Diazomethyl Ketone Substrate Derivatives as Active-Site-Directed Inhibitors of Thiol Proteases. Papain†

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ABSTRACT: The diazomethyl ketones of z-Phe and z-Phe-Phe inactivate papain by a stoichiometric reaction at the active-center thiol. Since the reagents are stable in mercaptoethanol, their reaction with papain is judged to be the result of complex formation characteristic of affinity-labeling reagents. The diazomethyl ketones react by a mechanism different from that of chloromethyl ketones, since the pH dependence of their inactivation of papain is different, the rate increasing with decreasing pH. This relationship has been observed in other cases, such as in the reaction of azaserine with glutamine amidotransferases [Buchanan, J. M. (1973), *Adv. Enzmol. Relat. Areas Mol. Biol. 39*, 91], and is interpreted as an indication of reaction with a thiol group in its protonated form.

Thiol proteinases and serine proteinases comprise two major classes of proteolytic enzymes designated by the active-center functional group involved in covalent catalysis (Walsh, 1975). Within each group are proteases of specialized function. The number of these that are being identified is rapidly increasing and the clarification of their role in biological control is under study in diverse research contexts.

Inhibitors provide a valuable tool both for the characterization of purified enzymes as well as for deducing their role in vivo. Chloromethyl ketone derivatives of amino acids and of peptides have been shown to be extremely selective and effective inhibitors when comparisons are made among the serine proteinases (Shaw, 1975). Reagents of this group are available which discriminate among the major specificity classes of the serine proteinases, i.e., chymotryptic, tryptic, and elastolytic, but also within a given specificity class, such as the tryptic-like enzymes (Coggins et al., 1974; Kettner and Shaw, 1977). However, thiol proteases are also readily inactivated by chloromethyl ketones. The wide-spread occurrence of cathepsin B_1 in tissues makes it impossible in the presence of this enzyme to ascribe an effect produced by chloromethyl ketones to a serine proteinase or to cathepsin B_1. The same ambiguity accompanies the use of the naturally occurring inhibitors chymostatin and leupeptin (Umezawa, 1972) as well as of certain tissue and plasma inhibitors (Starkey and Barrett, 1973). We therefore undertook the examination of a different reactive grouping, diazomethyl ketones, for use in affinity labeling with the hope of obtaining reagents selective for thiol protease but without action on serine proteinases. Such compounds should at least permit the unambiguous examination of the role of cathepsin B and other thiol proteases in normal and pathological conditions.

The possibility that diazomethyl ketones might provide a useful reactive grouping to incorporate into an affinity label for thiol proteases was suggested by the work of Buchanan and his associates on the mechanism of action of the antibiotic, azaserine, a diazoacetyl derivative (Buchanan, 1973). These investigations demonstrated that the irreversible inhibition of 2-formamido-*N*-ribosylacetamide 5′-phosphate:L-glutamine amide ligase by azaserine results from the alkylation of a particular cysteine residue of the enzyme (Dawid et al., 1963) which has been proposed to participate in thioacyl formation in the normal mechanism of action of this enzyme (Mizobuchi and Buchanan, 1968). Diazomethyl ketone glutamine analogues have been utilized as effective inhibitors of a variety of glutamine-utilizing enzymes (Roberte et al., 1972).

Diazomethyl ketone derivatives have also been used as active-site-directed inhibitors of acid proteases (Delpierre and Fruton, 1966; Ong and Perlmann, 1967) as have diazoacetyl derivatives. The inactivation of pepsin by diazomethyl ketones has been shown to result from the esterification of an aspartate residue (Fry et al., 1968; Bayliss and Knowles, 1968; Lunblad and Stein, 1969) apparently with a requirement for copper ion. As in the case of the glutamine amide ligase, the substituted residue has been proposed to participate in the catalytic mechanism of pepsin (Fruton, 1971).

The sulfhydryl proteases have been shown to possess a highly reactive cysteine residue as an essential component of their active sites (Glazer and Smith, 1971) and might thus serve as target enzymes for active-site-directed diazomethyl ketones. Allusion has been made to the reaction of papain with Tos-GlyCHN_2 (Hussain and Love, 1965), but no details have been forthcoming. Papain, available commercially and obtainable in a highly purified and active form, was chosen as a model for this class of proteases prior to the study of cathepsin B_1. z[1]-Phenylalanyl diazomethyl ketone and z-phenylalanylphenylalanyl diazomethyl ketone were synthesized and tested as potential active-site-directed reagents.

Materials and Methods

Recrystallized papain (Sigma Chemical Co.) was further purified using an organomercurial Sepharose column prepared as described by Sluyterman and Wijdenes (1970). The active papain, retained by the column, was eluted with 0.001 M $HgCl_2$, and stored as the mercuri derivative at 4 °C. Papain concentrations, as reported in this paper, were determined

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[1] Abbreviations used are: z, benzyloxycarbonyl; EDTA, (ethylenedinitrilo)tetraacetic acid; TLC, thin-layer chromatography; HPLC, high-pressure liquid chromatography.

spectrophotometrically from the value $E_{1cm}^{1\%}$ equal to 25.0 at 278 nm and on the basis of a molecular weight of 23 400 (Glazer and Smith, 1971). The concentration of activatable papain was calculated using a secondary standard rate assay with N^{α}-z-lysine nitrophenyl ester (Bender and Brubaker, 1966). These two methods were in excellent agreement, indicating that the purification procedure employed resulted in a fully activatable form of papain. The papain in the inactivation experiments reported in this paper was maintained in a fully active form by a large excess of thiol.

Papain was assayed spectrophotometrically with N^{α}-z-N-lysine nitrophenyl ester by the addition of enzyme to 1.5 mL of 0.10 M acetate buffer, 0.0025 M mercaptoethanol, 0.001 M EDTA (pH 5.4), followed by the addition of 20 μL of 10^{-2} M substrate in 95% acetonitrile solution. The increase in absorbance at 340 nm was measured with a Beckman DB spectrophotometer attached to a Leeds Northrup recorder (Model 69800) calibrated to a full deflection of 0.10 absorbance unit. A cuvette containing buffer and substrate was used as a control, and the enzyme activity was expressed as change in absorbance at 340 nm min^{-1} mL^{-1} enzyme solution.

The following chemicals were obtained from commercial sources: mercaptoethanol from Eastman Kodak; Sephadex G-25 from Pharmacia; Partisil 20 from Whatman, Inc.; Aquasol from New England Nuclear; N^{α}-z-lysine nitrophenyl ester from Cyclo Chemical Co.; and methanol from Fisher Scientific Co. Acetonitrile (Aldrich Chemical Co.) was redistilled before use. RNase, obtained from Worthington Biochemicals (RNase), was dialyzed for 24 h against two changes of 7 L of water and then lyophilyzed for use in the performic acid oxidation experiments.

z-PheCH_2Cl (ZPCK) was prepared by the literature method (Shaw, 1967); z-PheCHN_2 is an intermediate in this synthesis. To ensure freedom from the chloromethyl ketone,[2] 50 mg of crystalline z-PheCHN_2 was purified by HPLC on a Partisil 20 column (0.9 × 20 cm) developed with hexane–ethyl acetate, 3:1 (v/v), and monitored at 280 nm. An initial peak, 3 mg, was identified as chloromethyl ketone (R_f 0.40, TLC in the same solvent) and was followed by pure diazomethyl ketone, 45 mg (R_f 0.20, TLC as above). Crystallized samples had mp 81–83 °C in agreement with the literature value (Ong and Perlmann, 1967); however, melting point is not an adequate criterion for purity.

Preparation of L-1-Diazo-3-N-carbobenzyloxy-L-phenylalanylamino-4-phenyl-2-butanone (z-Phe-PheCHN_2). z-Phenylalanylphenylalanine (4.47 g, 10 mmol) was dissolved in dry tetrahydrofuran (50 mL), treated with N-methylmorpholine (1.12 mL) for 5 min at room temperature, and cooled to at least −10 °C under anhydrous conditions. Isobutyl chloroformate (1.31 mL) was then added with vigorous stirring. After 5 min, the reaction mixture was filtered rapidly into a dropping funnel and diluted with 100 mL of cold tetrahydrofuran. The filtrate, under dry nitrogen, was added dropwise to cold (−10 °C) 0.3 M diazomethane in ether (250 mL) with stirring. When addition was complete, the reaction mixture was allowed to warm to room temperature for 1 h. After removal of the solvent under reduced pressure, the residual crystals were dissolved in chloroform and applied to a column (3.8 × 19.5 cm) of silica gel 60 (ca. 70–230 mesh ASTM, Merck Co., 150 g). Elution was carried out with a chloroform–methanol–water mixture (97:2.8:0.2, v/v).

The main peak was collected and allowed to stand 1 day for crystallization, yielding 2.44 g (51.9%): mp 151.5–152.0 °C; IR (KBr) 2100 cm^{-1} (C=N=N); NMR ($CDCl_3$) δ 7.28 (5 H, s), 7.03 (10 H, m), 6.52 (1 H, m), 5.28 (1 H, m), 5.03 (2 H, s), 4.93 (1 H, s), 4.62 (1 H, q), 4.52 (1 H, q), 2.98 (2 H, d), 2.93 (2 H, d). Anal. Calcd for $C_{27}H_{26}N_4O_4$: C, 68.92; H, 5.57; N, 11.91. Found: C, 69.06; H, 5.70; N, 11.83.

Preparation of Labeled Inhibitor, z-Phe-Phe-^{14}CHN_2. For the preparation of [^{14}C]diazomethane, N-nitroso-N-[^{14}C]-methyl-β-toluenesulfonamide was synthesized. p-Toluenesulfonamide (2.44 g) in dry methanol (15 mL) was treated with 1 N methanolic sodium methylate (14.4 mL). [^{14}C]-Methyl iodide (7 mCi, New England Nuclear) was taken up in methanol (1 mL) containing carrier methyl iodide (0.88 mL) and added to the above solution. After 4.5 days at room temperature, the reaction mixture was partitioned between water and ethyl acetate. The dried residue from the organic layer, N-[^{14}C]methyl-p-toluenesulfonamide (1.89 g), was dissolved in acetic acid (14 mL) and water (2.8 mL) and treated slowly at 0 °C with a solution of sodium nitrite (1.064 g) in water (2.8 mL). After 10 min, additional water was slowly added to induce crystallization of N-nitroso-N-[^{14}C]methyl-p-toluenesulfonamide. This was converted to [^{14}C]diazomethane by the standard methylcellusolve–KOH procedure.

The total preparation was treated with the mixed anhydride obtained from 200 mg of z-Phe-Phe by following the procedure above for the nonradioactive preparation. The product was twice recrystallized providing 100 mg (TLC, a single spot R_f 0.22), indicating the absence of the corresponding chloromethyl ketone, R_f 0.36. The specific activity was 8.74×10^5 dpm/μmol.

Inactivation Studies. z-PheCHN_2 and the corresponding chloromethyl ketone were maintained as 10^{-2} M stock solutions in methanol, and any further dilutions were made with methanol. The dipeptide derivative, on the other hand, was prepared as a 5×10^{-3} M stock solution in acetonitrile with further dilutions as needed in acetonitrile.

The inactivation experiments involved preincubation of papain with various concentrations of inhibitor at room temperature. Conditions are described in Table I. The final concentration of organic solvent was 10%. At various times the extent of enzyme inactivation was determined by assaying an aliquot of the reaction by the procedure described above. A control mixture containing all of the components of the reaction mixture except the inhibitor was run with each inactivation experiment.

The results of the kinetic inactivation experiments are presented graphically or as pseudo-first-order rate constants calculated in the usual way using the equation $k_1 = 0.693/t_{1/2}$. The half-times of inactivation were obtained from first-order plots (log of percent enzyme activity). In the case of enzyme inactivations by chloromethyl ketones which did not conform to pseudo-first-order kinetics due to reaction of the inhibitor with the large excess of mercaptoethanol, the half-times were estimated by extrapolation of the initial part of the curve obtained in the first-order plot. In all cases the reported first-order rate constants are corrected for the small decreases in enzyme activity observed in the appropriate control mixture.

Preparative-scale inactivation experiments were also carried out to permit determination of the site of attack of the z-Phe diazomethyl ketone by amino acid analysis and the stoichiometry of inactivation using ^{14}C-labeled z-Phe-Phe diazomethyl ketone. The reaction mixture for the amino acid analysis experiment contained 1.0×10^{-5} M papain and 2.5×10^{-4} M z-Phe diazomethyl ketone in 20 mL of 0.9 M acetate, 0.0020 M mercaptoethanol, 0.00090 M EDTA (pH 5.4)

[2] In initial experiments it was not appreciated that diazomethyl ketones prepared by addition of acid chlorides to excess diazomethane usually contain some chloromethyl ketone which may be present even after recrystallization. Typical analytical procedures are much less sensitive than enzymes for the detection of such materials.

with 10% ethanol. The control mixture contained all of the components of the reaction mixture except the inhibitor. At the end of the inactivation experiment, the control and reaction mixtures were dialyzed separately for 12 h vs. 2 L of 5% methanol in water and then against two changes of 4 L of water each. After this, the samples were lyophilyzed in preparation for performic acid oxidation and amino acid analysis.

The inactivation mixture used to determine the stoichiometry of binding of z-Phe-Phe diazomethyl ketone to papain contained 1.45×10^{-5} M papain in a total volume of 20 mL of 0.90 M acetate buffer, 0.0019 M mercaptoethanol, 0.0009 M EDTA (pH 5.4) with 10% methanol at room temperature. The reaction was initiated by the addition of the ^{14}C-labeled diazomethyl ketone. The final concentration of the inhibitor used in this experiment was 2.5×10^{-5} M. Under these conditions, the reaction was complete within 15 min as measured by the complete loss of esterase activity. The reaction mixture was applied to a Sephadex G-25 column (2.5 × 35 cm) and eluted with 0.10 M acetate buffer, 0.001 M EDTA (pH 5.4) containing 10% methanol; fractions of 5 mL were collected.

Performic acid oxidations were carried out as described by Hirs (1956). The lyophilyzed protein samples (3–5 mg) from the z-Phe diazomethyl ketone inactivation experiment were each dissolved in 0.50 mL of 97 to 100% formic acid. The protein samples and the previously prepared performic acid reagent (0.7 mL) were cooled to 0 °C, mixed, and kept 4 h at 0 °C. The samples were then transferred to lyophilyzation flasks containing 100 mL of ice water and lyophilized.

Quantitative amino acid analyses were carried out on 24-hr hydrolysates (6 N HCl). The number of residues of cysteic acid per mole or papain was established by determining the latter using the average values based on the yield of proline, glycine, alanine, aspartic acid (aspartic plus asparagine), and glutamic acid (glutamic plus glutamine), and the known content of these residues (Mitchel et al., 1970). In order to correct for the efficiency of oxidation and overall recovery of cysteic acid, a parallel performic acid oxidation and analysis of RNase was carried out. The correction factor of 0.92 for efficiency of performic acid oxidation and recovery of cysteic acid was determined by dividing the calculated yield of cysteic acid per mole of RNase by the theoretical value.

Radioactivity was determined by adding aliquots of solution, up to 0.50 mL, to 12 mL of Aquasol in a scintillation vial; the samples were counted using a Beckman liquid scintillation counter (Model LS 233).

Results

The reaction of activated papain with z-Phe diazomethyl ketone was examined at pH 6.5 and was found to be irreversible and to follow pseudo-first-order inactivation kinetics with respect to enzyme. The pseudo-first-order rate constants calculated from the data of this experiment are shown in Table I. Table I also shows the pseudo-first-order rate constants calculated for the more rapid reaction of z-Phe chloromethyl ketone with papain determined at pH 5.4. The second-order rate constant for the reaction of the chloromethyl ketone derivative with papain calculated from the initial rate of inactivation of papain was 1670 M^{-1} s^{-1}.

A comparison of the results obtained with the diazomethyl and chloromethyl ketone derivatives of z-Phe indicates the possibility that contamination of the diazomethyl ketone with trace amounts (less than 1%) of the chloromethyl ketone could acccount for the inactivation of papain observed with z-Phe diazomethyl ketone which might have no effect if pure. In order to eliminate the possibility of contamination from this source,[2] the effect of preincubation at alkaline pH on the two derivatives was compared. The z-Phe chloromethyl ketone derivative was preincubated at a concentration of 1.0×10^{-5} M at pH 10.9 in 0.10 M CO_3–HCO_3 buffer containing 40% methanol, and then tested for its ability to inactivate papain under standard conditions at pH 5.4 (Table I) at a concentration of 1.0×10^{-6} M. Under these preincubation conditions, as expected, the inhibitory activity of the chloromethyl ketone derivative for papain was totally lost within 5 min. On the other hand, the z-Phe diazomethyl ketone derivative preincubated 30 min under the same conditions at 1.25×10^{-3} M and then tested for its ability to inactivate papain at 1.25×10^{-4} M showed no significant change in its inhibitory activity.

TABLE I: Relative Inhibitory Activity[a] of Substrate Analogues in the Affinity Labeling of Papain.[b]

Reagent	pH of inactivation	$t_{1/2}$ (min)	$10^4 \times K_I$ (s^{-1})	$k_{1/[I]}$ (M^{-1} s^{-1})
z-PheCHN$_2$				
2.50×10^{-4} M	6.5	4.5	25.67	10.3
1.25×10^{-4} M		8.8	13.17	10.5
6.25×10^{-5} M		17.3	6.67	10.7
z-PheCHN$_2$				
1.25×10^{-4} M	5.4	9.6	10.2	8.16
z-PheCH$_2$Cl				
1.0×10^{-6} M	5.4	6.4	16.7	1670
z-Phe-PheCHN$_2$				
1.0×10^{-6} M	6.5	5.3	21.8	2180
5×10^{-7} M		11.4	10.13	2030
3.75×10^{-7} M		15.1	7.65	2040
2.5×10^{-7} M		25.5	4.53	1810

[a] k_I divided by inhibitor concentration (last column) used as index of relative inhibitory activity. [b] Conditions for inactivation experiments: 0.085 M buffer (acetate for pH 5.4 and phosphate for pH 6.5), 0.0021 M mercaptoethanol, 0.0009 M EDTA with 10% organic solvent (acetonitrile for z-Phe-PheCHN$_2$, methanol for all others). Papain concentration = 2.75×10^{-7} M for z-Phe derivatives, 3.33×10^{-8} M for z-phe-PheCHN$_2$; all inactivations performed at room temperature.

The effect of pH on the pseudo-first-order rate constant for the inactivation of papain by z-Phe diazomethyl ketone was measured. The rate of inactivation was constant between pH 4.0 and 5.5, increased to maximum at pH 6.5, and then decreased sharply with further increases in pH.

A preparative inactivation of papain was carried out in order to obtain material for examination of the possibility that the active-center cysteine residue was the site of modification by z-Phe diazomethyl ketone. The reaction was carried out at room temperature for 2 h at which time the z-Phe diazomethyl ketone treated sample had dropped to less than 0.5% of its initial activity, whereas the control retained 97% of its initial activity. The amino acid composition, determined following performic acid oxidation, indicated that the control papain contained 6.15 residues of cysteic acid per mole of papain, whereas the z-Phe diazomethyl ketone inactivated sample contained 5.35 residues of cysteic acid per mole of papain. When these values were corrected for the efficiency of oxidation and recovery of cysteic acid (92%), the value of 6.68 residues per mole of protein was calculated for the control and 5.81 residues per mole of protein for the z-Phe diazomethyl ketone treated sample.

The reaction of the dipeptide derivative, z-Phe-Phe diazomethyl ketone, with papain was also investigated, and the results of one such experiment are shown in Figure 1. The inactivation of papain by z-Phe-Phe diazomethyl ketone was also irreversible and followed pseudo-first-order inactivation ki-

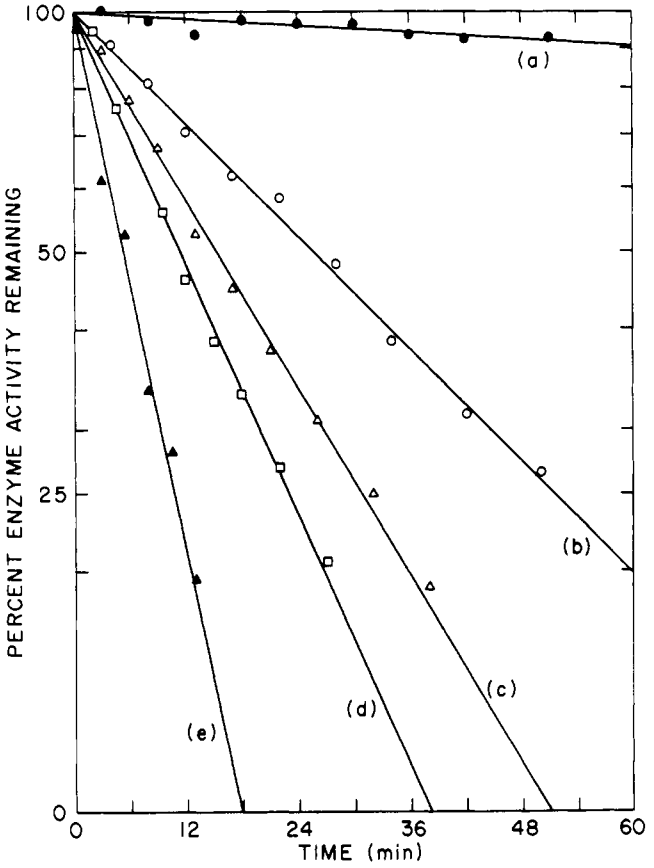


FIGURE 1: Inactivation of papain by z-phenylalanylphenylalanyl diazomethyl ketone at room temperature in 0.085 M phosphate, 0.0021 M mercaptoethanol, 0.0009 M EDTA (pH 6.5) with 10% acetonitrile; concentration of papain 3.33×10^{-8} M; concentration of z-Phe-PheCHN$_2$: (a) 0; (b) 2.50×10^{-7} M; (c) 3.75×10^{-7} M; (d) 5×10^{-7} M; (e) 1×10^{-6} M.

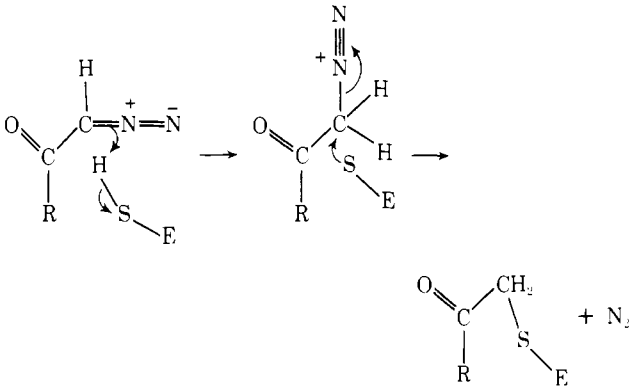
netics with respect to enzyme. However, as can be seen from the results in Table I, the dipeptide derivative is approximately 200-fold more effective an inhibitor than z-Phe diazomethyl ketone. As in the case of z-Phe diazomethyl ketone, the dipeptide retained all of its inhibitory activity toward papain following 30-min preincubation at pH 10.9 in 0.10 M CO_3-HCO containing 40%, v/v, of methanol. The pH vs. rate of inactivation curve for the reaction of z-Phe-Phe diazomethyl ketone was of the same type as that observed for z-Phe diazomethyl ketone. Thus, at 25 °C, the rate, $k_{1st} \times 10^3$ (s^{-1}), of inactivation in 2×10^{-6} M inhibitor diminished as follows: (pH) k_{1st} (6.5) 3.4; (8.0) 1.93; (8.5) 1.36; (9.0) 0.64. This represents a drop of 81% over this range. For both inhibitors, the maximum rate was observed at pH 6.5. As in the earlier case, these last two observations provide evidence that the observed inactivation of papain by z-Phe-Phe diazomethyl ketone was the result of the dizaomethyl ketone function and not due to contamination with the chloromethyl ketone derivative.

The stoichiometry of binding of z-Phe-Phe diazomethyl ketone was determined with the use of labeled inhibitor. The ratio of radioactivity incorporated to papain concentration, in the five successive protein-containing fractions from the Sephadex G-25 column, range from 0.96 to 1.05 (average 1.02) mol of ^{14}C incorporated per mol of enzyme, indicating a stoichiometric reaction.

Discussion

The results obtained in the present study demonstrate that

Scheme I



both z-Phe and z-Phe-Phe diazomethyl ketones serve as effective irreversible inhibitors of the sulfhydryl protease papain. Amino acid analysis of z-Phe diazomethyl ketone treated papain showed that inactivation resulted from alkylation of the active-center cysteine residue.

The reaction of a diazomethyl ketone with a cysteine residue has been previously demonstrated in the reaction of azaserine with the active-center cysteine of 2-formamido-*N*-ribosylacetamide 5′-phosphate:L-glutamine amide ligase (Dawid et al., 1963). It was proposed (French et al., 1963) that the reaction occurs by protonation of the diazomethyl carbon atom by the sulfhydryl of the active-center cysteine residue, resulting in concomitant formation of a diazonium salt and the thiolate anion. The diazonium salt is then thought to be subject to nucleophilic attack at the C_1 carbon atom of the inhibitor by the sulfhydryl anion, resulting in the release of nitrogen and alkylation of the active-center cysteine residue. The mechanism is shown in Scheme I. The observed decrease in reaction rate of z-Phe-Phe diazomethyl ketone with papain above pH 6.5 suggests that the loss of reactivity accompanies the ionization of the active-center cysteine residue (pK ~8.5) (Glazer and Smith, 1971). This observation is consistent with the alkylation mechanism discussed above, although the active-center thiol may, in fact, be H bonded to a histidine residue, and the precise nature of the ionization steps as a result is not certain (Lewis et al., 1976). However, it is important that the observed pH dependence is strikingly different from that of the alkylation of papain by substrate-derived chloromethyl ketones (Bender and Brubacher, 1966; Whitaker and Perez-Villasenor, 1968) or by chloroacetamide (Chaiken and Smith, 1969) the rates for which increase above pH 6.5 in an S-shape curve, suggesting the participation of the anionic form of the essential thiol. For example, the rate of inactivation by Tos-PheCH$_2$Cl increases 34-fold in the pH range 6.5–9.0 (Bender and Brubacher, 1966), whereas the rate of inactivation by z-Phe-PheCHN$_2$ drops 81%. The inactivation by diazomethyl ketones thus cannot be ascribed to contamination by chloromethyl ketone.[2] In addition, the retention of inhibitory activity of the diazomethyl ketone preparations after preincubation at pH 10.9 for 30 min also eliminated this possibility, since chloromethyl ketones are destroyed under such conditions.

Examination of the inactivation of papain by Tos-PheCH$_2$Cl and Tos-LysCH$_2$Cl led to the conclusion (Wolthers, 1969) that only in the latter case does the reaction appear to be active-site directed, since it may be blocked by a substrate, benzoylarginine ethyl ester, whereas the rate of inactivation by Tos-PheCH$_2$Cl is independent of the concentration of the ester substrate. It was also noted that the reaction of papain with Tos-LysCH$_2$Cl was 20 times faster than with Tos-PheCH$_2$Cl.

If the conclusion that Tos-PheCH_2Cl is not acting by affinity labeling is valid, there may be some doubt about whether or not z-PheCHN_2 is inactivating papain as the result of prior complex formation. This was not examined by kinetic studies. However, it has been shown (Dawid et al., 1963) that azaserine, a representative diazomethyl ketone, does not react with either free cysteine or activated papain in contrast to chloroacetate, which reacts with both papain and cysteine (Chaiken and Smith, 1969). These later observations strongly suggest that the reaction of z-L-Phe diazomethyl ketone with papain requires prior complex formation. The initial binding must result in an orientation of the inhibitor at the active site of the enzyme such that the chemical potential of the essentially inert diazomethyl ketone function is evoked by proximity to the -SH group and alkylation of the active-center cysteine residue occurs. Although the rate enhancement due to proximity has not been measured in this reaction, it is apparently large, since the diazomethyl ketones are not affected by preincubation in 2×10^{-3} M mercaptoethanol but react rapidly with 10^{-7} M papain. It may be worth recalling that in the alkylation of chymotrypsin by z-PheCH_2Cl the effect of proximity results in a rate enhancement of 10^6 (Shaw and Ruscica, 1971). Furthermore our observations that the estimated second-order rate constant for the reaction of z-Phe chloromethyl ketone with papain (1670 M^{-1} s^{-1}) is approximately 150-fold greater than that for the reaction of Tos-L-Phe chloromethyl ketone with papain under similar conditions (Whitaker and Perez-Villasenor, 1968) suggest that the *N*-carbobenzoxy substituent confers substrate-like qualities on both the diazomethyl ketone and chloromethyl ketone derivatives of z-Phe, and, therefore, both reagents are considered to be active-site-directed inhibitors.

The finding that the dipeptide derivative z-Phe-Phe diazomethyl ketone is an extremely potent inhibitor of papain, 200-fold greater than z-Phe diazomethyl ketone (Table I), was not unexpected, since it has been previously shown that the S_2 subsite of papain shows a strong preference for a phenylalanine residue (Schachter and Berger, 1968). This observation again indicates that usefulness of utilizing enzyme specificity in the design of active-site-directed reagents.

These studies with papain were intended to provide preliminary observations for eventual extension to cathepsin B_1 if successful. Initial observations with bovine spleen cathepsin B_1 have confirmed the applicability of this method of affinity-labeling to that thiol protease also (Leary and Shaw, unpublished results). Because of relative chemical inertness, peptide diazomethyl ketones should be useful for the study of the role of sulfhydryl proteases in normal and pathological processes. The extent to which these peptitide derivatives are inert to serine proteases must be explored more fully as inhibitor sequence variations are made; however, it is encouraging that z-Phe diazomethyl ketone as well as the dipeptide derivative are inert to chymotrypsin (Watanabe and Shaw, unpublished results). Although, judging from initial results, a diazomethyl ketone is less reactive in the affinity labeling of papain than the corresponding chloromethyl ketone, the gain in selectivity is the desirable result.

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