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# 2-Chloromethyl-4-nitrophenyl (*N*-Carbobenzoxy)glycinate. A New Reagent Designed to Introduce an Environmentally Sensitive Conformational Probe near the Active Site of Papain†

John E. Mole‡ and H. Robert Horton*

ABSTRACT: A specific quasisubstrate, 2-chloromethyl-4-nitrophenyl (*N*-carbobenzoxy)glycinate (Z-Gly-ONB-Cl), was synthesized in order to introduce the environmentally sensitive 2-hydroxy-5-nitrobenzyl (HNB) conformational probe into the covalent structure of papain near its active site, through the technique of affinity labeling. Papain, which had been obtained in fully active form by affinity chromatography, exhibited similar activities toward Z-Gly-ONB-Cl and the substrate *p*-nitrophenyl (*N*-carbobenzoxy)glycinate. Identical catalytic coefficients of 6.6 $sec^{-1}$ were obtained for the papain-catalyzed hydrolysis of *p*-nitrophenyl (*N*-carbobenzoxy)glycinate and Z-Gly-ONB-Cl, but their Michaelis constants were 6.2 and 69.2 $\mu$M, respectively. Reaction of papain, at pH 5.0, with a 200-fold molar excess of Z-Gly-ONB-Cl resulted in the incorporation of 1 mol of HNB groups/mol of enzyme. Amino acid analysis and measurements of fluorescence emission in 6 M guanidine hydrochloride indicated modification of 1 tryptophan. Cysteine residue 25 was not alkylated during hydroxynitrobenzylation of the enzyme as evidenced by sulfhydryl group titrations. Spectral properties of the bound "reporter group" were examined in a study of the group's microenvironment. The spectral characteristics of the modified enzyme in solutions of varied pH showed that the hydroxynitrobenzyl group in activated HNB–papain was exposed to a polar medium readily accessible to water. Upon interaction of the enzyme with the competitive inhibitor, benzamidoacetonitrile, the HNB group appeared to be displaced to a slightly less polar environment; similar movement was observed upon inactivation of the HNB–enzyme by mercuric ions or by iodoacetate. Circular dichroic spectra revealed a slight dissymmetry in the environment of the "reporter group" which was somewhat increased upon enzyme inactivation. These findings are interpreted as evidence that the 2-hydroxy-5-nitrobenzyl probe is bound to a tryptophyl residue (or residues) situated in a largely aqueous environment in close spatial proximity to the cysteinyl-25 residue of the active site of papain. Changes in the HNB group's spectral properties reflect changes in the state of enzyme activation and in the binding of inhibitors to the enzyme in solution.

Apart from X-ray crystallography, the most direct evidence for the presence of an amino acid residue in the active site of an enzyme has been provided by the application of active-site-directed irreversible inhibitors. A milestone in the establishment of this methodology was the introduction of the site-specific reagent, diisopropyl phosphofluoridate, which was found to combine with the active seryl residues of certain hydrolytic enzymes such as cholinesterase and chymotrypsin (Wilson *et al.*, 1950; Schaffer *et al.*, 1954). Subsequently, reagents such as L-1-tosylamido-2-phenylethyl chloromethyl ketone and its lysyl side-chain analog were designed for the affinity labeling of other nucleophilic residues present in the active sites of various proteases (Schoellmann and Shaw, 1962; Whitaker and Perez-Villaseñor, 1968; Shaw, 1972). Prior to the establishment of a covalent bond, the enzyme and such an active-site-directed irreversible inhibitor must form an enzyme–inhibitor complex (Main, 1964). In order to facilitate such binding, the reagent, which may be either a pseudosubstrate or a quasisubstrate, is designed so as to appear structurally similar to synthetic substrates for the enzymes in question.

Since their introduction, 2-hydroxy-5-nitrobenzyl halides and their derivatives have found wide application in protein modification studies (Horton and Koshland, 1965, 1972).

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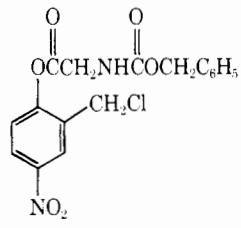
‡ Present address: Departments of Microbiology and Medicine, The University of Alabama in Birmingham, Medical Center, Birmingham, Ala. 35233.

They have generally been found to possess striking selectivity in modifying tryptophyl residues, although some alkylation of sulfhydryl groups has also been noted, particularly at elevated pH where cysteine exists as the sulfide anion. The *p*-nitrophenolic chromophore, which can thus be bound to an enzyme, may serve as an environmentally sensitive "reporter group" which provides spectral data useful in interpreting interactions with residues in its vicinity (Burr and Koshland, 1964; Kirtley and Koshland, 1970; Naik and Horton, 1973).

A class of reagents in which the hydroxyl group in the ortho position of a benzyl halide is chemically "masked" and then generated *in situ* to provide affinity labeling of a tryptophan near the active site of an enzyme was exemplified by the synthesis of 2-acetoxy-5-nitrobenzyl chloride as a noninhibitory environmentally sensitive reagent for chymotrypsin (Horton and Young, 1969).

Papain (EC 3.4.4.10) serves as an attractive model for introducing the environmentally sensitive 2-hydroxy-5-nitrobenzyl (HNB)[1] moiety into the active-site region in order to probe interactions of the substrate-binding site and catalytic site of a proteolytic enzyme in solution, since this enzyme contains several tryptophyl residues in the vicinity of the active site in its crystalline structure (Drenth *et al.*, 1968). In order to facilitate introduction of the HNB grouping into this region of the papain molecule, the carbobenzoxyglycyl ester of 2-hydroxy-5-nitrobenzyl chloride, 2-chloromethyl-4-nitrophenyl (*N*-carbobenzoxy)glycinate was synthesized. This reagent resembles the *p*-nitrophenyl derivative of carbobenzoxyglycine, Z-Gly-ONp, which has been shown to be an excellent substrate for papain (Kirsch and Igelström, 1966; Williams and Whitaker, 1967). Enzymatic hydrolysis of Z-Gly-ONB-Cl could be expected to release the highly reactive HNB-Cl, *in situ*, to provide selective alkylation of one or more tryptophyl residues present at the active site of papain.




## Experimental Procedure

*Enzyme.* Papain was obtained from Worthington Biochemicals and from P-L Laboratories as twice-crystallized preparations suspended in 0.05 M acetate buffer (pH 5.0). Each preparation was subjected to additional purification by affinity chromatography on a mercurial column containing covalently bound *p*-aminophenyl mercuric acetate (Sluyterman and Wijdenes, 1970; Mole and Horton, 1973a,b). A quantity of suspension containing 1 g of papain was diluted with an equal volume of a solution to provide final concentrations of 0.05 M acetate (pH 5.0), 10% dimethyl sulfoxide, 0.5% 1-butanol, 0.1 M KCl, 1 mM EDTA, and 0.3 M β-mercaptoethanol, with an enzyme concentration of 13–20 mg/ml. After 1-hr activation, the solution was cleared of precipitate by centrifugation, and the supernatant (*ca.* 60 ml) was applied to a column (4 cm × 44 cm) of Sephadex G-25 (Pharmacia Fine Chemicals) which had been equilibrated with the same buffer except with 10 mM $Na_2SO_3$ substituted for β-mercaptoethanol. The remainder of the purification procedure was as previously described (Sluyterman and Wijdenes, 1970). After dialysis the purified papain was concentrated on a PM-10 Diaflo membrane (Amicon Corp.) and stored at 5° in stock solutions of 25 mg/ml in 0.05 M acetate (pH 5.0), containing 0.5 mM $HgCl_2$.

*Substrate and Inhibitors.* *p*-Nitrophenyl (*N*-carbobenzoxy)-glycinate (lot 58B-1000) was purchased from Sigma Chemical Co. Benzamidoacetonitrile (Bz-$NHCH_2CN$) was synthesized according to the procedure of Klages and Haack (1903). Iodoacetate was obtained from Calbiochem and was recrystallized from ethanol–water prior to use. Mercuric chloride was from Baker Chemical Co.

*2-Chloromethyl-4-nitrophenyl (N-Carbobenzoxy)glycinate.* A quantity of 4.18 g of carbobenzoxyglycine was dissolved in 100 ml of anhydrous ethyl acetate, and the flask containing the solution was packed in ice to lower the temperature to 5°; 4.5 g of HNB-Cl (Buehler *et al.*, 1940) and then 4.13 g of dicyclohexylcarbodiimide (Mann) were added and the flask was stoppered and connected to a $CaCl_2$-drying tube. After an initial period of 30 min at 5°, the contents were brought to room temperature and the reaction was allowed to continue for a total of 2 hr. The precipitated dicyclohexylurea was removed by filtration with suction and the pale yellow solution was flash evaporated to a thick syrup which could be dried by repeated addition and evaporation of anhydrous ether. The crude product (9.3 g) was recrystallized from 25 ml of dry methanol; yield 3.8 g (42%), mp 89–90°. Higher yields could be readily obtained by further crystallizations of the mother liquor. *Anal.* Calcd for $C_{17}H_{15}N_2O_6Cl$: C, 53.91; H, 3.99; N, 7.40; Cl, 9.36. Found: C, 53.77; H, 3.97; N, 7.23; Cl, 9.50.

*Other Reagents.* L-Cysteine hydrochloride (lot 54299) and $NBS_2$ (lot 010022) were purchased from Calbiochem. Acetonitrile (reagent grade) was from Baker Chemical Co. *p*-Chloromercuribenzoate (control 6209) was from Nutritional Biochemicals; β-mercaptoethanol was from Matheson Coleman and Bell; EDTA and carbobenzoxyglycine (lot 17B-0320) were from Sigma Chemical Co. Guanidine hydrochloride (lot 4239-2) was from Pierce Chemicals; *p*-aminophenyl mercuric acetate (lot 082791) was from Aldrich. Reagent grade chemicals were used in the preparation of all buffers. Where ionic strength was maintained at 0.3 M, reagent grade KCl was used.

*Enzyme Modification.* Freshly activated papain was treated at pH 5.0 with a 200-fold molar ratio of Z-Gly-ONB-Cl, added slowly as a solution in anhydrous acetonitrile with continuous stirring; final concentrations: 0.23 M acetate, 2.2 mM EDTA, 2.16 mM cysteine, 21.7 μM mercuripapain, 4.3 mM Z-Gly-ONB-Cl, 12% (v/v) acetonitrile. (A preincubation period of 15 min was sufficient to fully activate the mercuripapain.) Progress of hydrolysis could be followed spectrophotometrically by appropriate dilution into 0.05 M Tris-acetate buffer (pH 7.5); under these conditions, $\Delta\epsilon$ for 2-hydroxy-5-nitrobenzyl alcohol (HNB-OH) at 405 nm is 14,950 $M^{-1}$ $cm^{-1}$. After reaction was complete, a 2-fold molar excess of $HgCl_2$ (over all sulfhydryl groups) was added to inactivate the HNB-papain; the precipitate was centrifuged, and hydroxynitrobenzylated mercuripapain was separated from excess HNB-OH by passage through a column (4 cm × 44 cm) of Sephadex G-25 equilibrated at pH 7.5 with 0.05 M Tris-

[1] Abbreviations used are: HNB, 2-hydroxy-5-nitrobenzyl; Z-Gly-ONB-Cl, 2-chloromethyl-4-nitrophenyl (*N*-Cbz)glycinate, *i.e.*, the carbobenzoxyglycyl ester of HNB-Cl; Z-Gly-ONp, *N*-Cbz-glycine *p*-nitrophenyl ester; Bz-$NHCH_2CN$, benzamidoacetonitrile; $NBS_2$, 5,5′-dithiobis(2-nitrobenzoic acid).

acetate. To achieve complete removal of HNB-OH, it was necessary to concentrate the solution to *ca.* 30 ml and dialyze it against three changes (2 l. each) of 0.01 M Tris-acetate (pH 5.0), containing 0.5 mM $HgCl_2$, for a period of 36 hr, and finally against two changes (2 l. each) of 0.01 M sodium acetate (pH 5.0), containing 0.5 mM $HgCl_2$, for 5 hr. The modified protein was then concentrated, centrifuged, and stored at 5° as stock solutions of approximately 5 mg/ml for use in subsequent studies.

*Kinetic Measurements.* A Cary Model 15 spectrophotometer with thermostated cell compartment (25°) was used to follow the hydrolysis of Z-Gly-ONp and Z-Gly-ONB-Cl at pH 5.11; assays were based on spectrophotometric measurements of the release of *p*-nitrophenol ($\Delta\epsilon_{320\ m\mu} = 8700\ M^{-1}\ cm^{-1}$) and HNB-OH ($\Delta\epsilon_{320\ m\mu} = 8200\ M^{-1}\ cm^{-1}$), respectively. Both assays contained 5.8 mM β-mercaptoethanol, 2.2 mM EDTA, 0.23 M acetate buffer, and 12% (v/v) acetonitrile; $[S]_0$ was varied from 0.02 to 0.10 mM, and $[E]_0$ from 0.02 to 0.11 μM.

Kinetic data were analyzed for $k_{cat}$ and $K_m$(app) values by computer fitting to the hyperbolic Michaelis–Menten equation according to a modification of the program of Cleland (1967).

*Ultraviolet–Visible Spectral Characterization.* The pH dependence of ultraviolet–visible absorption spectra of hydroxynitrobenzylated papain was determined using the Cary Model 15 spectrophotometer to record spectra in 0.1 M buffers, 5 mM in β-mercaptoethanol, 1 mM in EDTA, and 10.4 μM in enzyme. The p$K_a$ of the hydroxynitrobenzyl reporter group was evaluated from a plot of absorbance at 410 nm *vs.* pH.

Molar absorption coefficients for mercuri-HNB-papain and activated HNB-papain were determined from spectra obtained in 0.1 M Tris-acetate (pH 7.5) at enzyme concentrations of 20.0 μM; in the case of activated enzyme, the buffer included 5 mM β-mercaptoethanol and 1 mM EDTA. The Δε associated with enzyme activation was evaluated under the same conditions except at an enzyme concentration of 74.8 μM.

Difference spectra arising from the interaction of HNB-enzyme with various concentrations of the competitive inhibitor, benzamidoacetonitrile (Bz-$NHCH_2CN$) in 0.1 M Tris-acetate (pH 7.5) were recorded using matched quartz double-sector mixing cells of 1.0-cm nominal light path (0.4375 cm, each compartment; Hellma 239-QS). HNB-papain was placed on one side of each cuvette; and the inhibitor, in the same buffer, was placed on the other side. (In similar experiments, buffer alone was placed on the other side, or activator and buffer were placed on the other side, as indicated.) With one cell in the sample compartment and the other in the reference compartment of the spectrophotometer, a spectral base line was recorded. The contents of the sample cuvette were then mixed by inversion, and difference spectra were recorded. Appropriate corrections were made for enzyme dilution, and accuracy of pipetting and of the spectral measurements was confirmed by mixing the contents of the cuvette in the reference compartment to regenerate the spectral base line.

An attempt was made to estimate the dissociation constant, $K_I$, from the difference spectra generated by interaction of HNB-papain with benzamidoacetonitrile. If there were a single class of binding sites which could be represented by a single value for $K_I$, defined by eq 1

$$K_I = [\text{free sites on enzyme}][\text{free Bz-NHCH}_2\text{CN}]/[\text{Bz-NHCH}_2\text{CN-complexed sites}] \quad (1)$$

then $K_I$ could be evaluated using eq 2

$$K_I = (\Delta A_{max} - \Delta A)(M)/\Delta A \quad (2)$$

in which $\Delta A_{max}$ represents the maximum change in the hydroxynitrobenzyl chromophore's absorbance at 430 nm (empirically determined to be 0.041 for a concentration of HNB-papain of $6.82 \times 10^{-5}$ M); $\Delta A$ is the measured change in absorbance at 430 nm at each concentration of inhibitor employed; and (M) is the molar concentration of free Bz-$NHCH_2CN$ (calculated as [total Bz-$NHCH_2CN$][1 − fraction complexed]).

The differential absorption of HNB-papain induced by its interaction with iodoacetate was evaluated from difference spectra obtained using 5 mM β-mercaptoethanol, 1 mM EDTA, 2.5 mM iodoacetate, and 56.1 μM HNB-papain in the sample cuvette. The reference cuvette contained an identical mixture except that it lacked iodoacetate. The difference in molar absorption coefficient induced by iodoacetate was calculated per HNB residue.

*Circular Dichroism.* Circular dichroic spectra were recorded on a Cary Model 60 spectropolarimeter equipped with the Model 6001 CD unit. The scale setting and time constant employed for all spectra were 0.02° and 10, respectively, and quartz cells of 1.0-cm light path were used throughout. The concentration of HNB-papain was 3 mg/ml. Spectra of mercuri-HNB-papain were obtained using 0.1 M Tris-acetate buffer (pH 7.5); those of activated HNB-papain employed 0.1 M Tris-acetate (pH 7.5), containing 5 mM β-mercaptoethanol and 1 mM EDTA. Circular dichroic spectra were also obtained for HNB-papain in 0.1 M phosphate (pH 7.5), containing 5 mM β-mercaptoethanol and 1 mM EDTA in the absence and presence of 0.46 mM benzamidoacetonitrile.

Ellipticity, θ, was recorded directly in degrees, and the molecular ellipticity, [θ], was calculated according to eq 3,

$$[\theta] = \theta \times \text{time constant} \times M/10lc \quad (3)$$

where $M$ = gram-molecular weight of papain (23,350), $l$ = length of light path in cm, and $c$ = protein concentration in g/cm³.

The differential molecular ellipticity arising from the activation of mercuri-HNB-papain, Δ[θ], was calculated by subtracting [θ] of mercuri-HNB-papain from that of activated HNB-papain. Values of [θ] and Δ[θ] are expressed as (deg cm²)/dmol of HNB groups.

*Fluorescence.* Florescence measurements were made at room temperature using matched 1.0-cm cuvettes in an Aminco-Bowman spectrophotofluorometer equipped with an X–Y recorder. Emission spectra were recorded for mercuripapain, activated papain, mercuri-HNB-papain, and activated HNB-papain in 0.1 M Tris-acetate (pH 7.5), employing an excitation wavelength of 280 nm. The concentration of the native papain samples was 5.0 μM and that of the HNB-papain was 4.6 μM. The activated preparations contained, in addition to enzyme and Tris-acetate, 5 mM β-mercaptoethanol and 1 mM EDTA. Emission spectra were normalized with respect to protein concentration and related to that of activated, native papain (recorded as "100%").

Emission spectra of denatured papain and denatured HNB-papain in 6 M guanidine hydrochloride containing 0.1 M β-mercaptoethanol were also obtained, after incubating each enzyme preparation 8 hr at 25°. These spectra were also normalized with respect to protein concentration, and that

of denatured, native enzyme was recorded as "100%" relative fluorescence. The native papain was assumed to contain 5.0 mol of tryptophyl residues per mol (Drenth *et al.*, 1971).

*Analytical Methods.* The concentrations of solutions containing native papain were determined from their absorbance at 280 nm employing an absorption coefficient of 25.0 for a 1.0% solution (Glazer and Smith, 1965), a value which had been verified through amino acid analyses (Mole and Horton, 1973a,b). The concentrations of HNB-papain solutions were determined by diluting samples with strong base (pH >12) and measuring absorbance of the protein at 290 nm. A value of 81,300 $M^{-1}$ $cm^{-1}$ for $\epsilon_{290}$ was determined for HNB-papain based on results of spectral measurements and amino acid analyses. The HNB content of such preparations was also determined from their spectra in alkali, employing $\epsilon_{410}$ = 18,450 $M^{-1}$ $cm^{-1}$ for the hydroxynitrobenzyl chromophore (Horton and Koshland, 1972).

Amino acid analyses were performed on samples hydrolyzed at 110° for 20 hr in evacuated, sealed Pyrex tubes using 6 N HCl containing 4% thioglycolic acid (Matsubara and Sasaki, 1969), using a Beckman Model 120C amino acid analyzer.

Sulfhydryl group titrations were conducted using slight modifications of the procedures of Ellman (1959) and Boyer (1954). The mercurial derivatives of papain and HNB-papain (approximately 4 mg in 2 ml of 0.05 M sodium acetate, pH 5.0) were each adjusted to 0.35 M in β-mercaptoethanol and 5 mM EDTA, and their pH was raised to 8.2 with one drop of saturated Tris. Activation was allowed to proceed for 30 min, whereupon excess mercaptoethanol was removed from the proteins by gel filtration using a 1 × 15 cm column of G-25 Sephadex which had been equilibrated with 1.0 mM HCl. The thiol contents of aliquots of each protein were determined by titrations with $NBS_2$ and with *p*-chloromercuribenzoate from measurements of absorbance at 412 and 255 nm, respectively.

## Results

*Reaction of Papain with Z-Gly-ONB-Cl.* Rates were recorded for the papain-catalyzed hydrolysis of the ester substrate, Z-Gly-ONp, and of the quasisubstrate, Z-Gly-ONB-Cl, which contains the tryptophan-modifying reagent, HNB-Cl, as the alcohol moiety. At pH 5.0, in the absence of papain, no spontaneous hydrolysis of either ester could be detected; and even in the presence of 5.8 mM β-mercaptoethanol, hydrolysis was negligible.

When treated at pH 5.0 with a 200-fold molar excess of Z-Gly-ONB-Cl, fully activated papain (1.0 mol of SH groups/mol) incorporated 1 mol of HNB residues/mol. By contrast, commercial preparations of papain containing 0.3–0.5 mol of SH groups/mol incorporated only 0.3–0.5 mol of HNB residues/mol. Moreover, purified mercuripapain which was not activated (treated identically except that cysteine was omitted from reaction medium) failed to incorporate HNB groups, even during prolonged incubation (3 hr) with Z-Gly-ONB-Cl. Thus, enzymatic cleavage of Z-Gly-ONB-Cl to release HNB-Cl *in situ* was found to be prerequisite to hydroxynitrobenzylation of papain by this reagent, and it is necessary that only fully active papain preparations be used in order to achieve uniform labeling.

Results of a kinetic comparison of the action of papain on Z-Gly-ONp and on Z-Gly-ONB-Cl are presented in Table I. Identical values for $k_{cat}$ provide evidence for the validity of the familiar three-step hydrolytic mechanism for ester hydrolysis which involves an acyl-enzyme intermediate. Thus, $k_{cat}$ may be presumed to reflect the rate constant for deacylation of the common carbobenzoxyglycylpapain intermediate generated in both instances. By contrast, there is a 10-fold difference in the values of the apparent Michaelis constants, seemingly reflecting the effect of the bulky chloromethyl side chain of Z-Gly-ONB-Cl on binding and/or acylation steps of its hydrolysis by papain.

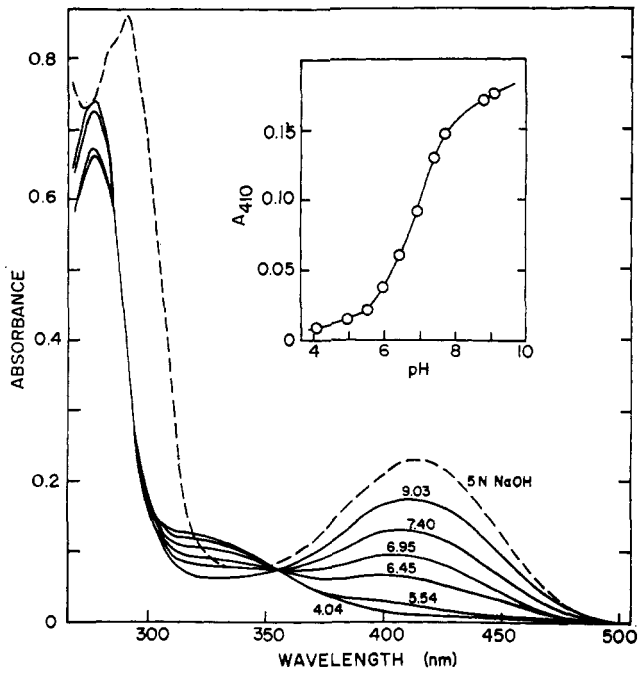



FIGURE 1: The pH dependence of HNB-papain's absorption spectra. Enzyme concentration = 10.4 μM; other conditions given in text. *Inset*: spectrophotometric evaluations of $pK_a$.

Knowledge of the extreme reactivity of cysteinyl residue 25 of papain towards a variety of alkylating agents including hydroxynitrobenzyl reagents (Whitaker and Perez-Villaseñor, 1968; Sluyterman, 1968; Morihara and Nagami, 1969; Furlanetto and Kaiser, 1970; Wallenfels and Eisele, 1968) necessitated verification that this sulfhydryl group was not the site of HNB group incorporation by Z-Gly-ONB-Cl. $NBS_2$ and *p*-chloromercuribenzoate titrations of papain and of HNB-papain revealed the presence of 1.0 mol of sulfhydryl groups/mol of enzyme, both before and after its hydroxynitrobenzylation by reaction with Z-Gly-ONB-Cl. Moreover, amino acid analysis (11 analyses on three separate HNB-papain preparations) revealed that the only amino acid modified by such HNB group incorporation was 1 mol of tryptophyl residues/mole of enzyme.

*Spectral Characteristics of HNB-Papain.* Absorption spectra of activated HNB-papain as a function of pH are recorded in Figure 1. The bound hydroxynitrobenzyl group exhibits a spectral sensitivity to pH which resembles that of

TABLE I: Kinetics of Papain-Catalyzed Hydrolysis of Z-Gly-ONp and Z-Gly-ONB-Cl at pH 5.11.[a]

| Substrate | $K_m$ (app) (× $10^6$ M) | $k_{cat}$ ($sec^{-1}$) | Δε ($M^{-1}$ $cm^{-1}$) |
|---|---|---|---|
| Z-Gly-ONp | 6.2 ± 1.5 | 6.5 ± 0.3 | 8700 |
| Z-Gly-ONB-Cl | 69.2 ± 5.7 | 6.7 ± 0.3 | 8200 |

[a] For conditions of assay, see text.

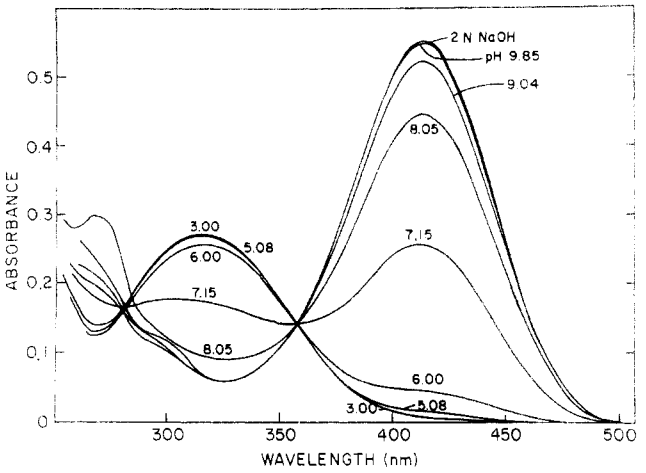



FIGURE 2: Absorption spectra of hydroxynitrobenzylated *N*-acetyl-L-tryptophan ethyl ester at various pH values as indicated.

model compounds such as 2-hydroxy-5-nitrobenzyl alcohol (Naik and Horton, 1973) or the noncyclic adduct of *N*-acetyl-L-tryptophan ethyl ester (Figure 2). The spectra of HNB-papain are characterized by a $pK_a$ of 6.9 and an isosbestic point at 354 nm (Figure 1). Thus, it appears that the papain-bound hydroxynitrobenzyl chromophore resides in a polar environment which is readily accessible to water throughout the range of pH 4–9.

Absorption spectra of mercuri-HNB-papain and activated HNB-papain were compared at pH 7.5. A 9.2% decrease in the molar absorptivity and a shift in the $\lambda_{max}$ of the *p*-nitrophenoxide anion, from 409 to 413 nm, was found to accompany the activation of the mercuri-enzyme. Thus the activation process, which chelates mercuric ions and releases the sulfhydryl group of HNB-papain, appears to expose the hydroxynitrobenzyl probe to a medium of somewhat greater polarity.

Examination of the spectral sensitivity of HNB-papain to the presence of the reversible inhibitor, Bz-$NHCH_2CN$, led to the difference spectra recorded in Figure 3. The interaction of the labeled enzyme with Bz-$NHCH_2CN$ is not simple, as can be seen by the complexity of the difference spectra. A pro-

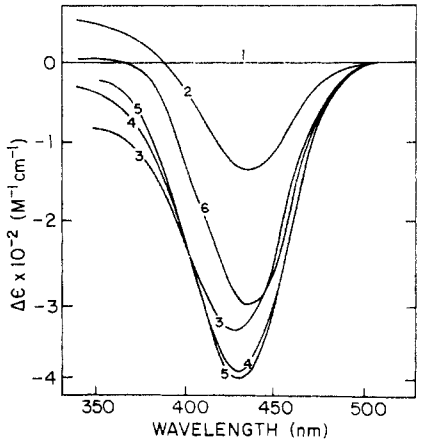



FIGURE 3: Differential molar absorptivities arising from interaction of activated HNB-papain ($6.8 \times 10^{-5}$ M) at pH 7.5 with various concentrations of the competitive inhibitor, Bz-$NHCH_2CN$. Curve 1, no inhibitor, is recorded as zero, and other curves are recorded relative to this value. Curve 2, 0.1 mM Bz-$NHCH_2CN$; curve 3, 0.2 mM; curve 4, 0.5 mM; curve 5, 0.83 mM; curve 6, 1.0 mM.

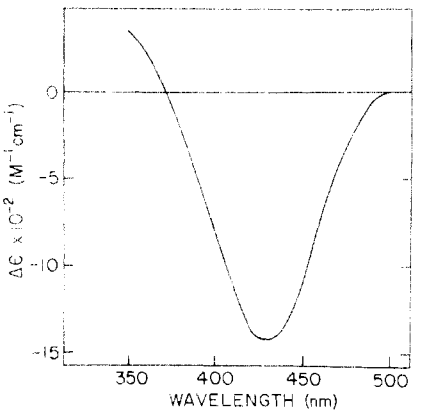



FIGURE 4: Iodoacetate-induced change in molar absorptivity of HNB-papain (56.1 μM) at pH 7.5, as a function of wavelength.

gressive decrease in the HNB group's absorbance at *ca.* 440 nm is observed with increasing inhibitor concentrations up to 0.83 mM, but still higher levels of the inhibitor result in a partial reversal of this phenomenon (Figure 3). Evaluation of the difference spectra according to eq 2 (Experimental Procedure) provides apparent $K_I$ values of 0.20, 0.05, 0.07, and 0.12 mM at total Bz-$NHCH_2CN$ concentrations of 0.10, 0.20, 0.50, and 0.83 mM, respectively, and an apparent $K_I$ of 0.50 mM at 1.0 mM Bz-$NHCH_2CN$. Clearly, the assumption of a single class of binding sites for this inhibitor with respect to its interaction with the HNB reporter group is not warranted. A separate kinetic analysis of the inhibition of HNB-papain-catalyzed hydrolysis of *N*-benzoyl-L-arginine-*p*-nitroanilide by Bz-$NHCH_2CN$ provided a $K_I$ of 0.18 mM. A value of 0.4 mM had been previously reported for the inhibition of papain-catalyzed hydrolysis of methyl hippurate by Bz-$NHCH_2CN$ (Lucas and Williams, 1969).

Inactivation of HNB-papain by treatment with iodoacetate at pH 7.5 resulted in a decrease in absorbance at 430 nm (Figure 4), similar to that exhibited during interaction of HNB-papain with the reversible inhibitor, Bz-$NHCH_2CN$.

Fluorescence emission spectra of activated papain, mercuri-papain, activated HNB-papain, and mercuri-HNB-papain at pH 7.5 are presented in Figure 5a. Hydroxynitrobenzylation led to 38% quenching of the fluorescence of papain excited at 280 nm (compare curve 3, Figure 5a, with curve 1), but no shift in $\lambda_{max}$ (341 nm). Complexation of either native or HNB-papain with mercuric ions led to quenching of fluorescence and a shift in $\lambda_{max}$ to 336 nm.

Figure 5b shows the fluorescence emission spectra of papain and HNB-papain reductively denatured in 6 M guanidine hydrochloride containing 0.1 M β-mercaptoethanol. Under these conditions, the relative fluorescence intensity of HNB-papain was 83% of that of native papain. Since HNB-tryptophyl residues have been shown to exhibit only 2% of the fluoresence intensity of unmodified tryptophyl residues in model compounds (Naik and Horton, 1973), the fluorescence of HNB-papain under denaturing conditions corresponds to that of 4.1 out of 5.0 tryptophyl residues. Thus, the fluorescent properties of the active and denatured HNB-enzyme support the results of amino acid analysis and provide evidence consistent with the modification of a single tryptophan by hydroxynitrobenzylation of papain using Z-Gly-ONB-Cl.

The hydroxynitrobenzyl chromophore of HNB-papain exhibits circular dichroism which is dependent, in part, on the state of activation of the enzyme. Figure 6 shows the effects of activation of mercuri-HNB-papain, interaction of

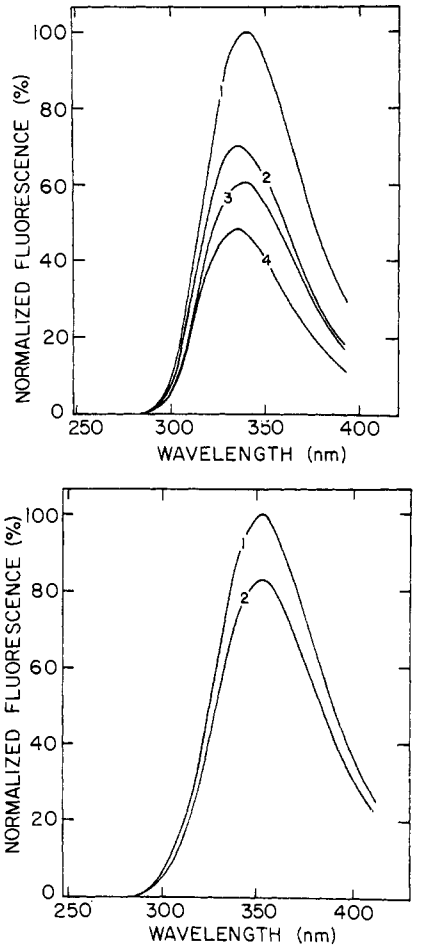



FIGURE 5: (a, top) Effects of hydroxynitrobenzylation on fluorescence emission of activated papain and mercuripapain at pH 7.5. Curve 1, activated papain, curve 2, mercuripapain; curve 3, activated HNB-papain; curve 4, mercuri-HNB-papain; excitation wavelength, 280 nm. (b, bottom) Fluorescence emission spectra of papain (curve 1) and HNB-papain (curve 2) denatured in 6 M guanidine hydrochloride containing 0.1 M $\beta$-mercaptoethanol; excitation wavelength, 280 nm.

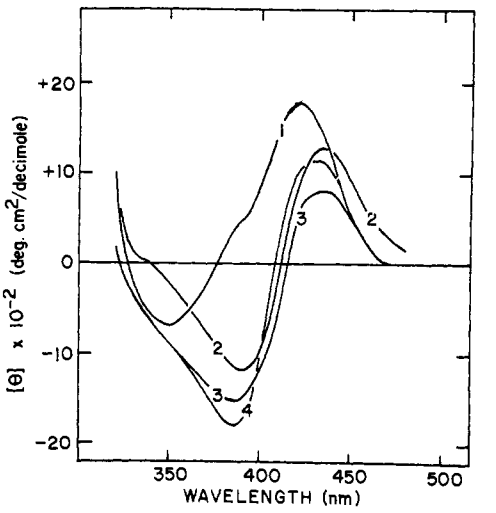



FIGURE 6: Molecular ellipticity of HNB-papain as a function of wavelength. Curve 1, mercuri-HNB-papain in Tris-acetate, pH 7.5; curve 2, activated HNB-papain in Tris-acetate, pH 7.5; curve 3, activated HNB-papain in phosphate, pH 7.5; curve 4, activated HNB-papain in phosphate, pH 7.5, 0.46 mM in Bz-NHCH$_2$CN.

HNB-papain with Bz-NHCH$_2$CN, and the nature of the buffer at pH 7.5. Activated HNB-papain in Tris-acetate and phosphate buffers, in the absence and presence of Bz-NHCH$_2$CN, exhibits negative ellipticity in the region of 385–390 nm and positive ellipticity at 430–435 nm. In contrast, mercuri-HNB-papain is characterized by a negative band at 350 nm and a positive ellipticity at 420 nm, with a shoulder occurring in the region of 390–400 nm. Although the molecular ellipticities are relatively small in magnitude, ranging from +800 to +1800 and −700 to −1800 (deg cm$^2$)/dmol of HNB residues, such dissymmetry in the environment of the 2-hydroxy-5-nitrobenzyl probe is a consequence of its attachment to the papain molecule, as evidenced by the lack of detectable circular dichroism of HNB-L-tryptophan ethyl ester in the 350–500-nm region under the same conditions of measurement.

## Discussion

Previous attempts to introduce a nitrophenolic "reporter" group into the structure of papain through reaction with 2-hydroxy-5-nitrobenzyl bromide (Morihara and Nagami, 1969) or $\alpha$-bromo-4-hydroxy-3-nitroacetophenone (Furlanetto and Kaiser, 1970) have resulted in irreversible inactivation of the enzyme. Such inactivation may be attributed to the susceptibility of cysteinyl residue 25, in the active site of papain, toward alkylation by a variety of reagents, as exemplified by its reaction with chloroacetate at pH 6, which proceeds at 30,000 times the rate of the reaction of free cysteine with the reagent (Sluyterman, 1968). Accordingly, in order to provide a means for investigating the participation of tryptophan in the activity of papain and to permit the incorporation of an environmentally sensitive reporter group whose spectral responses could relate to interactions of a functionally active enzyme, it was necessary to design a reagent which would specifically hydroxynitrobenzylate tryptophan without attacking such a strongly nucleophilic cysteinyl residue. Earlier findings regarding the role of the *o*-hydroxyl substituent in 2-hydroxy-5-nitrobenzylating reagents (Horton *et al.*, 1965; Horton and Young, 1969), together with the known effectiveness of the *p*-nitrophenyl ester of carbobenzoxyglycine as a substrate for papain (Kirsch and Igelström, 1966) led to the design of Z-Gly-ONB-Cl as a potentially suitable reagent for selectively hydroxynitrobenzylating papain at a tryptophan residue in the active-site region.

Papain possesses several characteristics which are worth noting in considering its reaction with Z-Gly-ONB-Cl. First, the pH optimum of its catalytic activity is slightly acidic; hence, conditions for maximal reactivity of the enzyme are those in which nonspecific hydroxide ion-catalyzed hydrolysis of the ester bond of Z-Gly-ONB-Cl should be minimal. Second, papain contains no methionine, an amino acid which can react with active benzyl halides to modify their reaction with tryptophan (Horton and Koshland, 1965). Third, X-ray crystallographic analysis has revealed the presence of several tryptophyl residues in the vicinity of papain's active site (Drenth *et al.*, 1968), one or more of which could be susceptible to alkylation by HNB-Cl generated at the active site. Finally, papain contains only one cysteinyl residue capable of reacting, residue 25; and if papain-catalyzed esterolysis does, in fact, proceed by the generally accepted double displacement mechanism, then it could be protected from alkylation by the HNB-Cl generated *in situ* by virtue of its transient acylation by the carbobenzoxyglycyl moiety. Such protection would, of course, depend on the relative rates of enzyme deacylation

and HNB-Cl hydrolysis and/or reaction with a nearby tryptophan.

The results affirm the predicted value of Z-Gly-ONB-Cl as a selective hydroxynitrobenzylating reagent and provide direct support for papain's double displacement mechanism of ester hydrolysis involving an acylated enzyme intermediate. Z-Gly-ONB-Cl is virtually nonreactive in aqueous solutions at pH 5, in contrast to the highly reactive HNB-Cl. However, upon addition of papain, there is a steady rate of hydrolysis of the ester bond. Comparison of the kinetics of hydrolysis of Z-Gly-ONB-Cl by papain with those of Z-Gly-ONp (Table I) revealed identical values for $k_{cat}$ (6.6 $sec^{-1}$), although $K_m$ values differed by an order of magnitude (69.2 and 6.2 $\mu$M, respectively). Thus, $k_{cat}$ appears to be governed by the rate of deacylation of the carbobenzoxyglycyl-papain thio ester intermediate which would be generated by both substrates in a double displacement mechanism. Moreover, the complete lack of detectable alkylation of cysteine-25, as evidenced by both $NBS_2$ and *p*-chloromercuribenzoate titrations, reflects its apparent protection from reaction with the short-lived HNB-Cl generated *in situ* from Z-Gly-ONB-Cl by the enzyme's transient acylation with the carbobenzoxyglycyl moiety.

In our initial attempts, a consistently low level of HNB group incorporation into papain was obtained upon incubation of commercial preparations of the enzyme with Z-Gly-ONB-Cl at pH 5 (on the order of 0.3–0.5 mol/mol). Since enzymatic cleavage to release HNB-Cl at the active site is prerequisite to hydroxynitrobenzylation of the protein, and since approximately one-half of the protein in commercial preparations of papain is inactive (Sluyterman and Wijdenes, 1970), affinity chromatography was utilized to obtain preparations which were fully active. Thus purified, papain incorporated the HNB moiety of Z-Gly-ONB-Cl to a greater extent, resulting in a modified enzyme which contained 1 mol/mol, as anticipated on the basis of the reagent's design and reaction kinetics.

Incorporation of the HNB group into papain was accompanied by the modification of 1 mol of tryptophyl residues/mol as evidenced by amino acid analysis and by measurements of fluorescence emission under denaturing conditions. Spectral properties of the hydroxynitrobenzylated enzyme provide information concerning the nature of the environment of the reporter group. The similarity between the spectral sensitivity of HNB-papain to pH (Figure 1) and that of the model compounds, HNB alcohol (Naik and Horton, 1973) and hydroxynitrobenzylated *N*-acetyl-L-tryptophan ethyl ester (Figure 2), suggests that the reporter group is bound to a tryptophyl residue in a noncyclic fashion such that it is exposed to a polar medium ($\lambda_{max}$ 413 nm) readily accessible to water ($pK_a$ = 6.9). The simplicity of the family of spectra generated by variation in pH from 4 to 9, characterized by an isosbestic point at 354 nm, is in marked contrast to the complexity observed in spectra of HNB-carboxypeptidase A (Naik and Horton, 1973), in which the reporter group appears to be situated in a generally hydrophobic environment (characterized by $\lambda_{max}$ 388 nm over the pH range of enzymatic activity and a $pK_a$ = 7.1). Circular dichroic spectra (Figure 6) reveal the existence of some degree of dissymmetry in the molecular environment of the reporter group in HNB-papain. The molecular ellipticity values per HNB residue are only of the order of one-fifth the magnitude of those exhibited by HNB-carboxypeptidase A, but they are attributable to the spatial relationships of the papain molecule as evidenced by the absence of such extrema in the 350–500-nm region in the circular dichroic spectra of the isolated diastereomer (Tucker *et al.*, 1971) of HNB-L-tryptophan ethyl ester. Thus the observed rotation in the visible region does not arise from spatial interaction of the HNB ring and the indole moiety of tryptophan *per se*. Moreover, no circular dichroism in the 350–500-nm region was exhibited by a 1:1 mixture of native papain and HNB alcohol. These data indicate that attachment of the HNB probe to papain is such that, although its ionization is influenced by an aqueous medium, it is not completely exposed for random interactions with solvent.

Both the ultraviolet-visible absorption spectra and the circular dichroism of the reporter group are sensitive to changes accompanying activation of mercuri-HNB-papain and those accompanying inhibition of the enzyme by Bz-$NHCH_2CN$ or inactivation of the enzyme by iodoacetate. In mercuri-HNB-papain, the reporter group appears to reside in a somewhat less polar environment at pH 7.5 ($\lambda_{max}$ 409 nm) than in the activated enzyme. Difference spectra generated at pH 7.5 by interaction of the HNB-enzyme with the reversible inhibitor, Bz-$NHCH_2CN$, and with the irreversible inactivator, iodoacetate, reveal a sizeable decrease in the HNB group's absorbance at 430 nm. Accompanying the shift observed in the absorption spectra upon activation of mercuri-HNB-papain is a shift in $[\theta]_{max}$ toward longer wavelengths (Figure 6). The observed spectral sensitivity of the reporter group to mercaptide formation, inhibitor binding, and carboxymethylation of the active site of HNB-papain lends support to the postulation that hydroxynitrobenzylation of papain by Z-Gly-ONB-Cl would occur at a tryptophyl residue which is proximal to cysteinyl residue 25 in the solution conformation of the enzyme.

Fluorescence measurements at pH 7.5 indicate that there are at least two residues which are quenched upon interaction of native papain with mercuric ions, and not one as had been suggested by Steiner (1971). Although quenching was observed upon hydroxynitrobenzylation of papain, further quenching of HNB-papain resulted from its interaction with mercuric ions (Figure 5a). It is interesting that Steiner had concluded that the dominant tryptophan, which was accessible to oxidation by *N*-bromosuccinimide, was situated in a highly polar environment (Steiner, 1971). The fluorescence data obtained in the present studies indicate that the tryptophyl residue which was hydroxynitrobenzylated by papain's reaction with Z-Gly-ONB-Cl may have been the dominant tryptophan (evidenced by 40% reduction in fluorescence), while the absorption spectral data indicate the bound HNB reporter group is located in a highly polar, largely aqueous environment. However, location of the position(s) of the HNB group in the primary sequence of HNB-papain will be necessary to directly distinguish between a single modified tryptophan residue and the partial modification of two or more tryptophan residues in papain.

The effect of hydroxynitrobenzylation on the catalytic activity of papain is considered in the following article (Mole and Horton, 1973b).

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# A Kinetic Analysis of the Enhanced Catalytic Efficiency of Papain Modified by 2-Hydroxy-5-nitrobenzylation†

John E. Mole‡ and H. Robert Horton*

ABSTRACT: The incorporation of a 2-hydroxy-5-nitrobenzyl (HNB) group into the covalent structure of papain, through reaction of the enzyme with 2-chloromethyl-4-nitrophenyl (*N*-carbobenzoxy)glycinate, resulted in altered kinetics of hydrolysis of benzoyl-L-arginyl substrates. Comparison of $k_{cat}/K_m$ ratios at pH 6.5 revealed that hydroxynitrobenzylation of papain led to a 24% increase in activity toward benzoyl-L-arginine ethyl ester (BzArgOEt) and a 240% increase in activity toward benzoyl-L-arginine *p*-nitroanilide (BzArgNan). These changes in activity reflected a decrease in the apparent Michaelis constants for both substrates, coupled with a 28% decrease and a 27% increase in $k_{cat}$ values for BzArgOEt and BzArgNan, respectively. Analysis of the pH-dependent steady-state kinetics of BzArgNan hydrolysis revealed that hydroxynitrobenzylation had resulted in a shift in $pK_a$ values of papain's functional groups from 4.3 and 8.2 to 4.6 and 7.7, respectively. The increased efficiency of BzArgNan hydrolysis could be attributed to a sevenfold increase in $k_2$(lim), the rate constant governing the enzyme "acylation" step. By contrast, no significant change in the rate constant governing "deacylation" resulted from hydroxynitrobenzylation of papain.

In their X-ray crystallographic analyses of papain (EC 3.4.4.10), Drenth *et al.* (1968, 1970, 1971) discovered the presence of several tryptophyl residues in the immediate vicinity of the active site of the enzyme. In particular, the indole side chain of Trp-177 is located at the surface of the enzyme molecule next to the imidazole ring of His-159 and at a distance of about 6 Å from the sulfur atom of Cys-25 in the crystalline state. Involvement of tryptophyl residues in the catalytic activity of papain has been implicated through a variety of chemical modifications, including proflavine-sensitized photooxidation (Jori and Galiazzo, 1971), oxidation with *N*-bromosuccinimide (Kirschenbaum, 1971), and reaction of papain with sodium bisulfite, tetranitromethane, and 2-hydroxy-5-nitrobenzyl bromide (Morihara and Nagami, 1969). In addition, the fluorescence of papain has been

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‡ Present address: Departments of Microbiology and Medicine, The University of Alabama in Birmingham, Medical Center, Birmingham, Ala. 35233.