Cleavage of One Specific Disulfide Bond in Papain*

EMMANUEL SHAPIRA AND RUTH ARNON

From The Department of Chemical Immunology, The Weizmann Institute of Science, Rehovot, Israel

SUMMARY

Conditions leading to cleavage of all of the disulfide bridges in ribonuclease and several other proteins (0.32 M 2-mercaptoethanol in 8 M urea) caused in papain only partial reduction of the disulfide bonds. Electrophoretic studies indicated that alkylation of the partially reduced derivative yielded a unique molecular species (3-RCM papain) in which one specific disulfide bond had been split, rather than a mixture of unreduced and open chain papain. On the other hand, reduction of papain in 0 M guanidine hydrochloride, followed by alkylation, yielded a completely open polypeptide mixture of unreduced and open chain papain. On the other hand, reduction of papain in 6 M guanidine hydrochloride, followed by alkylation, yielded a completely open polypeptide chain (7-RCM papain). Alkylation of native papain after reduction in aqueous solution resulted in the sole blocking of the active sulfhydryl group (1-RCM papain). Immunological interactions with antipapain serum indicated identity between 1-RCM papain and native papain, whereas 7-RCM papain was completely unreactive. 3-RCM papain cross-reacted with 60% of the antibodies. The single cleaved disulfide bond in 3-RCM papain was identified by isolation and analysis of the peptides containing carboxymethylcysteine, obtained from a tryptic digest. It was shown to be the bond connecting the cysteine residues in positions 43 and 152 in the amino acid sequence of papain. It was also shown that during the reduction of native papain in 8 M urea autodigestion takes place. This phenomenon was avoided when 1-RCM papain was reduced instead of the native enzyme; in both cases the product of reduction and alkylation was 3-RCM papain.

A native protein molecule has a unique conformation which is dictated by the intramolecular forces stemming from the primary sequence (1, 2) and this conformation is stabilized by the intrachain disulfide bonds. The role of the disulfide cross-linkages in maintaining the three-dimensional molecular configuration and their prime importance for the biological activity has been shown for many proteins. Thus, the total reduction of the S–S bonds brings about complete loss of the catalytic activity in several enzymes (3–7) and abolishes all of the immunological activity of immunoglobulins, both as antibodies and as antigens (8–10).

Not all of the disulfide bonds present in a molecule are equally important for the three-dimensional structure, and some of them are nonessential for preservation of the biological functions. It has been shown that in several proteins, such as immunoglobulins (11, 12), ribonuclease (3), and lysozyme (13, 14), partial opening of the disulfide bonds does not significantly affect the biological activity. In a recent study (15), it was reported that in ribonuclease the selective opening of two out of the four disulfide bonds led to a unique molecular species which retained the full catalytic activity toward RNA, was more active than native ribonuclease toward cytidine 2′,3′-cyclic phosphate, and was immunologically indistinguishable from the native enzyme.

In all of the studies mentioned above, the conditions used in the partial reduction were relatively mild, mostly comprising the reducing agent dissolved in buffered media in the absence of urea. Thus, papain retains its catalytic activity in water-methanol solutions, and only when the concentration of methanol is raised to 50% or above is a drop in activity observed (16). In 8 M urea, only slight changes in conformation could be detected by optical rotatory dispersion and viscosity measurements. Moreover, the catalytic activity of papain was also preserved in the presence of 8 M urea. On the other hand, more drastic denaturing conditions such as 6 M guanidine or trichloracetic acid were found to cause complete and irreversible inactivation of papain (16, 17). In view of the stability of papain in 8 M urea, the question arose whether this medium is sufficient for inducing in the papain molecule the conformational changes which are probably essential for a full reduction, and thus whether reduction of papain in 8 M urea will lead to a fully reduced derivative, as was the case in many other proteins.

The results presented in this report show that only partial reduction of native papain is attained in 8 M urea, involving the opening of only one specific disulfide bond. On the other hand,
if the papain is previously denatured by stronger agents, its subsequent reduction in 8 M urea leads to complete rupture of all three disulfide bonds.

**EXPERIMENTAL PROCEDURE**

**Materials**

Papain (twice crystallized, Lots 6HP, 6DB, 7CE, 7FA, and 7KA), chymopapain (Lots 5541), and trypsin (twice crystallized, lyophilized, Lot TRL 6263) were purchased from Worthington. Benzoyl-L-arginine ethyl ester was obtained from Yeda Company, Rehovot. Sephadex G-25 coarse, G-25 fine, and C-10 were from Pharmacia. Urea (British Drug House, analytical reagent grade) and guanidine hydrochloride (Eastman, White Label) were recrystallized from 95% ethanol prior to use, and dried in a vacuum. Solutions of recrystallized urea and guanidine hydrochloride were prepared immediately before use. The 2-mercaptoethanol (Organic Research Chemicals, Ltd.) was purified prior to use by distillation in a nitrogen atmosphere. Dithiothreitol (Cleland’s reagent) was from Calbiochem. Iodoacetic acid (Fluka Chemicals, Switzerland) was crystallized from hot petroleum ether and stored in the dark. \(^{14}C\)-Labeled iodoacetic acid (2-\(^{14}C\)) was purchased from Amersham, England, and was crystallized in the same manner with excess of unlabeled material. The specific activity of the preparation used was 1.4 \(\times\) 10\(^5\) cpm per \(\mu\) mole. All other reagents were either reagent grade or the best grade available.

Antiserum against papain was prepared as described elsewhere (18).

**Methods**

**Reduction of Papain**—The reduction of papain was carried out either in aqueous solution, 8 M urea, or 6 M guanidine hydrochloride. The pH in all cases was adjusted to pH 8.2 with Tris-HCl buffer. In the case of \(H_2O\) or 8 M urea addition of 0.05 M Tris-HCl buffer to a final concentration of 0.05 M ethanol prior to use, and dried in a vacuum. Solutions of recrystallized urea and guanidine hydrochloride were prepared immediately before use. The 2-mercaptoethanol (Organic Research Chemicals, Ltd.) was purified prior to use by distillation in a nitrogen atmosphere. Dithiothreitol (Cleland’s reagent) was from Calbiochem. Iodoacetic acid (Fluka Chemicals, Switzerland) was crystallized from hot petroleum ether and stored in the dark. \(^{14}C\)-Labeled iodoacetic acid (2-\(^{14}C\)) was purchased from Amersham, England, and was crystallized in the same manner with excess of unlabeled material. The specific activity of the preparation used was 1.4 \(\times\) 10\(^5\) cpm per \(\mu\) mole. All other reagents were either reagent grade or the best grade available.

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**Alkylation of Reduced Papain**—Alkylation of the free sulfhydryl groups formed during the reduction was carried out by the addition of iodoacetic acid. The pH was then adjusted to pH 8.2 by adding a few drops of concentrated Tris solution. The mixtures were allowed to react for 30 min at 4°C, and were then chromatographed on a Sephadex G-25 (coarse) column, in 0.05 M ammonium bicarbonate. In several cases \(^{14}C\)-labeled iodoacetic acid was used.

Alkylated papain (1-RCM papain) and partially reduced and alkylated papain (3-RCM papain) were water-soluble after elution from the column, and remained so for several weeks if stored in solution 4°C. However, after lyophilization they could not be redissolved in aqueous solution, at pH range 6 to 9. The fully reduced and alkylated papain (7-RCM papain) was water-soluble in this pH range before as well as after lyophilization.

**Tryptic Digest of 3-RCM Papain**—3-RCM papain, 120 mg (approximately 6 \(\mu\)M) was dissolved in 3 ml of 8 M urea. To this solution 0.2 ml containing 1.2 mg of trypsin dissolved in 8 M urea was added. The trypsin was previously maintained in 8 M urea at pH 4.5 for 30 min to effect complete inactivation of the chymotrypsin (19). The pH were then brought to 9.1 in the pH-stat. Digestion was initiated by adding 9 ml of \(H_2O\) (dilution to 2 x urea, the concentration at which trypsin is active (19)). A voluminous white precipitate formed, which disappeared within 5 min. Alkali uptake (amounting to a total of 115 \(\mu\)moles) was very rapid in the first 20 min and ceased completely after 45 min. Addition of 0.3 mg more of trypsin had no measurable effect upon alkali uptake. The digest was stored in the frozen state and aliquots of it were subjected to column chromatography under various conditions.

**High Voltage Electrophoresis**—Electrophoresis was performed on Whatman No. 1 and No. 3MM papers at 30 volts per cm according to Katz, Dreyer, and Anfinsen (20) with pyridine-acetic acid-water buffers at pH 6.5 and 3.5. At pH 1.9, 98% formic acid (20.5 ml) and 99% acetic acid (74.5 ml) were mixed completely after 45 min. Addition of 0.3 mg more of trypsin had no measurable effect upon alkali uptake. The digest was stored in the frozen state and aliquots of it were subjected to column chromatography under various conditions.

**Electrophoresis in Polyacrylamide Gel**—This technique was carried out according to Davis (21). The sample containing 100 \(\mu\)g of protein in 0.1 ml of 8 M urea was applied to a polyacrylamide gel column 6 cm in length. A constant current of 4 ma per gel was passed for 75 min. The gels were then stained with 1% Amido black in 7% acetic acid.

**Enzymatic Activity Assay**—Papain activity was assayed by following the rate of hydrolysis of benzoyl-L-arginine ethyl ester (0.1 M) in a pH-stat as previously described (18).

**Immunochemical Assay**—Quantitative precipitin analyses with antipapain serum were carried out as previously described (18).

**Double Diffusion in Agar Gel**—According to Ouchterlony (22).

**Physical Methods**—Spectrophotometric measurements were made on a Zeiss model PMQII spectrophotometer, at approximately 25°C, with quartz cells of 1-cm light path. pH measurements, determination of alkaline consumption during trypsic digest of papain, or benzoyl-L-arginine ethyl ester hydrolysis by papain was carried out with an autotitrator (Radiometer, Copenhagen,
The reduction of papain was carried out in the different solvents, at pH 8.2. In the case of aqueous or 8 M urea solutions, addition of 0.5 M Tris-HCl buffer to a final concentration of 0.05 M was applied, whereas in the case of 6 M guanidine-HCl solid Tris powder was added. In all experiments, except No. 5, reduction was performed with 0.32 M 2-mercaptoethanol. Alkylation was carried out with either unlabeled or 14C-labeled iodoacetic acid.

<table>
<thead>
<tr>
<th>Experiment and starting material</th>
<th>Reduction medium</th>
<th>No. of independent preparations</th>
<th>Average yield(^a)</th>
<th>Carboxymethyl cysteine residues(^b)</th>
<th>Labeled iodoacetic acid bound</th>
<th>Designation of product</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Papain</td>
<td>0.05 M Tris buffer</td>
<td>7</td>
<td>91</td>
<td>0.63-0.88</td>
<td>0.78-1.1</td>
<td>1-RCM-papain</td>
</tr>
<tr>
<td>2. Papain</td>
<td>8 M urea</td>
<td>4</td>
<td>12</td>
<td>2.51-2.76</td>
<td></td>
<td>3-RCM-papain</td>
</tr>
<tr>
<td>3. Papain</td>
<td>6 M guanidine HCl</td>
<td>3</td>
<td>76</td>
<td>6.1-6.3</td>
<td></td>
<td>7-RCM-papain</td>
</tr>
<tr>
<td>4. 1-RCM-papain(^c) reduced with 0.03 M dithiothreitol</td>
<td>8 M urea</td>
<td>3</td>
<td>84</td>
<td>2.73-3.0</td>
<td>2.1</td>
<td>3-RCM-papain</td>
</tr>
<tr>
<td>5. 1-RCM-papain(^c) reduced with 0.03 M dithiothreitol</td>
<td>8 M urea</td>
<td>4</td>
<td>80</td>
<td>2.59-2.97</td>
<td>1.96-2.2</td>
<td>3-RCM-papain</td>
</tr>
<tr>
<td>6. Papain after exposure to guanidine HCl</td>
<td>8 M urea</td>
<td>2</td>
<td>72</td>
<td>6-2.6</td>
<td>1</td>
<td>7-RCM-papain</td>
</tr>
</tbody>
</table>

\(^a\) The yield of the reduced protein was calculated from the amount of protein recovered after alkylation and isolation on a Sephadex column.

\(^b\) Calculated from amino acid analyses, according to the average ratios to glutamic acid and alanine. The values in the table were not corrected for destruction during hydrolysis.

\(^c\) In Experiments 4 and 5, 1-RCM-papain was unlabeled, and only after its reduction was alkylation carried out with labeled iodoacetic acid. Only 2 out of the 3 carboxymethylcysteine residues in the product are, therefore, labeled.

Fig. 1. Catalytic activity of papain as a function of the time of incubation in various solvents, in pH 8.2 at 37\(^\circ\), in the presence or absence of reducing agent. ▲, aqueous solution (0.05 M Tris buffer); △, the same solvent in the presence of 0.32 M 2-mercaptoethanol; ○, in 8 M urea; □, in 8 M urea containing 0.32 M 2-mercaptoethanol; ■, in 6 M guanidine hydrochloride; □, in 6 M guanidine hydrochloride containing 0.32 M 2-mercaptoethanol. The activity in all cases was assayed by diluting the particular sample containing 75 \(\mu\)g of papain with 4 ml of a solution containing 0.1 M benzoyl-L-arginine ethyl ester, 0.005 M cysteine, and 0.002 EDTA, and following the rate of hydrolysis in the pH-stat.

model TTT 1), with a combined electrode. Radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer, model 3324.

Amino Acid Analyses—Amino acid analyses were performed on the Beckman-Spinco automatic amino acid analyzer, model 120B, after hydrolysis under reduced pressure in constant boiling hydrochloric acid for 22 hours at 110\(^\circ\) (23).

RESULTS

Reduction of Papain—Native papain was reduced by 2-mercaptoethanol under various conditions, namely, in an aqueous solution (0.05 M Tris buffer, pH 8.2), in 8 M urea, and in 6 M guanidine hydrochloride solutions. Similar papain solutions, in the absence of the 2-mercaptoethanol, served as controls. The six samples were incubated at 37\(^\circ\), and the extent of reduction was determined after alkylation of the products and assay of the resultant carboxymethylcysteine residues in the chromatographically separated proteins. The results are given in Table I. As shown, after reduction of papain in aqueous solution only one free sulfhydryl group was detected, presumably the one which is involved in the active site. When the reduction was performed in the presence of 6 M guanidine hydrochloride, all three disulfide bonds were disrupted. However, when the reduction was carried out in 8 M urea, only three carboxymethylcysteine residues were present in the derivative, indicating that only one disulfide bond per molecule was opened, on the average. All of these derivaties were enzymatically inactive because of the blocking of the active sulfhydryl group. As is also apparent from Table I, the yield of the reduced product in 8 M urea was very low, in contrast to the reduced and alkylated derivatives from the aqueous and guanidine solutions, which were obtained in high yield.

In parallel to the assessment of the extent of reduction, the effect of the various reducing conditions on the catalytic activity of papain was also measured. For this purpose 25-\(\mu\)l aliquots (containing 75 \(\mu\)g of enzyme) were taken from each reaction mixture after different incubation times and immediately diluted with 4 ml of a solution containing 0.1 M benzoyl-L-arginine ethyl ester, 0.005 M cysteine, and 0.002 EDTA. The catalytic activity was then measured by following the rate of hydrolysis of the benzoyl-L-arginine ethyl ester. The results are given in Fig. 1. The incubation of papain in buffered aqueous solution either in the absence or in the presence of 2-mercaptoethanol did not bring about any decrease in its enzymatic activity.

After exposure to 8 M urea, it was observed that in the absence of reducing agent the activity of papain was preserved in its full extent. Under reducing conditions, however, a gradual decrease
activity and, if at that stage it is dissolved in 8 M urea or even just suspended in water and reduced by 2-mercaptoethanol, it yields a completely reduced papain molecule (Experiment 6 in Table I).

The partially reduced and alkylated derivative of papain prepared by reduction in 8 M urea (denoted 3-RCM papain) was tested for homogeneity and compared to the other derivatives by electrophoresis in polyacrylamide gel (Fig. 4). As shown, each of the reduced and alkylated derivatives is a homogeneous material with distinct electrophoretic mobility. This proves that the 3-RCM papain is a unique derivative and not a mixture of fully reduced and unreduced derivatives of papain.

**Immunological Properties of Reduced and Alkylated Derivatives**

— All three reduced and alkylated derivatives, namely, 1-RCM papain, 3-RCM papain, and 7-RCM papain, were tested for their capacity to react with antiserum against native papain. In the quantitative precipitin test 1-RCM papain gave a precipitin curve which was identical with that of papain (Fig. 5), whereas the 3-RCM papain derivative gave a lower precipitin curve, showing a shift to the left in the equivalence zone. 7-RCM papain did not give any precipitation with the antiserum, and did not possess any capacity to inhibit the homologous precipitation of the antiserum with native papain. In double gel diffusion (Fig. 6), 3-RCM papain showed partial identity with native papain, as indicated by a spur formation. These experiments indicate that 1-RCM papain retains the full immunological reactivity of papain, 3-RCM papain contains only part of the antigenic deter-

in activity occurred over a period of several hours. After 3 hours only 13% of the original activity was left in the solution. A sample of this reaction mixture after 3-hour incubation was chromatographed on a Sephadex G-25 column in 0.1 M acetic acid (Fig. 2). The first peak containing the protein was obtained in a yield of only 12%, based on the total amount of protein in the reduction mixture applied to the column. The catalytic activity of this material after reoxidation was identical with that of native papain. The protein peak was followed by a second peak which, in addition to the urea and 2-mercaptoethanol, contained a large number of peptides of different sizes, as shown by paper electrophoresis (Fig. 3).

These findings led to the assumption that the cause for both the decrease in the enzymatic activity and the low yield of the reduced protein is the autodigestion of reduced papain in 8 M urea. This assumption was validated by performing the reduction in 8 M urea on the inactive papain derivative obtained after alkylating the free sulphydryl group (1-RCM papain), instead of on the native enzyme. In this case again only one disulfide bond per molecule was reduced, but autodigestion did not take place and the reduced and alkylated derivative containing 3 carboxymethylcysteine residues was obtained in the expected yield (Experiment 4 in Table I). In most of the later experiments dithiothreitol was used as reducing agent instead of 2-mercaptoethanol, with the same results (Experiment 5 in Table I).

The exposure of papain to 6 M guanidine HCl in the presence, or even in the absence, of reducing agent causes immediate and complete loss of enzymatic activity (Fig. 1). The changes that occur in the papain molecule under these conditions are irreversible—after exhaustive dialysis of the guanidine, whether directly against aqueous buffered solution or first against 8 M urea, the papain precipitates out of solution, does not regain its enzymatic activity

![Fig. 2. Gel filtration on a Sephadex G-25 column (2.8 x 55 cm) in 0.1 M acetic acid of the reduction product obtained after incubation of papain (150 mg) for 3 hours in 8 M urea and 0.32 M 2-mercaptoethanol. The fraction marked by the arrow was subjected to paper electrophoresis (see Fig. 3).](http://www.jbc.org/)

![Fig. 3. Paper electrophoresis (36 volts per cm) at pH 3.5 of a fraction obtained from the second peak of the gel filtration of reduced papain (marked by the arrow in Fig. 2).](http://www.jbc.org/)

![Fig. 4. Polyacrylamide gel electrophoresis (21) of the various reduced and alkylated derivatives of papain. A, a mixture of 1-RCM papain, 3-RCM papain and 7-RCM papain; B, 1-RCM papain C, 3-RCM papain; D, 7-RCM papain. See Table I for description of products.](http://www.jbc.org/)
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60 90 120

FIG. 5. Quantitative precipitin test of antipapain serum with papain (○—○); 1-RCM papain (△—△); 3-RCM papain (□—□) and 7-RCM papain (×—×). See Table I for description of the derivatives.

FIG. 6. Agar gel diffusion (22) of native papain (upper left well) and 3-RCM papain (upper right well) against antipapain serum (lower well).

minants, and the completely reduced and alkylated derivative has lost all of the antigenic determinants.

Identification of Reduced Disulfide Bond in 3-RCM Papain—The homogeneity of this derivative in electrophoresis shows that only one disulfide bond has been opened in each molecule. The following procedure was used for the identification of this bond. 1-RCM papain was reduced in 8 M urea by either 0.32 M 2-mercaptoethanol or 0.03 M dithiothreitol and alkylated with W-labeled iodoacetic acid. Monitoring of the radioactivity of this derivative indicated the presence of 2 moles of labeled carboxymethyl groups per mole of papain (see Table I). This derivative was digested by trypsin in 2 M urea at pH 9.1. An aliquot of the digest was applied to a Sephadex G-25 (fine) column in 0.05 M ammonium carbonate, pH 7.5, and the fraction containing the radioactive material, in which the recovery of radioactivity was 50%, was subjected to paper electrophoresis at pH 3.5 and pH 6.5. At both pH values only one of the peptides in the mixture was radioactive. The radioactive peptide obtained upon electrophoresis at pH 3.5 was eluted from the paper and its amino acid composition was determined; it is given in Table II (Peptide 1). From the fact that only one labeled peptide was recovered, and from the low yield of radioactivity, it was deduced that only one peptide out of the pair formed in the reduction was eluted from the column under these conditions.

A second aliquot of the tryptic digest of 3-RCM papain was applied to a Sephadex G-10 column in 0.01 M HCl (Fig. 7B). Two radioactive peaks were obtained with an over-all recovery of 86% of the total radioactivity applied to the column. The fractions of the first radioactive peak were pooled, applied to a Sephadex G-25 column in 0.05 M ammonium carbonate, pH 7.5, and yielded one radioactive peak (Peptide 2) which had a very similar amino acid composition to Peptide 1 (Table II). The second radioactive peak from the Sephadex G-10 column was rechromatographed on the same column, in the same eluent, and the narrow radioactive peak obtained was subjected to electrophoresis at pH 1.9. Out of the seven peptides present in this fraction only one was radioactive (Peptide 3), and after elution it was analyzed for its amino acid composition (Table II). The composition of Peptides 1 and 2 is in good agreement with

Fig. 7. A, chromatographic separation of the peptides obtained upon tryptic digestion of 5 μM 3-RCM papain in which only the cysteine residues composing the split disulfide bond were converted into 14C-labeled carboxymethylcysteine residues. The Sephadex G-25 (fine) column (3 X 150 cm) was equilibrated and eluted with 0.05 M ammonium bicarbonate, pH 7.5. The solid line designates the optical density, whereas the broken line expresses the radioactivity. The shaded fraction was further analyzed. B, chromatographic separation of 2 μM of the same peptide mixture described in A on a Sephadex G-10 column (0.9 X 150 cm) equilibrated with 0.01 M HCl. The shaded fractions were pooled and rechromatographed on the same column, to yield a much narrower radioactive peak at the same position.
TABLE II

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Peptide 1</th>
<th>Peptide 2</th>
<th>Calculated for 136-153</th>
<th>Peptide 3</th>
<th>Calculated for 42-44</th>
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<tbody>
<tr>
<td>Lys</td>
<td>1.91</td>
<td>1.75</td>
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<td>1.18</td>
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<td>Tyr</td>
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<td>Phe</td>
<td></td>
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</tbody>
</table>

that of the peptide containing cysteine residue 152 in the amino acid sequence of papain, and that of Peptide 3 agrees with the sequence Tyr-Cys-Arg of the peptide containing cysteine residue 43 (24). The only disagreement is in one glycine and one threonine which are missing in Peptides 1 and 2 but are reported by Kimmel, Rogers, and Smith (24) to be present in the peptide containing cysteine residue 152.

DISCUSSION

The main finding described in this paper is that reduction of papain in the presence of 8 M urea leads to a selective cleavage of only one of the disulfide bridges of the molecule. These findings seem, on first impression, to stand in contradiction to the results of several studies carried out by Kimmel et al. (24) and Light and Greenberg (25) in connection with the determination of the amino acid sequence of papain. They found that a total reduction of papain or of its carboxymethylated derivative was achieved in 8 M urea. It should be remembered, however, that in their studies the reduction was performed either after complete denaturation and inactivation of the papain by prolonged dialysis against HCl (25) or after carboxymethylation of the active sulfhydryl groups and precipitation of the derivative by 10% trichloroacetic acid (24). In our study we encountered a similar phenomenon, namely, that if the reduction was performed after a previous exposure of papain to 6 M guanidine and removal of the reagent a full reduction of the disulfide bonds was obtained, in contrast to the partial reduction in 8 M urea. Bearing in mind that guanidine has a strong denaturing effect on papain (17), it is apparent from the above findings that a total reduction of the molecule is feasible only when its conformation has been impaired, whereas as long as the native conformation is preserved subjection to reducing agents leads only to partial opening of the disulfide bonds, probably the more accessible ones.

As shown by Sluyterman (16) and also observed in the studies reported above, papain is enzymatically active in 8 M urea. On the other hand, it does not undergo autodigestion unless subjected to reducing conditions (Fig. 1). It can thus be assumed that the autodigestion occurs only when at least part of the molecules are in the partially reduced form and serve as substrate either for intact papain or for the papain with one disulfide bond open. On the other hand, the possibility that the partially reduced papain can digest intact papain has not been excluded. This autolysis splits the molecule into small fragments and abolishes the activity. A similar phenomenon of autodigestion either at pH values above 5 or at relatively low concentrations of urea was reported for trypsin as well (26, 27). It was explained by the fundamental assumption (28) that in solution the enzyme is in equilibrium between two forms, one of which is active and attacks the other, thus leading to a shift of the equilibrium and continuation of the process. The selectivity of the partial reduction of papain in 8 M urea was established in several ways. (a) The product resulting from the partial reduction and alklylation (3-RCM papain) was homogeneous, as shown by polyacrylamide gel electrophoresis; its mobility was distinctly different from that of 1-RCM papain or 7-RCM papain. (b) The immunological properties of papain were partially retained in 3 RCM papain, whereas in 7 RCM papain they were lost completely. The shape of the precipitin curve with 3-RCM papain (a smaller amount of precipitated antibodies and a shift to the left in the equivalence zone) indicates that it is a distinct derivative which contains part of the antigenic determinants, and not a mixture of active and completely inactive derivatives. The latter would have yielded a precipitin curve in which the maximum amount of precipitated antibodies would have been similar to that in the homologous precipitin curve, but would have been reached at a much higher antigen concentration. That only a part of the antigenic determinants are retained in 3-RCM papain was shown also by gel diffusion. (c) The particular disulfide bond split during the reduction was unequivocally identified by specific labeling of the sulfhydryl groups formed during the partial reduction, followed by the isolation of the radioactive peptides and their amino acid analysis. Moreover, by applying the “diagonal map” technique (29, 30) only two pairs of off diagonal peptides could be detected in a digest of 3-RCM papain, whereas a similar digest of 1-RCM papain contained three such peptide pairs.

The peptides obtained upon tryptic digestion of such a 3-RCM papain derivative, in which only the reduced and alkylated sulfhydryl groups were radioactive, were isolated and analyzed. As Table II indicates, one of these peptides is composed of only three amino acids, namely, arginine, carboxymethylcysteine, and tyrosine. This peculiar composition explains the difficulty of its removal from the Sephadex column in basic or neutral conditions and the relatively low yield in which it was ultimately...
The enzymatic properties of a partially reduced derivative of this type, where only one S-S bond is opened, are of great interest. Unfortunately, no studies in this direction could be performed with the 3-RCM papain derivative, as a result of the blocking of the active sulfhydryl group and the consequent loss of activity. On the other hand, the removal of the urea, under conditions which are suitable for activity assay, brings about the reoxidation and reformation of the native enzyme. An attempt to prevent reoxidation of the partially reduced papain to the native enzyme was undertaken by the reversible “blocking” of the sulfhydryl groups by forming a mercury bridge, of the —SH-Hg-S— type (33), between them. The preparation and properties of this derivative are described in the following publication (34).

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