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Exo-Site Affinity Labeling of C1s̄, a Subcomponent of the First Component of Complement, by *m*-[*o*-(2-Chloro-5-fluorosulfonylphenylureido)phenoxybutoxy]benzamidine†

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ABSTRACT: Fully activated two chain human C1s̄, a subcomponent of C1̄, the first component of complement, was selectively and irreversibly inactivated with *m*-[*o*-(2-chloro-5-fluorosulfonylphenylureido)phenoxybutoxy]benzamidine [*m*CP(PBA)-F]. The time course of inactivation followed pseudo-first-order kinetics (k_L = 1.52 × 10^{-4} s^{-1}, 23 °C), while the initial rate of inactivation at various inhibitor concentrations followed a rectangular hyperbolic function predicted for affinity labeling kinetics (K_L = 87.3 μM; k_2 = 3.06 × 10^{-3} s^{-1}). The hydrolyzed *m*CP(PBA)-F in which a sulfonic acid replaces the reactive sulfonyl fluoride group was a competitive inhibitor of C1s̄ (K_i = 4.6 μM) implicating active site binding. The analogue *o*-(2-chloro-5-fluorosulfonylphenylureido)methoxybenzene which lacks the active-site-directed benzamidine only slowly inactivated C1s̄ (k_L = 1.35 × 10^{-5} s^{-1}). At a tenfold molar excess of [^{3}H]*m*CP(PBA)-F to C1s̄, 1.078 mol of label was incorporated per mol of C1s̄. Diisopropyl fluorophosphate (Dip-F) and *m*CP(PBA)-OH protected C1s̄ from incorporation of [^{3}H]*m*CP(PBA)-F. In contrast to [^{14}C]Dip-F which labels only the light chain of C1s̄, the [^{3}H]*m*CP(PBA)-F label was distributed 57.6% and 42.3% on the heavy and light chain, respectively. C1s̄ fully inactivated with Dip-F retained the ability to inhibit formation of EAC4,2̄, the cell bound C3 convertase enzyme, whereas *m*CP(PBA)-F inactivated C1s̄ had partially lost this activity. From the dimensions of the *m*CP(PBA)-F reagent we infer the proximity of the heavy chain of C1s̄ to portions of the light chain comprising the catalytic center and suggest that these regions contain those portions on the C1s̄ molecule which specifically bind the complement component C4.

C1s̄[1] is a serine protease which is bound in a Ca(II)-dependent complex with two other proteins C1q and C1r to form C1, the first component of complement. Activated C1s̄ consists of a heavy chain (53 000 daltons) and light chain (29 000 daltons) linked by one or more disulfide bonds. The light chain contains the active site serine within the sequence Ala-Cys-Gly-Lys-Asp-Ser-Gly-Glu(Gly)-Arg. C1s̄ is activated by C1r̄ following the interaction of C1q with immune complexes or other complement activators. The activation proceeds by limited proteolysis of a single polypeptide chain with no apparent decrease in molecular weight. C1s̄ is the enzyme in the C1̄ complex responsible for formation of C3 convertase enzyme via limited proteolysis of the protein substrates C4 and C2 (Reid & Porter, 1975; Cooper & Ziccardi, 1976).

C1s̄ hydrolyzes a number of synthetic substrates. The esters *N*-Ac-Gly-L-Lys-OMe, *N*-Ac-L-Lys-OMe, *N*-Ts-L-Arg-OMe, *N*-Ac-Tyr-OEt, *N*-Z-Lys-ONp, and *N*-Z-L-Tyr-ONp and a tripeptide anilide Boc-Phe-Val-Arg-Na are hydrolyzed (Bing, 1969; Cooper & Ziccardi, 1976; Harpel, 1970; Nagaki & Stroud, 1969; Morgan & Nair, 1977). The turnover rate of these substrates by C1s̄ is very slow compared with the activity of other enzymes such as trypsin, thrombin, and plasmin toward the same compounds (Cooper & Ziccardi, 1976; Morgan & Nair, 1977, Andrews et al., 1978). Like these enzymes, however, C1s̄ is competitively inhibited by substituted benzamidines and phenylguanidine as well as a variety of N-*a*-blocked tyrosines (Bing, 1969; Andrews et al., 1978), further illustrating the hydrophobic and ionic nature of the active site.

The extent to which secondary binding sites define the substrate specificity of C1s̄ is largely unknown. Several studies on the C4 and C2 binding sites in C1̄ have demonstrated competitive inhibition of C2 activity by *N*-Ts-L-Arg-OMe

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[1] Abbreviations used: Dip-F, diisopropyl fluorophosphate; *m*CP(PBA)-F, *m*-[*o*-(2-chloro-5-fluorosulfonylphenylureido)phenoxybutoxy]benzamidine; *m*CP(PBA)-OH, *m*-[*o*-(2-chloro-5-sulfonic acid phenylureido)phenoxybutoxy]benzamidine; *m*CP(MB)-F, *m*-[*o*-(2-chloro-5-fluorosulfonylphenylureido)methoxy]benzene; *N*-Ac-Gly-L-Lys-OMe, *N*-acetyl-glycyl-L-lysine methyl ester; *N*-Ts-L-Arg-OMe, *N*-tosyl-L-arginine methyl ester; *N*-Ac-L-Tyr-OEt, *N*-acetyl-L-tyrosine ethyl ester; *N*-Z-L-Lys-ONp, *N*-carbobenzyloxy-L-lysine *p*-nitrophenyl ester; *N*-Z-L-Tyr-ONp, *N*-carbobenzyloxy-L-tyrosine *p*-nitrophenyl ester; $NaDodSO_4$, sodium dodecyl sulfate; em, effective molecules; Gdn·HCl, guanidine hydrochloride. The terminology for the complement system is that previously suggested ((1968) *Bull. W.H.O. 39,* 935). The complement components are numbers C1, C2, C4, etc. An activated component is indicated by a line drawn across the top. Thus, C1s̄ is the activated form of the s subcomponent of the first component of complement. EA is sheep erythrocytes sensitized with rabbit anti-sheep erythrocyte antibody. VBS is 0.15 ionic strength Veronal buffered saline, pH 7.4. S-VBS is 0.065 ionic strength VBS made to 300 mosM by addition of sucrose.

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implicating the importance of the $C1\bar{s}$ esteratic site (Stroud et al., 1965). Gigli & Austen (1969) found that the C4 binding site in $C\bar{1}$ is heat labile and distinguishable from the Dip-F sensitive site. The site has been assigned to the $C1\bar{s}$ subcomponent because (i) in solution $C1\bar{s}$ alone causes limited proteolysis of C4 or C2 and (ii) the presence of C1r inhibits this action of $C1\bar{s}$ with regard to C2, but addition of C4 overcomes this inhibition. Furthermore, binding of C4 to $C1\bar{s}$ in the C1 complex creates an additional binding site for C2, and leads to formation of the $C\overline{4,2}$ (C3 convertase) enzyme. The work of Strunk & Colten (1974) supports the concept of separate binding sites for C4 and C2 in $C1\bar{s}$, but to date no one has ever demonstrated the direct binding of C4 to $C1\bar{s}$. In the present report, it is shown that *m*-[*o*-(2-chloro-5-fluorosulfonylphenylureido)phenoxybutoxy]benzamidine [*m*CP(PBA)-F] is a potent affinity labeling reagent for $C1\bar{s}$. Furthermore, the reagent labels both chains of the protease and partially blocks the C4 binding site on the enzyme.

Materials and Methods

Enzyme Preparations. $C1\bar{s}$ was purified from Cohn fraction I as follows. Approximately 2 kg of Cohn fraction I (generously provided by Mr. L. Larson, Biologic Laboratories, Jamaica Plain, Mass.) was solubilized by overnight stirring with 1 L of 0.5 M NaCl. The large insoluble material was removed by filtration through cheesecloth and the liquid portion dialyzed overnight against 4 L of 0.15 ionic strength VBS–3 mM $CaCl_2$ pH 7.4 at 4 °C. The material formed a solid clot which was broken up by brief homogenization in a Waring blender. Insoluble material was removed by centrifugation (10 000*g*, 20 min, 4 °C) and the material redialyzed against 4 L of 0.15 ionic strength VBS–0.1 mM $MgCl_2$–0.015 mM $CaCl_2$, 4 °C. The $C\bar{1}$ was removed by affinity chromatography on a 500-mL bed volume of (2-hydroxy-5-nitrobenzyl-IgG)-*p*-azo-benzyloxyethylsulfonoethyl-Sepharose 6B resin as previously described (Andrews et al., 1978). The $C1\bar{s}$ was further purified by DEAE ion-exchange chromatography (DE-32, Whatman) (Taylor et al., 1977) and gel filtration on Ultra-Gel ACA 44 (LKB) in 0.1 M EDTA. The yield was approximately 20 mg of highly purified protein. The purified $C1\bar{s}$ had an esterolytic specific activity vs. *N*-Z-L-Tyr-ONp of 2200 U/mg assuming an $E^{1\%}_{280} = 9.3$ (Bing, 1969). It migrated as a single band when 100 μg was electrophoresed on $NaDodSO_4$–polyacrylamide gel electrophoresis (5% gels) with an apparent molecular weight of 83 000 ± 2000. The $C1\bar{s}$ was judged to be totally activated according to the following criteria: (i) based on the V_{max} and k_{cat} with respect to *N*-Z-L-Tyr-ONp (Bing, 1969), the maximum specific activity for $C1\bar{s}$ is calculated to be 2600 U/mg. The $C1\bar{s}$ used in these studies had 84.6% of the theoretically possible activity. (ii) It was fully converted to heavy and light chains upon reduction with 1% mercaptoethanol (redistilled) and S-pyridylation with 4-vinylpyridine (Freidman et al., 1970) (see Figure 5 insert in panels A and D). This latter criteria has been used by Cooper & Ziccardi (1978) to define full activation of zymogen $C\bar{1}$.[2] A molecular weight of 83 000 was used to calculate molarity of $C1\bar{s}$. This $C1\bar{s}$ was functionally active. In the assay with C2 and EAC4 (Nagaki & Stroud, 1969), it had an activity of 3.18×10^{11} em/μg.

Proteins. Molecular weight markers for $NaDodSO_4$–polyacrylamide gel electrophoresis were as follows: phosphorylase a (Worthington Biochemical) 94 000, bovine serum albumin (Sigma) 68 000, carbonic anhydrase (Sigma) 54 000, glutamic dehydrogenase (Sigma) 40 000, and creatine kinase (Sigma) 29 000.

Synthesis of mCP(PBA)-F and [^{3}H]mCP(PBA)-F. *m*CP(PBA)-F was prepared as described by Baker & Cory (1971). The [^{3}H]*m*CP(PBA)-F (0.426 Ci/mmol) was prepared by tritiation of the intermediate *m*-(*o*-aminophenoxybutoxy)benzamidine (New England Nuclear) with 10 Ci of ^{3}H by catalytic exchange prior to condensation with *O*-(*p*-nitrophenyl)-*N*-(2-chloro-5-fluorosulfonylphenyl)carbamate. The reagent [^{14}C]Dip-F was obtained with a specific activity of 100 mCi/mmol (New England Nuclear). Unless otherwise specified, other organic compounds were purchased as reagent grade (Aldrich). Buffered solutions calibrated against reference pH standard were made with deionized water and salts meeting the American Chemical Society specifications.

Examination of Inactivation Kinetics. The inactivation time course was analyzed in the following manner. Duplicate 500-μL samples of 16.5 μM $C1\bar{s}$ were prepared in 60 mM NaCl/5 mM Tris–acetate at pH 8.1. These were mixed with 150 μL of 1.01 mM *m*CP(PBA)-F or 9.62 mM *o*CP(MB)-F in methanol or solvent alone for controls and then incubated at 24 °C, and 50-μL samples were withdrawn at 4- or 5-min intervals. Residual $C1\bar{s}$ enzymic activity was measured with *N*-Z-L-Tyr-ONp (Nutritional Biochemical) by measuring the absorbancy of *p*-nitrophenol released at 410 nm with a Gilford 240 spectrophotometer (Bing, 1969). The log of the activity remaining per incubation time thus gave the pseudo-first-order labeling rate constant (k_L) for inactivation.

In other experiments, the initial velocity (v_0) of inactivation was determined essentially by the method of Mares-Guia & Shaw (1967) as modified by Childs & Bardsley (1975). The preceding protocol was followed except that the concentration of the reagent *m*CP(PBA)-F in methanol was varied from 10.1 to 91.9 μM. These data were analyzed on the basis that the enzyme $C1\bar{s}$ (E) initially reacts with the inhibitor affinity-labeling reagent (I) to form a reversible enzyme–inhibitor complex (EI) and subsequently an irreversible complex (EI′), as a consequence of forming a covalent bond (eq 1)

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \xrightarrow{k_2} EI' \qquad (1)$$

Assuming steady-state conditions $d(EI)/dt = 0$ and $k_1 \gg k_{-1}$, then the loss of enzyme activity per unit time $[-d(E)/dt]$ approximates the velocity $[v = d(EI')/dt = k_2(EI)]$ in the initial stages of the reaction $[v \sim v_0, (E) \sim (E_0), (I) \sim (I_0)]$. This may be expressed by a simple rectangular hyperbolic function (eq 2)

$$v_0 = \frac{-d(E_0)}{dt} \cong \frac{k_2(E)(I)}{K_L + (I)} \qquad (2)$$

which contains the affinity labeling constant $K_L = (k_{-1} + k_2)/k_1$, analogous to the Michaelis–Menten constant of simple enzyme–substrate kinetics (Morris, 1975). The plot of the reciprocal velocity $(1/v)$ against the reciprocal reagent concentration $[1/(I)]$ consequently has an intercept of $1/k_2(E)$ and a slope of $K_L/k_2(E)$. The slope/intercept is K_L, whereas the reciprocal product of the intercept and the initial enzyme concentration (E_0) gives k_2.

The inhibition constant (K_i) for benzamidine against *N*-Z-L-Tyr-ONp hydrolysis was measured at 1 to 0.1 mM concentrations. The K_i for *m*CP(PBA)-OH [hydrolyzed *m*CP(PBA)-F] was similarly measured at 0.55 to 4.38 μM concentrations and was assumed to approximate k_{-1}/k_1 (eq

[2] To date, there is no known active site titrant for $C1\bar{s}$. In fact, NPGB, while it reacts with $C1\bar{r}$, does not titrate $C1\bar{s}$ (Tamura et al., 1977; Andrews, J. M., & Bing, D. H., manuscript in preparation).

1), which permitted a second estimate (k_2') of k_2 from the initial inactivation rate (eq 3).

$$k_2' = K_i \frac{v_0}{(E_0)(I_0)} \quad (3)$$

Kinetic constants and statistical errors were determined on the NIH PROPHET computer system.[3]

Examination of Labeling Products. C1$\bar{s}$ reacted with isotopically labeled reagents was dissolved in 10 mL of Biofluor (New England Nuclear) scintillation fluid. Radioactivity was measured with a Searle Mark III scintillation counter, and isotope specific activities were determined for a minimum of four samples.

The distribution of labeled material was examined in NaDodSO$_4$–5% PAA gels (8 cm) according to Weber & Osborn (1975), except that 20 μL of *N,N,N',N'*-tetraethylenediamine was used to catalyze gel polymerization. Gel calibrations were based on the mobilities of standards of known molecular weights in 5% PAA gels. Protein reduction and S-pyridylation were carried out with 1% mercaptoethanol (redistilled) and 4-vinylpyridine (redistilled) (Freidman et al., 1970), except the products were dialyzed overnight against 1 L of 0.1% NaDodSO$_4$–100 mM sodium phosphate, pH 7.4, prior to measuring radioactivity and electrophoresis in NaDodSO$_4$–PAA gels. Protein was measured by Folin reaction as described by Lowry et al. (1951) and bovine serum albumin was used to establish a standard curve. Protein components in the gels were assessed by staining with Coomassie Blue (Schwarz/Mann). Radioactivity distributions were determined by sectioning gels into 35–45 fractions with a Savant Autogel Divider and the pulverized gel slices were suspended directly in Biofluor scintillation fluid.

Binding of [^{3}H]mCP(PBA)-F to C1$\bar{s}$. Duplicate samples of 200 μL of 18.1 μM C1$\bar{s}$ in 60 mM NaCl/5 mM Tris-acetate, pH 8.1, were incubated for 3 h at 23 °C with 50 μL of 100 mM Dip-F, 50 μL of 0.86 mM *m*CP(PBA)-OH or 200 μL of 8 M urea in a final volume of 500 μL. Then 50 μL of 1.29 mM [^{3}H]*m*CP(PBA)-F was added, samples were withdrawn at 5-min intervals, and the protein was precipitated with 1 mL of cold 10% Cl_3CCOOH. The precipitate was collected on 0.45-μm Millipore filters, washed three times with 5 mL of ice-cold 5% Cl_3CCOOH, and then 5 mL of ice-cold absolute ethanol. The filters were suspended in 10 mL of Biofluor and radioactivity was determined.

Complement Assays. The 0.15 ionic strength Veronal-buffered saline–0.1 mM $MgCl_2$–0.015 mM $CaCl_2$–0.1% gelatin, pH 7.4 (VBS), 0.065 ionic strength sucrose–Veronal buffer–0.15 mM $MgCl_2$–0.015 mM $CaCl_2$, 0.1% gelatin, pH 7.4 (S-VBS), EA, EAC4 intermediate and CEDTA were prepared as previously described (Andrews et al., 1977). The C1$\bar{s}$ was assayed for ability to catalyze hemolysis of the EAC4 in the presence of C2 and CEDTA (Nagaki & Stroud, 1969). To determine inhibition of C1$\bar{s}$ catalysis of EAC$\overline{4,2}$ formation by Dip-F or *m*CP(PBA)-F inhibited C1$\bar{s}$, the following protocol was used: 4.5 mg of C1$\bar{s}$ in 2.5 mL of 100 mM Tris-acetate, pH 8.1, was completely inactivated (<10 esterase units/mL) at 37 °C by addition of five separate aliquots of 50 μL of 100 mM Dip-F or 25-μL aliquots of 1 mM *m*CP(PBA)-F over a period of 4 h. The Dip-C1$\bar{s}$ and *m*CP(PBA)-C1$\bar{s}$ were then dialyzed separately overnight at 4 °C against 1 L of Veronal-buffered saline–1 mM $MgCl_2$–0.15 mM $CaCl_2$, pH 7.4 (VBS), of 0.15 ionic strength to remove excess inhibitor. To determine inhibition of EAC$\overline{4,2}$ formation, 200 μL of Dip-C1$\bar{s}$ or *m*CP(PBA)-C1$\bar{s}$ was incubated at 32 °C with shaking for 30 min with 1.5 mL of EAC4 [1.5×10^8/mL in 0.065 ionic strength sucrose Veronal-buffered saline–1 mM $MgCl_2$–0.15 mM $CaCl_2$ (S-VBS)]. Then 1.5 mL of C2 diluted in S-VBS to give 100 em/cell (Rapp & Borsos, 1970) was added simultaneously with 50 μg of C1$\bar{s}$ and incubated at 32 °C with shaking. Samples of 200 μL were then removed at 0, 1, 2, 4, 8, 10, 15, and 20 min and added to 300 μL of CEDTA followed by 1-h incubation at 37 °C with occasional shaking. The degree of lysis was determined following addition of 1 mL of cold 150 mM NaCl, centrifugation, and measurement of A_{412} of the supernatants. EAC$\overline{4,2}$ sites were calculated as described by Rapp & Borsos (1970).

Results and Discussion

Affinity Labeling of C1$\bar{s}$ with mCP(PBA)-F. Criteria for demonstrating affinity labeling have been set forth in detail by Baker (1967), Singer (1963), and Shaw (1970). The technique uses a reagent which (1) achieves a high local concentration because it is selectively and reversibly bound by a protein (enzyme, antibody, receptor protein, etc.) at the active site, and (2) is capable of forming an irreversible covalent bond with the protein within or adjacent to the binding site. The local concentration of the reagent greatly increases its probability of reacting with contacted residues neighboring a high-affinity-binding site (specific labeling) in contrast to residues of equivalent chemical reactivity located elsewhere on the protein surface (nonspecific labeling). Hence, the term "affinity labeling" was coined by Wofsy et al. (1962) to distinguish this type of selective labeling from those taking advantage of chemically hyperreactive residues (e.g., Dip-F reaction with the uniquely reactive serine 195 in the active site of trypsin or the light chain of C1$\bar{s}$).

Benzamidine and related compounds are well-established competitive inhibitors of C1$\bar{s}$; extending side chains, moreover, substantially increase the inhibitory activity of such compounds for C$\bar{1}$ (Bing et al., 1974). The compound *m*CP(PBA)-F possesses both a benzamidine moiety and an extended side chain (Figure 1), which should make it a potent inhibitor. Indeed, the alkaline hydrolyzed compound *m*CP(PBA)-OH with the reactive sulfonyl fluoride of *m*CP(PBA)-F converted to a nonreactive sulfonic acid group, was a strong competitive inhibitor of C1$\bar{s}$ esterase activity and exhibited an inhibition constant (K_i = 4.6 μM) at least two orders of magnitude lower than that of benzamidine (K_i = 0.63 mM (Bing, 1969)). As this constant is a reciprocal measure of the binding affinity (Morris, 1975), the side chain contributes considerably to binding. Furthermore, since *m*CP(PBA)-OH is a competitive inhibitor, it binds at the active site, and since it differs only slightly from *m*CP(PBA)-F, the latter is, with little question, active-site bound upon reacting with C1$\bar{s}$. Indeed, *m*CP(PBA)-OH protects the enzymes from [^{3}H]-*m*CP(PBA)-F binding (Table I); the specificity of binding of [^{3}H]*m*CP(PBA) was further demonstrated as C1$\bar{s}$ reacted with the active-site-directed reagent Dip-F bound only about one-fourth the amount of [^{3}H]*m*CP(PBA)-F as C1$\bar{s}$ alone and denatured C1$\bar{s}$ (4 M urea) bound no reagent (see Figure 2).

Sulfonyl fluorides are known to react with the Dip-F-sensitive serine of the pancreatic seryl proteases (Gold & Fahrney, 1964), and like Dip-F do so because of the increased reactivity of this serine through its involvement in the enzyme catalytic mechanism. On the other hand, Cardinaud & Baker (1970) found evidence that an extended-chain sulfonyl fluoride compound α-(2-carboxy-4-chlorophenoxy)-*N*-[*m*-(*m*-fluorosulfonylphenylureido)benzyl]acetamide could react with

[3] The NIH PROPHET computer system has been described ((1978) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 32*, 1744).

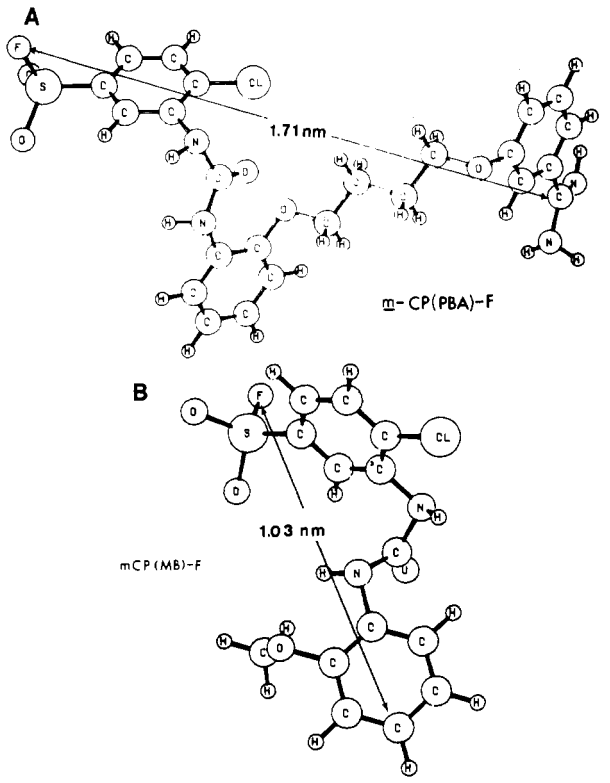

FIGURE 1: Models of *m*CP(PBA)-F (A) and *m*CP(MB)-F (B). The models were constructed with aid of the NIH PROPHET computer[3] and have been put in the most probable extended conformations.

TABLE I: Incorporation of [^{3}H]*m*CP(PBA)-F and Dip-F into C1s.[a]

pretreatment	time (min)	mol of [^{3}H]*m*CP-(PBA)/mol of C1s
none	30	0.576
	60	0.984
	120	1.078
0.87 mM *m*CP(PBA)-OH	60	0.512
1 mM Dip-F	60	0.372

[a] Two milliliters of C1s, 3 μM, in 100 mM Tris-acetate, pH 8.1, was reacted for indicated times with 39.9 μM [^{3}H]*m*CP(PBA)-F. The reaction was stopped by addition of 2 mL of 6 M Gdn·HCl. The reaction mixtures were dialyzed successively over 72 h against 1 L of 6 M Gdn·HCl and two changes of 1 L of H_2O. The mixtures were lyophilized and dissolved in 2 mL of 0.2% $NaDodSO_4$–200 mM sodium phosphate, pH 7.4. Half was removed and reacted at 37 °C for 2 h with 1% mercaptoethanol and then S-pyridylated for 2 h at 37 °C with 4-vinylpyridine. The reagents were removed by dialysis against the 0.1% $NaDodSO_4$–100 mM sodium phosphate, and 50-μL aliquots analyzed in triplicate for protein and radioactivity incorporation.

residues other than the Dip-F-sensitive serine in chymotrypsin and affinity labeling of thrombin by *m*CP(PBA)-F results in covalent modification of both chains (Bing et al., 1977a). In both instances the reactive group (e.g., the sulfonyl fluoride) was well removed from the active-site-directed ligand moiety (the benzamidine). This should prevent its reaction with the Dip-F-sensitive serine, if the compound was specifically bound within the active site. For *m*CP(PBA)-F, the distance between the reactive fluoride and the amidino cation has been calculated to be approximately 1.7 nm assuming that the compound is in its most probable low energy configuration. For comparison, in the C1s nitroanilide substrate Boc-Phe-Val-Arg-Na (Morgan & Nair, 1977), the scissile bond is 0.65 nm from the cationic guanidinium group and thus approximates the size of catalytic site of C1s. The high binding affinity of *m*CP(PBA)-OH, compared with benzamidine, implies that *m*CP(PBA)-F binds not only via its specific ligand but also via interactions of the extended side chain and that this chain extension has restricted orientations possibly within the peptide binding groove (Feldmann et al., 1978).

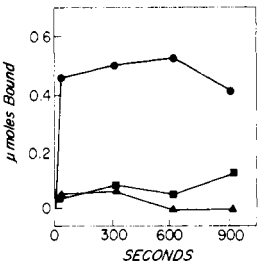

FIGURE 2: Binding of [^{3}H]*m*CP(PBA)-F to C1s in the presence of Dip-F (▲) and 6 M urea (■). The control (●) contained buffer in place of inhibitors.

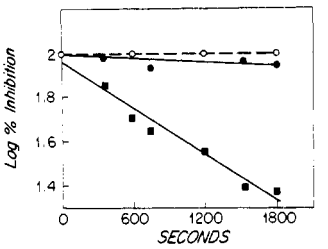

FIGURE 3: Inactivation of 16.5 μM C1s by methanol (○), *m*CP(PBA)-F (■), and *m*CP(MB)-F (○). The inactivation was performed as described in Materials and Methods. The pseudo-first-order rate constants $k_L = 6.3 \times 10^{-7}$ s^{-1} for the methanol control 1.35×10^{-5} s^{-1} for 222 μM *m*CP(MB)-F and 1.5×10^{-4} s^{-1} for 233 μM *m*CP(PBA)-F.

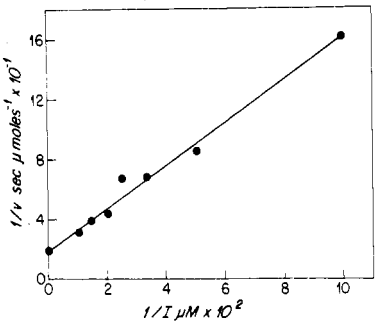

FIGURE 4: Steady-state treatment of inactivation kinetics of C1s by *m*CP(PBA)-F. The inhibitor was varied from 10.1 to 91.1 μM. The V_{max} = 5.3 nmol/s and the K_m/V_{max} slope = 10.4 (r = 0.96; N = 5).

Four independent methods were used to establish affinity labeling of C1s with *m*CP(PBA)-F. First, a 14-fold molar excess of the compound caused a rapid inactivation of C1s (Figure 3). This inactivation followed pseudo-first-order kinetics ($k_L = 1.52 \times 10^{-4}$ s^{-1}, 23 °C), as predicted for the time course reaction with an affinity labeling reagent. Further, by varying the reagent concentration, the initial inactivation velocity followed the double-reciprocal relationship (Figure 4) for steady-state expression (eq 2), analogous to that for simple enzyme-substrate kinetics (Morris, 1975). The rectangular hyperbolic constant K_L was 87.3 μM, while the k_2 was 3.06×10^{-3} s^{-1} (V_{max} for 16.8 μM C1s was 5.3 nM enzyme/s). A value of 4.6 μM for the K_i of *m*CP(PBA)-OH was used to calculate k_2' as 3.62×10^{-3} s^{-1} (eq 3, see above) and dem-

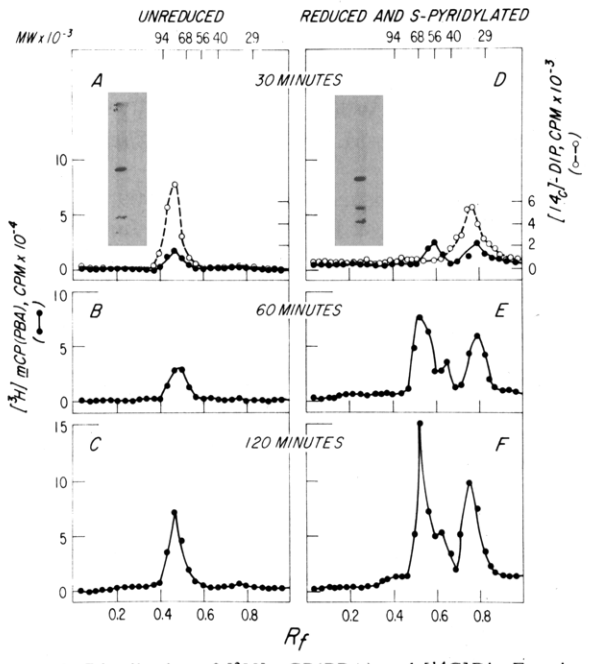

FIGURE 5: Distribution of [^{3}H]*m*CP(PBA) and [^{14}C]Dip-F on intact and heavy and light chains of C1s̄. A, B, and C are C1s̄ labeled for 30, 60, and 120 min with [^{3}H]*m*CP(PBA)-F. D, E, and F are reduced and S-pyridylated C1s̄. Each gel contained 186 to 140 μg of [^{3}H]*m*CP(PBA)–C1s̄. A constant amount of [^{14}C]Dip–C1s̄ (57 μg) unreduced or reduced and S-pyridylated was added to each sample, as an internal marker of the intact light chain of C1s̄. The R_fs of molecular weight markers are indicated at the top of the panel. The insert contains a picture of the stained gel. The minor bands evident in panels E and F arise from the presence of a lower molecular weight derivative of C1s̄ which arises during purification (see footnote 2).

onstrated the internal consistency of the method. Second, the high-affinity competitive inhibitor *m*CP(PBA)-OH protected C1s̄ from [^{3}H]*m*CP(PBA)-F incorporation, demonstrating that the active site was indeed labeled (Table I).

Third, at approximately the same twofold molar excess, the analogue *o*CP(MB)-F (Figure 1) caused no inactivation of C1s̄ in contrast to over 90% inactivation of C1s̄ with *m*CP(PBA)-F (Figure 2). Thus, the sulfonyl fluoride group alone could not explain the more extensive inactivation of *m*CP(PBA)-F.

Fourth, [^{3}H]*m*CP(PBA)-F was stoichiometrically incorporated into C1s̄ by 1 h and further incubation resulted in no further uptake (Table I).[4] That [^{3}H]*m*CP(PBA)-F forms a covalent bond with C1s̄ was demonstrated by the stability of the labeled products to reduction and S-pyridylation (Table I) and to the protein-dissociating agent $NaDodSO_4$ upon electrophoresis (Figure 5). These experiments collectively demonstrate that *m*CP(PBA)-F behaves with C1s̄ in a manner predicted for an active-site directed affinity-labeling reagent.

Labeled Products of C1s̄ with [^{3}H]mCP(PBA)-F. The reagent [^{3}H]*m*CP(PBA)-F has dimensions such that, when the benzamidine moiety is specifically bound, its reactive group should extend beyond the classical active site (catalytic center and cationic binding site region) and should not be in a position to readily react with the Dip-F-sensitive serine in the enzyme active site (see above). This serine, however, could potentially react if the reagent nonspecifically entered the site or if the reagent folded back on itself (prior to or upon binding at the site) making the reactive group accessible to the serine. That

[4] The term stoichiometric is used as defined by Wofsy et al. (1962). It refers to moles of label bound per mole of protein, and infers nothing about the site(s) of reaction in the protein.

TABLE II: Distribution of [^{3}H]*m*CP(PBA)-F on Heavy and Light Chains of C1s̄.[a]

time (min)	heavy chain (%)	light chain (%)
30	44.3	55.7
60	58.8	41.2
120	57.6	42.3

[a] C1s̄ was labeled as described in Table I and electrophoresed as described in Materials and Methods. The percent distribution on the chains was calculated from the gels containing reduced and S-pyridylated C1s̄ (Figure 5D–F).

C1s̄ partially inactivated by Dip-F failed to stoichiometrically incorporate [^{3}H]*m*CP(PBA)-F (Table I) implies the involvement of the Dip-F sensitive serine, but the result does not establish that both compounds react with this residue. Rather the derivatization of the serine with Dip-F could prevent binding of [^{3}H]*m*CP(PBA)-F (e.g., steric blocking of the active site or enzyme conformational rearrangements upon derivatization). The two experiments together clearly show that the labeling with either compound involves a common region of the enzyme molecule (its active site) or regions which function in concert with one another.

Although the Dip-F sensitive serine is a hyperreactive residue, [^{3}H]*m*CP(PBA)-F because of its reactive sulfonyl fluoride should be capable of reacting with the hydroxyl groups of other serines and threonines (Baker, 1967). Reaction of [^{3}H]*m*CP(PBA)-F with residues other than the Dip-F sensitive serine was demonstrated by the labeling incorporation among C1s̄ chains. With labeled and subsequently reduced and S-pyridylated C1s̄ (Figure 5),[5] the [^{3}H]*m*CP(PBA)-F products were distributed on the heavy chain (57.6%) and on the light chain (42.3%) after 2 h of reaction. At 30 min, there was preferential incorporation into the light chain at a point where a little over one half of the sites were modified (see Table II). This ratio was reversed by 1 h when the C1s̄ was virtually completely inactivated. Furthermore, the ratio of label on heavy and light chains was not changed between 1 and 2 h. By weight the heavy chain is 65% and the light chain is 35% (Sim et al., 1977). The final percent distribution of [^{3}H]-*m*CP(PBA)-F on the chains coupled with the apparent difference in rate of uptake of the chains strongly supports the concept that the reagent is bound and incorporated in a specific fashion into C1s̄. The difference between K_L for *m*CP(PBA)-F and K_i for *m*CP(PBA)-OH further implies it is the formation of the covalent bond that is the rate-limiting step.

Finally, the specific nature of inactivation of C1s̄ was tested in a functional assay for C1s̄ activity. The report of Gigli & Austen (1969) suggested that the specific binding site for C4 resides in the C1s̄ subcomponent of C1 because this site was heat labile but not blocked by interaction of C1̄ with Dip-F. As C4 is a specific substrate for C1s̄, they implied there should be a secondary binding site for C4 on C1s̄. It follows, therefore, that Dip-C1s̄ (e.g., C1s̄ completely inactivated with Dip-F) should be able to compete with unmodified C1s̄ for C4, and inhibit C1s̄ activity in a functional complement assay for for-

[5] In the $NaDodSO_4$–polyacrylamide gel electrophoresis of C1s̄ there is a minor component at a molecular weight of about 75 000 in the unreduced C1s̄ and 50 000 in reduced and S-pyridylated C1s̄ which incorporates the [^{3}H]-*m*CP(PBA)-F. This is particularly evident in panels E and F of Figure 5. This material is a derivative form of C1s̄ which arises during purification. It retains full esterolytic activity and incorporates [^{14}C]DIFP (Bing & Attisano, unpublished results).

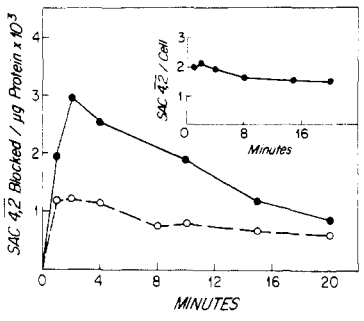

FIGURE 6: Inhibition of EAC$\overline{4,2}$ formation by Dip-C1$\bar{s}$ (●) and *m*CP(PBA)-C1$\bar{s}$ (○). The C1$\bar{s}$ was inactivated as described, and assay for EAC$\overline{4,2}$ formation was measured as described in Materials and Methods. The insert shows EAC$\overline{4,2}$ formation as a function of time in absence of Dip-C1$\bar{s}$ or *m*CP(PBA)-C1$\bar{s}$.

mation of the C$\overline{4,2}$ enzyme. The hypothesis tested in the present experiment was as follows: if *m*CP(PBA)-F covalently modifies to any degree the C4 binding site on C1$\bar{s}$, then Dip-C1$\bar{s}$ should inhibit C1$\bar{s}$ catalysis of EAC$\overline{4,2}$ formation but *m*CP(PBA)-C1$\bar{s}$ should not. As the assay for EAC$\overline{4,2}$ formation requires a large excess of C2, which would favor EAC$\overline{4,2}$ formation with time, the inhibition was determined at various times over a period of 20 min. The results of this experiment (Figure 6) confirmed that Dip-C1$\bar{s}$ could inhibit C4 functional activity and thus retained the C4 binding site. Furthermore, as expected, there was a kinetic effect; at 2 min Dip-C1$\bar{s}$ caused almost twice the inhibition as did *m*CP(PBA)-C1$\bar{s}$. This effect virtually disappeared by 20-min incubation, indicating the predicted reversible nature of the interaction of Dip-C1$\bar{s}$ with C4. That *m*CP(PBA)-C1$\bar{s}$ could not inhibit EAC$\overline{4,2}$ formation as well as Dip-C1$\bar{s}$ implies that the reagent derivatized or blocked the secondary binding sites which are responsible for the narrow protein substrate specificity of C1$\bar{s}$. The 60% of the reagent which was distributed on the heavy chain correlated with twofold difference in inhibitory capacity between Dip-C1$\bar{s}$ and *m*CP(PBA)-C1$\bar{s}$. It is tempting to speculate that these secondary binding sites reside on that portion of the molecule.

In any case, the present studies provide the first direct evidence that the heavy chain of C1$\bar{s}$ is located near the classical active site of the enzyme and suggests that both chains participate in defining enzyme specificity. Whether or not the structural basis for the unique specificity of C1$\bar{s}$ resides entirely within the area of the protein chemically defined as the active site remains to be determined and cannot be ascertained from these studies. Based on studies of exo-site affinity labeling on clotting and nonclotting thrombins, factor Xa and plasmin (Bing et al., 1977b), an attractive hypothesis is that the active site of C1$\bar{s}$ coevolved with the cleavage site in C4 and C2 to create a uniquely reactive highly specific system of protein-protein interaction and proteolysis.

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