Completion of the Amino Acid Sequence of Papain

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Papain was inhibited with bromo[2-¹⁴C]acetic acid, the tertiary structure of the inhibited enzyme was unfolded and the disulphide bridges were reduced with mercaptoethanol and aminoethylated. Digestion with trypsin gave a radioactive peptide consisting of residues 18–58 inclusive and containing therefore the sequence of the thirteen unknown residues 29–41 in the primary sequence of papain. This peptide was digested with pepsin to give a radioactive peptide consisting of residues 18–47, which after digestion with 0.4 M-hydrochloric acid gave a radioactive peptide consisting of residues 24–43 inclusive. Further digestion with 6 M-hydrochloric acid gave peptides that were used to determine the sequence:

Ser-Ala-Val-Val-Thr-Ile-Glx-Gly-Ile-Ile-Lys-Ile-Arg

for the residues 29–41, so completing the amino acid sequence of papain.

The X-ray crystal-structure analysis of papain, the cysteine proteinase from papaya latex (Drenth, Jansonius, Koekoek, Swen & Wolthers, 1968), in addition to elucidating the tertiary structure of the enzyme, showed that the tentative amino acid sequence (Light, Frater, Kimmel & Smith, 1964) required considerable revision. It also indicated that the amino acids from residue 29 to residue 41 (with the exception of the dipeptide isoleucylisoleucine) had not been isolated during the degradative studies. It seemed significant that the sequence from residue 26 to residue 41 formed an extensive piece of α -helix running from the surface of the molecule right into the core of the tertiary structure, and probably consisted mainly therefore of hydrophobic residues.

The active-site cysteine residue of papain has been identified as residue 25 (Light *et al.* 1964; Husain & Lowe, 1965) and is therefore conveniently placed for specifically labelling the molecule close to the sequence of the thirteen unknown residues. By taking advantage of this fact, a radioactive peptide containing the sequence of thirteen unknown residues was readily obtained. The sequence of these residues has now been determined, so completing the amino acid sequence of papain.

EXPERIMENTAL

Detection of peptides. All radioactivity measurements were made in solution in a Beckman DPM-100 liquid-scintillation counter.

Non-radioactive peptides were detected either by measuring the extinction at 280 nm. or by measuring the

extinction at 570 nm. after reaction of the alkaline hydrolysate with ninhydrin (see Hirs, Moore & Stein, 1956).

Paper chromatography. All paper chromatograms were run on Whatman 3MM paper with butan-1-ol-acetic acid-water (40:6:15, by vol.) as solvent.

Amino acid analyses. Peptides (approx. 9.1 μ mole) were hydrolysed with 6 M-HCl (2 ml.) at 110° in a sealed evacuated tube for 18–96 hr. as indicated. The hydrolysate was evaporated to dryness under reduced pressure and the residue taken up in 0.1 M-HCl. The amino acid analyses were performed on a Technicon AutoAnalyzer with the buffer system of Thomson & Miles (1964). The analyses are not corrected for decomposition during hydrolysis.

Edman degradations. These were performed as described by Gray (1967).

Papain. Twice-crystallized papain was prepared from granular papaya latex (we are grateful to the Wallerstein Co., New York, N.Y., U.S.A., for a generous gift of this material) by the method of Kimmel & Smith (1954). Enzyme concentrations were determined from the extinction at 278 nm. (Glazer & Smith, 1961). Enzyme activity was assayed by the rate of hydrolysis of methyl hippurate on a Radiometer pH-stat (Lowe & Williams, 1965).

Inhibition of papain with bromo[2-¹⁴C]acetic acid. Papain (0.81 g.) in 0.025 M-sodium citrate buffer, pH 5.5 (150 ml.), was activated with cysteine hydrochloride (0.12 g.) and EDTA (0.06 g.), and then completely and irreversibly inhibited with bromo[2-¹⁴C]acetic acid (0.1 mc; 18.5 mg.). The excess of reagents was removed by gel filtration on a column (45 cm. $\times$ 4 cm.) of Sephadex G-25 (fine grade).

Reduction and aminoethylation of the inhibited papain. The solution of the inhibited enzyme was evaporated to dryness in a rotary evaporator and the residue dissolved in 6 M-guanidinium chloride (75 ml.) in 1.0 M-tris-HCl buffer, pH 8.6. Mercaptoethanol (0.8 ml.) was added and the solution kept under N₂ at 25° for 4 hr. Ethyleneimine (3 ml.) was added in three 1 ml. portions at 10 min. intervals

(see Raftery & Cole, 1966). After 30 min. the solution was concentrated to 60 ml. in a rotary evaporator and the protein separated from reagents on a column of Sephadex G-25 with aq. 50% (v/v) acetic acid.

Digestion with trypsin. Most of the acetic acid was removed from the protein solution in a rotary evaporator by concentrating, adding water and again concentrating several times. The solution was made up to 50 ml. with 0.1M-KCl. The solution was brought to pH 9.4 with 2M-NaOH solution, 0.5M-calcium acetate (1 ml.) was added and the papain was digested with trypsin that had been treated with diphenylcarbamoyl chloride (20 mg.; Seravac Laboratories Ltd., Maidenhead, Berks.). The pH was maintained by adding 0.5M-NaOH in a pH-stat. After 2 hr., more trypsin (4.5 mg.) was added and the digestion continued for a further 1 hr. The digestion was stopped by adjusting the pH to 3.8 with acetic acid. The digest was concentrated in a rotary evaporator to approx. 3 ml., and taken up in aq. 50% (v/v) acetic acid (20 ml.).

Gel filtration of the tryptic digest. The digest was passed through a column (45 cm. $\times$ 3.5 cm.) of Sephadex G-25 (fine grade) with aq. 50% (v/v) acetic acid containing 0.2% of thiodiglycol. All the radioactivity emerged immediately after the void volume of the column. The radioactive fractions were combined, concentrated to 5 ml. in a rotary evaporator, and passed through a column (150 cm. $\times$ 1 cm.) of Sephadex G-50 (fine grade) with aq. 50% (v/v) acetic acid containing 0.2% of thiodiglycol. The chromatogram is shown in Fig. 1.

Fractions 49–61 were combined and a sample, run on paper chromatography, gave a single spot at the origin. The amino acid analysis is shown in Table 1. Fractions 76–86 were combined and the radioactive peptide separated

from several non-radioactive peptides by paper chromatography. The radioactive material was eluted from the paper with aq. 75% (v/v) formic acid. The eluate was concentrated to 1 ml. and passed through a column (150 cm. $\times$ 1 cm.) of Sephadex G-50 with aq. 50% (v/v) acetic acid containing 0.2% of thiodiglycol. The radiochromatogram is shown in Fig. 2. The amino acid analysis of the combined fractions 46–52 is shown in Table 1.

Treatment of the radioactive peptide (0.1 μ mole) with carboxypeptidase A at pH 7.8 and 25° for 24 hr. did not release any amino acids, but with carboxypeptidase B under the same conditions 1 residue of arginine/mol. was released.

Digestion of the radioactive peptide (I) (fractions 49–61, Fig. 1) with pepsin. The radioactive peptide (6 μ moles) in 0.1M-HCl (10 ml.) was digested with pepsin (1 mg., thrice-crystallized; Seravac Laboratories Ltd.) for 24 hr. at 30°. The digestion was stopped by immersing the solution in boiling water for 2 min. The solution was concentrated to 4 ml. and passed through a column (160 cm. $\times$ 2 cm.) of Sephadex G-25 (fine grade) with aq. 50% (v/v) acetic acid containing 0.2% of thiodiglycol. The chromatogram is shown in Fig. 3. The following fractions were combined: 40–44, 59–64 and 68–72. The amino acid analyses for their hydrolysates are shown in Table 2.

Digestion of the radioactive peptides (II) (fractions 40–44, Fig. 3) with 0.4M-hydrochloric acid. Fractions 40–44 (Fig. 3) were evaporated to dryness in a rotary evaporator and the residue was taken up in 0.4M-HCl (5 ml.). The solution was kept at 110° for 5 hr. in a sealed evacuated tube. The solution was evaporated to dryness in a rotary evaporator, and the residue dissolved in aq. 50% (v/v) acetic acid (1 ml.) and passed through a column (140 cm. $\times$ 1 cm.) of Sephadex G-25 (fine grade) with aq. 50% (v/v) acetic acid containing 0.2% of thiodiglycol. The chromatogram is shown in Fig. 4. The following fractions were combined: 42–48, 82–88, 103–107 and 114–120. The combined fractions 82–88 and 103–107 were purified by paper chromatography before hydrolysis and amino acid analyses. Fractions 114–120 contained mainly aspartic acid with some serine and glycine. The amino acid analyses are shown in Table 3.

Digestion of the radioactive peptide (III) (fractions 42–48, Fig. 4) with 6M-hydrochloric acid. Fractions 42–48 (Fig. 4) were combined and evaporated to dryness in a rotary evaporator. The residue in 6M-HCl (2 ml.) was kept at 110° for 2 hr. in a sealed and evacuated tube. The solution was evaporated to dryness and the residue in aq. 50% (v/v) acetic acid (1 ml.) passed through a column (140 cm. $\times$ 1 cm.) of Sephadex G-25 with aq. 50% (v/v) acetic acid. The chromatogram is shown in Fig. 5.

(a) Fractions 40–46 (Fig. 5). This material showed on paper chromatography a single spot at the origin. The amino acid analysis of the hydrolysate is shown in Table 4. Treatment with carboxypeptidase A or B did not release any amino acid residues. However, if the peptide (0.2 μ mole) was kept at 95° in 0.1M-sodium phosphate buffer, pH 7.8, containing 1.6% (w/v) of sodium dodecyl sulphate for 24 hr., then cooled rapidly to 25° and treated with carboxypeptidase B that had been freshly purified, arginine (0.2 residue/mol.) and a smaller proportion of isoleucine were released.

(b) Fractions 52–56 (Fig. 5). The peptide after acid hydrolysis for 48 hr. gave the following amino acid analysis (residues/mol.): Gly (0.6), Ile (2.5; 3.0 after 96 hr.), Lys

Table 1. *Amino acid analyses of the acid hydrolysates of the radioactive peptide (I)*

Both combined fractions gave a positive reaction with Ehrlich reagent. The period of digestion is given above each analysis. Abbreviations: CMCys, *S*-carboxymethylcysteine; AECys, *S*-aminoethyleysteine.

Amino acid	Fractions 49–61 (Fig. 1)		Fractions 46–52 (Fig. 2)	
	18 hr.	90 hr.	24 hr.	90 hr.
CMCys	0.7	0.7	0.8	0.7
Asp	4.7	4.5	5.0	4.7
Thr	2.0	1.9	1.7	1.9
Ser	4.0	3.3	3.4	2.6
Glu	6.0	6.1	6.2	6.0
Pro	~0.1	~0.1	0	0
Gly	3.9	4.0	4.1	4.2
Ala	2.3	2.3	2.0	2.3
Val	1.7	2.3	1.0	2.1
Ile	2.7	4.2	2.0	3.9
Leu	3.1	3.1	2.9	2.8
Tyr	1.2	1.2	1.0	0.9
Phe	1.2	1.2	1.0	1.0
AECys	1.3	1.6	1.6	1.6
Lys	1.3	1.3	0.9	1.1
Arg	1.7	1.8	1.6	1.8

Fig. 1. Gel filtration in aq. 50% (v/v) acetic acid on Sephadex G-50 of the tryptic digest of bromo[2- ^{14}C]acetic acid-inhibited and aminoethylated papain, showing radioactivity ($\blacktriangle$) and E_{280} ($\bullet$).

Fig. 2. Gel filtration in aq. 50% (v/v) acetic acid on Sephadex G-50 of the radioactive material after paper chromatography of fractions 76-86 (Fig. 1).

mol.): Ile (2.5), Lys (1.0), Arg (1.0). The peptide can be assigned therefore the partial sequence:

(c) Fractions 60-68 (Fig. 5). These fractions contained two major peptides, which were purified by paper chromatography. The amino acid analysis of one peptide (22 cm. from the origin) after acid hydrolysis for 48 hr. was (residues/mol.): Ile (1.6; 2.1 after 96 hr. digestion), Lys (1.0). After one cycle of Edman degradation the residual peptide after acid hydrolysis for 96 hr. gave the following amino acid analysis (residues/mol.): Ile (1.45), Lys (1.0). Digestion of the peptide with carboxypeptidase B for 4 hr. released 1 residue of lysine/mol. The peptide must therefore have the sequence:

(1.0), Arg (0.9). Digestion with carboxypeptidase B in 0.1M-sodium phosphate buffer, pH 7.8, at 25° for 24 hr. released 1 residue of arginine/mol. After one cycle of Edman degradation the residual peptide gave the following amino acid analysis after acid hydrolysis for 96 hr. (residues/

The amino acid analysis of the other peptide (30 cm. from the origin) after acid hydrolysis for 48 hr. was (residues/mol.): Thr (0.6), Glu (1.0), Val (1.7), Ile (0.9). Carboxypeptidase A did not effect any digestion. After one cycle of Edman degradation the amino acid analysis of the residual peptide after acid hydrolysis for 48 hr. was

Fig. 3. Gel filtration in aq. 50% (v/v) acetic acid on Sephadex G-25 of the peptic digest of fractions 49-61 (Fig. 1), showing radioactivity (▲) and E_{570} (●) after alkaline hydrolysis and reaction with ninhydrin.

Fig. 4. Gel filtration in aq. 50% (v/v) acetic acid on Sephadex G-25 of the 0.4M-HCl digest of fractions 40-44 (Fig. 3), showing radioactivity (▲) and E_{570} (●) after alkaline hydrolysis and reaction with ninhydrin.

(residues/mol.): Thr (0.6), Glu (1.0), Val (1.0), Ile (0.8).
The partial sequence must therefore be:

(d) Fractions 70-74 (Fig. 5). The amino acid analysis of

this peptide after acid hydrolysis for 48 hr. and 96 hr. was (residues/mol.): Ile (1.2), Arg (1.0). One cycle of Edman degradation released free arginine. The sequence of this peptide must therefore be:

Ile-Arg

Table 2. *Amino acid analyses of the acid hydrolysates of the three peptides obtained by digestion of the radioactive peptide (I) with pepsin*

The period of digestion is given above each analysis. Peptide (II) gave a positive reaction with Ehrlich reagent. Abbreviations: CMCys, *S*-carboxymethylcysteine; AECys, *S*-aminoethylcysteine.

Fractions 40-44 (Fig. 3)		Fractions 59-64 (Fig. 3)		Fractions 68-72 (Fig. 3)	
Amino acid	Peptide (II) 48 hr.	Amino acid	24 hr.	Amino acid	24 hr.
CMCys	0.7	Asp	2.1	Ser	1.0
Asp	3.0	Leu	1.2	Glu	3.0
Thr	2.1	AECys	1.0	Leu	0.9
Ser	2.8	Arg	0.9	Tyr	1.0
Glu	3.1				
Gly	4.3				
Ala	2.1				
Val	1.7				
Ile	2.9 (3.9 after 96 hr.)				
Leu	1.1				
Tyr	0				
Phe	1.0				
AECys	0.8				
Lys	1.1				
Arg	0.8

Fig. 5. Gel filtration in aq. 50% (v/v) acetic acid on Sephadex G-25 of the 6M-HCl digest of fractions 42-48 (Fig. 4), showing radioactivity (Δ) and E_{570} ($\bullet$) after alkaline hydrolysis and reaction with ninhydrin.

(e) Fractions 75-79 (Fig. 5). These fractions were purified by paper chromatography. The amino acid analysis of the major peptide (41 cm. from the origin) after acid hydrolysis for 48 hr. showed the presence of isoleucine only. Amino acid analysis before hydrolysis showed no isoleucine, and one cycle of Edman degradation released free isoleucine. The peptide must therefore be:

Ile-Ile

The amino acid analysis of the minor peptide (37 cm. from the origin) after acid hydrolysis for 48 hr. was (residues/mol.): Ala (1.0), Val (1.7). After one cycle of Edman degradation, the amino acid analysis of the resultant peptide after acid hydrolysis for 48 hr. showed only valine. The peptide must therefore be:

Ala-Val-Val

(f) Fractions 80-83 (Fig. 5). These fractions were

Table 3. *Amino acid analyses of the acid hydrolysates of the peptides obtained by digestion of the radioactive peptide (II) with 0.4 M-hydrochloric acid*

The period of digestion is given above each analysis. Abbreviations: CMCys, *S*-carboxymethylcysteine; AECys, *S*-aminoethylcysteine.

Amino acid	Fractions 42-48 (Fig. 4)	Amino acid	Fractions 82-88 (Fig. 4)	Amino acid	Fractions 103-107 (Fig. 4)
	Peptide (III) 48 hr.		18 hr.		18 hr.
CMCys	0.9				
Asp	0	Ser	0.9	Glu	1.1
Thr	1.9	Gly	1.0	Gly	1.0
Ser	1.9	AECys	1.1		
Glu	1.2				
Gly	2.3				
Ala	2.0				
Val	1.5 (2.0 after 96 hr.)				
Ile	3.1 (4.1 after 96 hr.)				
Leu	0				
Tyr	0				
Phe	1.1				
AECys	0				
Lys	1.0				
Arg	1.0				

Table 4. *Amino acid analysis of the acid hydrolysate of peptide (IV)*

The peptide was digested with 6 M-HCl at 110° for 48 hr. Abbreviations: CMCys, *S*-carboxymethylcysteine; AECys, *S*-aminoethylcysteine.

Amino acid	Fractions 40-46 (Fig. 5)
	Peptide (IV)
CMCys	0.7
Asp	0
Thr	1.0
Ser	1.3
Glu	1.0
Gly	1.0
Ala	2.0
Val	1.6 (2.0 after 96 hr.)
Ile	3.3 (3.9 after 96 hr.)
Leu	0
Tyr	0
Phe	0.9
AECys	0
Lys	0.9
Arg	1.0

separated into three peptides by paper chromatography. The major peptide (12 cm. from the origin) gave the following amino acid analysis after acid hydrolysis for 48 hr. (residues/mol.): Thr (0.7), Glu (1.0), Ile (1.0, and does not increase after 96 hr. hydrolysis). After one cycle of Edman degradation the residual peptide had the following amino acid analysis after acid hydrolysis for 24 hr. (residues/mol.): Thr (0.4), Glu (1.0), Ile (0.9). After a second cycle of Edman degradation free glutamic acid (0.6) was released. The peptide must therefore be:

Thr-Ile-Glu

The second peptide (25 cm. from the origin) showed only valine on amino acid analysis after acid hydrolysis for 48 hr. There was no free valine before acid hydrolysis, and one cycle of Edman degradation released free valine. The peptide must therefore be:

Val-Val

The third peptide (35 cm. from the origin) showed only isoleucine on amino acid analysis after acid hydrolysis for 48 hr. One cycle of Edman degradation also released free isoleucine. The peptide must therefore be:

Ile-Ile

(g) Fractions 86-90 (Fig. 5). The fractions were separated by paper chromatography. Free valine, isoleucine and arginine were found together with two peptides. The amino acid analysis of the first peptide after acid hydrolysis for 48 hr. was (residues/mol.): Ala (1.1), Phe (1.0). One cycle of Edman degradation released free phenylalanine. The peptide must therefore be:

Ala-Phe

The amino acid analysis of the second peptide after acid hydrolysis for 24 hr. was (residues/mol.): Ser (0.8), Phe (1.0). One cycle of Edman degradation released free serine. The peptide must therefore be:

Phe-Ser

(h) Fractions 93-97 (Fig. 5). These fractions contained small amounts of peptides, which were inseparable by paper chromatography, but mainly consisted of valine and isoleucine.

(i) Fractions 98-104 (Fig. 5). These fractions contained a mixture of amino acids and dipeptides, which were not separable by paper chromatography.

(l) Fractions 111-116 (Fig. 5). These fractions contained *S*-carboxymethylcysteine, glycine, serine and threonine.

Fig. 6. Schematic summary of the degradation of peptide (I), the radioactive peptide obtained from the tryptic digest of bromo[2-¹⁴C]acetic acid-inhibited and aminoethylated papain. The sequence of the thirteen previously unknown residues is shown in **bold type**. The active-site cysteine (shown as CMCys, *S*-carboxymethylcysteine) is indicated by an asterisk (*). AECys, *S*-aminoethylcysteine.

Fig. 7. Amino acid sequence of papain.

DISCUSSION

The proximity of the thirteen amino acid residues 29–41 inclusive to the active-site cysteine-25 in the primary sequence of papain greatly facilitated the isolation of these unknown residues. The active-site cysteine-25 was labelled by inhibition of papain with radioactive bromoacetic acid. The tertiary structure of the inhibited enzyme was opened, the disulphide bridges were reduced with mercaptoethanol and aminoethylated, and the protein was digested with trypsin. The tryptic digest was taken up in aq. 50% (v/v) acetic acid (it formed a gel in aqueous solution) and chromatographed on Sephadex G-25. All the radioactivity was eluted at the front (i.e. it emerged immediately after the void volume of the column). When the radioactive material was chromatographed again on Sephadex G-50 (Fig. 1) most of the radioactivity was eluted at the front. The radioactive peptide must therefore have an apparent molecular weight greater than 10^4 , and was presumably aggregating. It was not possible, however, to break the aggregate in 6M-guanidinium chloride or 8M-urea solution. Paper chromatography showed no contaminating peptides. The other main radioactive peak (fractions 76–86, Fig. 1) was separated from several non-radioactive peptides on paper and then chromatographed again on Sephadex G-50 (Fig. 2). Elution of radioactive material at the front clearly indicated that further aggregation had occurred. The amino acid analysis of this material was essentially indistinguishable from that of the material eluted at the front from the first Sephadex G-50 column (Fig. 1 and Table 1). It was also completely compatible with the amino acid sequence of papain from residues 18–28 inclusive and 42–58 inclusive, leaving exactly thirteen residues, assuming the presence of only one tryptophan residue. The amino acid composition of the unknown sequence was thus established, and the overlap on the *N*- and *C*-termini confirmed the revised amino acid sequence proposed by Drenth *et al.* (1968). The peptide (I) (Fig. 6) was obtained in at least 60% yield.

To facilitate the determination of the sequence of the thirteen unknown residues, this large peptide (I) (41 residues) was first digested with pepsin, which removed eleven residues from the *C*-terminus. The hexapeptide and pentapeptide removed were isolated (Fig. 3) and their amino acid compositions confirmed (Table 2). The radioactive peptide (II) was eluted at the front from a Sephadex G-25 column (Fig. 3), and its amino acid analysis confirmed the composition of the unknown sequence (Table 2).

Digestion of the radioactive peptide (II) with 0.4M-hydrochloric acid at 110° for 5 hr. removed

residues from both the *N*- and *C*-termini, leaving a radioactive peptide (III) containing only twenty amino acid residues (Table 3, assuming one tryptophan residue). Again it was eluted at the front from a Sephadex G-25 column (Fig. 4). A dipeptide and a tripeptide were also obtained after further purification by paper chromatography (Table 3 and Fig. 6).

The unknown sequence of thirteen residues was now contained in the radioactive peptide (III), but this peptide was so insoluble in aqueous solution that enzymic digestion could not be achieved. Instead the peptide was digested with 6M-hydrochloric acid, in which it was soluble, at 110° for 2 hr. The digest was chromatographed on Sephadex G-25 in aq. 50% (v/v) acetic acid (Fig. 5). The only radioactive fractions were free *S*-carboxymethylcysteine and the radioactive peptide (IV), now containing only seventeen amino acid residues, including the sequence of thirteen unknown residues. The other peptide-containing fractions were chromatographed on paper, from which ten peptides were isolated (Fig. 6). Determination of the complete sequence of some and the partial sequence of other peptides led to the assignment of serine to residue 29, but no overlap was obtained between this residue and the *N*-terminus of either of the two hexapeptide sequences, Ala-Val-Val-Thr-Ile-Glx and Gly-Ile-Ile-Lys-Ile-Arg, or between these hexapeptide sequences. Peptide (IV) was therefore treated with carboxypeptidase A and carboxypeptidase B, but no amino acid was released. If, however, peptide (IV) was kept at 95° for 24 hr. in buffer containing sodium dodecyl sulphate and then cooled rapidly to 25°, and carboxypeptidase B was added, arginine and a smaller proportion of isoleucine were released. The sequence of residues 29–41 was therefore established (Fig. 6), so completing the amino acid sequence of papain (Fig. 7). This sequence has an additional residue (Val-130) from the published sequence (Drenth *et al.* 1968) and arises from a reinterpretation of the refined electron-density map (Drenth, Jansonius, Koekoek, Sluyterman & Wolthers, 1969); residue 73 is now glutamine rather than glutamic acid (E. L. Smith, unpublished work). The papain molecule therefore has 212 amino acid residues. The active-site cysteine residue remains as residue 25, but the active-site histidine residue becomes residue 159.

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