Structure of Papain Modified by Reaction with 2-Chloromethyl-4-nitrophenyl *N*-Carbobenzoxyglycinate†

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ABSTRACT: Papain (EC 3.4.22.2), which had been obtained in fully active form by mercurial column chromatography, was treated with 2-chloromethyl-4-nitrophenyl *N*-carbobenzoxyglycinate (Z-Gly-ONB-Cl) at pH 5.0 to introduce the 2-hydroxy-5-nitrobenzyl (HNB) reporter group into the vicinity of the enzyme's active site, in a manner leading to a doubling of its catalytic activity toward *N*-benzoyl-L-arginine *p*-nitroanilide. The HNB-papain, which contained 1.0 ± 0.1 mol of HNB/mol of protein, was digested with trypsin following either thermal and acid denaturation or reduction and carboxymethylation, and the resulting HNB-containing peptides were chromatographically isolated and purified. In each case, the HNB chromophore was found to reside in the tryptic fragment consisting of residues 175–188, which contains two of papain's five tryptophyl residues, Trp-177 and Trp-181. Analysis of the peptide samples revealed the presence of 1 mol of unmodified Trp (0.86–0.89 mol recovery) and 1 mol of HNB (0.93–0.96) groups per mol of peptide, suggesting that one of the Trp residues had been selectively modified. Subtractive Edman degradation of the HNB-peptide revealed Asx as the N terminus, i.e., Edman cleavage of the Asn-175–Ser-176 peptide bond had occurred; however, additional cycles of Edman treatment failed to produce further peptide bond cleavage. It appears that hydroxynitrobenzylation of Trp-177 renders the Ser-176–Trp-177 peptide bond resistant to normal Edman degradation. Trp-177 was confirmed as the site of hydroxynitrobenzylation through timed enzymatic digestion of the HNB peptide with leucine aminopeptidase. Spectral properties of the "reporter group" of HNB-papain, which had revealed that the chromophore resides in a polar medium readily accessible to solvent, are consistent with the steric relationships of Trp-177 in the crystallographic structure of papain. The finding that Trp-177 is the unique tryptophyl residue which is modified upon reaction of papain with Z-Gly-ONB-Cl confirms the validity of the design of this reagent as an active-site-directed reporter group and provides additional evidence for transient acylation of the thiol group of Cys-25, which is "protected" during enzyme-catalyzed cleavage of the ester bond of Z-Gly-ONB-Cl.

The presence of several tryptophyl residues in the vicinity of the active site of papain (EC 3.4.22.2) was discovered by Drenth et al. (1971a,b) in their crystallographic analysis of the enzyme's structure. Of the five Trp residues in papain, the indole rings of residues 26 and 177 are virtually equidistant from the active site thiol (Cys-25), but Trp-26 is buried within the tertiary structure, whereas Trp-177 is exposed to solvent, "covering" the hydrogen bond between Asn-175 and His-159 (Drenth et al., 1971b). Trp-69 is exposed in the crystallographic structure but comprises part of the substrate-binding site ("subsite S_2", see Schechter & Berger, 1967), while Trp-181 is mostly buried, essentially perpendicular to the enzyme's surface, farther from Cys-25 than Trp-177. The fifth tryptophyl residue, Trp-7, lies farthest from the active site. Involvement of one or more tryptophyl residues in the catalytic activity of papain has been implicated through a variety of chemical modifications, including photooxidation (Jori & Galiazzo, 1971; Jori et al., 1971) and reaction with *N*-bromosuccinimide (Kirschenbaum, 1971; Steiner, 1971; Lowe & Whitworth, 1974).

Upon reaction with 2-chloromethyl-4-nitrophenyl *N*-carbobenzoxyglycinate (Z-Gly-ONB-Cl),[1] papain became hydroxynitrobenzylated (Mole & Horton, 1973b) and, concomitantly, its catalytic activity increased substantially (Mole & Horton, 1973c). Provided the papain had been purified and fully activated to yield 1.0 mol of active site thiol/mol (Mole & Horton, 1973a), prior to exposure to Z-Gly-ONB-Cl, such reaction led to incorporation of 1.0 ± 0.1 mol of HNB/mol of protein, while retaining 1.0 mol of thiol/mol. Less pure or less active preparations resulted in proportionately lower degrees of hydroxynitrobenzylation (Mole & Horton, 1973b). Although Trp appeared to be the site of papain's modification, it had not been determined which of the five Trp residues was bound to the HNB chromophore.

The present paper presents evidence that Trp-177 is the site of hydroxynitrobenzyl group incorporation in papain upon enzyme-catalyzed generation of HNB-Cl from the active site-directed reagent, Z-Gly-ONB-Cl.

Materials and Methods

Papain was obtained from P-L Biochemicals (lot no. 0588-7) and was further purified by mercurial column chromatography (Sluyterman & Wijdenes, 1970; Mole & Horton, 1973a). Trypsin (Tos-PheCH_2Cl-treated, lots 33J790 and 36D927) and leucine aminopeptidase (lot 57A535P) were purchased from Worthington Biochemical Corp. Z-Gly-ONB-Cl was synthesized as previously described (Mole & Horton, 1973b); α-*N*-benzoyl-L-arginine *p*-nitroanilide was purchased from Bachem. Before use, iodoacetic acid, from Eastman Kodak, was recrystallized from hexane; triethylamine, also from Eastman, was redistilled; pyridine, from Fisher Scientific, was distilled from ninhydrin. Ultrapure GdmCl was obtained from Schwarz/Mann; Edman sequencer reagents (sequenal grade) were purchased from Pierce Chemical Co. All other chemicals

† From the Department of Biochemistry, North Carolina State University, Raleigh, North Carolina 27650. *Received November 15, 1978.* This investigation was supported in part by the National Science Foundation under Grant BMS 72-01999 A01. Any opinions, findings, and conclusions or recommendations expressed in this publication are those of the authors and do not necessarily reflect the views of the National Science Foundation. This is paper 5825 of the Journal Series of the North Carolina Agricultural Experiment Station, Raleigh, NC.

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[1] Abbreviations used: HNB, 2-hydroxy-5-nitrobenzyl; Z-Gly-ONB-Cl, 2-chloromethyl-4-nitrophenyl *N*-carbobenzoxyglycinate; GdmCl, guanidinium chloride; Tos-PheCH_2Cl, L-1-tosylamido-2-phenylethyl chloromethyl ketone; CM, carboxymethyl; Pth, phenylthiohydantoin.

0006-2960/79/0418-1559$01.00/0

were of reagent grade. Sephadex G-25 was a product of Pharmacia; Bio-Gel P-2 was obtained from Bio-Rad.

Hydroxynitrobenzylation of Papain. Freshly activated papain was treated with a 200-fold molar ratio of Z-Gly-ONB-Cl at pH 5.0 (Mole & Horton, 1973b). 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 were estimated from absorbance at 290 nm (ϵ_{290} = 81 300 M^{-1} cm^{-1} at pH >12). The concentrations of HNB moieties in HNB-papain and HNB peptides were determined from absorbance measurements at 410 nm at pH >12 (ϵ_{410} = 18 450 M^{-1} cm^{-1}). Kinetic assays of native and HNB-enzyme preparations were conducted as described by Mole & Horton (1973a).

Tryptophan contents of native papain, HNB-papain, and HNB-containing peptides were determined by amino acid analysis following hydrolysis in 6 M HCl containing 4% (v/v) thioglycolic acid (Matsubara & Sasaki, 1969).

Tryptic Digestion and Peptide Fractionation. Preparations of HNB-papain (containing 0.93–1.1 mol of HNB/mol) were subjected to either thermal denaturation or reduction and carboxymethylation prior to tryptic digestion. Denaturation was achieved by acidifying papain solutions to pH 2.8 with HCOOH, heating in a boiling water bath for 3 min, and then neutralizing with 4 M NaOH. Precipitated protein was collected by centrifugation. Reduction and alkylation of disulfide bonds were achieved by the three-step procedure of Mitchel et al. (1970). The product, hepta-*S*-carboxymethyl-HNB-papain, was recovered as a yellow precipitate following dialysis against water.

Samples of thermally denatured HNB-papain and carboxymethylated HNB-papain were subjected to digestion with Tos-PheCH_2Cl-treated trypsin (Smyth, 1967). Digestion at pH 8.4 (pH-stat) and 37 °C was initiated by adding 3% (w/w) trypsin to the suspended protein; second and third additions of trypsin (each 1% w/w) were made after 2 and 4 h, respectively, and hydrolysis was allowed to continue for 16 h. The digests were adjusted to pH 10.4 with NH_4OH, whereupon most of the precipitate dissolved; the soluble portion was lyophilized, dissolved in 0.1 M NH_4OH, and fractionated on a 2.5 × 100 cm column of Sephadex G-25 (0.1 M NH_4OH, pH ~10.5). Peptides were further fractionated by passage through a 2.0 × 150 cm column of Sephadex G-25 (0.1 M NH_4HCO_3, pH ~8). Peaks containing the HNB chromophore (A_{410}) were collected, and aliquots were subjected to amino acid analysis (Beckman Models 120C and 118 amino acid analyzers with scale amplifiers).

Subtractive Edman Degradation. N-Terminal sequences of peptides were examined using a modification of the subtractive Edman degradation, as discussed by Konigsberg (1967, 1972).

Aminopeptidase Digestion. Digestion of 0.05 μmol of HNB-labeled peptide with leucine aminopeptidase (0.5 mg/mL) was performed at 37 °C in 0.1 M Tris-Cl, pH 8.5, containing 2.5 mM $MgCl_2$ (Light, 1972). After various intervals, aliquots (ca. 0.005 μmol) were removed, adjusted to pH 2.2, and analyzed for free amino acids. Control samples, lacking the HNB peptide, were similarly analyzed.

Contents of serine and asparagine, which coeluted in amino acid analysis, were determined by comparing apparent amino acid compositions before and after partial acid hydrolysis (Hill & Smith, 1959).

Results

Incorporation of 1.00 mol of HNB groups/mol of papain through reaction of the enzyme with Z-Gly-ONB-Cl resulted in a 238% enhancement of catalytic activity toward α-*N*-benzoyl-L-arginine *p*-nitroanilide, from 2.36 to 5.61 units/mg. Amino acid analysis of carboxymethylated HNB-papain and of native papain revealed 7.0 and 0.0 residues of CM-cysteine, respectively, and 3.8 and 4.8 residues of tryptophan, respectively, per molecule. No other amino acid recoveries were affected by hydroxynitrobenzylation and carboxymethylation. Thus, Trp appeared to be the only site of hydroxynitrobenzylation. The fact that all seven half-cystine residues were carboxymethylated confirms the earlier titration data with *p*-mercuribenzoate and 5,5′-dithiobis(2-nitrobenzoate), viz., that the active site thiol had not reacted with the HNB group (Mole & Horton, 1973b).

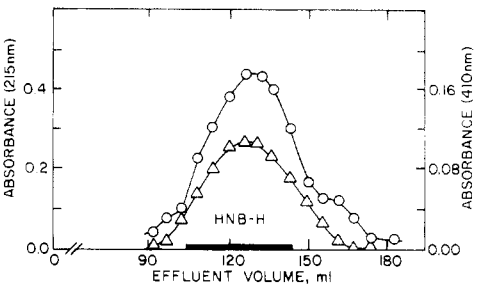

FIGURE 1: Purification of peptide HG-25_2G-25_1 on Bio-Gel P-2. Peptide HG-25_2G-25_1 (first yellow peak from second Sephadex G-25 chromatogram) was dissolved in 1.0 mL of 0.1 M NH_4HCO_3 and applied to a 2.0 × 50 cm column of Bio-Gel P-2 (equilibrated and eluted with 0.1 M NH_4HCO_3). Absorbance was measured at 410 nm (△) and at 215 nm (○). The bar indicates the fractions pooled and analyzed as peptide HNB-H.

The characteristic interaction of hydroxynitrobenzyl moieties with dextran gels (Horton & Koshland, 1972) facilitated fractionation of HNB peptides by gel chromatography on Sephadex G-25. Ion-exchange celluloses and polystyrenes were avoided since HNB-containing species are adsorbed too tightly to such matrixes to permit elution under nondestructive conditions (Dopheide & Jones, 1968; Robinson, 1970). Initial fractionation of tryptic digests of heat-denatured (designated H) and carboxymethylated (designated C) HNB-papain on Sephadex G-25 in 0.1 M NH_4OH resulted in two major yellow peaks in each case. The first peaks, which eluted near the void volume, had amino acid compositions like those of HNB-papain and CM-HNB-papain, respectively, and were thus identified as undigested protein. The second yellow peaks (HG-25_2 and CG-25_2), which eluted late in the chromatograms (at 400 mL effluent volume and 220 mL effluent volume, respectively), were collected, lyophilized, and rechromatographed on Sephadex G-25 in 0.1 M NH_4HCO_3. The Sephadex matrix was observed to retard the HNB peptides significantly, as the first yellow bands did not emerge prior to the position expected for dipeptides and free amino acids; the volume required to remove the final HNB-labeled peptides was 1.5 times the bed volume of the column. The first yellow peptide fraction from denatured HNB-papain (HG-25_2G-25_1) was further purified by chromatography on a Bio-Gel P-2 column in 0.1 M NH_4HCO_3, as shown in Figure 1. The isolated HNB-containing tryptic peptides from heat-denatured HNB-papain (peptide HNB-H) and carboxymethylated HNB-papain (peptide HNB-C) were subjected to amino acid analysis, which revealed only traces of impurities and proved suitable for establishing the identity of the HNB-labeled Trp.

As indicated by the results given in Table I, the composition of each peptide corresponded to that of the tryptic fragment of papain containing residues 175–188 (Mitchel et al., 1970). Thus, both heat denaturation and carboxymethylation procedures were effective in providing for trypsin-catalyzed

Table I: Amino Acid Composition of HNB Peptides

amino acid	HNB peptides HNB-H	HNB-C	theor no. of residues in peptide 175–188
Lys	0.14	0.18	0
His	0.12	0.11	0
Arg	1.15	0.98	1
Asp	1.82	2.08	2
Thr	0.95	1.00	1
Ser	1.32	1.31	1
Glu	1.09	1.33	1
Gly	3.82	4.00	4
Ala	0.25	0.32	0
Val	0.18	0.21	0
Ile	0.78	1.10	1
Leu	0.11	0.26	0
Tyr	0.94	0.92	1
Phe	0.01	0.13	0
Trp[a]	0.86	0.89	2
HNB moiety[b]	0.93	0.96	
amino terminus (Edman)	Asx	Asx	Asn
amino terminus (Leu aminopeptidase)	Asn	Asn	Asn

[a] Recovery following acid hydrolysis of the peptide in the presence of 4% thioglycolic acid. [b] Calculated from A_{410} of the peptide in 0.1 M NaOH.

cleavage of the Lys-174 and Arg-188 peptide bonds. The fact that each of the two peptides contained one HNB group (A_{410} in alkali) and one unmodified Trp (acid hydrolysis in the presence of 4% thioglycolic acid) implied that a total of two Trp moieties had been present in each case; this facilitated identification of the unique tryptic fragment in papain which contains two Trp resides. Moreover, the lack of Cys residues in this tryptic fragment is consistent with the identity of HNB-H and HNB-C (whereas Trp-26 and Trp-69 would appear in disulfide-bonded tryptic fragments isolated from heat-denatured papain).

Since the HNB-labeled tryptic peptide contained two Trp residues (Trp-177 and Trp-181), subtractive Edman degradation was used in an attempt to identify the site of HNB attachment. Unfortunately, the Edman degradation procedure, when applied to the HNB peptide under investigation, succeeded only in establishing the N-terminal residue as Asx. Further chemical degradation of the peptide chain was not achieved by subsequent exposure to repeated coupling and cleavage cycles, as revealed by the recovery data in Tables II and III. The finding that peptides HNB-H and HNB-C had the same N terminus as well as the same amino acid composition confirmed their identity. Moreover, of the four Trp-containing tryptic fragments of papain, only the one comprising residues 175 through 188 contains N-terminal Asn and C-terminal Arg.

The finding that the first peptide bond, between Asn-175 and Ser-176, was cleaved in a normal manner by Edman degradation, whereas the next bond, between Ser-176 and residue 177, was resistant to cleavage on repeated cycles, indicated that Trp-177 was probably the site of hydroxynitrobenzylation. Proof that Trp-177 was indeed modified and that Trp-181 was not hydroxynitrobenzylated was obtained by enzymatic digestion of the HNB-labeled peptide with

Table II: Subtractive Edman Degradation of Peptide HNB-H

175–188 sequence:	175 Asn	-	Ser	-	177 Trp	-	Gly	-	Thr	-	180 Gly	-	181 Trp	-	Gly	-	Glu	-	Asn	-	185 Gly	-	Tyr	-	Ile	-	188 Arg	
AA[c] composition:	Asx		Ser		Trp		Gly		Thr								Glu						Tyr		Ile		Arg	HNB
	1.82		1.32		0.86		3.82		0.95								1.09						0.94		0.78		1.15	0.93
step 1 (70%)[a]	1.23		1.36		*b*		3.73		0.83								1.03						0.97		0.58		1.05	0.89
step 2 (56%)	1.20		1.43		*b*		3.77		0.86								1.02						0.89		0.50		0.89	0.88
N terminus determined	Asx																											

[a] Recovery. [b] Not estimated. [c] AA, amino acid.

Table III: Subtractive Edman Degradation of Peptide HNB-C

175–188 sequence:	175 Asn	-	Ser	-	177 Trp	-	Gly	-	Thr	-	180 Gly	-	181 Trp	-	Gly	-	Glu	-	Asn	-	185 Gly	-	Tyr	-	Ile	-	188 Arg	
AA[c] composition:	Asx		Ser		Trp		Gly		Thr								Glu						Tyr		Ile		Arg	HNB
	2.08		1.31		0.89		4.00		1.00								1.33						0.92		1.10		0.98	0.96
step 1 (90%)[a]	1.36		1.28		*b*		3.86		0.96								1.34						0.72		0.97		0.96	0.88
step 2 (75%)	1.24		1.47		*b*		3.98		0.97								1.10						0.72		0.91		0.85	0.86
N terminus determined	Asx																											

[a] Recovery. [b] Not estimated. [c] AA, amino acid.

Table IV: Elucidation of HNB-Labeled Trp through Aminopeptidase Digestion of HNB-C

175–188 sequence:	175 Asn	-	Ser	-	177 Trp (HNB)	-	Gly	-	Thr	-	180 Gly	-	181 Trp	-	Gly	-	Glu	-	Asn	-	185 Gly	-	Tyr	-	Ile	-	188 Arg
AA[b] composition:	Asx		Ser		Trp		Gly		Thr								Glu						Tyr		Ile		Arg
	2.08		1.31		0.89		4.00		1.00								1.33						0.92		1.10		0.98
AA released after digestion for	Asn[a]		Ser		Trp		Gly		Thr								Glu						Tyr		Ile		Arg
2 h	1.06		0.82		0.09		0.75		0.20								0.20						0.12		0.11		0.30
5 h	0.89		0.86		0.05		0.75		0.24								0.32						0.20		0.20		0.35
6 days	2.20		1.00		0.89		3.30		0.81								1.10						1.10		1.20		1.20

[a] Calculated from the difference in apparent Ser content before and after partial acid hydrolysis following leucine aminopeptidase digestion. [b] AA, amino acid.

leucine aminopeptidase. The results are presented in Table IV as moles of each amino acid recovered per mole of peptide (corrected for trace contaminants found in the controls). Note that glycine is released by the action of the aminopeptidase substantially before free tryptophan appears in the digest, evidence that the residue preceding Gly in the peptide sequence (i.e., residue 177) was HNB-Trp rather than unmodified Trp. Extended digestion with the aminopeptidase released free tryptophan, corresponding to unmodified Trp-181. Thus, in situ hydroxynitrobenzylation of papain through reaction with the quasisubstrate Z-Gly-ONB-Cl occurs at Trp-177, which renders the Ser-176 to Trp-177 peptide bond inert to Edman degradation.

Discussion

Identification of tryptic peptide 175–188 as the site of in situ hydroxynitrobenzylation of papain, through its interaction with the active-site-directed "reporter group," Z-Gly-ONB-Cl, is based on three classes of data, each of which excludes the other Trp-containing peptides: amino acid composition; N-terminal Asn and C-terminal Arg; and recovery of one unmodified Trp (0.86–0.89 mol/mol) in addition to one HNB group (0.93–0.96 mol/mol).

In attempting to identify which of the two Trp residues within the peptide (177 or 181) had been hydroxynitrobenzylated, subtractive Edman degradation was employed. It is interesting that, in the second and subsequent cycles of Edman degradation, no amino acid was removed from the HNB-labeled peptide. The reason that the Edman procedure fails to cleave the peptide further has not been established, but through use of the subtractive method (Konigsberg, 1972) it is possible to avoid artifacts of other methodology. (Thus, for example, it is not an artifact of back hydrolysis of Pth-Ser, which is readily destroyed in acid, nor is it simply an extraction problem, as might be encountered in direct Edman procedures.) Such lack of further cleavage had been encountered by Dopheide & Jones (1968) in one of four HNB-Trp-containing peptides from pepsin, whereas the other peptides provided normal Edman degradations. In the present analysis, the fact that Trp-177 is hydroxynitrobenzylated (as determined by leucine aminopeptidase digestion) adds insight to the Edman findings. The presence of the HNB group bound to Trp-177 might either prevent access of the phenyl isothiocyanate to the α-amino group of Ser-176 or impede subsequent cyclization of the phenylthiocarbamyl-Ser peptide to the thiazolinone and cleavage of the Ser–Trp peptide bond. Konigsberg (1972) has observed that, when an internal Gln is reached during Edman degradation, it often cyclizes to form a pyrrolidonecarboxylic acid residue, stopping any further degradation. It is interesting to speculate a relationship to certain HNB-Trp residues, such as that encountered in papain. Spectral analysis (Mole & Horton, 1973b) had revealed that the HNB chromophore was bound to papain in a noncyclic adduct, which may well persist through tryptic cleavage and ammoniacal chromatography. Following Edman cleavage of the terminal Asn, however, the proclivity of certain HNB–indole adducts to cyclize (Chan & Schellenberg, 1968; Tucker et al., 1971; Horton & Koshland, 1972) might have resulted in an N-cyclic structure through the α-amino group of Ser-176, thereby blocking further Edman coupling and cleavage of the peptide. Since two diastereomers of HNB-L-tryptophan ethyl ester can be formed (Tucker et al., 1971), the lack of Edman cleavage of HNB-Trp peptides in some cases and normal cleavage in others (Dopheide & Jones, 1968) may reflect differences in steric relationships which result in N-cyclization or noncyclization, respectively. These results offer interesting contrast with the "double-stepping" noted in Edman degradation of α-*N*-HNB-Asn-labeled polypeptide chains (Wu & Horton, 1979), which resembles that encountered with N-terminal His (Schroeder, 1972).

The finding that Trp-177 of papain is hydroxynitrobenzylated, rather than Trp-181, contrasts with the site of hydroxynitrobenzylation in a homologous peptide fragment of streptococcal proteinase elucidated by Robinson (1970). It also provides evidence for the validity of the design of Z-Gly-ONB-Cl as an active-site-directed reagent and affirms the double displacement mechanism of papain-catalyzed hydrolysis (Stockell & Smith, 1957). In contrast with the conclusion of Jori & Galiazzo (1971) "that the solution conformation of some areas [the Trp-177 region] of the papain molecule may be different from that in the crystal state as deduced from X-ray diffraction studies", our data provide evidence that the conformation of this region in solution is like that in the crystallographic structure. Trp-177, which appears to form part of the S_1' subsite (Lowe & Whitworth, 1974), would be the tryptophyl residue positioned to interact with the leaving group (P_1) of an ester substrate during the first nucleophilic displacement step. In the case of Z-Gly-ONB-Cl, such displacement would produce transient acylation of Cys-25 (Z-glycyl thioester) at the active site of papain and release of HNB-Cl in the vicinity of Trp-177, based on the conformational features deduced from X-ray crystallography (Drenth et al., 1971b). Thus, Trp-177 reacts with the active-site-generated HNB-Cl, while the reactive thiol group of Cys-25 is "protected" as the transient thioester. In contrast, inactivation of papain and hydroxynitrobenzylation of Cys-25 occurs when the enzyme is treated with HNB-Br or HNB-Cl, per se (Morihara & Nagami, 1969; Mole & Horton, 1973b). Although Trp-69 is also exposed to solvent in the crystallographic structure of papain (Drenth et al., 1971b), it is located in the S_2 subsite of the molecule which would be expected to interact with the carbobenzoxy moiety of both Z-Gly-ONB-Cl, per se, and the acyl-enzyme intermediate.

In solution, HNB-papain's spectral characteristics have revealed that the reporter group "was exposed to a polar medium readily accessible to water" (Mole & Horton, 1973b), thus confirming the environment of Trp-177 in the active enzyme to be like that deduced crystallographically, whereas proflavin-sensitized photooxidation, accompanied by loss of catalytic activity, had led to the earlier conclusion that, in solution, Trp-177 "appears to be largely buried" (Jori & Galiazzo, 1971).

Finally, the finding that Trp-177 is the alkylated residue in HNB-papain reveals that it was indeed the dominant residue which was found to contribute approximately 40% of the total indole fluorescence of papain in solution (Mole & Horton, 1973b); this agrees with the conclusion of Lowe & Whitworth (1974), from their investigations based on reaction of Trp-177 with *N*-bromosuccinimide. However, the kinetic consequences of *N*-bromosuccinimide oxidation of Trp-177, which leads to a marked decrease in the acylation rate constant of papain catalysis, contrast with the notable elevation in the acylation rate constant which results from hydroxynitrobenzylation of Trp-177 (Mole & Horton, 1973c).

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Carboxypeptidase of *Streptomyces griseus*. Implications of Its Characteristics†

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ABSTRACT: Carboxypeptidase from *Streptomyces griseus* has been isolated by use of a new, highly efficient affinity chromatographic procedure. The enzyme isolated in this manner consists of a single polypeptide chain with a molecular weight of 41 200. It is reversibly inhibited by *o*-phenanthroline and other metal chelators and contains 1 g-atom of zinc/mol. The absorption and magnetic circular dichroic spectra of the cobalt-substituted enzyme are virtually identical with those previously observed for bovine carboxypeptidase A. Moreover, chemical modification studies suggest the importance of tyrosyl, arginyl, and glutamyl residues for catalytic activity, all of which have been demonstrated to be essential for the activity of bovine carboxypeptidase A. Importantly, the *S. griseus* carboxypeptidase exhibits unique properties not previously observed in other zinc carboxypeptidases. It contains 2 g-atoms of tightly bound calcium which appears to function in protein stabilization in concert with two disulfide bridges. In marked contrast to any of the metallocarboxypeptidases known presently, the *S. griseus* enzyme hydrolyzes C-terminal basic peptide substrates and their exact ester analogues with kinetic parameters comparable to those of the corresponding neutral C-terminal substrates. These properties of this bacterial enzyme, combined with its close mechanistic similarity to bovine carboxypeptidase A, suggest that it may be the postulated but yet to be identified intermediate between endopeptidases and the carboxypeptidases.

Proteolytic enzymes are known to operate by at least four different mechanisms, each characterized by a distinctive constellation of functional amino acid residues (Hartley, 1960; Neurath & Bradshaw, 1970). However, the data presently available cannot resolve whether each of the four classes originated separately and independently of the others or whether some had a common ancestor. Moreover, in those functional classes which contain both exo- and endopeptidases, an evolutionary relationship between them has not yet been demonstrated.

A metalloendopeptidase has been postulated to have given rise to both the vertebrate carboxypeptidases A and B via a single intermediate carboxypeptidase possessing the combined specificities of the A and B enzymes (Neurath & Bradshaw, 1970). However, such an intermediate has not been isolated and hence the evolutionary link between the metalloendopeptidases and the metallocarboxypeptidases has not been established. Why or when in the course of evolution a single carboxypeptidase with A and B specificities was abandoned in favor of multiple enzymes with more limited specificities is not known. If on an evolutionary time scale this event occurred late, a single functionally homologous exopeptidase

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0006-2960/79/0418-1563$01.00/0