ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS
Vol. 206, No. 2, February, pp. 362–371 1981

Effect of Hydroxynitrobenzylation of Tryptophan-177 on Reactivity of Active Site Cysteine-25 in Papain[1]

BETTIE L. B. EVANS,[2] JAMES A. KNOPP, AND H. ROBERT HORTON

Department of Biochemistry, North Carolina State University, Raleigh, North Carolina 27650

Received July 25, 1980

It is known that the enzymatic activity of papain (EC 3.4.22.2) toward α-*N*-benzoyl-L-arginine *p*-nitroanilide can be substantially increased by hydroxynitrobenzylation of Trp-177 through reaction of the enzyme with the active site-directed reagent, 2-chloromethyl-4-nitrophenyl (*N*-carbobenzoxy)glycinate (S.-M. T. Chang and H. R. Horton, 1979, *Biochemistry* 18, 1559–1563). To determine the effect of such hydroxynitrobenzylation on the nucleophilicity of the essential thiol group at the active site of the enzyme, rates of inactivation by S_N2 reactions of Cys-25 with chloroacetamide and chloroacetate and by Michael addition of Cys-25 to *N*-ethylmaleimide were monitored. The kinetics revealed that, at pH 6.5, the reactivities of the sulfhydryl group of hydroxynitrobenzylated papain with chloroacetamide and with *N*-ethylmaleimide are 24 and 27% greater than those of the sulfhydryl group of native papain. At pH 7.1, the rate enhancements are 34 and 39%, respectively. These increases in reactivity of Cys-25 as an attacking nucleophile appear to account for the increased catalytic activity of hydroxnitrobenzyl-papain toward an oligopeptide substrate, α-*N*-benzoyl-L-phenylalanyl-L-valyl-L-arginine *p*-nitroanilide, and toward an ester substrate, *N*-carbobenzoxyglycine *p*-nitrophenyl ester. However, the presence of the hydroxynitrobenzyl reporter group provides substantially greater improvement (250%) in enzymatic efficiency toward α-*N*-benzoyl-L-arginine *p*-nitroanilide, apparently by blocking nonproductive binding of this substrate to the enzyme. Fluorescence changes accompanying the various chemical modifications are interpreted in terms of a charge–transfer interaction between the imidazolium ion of His-159 and the indole moiety of Trp-177 in the active form of native papain, which should help to stabilize the catalytically essential mercaptide-imidazolium ion-pair (Cys-25, His-159).

Selective chemical modifications of a protein's functional groups have provided considerable information relating structure to biological function, in many diverse systems. It is rare that chemical modification of an enzyme's active site region enhances catalytic activity, however.

Tryptophan has been implicated in the action of papain (EC 3.4.22.2) through photooxidation (1, 2), reaction with *N*-bromosuccinimide (3–5), and reaction with the active site-directed reagent, 2-chloromethyl-4-nitrophenyl (*N*-carbobenzoxy)-glycinate (Z-Gly-ONB-Cl)[3] (6, 7). The presence of several of papain's five tryptophyl residues within the vicinity of the enzyme's active site had been discovered by X-ray crystallography (8–10). The location of Trp-177 in the crystallographic structure is of particular interest, since it appears to form

[1] This investigation was supported in part by the National Science Foundation under Grant BMS 72-01999 A01. Any opinions, findings, and conclusions are those of the authors and do not necessarily reflect the views of the National Science Foundation. This is Paper No. 5956 of the Journal Series of the North Carolina Agricultural Research Service, Raleigh, N.C.

[2] Present address: Biology Department, Brookhaven National Laboratory, Upton, L. I., N. Y. 11973.

[3] Abbreviations used: Z-Gly-ONB-Cl, 2-chloromethyl-4-nitrophenyl (*N*-carbobenzoxy)glycinate; HNB-, 2-hydroxy-5-nitrobenzyl-; BzArgNan, α-*N*-benzoyl-L-arginine *p*-nitroanilide; BzPheValArgNan, α-*N*-benzoyl-L-phenylalanyl-L-valyl-L-arginine *p*-nitroanilide; Z-Gly-ONp, *N*-carbobenzoxyglycine *p*-nitrophenyl ester; NEM, *N*-ethylmaleimide; Tos-Phe-CH_2Cl, L-1-tosylamido-2-phenylethyl chloromethyl ketone; Tos-LysCH_2Cl, L-1-tosylamido-5-aminopentyl chloromethyl ketone.

0003-9861/81/020362-10$02.00

part of the S_1' subsite (5) and lies close to the hydrogen bond linking Asn-175 with His-159 (10).

The tryptophyl residue occupies a unique position in protein spectroscopy because it has a high absorption coefficient and a reasonable fluorescence quantum yield (11). As a consequence of energy transfer from Tyr to Trp in proteins containing tryptophan, observed fluorescence spectra are due almost exclusively to emission from the indole side chains of Trp residues. The monitoring of protein emission spectra is a useful technique for detecting changes, due to the sensitivity of tryptophyl emission to local environment and to the sizable contribution of energy transfer to the resultant fluorescence emission. Intramolecular interaction between an indole side chain and an imidazolium ion (protonated His side chain) results in marked quenching of the fluorescence intensity of Trp-containing peptides and proteins (12).

The fluorescence emission spectrum of papain is attributable to its Trp residues with little contribution from its 19 tyrosyl residues (13). Alkylation of the enzyme's single sulfhydryl group (Cys-25 at the active site) affects papain's intrinsic fluorescence: reaction with iodoacetate or iodoacetamide was found to enhance fluorescence intensity at 352 nm, whereas reaction with Tos-Phe-CH_2Cl or with Tos-LysCH_2Cl produced significant quenching (13). Steiner observed that the fluorescence of papain is dominated by a Trp residue which is susceptible to oxidation by *N*-bromosuccinimide (4); Lowe and Whitworth subsequently identified this residue as Trp-177 (5). Both studies revealed that the dominant Trp could be quenched by protonation of a His residue, identified as His-159 based on a study of 1,3-dibromoacetone-modified papain (5). Hydroxnitrobenzylation of papain was also observed to lead to quenching of the dominant Trp (6); the hydroxynitrobenzylated residue has subsequently been identified as Trp-177 (14).

Whereas photooxidation of Trp-177 (1) or its reaction with *N*-bromosuccinimide (5) results in marked loss of enzymatic activity, *in situ* hydroxynitrobenzylation of Trp-177 by reaction with Z-Gly-ONB-Cl leads to a substantial increase in catalytic activity (7). The magnitude of the increased enzymatic efficiency was observed to vary considerably, depending on the substrate chosen; thus, at pH 6.5, enzymatic activity toward *N*-benzoyl-L-arginine ethyl ester appeared to have increased 24%, whereas activity toward *N*-benzoyl-L-arginine *p*-nitroanilide (BzArgNan) was increased by 240%.

Kinetic analysis suggested that the increased efficiency of HNB-papain's action on BzArgNan resulted from enhancement of the enzyme's "acylation" rate constant (7), whereas *N*-bromosuccinimide-oxidation of Trp-177 was observed to result in a marked decrease in the "acylation" rate constant (5). Thus, it was of interest to evaluate the effect of hydroxynitrobenzylation of Trp-177 on the nucleophilicity of the essential thiol group of Cys-25, in an effort to determine the mechanism by which the HNB group enhances papain's catalytic activity toward a variety of substrates, and to further delineate the nature of interactions among Cys-25, His-159, and Trp-177 in native and HNB-papain.

EXPERIMENTAL PROCEDURES

Materials

Papain was obtained from P-L Biochemicals (Lot No. 0588-7) and from Worthington Biochemical Corporation (Lot No. 3DB) and was further purified by mercurial column chromatography (15). BzArgNan was purchased from Protein Research Foundation, Osaka, Japan, and from Bachem Fine Chemicals (Lot Nos. 4919 and 4967); BzPheValArgNan (S-2160) was obtained from AB Bofors, Nobel Division, Peptide Research, Mölndal, Sweden and from Vega Biochemicals. Z-Gly-ONp (Lot No. 84C-0107) and *N*-ethylmaleimide were purchased from Sigma Chemical Company; 2-chloroacetic acid and 2-chloroacetamide were products of Matheson, Coleman and Bell, and Aldrich Chemical Company, respectively. Z-Gly-ONB-Cl was synthesized as previously described (6). All other chemicals were of reagent grade.

Methods

Hydroxynitrobenzylation of papain. Freshly activated papain was treated with Z-Gly-ONB-Cl at pH 5.0 as previously described (6). Concentrations of native papain were estimated from absorbance at 278 nm (ϵ_{278} = 57,500 M^{-1} cm^{-1} at pH 7.0); those of HNB-papain from absorbance at 290 nm (ϵ_{290} = 81,300 M^{-1} cm^{-1} at pH > 12).

Kinetic measurements. Solutions of papain and HNB-papain were activated in 5.0 mM L-cysteine, 0.1 mM EDTA. A Beckman DB-G spectrophotometer with thermostatted cell compartment (26°C) was used to monitor the hydrolysis of BzArgNan, BzPheValArgNan, and Z-Gly-ONp, respectively, and also to determine the rate of loss of papain activity during chemical inactivation experiments (alkylation of Cys-25). The pH of each solution was verified using Radiometer Model 25-SE and Corning Model 10 pH meters.

Spectrophotometric assays for hydrolysis of BzArgNan involved the continuous monitoring of the *p*-nitroaniline released ($\Delta\epsilon_{410}$ = 8800 M^{-1} cm^{-1}), as previously described (15). Similar assays for hydrolysis of BzPheValArgNan employed a $\Delta\epsilon_{410}$ value of 9400 M^{-1} cm^{-1}. The solubility of the tripeptide nitroanilide was found to be quite low at high ionic strength; accordingly, solutions of 0.002 M sodium phosphate were used at each pH. BzPheValArgNan concentrations ranged from 0.03 to 0.3 mM; exact concentrations were determined from absorbance at 301 nm (ϵ_{301} = 14,600 M^{-1} cm^{-1}). Assays of Z-Gly-ONp hydrolysis were based on spectrophotometric measurements of the release of *p*-nitrophenol ($\Delta\epsilon_{320}$ = 8700 M^{-1} cm^{-1}).

Fluorescence and inactivation of papain. Fluorescence measurements (280 nm excitation, 365 nm emission) were made using 1.0-cm cuvettes in the thermostatted cell compartment of an Aminco-Bowman spectrophotofluorometer, equipped with a Beckman stripchart recorder. Papain or HNB-papain (at concentrations corresponding to an A_{278} of 0.1 to 0.2) was activated in 5.0 mM L-cysteine, 0.1 mM EDTA, In some instances (where warranted by the observed kinetics), after the enzyme was activated, it was freed from cysteine and EDTA by passage through a 2.5 × 43.5-cm column of Sephadex G-25 under N_2. To initiate inactivation of enzyme samples through alkylation of Cys-25, the specific inactivator (chloroacetamide, chloroacetate, or *N*-ethylmaleimide) in 0.105 M sodium phosphate at the designated pH was added to the enzyme solution. Rates of alkylation were determined by measuring either residual enzymatic activity of suitable aliquots after various intervals, or changes in fluorescence intensity of the protein (see Results). In the latter case, the fluorescence intensity extrapolated to zero time was taken as the initial emission intensity. The extent of alkylation was calculated from the fractional change in fluorescence intensity (between initial and final values) or from the fractional loss of enzymatic activity toward BzArgNan.

Kinetics of alkylation of small thiols. Rates of alkylation of L-cysteine and of glutathione with chloroacetamide and with chloroacetate were monitored using a Radiometer 25/TTT 11/SBR 2c/ABU 1b pH stat as described by Sluyterman (16). Rates of reaction with *N*-ethylmaleimide were determined from continuous spectrophotometric measurements at 305 nm (17).

RESULTS

Enzymatic Rate Enhancement Resulting from Hydroxynitrobenzylation

As previous results had revealed that the magnitude of the increase in enzymatic efficiency of papain resulting from hydroxynitrobenzylation of Trp-177 varied considerably depending on substrate, it was of interest to examine the relative activities toward anilide and ester bonds of other substrates. Kinetics of enzymatic hydrolysis of BzPheValArgNan and Z-Gly-ONp are compared with those of BzArgNan in Table I. The data confirm the earlier observation that the activity of HNB-papain (k_{cat}/K_m) in catalyzing hydrolysis of BzArgNan is 250% greater than that of native papain at pH 6.5; the rate enhancement increases to 280% at pH 7.1. By contrast, hydroxynitrobenzylation of papain provides 24% enhancement of catalytic activity toward the oligopeptide-anilide substrate, BzPheValArgNan, at pH 6.5, which increases to 39% at pH 7.15. Hydrolysis of the nitrophenyl ester substrate, Z-Gly-ONp, is similarly enhanced; thus, it is not the anilide bond per se which leads to the unique level of improved enzymatic efficiency observed with BzArgNan as substrate.

Sulfhydryl group alkylation reactions. To ascertain the relative nucleophilicities of the essential sulfhydryl groups (Cys-25) of HNB-papain and papain, the kinetics of inactivation by S_N2 reactions with chloroacetamide and chloroacetate, and by Michael addition with NEM were examined. It was found that, during reaction of native papain with chloroacetamide, chloroacetate, or NEM, the intrinsic fluorescence of the protein markedly increased, as can be seen in Fig. 1. The emission wavelength monitored in these studies, 365 nm, was chosen from determination of the maximum change in fluorescence intensity upon inactivation. The enhancement in fluorescence emission (ΔF_{365}^{280}) of papain during alkylation of its thiol group mirrors the loss of enzymatic activity (Fig. 1). Semilogarithmic plots of the kinetic data, such as those in Fig. 2, reveal that fluorescence change and loss of enzymatic activity are precisely equivalent and exhibit pseudo-first-order kinetics over

TABLE I

KINETICS OF NATIVE PAPAIN- AND HNB-PAPAIN-CATALYZED REACTIONS

Substrate	pH	K_m (mM)		V (μmol min^{-1} mg^{-1})		k_{cat}/K_m (HNB-papain) / k_{cat}/K_m (native papain)
		Native	HNB	Native	HNB	
BzArgNan	6.5	2.95	1.09	0.31	0.40	3.5
	7.1	3.72	1.27	0.30	0.39	3.8
BzPheValArgNan	6.5	0.139	0.101	31.3	28.2	1.24
	7.15	0.177	0.149	21.2	24.8	1.39
Z-Gly-ONp	6.5	0.075	0.056	11.0	10.0	1.22
	7.15	0.045	0.035	8.0	8.0	1.29

at least 95% of the reaction. Similar equivalence was observed for reactions of native papain with NEM and with chloroacetate; Fig. 3 shows the correlation during reaction of the enzyme with 1.0 mM chloroacetate at pH 6.5 and 26°C.

By contrast, changes in fluorescence of HNB-papain did not exhibit direct correlation with enzymatic inactivation upon alkylation with chloroacetamide, NEM, or chloroacetate (see below), apparently reflecting the participation of Trp-177 in the fluorescence response of the *native* enzyme. Accordingly, the kinetics of alkylation of native papain were monitored both by fluorescence (ΔF_{365}^{280}) and by enzymatic activity (BzArgNan hydrolysis) measurements, whereas the kinetics of alkylation of HNB-papain were determined solely by enzymatic activity measurements.

From semilogarithmic plots of kinetic data, second-order rate constants governing alkylation of the essential thiol group (Cys-25) of papain and that of HNB-papain were evaluated. They are presented, together with second-order rate constants for alkylation of the thiol groups of L-cysteine and glutathione, in Table II. It is evident that the reactivities of the thiol groups of papain and HNB-papain in the S_N2 reactions (chloroacetamide and chloroacetate) are orders of magnitude greater than the reactivities of the thiol groups of cysteine and glutathione, whereas the opposite order of reactivities is observed in the Michael addition reactions with NEM. As a result, the measured rates of reaction of the chloroalkylating species with the enzymes were found to be the same in the presence as in the absence of "activation solution" (5.0 mM L-cysteine, 0.1 mM EDTA). In contrast, measurements of the rates of NEM's reactions with papain and HNB-papain required the removal of free cysteine from the activated enzyme preparations (gel filtration under N_2 as described under Experimental Procedures).

Comparisons of rates of reactions of papain and HNB-papain revealed that, with neutral alkylating agents at pH 6.5, the hydroxynitrobenzylated enzyme reacted faster than the native enzyme. Thus, reac-

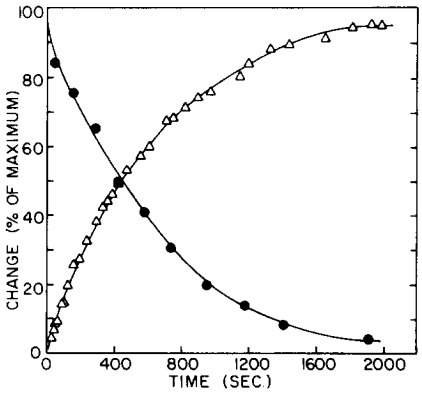


FIG. 1. Changes in enzymatic activity and fluorescence of papain during reaction with chloroacetate. (●) Activity toward BzArgNan; (△) fluorescence (F_{365}^{280}). Conditions: 0.105 M sodium phosphate, pH 6.5, 26°C, 1.0 mM chloroacetate.

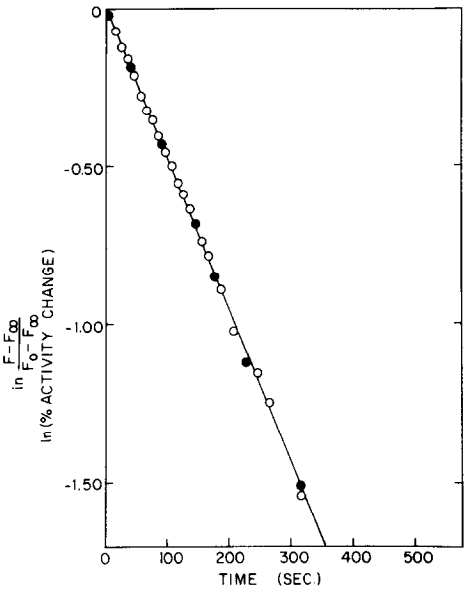


FIG. 2. Pseudo-first-order plot of alkylation of papain with chloroacetamide at 15.5°C. (●) Activity toward BzArgNan; (○) fluorescence (F^{280}_{365}). Conditions: 0.105 M sodium phosphate, pH 6.5, 90.9 mM chloroacetamide.

tivities with chloroacetamide and with NEM were 24 and 27% greater at pH 6.5 (Table II); at pH 7.1, the corresponding rate enhancements were 34 and 39%, respectively. The enhancement in nucleophilicity of the thiol group of papain as a result of hydroxynitrobenzylation of Trp-177 increases as pH is raised to 8.5, as evidenced from plots of second-order rate constants governing the reactions with chloroacetamide (Fig. 4). When the *difference* in measured rate constants ($k_{\text{HNB-papain}} - k_{\text{papain}}$) was plotted as a function of pH, a complex curve was obtained which revealed the influence of a functional group in the HNB-enzyme whose pK_a is 6.95. This functional group can be identified as the hydroxyl moiety of the HNB reporter group, itself, based on identity with the pK_a of 6.9 which was previously measured by spectrophotometric titration (ΔA_{410}) of HNB-papain (6). Thus, in its ionized (conjugate base) form, the *p*-nitrophenoxide substituent which is covalently bound to Trp-177 enhances the nucleophilic character of Cys-25.

The relative reactivities of papain and HNB-papain with the negatively charged alkylating agent, chloroacetate, contrasted with their relative reactivities with the neutral alkylating agents, chloroacetamide and NEM (Table II). The second-order rate constant governing carboxymethylation of HNB-papain at pH 6.5 and 26°C is only 56% of that of native papain. The rate constants for reaction of each enzyme with chloroacetate rise with increasing pH, but peak at pH 6.5, whereas the corresponding rate constants for reaction with chloroacetamide peak at pH 8.4 (Fig. 4). It is clear that the ionic charge of chloroacetate exerts an effect on measured alkylation rates which is superimposed on the effect of nucleophilic strength of the thiol group, per se, as evidenced by the reversal of reaction rates of cysteine and glutathione with this reagent (also seen in Table II).

Fluorescence Changes during Alkylation of Enzymes

When native papain was treated with chloroacetate, the intensity of its emission at 365 nm increased by a factor of 3 to 4, with a concomitant shift in wavelength of maximum emission from 343 to 347 nm. Similar, though not identical, changes were observed upon carboxymethylation of HNB-papain; intensity increases were smaller in magnitude, but the shift in maximum emission wavelength was more pronounced, from 338

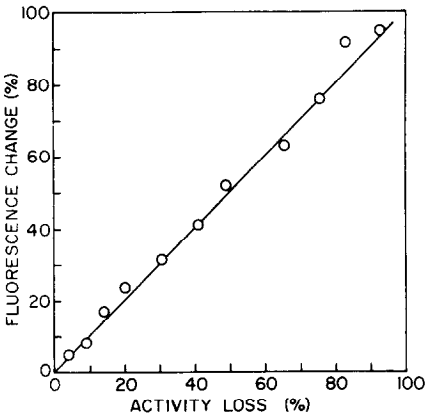


FIG. 3. Correlation of enhanced fluorescence emission with loss of enzymatic activity during reaction of papain with 1.0 mM chloroacetate. Activity measured toward BzArgNan; fluorescence emission measured at 365 nm (excitation wavelength, 280 nm). Conditions: 0.105 M sodium phosphate, pH 6.5, 26°C.

TABLE II

SECOND-ORDER RATE CONSTANTS GOVERNING ALKYLATION OF VARIOUS THIOLS WITH CHLOROACETAMIDE, CHLOROACETATE, AND *N*-ETHYLMALEIMIDE AT pH 6.5, 26°C

Reactants	k (M^{-1} s^{-1})
$Cl\text{-}CH_2CONH_2$:	
L-Cysteine	0.00412
Glutathione	0.00157
Papain	0.144
HNB-papain	0.179
$Cl\text{-}CH_2COO^-$:	
L-Cysteine	0.00057
glutathione	0.00108
Papain	2.75
HNB-papain	1.53
N-Ethylmaleimide:	
L-Cysteine	593
Glutathione	263
Papain	2.55
HNB-papain	3.24

to 347 nm. When NEM was used as the inactivator, even greater spectral shifts were observed for both enzymes. Table III presents a summary of fluorescence data. The calculated spectral bandwidths of the various protein-containing samples were equal to or less than that of the reference compound, *N*-acetyltryptophan ethyl ester,

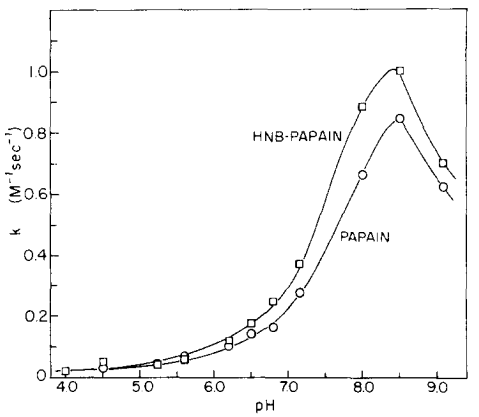


FIG. 4. Effect of pH on second-order rate constants governing inactivation of papain and HNB-papain by chloroacetamide at 26°C. Rate constants for native papain (○) were determined from both fluorescence (F_{365}^{280}) and enzymatic activity (BzArgNan) data; those for HNB-papain (□) were based solely on measurements of activity (BzArgNan).

with the exception of activated enzymes in the presence of dithiothreitol and EDTA.

The equivalence observed between fluorescence enhancement and loss of enzymatic activity during alkylation of native papain at pH 6.5 (Fig. 3) continued over a broad range of pH, as shown in Fig. 5, which presents data for reactions with chloroacetate. Similar equivalence was observed with the other inactivators. In contrast to these findings, no correlation existed between residual enzymatic activity and the increases in protein fluorescence when HNB-papain was treated with any of the alkylating agents. Such lack of correlation is exemplified by the apparent rate constants for carboxymethylation of HNB-papain, plotted as a function of pH in Fig. 6.

DISCUSSION

Reactivites of Cys-25

One of the most sensitive methods for delineating the effect of molecular environ-

TABLE III

FLUORESCENCE EMISSION OF PAPAIN AND HNB-PAPAIN UNDER VARIOUS CONDITIONS[a]

Sample	λ_{max} (nm)	Spectral bandwidth[b] (nm^{-1})
α-*N*-Acetyl-L-tryptophan ethyl ester	355	0.000502
Mercuripapain	336	0.000502
N-Ethylmaleimide-treated papain	353	0.000455
Chloroacetate-treated papain	347	0.000483
Papain + 1 mM dithiothreitol + 1 mM EDTA	343	0.000513
Mercuri-HNB-papain	335	0.000500
N-Ethylmaleimide-treated HNB-papain	350	0.000478
Chloroacetate-treated HNB-papain	347	0.000505
HNB-papain + 1 mM dithiothreitol + 1 mM EDTA	338	0.000511

[a] Uncorrected emission spectra of protein solutions ($A_{280} \leq 0.10$) were determined at 25°C; excitation at 280 nm. Spectra of inactivated enzymes were obtained after alkylation reactions were completed.

[b] Spectral bandwidths represent the difference in the reciprocal wavelengths determined at half-maximum intensity.

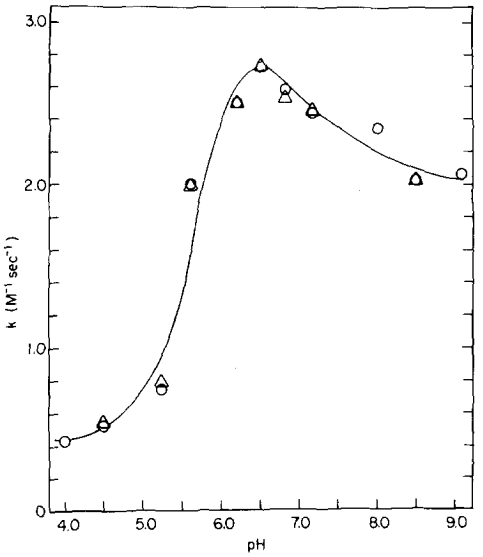


FIG. 5. pH-Dependence of second-order rate constants governing reactions of native papain with 1.0 mM chloroacetate. (△) Values obtained from measured rates of 365 nm fluorescence enhancement (excited at 280 nm); (○) values obtained from measured rates of enzyme inactivation (BzArgNan assays).

ment on a given functional group is to study its chemical reactivity. Sluyterman (16, 18) observed that the single thiol group of papain reacted far more rapidly with KCNO and with chloroacetate than did the thiol group of cysteine. At pH 6.0 and 25°C, he observed a second-order rate constant of 171 M^{-1} min^{-1} (i.e., 2.85 $M^{-1}s^{-1}$) for the reaction of papain with chloroacetate, a value comparable to that found in the present investigation (2.75 $M^{-1}s^{-1}$ at pH 6.5). It is also clear that Cys-25 of papain is more reactive than the simple thiol groups of cysteine and glutathione in other S_N2 reactions, as evidenced by reactivities with chloroacetamide (Table II), α-bromo-β-(5-imidazolyl)-propionate (19), and 2-bromoacetamido-4-nitrophenol (20). Interestingly, in Michael-type additions to maleimides, the reverse is true; that is, the Cys-25 of papain reacts at less than 1% of the rate of cysteine or glutathione (Table II). Reported rate constants governing the reaction of papain with NEM have varied from 0.661 M^{-1} s^{-1} at pH 6.0 (21) to 6.0 M^{-1} s^{-1} at pH 6.8 (22); the present value of 2.55 M^{-1} s^{-1} at pH 6.5 falls within the reported range. As pointed out by Brubacher and Glick (23), the thiol groups of a wide variety of proteins react more slowly with NEM than does that of cysteine, presumably reflecting the steric constraints of addition to the double bond of the large, planar maleimide molecule. That the free energy of activation of thiol addition to *N*-ethylmaleimide is actually lower than that of S_N2 displacement of chloride from chloroacetamide is evident from the absolute rates of alkylation of small thiols. (For example, compare the second-order rate constants of glutathione's or cysteine's reactions with NEM and with chloroacetamide, given in Table II.)

Yet, despite the steric constraints on absolute reaction rates imposed by the restrictive trajectory of addition to the π-bond, NEM serves as a useful reagent for comparing the effective nucleophilic character of Cys-25 in HNB-papain with that of Cys-25 in papain. At pH 6.5, the second-order rate constants for reactions of HNB-papain with NEM and with chloroacetamide were enhanced 27 and 24%, respectively, over those for reactions of native papain. Such enhanced nucleophilicity of Cys-25 due to hydroxynitrobenzylation of Trp-177 parallels the enhanced enzymatic activity of HNB-papain toward α-*N*-benzoyl-L-arginine ethyl ester (7), Z-Gly-ONp (Table I) and BzPheValArgNan (Table I) at pH 6.5.

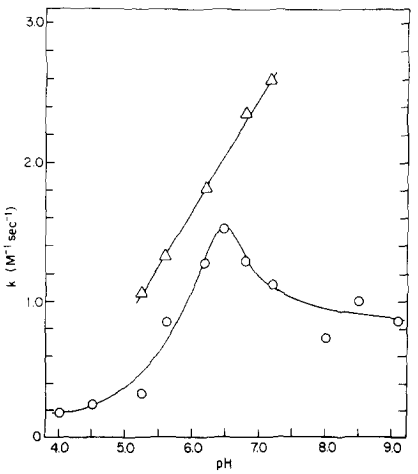


FIG. 6. pH-Dependence of apparent rate constants governing reactions of HNB-papain with 1.0 mM chloroacetate. (△) Values obtained from fluorescence measurements (280 nm:365 nm); (○) values obtained from activity measurements (BzArgNan).

The kinetics of hydrolysis of the latter substrate are of special interest, in view of the 250% increase in k_{cat}/K_m for hydrolysis of BzArgNan which results from hydroxynitrobenzylation of papain. Previous analyses had demonstrated significant differences in the kinetics of papain's action on the ethyl ester and the nitroanilide of benzoyl-L-arginine (15). Furthermore, Hall and Anderson (24) reported that proflavine, which binds to papain in a 1:1 complex with a K_{diss} of approx 10^{-4} M, enhances the enzyme's activity toward the ethyl ester, but inhibits enzymatic activity with respect to BzArgNan. Indeed, the question was raised as to whether hydrolysis of the anilide bond might involve a different catalytic mechanism than hydrolysis of the ester bond. The findings that the anilide bond in the oligopeptide substrate, BzPheValArgNan, is hydrolyzed by papain more efficiently than that of BzArgNan, and that the effect of hydroxynitrobenzylation of papain is to enhance the oligopeptide nitroanilide's rate of hydrolysis by 24% at pH 6.5 (Table I) provide convincing evidence that the anilide bond per se is not the source of the unique kinetic properties of BzArgNan. Rather, the presence of the hydroxynitrobenzyl group, attached to Trp-177, appears to block access of BzArgNan to a nonproductive mode of binding to one (or more) of the subsites of papain (25, 26), thereby enhancing the efficiency of its hydrolysis by HNB-papain.

Reversal of the order of reactivity of papain and HNB-papain with chloroacetate appears to be the result of coulombic repulsion of the negatively charged alkylating agent and the added negative charge of the ionized hydroxynitrobenzyl reporter group bound to Trp-177 in the modified enzyme. A similar reversal in reactivity of the thiol groups of cysteine and glutathione is observed: whereas the ratio of second-order rate constants for cysteine:glutathione is 2.6:1 and 2.3:1 for reactions with chloroacetamide and NEM, respectively, at pH 6.5, the corresponding ratio for reactions with chloroacetate is 0.53:1. The ratio observed for HNB-papain:papain with chloroacetate is 0.56:1. Thus, it appears that relative reactivities with ionically charged alkylating agents do not provide a direct measure of relative nucleophilicities, whereas reactivities with neutral molecules can provide a good index of nucleophilic character.

It is concluded that hydroxynitrobenzylation of Trp-177 in papain enhances the nucleophilic strength of Cys-25, which accounts for the improved catalytic efficiency of the modified enzyme toward various ester and oligopeptide-amide substrates. Superimposed on this general effect is an added increment of enzymatic enhancement specifically observed with BzArgNan, which seemingly results from blocking one or more nonproductive binding modes available to this small substrate (containing two aromatic moieties) in the unmodified enzyme (21, 25).

Fluorescence of Papain and HNB-Papain

If Trp-177 were acting as a chromophore but not as a fluorophore, then the loss of indole resonance in this residue through chemical modification would result in a 0 to 20% decrease in fluorescence intensity of the enzyme.[4] The observed 40% decrease in fluorescence emission of papain upon hydroxynitrobenzylation of Trp-177 (6) is thus inconsistent with such a model. Lowe and Whitworth (5) concluded that Trp-177 contributes 46% of the protein's fluorescence, based on studies of *N*-bromosuccinimide-oxidized papain (four equivalents yielding a derivative modified at both Trp-69 and Trp-177); the present observations with HNB-papain (modified only at Trp-177) confirm the interpretation that Trp-177 is a dominant fluorophore in the native enzyme. Since hydroxynitrobenzylation renders tryptophan nonfluorescent (27), the fluorescence of HNB-papain must arise from the four remaining Trp residues.

Although Trp-177 can now be identified as the dominant tryptophan cited by Steiner (4), whose fluorescence can be "quenched by protonation of a histidine in its vicinity" (i.e., His-159), it is also now clear that Trp-177 is *not* the principal fluorophore which

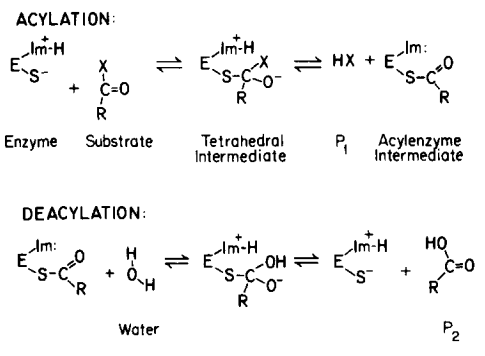
[4] This range reflects the range in extent of energy transfer from one of five tryptophyl residues in the papain molecule to the dominant fluorescing residue (0 to 100%).

is quenched by the Hg of mercuripapain. This conclusion is based on the finding that the fluorescence of HNB-papain is quenched upon complexing the enzyme with Hg^{2+}, as is the fluorescence of native papain (Ref. 6, see Fig. 5a). Thus, it is one of the remaining Trp residues, present as a contributing fluorophore in both the native enzyme and HNB-papain, whose interaction with the Cys-25-bound Hg ion (either direct or through some indirect change in environment) leads to quenching of fluorescence in mercuripapain.

Implications Regarding the Role of Trp-177

As noted by Drenth *et al.* (28), the imidazole ring of His-159 and the indole of Trp-177 are in close proximity in the crystallographic structure of papain. A monoprotonated species, involving the pairing of Cys-25 with His-159, is the catalytically active form of papain (29). Although considerable controversy has surrounded the assignment of pK_a values to Cys-25 and His-159 (5, 19, 30), the fluorescence behavior of active papain exhibits pH-dependent quenching which corresponds to the descending or alkaline limb of the pH-activity curve of the enzyme (31). Drenth *et al.* pointed out that one must conclude that the imidazolium ion of His-159 is the quenching species in activated papain (28); thus the His-159:Cys-25 couple must be in the imidazolium:mercaptide ion-pair form (29).

Such an ion-pair in the active site of papain could catalyze substrate hydrolysis according to the following scheme:



SCHEME 1

A charge transfer interaction between Trp-177 and the imidazolium ring of His-159 would help to stabilize the essential mercaptide-imidazolium charge separation in active, native papain, and thereby increase the nucleophilicity of the S atom of Cys-25. When Cys-25 is alkylated, the effective pK_a of His-159 falls from about 8.5 (in active, native papain) to about 4.1 (in carboxamidomethyl-papain) (28). His-159 loses its proton, thus being converted from the imidazolium ion to an imidazole species at neutral pH; quenching of fluorescence of Trp-177 is thereby released, as the charge transfer interaction is disrupted. This model is thus consistent with the observed enhancement of fluorescence emission of native papain which accompanies alkylation of Cys-25. Indeed, the precise correlation observed between increased fluorescence intensity and loss of enzymatic activity (Fig. 3) would be predicted on the basis of this model.

In the case of HNB-papain, however, Trp-177 is no longer a fluorophore, and changes in fluorescence intensity of the protein are not directly correlated with chemical modification of Cys-25 (Fig. 6). Moreover, the pH-sensitivity of native papain's fluorescence is not exhibited by HNB-papain (data not shown), nor is it shown by other derivatives of papain in which Trp-177 is oxidized (4, 5). By the same token, chemical modification of His-159 abolishes the characteristic pH-sensitivity of papain's fluorescence (5).

It is curious that hydroxynitrobenzylation of Trp-177 in papain increases the measured nucleophilicity of Cys-25 and thereby increases the apparent acylation rate constant (7). It would appear that the *p*-nitrophenoxide ion (conjugate base form of the HNB reporter group) is in a position to interact with the imidazolium moiety of His-159 so as to further facilitate charge separation in the mercaptide-imidazolium ion-pair; our kinetically determined pK_a of 6.95 is consistent with such interaction. Considering the precision of interactions which are usually associated with residues at the active sites of enzymes, such fortuitous alteration to *enhance* enzymatic efficiency seems surprising, and is certainly a rare event. However, Drenth *et al.* (28) have presented evidence, based

on X-ray analysis of papain derivatives involving chloromethylketones of specific peptides, that the imidazole group of His-159 possesses unique rotational freedom, which does not disturb the rest of the papain molecule, but permits participation in the kind of nonrepetitive mechanism depicted in Scheme 1. Subsequently, cryoenzymology has led Angelides and Fink to conclude that conformational flexibility of His-159 is a key feature of papain's mechanism of action (32). Such rotational freedom may provide just the flexibility needed to accommodate the activating effect of the hydroxynitrobenzyl moiety attached to Trp-177 in HNB-papain.

REFERENCES

1. JORI, G., AND GALIAZZO, G. (1971) *Photochem. Photobiol.* **14**, 607–619.
2. JORI, G., GENNARI, G., TONIOLO, C., AND SCOFFONE, E. (1971) *J. Mol. Biol.* **59**, 151–168.
3. KIRSCHENBAUM, D. M. (1971) *Biochim. Biophys. Acta* **235**, 159–163.
4. STEINER, R. F. (1971) *Biochemistry* **10**, 771–778.
5. LOWE, G., AND WHITWORTH, A. S. (1974) *Biochem. J.* **141**, 503–515.
6. MOLE, J. E., AND HORTON, H. R. (1973) *Biochemistry* **12**, 5278–5285.
7. MOLE, J. E., AND HORTON, H. R. (1973) *Biochemistry* **12**, 5285–5289.
8. WOLTHERS, B. G., DRENTH, J., JANSONIUS, J. N., KOEKOEK, R., AND SWEN, H.M. (1970) *in* Structure-Function Relationships of Proteolytic Enzymes (Desnuelle, P., Neurath, H., and Ottesen, M., eds.), pp. 272–288, Academic Press, New York.
9. DRENTH, J. JANSONIUS, J. N., KOEKOEK, R., AND WOLTHERS, B. G. (1971) *in* The Enzymes (Boyer, P. D., ed.) 3rd ed., Vol. 3, pp. 485–499, Academic Press, New York.
10. DRENTH, J., JANSONIUS, J. N., KOEKOEK, R., AND WOLTHERS, B. G. (1971) *Advan. Protein Chem.* **25**, 79–115.
11. TEALE, F. W. J., AND WEBER, G. (1957) *Biochem. J.* **65**, 476–482.
12. SHINITZKY, M., AND GOLDMAN, R. (1967) *Eur. J. Biochem.* **3**, 139–144.
13. BAREL, A. O., AND GLAZER, A. N. (1969) *J. Biol. Chem.* **244**, 268–273.
14. CHANG, S.-M. T., AND HORTON, H. R. (1979) *Biochemistry* **18**, 1559–1563.
15. MOLE, J. E., AND HORTON, H. R. (1973) *Biochemistry* **12**, 816–822.
16. SLUYTERMAN, L. A. AE. (1968) *Biochim. Biophys. Acta* **151**, 178–187.
17. RIORDAN, J. F., AND VALLEE, B. L. (1972) *Methods Enzymol.* **25**, 449–456.
18. SLUYTERMAN, L. A. AE. (1967) *Biochim. Biophys. Acta* **139**, 439–449.
19. JOLLEY, C. J., AND YANKEELOV, J. A., JR. (1972) *Biochemistry* **11**, 164–169.
20. LEWIS, S. D., AND SHAFER, J. A. (1974) *Biochemistry* **13**, 690–698.
21. GLICK, B. R., AND BRUBACHER, L. J. (1974) *Canad. J. Biochem.* **52**, 877–883.
22. HALL, P. L., AND ANDERSON, C. D. (1974) *Biochemistry* **13**, 2082–2087.
23. BRUBACHER, L. J., AND GLICK, B. R. (1974) *Biochemistry* **13**, 915–920.
24. HALL, P. L., AND ANDERSON, C. D. (1974) *Biochemistry* **13**, 2087–2092.
25. BROCKLEHURST, K., CROOK, E. M., AND WHARTON, C. W. (1968) *FEBS Lett.* **2**, 69–73.
26. HINKLE, P. M., AND KIRSCH, J. F. (1971) *Biochemistry* **10**, 2717–2726.
27. NAIK, V. R., AND HORTON, H. R. (1973) *J. Biol. Chem.* **248**, 6709–6717.
28. DRENTH, J., SWEN, H. M., HOOGENSTRAATEN, W., AND SLUYTERMAN, L. A. AE. (1975) *Proc. Koninkl. Nederl. Akademie van Wetenschappen* **C78**, 104–110.
29. POLGÁR, L. (1977) *Int. J. Biochem.* **8**, 171–176.
30. LOWE, G. (1976) *Tetrahedron* **32**, 291–302.
31. SLUYTERMAN, L. A. AE., AND DEGRAAF, M. J. M. (1970) *Biochim. Biophys. Acta* **200**, 595–597.
32. ANGELIDES, K. J., AND FINK, A. L. (1978) *Biochemistry* **17**, 2659–2668.