destroy nitroxide radicals covalently attached to positions 4 or 5 of the pyrimidine bases.

Registry No. DM, 20830-81-3; $(1s^4U,C)_n$, 104532-53-8; (DUAT, dT-dA)$_n$, 104548-86-9; (DUAP, dT)$_n$, 104532-54-9.

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Disulfide Bond Formation between the Active-Site Thiol and One of the Several Free Thiol Groups of Chymopapain

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ABSTRACT: Chymopapain, a cysteine protease of papaya latex, has been purified with the use of fast protein liquid chromatography. Two homogeneous fractions were analyzed for thiol content and thiol reactivity. It was found that peak 1 and peak 2 contained two and three thiol groups, respectively, per mole of enzyme. This result is inconsistent with the general belief that chymopapain contains one essential and one nonessential thiol group and suggests that a significant portion of the thiol groups was oxidized in the previous preparations. Such an oxidation can account for some of the inconsistent results reported in the literature. An irreversibly oxidized nonessential thiol group may modify the catalytic function of chymopapain especially if it is close to the active site. That one thiol group resides indeed in the vicinity of the essential thiol group is clearly demonstrated by the biphasic reactions of chymopapain with disulfide compounds such as 2,2′-dipyridyl disulfide and 5,5′-dithiobis(2-nitrobenzoate). In the first step of these reactions a mixed disulfide is formed between the enzyme and the reactant, which is followed by a first-order, intramolecular reaction leading to the liberation of the second half of the disulfide compound. Furthermore, on addition of one Hg^{2+} ion, 2 mol of thiol group, one essential and one nonessential, disappears concomitantly. Formation of a disulfide bond between the catalytically competent thiol group and another free thiol group of chymopapain under physiological conditions may be of regulatory importance.

Chymopapain has been distinguished from the other cysteine proteases of papaya latex by its additional thiol group and classified as a dithiol cysteine protease (Brocklehurst & Salih, 1983). In the course of chromatography of papaya latex it is eluted from a cation-exchanger column as a broad peak between papain and papaya proteinase A. The broad chymopapain peak can be separated into several fractions, all containing essential and nonessential thiol groups, but less than

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two thiol groups per mole of enzyme. The first preparation that contained two thiol groups per mole of chymopapain was produced from the least basic component of the broad peak with further purification on agarose–mercurial and thiol–Sepharose columns (Khan & Polgár, 1983). The origin of the heterogeneity of chymopapain has not yet been established. Presumably, proteolytic cleavages and various levels of oxidation of the thiol groups, as well as chymopapains encoded by different genes, may account for the multiple enzyme forms (Polgár, 1984; Brocklehurst & Salih, 1985; Barrett & Buttle, 1985). Heterogeneity in the thiol groups is supported by our preliminary analysis of the SH peptides of chymopapain, which indicated that there are at least two other cysteine peptides in addition to the active-site cysteine peptide (L. Polgár and M. Sajgó, unpublished result).

By means of high-performance protein chromatography, such as the FPLC[1] system, distinct peaks of homogeneous chymopapain have recently been obtained, but the thiol content and thiol reactivity of the small amount of the enzyme produced have not been studied (Buttle & Barrett, 1984; Zucker et al., 1985). Therefore, we have undertaken to prepare homogeneous chymopapain by FPLC chromatography in a quantity sufficient for studying the reactivity characteristics of its thiol groups. For these studies we have employed the disulfide compound, PDS, which is known to react specifically with the catalytic thiol group (SH_C) at pH 4 and with the total amount of the thiol groups (SH_T) at pH 8 [cf. Brocklehurst (1982)]. This compound can be used to differentiate the two types of chymopapain, forms A and B, since the pH dependence and the value of the rate constants are different for the two forms [see, e.g., Brocklehurst et al. (1984)]. Our results indicate that chymopapain is not a two-thiol-containing enzyme as it was classified, but exhibits more than two free thiol groups, one of which is close to the active-site thiol group so that a disulfide bond between them can be formed.

Experimental Procedures

Materials. Chymopapain was purified from papaya latex supplied by Sigma (papain, type I, crude) according to the procedure described earlier (Khan & Polgár, 1983) except that the last step, thiol–Sepharose chromatography, was omitted. Instead, the mercuric salt of the enzyme was purified by FPLC (Pharmacia system) at high pH. We adapted the method of Buttle and Barrett (1984) with slight modifications as follows. Mercurichymopapain was activated by 20 mM cysteine at pH 5.0 for about 20 min. Then the enzyme solution was gel-filtered on a Sephadex G-25 column in a 0.01 M phosphate buffer, pH 8.0, containing 1 mM EDTA. A sample of 200 μL of about 0.5 mM solution of the enzyme was applied to a Mono S HR 5/5 (cation-exchange) column, which had previously been equilibrated with 0.008 M sodium tetraborate, pH 9.5, containing 1 mM DTE and 1 mM EDTA (buffer A). After injection of the sample, the column was reequilibrated with 5 mL of buffer A, and a linear gradient of NaCl was developed with buffer B (0.4 M NaCl in buffer A) at a flow rate of 1.1 mL/min. At 10% B (0.040 M NaCl) elution was continued at the same NaCl concentration until the total protein was practically eluted from the column. The fractions were collected in tubes containing 0.5 mL of 1.5 M acetate buffer, pH 5.0, 1 mM DTE, and 1 mM EDTA, sufficient to adjust the pH of the eluted enzyme to 5.0. Usually several identical runs were performed with collection into the same set of tubes. The peaks were concentrated about tenfold on an Amicon UM-10 membrane, and small molecular weight thiol compounds were removed by gel filtration on Sephadex G-25. The main fraction (peak 1 on Figure 1A) was about 30% of the protein applied to the column and gave pure chymopapain on rechromatography in the above system. It contained 0.97 ± 0.05 SH_C and 1.9 ± 0.1 SH_T per mol of protein as determined by PDS titrations at pH 4.0 and 8.0, respectively. This material was used in all experiments except when otherwise stated.

Enzyme Assays. The reactions with *N*-benzoyl-L-arginine ethyl ester and *N*-Z-phenylalanylglycine ethyl ester were followed under pseudo-first-order conditions, i.e., at substrate concentrations much less than K_m. The apparent first-order rate constant obtained was divided by the active enzyme concentration to obtain the second-order rate constant, $k_2/K_s = k_{cat}/K_m$ [cf. Bender and Kézdy (1965)]. The reaction with *N*-benzoyl-L-arginine ethyl ester was followed spectrophotometrically at 253 nm (Schwert & Takenaka, 1955). The hydrolysis of *N*-Z-phenylalanylglycine ethyl ester was measured in a Radiometer pH-stat system as described previously (Asbóth & Polgár, 1977). The reaction of Z-glycine *p*-nitrophenyl ester was followed spectrophotometrically at 340 nm at substrate concentrations around K_m. The second-order rate constant, k_{cat}/K_m, was then calculated from values of K_m and k_{cat} determined by computer fit of the experimental points to the hyperbolic form of the Michaelis–Menten equation.

Thiol Determinations with PDS. Samples were diluted in 0.1 M citrate, pH 4.0, and 0.1 M phosphate, pH 8.0, for titrations of SH_C and SH_T, respectively, with about a tenfold molar excess of PDS. For the determination of protein concentration, $A_{280}^{1\%} = 15.5$ and M_r 24 200, obtained by SDS–PAGE on gradient (4–26%) gels (not demonstrated), were used.

Reaction of Chymopapain with $HgCl_2$. Subequivalent portions of a 1 mM $HgCl_2$ stock solution were added to about 0.1 mM solution of the enzyme in 0.1 M phosphate buffer, pH 8.0. The concentration of $HgCl_2$ in the reaction mixture increased by 0.01–0.02 mM after each addition. The remaining free thiol groups were titrated with PDS.

Reaction of Excess Chymopapain with PDS. Portions of a 1 mM PDS stock solution were added to a ~20 μM solution of active chymopapain (2.0 SH_T per mole of protein) in a 0.1 M phosphate buffer, pH 8.0. Each addition resulted in a 5 μM increase of PDS concentration in the cell. The absorbancy at 343 nm was continuously recorded.

pH Dependency of Disulfide Interchange. About 0.1 mM enzyme solution in a 0.03 M citrate buffer, pH 4.0, was reacted with an equimolar concentration of PDS. The reaction was followed at 343 nm, and when it was completed, the reaction mixture was gel-filtered on a Sephadex G-25 column. The protein was then diluted into buffers of appropriate pH in the cell, and the change in absorbancy at 343 nm was recorded.

SDS–PAGE was performed on 15% slab gels (1 × 150 × 100 mm) by using the discontinuous buffer system of Laemmli (1970). The upper and lower buffer reservoirs contained 40 mM Tris-borate, pH 8.6, containing 0.1% (w/v) SDS, and 0.4 M Tris-HCl, pH 9.2, respectively. The samples containing chymopapain were precipitated at 5% trichloroacetic acid concentration, and allowed to stand for 15 min. After centrifugation, the precipitates were dissolved in 40 mM Tris-borate buffer, pH 8.6, containing sucrose (5% w/v), SDS (2% w/v), 0.6 M 2-mercaptoethanol, and Bromophenol Blue (0.005% w/v). Five to twenty-five microliters of this solution

[1] Abbreviations: DTE, dithioerythritol; EDTA, ethylenediaminetetraacetate; FPLC, fast protein liquid chromatography; Nbs_2, 5,5′-dithiobis(2-nitrobenzoate); PAGE, polyacrylamide gel electrophoresis; PDS, 2,2′-dipyridyl disulfide; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; Z, *N*-benzyloxycarbonyl.

(~0.5 mg/mL protein) was put into a well. A constant voltage of 100 V was applied until the sample penetrated the separation gel; then electrophoresis was continued at 200 V for 2–2.5 h. Gels were stained for 2 h in 0.05% (w/v) Coomassie Brilliant Blue R250 in methanol/acetic acid/water (5:1:4 by volume), and destaining was done in methanol/acetic acid/water (1:1:8 by volume).

Results and Discussion

Chromatography of Chymopapain on FPLC. In a recent study chymopapain was purified to homogeneity by two successive chromatographies on FPLC (Buttle & Barrett, 1984). The first run was carried out at pH 5.0 as in the usual carboxmethylcellulose or carboxymethyl-Sepharose chromatography. One selected peak of the most basic region (corresponding to chymopapain B) was then rechromatographed at pH 9.5. In the present purification we made the following modifications. (1) In order to obtain an appropriate amount of enzyme, the first chromatography at pH 5.0 was carried out on a carboxymethylcellulose column as described earlier (Khan & Polgár, 1983). (2) We selected the least basic component of the chymopapain peak (corresponding to chymopapain A) because this fraction had the highest thiol content. (3) Further purification was effected on an agarose–mercurial column. The thiol-containing enzymes bound to the column were eluted with $HgCl_2$ and stored in the mercuric form. At this stage of purification chymopapain is not homogeneous, as indicated by a gel electrophoretic pattern of the enzyme (Khan & Polgár, 1983). Since we have found that under the alkaline conditions employed with the FPLC chromatography chymopapain lost a significant amount of its thiol groups, we (4) added 1 mM DTE to all solutions used in the chromatographic procedure, and (5) the chymopapain fractions from the column were collected in tubes containing 1.5 M acetate buffer, pH 5.0, 1 mM DTE, and 1 mM EDTA that provided an appropriate medium for the purified enzyme. Under these conditions no significant loss in the thiol content of chymopapain was observed.

A typical elution profile of chymopapain is shown by Figure 1A. It is seen that only peaks 1 and 2 may serve as homogeneous protein fractions. Indeed, rechromatography of peak 1 under similar conditions exhibited a symmetrical peak (Figure 1B), indicating the homogeneity of chymopapain. For most of our studies this larger fraction was used.

Reaction of Chymopapain with PDS. For both peak 1 and peak 2, titration of the active-site thiol group with PDS at pH 4.0 always resulted in more than 1 equivalent of thiol group per mole of protein. Specifically, we obtained 1.15 ± 0.1 SH/mol for 12 different preparations by using $A_{280}^{1\%} = 18.3$ (Robinson, 1975) and M_r 24 200 (determined by SDS–PAGE). Consequently, this generally accepted value is not applicable for our homogeneous chymopapain preparation. Therefore, we have used, throughout this paper, $A_{280}^{1\%} = 15.5$, which gave rise to 0.97 ± 0.1 SH_C/mol of chymopapain.

For the total thiol content of peak 1 protein, 1.9 ± 0.1 SH_T/mol of chymopapain was found, as anticipated for a good dithiol enzyme preparation. For the peak 2 protein, however, we obtained 2.7 ± 0.2 SH_T mol of chymopapain, a high value not measured so far. This suggests that chymopapain contains more than two free thiol groups, in agreement with other findings to be discussed later.

The above thiol titrations were performed with an excess of PDS. When the reaction conditions were changed so that PDS was added to an excess of enzyme at pH 8.0, a biphasic curve, as shown in Figure 2, was obtained. The first phase was instantaneous in the conventional spectrophotometric assay, and the second phase followed a first-order kinetics. It is seen from Table I that 2-thiopyridone liberated instantaneously is equivalent to the amount of PDS added, and the second part of 2-thiopyridone liberated amounts to a similar quantity. In other words, from 1 mol of PDS 2 mol of 2-thiopyridone are formed. The two-step liberation of 2-thiopyridone can be explained in terms of disulfide interchange: the mixed disulfide formed in the reaction between PDS and one thiol group in the fast step reacts with another free thiol

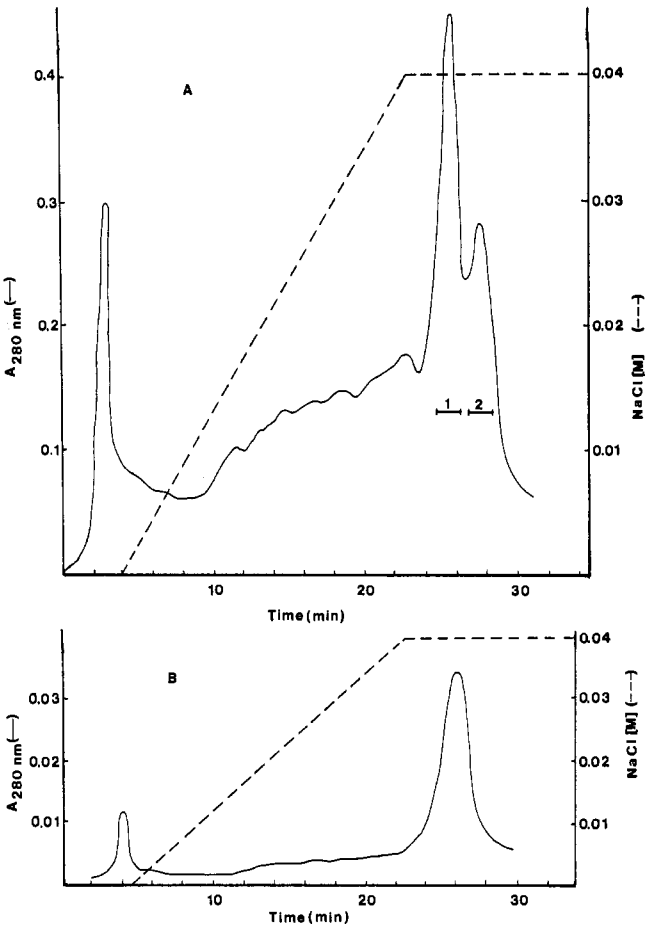

FIGURE 1: FPLC chromatography of chymopapain on Mono S with a NaCl gradient in 0.008 M sodium tetraborate, pH 9.5. (A) Approximately 2 mg of chymopapain was applied to the column as described under Experimental Procedures. Fractions corresponding to the bars were pooled and analyzed further. (B) Chromatography of peak 1 from Figure 1A in the same system.

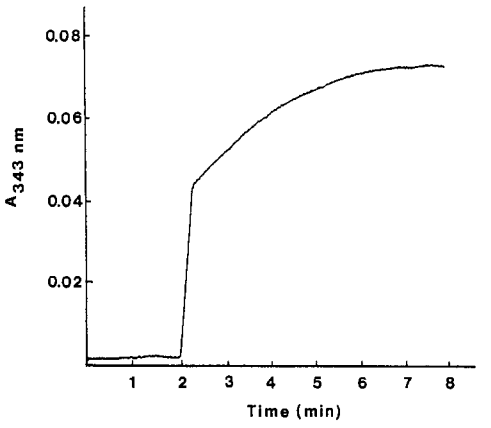

FIGURE 2: Liberation of 2-thiopyridone on addition of PDS to an excess of chymopapain at pH 8.0. To a 20 μM solution of the enzyme in 0.1 M phosphate buffer, pH 8.0, 4.5 μM PDS was added. Absorbance at 343 nm was recorded, starting 2 min before addition of PDS.

Table I: Reaction of Chymopapain with Subequivalent Portions of PDS Added Repeatedly[a]

PDS added (μM)	2-thiopyridone liberated in phase 1 (μM)	2-thiopyridone liberated in phase 2 (μM)	SH_T[b] (μM)	SH_C[c] (μM)
0			38	19
5.0	5.0	5.0		
+5.0	5.0	5.0	17	10
+5.0	4.9	4.8		
+5.0	4.2	4.2	0	0
+5.0	0	0		

[a] At pH 8.0, as described under Experimental Procedures. Data are the averages of two separate experiments where the values varied less than 10%. [b] Determined from PDS titration at pH 8.0. [c] Determined from PDS titration at pH 4.0.

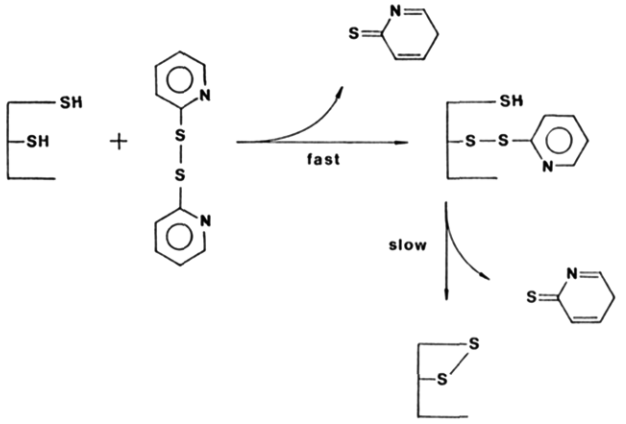

FIGURE 3: Scheme of the reaction of PDS with an excess of chymopapain.

in the subsequent slow step (Figure 3). As it is seen from Table I, in the course of disulfide interchange the decrease in the concentration of SH_T is twice that of PDS added, while the decrease in the concentration of SH_C is equal to the PDS added; i.e., the concentrations of the catalytically essential and nonessential thiols decrease in a parallel way. This implies that one type of thiol group reacts in the fast step, the other in the slow, and the disulfide bond formed involves the active-site thiol of chymopapain. The involvement of the active-site thiol in the disulfide interchange is substantiated by the observation that the enzyme after complete disulfide interchange is inactive, but catalytic activity can be restored on incubation with DTE.

Intramolecularity of Disulfide Interchange in Chymopapain. That the second, slow reaction between PDS and chymopapain is an intramolecular process (Figure 3) is indicated by several lines of evidence.

(1) The reacton is true first order, independent of the concentration of chymopapain within a fivefold range (Table II). Were the reaction intermolecular, its rate should be proportional to the concentration of the enzyme in excess. The finding that at a higher concentration of the mixed disulfide, i.e., at a threefold increase in PDS concentration, the rate constant does not increase (cf. Table II) rules out the possibility that two molecules, each containing a mixed disulfide and a free thiol group, would react with each other, so that the mixed disulfide of one molecule reacts with the free thiol group of the other.

(2) If the disulfide interchange takes place between two chymopapain molecules within a preformed dimer, a first-order rate constant may also be obtained, though the resulting disulfide bridge would be intermolecular. This possibility is excluded by the results obtained with SDS–PAGE. As seen

Table II: Effect of Changes in the Concentration of PDS and Chymopapain on the Rate of Disulfide Interchange[a]

SH_T (μM)	PDS (μM)	$10^3 \times k$ (s^{-1})
5.2	2.6	6.7 ± 0.8
26	2.6	7.8 ± 0.8
30	8	6.4 ± 0.8

[a] Measured at pH 8.0, as described under Experimental Procedures.

FIGURE 4: SDS–PAGE of chymopapain on 15% gel before and after disulfide interchange reaction with PDS: (a) chymotrypsinogen A; (b) chymopapain of peak 1, 10 μL; (c) chymopapain of peak 1, 5 μL; (d) chymopapain of peak 1 reacted with equimolar PDS after complete disulfide exchange, 5 μL; (e) the same as on lane d but the sample buffer contained no mercaptoethanol; (f) chymopapain of peak 2, 10 μL; (g) chymopapain of peak 2, 5 μL; (h) ovalbumin; (i) the same as on lane d, 10 μL; (j) the same as on lane e, 10 μL; (k) low molecular weight standard (Pharmacia) consisting of (from bottom upward) α-lactalbumin of M_r 14 400, soybean trypsin inhibitor of M_r 20 100, carbonic anhydrase of M_r 30 000, ovalbumin of M_r 43 000, bovine serum albumin of M_r 67 000, and phosphorylase *b* of M_r 94 000.

from Figure 4, native chymopapain (lanes b and c) and chymopapain that has undergone disulfide interchange (lane d) run together, and neither displays a molecular mass characteristic of a dimer. When chymopapain with interchanged disulfide bond was applied under nonreducing conditions (lane e), the apparent molecular mass was not appreciably different from that run under reducing conditions (lane d). This clearly shows that disulfide bond formation is not intermolecular.

(3) By using another technique, gel filtration on a Sephadex G-75 column, we could not find any protein fraction that would indicate the formation of a chymopapain dimer (not demonstrated). Consequently, the second phase of the reaction between chymopapain and PDS can only be interpreted in terms of an intramolecular disulfide bond formation.

pH Dependence of the Disulfide Interchange. The mechanism of disulfide interchange can be analyzed by determining its pH dependence. Figure 5 shows the pH rate profile of the disulfide interchange between a free thiol group of chymopapain and the mixed disulfide formed with the catalytic thiol group. The experimental points conform to a theoretical curve calculated for the dissociation of an ionizing group with a pK_a of 8.25. This value is characteristic of the pK_a of an ordinary cysteine residue not significantly affected by the protein environment. Accordingly, the disulfide interchange reaction is controlled by the dissociation of the reacting thiol group of chymopapain.

Reaction of Chymopapain with Nbs_2. PDS is a special reagent of cysteine proteases, inasmuch as it reacts with the active-site thiol group at an unusually high rate at low pH. This high reactivity may be partly accounted for by the protonation of one of the pyridine rings of PDS. Another chromogen thiol reactant of disulfide nature, Nbs_2, possesses

Table III: Decrease of Thiol Content of Chymopapain on Repeated Addition of $HgCl_2$[a]

portions of $HgCl_2$ added (μM)	SH_T		SH_C	
	concn (μM)	decrease (μM)	concn (μM)	decrease (μM)
0	157		86	
15	133	24	70	16
+15	100	33	56	14
+15	70	30	41	15

[a] Reaction was carried out at pH 8.0, as described under Experimental Procedures. Concentration of chymopapain: 80 μM. The data are averages of two separate experiments where the values varied less than 10%.

negatively rather than positively charged groups and does not show the enhanced reactivity toward cysteine proteases. Therefore, we examined whether the disulfide interchange can occur also with this normal reactant. We have found no significant difference between PDS and Nbs_2 in this respect. Disulfide interchange with Nbs_2 does take place, and the rate constant measured at pH 8.0 was found to be similar (8.1×10^{-3} s^{-1}) to that obtained with PDS (8.8×10^{-3} s^{-1}). The relationship of the reactivity of the two disulfide compounds is also similar in their reactions with cysteine.

Reaction of Chymopapain with Hg^{2+} Ions. A further evidence that supports the proximity of two thiol groups in chymopapain comes from the reaction between the enzyme and $HgCl_2$. Table III shows that on addition of subequivalent portions of Hg^{2+} ions one inhibitor ion abolishes two thiol groups as measured with PDS at pH 8.0. Similar to the disulfide interchange reaction, the active-site thiol and another free thiol group disappear simultaneously. This is consistent with cross-linking of the two thiol groups by the Hg^{2+} ions. The intramolecularity of the reaction is supported by gel chromatography demonstrating the same apparent molecular mass for chymopapain with and without the Hg^{2+} ion (not shown). When the monothiol enzyme, papain, was similarly reacted with Hg^{2+} ions, the increase in molecular mass resulting from the intermolecular cross-link was observed (Sluyterman et al., 1977). Hence, it is not likely that dimer formation in our case would remain undetected.

Thiol Groups of Chymopapain. The FPLC-purified chymopapain of peak 2 contains 3 thiol groups/mol, which is inconsistent with the previous classification of the enzyme as a dithiol protease (Brocklehurst & Salih, 1983). The higher thiol content of peak 2 may not result from the reduction of a disulfide bridge of native chymopapain during the chromatographic process. This is supported by the fact that the presence of the enzyme form with three thiol groups is independent of the reducing strength of the chromatography media. Specifically, variation of the concentration of DTE or the less effective reducing agent 2-mercaptoethanol between 0.1 and 1 mM did not affect the thiol contents of peaks 1 and 2.

Despite their different thiol contents, peak 1 and peak 2 enzyme forms represent the same type of chymopapain. This is indicated by the fact that the rate constant for the reaction

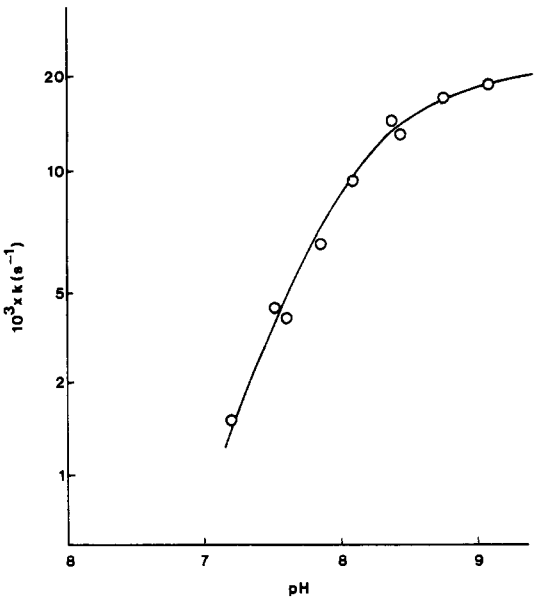

FIGURE 5: pH dependence of disulfide interchange reaction in chymopapain. The buffers used were 0.2 M phosphate and 0.2 M borate. The pK_a of the theoretical curve is 8.25.

of SH_C of both enzymes with PDS is practically pH-independent between pH 4 and 5.5 (approximately 2000 m^{-1} s^{-1}), which is characteristic of chymopapain A (Brocklehurst et al., 1984). However, as seen from Table IV, the two enzyme forms differ in catalytic abilities from each other as well as from chymopapain purified by thiol–Sepharose chromatography (Khan & Polgár, 1983). It may be surprising that two similar enzyme forms, not easily separable even by FPLC, exhibit considerable differences in their rate constants. This questions the reliability of all studies to date pertinent to the catalytic properties of chymopapain.

That chymopapain contains more than one nonessential thiol is supported by two additional pieces of evidence. (1) From a peptic hydrolyzate of chymopapain carboxymethylated on its essential thiol group, we have isolated two different SH peptides (L. Polgár and M. Sajgó, unpublished result) by a method described previously (Polgár & Sajgó, 1981). (2) A preliminary amino acid sequence of chymopapain has recently been reported (Lynn et al., 1985). This shows a distribution of cysteine residues in chymopapain different from that found in papain and actinidin, both enzymes possessing three disulfide bridges. If similar steric structures for the three enzymes are assumed, chymopapain may form only one disulfide bridge that involves residues 22 and 63, because the corresponding pairs of Cys-56 and Cys-153 (papain numbering) are not cysteine residues in chymopapain. However, chymopapain possesses an additional cysteine, Cys-117, which is absent from papain. Thus, chymopapain appears to contain three nonessential thiols. In fact, the two SH peptides we have isolated from the peptic hydrolyzate of chymopapain perfectly conform to the amino acid sequence around Cys-117 and Cys-153, respectively. Specifically, a heptapeptide containing Lys, Arg,

Table IV: Second-Order Rate Constants of Acylation of Chymopapains[a]

	Z-glycine *p*-nitrophenyl ester[b] (M^{-1} s^{-1})	*N*-benzoyl-L-arginine ethyl ester[c] (M^{-1} s^{-1})	*N*-Z-phenylalanylglycine ethyl ester[d] (M^{-1} s^{-1})
peak 1	140000 ± 10000	15 ± 3	9000 ± 800
peak 2	380000 ± 20000	20 ± 3	35000 ± 3000
chymopapain[e]	45000 ± 4000	28 ± 3	10000 ± 1000

[a] At pH 6.3, 25 °C, as described under Experimental Procedures. [b] In 0.1 M phosphate buffer; enzyme concentration about 0.5 μM, substrate concentration range 5–50 μM. [c] In 0.1 M phosphate buffer; enzyme concentration about 10 μM, substrate concentration 0.1 mM. [d] In 0.2 M KCl; enzyme concentration about 0.5 μM, substrate concentration 50 μM. [e] Prepared according to Khan and Polgár (1983).

Val, Pro, Ser, Asx, and Ser has the same amino acid composition as that of the chymopapain portion encompassing residues 111-117; an octapeptide containing Gly, Pro, Cys, Gly, Thr, Lys, Leu, and Asx fits exactly the polypeptide segment holding residues 151-158 (papain numbering).

The occurrence of two or three nonessential thiol groups in chymopapain with one of them close to the active site can account for some of the heterogeneity of the enzyme and the contradictory results on thiol reactivity (Brocklehurst et al., 1984; Polgár, 1984). In the different enzyme preparations the nonessential thiol groups can be oxidized to various extents. Indeed, before FPLC purification we have also found chymopapain containing 1.0 SH_C and 2.0 SH_T, which did not show a complete disulfide interchange presumably because the necessary thiol group was partially oxidized. It is remarkable that the thiol group involved in the disulfide bond formation with the active-site thiol is present in both our peak 1 and peak 2 chymopapains. Structural analysis is in progress to determine the cysteine residue implicated in the disulfide bond formation. It may be of regulatory significance that the disulfide form of chymopapain can serve as an inactive "proenzyme" that becomes active on reduction.

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Registry No. PDS, 2127-03-9; Nbs_2, 69-78-3; Z-glycine *p*-nitrophenyl ester, 1738-86-9; *N*-benzoyl-L-arginine ethyl ester, 971-21-1; *N*-Z-phenylalanylglycine ethyl ester, 2778-34-9; chymopapain, 9001-09-6.

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Amino Acid Sequence of the a Subunit of Human Factor XIII†

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ABSTRACT: Factor XIII is a plasma protein that plays an important role in the final stages of blood coagulation and fibrinolysis. The complete amino acid sequence of the a subunit of human factor XIII was determined by a combination of cDNA cloning and amino acid sequence analysis. A λgtll cDNA library prepared from human placenta mRNA was screened with an affinity-purified antibody against the a subunit of human factor XIII and then with a synthetic oligonucleotide probe that coded for a portion of the amino acid sequence present in the activation peptide of the a subunit. Six positive clones were identified and shown to code for the a subunit of factor XIII by DNA sequence analysis. A total of 3831 base pairs was determined by sequencing six overlapping cDNA clones. This DNA sequence contains a 5′ noncoding region or a region coding for a portion of a pro-piece or leader sequence, the mature protein (731 amino acids), a stop codon (TGA), a 3′ noncoding region (1535 nucleotides), and a poly(A) tail (10 nucleotides). When the a subunit of human factor XIII was digested with cyanogen bromide, 11 peptides were isolated by gel filtration and reverse-phase HPLC. Amino acid sequence analyses of these peptides were performed with an automated sequenator, and 363 amino acid residues were identified. These amino acid sequences were in complete agreement with those predicted from the cDNA. The a subunit of factor XIII contained the active site sequence of Tyr-Gly-Gln-Cys-Trp, which is identical with that of tissue translguaminase. Six potential Asn-linked carbohydrate attachment sites are present in the a subunit. At least three of these sites have little or no carbohydrate as determined by amino acid sequence analysis. Little or no significant homology to other proteins was observed by computer-assisted amino acid sequence analysis employing the Dayhoff protein sequence data base.

Factor XIII (fibrin stabilizing factor, fibrinoligase, or plasma transglutaminase) is a plasma glycoprotein that circulates in blood as a proenzyme. During the final stages of blood coagulation, thrombin converts the proenzyme to an active form called factor $XIII_a$. Factor $XIII_a$ is a transglutaminase that catalyzes the cross-linking of fibrin monomers through the formation of intermolecular ε-(γ-glutamyl)lysine bonds. This

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