Leu-Ala-Arg-Sar appears to stimulate the cleavage of the substrate (Fig. 3). These data both in the presence and in the absence of the effector molecule were simultaneously analysed according to the velocity equation for the non-essential activation model described in the Experimental section, and the results of this analysis are given in Table 2. V_{max} in the absence of the activator molecule is 0.54 mol of 7-amino-4-methylcoumarin/min per mol of CVFBb. The

dissociation constant for substrate is $111 \mu\text{M}$ and that for the activator was $148 \mu\text{M}$. As shown in Fig. 3, the activator molecule increased the maximal rate of hydrolysis by 2.82-fold, from 0.54 to 1.52 mol of 7-amino-4-methylcoumarin/min per mol of CVFBb. The value for α or the fractional change in K_s and K_A in the presence of activator and substrate respectively was 1.69, but the 95% confidence limits for this number contain 1. In contrast, the 95% confidence limits for β do not contain 1, and hence the increase in V_{max} is statistically significant. Therefore the sarcosine-containing peptide is acting as a partial non-competitive activator.

To make the analysis of the data more graphic, the inset gives the double-reciprocal transform of the data. Here two things are apparent. First, the two lines intersect each other at the abscissa axis. As discussed by Segel (1981) and by Frieden (1964), this indicates that α is close to 1. In contrast, it is clear that the ordinate-axis intercept of the double-reciprocal plot in the presence of the activator is significantly decreased, suggesting that β is greater than 1. This analysis supports the conclusion that the binding of the effector increases k_{cat} , but has minimal effect on the binding of this substrate.

Fig. 2. Dixon plot for the determination of K_i for the inhibitor Leu-Gly-Leu-Ala-Arg-Gly

Increasing concentrations of inhibitor were added to enzyme before the addition of the substrate: $33 \mu\text{M}$ - (●), $41 \mu\text{M}$ - (□), $66 \mu\text{M}$ - (○) or $130 \mu\text{M}$ -Boc-Leu-Gly-Arg-NH-Mec (■). V is expressed as mol of 7-amino-4-methylcoumarin/min per mol of CVFBb.

Table 1. R_F values of Leu-Gly-Leu-Ala-Arg-Sar (LGLARSar) and Leu-Gly-Leu-Ala-Arg-Gly (LGLARG)

Solvent I: butan-1-ol/acetic acid/pyridine/water (15:3:10:12, by vol.). Solvent II: butan-1-ol/acetic acid/water (4:1:1, by vol.). Solvent III: butan-1-ol/acetic acid/water (2:1:1, by vol.).

	R_F value	
	LGLARSar	LGLARG
Solvent I	0.68	0.45
Solvent II	0.35	0.20
Solvent III	0.47	0.67

Fig. 1. Effect of addition of $0 \mu\text{M}$ - (○), $213 \mu\text{M}$ - (△), $352 \mu\text{M}$ - (□) and $705 \mu\text{M}$ -Leu-Gly-Leu-Ala-Arg-Gly (▽) on the rate of liberation of 7-amino-4-methylcoumarin from Boc-Leu-Gly-Arg-NH-Mec by CVFBb

Fig. 3. Plot of the rate of hydrolysis of the fluorogenic substrate Boc-Leu-Gly-Arg-NH-Mec in the presence (▲) and in the absence (■) of activator, $740 \mu\text{M}$ -Leu-Gly-Leu-Ala-Arg-Sar

The curves are drawn according to the model in Scheme 1 (see eqn. 1) with the calculated parameter values given in Table 2. The inset shows a double-reciprocal plot.

Table 2. Analysis of kinetic data

Parameters are as defined for Scheme 1 in the Experimental section.
The confidence limits are 95 % confidence intervals.

Parameter	Value	Confidence limits
V_{max}	0.54 mol of 7-amino-4-methylcoumarin/min per mol of CVFBb	0.38, 0.64
K_s	111 μ M	59, 169
K_A	148 μ M	70, 247
α	1.69	0.63, 3.29
β	2.82	1.85, 4.27

Haemolytic assays

CVF causes haemolysis by combining with Factor B present in serum, leading to the formation of the enzyme CVFBb. Cleavage of serum C5 by this enzyme, generating C5b, causes ‘bystander lysis’ of guinea-pig erythrocytes by membrane-attack complexes formed as a result of the presence of C5b.

At concentrations of Leu-Gly-Leu-Ala-Arg-Gly as high as 2 mM, we were unable to demonstrate significant inhibition of the haemolytic assay by this peptide. In contrast, as shown in Fig. 4, increasing concentrations of Leu-Gly-Leu-Ala-Arg-Sar cause increasing inhibition of the CVF-stimulated haemolysis of guinea-pig erythrocytes. Fig. 5 shows that increasing amounts of CVF are needed to produce 50 % haemolysis [$y/(100 - y) = 1$, where y is the percentage haemolysis] as the concentration of Leu-Gly-Leu-Ala-Arg-Sar in the assay was increased.

C5 cleavage

As shown in Fig. 6, the cleavage of the C5 α -chain to the α' -chain by CVFBb is inhibited by Leu-Gly-Leu-Ala-Arg-Sar. This result is consistent with that of the haemolytic assay.

DISCUSSION

Two peptides with sequences that mimic the site at which the complement protein C3 is cleaved by CVFBb were prepared by solid-phase synthesis and compared for their effectiveness of inhibition of this enzyme in several assay systems. We found that Leu-Gly-Leu-Ala-Arg-Gly serves as a competitive (or partially competitive) inhibitor of CVFBb cleavage of the synthetic substrate Boc-Leu-Gly-Arg-NH-Mec. In contrast, its methylated analogue, Leu-Gly-Leu-Ala-Arg-Sar, stimulated the enzyme in a

Fig. 4. Dose-response curve showing the percentage inhibition of CVFBb-mediated haemolytic activity as a function of the concentration of Leu-Gly-Leu-Ala-Arg-Sar in the assay

Fig. 5. Plot showing that the amount of CVF needed to produce 50 % haemolysis [$y/(100 - y)$, where y is the percentage haemolysis] increases as the concentration of Boc-Leu-Gly-Leu-Ala-Arg-Sar in the assay is increased: 0 μ M ($\square$) to 40 μ M ($\triangle$) 80 μ M ($\circ$)

Fig. 6. SDS/7.5%-PAGE showing the effect of 1.7 mM-Boc-Leu-Gly-Leu-Ala-Arg-Sar and on the cleavage of the C5 α -chain to the C5b α' -chain by CVFBb

non-competitive manner in this assay. The similarity in sequence of the activating and substrate peptides recalled the observations of substrate activation of trypsin (Trowbridge *et al.*, 1963).

We thought that a likely explanation for the observed stimulation of peptide substrate cleavage by the methylated peptide might be that it is stabilizing a catalytically more active conformation of the active site. If the methylated peptide stimulates by binding in an extended substrate-recognition site, then cleavage of a substrate larger than the tripeptide, one that would be expected to have a larger region of interaction with the enzyme, might be inhibited by this peptide. Thus we used a haemolytic assay, which involves the cleavage of the 180000 Da protein C5 by the enzyme. As expected, the methylated peptide was an effective inhibitor in the haemolytic assay.

Because the haemolytic assay system is complex, we also tested the ability of the peptide to inhibit the cleavage of C5 by CVFBb directly in a system containing only the purified proteins (Fig. 6). Again, the methylated peptide was an effective inhibitor in this system; the non-methylated peptide was far less effective than the methylated one in inhibiting action on the

macromolecular substrate, inhibiting neither C5 cleavage nor haemolysis at the concentrations tested.

These data illustrate that the choice of substrate may determine whether or not a test compound is found to be an enzyme inhibitor.

The active sites of serine proteinases have been proposed to undergo conformational alterations on binding of substrate (Fink, 1976; Kraut, 1977; Bizzozero & Dutler, 1981; Rodier & Ilgenfritz, 1982; Steitz & Shulman, 1982; Malthouse & Scott, 1983). Temperature-jump (Rodier & Ilgenfritz, 1982), n.m.r. (Steitz & Shulman, 1982), cryoenzymological (Malthouse & Scott, 1983; Fink, 1976) and X-ray-crystallographic (Steitz & Shulman, 1982) studies have indicated that, upon substrate binding, serine proteinase active-site residues move into a catalytically more active conformation. The ability of serine proteinases to distinguish among protein substrates may in part be explained by the apparent dependence of this movement upon the interaction of the substrate with the specificity pocket (for example, a basic side chain in the substrate forming a salt bridge with Asp-177 in the specificity pocket of trypsin) (Malthouse & Scott, 1983; Inagami & York, 1968).

Addition of methylamine or ethylamine greatly stimulates trypsin's poor cleavage of esters of glycine and alanine (Inagami & York, 1968; Antonini & Ascenzi, 1981). This improved catalysis has been shown to be due to an increased k_{cat} , (as is observed in the present work) without an alteration in K_m , and is thought to be due to binding of the positively charged amine to the specificity pocket of the enzyme, mimicking the behaviour of the side chain of tryptic substrates, without blocking the approach of the small alanine and glycine esters. That these amines are in fact binding in the specificity pocket is supported by the inhibition of cleavage of lysine-containing substrates, which presumably are sterically blocked by the amines from entering the specificity pocket, and by lack of tryptic cleavage of the alanine ester by longer amines such as propylamine and butylamine. Analogously, the methyl group, increasing the *cis/trans* ratio of the scissile bond, could direct the sarcosine-containing peptide to a different set of interactions on Bb's surface; just as ethylamine inhibits tryptic hydrolysis of larger substrates that would make the necessary contacts for activation of their own catalysis, the activating peptide inhibits cleavage of C5.

The coagulation and complement proteinases have much stricter specificity requirements than chymotrypsin and trypsin. Thus they must recognize more sequence than simply the basic residue to the left of their tryptic-like cuts. CVFBB cleaves small peptide substrates that mimic the cleavage site of its protein substrates, but not as efficiently as it cleaves C3 (Caporale *et al.*, 1981). Thus it is likely that interactions beyond those in contact with small peptides bound at the active site are important for stimulating catalysis. Three-dimensional models of these proteinases, based upon the crystal structures of chymotrypsin and trypsin, do reveal highly individualized surfaces surrounding the active site of each of these proteinases.

The role of secondary binding interactions in increasing the catalytic efficiency of certain proteinases has been pointed out (Fruton, 1970). Certain non-substrate peptides have been found to activate cleavage of small substrates by pepsin. This activation is also characterized by an increase in k_{cat} , rather than K_m (Wang & Hofmann, 1976) and has been found to be associated with a conformational change (Wang *et al.*, 1974). Certain antibodies directed against ribonuclease also have been demonstrated to affect the activity on different substrates differentially, activating the enzyme against the low-molecular-mass substrate cyclic CMP but inhibiting its action against polyribonucleotides (Cinader, 1977), being more inhibitory to larger substrates.

For the goal of designing a peptide to inhibit complement-

mediated haemolysis, the methylated peptide is a more effective inhibitor than its non-methylated analogue, indicating the potential value of preparing a series of methylated peptides.

We thank Dr. Bruce Gaber, of the Optical Probes Branch of the Naval Research Laboratories, for the use of the fluorimeter, and for other support of this work, and Dr. James Doherty for helpful discussions. Dr. Carl Hammer generously provided the purified C5 used in this work. We also thank those volunteers at the Nation's Capital Affiliate of the American Association whose fund-raising efforts support the Mamie Doud Eisenhower Postdoctoral Fellowship fund, and Mildred Rosenberg for her encouragement. A. B. was Mamie Doud Eisenhower Fellow of the American Heart Association Nation's Capital Affiliate. This work was supported by Grant HL29523 from the National Institutes of Health.

REFERENCES

- Antonini, E. & Ascenzi, P. (1981) *J. Biol. Chem.* **256**, 12449–12455
- Barany, G. & Merrifield, R. B. (1980) in *The Peptides*, 2nd edn., (Gross, E. & Meienhofer, J., eds.), pp. 1–284, Academic Press, New York
- Bizzozero, S. & Dutler, H. (1981) *Bioorg. Chem.* **10**, 46–62
- Caporale, L. H., Gaber, S.-S., Kell, W. & Gotze, O. (1981) *J. Immunol.* **126**, 1963–1965
- Chaudhuri, M. & Najjar, V. (1979) *Anal. Biochem.* **95**, 305–310
- Cinader, B. (1977) *Methods Immunol. Immunochem.* **4**, 313–374
- Coggins, J. R. & Benoiton, N. L. (1970) *J. Chromatogr.* **52**, 251–256
- Coy, D. H. & Branyas, N. (1979) *Int. J. Pept. Protein Res.* **14**, 339–343
- Ebata, M., Takahashi, Y. & Otsuka, H. (1966) *J. Chromatogr.* **25**, 1–10
- Fink, A. L. (1976) *Biochemistry* **15**, 1580–1586
- Frieden, C. (1964) *J. Biol. Chem.* **239**, 3522–3531
- Fruton, J. S. (1970) *Adv. Enzymol. Relat. Areas Mol. Biol.* **33**, 401–443
- Gish, D. T. & Carpenter, F. H. (1953) *J. Am. Chem. Soc.* **75**, 5872–5877
- Gisin, B. F. (1973) *Helv. Chim. Acta* **56**, 1476–1482
- Goldfarb, A. R., Saidel, L. J. & Mosovich, E. (1951) *J. Biol. Chem.* **193**, 397–404
- Gutierrez, J. C., Gotze, O. & Caporale, L. H. (1983) *Biochim. Biophys. Acta* **744**, 276–280
- Ham, J. S. & Platt, J. R. (1951) *J. Chem. Phys.* **20**, 335–336
- Hammer, C. H., Wirtz, G. H., Renfer, L., Gresham, H. D. & Tack, B. F. (1981) *J. Biol. Chem.* **256**, 3995–4006
- Inagami, T. & York, S. S. (1968) *Biochemistry* **7**, 4045–4052
- Inman, J. K. (1981) in *The Peptides: Analysis, Synthesis and Biology* (Gross, E. & Meienhofer, J., eds.), vol. 3, pp. 253–302, Academic Press, New York
- Itoh, M., Hagiwara, D. & Kamiya, T. (1975) *Tetrahedron Lett.* **49**, 4393–4394
- Johnson, M. L., Halvorson, H. R. & Ackers, G. K. (1976) *Biochemistry* **15**, 5363–5371
- Johnson, M. L., Correia, J. J., Yphantis, D. A. & Halvorson, H. R. (1981) *Biophys. J.* **36**, 575–588
- Kaiser, E., Colosco, R. L., Bessinger, C. D. & Cook, P. I. (1970) *Anal. Biochem.* **34**, 595–598
- Kam, C.-M., McRae, B. J., Harper, J. W., Niemann, M. A., Volanakis, J. E. & Powers, J. C. (1987) *J. Biol. Chem.* **262**, 3444–3451
- Kraut, J. (1977) *Annu. Rev. Biochem.* **46**, 331–358
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Laufer, R., Chorev, M., Gilon, C., Friedman, Z., Wormser, U. & Selinger, Z. (1981) *FEBS Lett.* **123**, 291–294
- Malthouse, J. P. G. & Scott, I. (1983) *Biochem. J.* **215**, 555–563
- Merrifield, R. B. (1964) *Biochemistry* **3**, 1385–1390
- Morita, T., Kato, M., Iwanga, S., Kimura, T. & Sakakibara, S. (1977) *J. Biochem. (Tokyo)* **82**, 1495–1498
- Müller-Eberhard, H. J. (1988) *Annu. Rev. Biochem.* **57**, 321–347
- Pena, C., Stewart, J. M. & Goodfriend, T. (1974) *Life Sci.* **14**, 1331–1336
- Photaki, I. & Yiotakis, A. (1976) *J. Chem. Soc. Perkins Trans. 1* 259–264
- Rodier, R. & Ilgenfritz, G. (1982) *Eur. J. Biochem.* **128**, 451–454
- Sandberg, B., Lee, C.-M., Hanley, M. & Iversen, L. (1981) *Eur. J. Biochem.* **34**, 595–598
- Segel, I. H. (1975) *Enzyme Kinetics*, p. 110, Wiley-Interscience, New York
- Segel, I. H. (1981) *Biophys. J.* **36**, 227–231

- Steitz, T. A. & Shulman, R. G. (1982) *Annu. Rev. Biophys.* **11**, 419–444
- Trowbridge, C., Krehbiel, A. & Laskowski, M. (1963) *Biochemistry* **2**, 843–850
- Vogel, C.-W. & Müller-Eberhard, H. J. (1982) *J. Biol. Chem.* **257**, 8292–8299
- Vogel, C.-W. & Müller-Eberhard, H. J. (1984) *J. Immunol. Methods* **73**, 203–220
- Wang, T.-T. & Hofmann, T. (1976) *Biochem. J.* **153**, 701–712
- Wang, T.-T., Dorrington, K. J. & Hofmann, T. (1974) *Biochem. Biophys. Res. Commun.* **57**, 865–869
- Weinstein, S., Engel, M. & Hare, P. (1982) *Anal. Biochem.* **121**, 370–377
- Westall, F. & Hesser, H. (1974) *Anal. Biochem.* **61**, 610–613
- Yajima, H., Fujii, N., Ogawa, H. & Kawatani, H. (1974) *J. Chem. Soc. Chem. Commun.* 197–108
- Zimmerman, M., Quigley, J. P., Ashe, B., Dorn, C., Goldfarb, R. & Troll, W. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 750–753

Received 6 December 1989/21 March 1990; accepted 29 March 1990