Inactivation of the First Component of Human Complement by *m*-[*o*-(2-Chloro-5-fluorosulfonylphenylureido)-phenoxybutoxy]benzamidine†

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ABSTRACT: The compound *m*-[*o*-(2-chloro-5-fluorosulfonylphenylureido)phenoxybutoxy]benzamidine has been shown to inactivate irreversibly the first component of human complement $C\bar{1}$ purified by affinity chromatography. At a concentration of 7×10^{-5} M at 30° in pH 7.4, 0.065 ionic strength triethanolamine-buffered NaCl–0.17 M sucrose, inactivation proceeded according to strict pseudo-first-order kinetics with a rate constant of 4.72×10^{-4} sec^{-1}. The inactivation occurred *via* formation of a specific complex, strictly analogous to an enzyme–substrate complex. The site of action of *m*-[*o*-(2-chloro-5-fluorosulfonylphenylureido)phenoxybutoxy]-benzamidine on $C\bar{1}$ was the esteratic site which is sensitive to the naturally occurring serum protein inhibitor of $C\bar{1}$. The inhibitor at the same concentration also irreversibly inactivated partially purified human $C\bar{1}$.

Affinity-labeling compounds for the investigation of structure–function relationships of proteins has had wide applicability in studies on proteases and antibodies. Because such reagents form covalent bonds with the protein, it is possible to obtain detailed information on important functional groups critical to the activity of the particular protein (Singer, 1967). The possibility of applying this experimental technique to the study of structure–function relationships of the first component of complement is suggested by the observation that DFP[1] can inhibit guinea pig $C\bar{1}$ and the site of action is an esterase activity associated with $C\bar{1}$ (Becker, 1956a,b). This DFP-sensitive esterase activity has subsequently been shown to be associated with the $C1\bar{s}$ subunit (Haines and Lepow, 1964). The $C1\bar{s}$ subunit has also been shown to be inhibitable by a series of aromatic and alkyl phosphonate esters (Becker and Austen, 1966).

In a series of papers, Baker and coworkers (Baker and Erickson, 1969; Baker and Cory, 1969a,b, 1971a,b) reported the synthesis and screening of a large series of potential specific, active-site-directed irreversible inhibitors of the first component of guinea pig complement. These compounds contain a cationic benzamidine residue which specifically binds to the $C\bar{1}$ anionic binding site and a sulfonyl fluoride group that reacts to form a covalent bond with the protein. In those studies, inhibitory activity was measured as per cent inhibition of lysis of EA by whole complement and/or per cent inhibition of functionally pure guinea pig $C\bar{1}$ lysis of EA with an R1 reagent. Several of the compounds were quite active being inhibitory in both assays at concentrations of 3×10^{-4} M (Baker and Cory, 1971b). The present experiments are a detailed study of the reaction between affinity chromatographically purified human $C\bar{1}$ (Bing, 1971) and a potent guinea pig $C\bar{1}$ inhibitor *m*-[*o*-(2-chloro-5-fluorosulfonylphenylureido)-phenoxybutoxy]benzamidine (Baker and Cory, 1971b). Evidence is presented that $C\bar{1}$ is inactivated *via* the formation of a specific complex and the site of action is the esteratic site on the $C1\bar{s}$ subunit.

Materials and Methods

Proteins, Antiserum. Rabbit sheep hemolysin was purchased from Behring Diagnostics (Woodbury, N. Y.). Sheep erythrocytes were in Alsevers solution (Mayer, 1961) and aged 2 weeks prior to use. Pooled human serum and guinea pig serum were donated by the Michigan State Public Health Laboratories (Lansing, Mich.). Highly purified human $C\bar{1}$ was prepared by affinity chromatography on gels of Sepharose to which human IgG immunoglobulin had been covalently bound (Bing, 1971). Functionally pure human $C\bar{1}$ was prepared by precipitation of the euglobulin fraction of serum at a final ionic strength of 0.03 and pH 6.4 (Lepow *et al.*, 1963). Purified C1 inactivator was prepared by the method of Haupt *et al.* (1970). Functionally pure guinea pig $C\bar{2}$ was prepared according to the method of Nelson *et al.* (1966).

Chemicals, Buffers. DFP was a gift of Dr. H. L. Sadoff, Department of Microbiology and Public Health, Michigan State University, East Lansing, Mich. It was diluted to 1×10^{-3} M in isopropyl alcohol just prior to use. Poly (anetholesulfonic acid), sodium salt (Liquoid, Calbiochemicals, La Jolla, Calif.), was made up to 1 mg/ml in pH 7.4, 0.15 ionic strength sodium phosphate buffer (Chase and Williams, 1968). Phenylmethanesulfonyl fluoride (Calbiochemicals, La Jolla, Calif.) was made to 1.4×10^{-4} M in CH_3OH. The late Dr. B. R. Baker, Department of Chemistry, University of California at Santa Barbara, Santa Barbara, Calif., gener-

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[1] Terminology for the complement system is conventional (*Bull. W. H. O. 39*, 935 (1968)). Thus, $C\bar{1}$ designates the activated form of C1, the first component of complement. The subunits of $C\bar{1}$ are C1q, C1r, and C1s. $C1\bar{s}$ is the enzymatically active form of the C1s subunit. C2 and C4 are the second and fourth components of complement. EA is sheep erythrocytes treated with anti-sheep erythrocyte antiserum; C1 inactivator is the naturally occurring serum inhibitor of $C\bar{1}$. CEDTA is fresh serum treated with EDTA. Other abbreviations used in this report are MCFPB, *m*-[*o*-(2-chloro-5-fluorosulfonylphenylureido)-phenoxybutoxy]benzamidine; DFP, diisopropyl fluorophosphate; PMSF, phenylmethanesulfonyl fluoride; *N*-Z-L-TyrONp, *N*-carbobenzoxy-L-tyrosine *p*-nitrophenyl ester; eff mol, effective molecules. TBS, triethanolamine-buffered saline; TB-sucrose, triethanolamine-buffered saline sucrose.

TABLE I: Inactivation of Purified $C\bar{1}$ by DFP and MCFPB.

Compound Added	Enzymatic Activity: Units/ml	Enzymatic Activity: % Inhibn	$EA\bar{1},4$ Formation: Eff Mol/ml	$EA\bar{1},4$ Formation: % Inhibn
None	10		2.24×10^{11}	
DFP (9.09×10^{-5} M)	5.6	44.5	1.11×10^{11}	50.5
MCFPB (15.5×10^{-5} M)	0	100	0.0097×10^{11}	99.6

ously donated the *m*-[*o*-(2-chloro-5-sulfonylfluorophenylureido)phenoxybutoxy]benzamidine. The synthesis of this compound has been described (compound **25**, Baker and Cory, 1971b). It was made at 1.4×10^{-3} M in CH_3OH and stored at −20° until used.

TBS at pH 7.4, 0.75 ionic strength, and Tris-HCl buffer (pH 7.4), 0.15 ionic strength, were made according to Chase and Williams (1968). Triethanolamine-buffered NaCl, pH 7.4, 0.15 ionic strength, with a final concentration of 0.1% gelatin, 1×10^{-3} M $MgCl_2$, and 1.5×10^{-4} M $CaCl_2$, was made as follows: 100 ml of pH 7.5, 0.75 ionic strength TBS was mixed with 0.5 g of gelatin (Mann Biochemicals) and 0.5 ml of a 1 M $MgCl_2$–0.15 M $CaCl_2$ solution and diluted to 500 ml. Triethanolamine-buffered saline sucrose (TB-sucrose), pH 7.4, 0.065 ionic strength, containing 0.17 M sucrose, 1×10^{-3} M $MgCl_2$, 1.5×10^{-4} M $CaCl_2$, and 0.1% gelatin, was made as follows: 60 ml of pH 7.4, 0.75 ionic strength TBS was mixed with 40 ml of a solution consisting of 1.421 M sucrose, 1 M HCl, 0.0453 M triethanolamine, 0.5 g of gelatin, and 0.5 ml of a 1 M $MgCl_2$–0.15 M $CaCl_2$ solution and the buffer was diluted to a final volume of 500 ml. Tris-acetate (pH 8.1, 0.005 ionic strength)–0.09 M NaCl was made by appropriate dilution from 3 M NaCl and pH 8.1, 0.1 ionic strength Tris-acetate buffer (Bing, 1971). All buffers, salts, solvents, and chemicals were reagent grade. Triple-distilled water was used for all solutions.

Assays. Esteratic activity was measured with the substrate *N*-Z-L-TyrONP (Bing, 1969). The formation of the $EAC\bar{1},4$ intermediate was measured by a modification of the transfer test described by Borsos and Rapp (1965). The $C\bar{1}$ was added to EAC4 (Mayer, 1961) in 0.065 ionic strength TB-sucrose, incubated 10 min at 30°, and then the cells were centrifuged and washed three times with 0.065 ionic strength TB-sucrose. The cells were then transferred to a new tube and the functionally pure C2 was added. Incubation was continued for 30 min and then guinea pig serum diluted 1:25 in 0.15 ionic strength TBS with a final concentration of 0.01 M EDTA (CEDTA) was added. The formation of the $EAC\bar{4},\bar{2}$ intermediate was measured with the method described by Nagaki and Stroud (1969). Effective molecules were calculated according to the method outlined by Borsos *et al.* (1961). Protein concentration was determined by the method of Lowry *et al.* (1951); crystalline bovine serum albumin (Nutritional Biochemical Corp., Cleveland, Ohio) was used as a standard.

Results

Inactivation of $C\bar{1}$ by MCFPB. In a preliminary study, it was found that MCFPB irreversibly inactivated purified $C\bar{1}$. The experimental design was as follows. Four-tenths of a milliliter of purified $C\bar{1}$ (3.4×10^{11} eff mol/ml, 57.5 μg/ml) and a 0.045 M NaCl–0.005 ionic strength Tris-acetate buffer (pH 8.1) was mixed with 0.05 ml of 1.4×10^{-3} M MCFPB and incubated 18 hr at 0° followed by incubation at 37° for 30 min. The sample was dialyzed against two changes of 1 l. of 0.09 M NaCl–0.005 ionic strength Tris-acetate buffer (pH 8.1) at 4° over a period of 24 hr. The sample was then assayed for enzymatic activity with *N*-Z-L-TyrONP and for the ability to form the $EAC\bar{1},4$ intermediate. Two controls consisted of 0.5 ml of $C\bar{1}$ which received 0.05 ml of CH_3OH and 0.05 ml of 1×10^{-3} M DFP, respectively. These results are presented in Table I. Compared to the control, DFP caused between 44.5 and 50.5% inhibition and the MCFPB treatment resulted in complete inhibition. Furthermore, the degree of inhibition was essentially the same according to either assay. These results indicated the MCFPB irreversibly inactivated the $C\bar{1}$.

Kinetic Studies on Inactivation of $C\bar{1}$ by MCFPB. To gain further insight into the mechanism of inactivation of $C\bar{1}$ by MCFPB, the kinetics of the reaction were investigated under conditions where the order could be determined. Two-tenths of a milliliter of purified $C\bar{1}$ (7.42×10^{12} eff mol/ml, 1.25 mg/ml) was diluted to 2 ml in pH 7.4, 0.065 ionic strength TB-sucrose, warmed to 30°, and mixed with 0.1 ml of 1.4×10^{-3} M MCFPB. Every 30 min a sample was removed and diluted 1:100, 1:1,000, and 1:10,000 in the same buffer, and each dilution assayed for $EAC\bar{4},\bar{2}$ formation (see Materials and Methods). A control consisted of $C\bar{1}$ treated identically with 0.1 ml of CH_3OH. The results are presented in Figure 1A. The inactivation followed strict psudo-first-order kinetics. The first-order rate constant was calculated to be 4.73×10^{-4} sec^{-1}. A similar experiment was done where the decrease in $C\bar{1}$ enzymatic activity toward *N*-Z-L-TyrONP was measured. The reaction mixture in this instance contained 0.4 ml of purified $C\bar{1}$ (520 units of enzyme/ml, 5 mg/ml) and 0.05 ml of 1.4×10^{-3} M MCFPB in pH 8.1, 0.15 ionic strength Tris-HCl buffer containing 20% glycerol (v/v). The control was identical except it contained 0.05 ml of CH_3OH in place of the MCFPB. Every 15 min, samples were withdrawn, diluted 1:30 in 0.09 M NaCl, and assayed with *N*-Z-L-TyrONP. These results are presented in Figure 1B and are essentially identical with those in Figure 1A. In this instance the first-order rate constant was calculated to be 2.74×10^{-4} sec^{-1}.

The possibility was considered that the sulfonyl fluoride group was solely responsible for the inactivation and the phenoxybutoxybenzamidine was not directly related to the observed inactivation. This hypothesis was tested by comparing the ability of MCFPB and phenylmethanesulfonyl fluoride to inactivate $C\bar{1}$. The same preparation of $C\bar{1}$ in pH 7.4, 0.065 ionic strength TB-sucrose was warmed to 30° and mixed with MCFPB and PMSF to give final concentrations of 7×10^{-5} and 9×10^{-5} M, respectively. Samples were removed every 30 min and assayed for formation of the $EAC\bar{4},\bar{2}$ intermediate. The results are presented in Table II. By 90 min, the sample treated with MCFPB was totally inactivated, whereas the sample treated with PMSF remained essentially the same as the control for up to 2 hr.

The mechanism of the inactivation by MCFPB was next investigated to determine if the reaction of MCFPB with $C\bar{1}$ was proceeding *via* formation of a specific enzyme–inhibitor complex as described by

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

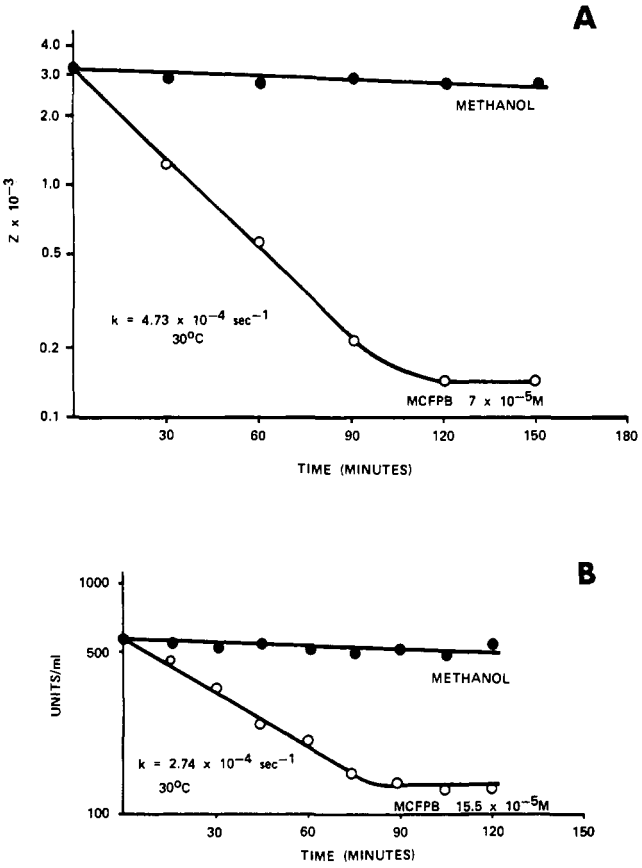

FIGURE 1: Inactivation of purified human $C\bar{1}$ by MCFPB. (A) Inhibition of $EAC\overline{4,2}$ formation, $Z = -\ln(1 - y)$, where y is the degree of lysis. See Results for experimental details. (B) Inhibition of esteratic activity of $C\bar{1}$ as assayed with *N*-Z-L-TryONp. See Results for experimental details.

As pointed out by Mares-Guia and Shaw (1967), such a mechanism is strictly analogous to the formation of an enzyme–substrate complex. Thus, if the concentration of the enzyme is held constant and the concentration of inhibitor is increased, the rate of inactivation of the enzyme will increase nonlinearly, approaching a maximum value. A steady-state treatment of equation (1) (Fahrney and Gold, 1963) leads to a solution for the rate of decrease of enzyme activity

$$-\frac{d(E)}{dt} = \frac{k_2(E)(I)}{K_m + (I)} \quad (2)$$

where $K_m = (k_{-1} + k_2)/k_1$, the Michaelis constant. Equation 2 indicates a nonlinear dependence of the rate of inactivation in eq 1, a limiting value being approached as E is being completely converted to the E–I complex. Rearrangement of eq 2, substituting v for $-d(E)/dt$ gives

$$\frac{1}{v} = \frac{1}{k_2(E)} + \frac{K_m}{k_2(E)}\frac{1}{(I)} \quad (3)$$

which has the same form as the Lineweaver–Burk equation for an enzyme substrate process (Dixon and Webb, 1958).

This mechanism was tested by measuring the initial rate of inactivation of $C\bar{1}$ at various concentrations of MCFPB at 30° in pH 8.1, 0.15 ionic strength Tris-HCl buffer containing 20% glycerol. The method of Mares-Guia and Shaw

TABLE II: Inactivation of Purified Human $C\bar{1}$ by PMSF and MCFPB.

Time (min)	$EAC\overline{4,2}$ Formation (Eff Mol/ml)[a] Control	PMSF[b]	MCFPB[c]
0	1.24×10^{11}	1.24×10^{11}	1.24×10^{11}
30	1.16×10^{11}	1.22×10^{11}	0.003×10^{11}
60	1.23×10^{11}	1.22×10^{11}	0.0008×10^{11}
90	1.24×10^{11}	1.09×10^{11}	$<10^4$
120	0.99×10^{11}	0.99×10^{11}	$<10^4$
150	0.94×10^{11}	0.82×10^{11}	$<10^4$

[a] See Results for experimental details. [b] PMSF = 7×10^{-5} M. [c] MCFPB = 7×10^{-5} M.

(1967) was used to evaluate v, the initial rate of inactivation. The disappearance of $C\bar{1}$ was measured by assaying every 5 min with *N*-Z-L-TyrONp. The results, presented in Figure 2, indicate strict adherence to eq 3 and are consistent with inactivation proceeding *via* formation of a specific complex. The K_m for inactivation of $C\bar{1}$ by MCFPB was calculated to be 2.62×10^{-5} M and V_{max} was determined to be 0.74 unit sec^{-1}. When (I) is much smaller than K_m, eq 3 simplifies to

$$-\frac{d(E)}{dt} = \frac{k_2(E)(I)}{K_m} \quad (4)$$

and k_2/K_m corresponds to k_{2nd} in the rate equation

$$\frac{dx}{dt} = k_{2nd}(a - x)(b - x) \quad (5)$$

where x is the product and a and b are the initial concentrations of $C\bar{1}$ and MCFPB. The k_{2nd} for MCFPB inactivation of $C\bar{1}$ was determined to be 2.52 M^{-1} sec^{-1} by evaluating $t_{1/2}$, the time for disappearance of half of the enzyme, at several concentrations of MCFPB (Aldridge, 1950). This compares favorably with the k_{2nd} determined for PMSF inactivation of chymotrypsin and trypsin (Table III).

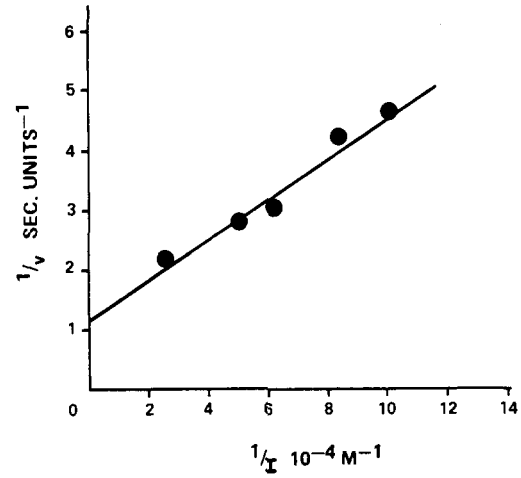

FIGURE 2: $C\bar{1}$ inactivation by MCFPB. The inital rate of inactivation is v; I is MCFPB. See Results for experimental details.

TABLE III: Comparison of Second-Order Rate Constants of PMSF Inactivation of Trypsin and Chymotrypsin and MCFPB Inactivation of $C\bar{1}$.

Enzyme	Inhibitor	pH	k_{2nd} (M^{-1} sec^{-1})	Temp (°C)	Ref
Trypsin	Phenylmethane-sulfonyl fluoride	7.2	4.52	25	Fahrney and Gold (1962)
Chymo-trypsin	Phenylmethane-sulfonyl fluoride	7.0	2.48	25	Fahrney and Gold (1962)
$C\bar{1}$	MCFPB	8.1	2.52	30	This work

Site of Action of MCFPB on $C\bar{1}$. Three of the defined biochemical activities of $C\bar{1}$ were tested as possible sites of action of MCFPB, namely, the esteratic activity associated with the $C1\bar{s}$ subunit, EAC4 binding activity associated with the C1q subunit and the poly(anetholesulfonate) (liquoid) sensitive activity associated with the C1r subunit.

The ability of MCFPB to inhibit $C\bar{1}$ hydrolysis of the substrate *N*-Z-L-TryONp suggested that the esteratic site on $C\bar{1}$ was being inhibited (Figure 1B). This hypothesis was further verified by measuring the ability of MCFPB inhibited $C\bar{1}$ ($C\bar{1}i$) to bind C1 inactivator, the naturally occurring protein inhibitor of $C\bar{1}$. Previous studies on the mechanism of interaction of $C\bar{1}$ with C1 inhibitor indicated that C1 inhibitor combines stoichiometrically with $C\bar{1}$ at or near the esteratic site associated with the $C1\bar{s}$ subunit. Furthermore, if this site is destroyed with DFP, $C1\bar{s}$ can no longer bind $C\bar{1}$ inactivator (Haines and Lepow, 1964; Gigli and Austen, 1967; Bing, 1969).

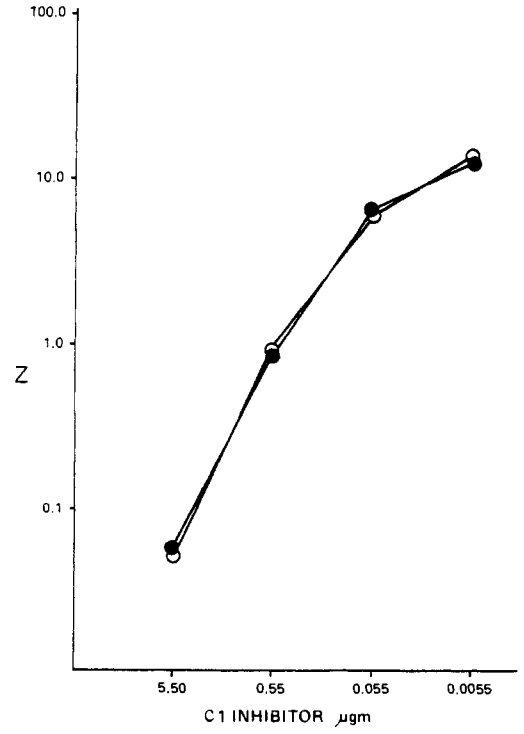

FIGURE 3: The inability of $C\bar{1}$ inhibitor to react with $C\bar{1}i$. (●) buffer + C1 inhibitor + $C\bar{1}$; (○) $C\bar{1}i$ + $C\bar{1}$ inhibitor + $C\bar{1}$. $Z = -\ln(1 - y)$, where y is the degree of lysis. See Results for experimental details.

TABLE IV: Inhibition of $EAC\bar{1},4$ Formation by $C\bar{1}$ Treated with MCFPB.

Mixture	% Lysis[a]	% Inhibition Obsd	% Inhibition Calcd
EAC4 + $C\bar{1}$	78.3		
EAC4 + $C\bar{1}$ inhibited + $C\bar{1}$	38.0	51.4	49.7

[a] After addition of C2 and CEDTA; see Results for experimental details.

Two-tenths of a milliliter of $C\bar{1}$ (3.05×10^{13} eff mol/ml, 3.1 mg/ml) was diluted 1:10 in pH 7.4, 0.065 ionic strength TB-sucrose and incubated with 0.2 ml of 1.4×10^{-3} M MCFPB for 2 hr at 30°. At the end of that time another 0.2 ml of 1.4×10^{-3} M MCFPB was added and incubation was continued for 2.5 hr at 30°. After dialysis to remove excess inhibitor, the $C\bar{1}$ was assayed and determined to be inhibited 99.8% (0.00756×10^{13} eff mol/ml). The inhibited $C\bar{1}$ (0.751 μg) was incubated with 5.5, 0.55, 0.055, and 0.0055 μg of $C\bar{1}$ inactivator for 10 min at 37°. Then free C1 inactivator was assayed with $C\bar{1}$ and EAC4 according to the procedure described by Gigli and Austen (1967). A control consisted of buffer containing no inhibited $C\bar{1}$. These results are presented in Figure 3. The inhibited $C\bar{1}$ was incapable of binding any C1 inactivator, indicating that MCFPB had destroyed the esteratic site on $C\bar{1}$. The same preparation of inhibited $C\bar{1}$ was tested for ability to block the uptake of $C\bar{1}$ by EAC4. The inhibited $C\bar{1}$ (3.1 μg) was incubated with 1.2 ml of EAC4 (1.5×10^8 cells/ml) in pH 7.4, 0.065 ionic strength TB-sucrose for 15 min at 30°. The cells were washed two times in the same buffer, suspended to 1.5×10^8 cells/ml, and 0.2 ml was transferred to a new tube. Then $C\bar{1}$ (3.03 μg) was added to provide 100 eff mol/cell; the cells were incubated at 30° for 10 min and assayed for formation of the $EAC\bar{1},4$ intermediate. The results are presented in Table IV. The presence of inhibited $C\bar{1}$ caused a 51.4% reduction in lysis, by intact $C\bar{1}$, indicating inhibition of uptake of intact $C\bar{1}$ by $C\bar{1}i$. This compares favorably with the calculated value of 49.7% reduction which would be expected on mixing equal volumes of fully active and inactivated $C\bar{1}$.

Finally, poly(anetholesulfonate) had no effect on the ability of MCFPB to inhibit $C\bar{1}$. Two-tenths of a milliliter of purified $C\bar{1}$ (1.105×10^{13} eff mol/ml) was diluted 1:10 in 0.065 ionic strength TB-sucrose and reacted with 50 μg of Liquoid for 10 min at 30°. Then 0.2 ml of 1.4×10^{-3} M MCFPB was added and inactivation of $C\bar{1}$ was measured for the next 2 hr. The results are presented in Table V. It is readily apparent that Liquoid had no effect on the inactivation by MCFPB.

Inhibition of Partially Purified $C\bar{1}$ by MCFPB. Partially purified $C\bar{1}$ prepared by precipitation of serum at a final ionic strength of 0.03 and pH of 6.4 was tested for inactivation by MCFPB. The disappearance of $C\bar{1}$ was followed by measuring $EAC\bar{4},\bar{2}$ formation with the conditions and concentration of MCFPB which were used for measuring inactivation of purified $C\bar{1}$. The results are presented in Figure 4. At 7×10^{-5} M MCFPB, the inactivation follows strict pseudo-first-order kinetics and the rate constant is 3.36×10^{-4} sec^{-1},

TABLE V: Effect of Poly(anetholesulfonate) on Inactivation of $C\bar{1}$ by 7×10^{-4} M MCFPB.

Time (min)	$C\bar{1}$ + 50 μg of Poly(anetholesulfonate) (Eff Mol/ml $\times 10^{12}$)	$C\bar{1}$ Alone (Eff Mol/ml $\times 10^{12}$)
0	11.0	11.1
20	1.69	1.66
40	0.589	0.548
60	0.355	0.346
120	0.216	0.202

which is very similar to the findings for purified $C\bar{1}$. In contrast, 9×10^{-5} M DFP caused no inactivation of partially purified $C\bar{1}$ under these conditions.

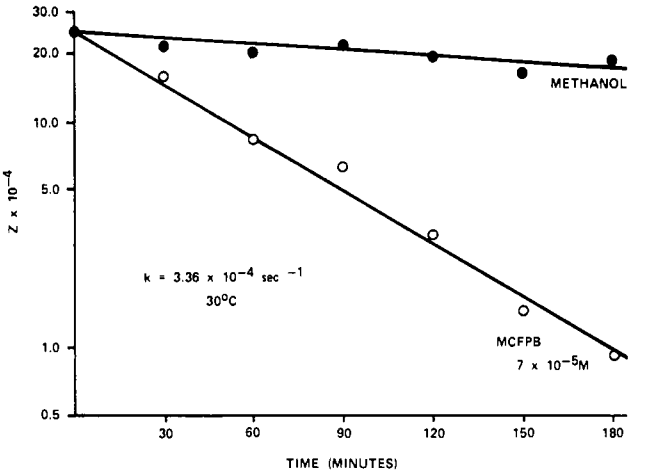

FIGURE 4: Inactivation of functionally pure human $C\bar{1}$ by MCFPB, as assayed by its inability to form the $EAC\overline{4,2}$ intermediate. $Z = -\ln(1 - y)$, where y is the degree of lysis. See Results for experimental details.

Discussion

The results of the present experiments have led to the following conclusions about the inactivation of human $C\bar{1}$ by MCFPB. First, MCFPB irreversibly inactivates purified human $C\bar{1}$ and dialysis of $C\bar{1}$ treated with MCFPB fails to restore activity. Furthermore, MCFPB is apparently more active than DFP, a known irreversible inhibitor of $C\bar{1}$, against either purified or partially purified $C\bar{1}$. On a molar basis the benzamidine compound is at least 30 times as effective as DFP; 15×10^{-5} M MCFPB will completely inactivate $C\bar{1}$, whereas it requires 5×10^{-3} M DFP to cause complete inactivation of $C\bar{1}$ (Becker, 1956a; Haines and Lepow, 1963). The irreversible inactivation caused by MCFPB is related to structural features other than the sulfonyl fluoride group within the benzamidine molecule; phenylmethanesulfonyl fluoride, a potent inhibitor of trypsin and chymotrypsin, did not inhibit $C\bar{1}$ (Tables II and III).

Second, the mechanism of inactivation of $C\bar{1}$ by MCFPB is via the formation of a specific complex. The kinetic studies indicate that the nature of this complex is similar to an enzyme–substrate complex. The exact portion of the MCFPB molecule which confers this specificity has yet to be determined, but the work of Baker and Cory (1969a,b, 1971a,b) would indicate that the length of the alkyl chain between the benzamidine and phenyl group and the position of the phenylureidosulfonyl fluoride portion are both critical.

Third, the site of action of the MCFPB molecule is almost certainly the esteratic site on the $C1\bar{s}$ subunit of the $C\bar{1}$ macromolecular complex. The concomitant loss of ability to catalyze the formation of $EAC\overline{4,2}$ and esteratic activity indicates that inactivation is due to loss of $C1\bar{s}$ esteratic activity. The inability of MCFPB inactivated $C\bar{1}$ to bind C1 inhibitor provides additional proof of inactivation of the $C1\bar{s}$ esteratic site by MCFPB. There may be other sites on the $C\bar{1}$ molecule affected by MCFPB, but the EAC4 binding activity of $C\bar{1}$ is not lost and a poly(anetholesulfonate) treatment had no effect on the ability of MCFPB to inactivate the ability of $C\bar{1}$ to catalyze formation of the $EAC\bar{1},4$ intermediate. These two observations suggest that neither C1q nor C1r is affected by MCFPB treatment.

Fourth, on a molar basis, MCFPB is equally effective against partially and highly purified $C\bar{1}$ (Figures 1 and 4). This is in direct contrast to the effect of DFP on $C\bar{1}$; 9×10^{-5} M DFP caused only 50% inactivation of purified $C\bar{1}$ and had no effect on the preparation of partially purified $C\bar{1}$. This latter observation further supports the conclusion that MCFPB is a specific inhibitor and indicates it could be used in a variety of studies where it is desirable to inactivate $C\bar{1}$ in the presence of other complement components.

Progress in delineating fine structural features of the complement proteins has been thwarted by the difficulty of isolating these proteins in a homogeneous state and by the lack of specific reagents which can be used as probes of the complement enzyme structure–function relationships. The present set of experiments with a highly purified preparation of $C\bar{1}$ indicates that it is possible to inactivate this complement component under mild conditions with a defined reagent, *m*-[*o*-(2-chloro-5-sulfonylfluorophenylureido)phenoxybutoxy]-benzamidine. The inactivation process is evidently specific and fulfills the criteria established by Wofsy *et al.* (1962) for an affinity-labeling reagent. There is specific uptake of MCFPB and an enhancement in the reaction of MCFPB with $C\bar{1}$ as compared to PMSF, a reagent with a similar functional group but otherwise structurally unrelated. Furthermore, the rate of inactivation of $C\bar{1}$ by MCFPB is similar to rates observed with similar compounds with other proteolytic enzymes (Table III). The exact relationship between structural features of the MCFPB molecule and the $C\bar{1}$ macromolecular complex have yet to be defined. It does appear, however, that the $C1\bar{s}$ subunit is the site of reaction. Thus, a start has been made toward obtaining detailed chemical information and the MCFPB reagent should prove very useful in future studies in the area of structure–function relationships of the first component of complement.

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Inactivation of Staphylococcal Nuclease by the Binding of Antibodies to a Distinct Antigenic Determinant†

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ABSTRACT: The interaction of antibodies with a distinct antigenic determinant in the region (99–126) of staphylococcal nuclease has been found to produce a soluble, inactive antibody–nuclease complex. The reaction has been followed spectrophotometrically, using the rate of hydrolysis of substrate DNA as a measure of residual, free nuclease concentration. This analysis has provided the following kinetic and equilibrium constants for the antibody–antigen interaction: $K_{ass} = 8.3 \times 10^8\ M^{-1}$, $k_{on} = 4.1 \times 10^5\ M^{-1}\ sec^{-1}$, $k_{off} = 4.9 \times 10^{-4}\ sec^{-1}$. Conversely, measurement of changes in nuclease activity can be used as a rapid and sensitive assay of antibody concentration. The Scatchard plot of the equilibrium inactivation data bends sharply near $r = 1$, which may reflect heterogeneity of the antibodies or may indicate possible steric interference in the binding of a second nuclease molecule to the bivalent antibody molecule.

We have recently reported the preparation of a population of antibodies specific for an antigenic determinant formed by amino acids in the region (99–126)[1] of staphylococcal nuclease (Sachs *et al.*, 1972). These antibodies, obtained by fractionation of goat anti-nuclease serum on immunoabsorbents bearing selected polypeptide fragments of nuclease, constituted approximately 3% of the total antibody of that serum. They have been designated as anti-$(99–126)_n$, in which the subscript, "*n*," indicates that the antibodies were obtained by immunization with the intact enzyme and are therefore presumably directed against the native conformation of this polypeptide sequence of nuclease.

The molecular location of this determinant is illustrated in Figure 1, which is an artist's representation of the crystallographic structure of nuclease in which the sequence (99–126) is darkened. Since DNA, the natural substrate of nuclease, is thought to fit into a "cleft" in the front of the molecule (Arnone *et al.*, 1971), as it is viewed in Figure 1, the presence of a bulky antibody molecule attached to a determinant in the region (99–126) might be expected to inhibit the enzyme's activity on DNA. Furthermore, because the component antibodies of anti-$(99–126)_n$ can combine with only a single site per nuclease molecule the resultant interaction does not lead to precipitation (Sachs *et al.*, 1972). Both of these considerations suggested the feasibility of a study of the combination of anti-$(99–126)_n$ with nuclease using the spectrophotometric assay of nuclease to monitor the interaction. We have found that the antibody does inhibit the enzymatic activity and this paper reports kinetic and equilibrium aspects of the antibody-induced inactivation.

Materials and Methods

Preparation and purification of nuclease, immunization procedures, and fractionation of the anti-nuclease serum have

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[1] Nuclease consists of a single polypeptide chain of 149 amino acids, numbered 1–149 from amino to carboxyl-terminal ends. 99–126 thus refers to a limited region of this polypeptide chain.