Enzymatic Activity of the Second Component of Complement†

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ABSTRACT: Isolated C2 and C2i preparations were able to hydrolyze a number of synthetic esters containing basic amino acids, among which *N*-α-acetylglycyl-L-lysine methyl ester (AcGlyLysOMe) was most susceptible. The cleaving activity was a property of the C2 molecule, since it correlated with the presence of C2 on analyses of C2 preparations by ultracentrifugation in sucrose gradients, filtration through Sephadex G-200 columns, and on electrophoresis in acrylamide gels. Furthermore, acrylamide gel electrophoretic studies showed a shift in hydrolytic activity from the position occupied by C2 to that characteristic of C2i after incubation of C2 with $C\bar{1}\bar{s}$. The action was enzymatically mediated as evidenced by a bell-shaped pH activity curve, a linear dependence on C2 concentration, and the presence of Michaelis–Menten kinetics. The Michaelis constant for cleavage of AcGlyLysOMe by C2 was 1.8×10^{-2} mol. Cleavage of C2 by $C\bar{1}\bar{s}$ increased C2 enzymatic activity, yet chemical oxidation of the molecule, although enhancing hemolytic activity, failed to increase C2 hydrolytic activity. The observed enzymatic activity of C2 was found to be relevant to the function of C2 in the $C\bar{4}\bar{2}$ complex, since AcGlyLysOMe competitively inhibited the $C\bar{4}\bar{2}$ mediated cleavage of C3 in free solution and the $C\bar{4}\bar{2}$ dependent binding of C3 to cells.

The second component of complement (C2) is an essential constituent of the key complement enzyme $C\bar{4}\bar{2}$, or C3 convertase. $C\bar{4}\bar{2}$ is generated from two inactive precursor molecules, C2 and C4, by the enzymatic action of $C\bar{1}\bar{s}$ (Müller-Eberhard et al., 1967). The natural substrate of $C\bar{4}\bar{2}$ is C3, which is cleaved by $C\bar{4}\bar{2}$ into two fragments, C3a and C3b (Müller-Eberhard et al., 1967, Dias da Silva et al., 1967). The smaller fragment, C3a, has anaphylatoxin activity (Dias da Silva et al., 1967, Bokisch et al., 1969) while the larger fragment, C3b, interacts with $C\bar{4}\bar{2}$ in an unknown manner to generate another proteolytic enzyme which has C5 as its substrate (Cochrane and Müller-Eberhard, 1968; Shin et al., 1968). Several observations suggest that the enzymatic site of $C\bar{4}\bar{2}$ resides in the C2 molecule. First, the ability to cleave C3 is acquired only on union of C2 with C4 and lost on dissociation of C2 from the complex (Müller-Eberhard et al., 1967). Second, the extent of $C\bar{4}\bar{2}$ activity is directly proportional to the amount of C2 employed to prepare the complex (Müller-Eberhard et al., 1966), and third, chemical oxidation of C2 prior to incorporation into $C\bar{4}\bar{2}$ enhances the activity of the enzyme formed with the altered molecule (Müller-Eberhard et al., 1967).

The present studies show that C2 is an enzyme able to hydrolyze certain synthetic esters containing lysine. The reaction exhibited Michaelis–Menten kinetics and a dependence on pH and C2 concentration. Furthermore, the ability of $C\bar{4}\bar{2}$ to cleave C3 was competitively inhibited by a synthetic C2 substrate, indicating that the functional activity of $C\bar{4}\bar{2}$ in the complement sequence is dependent on the enzymatic activity of C2.

Materials and Methods

Chemicals and Reagents. Reagent grade sodium phosphate (J.T. Baker Chemical Co., Phillipsburg, N.J.), sodium barbital (Sigma Chemical Co., St. Louis, Mo.), and Tris (Sigma Chemical Co., St. Louis, Mo.) were used to prepare buffers. Phenylmethanesulfonyl fluoride ($PhCH_2SO_2F$) and diisopropyl fluorophosphate (Dip-F) were purchased from Calbiochem, San Diego, Calif., and Boots Pure Drug Co., Ltd., Nottingham, England, respectively. Dithiothreitol and reagents for polyacrylamide gel electrophoresis were obtained from Bio-Rad Laboratories, Richmond, Calif. The following compounds were obtained from Cyclo Chemical, Los Angeles, Calif., or Schwarz/Mann, Orangeburg, N.Y.: *N*-acetyl-L-arginine methyl ester (AcArgOMe), *N*-α-acetylglycyl-L-lysine methyl ester (AcGlyOMe), *N*-α-acetyl-L-lysine methyl ester (AcLysOMe), *N*-α-acetyl-L-ornithine methyl ester (AcOrnOMe), L-arginylglycine (Arg-Gly), L-arginyl-L-glutamic acid (Arg-Glu), L-arginyl-L-leucine (Arg-Leu), L-arginine methyl ester (ArgOMe), *N*-benzoyl-L-arginine methyl ester (BzArgOMe), *N*-benzoylglycine methyl ester (BzGlyOMe), *N*-α-benzoyl-L-lysine methyl ester (BzLysOMe), *N*-α-carbobenzoxy-L-arginine methyl ester (CbzArgOMe), *N*-α-carbobenzoxyglycine methyl ester (CbzGlyOMe), *N*-α-carbobenzoxy-L-lysine methyl ester (CbzLysOMe), L-lysyl-L-aspartic acid (Lys-Asp), L-lysine ethyl ester (LysOEt), L-lysyl-L-glutamic acid (Lys-Glu), L-lysyl-L-glutamyl-α-glycine (Lys-Glu-Gly), L-lysyl-L-leucinamide (Lys-Leu-amide), L-lysyl-L-α-lysine (Lys-Lys), L-lysine methyl ester (LysOMe), L-lysyl-L-serine (Lys-Ser), *N*-α-tosyl-L-arginine methyl ester (Tos-ArgOMe), and *N*-α-tosyl-L-lysine methyl ester (Tos-LysOMe). The composition of Veronal-buffered saline containing 1.5×10^{-4} *M* calcium and 5×10^{-4} *M* magnesium with or with-

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[1] Abbreviations used are: $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; Dip-F, diisopropyl fluorophosphate; AcArgOMe, *N*-acetyl-L-arginine methyl ester; AcGlyLysOMe, *N*-α-acetylglycyl-L-lysine methyl ester; AcLysOMe, *N*-α-acetyl-L-lysine methyl ester; AcOrnOMe, *N*-α-acetyl-L-ornithine methyl ester; ArgOMe, L-arginine methyl ester; BzArgOMe, *N*-benzoyl-L-arginine methyl ester; BzGlyOMe, *N*-benzoylglycine methyl ester; BzLysOMe, *N*-α-benzoyl-L-lysine methyl ester; CbzArgOMe, *N*-α-carbobenzoxy-L-arginine methyl ester; CbzGlyOMe, *N*-α-carbobenzoxyglycine methyl ester; CbzLysOMe, *N*-α-carbobenzoxy-L-lysine methyl ester; LysOEt, L-lysine ethyl ester; LysOMe, L-lysine methyl ester; Tos-ArgOMe, *N*-α-tosyl-L-arginine methyl ester; Tos-LysOMe, *N*-α-tosyl-L-lysine methyl ester.

out 0.1% gelatin (GVB) has been described (Mayer, 1961).

Carboxymethylated human albumin was prepared by a published method (Polley and Müller-Eberhard, 1968). Soybean and lima bean trypsin inhibitors were purchased from Worthington Biochemical Corp., Freehold, N.J. C1 inactivator, α_1 anti-trypsin, and α_1 anti-chymotrypsin were obtained through the courtesy of Drs. K. Dräger and H. Schwick, Behringwerke AG, Marburg, Germany.

Complement and Complement Reagents. Fresh human serum was employed for the isolation of $C\bar{1}\bar{s}$, C2, C3, and C4. $C\bar{1}\bar{s}$ was isolated by a modification (Harpel and Cooper, 1975) of the previously published method (Valet and Cooper, 1974), while C2 (Polley and Müller-Eberhard, 1968), C3 (Nilsson and Müller-Eberhard, 1965), and C4 (Schreiber and Müller-Eberhard, 1975) were isolated as has been described. C2 was oxidized by treatment with iodine (Polley and Müller-Eberhard, 1967). Isolated C3 was radiolabeled with ^{125}I by the method of McConahey and Dixon (1966). The cellular intermediates EAC1, EAC14, and EAC142 were prepared by published methods (Cooper and Müller-Eberhard, 1968; Cooper et al., 1970).

Measurement of Complement Components. $C\bar{1}\bar{s}$, C2, C3, and C4 were directly quantitated by using the Lowry technique (Lowry et al., 1951) with standard curves calibrated by micro Kjeldahl determinations. Specific hemolytic titrations were employed for the quantitative measurement of C2, C3, and C4 activities (Cooper et al., 1970; Cooper and Müller-Eberhard, 1968, 1970). $C\bar{1}\bar{s}$ activity was measured by incubating samples with 10 μg of isolated C4 for 30 min at 37° in a reaction volume of 50 μl after which remaining hemolytically active C4 was determined by effective molecule titration. Calibration curves relating $C\bar{1}\bar{s}$ concentration to inactivation of C4 were linear and 50% inactivation occurred with 1.6 ng of $C\bar{1}\bar{s}$.

Formation of C2i. C2i was formed by incubation of C2 with a 1:100 molar ratio of $C\bar{1}\bar{s}$ for 30 min at 37°. C2 hemolytic activity was measured to verify formation of C2i.

Detection of C2 and C2i. C2 and C2i in fractions from Sephadex G-200 columns and sucrose density gradients and in eluates of acrylamide gels were detected by the Ouchterlony technique with monospecific antiserum to human C2 (Polley and Müller-Eberhard, 1968).

Generation of $C\bar{4}\bar{2}$. $C\bar{4}\bar{2}$ was formed after incubating 10 μg of C4 and 11 μg of oxidized C2 with 1 μg of $C\bar{1}\bar{s}$ for 20 min at 37° in a total volume of 0.6 ml. After addition of Dip-F (see below) and additional incubation for 20 min at 37°, dilutions of the complex were incubated with 20 μg of C3 in a reaction volume of 0.2 ml for 30 min at 37° to determine the activity of the $C\bar{4}\bar{2}$ complex. Fifty percent inactivation of the hemolytic activity of C3 was generally accomplished by 0.1 ml of a 1:40 dilution of the complex. For the study of the effect of AcGlyLysOMe on the $C\bar{4}\bar{2}$-mediated cleavage of C3, the $C\bar{4}\bar{2}$ preparation was diluted 20-fold and 0.1-ml aliquots were incubated with varying amounts of C3 in the presence or absence of AcGlyLysOMe as specified in the Results in a total reaction volume of 0.3 ml.

Enzymatic Studies. All substances to be examined for enzymatic activity were incubated for 30 min at 37° with 2×10^{-2} *M* Dip-F added directly to the samples from a 5 *M* stock. The samples were then dialyzed overnight in the cold against a 0.1 *M* sodium phosphate buffer (pH 7.5) before incubation with substrates dissolved in the same buffer. Typical reaction mixtures consisted of 0.2 ml of enzyme solution and 50 μl of 5×10^{-2} *M* substrate. Unless otherwise specified, the reactions mixtures were incubated for 2 hr at 37° and then examined for cleavage by high voltage paper electrophoresis or the Siegelman technique for assay of methyl alcohol.

High Voltage Paper Electrophoresis. Ten-microliter samples containing 0.1 μM of substrate were spotted on 46 × 57 cm sheets of Whatman Chromatography paper No. 1. The paper was moistened with pyridine acetate buffer (pH 6.4) and electrophoresis carried out to the cathode at 1200 V for 75 min. The paper was dried at 65° and stained with a solution of 2% ninhydrin in acetone.

Quantitation of Methyl Alcohol. A slight modification of the Siegelman technique for measurement of methyl alcohol was employed (Siegelman et al., 1962); 50 μl of 0.75 *M* perchloric acid was added to a 250-μl sample containing 2.5 μM of substrate. Next, 25 μl of 2% $KMNO_4$ followed by 25 μl of 10% Na_2SO_3 was added. The decolorized solutions were next incubated with 1 ml of 0.2% chromatopic acid (Eastman Kodak, Rochester, N.Y.) in 10.8 *N* H_2SO_4 in a boiling water bath for 30 min. After the solution was cooled, 2 ml of H_2O was added and the optical density of the solutions at 580 nm determined. A standard methyl alcohol optical density curve was obtained from methanol standards included in each experiment.

Polyacrylamide Gel Electrophoresis. Disc electrophoresis in 6% running gels was performed by the method of Davis (1964) in Tris-HCl-glycine buffer (pH 8.7). After electrophoresis, the gels were either stained with Amido Black or sectioned longitudinally and one-half was stained while the other half was sectioned at 2-mm intervals. The segments were placed in siliconized tubes and eluted with 50 μl of Tris-NaCl buffer (pH 7.5) containing 500 μg of carboxymethylated albumin/ml. Scanning of stained gels was performed on a Gilford linear transport device attached to a Gilford spectrophotometer (Gilford Instruments, Oberlin, Ohio).

Sucrose Density Gradient Ultracentrifugation. Samples were sedimented in 7–31% linear sucrose density gradients prepared in 8×10^{-2} *M* sodium phosphate, 1.5×10^{-1} *M* NaCl and 1×10^{-3} *M* EDTA (pH 5) in an SW 50.1 rotor at 40,000 rpm for 18 hr in a Beckman L 2-50 ultracentrifuge. A constant ionic strength was maintained throughout the gradient.

Chromatography on Sephadex G-200. Samples were filtered through 2 × 70 cm collumns of Sephadex G-200 in the pH 5.0 buffer described immediately above.

Results

Cleavage of AcGlyLysOMe by C2. Highly purified preparations of human C2 hydrolyzed several synthetic amino acid esters of which AcGlyLysOMe was most susceptible. On analysis of reaction mixtures by high voltage paper electrophoresis the ninhydrin reactive spot produced by AcGlyLysOMe partially or completely disappeared, and a new ninhydrin reactive product which had the same mobility as acetylglycyllysine appeared. Cleavage of the ester bond of AcGlyLysOMe was confirmed by the Siegelman technique which showed that the action of C2 on AcGlyLysOMe generated methyl alcohol. Representative results for cleavage of AcGlyLysOMe from separate experiments performed with seven different purified C2 preparations are shown in Figure 1. In these experiments 3.6–11 μg of C2 were incubated with 1×10^{-2} *M* AcGlyLysOMe for 2 hr at 37°. Although these studies were performed over a 1-year period with preparations of C2 which contained variable amounts

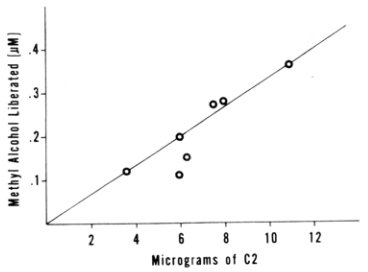

FIGURE 1: Cleavage of *N*-α-acetylglycyl-L-lysine methyl ester (abbreviated AGLMe in figures) by C2 preparations. Seven different isolated C2 preparations were examined separately for their ability to hydrolyze AGLMe in 2 hr of incubation at 37°.

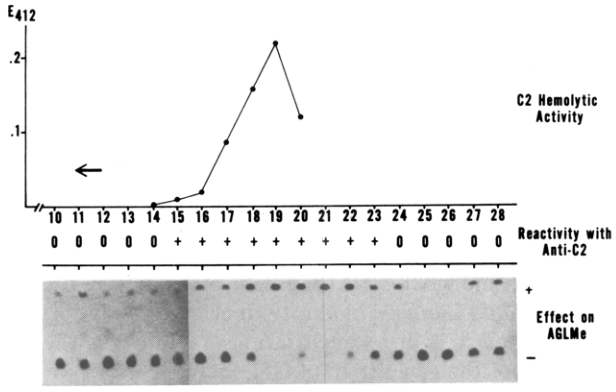

FIGURE 2: Sucrose gradient ultracentrifugal analysis of C2 and analysis of fractions for C2 and ability to cleave AGLMe; 50 μg of C2 was treated with 2×10^{-2} *M* Dip-F and sedimented in a sucrose density gradient. A good correlation was observed between the presence of C2 hemolytic activity (upper panel), C2 protein by Ouchterlony analysis with anti-C2 (middle panel), and ability to cleave AGLMe as determined by high voltage electrophoresis (lower panel).

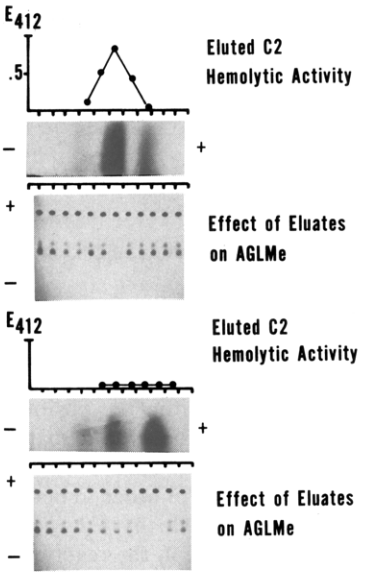

FIGURE 3: Acrylamide gel electrophoresis of C2 (upper three panels) and C2i (lower three panels). The upper three panels show the results obtained on testing the eluates of a gel to which 30 μg of Dip-F-treated C2 was applied for hemolytic activity, the stained acrylamide gel, and an analysis of the ability of the eluates to cleave AGLMe as assessed by high voltage paper electrophoresis. A good correlation between the presence of C2 activity, the stained C2 protein band, and ability to cleave AGLMe was obtained. The lower three panels show the results obtained with 30 μg of Dip-F-treated C2i. Considerable conversion of C2 to C2i which is devoid of hemolytic activity and a shift of AGLMe cleaving activity to the position occupied by C2i are evident. The heavy spot near the origin is due to glycine in the electrophoresis buffer.

of C2i, a cleaved biologically inactive form of C2 which has increased enzymatic activity as shown below, a fair relationship between protein concentration, and ability to hydrolyze AcGlyLysOMe was observed.

Three types of studies were performed to show that hydrolytic activity was a property of the C2 molecule and not due to a trypsin-like enzyme present as a contaminant in the C2 preparations. First, C2 was sedimented in sucrose density gradients in order to determine if there was a correlation between the location of C2 and the ability to cleave AcGlyLysOMe. Since the C2 isolation procedure does not include a separation based on size, it was thought that this technique might detect a potential contaminant. In the experiment depicted in Figure 2, which is representative of the four studies of this type, a good correlation was observed between the presence of C2 protein, as assessed by Ouchterlony analysis, and the ability to cleave AcGlyLysOMe, as determined by high voltage paper electrophoresis. Maximal hydrolytic activity, as shown by disappearance of the AcGlyLysOMe spot, occurred at the midpoint of the fractions exhibiting reactivity with anti-C2. The heavier fractions reacting with anti-C2 represented native C2 as shown by the presence of hemolytic activity while the lighter fractions represented C2i, which has a lower sedimentation rate (Polley and Müller-Eberhard, 1968) and which is hemolytically inactive. Second, Dip-F-treated C2 preparations were passed through Sephadex G-200 columns and the fractions similarly assayed for C2 hemolytic activity, C2 protein, and ability to cleave AcGlyLysOMe. As was observed in the sucrose density gradient studies, the presence of C2 protein and the ability to cleave AcGlyLysOMe correlated well. Third, C2 and C2i generated from C2 by treatment with $C\bar{1}\bar{s}$ were subjected to electrophoresis in 6% polyacrylamide gels. Half of each gel was stained while the remaining half was sectioned and eluted. The eluates were then examined for C2 hemolytic activity, for ability to cleave AcGlyLysOMe, and for the presence of active $C\bar{1}\bar{s}$. As depicted in Figure 3, the C2 gel showed two bands, as is frequently observed with C2 preparations (Polley and Müller-Eberhard, 1968). The heavier, cathodal band is characteristic of the mobility of C2, and C2 hemolytic activity was eluted from segments corresponding to this band. The fainter, more anodal band had a mobility characteristic of C2i which is generally present in varying proportions in C2 preparations (Polley and Müller-Eberhard, 1968). Only eluates corresponding to the position of C2 cleaved AcGlyLysOMe (Figure 3). The other gel to which C2i had been applied showed a weaker band corresponding to the position of C2 and a stronger band having the mobility characteristic of C2i. C2 activity could not be eluted from this gel (Figure 3). AcGlyLysOMe cleavage activity was now evident in eluates corresponding to the location of the C2i protein band. $C\bar{1}\bar{s}$ activity was not detected in any of the eluates from either gel. Qualitatively identical results were obtained in three additional studies of this type with other C2 preparations. In two of these studies, however, AcGlyLysOMe hydrolyzing activity was present in two areas of the gel to which untreated C2 had been applied, one of which correlated with the band produced by native C2, while the other corresponded to the C2i band. These studies thus indicate that AcGlyLysOMe cleaving activity correlates with the presence of C2 on sucrose density gradient ultracentrifugation,

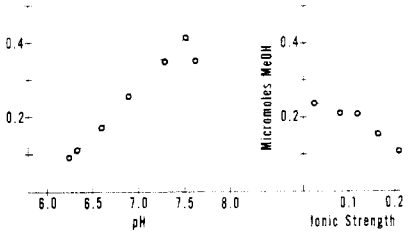

FIGURE 4: Effect of pH and ionic strength on the hydrolysis of AGLMe by C2.

Sephadex G-200 filtration and acrylamide gel electrophoresis. Furthermore, in the acrylamide gel electrophoretic studies this activity shifted from the position occupied by C2 to that characteristic of C2i after incubation with $C\bar{1}\bar{s}$.

Characteristics of AcGlyLysOMe Cleavage by C2. Several parameters were studied in order to determine whether the cleavage of AcGlyLysOMe by C2 was enzymatically mediated. As shown in Figure 4, the reaction was markedly dependent on pH, proceeding optimally at pH 7.5, and somewhat dependent on ionic strength. A linear dose response curve to at least 12 μg of C2 was obtained for cleavage of AcGlyLysOMe. In order to assess the effect of substrate concentration on hydrolysis, reaction mixtures containing a constant amount of C2 and varying concentrations of AcGlyLysOMe were examined at intervals for AcGlyLysOMe cleavage. Zero-order kinetics were obtained for each substrate concentration examined and the velocity of the reaction decreased as the concentration of substrate increased. The values, when graphed in the manner of Lineweaver and Burk (1934), demonstrated that a linear relationship prevailed between the reciprocal of the initial velocity of AcGlyLysOMe cleavage and the reciprocal of the AcGlyLysOMe concentration. A Michaelis constant (K_m) of 1.8×10^{-2} M was calculated from the results. The above studies, taken together, indicate that C2 enzymatically hydrolyzes AcGlyLysOMe.

Substrate Specificity of the C2 Enzyme. In a series of experiments a number of potential substrates were incubated with C2 or C2i isolated from purified preparations by sucrose density gradient ultracentrifugation. Analyses by high voltage paper electrophoresis revealed cleavage only of AcGlyLysOMe, CbzLysOMe, and AcyLysOMe (Table I). Quantitative measurements by the Siegelman technique for assay of methyl alcohol release were performed with a number of methyl esters containing arginine and lysine. As shown in Table I, AcGlyLysOMe was the most susceptible substrate followed by CbzLysOMe, Tos-LysOMe, CbzArgOMe, and AcLysOMe.

Effect of Enzyme Inhibitors on C2 Enzymatic Activity. The AcGlyLysOMe cleaving activity of 5 μg of isolated C2 was not inhibited by previous incubation for 1 hr at 37° with 2×10^{-2} M Dip-F or $PhCH_2SO_2F$, 100 μg of soybean or lima bean trypsin inhibitors, or by 200 μg of C1 inactivator, α_2 macroglobulin, α_1 anti-trypsin, or α_1 anti-chymotrypsin.

Effect of C2 Cleavage on Enzymatic Activity. Cleavage of C2 by $C\bar{1}\bar{s}$, a reaction termed activation, is necessary for formation of $C\bar{4}\bar{2}$ and for progression of an ongoing complement reaction. The larger fragment of cleaved C2 is able to bind to C4 for a brief period of time after activation, thus generating $C\bar{4}\bar{2}$. Cleaved C2 molecules which have lost the ability to bind to C4 are termed C2i. Since the enzymatic activity of C2 seemed increased after treatment with $C\bar{1}\bar{s}$, the enzymatic activity of C2i was investigated. A number of studies performed with identical amounts of Dip-F treated C2 and C2i indicated that treatment of C2 with $C\bar{1}\bar{s}$ led to a 1.5- to 2-fold increase in ability to cleave AcGlyLysOMe. Dip-F-treated $C\bar{1}\bar{s}$ in concentrations identical with those employed for the generation of C2i, included as a control in each experiment, was unable to cleave AcGlyLysOMe.

Table I: Compounds Examined as Substrates for C2.

High Voltage Paper Electrophoresis	Siegelman Assay (μmol of MeOH)	
Positive		
AcGlyLysOMe	AcGlyLysOMe	0.65
CbzLysOMe	CbzLysOMe	0.43
AcLysOMe	Tos-LysOMe	0.18
Negative		
ArgOMe	CbzArgOMe	0.10
AcOrnOMe	AcLysOMe	0.09
Arg-Gly	AcArgOMe	<0.07
Arg-Glu	AcOrnOMe	<0.07
Arg-Leu	BzArgOMe	<0.07
LysOMe	BzGlyOMe	<0.07
LysOEt	BzLysOMe	<0.07
Lys-Asp	CbzGlyOMe	<0.07
Lys-Glu	LysOMe	<0.07
Lys-Glu-Gly	Tos-ArgOMe	<0.07
Lys-Leu-Amide		
Lys-Lys		
Lys-Ser		

A number of experiments were performed to determine whether native C2 possessed enzymatic activity, or if treatment with $C\bar{1}\bar{s}$ was required in order to generate it. These experiments were complicated by the fact that most C2 preparations contained 20–30% of C2i and additional spontaneous generation of C2i occurred during the dialysis and prolonged incubation periods required to demonstrate enzymatic activity. In these studies, C2 preparations exhibiting varying proportions of C2i were generated by incubation with graded amounts of $C\bar{1}\bar{s}$ and assessed for ability to cleave AcGlyLysOMe. Conversion was quantitated by densitometric scanning of the stained gels. In the experiment depicted in Figure 5, 5 μg of untreated C2, which showed 23% conversion to C2i, had considerable enzymatic activity. Enzymatic activity was further enhanced 1.5-fold after treatment with $C\bar{1}\bar{s}$, however, the enzymatic activity remained constant after approximately 40% of the C2 was converted to C2i. In the same experiment, simultaneous hemolytic testing of an aliquot of the samples with 23, 41, 68, and 88% conversion to C2i showed 1.6×10^{12}, 1.4×10^{12}, 9.6×10^{11}, and 3.1×10^{11} effective molecules per ml, respectively, indicating that the increased generation of C2i observed in the acrylamide gel densitometric scan was also evident by hemolytic titration. The C2 preparations employed in the other three studies of this type, which contained between 23 and 33% of C2i before incubation with $C\bar{1}\bar{s}$, also showed enzymatic activity. In each case, enzymatic activity increased approximately 1.5-fold after treatment with $C\bar{1}\bar{s}$ until it reached a plateau which became evident at approximately 50% conversion of the molecule to C2i.

Effect of Oxidation of C2 on Enzymatic Activity. Oxidation of C2, although increasing hemolytic activity 7- to 15-fold in various experiments, produced no change in ability to hydrolyze AcGlyLysOMe.

Effect of AcGlyLysOMe on $C\bar{4}\bar{2}$ Mediated Cleavage of C3 in Free Solution. The ability of AcGlyLysOMe to in-

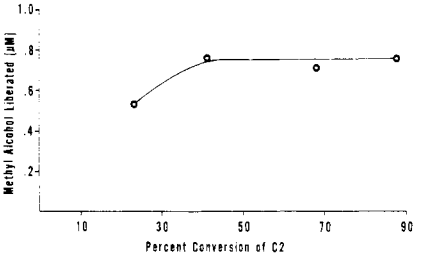

FIGURE 5: Relationship between conversion of C2 to C2i and ability to hydrolyze AGLMe.

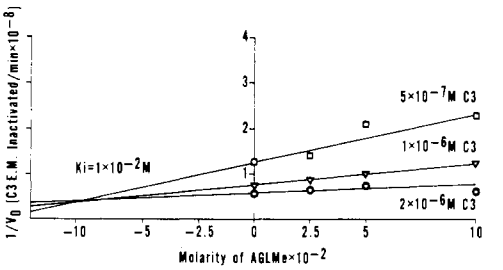

FIGURE 6: Inhibition by AGLMe of the $C\bar{4}\bar{2}$ mediated cleavage of C3 in free solution. Results are expressed by the method of Dixon which relates the initial velocity of C3 inactivation to the concentration of inhibitor for each of the several concentrations of C3 examined. The lines intersect above the abscissa and a K_i of 1×10^{-2} M was obtained.

hibit cleavage of C3 by $C\bar{4}\bar{2}$ was examined as follows. Aliquots of $C\bar{4}\bar{2}$ were incubated at 30° with 0.5 μg of C3 in the presence of either buffer or 1×10^{-2}, 5×10^{-3}, or 2.5×10^{-3} M AcGlyLysOMe. In the same manner $C\bar{4}\bar{2}$ was incubated with 1, 2, and 4 μg of C3 in the presence of buffer or the same concentrations of AcGlyLysOMe. In timed sequence, samples of the 16 reaction mixtures were taken after 3, 6, 9, and 12 min of incubation and delivered into a large volume of ice-cold buffer. After completion of the sampling, the reaction mixtures were further diluted and residual hemolytically active C3 was measured. Curves expressing C3 inactivation as a function of time were constructed for each of the reaction mixtures. Inactivation of C3 followed zero-order kinetics in the reaction mixtures containing 0.5 and 1 μg of C2 while first-order kinetics were observed with the two larger C3 concentrations. The initial velocity of C3 inactivation expressed as effective molecules per min obtained from each of these curves was graphed in reciprocal form against the reciprocal of the initial concentration of C3 in M. A family of straight lines resulted which intersected the ordinate at a common point, indicating competitive inhibition. From the line obtained in the absence of AcGlyLysOMe, a Michaelis constant of 1.8×10^{-6} M for the action of $C\bar{4}\bar{2}$ on C3 was calculated. When these values were graphed by the method of Dixon (1953) which related the reciprocal of the initial velocity of inactivation to the concentration of inhibitor for each of several concentrations of substrate, a series of lines was obtained (Figure 6) which intersected above the abscissa indicating competitive inhibition. From the intercept an inhibition constant (K_i) of 1×10^{-2} M of AcGlyLysOMe was calculated.

Effect of AcGlyLysOMe on Binding of C3 to EAC142. In order to determine whether AcGlyLysOMe interfered with the $C\bar{4}\bar{2}$ mediated binding of C3 to cells, 3×10^7 EAC142 was incubated at 30° with 10 μg of [^{125}I]C3 in the presence of either buffer or 1×10^{-2}, 5×10^{-3}, or 2.5×10^{-3} M AcGlyLysOMe. $C\bar{4}\bar{2}$ was also incubated with 20 and 40 μg of [^{125}I]C3 in the presence of buffer or AcGlyLysOMe. The 12 reaction mixtures were sampled after 3, 6, and 9 min of incubation into a large volume of ice-cold buffer, and radioactivity adherent to the cells after four washes was determined. A linear relationship expressing binding of C3 as a function of time was obtained for each C3 concentration and each AcGlyLysOMe concentration employed. The velocity of C3 binding observed with the highest concentration of C3 was 1.97×10^3 molecules per cell per min. AcGlyLysOMe produced a dose-related decrease in the velocity of C3 binding and a 30% decrease in velocity of binding was observed in the presence of 1×10^{-2} M AcGlyLysOMe. A family of straight lines which intersected the ordinate at a common point resulted when the results were graphed in the double reciprocal manner of Lineweaver and Burk (1934). The maximal velocity of C3 binding, obtained from the intercept, was 1×10^4 molecules per cell per min. An inhibition constant (K_i) of 1.9×10^{-2} M was calculated from the data.

Discussion

These studies show that C2 is able to cleave certain synthetic esters, such as AcGlyLysOMe, containing basic amino acids. Four lines of evidence indicate that AcGlyLysOMe cleaving activity is a property of the C2 molecule. First, all highly purified C2 preparations tested cleaved AcGlyLysOMe, and their ability to do so correlated reasonably well with their content of C2 protein as determined by chemical measurement. Second, a good correlation between the location of C2 protein, C2 hemolytic activity, and ability to cleave AcGlyLysOMe was found on sucrose density gradient ultracentrifugation, gel filtration on Sephadex G-200, and on acrylamide gel electrophoresis of highly purified C2 preparations. Third, whereas the ability to cleave AcGlyLysOMe correlated with the position of C2 on electrophoresis in polyacrylamide gels, this activity shifted to the position occupied by C2i after incubation of C2 with $C\bar{1}\bar{s}$. Fourth, hydrolytic activity increased after activation of C2 by treatment with $C\bar{1}\bar{s}$. These observations, taken together, strongly mitigate against a contaminant enzyme as being responsible for AcGlyLysOMe cleaving activity. Furthermore, plasma trypsin-like enzymes such as kallikrein, Hageman factor, and plasmin, which are able to cleave this compound, are inhibited by one or more of the enzyme inhibitors tested, all of which were unable to inhibit hydrolysis of AcGlyLysOMe by C2 preparations. Traces of $C\bar{1}\bar{s}$ are not responsible for the observed activity since $C\bar{1}\bar{s}$ is inhibited by the high concentrations of Dip-F routinely employed in all of the enzyme studies. In addition, the reaction mixtures employed in most of the studies were examined for the presence of active $C\bar{1}\bar{s}$ by testing for ability to cleave C4. This test, which is sensitive to the detection of less than 1 ng of $C\bar{1}\bar{s}$, failed to reveal active $C\bar{1}\bar{s}$. A minimum of 30 ng of $C\bar{1}\bar{s}$ is required to effect the cleavage of AcGlyLysOMe under the experimental conditions employed in these studies.

Cleavage of AcGlyLysOMe by C2 followed Michaelis-Menten kinetics and exhibited a bell-shaped pH activity curve with an optimum of pH 7.5. The rate of hydrolysis was linear with C2 concentration and showed a dependence on substrate concentration and a Michaelis constant of 1.8×10^{-2} mol. Insofar as studied, therefore, this reaction conforms to the characteristics of an enzymatic process. Although AcGlyLysOMe was not a particularly good substrate for C2 as evidenced by the high K_m value, it was the

most susceptible substrate found, although CbzLysOMe, Tos-LysOMe, CbzArgOMe, and AcLysOMe were all cleaved to some extent by C2. The spectrum and rank order of amino acids cleaved differ relatively little from that observed earlier by Naff and Ratnoff (1968) for $C\bar{1}\bar{s}$, plasmin, trypsin, and urokinase. Kallikrein, thrombin, PTA, and $C\bar{1}\bar{r}$ were found to exhibit a different spectrum and rank order of substrates.

Specific cleavage of C2 by treatment with $C\bar{1}\bar{s}$ consistently produced a 1.5- to 2-fold increase in enzymatic activity of the molecule. However, no further increase in enzymatic activity was observed after a level of 40–50% conversion of C2 to C2i was achieved. The reason for the plateau of enzymatic activity despite additional generation of C2i is not clear at present, but it may reflect a relative lability of C2i with regard to enzymatic activity with activity being lost at a rate comparable to the generation of additional C2i.

Multiple attempts were made to determine whether native C2 possessed enzymatic activity. Unfortunately, this was not possible because all of the C2 preparations examined exhibited significant concentrations of C2i after dialysis and the prolonged periods of incubation necessary to demonstrate this activity. The C2 preparation exhibiting the least proportion of C2i, 23% was able to cleave 0.54 μM of AcGlyLysOMe (Figure 5). While the question remains unresolved, it would not be surprising if C2 in native form possessed some ability to cleave AcGlyLysOMe, since a number of proenzymes or zymogens such as trypsinogen and pepsinogen among others (Kassel and Kay, 1973) have been found to have limited enzymatic activity which is increased after activation.

Earlier studies have shown that chemical oxidation of C2 leads to three distinguishable effects on the activity of this molecule. First, there is increased binding of oxidized C2 to C4, second, specifically bound oxidized C2 is hemolytically more efficient, and third, oxidized cell bound C2 exhibits a reduced rate of decay. The present studies reveal, somewhat surprisingly, that enzymatic activity of C2 for AcGlyLysOMe is not increased by oxidation. It may be inferred from this finding, therefore, that the increased hemolytic efficiency manifested by oxidized C2 is evident only on incorporation of the molecule into the $C\bar{4}\bar{2}$ complex. Among the possible mechanisms responsible for this effect are a stabilization of C2 enzymatic activity by C4, and an increased affinity of oxidized C2 in the $C\bar{4}\bar{2}$ complex for the natural protein substrate, C3.

The relevance of the enzymatic activity for synthetic substrates to the participation of C2 in the hemolytic sequence was examined through inhibition studies. First, AcGlyLysOMe was assessed for its ability to inhibit cleavage of C3 by $C\bar{4}\bar{2}$ in free solution. AcGlyLysOMe produced a dose-related inhibition in the ability of $C\bar{4}\bar{2}$ to hydrolyze C3, and the inhibition observed was competitive as evidenced by the common intercept on the ordinate when the reciprocal of the initial velocity of C3 inactivation obtained with each AcGlyLysOMe concentration was graphed against the reciprocal of the C3 molarity employed. Expression of these data by the graphical method of Dixon (1953) also indicated competitive inhibition as shown by the common intercept above the abscissa of the lines obtained with each of the C3 concentrations employed (Figure 6). A K_i of 1×10^{-2} mol was obtained. These data also allowed a determination of the K_m for the action of $C\bar{4}\bar{2}$ on C3 which was found to be 1.8×10^{-6} mol/l. Second, AcGlyLysOMe was found to produce a dose-related inhibition of the $C\bar{4}\bar{2}$ mediated binding of C3 to cells. The initial velocity of C3 binding was depressed approximately 30% in the presence of 1×10^{-2} M AcGlyLysOMe. Expression of these results by the method of Lineweaver and Burk (1934) yielded a series of straight lines intersecting the ordinate at a common point. Although the binding of C3 in this type of study is the result of a two-step reaction, involving first cleavage and activation of C3 and second, attachment of nascent C3b to cell-bound receptors, the linearity of the Lineweaver–Burk plots and other evidence (Shin and Mayer, 1968) indicate that the fission process and not the binding reaction constitutes the rate-limiting step in this reaction. This contention is also supported by the finding of an inhibition constant, K_i, of 1.9×10^{-2} mol, which is of the same order of magnitude as the K_i obtained for the effect of AcGlyLysOMe on cleavage of C3 by $C\bar{4}\bar{2}$ in free solution. Thus, these data quite probably indicate that AcGlyLysOMe competitively inhibits the $C\bar{4}\bar{2}$-mediated binding of C3 to cells.

The above studies taken together indicate that the enzymatic activity of C2, as evidenced by its ability to cleave AcGlyLysOMe, relates to the function of C2 in the $C\bar{4}\bar{2}$ complex in the cleavage of C3. The fact that the C-terminal residue of C3a cleaved from C3 by $C\bar{4}\bar{2}$ is arginine (Budzko et al., 1971) is also consistent with this interpretation. C2 and C2i are not proteolytic enzymes since neither protein was able to cleave C3 even when tested in a 1:10 molar ratio with C3. The reversible complex formed between C4 and native C2 was also unable to cleave C3 (Müller-Eberhard et al., 1967). Therefore, the ability to hydrolyze C3, which appears to be a property of the C2 enzyme, requires not only activation of C2 by $C\bar{1}\bar{s}$, but also incorporation into the $C\bar{4}\bar{2}$ complex. Whether attachment to C4 modifies the specificity of the C2 enzyme or, alternatively, whether the $C\bar{4}\bar{2}$ complex provides secondary binding sites which enable the enzyme to cleave C3 remain to be determined.

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p-Azidophenacyl Bromide, a Versatile Photolabile Bifunctional Reagent. Reaction with Glyceraldehyde-3-phosphate Dehydrogenase†

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ABSTRACT: The synthesis of the photochemically labile bifunctional reagent *p*-azidophenacyl bromide (**1**) is described. This compound may be covalently attached to a known site of an enzyme or other macromolecule by nucleophilic displacement at the α-bromo ketone moiety. Subsequent irradiation of the bound reagent gives a nitrene which may insert into a second portion of the enzyme forming a cross-link. Reagent **1** proved to be an excellent inhibitor of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase.

The use of specificially designed aryl azides as photochemical labeling reagents for investigating binding sites of macromolecules is an attractive concept (Knowles, 1972). However, recent results in various laboratories including our own have shown that there are severe problems and limitations with the method. When we used the labeling reagent 3-azidopyridine adenine dinucleotide, a loosely bound (K_i *ca.* 10^{-4} *M*) inhibitor of yeast alcohol dehydrogenase (YADH),[1] to probe the active site of this enzyme (Hixson and Hixson, 1973), much nonactive site labeling appeared to occur. Much of this random labeling is likely a result of the loose binding of the inhibitor to YADH. However, poor binding is not the only factor, for we have recently obtained strong evidence that when the reagent *p*-azidobenzenesulfonamide, a tightly bound inhibitor (K_i *ca.* 10^{-6} *M*) of bovine erythrocyte carbonic anhydrase (CA), is irradiated under conditions where >99% of the reagent is initially located (noncovalently) at the active site of CA, covalent labeling of the protein occurs largely at points removed from the active site (A. Lowrie and S. Burroughs, unpublished results).

This random labeling problem is almost certainly in large part a result of the rather low reactivity of aryl nitrenes. They do not react "instantaneously" upon generation, but instead have a sufficiently long lifetime that they may diffuse out of the binding site and react with more remote portions of the protein (Knowles, 1972). Other workers, notably Ruoho et al. (1973) and Richards et al. (1974), have encountered and discussed this problem in their work with other systems. Our conclusion has been that the use of noncovalently bound aryl azides as photochemical probes of binding sites of macromolecules is risky and apt to provide ambiguous information. Consequently we are turning to the use of bifunctional photochemical reagents which may be bound covalently at a known locus in the macromolecule prior to photolysis.

In this direction we have prepared the compound *p*-azidophenacyl bromide (**1**). This molecule contains the photochemically labile azide group along with a reactive α-bromo ketone moiety. α-Halo carbonyl compounds are known to label nucleophilic amino acids such as cysteine in proteins. Therefore reagent **1** should be particularly useful for investigating the active sites of the large number of sulfhydryl enzymes—those which possess a particularly reactive cysteine residue at the active site that may be selectively derivatized—and the compound was designed for this purpose. When **1** is irradiated after being covalently attached to the active site of the enzyme, any portion of the protein chain labeled by the resulting nitrene must be close in three-di-

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[1] Abbreviations used are: CA, bovine erythrocyte carbonic anhydrase; GPDH, rabbit muscle glyceraldehyde-3-phosphate dehydrogenase; YADH, yeast alcohol dehydrogenase.