Interaction of papain with derivatives of phenylalanylglycinal: Fluorescence studies

(transition-state analogues/stopped-flow kinetics)

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ABSTRACT  Fluorescence studies have been performed on the interaction of papain with active-site-directed inhibitors of the type Mns-(Gly)n-Phe-glycinal, where n = 0, 1, 2. It has been found that whereas the mansyl [6-(N-methylaminino)-2-naphthylmethylene] derivatives are not, although all three are equally effective as inhibitors of papain action. Measurements of fluorescence polarization and rotational relaxation time support the conclusion that the fluorescence probe group of the two longer mansyl compounds protrudes into the solvent to a greater degree than that of mansyl-Phe-glycinal. Considerable energy transfer from papain tryptophan to the mansyl group is evident for all three inhibitors, however, although it is most marked with mansyl-Phe-glycinal. Stopped-flow fluorescence measurements have shown that, after initial rapid interaction, the first-order conformational changes in the active site region of papain in the complex with mansyl-Phe-glycinal are approximately 1/10^4 those observed with comparable mansyl oligopeptide substrates, and approximately 1/10^3 those with acetyl-Phe-glycinal.

In a previous publication from this laboratory (1) it was reported that the intrinsic tryptophan fluorescence of papain is greatly enhanced upon the binding of the inhibitor Ac-Phe-glycinal (2) and of related compounds in which the acetyl group is replaced by benzoyloxy carbonyl (Z), Z-Gly, or Z-Gly-Gly. This fluorescence enhancement is largely abolished by the selective oxidation of Trp-69 (3) to yield a modified papain that retains its catalytic activity toward small synthetic substrates, such as Ac-Phe-Gly-NA (NA is p-nitroaniline). The results indicated that, in the binding of the above derivatives of Phe-glycinal at the active site of papain, there is significant interaction between the phenylalanine residue of the inhibitor and Trp-69 of the enzyme. To examine more closely the validity of this suggestion, we synthesized the series Mns-(Gly)n-Phe-glycinal (n = 0, 1, 2), as well as Mns-glycinal, for fluorescence studies on their interaction with papain. Mansyl [Mns, 6-(N-methylaminino)-2-naphthalene sulfonl] derivatives of suitable substrates and inhibitors of pepsin (4), papain (5), and thermolysin (6) have been used in earlier work to study stopped-flow fluorescence spectroscopy, the kinetics, and mechanism of the action of these enzymes. In the present investigation, the principal objective was to take advantage of the tight binding of the derivatives of Phe-glycinal at the active site of papain, as a consequence of thiohemiacetal formation by the reaction of the aldehyde group of the inhibitors with Cys-25 of the enzyme (7, 8), to examine the extent of energy transfer between tryptophan in the enzyme and the mansyl group of a series of inhibitors in which the fluorescence probe group is located at various known distances from the reactive aldehyde group. Also, the kinetics of the interaction of Mns-(Gly)n-Phe-glycinal with papain were studied by means of stopped-flow spectrophotometry.

MATERIALS AND METHODS

The mansyl derivatives of (Gly)n-Phe-glycinal (n = 0, 1, 2) were prepared by controlled acid hydrolysis of the corresponding diethyl acetal. The latter were obtained by catalytic hydrogenolysis (Pd black) of 1–2 mmol of the Z-compounds (1) in ethanol containing 1 equivalent of acetic acid, followed by treatment (20 hr, room temperature) with mansyl chloride (1.5 equivalents) in acetone, in the presence of N-methylmorpholine (3 equivalents). The solution was concentrated to dryness, the residue was dissolved in CHCl3 (3 ml), and the acetals were isolated by chromatography on a column (1.2 × 50 cm) of silica gel (20 g, 60–200 mesh) by using successively CHCl3, 1% MeOH in CHCl3, and 2% MeOH in CHCl3 as eluants. The fractions that contained the product were pooled and concentrated to dryness, and the residue was recrystallized from ethyl acetate/hexane. The yields were about 20%, based on the amount of Z-compound used. The products gave single spots upon thin-layer chromatography (Eastman Chromagram 6061 plates) with chloroform/methanol, 19:1 (vol/vol) as the solvent. Elemental analysis gave the following results. Mns-Phe-glycinal(OEt)2 (mp 140–141°C, Rf 0.21), calc. for C98H7N3O8S (575.7): C, 66.8; H, 6.5; N, 7.3; found: C, 66.9; H, 6.7; N, 7.4. Mns-Gly-Phe-glycinal(OEt)2 (mp 177–178°C, Rf 0.59), calc. for C98H7N3O8S (632.8): C, 64.5; H, 6.4; N, 9.1. Mns-Gly-Gly-Phe-glycinal(OEt)2 (mp 93–95°C, Rf 0.45), calc. for C98H7N3O8S (689.8): C, 62.7; H, 6.3; N, 10.15; found: C, 62.4; H, 6.3; N, 10.1. Mns-glycinal was prepared by treatment of glycinal(OEt)2 (Aldrich) with mansyl chloride in the manner described above, but a crystalline product was not obtained; the material used gave a single spot (Rf 0.65) upon thin-layer chromatography.

To convert the diethyl acetal to the corresponding aldehydes, we heated a 10 mM solution in 5 ml of acetone/water, 7:3 (vol/vol) under reflux in the presence of 2 g of Dowex 50W-X8 (Bio-Rad). The reaction was monitored by means of thin-layer chromatography, both for mansyl fluorescence and with 2,4-dinitrophenylhydrazine as the aldehyde reagent. Upon completion of the hydrolysis (2–6 hr), the reaction mixture was filtered and the filtrate was concentrated. With two of the acetals, crystalline aldehydes were obtained in yields of about 100%. Mns-Gly-Phe-glycinal (mp 155–156°C dec., Rf 0.26), calc. for C98H7N3O8S (558.6): C, 64.5; H, 5.4; N, 10.0; found: C, 64.7; H, 5.7; N, 10.0. Mns-Gly-Gly-Phe-glycinal (mp 139–141°C dec., Rf 0.15), calc. for C98H7N3O8S (615.7): C, 62.2; H, 5.4; N, 11.4; found: C, 62.4; H, 5.4; N, 11.4. Mns-Phe-glycinal (Rf 0.37) and Mns-glycinal (Rf 0.41) were not obtained in crys-

Abbreviations: Z, benzoyloxy carbonyl; NA, p-nitroaniline; Mns, mansyl, 6-(N-methylaminino)-2-naphthalene sulfonl; FU, fluorescence unit.
talline form; their concentrations in stock dioxane solutions were determined spectrophotometrically. Occasionally, difficulty was encountered in the complete hydrolysis of Mns-Phe-glycinal(OEt)$_2$, and the desired product was obtained by preparative thin-layer chromatography on silica gel. Monitoring of the hydrolysis of the manysyl peptide diethylacetals by means of thin-layer chromatography failed to reveal the appearance of ninyhdrin-positive components, indicating that the treatment with Dowex-50 did not result in any detectable cleavage of peptide bonds under the conditions used in this work.

The papain preparations were obtained by means of chromatography of commercial crystalline papain (Worthington) on a mercerarl agarose column (5) to yield mercapurapain, which was activated by 2-mercaptoethanol (25 mM) and EDTA (1 mM) in 0.02 M potassium phosphate buffer (pH 6.5). The concentration of papain solutions was estimated spectrophotometrically at 278 nm from a molar absorptivity of 59,000. The enzymic activity was assayed with Ac-Phe-Gly-NA, and this substrate was used to determine the relative extent of the inhibition of papain by the manysyl aldehydes, in the manner described (1) for the Z-derivatives of Phe-glycinal.

Unless otherwise stated, all absorbance and fluorescence measurements were performed with solutions containing 0.02 M potassium phosphate buffer (pH 6.5), 2.5 mM 2-mercaptoethanol, and 0.1 mM EDTA. Separate measurements showed that none of these constituents of the test solutions affected the absorption or emission spectra of the manysyl aldehydes to a significant extent. Absorbance measurements were performed with a Cary model 15 spectrophotometer. Fluorescence measurements were performed with a MFP-3 Hitachi-Perkin-Elmer spectrophotometer in its ratio mode, with 10-mm cells. The emission data reported here are derived from recorder tracings of complete spectra (approximately 300-560 nm) and are uncorrected for the variation with wavelength in the sensitivity of the detection system. In all fluorescence measurements, the emission spectrum of a 1.9 mM solution of quinine sulfate in 0.05 M H$_2$SO$_4$ (excitation at 360 nm; corrected emission maximum at 460 nm) was used as a standard, and its fluorescence intensity at the emission maximum was arbitrarily set at 1.0 fluorescence unit (FU). At the excitation maxima (near 285 nm for papain and near 325 nm for the manysyl compounds), the absorbance of the solutions in the 10-mm cells was less than 0.1 for the concentrations used in the fluorescence measurements. All absorbance and fluorescence measurements were conducted at 25°C, and the cell compartment was maintained at this temperature by means of a thermostatically controlled water bath.

Measurements of the fluorescence polarization ($p$) and rotational relaxation time ($\phi$) were performed with an SLM Instruments Inc. (Urbana, IL model 4800 LPF subnanosecond spectrophotometer. The polarization was calculated by means of the equation $p = (I_1 - I_2)/(I_1 + I_2)$, in which $I_1$ is the observed intensity and the subscripts refer to the relative orientation of the polarizer and analyzer. The polarization of Rhodamine 6G (5.4 mM and 34 mM) in glycerol was measured at 18°C (excitation at 496 nm), and the values were in satisfactory accord with those reported by Weber (9). The lifetime of the excited state ($\tau$) was determined at 30 MHz and calculated by means of the equation $\tau = (2\pi f)/tan\phi$, in which $f$ is the frequency of the exciting light and $\phi$ is the phase shift between the incident light and the emitted light. From $\tau$ and $p$ values, the rotational relaxation time ($\phi$) was calculated by means of the equation $[(I_1/p) - (I_2/p)] = [(1/p_0) - (1/p)](1 + (3\tau)/\phi)$, in which $p_0$ (assumed to be 0.4) is the polarization in the absence of rotatory Brownian motion and $\tau$ is the lifetime of the excited state. For the unliganded manysyl compounds, $\tau = 2.7$ nsec, whereas for the complexes with papain it ranged from 3.0 to 5.3 nsec.

The rapid kinetic measurements were made at 25°C with a Durrum Instrument Corp. (Palo Alto, CA) stopped-flow spectrophotometer (model D110) equipped with a Tectronix R-5103N storage oscilloscope, and the photomultiplier was mounted at 90° to the xenon light beam. To monitor the changes in fluorescence of the manysyl group, the excitation wavelength was 325 nm, and a Corning 3-74 filter in front of the phototube excluded light below about 400 nm.

RESULTS AND DISCUSSION

Inhibition of Papain. Westerik and Wolfenden (2) showed that Ac-Phe-glycinal is a potent competitive inhibitor of papain, with a $K_i$ of 0.046 mM at pH 5.5. Because of the tight binding of the derivatives of Phe-glycinal to the enzyme, kinetic data on the inhibition of papain action by these compounds cannot be evaluated by means of equations based on Michaelis-Menten kinetics, and alternative procedures such as those of Henderson (10) must be used. In this manner, a $K_i$ value of 0.01 mM was estimated for Ac-Phe-glycinal at pH 6.5 and 25°C, with Ac-Phe-Gly-NA as the substrate (7). However, with inhibitors of the type Z-(Gly)$_n$-Phe-glycinal (n = 0, 1, 2), it was found (1) that the 1:1 binding in the interaction of papain with Ac-Phe-glycinal did not hold and that equilibria involving higher powers (at least 2) of the inhibitor concentration were involved. In these circumstances, no reliable values of $K_i$ could be estimated for the inhibitors bearing an amino-terminal benzyl oxy carbonyl group, and the inhibition data were presented in the form of plots of $v_1/v_0$ against $[I]_0$, in which $v_1$ is the observed initial rate in the presence of inhibitor and $v_0$ is the rate in the absence of inhibitor. Comparison of the inhibitor concentrations required to effect 50% inhibition indicated that the three compounds of the series Z-(Gly)$_n$-Phe-glycinal are approximately equally effective as inhibitors of the hydrolysis of Ac-Phe-Gly-NA, and that they are about twice as effective in this regard as is Ac-Phe-glycinal. Examination of the members of the series Mns-(Gly)$_n$-Phe-glycinal as inhibitors of the action of papain on 0.2 mM Ac-Phe-Gly-NA showed that the inhibitor concentration required to effect 50% inhibition at pH 6.5 and 25°C was the same (approx. 0.07 mM) for the three inhibitors tested and the same as the value previously found for the analogous benzyl oxy carbonyl compounds. It is clear, therefore, that the manysyl aldehydes are bound tightly at the active site of papain.

On the other hand, with Mns-glycinal as the inhibitor, the kinetic data could be handled satisfactorily by means of a Dixon plot of $1/v_0$ against $[I]_0$ and the results indicated that this compound acts as a competitive inhibitor with a $K_i$ of 93 mM. It is evident, therefore, that the much tighter binding of the derivatives of Phe-glycinal is a consequence of the presence of the phenylalanyl residue next to the glycinal unit, in keeping with earlier knowledge of the primary specificity of papain (11, 12) and the observations of Westerik and Wolfenden (2) on the relative inhibitory capacity of various aldehydes. It should be noted that the diethyl acetal of Mns-Phe-glycinal does not inhibit the action of papain on 0.25 mM Ac-Phe-Gly-NA, even when tested at the highest concentration (0.25 mM) permitted by the solubility of the acetal.

Absorption and Emission Spectra of Mansyl Aldehydes. Under the conditions of our studies, the absorption spectra of the compounds in the series Mns-(Gly)$_n$-Phe-glycinal (n = 0, 1, 2) exhibit their principal absorption maximum in the near ultraviolet at about 320 nm, with values of $\epsilon_{max}$ = 18-22 M$^{-1}$cm$^{-1}$; for Mns-glycinal, the maximum is at 312 nm. When excited near their absorption maxima, the fluorescence of all the manysyl compounds in aqueous solution was characterized by a broad weak emission band with a maximum in the
Evidence for the view that the fluorescence enhancement of the mansyl group of Mns-Phe-glycinal upon the addition of excess papain is associated with the binding of the inhibitor at the active site of the enzyme is provided by the effect of the addition of 45 \mu M Ac-Phe-glycinal. Although a slightly weaker inhibitor of papain action than Mns-Phe-glycinal, when present in excess it may be expected to partially displace the mansyl aldehyde from the active site and thus reduce the mansyl fluorescence. This was in fact observed, and the mansyl fluorescence was reduced approximately 50%.

Determinations of the polarization and rotational relaxation time of the mansyl fluorescence in the three compounds Mns-(Gly)\textsubscript{3}-Phe-glycinal (n = 0, 1, 2) gave values near \( p = 0.06 \) and \( \rho = 1.3 \) nsec under the conditions of these studies. When mixed with excess papain (10 \mu M), the mansyl group of Mns-Phe-glycinal (1 \mu M) exhibits fluorescence that is polarized to a considerable degree (\( p = 0.27 \)) and whose rotational relaxation time (\( \rho = 28.6 \) nsec) suggests that the mansyl group does not have appreciable rotation independent of that of the papain–ligand complex. On the other hand, with the complex of papain with Mns-Gly-Phe-glycinal or Mns-Gly-Gly-Phe-glycinal, under comparable conditions the polarization of the mansyl fluorescence is less (\( 0.19 \) or \( 0.10 \), respectively) and the rotational relaxation times (5.8 nsec and 2.7 nsec, respectively) approach that of the mansyl group in the unliganded compounds. These data are consistent with the conclusion that the mansyl group of the two longer compounds protrudes to a greater degree from the active site than in the case of the complex of papain with Mns-Phe-glycinal.

Since the emission maximum of papain fluorescence under the conditions of this work is near 330 nm (1), the absorption maximum of the mansyl group, it was reasonable to examine the possibility that long-range nonradiative transfer of electronic excitation energy from excited state tryptophanyl residues of papain to the mansyl group of a bound aldehyde might be observed. Such energy transfer should make itself evident in the emission spectra of the papain–ligand complex upon excitation near the maximum of tryptophan absorption (290 nm) through a quenching of the tryptophan emission near 340 nm and an increase in the mansyl emission near 445 nm as compared with fluorescence of the unliganded aldehyde.

It will be seen in Fig. 3 that the tryptophan fluorescence of a 0.5 \mu M papain solution is strongly quenched upon the addition of Mns-Phe-glycinal, Mns-Gly-Phe-glycinal, and Mns-Gly-Gly-Phe-glycinal. To calculate the extent of quenching, it was necessary to determine the emission spectrum for papain in the absence of mansyl compound but in the presence of excess Ac-Phe-glycinal (10 \mu M). This is to take account of the known effect of the Phe-glycinal unit in enhancing the intrinsic tryptophan fluorescence of papain (1), which, under the conditions of this experiment, amounts to a 3-fold increase in fluorescence intensity at 340 nm, from 0.06 FU to 0.18 FU. The maximum extent of quenching (80%) indicates effective energy transfer from papain tryptophan to the mansyl group of bound Mns-Phe-glycinal. This value was calculated by means of the equation \( T = 1 - (F_i/F_0) \), in which \( T \) is the efficiency of energy transfer, and \( F_i \) and \( F_0 \) are the fluorescence intensities in the presence and absence of the energy acceptor. Similar experiments with Mns-Gly-Phe-glycinal and Mns-Gly-Gly-Phe-glycinal as the ligands showed a smaller degree of quenching of tryptophan fluorescence. It is evident, therefore, that although the three mansyl derivatives of Phe-glycinal are all bound tightly at the active site of papain by virtue of their Phe-glycinal unit, the successive addition of glycyl residues between that unit and the mansyl group greatly decreases the efficiency of energy transfer. In Fig. 2 is shown the enhancement of mansyl fluo-

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**FIG. 1.** Fluorescence spectra of mansyl derivatives of phenylalanylglycinal (2.5 \mu M) in the presence of papain (5 \mu M). Curve A, Mns-Phe-glycinal; curve B, Mns-Gly-Phe-glycinal; curve C, Mns-Gly-Gly-Phe-glycinal; curve D, all three mansyl compounds in the absence of papain. Excitation at 325 nm. The relative fluorescence intensity is expressed in units of 100 FU.

**FIG. 2.** Enhancement of mansyl fluorescence of Mns-Phe-glycinal (2.5 \mu M) at 445 nm upon the addition of papain. Curve A, excitation at 325 nm; curve B, excitation at 290 nm.
rescence at 445 nm of Mns-Phe-glycinal (2.5 µM) in the presence of various concentrations of papain (0-10 µM) upon excitation at 290 nm. Although there is clear evidence of energy transfer from tryptophan to the malsynl group, further studies are needed to allow reliable calculations of the extent of such transfer, especially since it appears that more than one of the five tryptophans of papain participates in the process.

**Stopped-Flow Studies.** It was suggested previously (1, 5) that the interaction of papain with oligopeptide substrates is a two-step process, E + S ←→ ES ←→ ES* ←→ E + products, with a conformational change at the active site in the conversion of ES to ES* (first-order rate constant, k=10³ sec⁻¹). On the other hand, in the binding of the same substrates to mercaptopapain, or papain in which Cys-25 is in the form of a mixed disulfide with 2-mercaptoethanol, the ES → ES* conversion was found to be much slower (k=10 sec⁻¹). A similar value was found for the corresponding EI → EI* conversion in the interaction of papain with Ac-Phe-glycinal (1). In the present work, stopped-flow measurements of the rate of the enhancement of the fluorescence of Mns-Phe-glycinal (1 µM) upon the interaction with papain (5 µM) showed an initial rapid rise (within 10 msec) to a fluorescence intensity about 5% of that ultimately attained, followed by a very slow first-order (0.1 sec⁻¹) increase. This finding supports the conclusion drawn earlier that chemical substitution of Cys-25, in this case by interaction of the aldehyde group of the inhibitor with the sulfhydryl group, markedly reduces the conformational mobility of the active site region of the enzyme.

The absence of significant enhancement of the malsynl fluorescence upon the binding of Mns-Gly-Gly-Phe-glycinal to papain is of interest in relation to the marked fluorescence increase observed (5) with the substrate Mns-Gly-Val-Glu-Leu-Gly, where the Glu-Leu bond is the site of enzymic cleavage. The distances along the peptide backbone from the fluorescent probe group to the carbonyl group that interacts with Cys-25 of the active site are the same for the two malsynl compounds and yet the environments of the probe group in the enzyme-substrate or enzyme-inhibitor complex are clearly different. Taken together with the significant differences in the rates at which reactive enzyme-substrate complexes are generated as compared with those for the interaction of specific substrates or inhibitors with inactive papain blocked at Cys-25, it would appear that chemical substitution of the sulfhydryl group is accompanied by extensive changes in the conformation of the active site region of the enzyme.

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