Specific Chemical Cleavage in High Yield at the Amino Peptide Bonds of Cysteine and Cystine Residues*

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SUMMARY

A protocol is presented for the quantitative conversion of cysteine and cystine residues in proteins to residues of S-cyanocysteine, using the reagent 2-nitro-5-thiocyanobenzoic acid (DEGANI, Y., AND PATCHORNIK, A. (1971) J. Org. Chem. 36, 2727). Cleavage of the amino peptide bond of the S-cyanocysteine residue is obtained upon exposure to 6 M guanidinium chloride-0.1 M sodium borate, pH 9.0, at 37° for 12 hours, with concomitant formation of 2-iminothiazolidine-4-carboxylic and ring closure, without the intermediate formation of an acyliminiothiazolidine.

Catsimpoolas and Wood (1) observed that exposure to CN− causes peptide bond cleavage in cystine-containing proteins and concluded that cleavage was a result of the formation of Cys-CN, which cyclized to an acyliminothiazolidine and subsequently cleaved to generate an amino-terminal peptide and COOH-terminal fragment. These reactions showed limited promise as a technique for generating specific protein fragments, since it was difficult to drive the formation of the cyano derivative to completion, with consequent random formation of SCN groups from both members of the disulfide and a resulting complex mixture of cleaved peptides. The recent development of techniques for quantitative formation of Cys-CN from cysteine residues in peptides has solved the problem of incomplete modification (2, 3). In this publication, we report the results of a study to obtain optimum conditions for cleavage and the application of the complete method to several proteins.

EXPERIMENTAL PROCEDURE

Materials

DFP-treated carboxypeptidase A was used as supplied by Worthington. The major spike protein from dX174 (eistron G product) was a gift of Dr. Marshall Edgel. The catalytic and regulatory subunits of Escherichia coli aspartate transcarbamylase were prepared as described by Gerhart and Holoubek (4). The regulatory subunit was further treated to remove residual mercury as described by Nellbach et al. (5). Potassium [14C]cyanide (New England Nuclear), 5,5'-dithiobis(2-nitrobenzoic acid) (Aldrich), guanidinium chloride (Heico), and 2-mercaptoethanol (Eastman) were used without further purification.

Acrylamide and N,N'-methylenebisacrylamide (Eastman) were recrystallized from chloroform and acetone, respectively, prior to use. Urea, prepared as a 10 M stock solution, was deionized immediately prior to use by passage through Amberlite MB-3. TNB-CN and its potassium salt were prepared essentially as described by Degani and Patchornik (6). Purity of the recrystallized products was assessed by melting point determinations and by spectrophotometric determinations of the formation of thionitrobenzoate dianion (ε₅₄₀ = 13,400) upon the addition of excess 2-mercaptoethanol at pH 7.0. [14C]TNB-CN was made by the same method, using K[14C]CN.

ITH was prepared according to the procedure of Schoberl and Hamm (7) as modified by Catsimpoolas and Wood (1). The white crystals decomposed at 230°. The yield was 27%.

C₇H₄N₂O₈S
Calculated: C 32.86, H 4.14, N 19.17
Found: C 32.86, H 4.16, N 19.04

AcITH was prepared as follows: 0.75 g of ITH was taken up in 35 ml of glacial acetic acid with heating. Acetic anhydride (2 ml) was added to this solution and the reaction mixture was allowed to stand at room temperature overnight. Upon re-

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frigeration, 0.4 g of white crystals formed; these decomposed at 246-248°. The yield was 41%.

\[ \text{C}_4\text{H}_8\text{N}_2\text{O}_8\text{S} \]

Calculated: C 38.28, H 4.25, N 14.89
Found: C 38.08, H 4.44, N 14.50

[\text{\textsuperscript{14}C}]\text{GSCN} was prepared by treatment of reduced glutathione with DTNB and then R\textsuperscript{14}CN in a manner analogous to the two-step procedure for modification of proteins described below. It was purified by ion exchange chromatography on a column of Spinco 50-A resin with a gradient of acetic acid-pyridine-water buffers, pH 2 to 3. The structure was confirmed by amino acid analysis and specific radioactivity. The yield was 70%.

**Methods**

**Modification of Proteins with TNB-CN**—Protein modifications with TNB-CN were carried out using one of the following two procedures. Procedure 1 is useful for preparation of cleavage fragments to be used in subsequent sequence analyses; Procedure 2 is more convenient for rapid analytical experiments.

For Procedure 1 the protein was dissolved in 6 M guanidinium chloride-0.2 M Tris-acetate buffer, pH 8, containing mercaptoan. For proteins which contain only cysteine, a slight excess of dithiothreitol was used and the solution was allowed to stand at room temperature for 1/2 hour. When disulfides were present the protein was incubated with at least a 10 mm excess of dithiothreitol for 2 hours at 37° before modification to ensure complete reduction. R-chain was routinely stored in 0.01 M KCl-O.2 M glacial acetic acid and dialyzed exhaustively into 50% acetic acid at 4°. Very little protein cleavage occurred during this procedure for modification of protein sulfhydryl groups, and no other reducing agent was used. Next, a 5- to 10-fold excess of \text{\textsuperscript{14}C}]\text{TNB-CN} over total thiol present was added and, if necessary, the pH was rapidly readjusted to 8 with NaOH. After 15 min at room temperature the reaction mixture was acidified by the addition of an equal volume of glacial acetic acid and dialyzed exhaustively into 50% acetic acid at 4°. Very little protein cleavage occurred during this brief incubation at pH 8, and no cleavage occurred after acidification (see “Results and Discussion”). The extent of modification was determined at this point by the loss of sulfhydryl groups reactive toward DTNB and by specific radioactivity. Since minor radioactive contaminants of the \text{\textsuperscript{14}C}]\text{TNB-CN preparation were not completely removed by dialysis (see below), we recommend a final desalting of the modified protein on Sephadex G-25 in 50% acetic acid if the extent of modification is to be determined carefully. Modified proteins were stored in 50% acetic acid at -20° until used in cleavage experiments; they were stable indefinitely under these conditions. For cleavage, aliquots of derivatized protein were removed and evaporated to dryness under a stream of nitrogen. The residue was then dissolved in the appropriate solution.

For Procedure 2, the protein was dissolved in a solution of 8 M urea-0.01 M 2-mercaptoethanol-0.1 M N-ethylmorpholine acetate buffer at pH 8.5 and incubated at 37° for 2 hours. A portion of this solution was then set aside as a control, and to the remainder enough \text{\textsuperscript{14}C}]\text{TNB-CN was added to give a 5-fold excess over total sulfhydryl groups. If necessary, the pH was readjusted to 8.5 by addition of 1 M NaOH. The mixture was then allowed to stand for 15 min at room temperature and then adjusted to pH 9.0 for cleavage. The cleavage reaction was quenched by making aliquots 1% each in sodium dodecyl sulfate and 2-mercaptoethanol; the mercaptoethanol removes uncleaved thiocyanate groups (2), and the sample is then ready to be loaded directly onto a gel.

**Modification with DTNB**—An alternative method of modification is the two-step procedure described by Vanaman and Stark (2). When this method was used, the protein was reduced in denaturant as above and a 5-fold excess of DTNB was added. After 15 min, K\textsuperscript{14}CN was added in a 10 fold excess over the DTNB. The solution was allowed to stand for 1/2 hour and then was acidified and treated as before (caution being taken because of the HCN). Since there was no particular advantage to this procedure, the simpler one-step modification was generally used.

**Rates of Cleavage**—The method of determining cleavage rates using Procedure 2 for modification is given above. When Procedure 1 was used, aliquots removed from the reaction mixture at various times were immediately made 50% in acetic acid and stored at -20°. Later, each aliquot was prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis by dialysis into 50% acetic acid and drying as described above.

**Analysis of Cleavage Products by Sodium Dodecyl Sulfate-Gel Electrophoresis**—Gel electrophoresis in sodium dodecyl sulfate was carried out as described by Davies and Stark (8) or by the procedure of Weiner et al. (9). Samples were prepared for the gels by boiling them for 2 min in 1% sodium dodecyl sulfate and 1% 2-mercaptoethanol. They were then mixed with an equal volume of 50% glycerol, 0.05% in bromphenol blue, and layered on top of the gels. Electrophoreses of \text{\textsuperscript{14}C}]\text{TNB-CN were carried out using one of the following two procedures. Procedure 1 is useful for preparation of cleavage fragments to be used in subsequent sequence analysis; Procedure 2 is more convenient for rapid analytical experiments.

**RESULTS AND DISCUSSION**

**Determination of Optimum Conditions for Modification and Cleavage**

The results of Degani et al. (3) suggest that essentially complete modification of protein sulfhydryl groups can be achieved in denaturing solvents with TNB-CN. Alternatively, complete modification can also be achieved by a two-step procedure with DTNB and KCN (4). In either case, we have found that the specific radioactivity of several modified proteins into which \textsuperscript{14}CN groups had been introduced was that expected from the specific radioactivity of the reagents and the known half-cystine content of the proteins (data not shown). Investigations of optimum conditions for cleavage were carried out with C-chain-CN and R-chain-CN.

**Analysis of Cleavage Products by Sodium Dodecyl Sulfate-Gel Electrophoresis**—Gel electrophoreses in sodium dodecyl sulfate...
It seemed possible that acidic conditions might catalyze cyclization, if the SCN group were more basic than an amide bond. R-chain-CN, prepared by Procedure 1, was exposed to a variety of acids, as shown in Table I. In no case was cleavage accomplished, as judged by analysis on sodium dodecyl sulfate-polyacrylamide gels, and no change was observed in the ultraviolet spectra of the samples, indicating that cyclization without cleavage had not occurred (see below). Therefore, alkaline conditions for cleavage were investigated.

R-chain-CN was incubated at 80°C for 2 hours in buffered guanidinium chloride solutions ranging in pH from 4 to 10. Sodium dodecyl sulfate-polyacrylamide gels of some of these samples are shown in Fig. 1. R-chain has a molecular weight of 17,000 and 4 cysteine residues, at positions 109, 114, 137, and 140. Cleavage at cysteine 109 or 114 generates an NH2-terminal fragment with molecular weight about 12,000, and this species appears as the lowest band on the gels (Fig. 1D). If cleavage occurs at position 137 or 140, but not at 109 or 114, then an NH2-terminal fragment with molecular weight of about 14,500 is formed. This species appears as the band second from the bottom. The top major band in Fig. 1 is uncleaved R-chain-CN; all the other cleavage fragments are too small to be seen on the gel. The faint upper bands are contaminants in the R-chain preparation. Therefore, cleavage is indicated by the appearance of the lower two bands and by disappearance of the R-chain band. Clearly, higher pH favors the cleavage reaction, and little or no cleavage occurs at pH 4. To investigate further the effect of alkaline pH on R-chain-CN, it was incubated in 6 M guanidinium chloride solutions in pH 9.0. The protein was incubated at 37°C for 12 hours, 0.3 ml of glacial acetic acid was then added, and the solution was passed onto a Sephadex G-25 column which was eluted with 50% acetic acid. The elution profile and the fractions included in the two pooled peaks are shown in Fig. 3. These two peaks contained 84% of the counts placed on the column. The remainder ran as a smear of radioactivity beyond the total volume of the column and was not associated with any amino acids. We believe this material came from a minor (with respect to mass) contaminant of the Na-3H]TNB-CN. The presence of a radioactive contaminant was supported by the finding that the peptides in Peaks I and II had specific activities lower than that determined for Na-3H]TNB-CN.

The amino acid composition of a hydrolysate from the first pool was consistent, within experimental error, with the composition of R-chain, as would be expected of a mixture of the three large cleavage fragments. The composition of the second pool is shown in Table II. Except for contamination by a small amount of alanine, it is consistent with an equimolar mixture of the two small fragments which should be generated by complete cleavage. Corrected for the radioactive contaminant, the extent of modification of the R-chain was 95% (±5%). If the sulfhydryl groups at each position undergo modification and cleavage to the same extent, one can calculate the yield of the cleavage reaction from the specific radioactivity of Peak I or from the extent of modification and the yield of the small fragments. By either method of evaluation the cleavage was 93% (±5%) complete at each site.

**Cleavage of Proteins**

**Cleavage of [cyano-3H]C-Chain-CN**—Since it is possible to derivatize the single cysteine of C-chain without denaturing agents, we were able to observe the stability of the cyano derivative in the native protein. No cleavage was observed over a period of months near neutral pH, and all the radioactivity could be recovered by treatment with 2-mercaptoethanol in a 6 M guanidinium chloride solution. However, when a solution of 5.2 mg of [cyano-3H]C-Chain-CN in 6 M guanidinium chloride-0.2 M Tris-chloride, pH 8.0, is incubated at 37°C, cleavage occurs readily, as shown by sodium dodecyl sulfate-polyacrylamide gels of aliquots of the solution taken at various times (Fig. 3). Nearly complete cleavage of the C-chain (mol wt 33,000) was observed after 8 hours (Fig. 3D), in contrast to R-chain-CN which is only partially cleaved under these conditions. The species at molecular weight 27,000 (±1,000) is the only new band appearing on the gels. Therefore, C-chain-CN cleaves into two fragments of molecular weight 27,000 and molecular weight 0,000, and the small fragment has run off the gel. To separate these fragments preparatively, 12 mg of cleaved [cyano-3H]C-Chain-CN in 6 M guanidinium chloride-0.04 M potassium phosphate, pH 7.6, was passed down a column (100 x 1.1 cm) of Sephadex G-100 equilibrated with the same buffer. The elution profile (from the column) clearly indicates that Peak I, the fragment of higher molecular weight, contains most of the radioactivity, while Peak II has essentially no counts. Amino acid analysis and sodium dodecyl sulfate-polyacrylamide gel electrophoresis of Peak I showed that it corresponds to the 27,000 molecular weight fragment. Peak II had an amino acid analysis consistent with a molecular weight of 5,000 to 6,000, and, since no 3H counts are associated with it, it must correspond to the amino-terminal peptide. Thus, the C-chain sulfhydryl group is located approximately 30 residues from the amino terminus. Peak III had no amino acids after acid hydrolysis, ran with the salt peak, and was assumed to be a contaminant.

**Cleavage of Carboxypeptidase A**—Pancreatic carboxypeptidase A has a molecular weight of about 34,000 and contains a single disulfide bridge linking half-cystine residues 138 and 161. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of Worthington DFP-treated carboxypeptidase A, shown in Fig. 6A, indicates that the commercial enzyme has an apparent molecular weight of 30,000 ± 1,000 when compared to standards. The protein was modified with Na-3H]TNB-CN, using Procedure 2, and was incubated at pH 6.0 for 12 hours at 37°C, together with an unmodified control. The results of sodium dodecyl sulfate-polyacrylamide gel analysis of these reaction mixtures are also shown in Fig. 6, together with a set of stand-
Fig. 1 to 3. See legends on p. 6587.
Fig. 4. Elution of cleaved [cyano-14C] C-chain-CN from a Sephadex G-100 column (100 x 1.1 cm) equilibrated with 6 M guanidinium chloride, 0.04 M potassium phosphate, pH 7.0. Absorbance at 230 nm (••••••) and counts per min per 25 µl, (○--○). I, II, and III indicate the fractions pooled.

Fig. 5. Elution of cleaved [cyano-14C] R-chain-CN from a Sephadex G-25 column (100 x 1.1 cm) equilibrated with 50% acetic acid. Counts per min per 25 µl are plotted. I and II indicate the fractions pooled.

Figs. 1 (top left), 2 (top right and bottom left). 10% sodium dodecyl sulfate-polyacrylamide gels of 65-µg portions of R-chain-CN incubated for 2 hours at 80° at different pH values: A, 6 M guanidinium chloride, 0.2 M citrate, pH 4.0; B, 6 M guanidinium chloride, 0.2 M succinate, pH 5.0; C, 6 M guanidinium chloride, 0.2 M cacodylate, pH 6.0; D, 6 M guanidinium chloride, 0.2 M imidazole, pH 7.0.

Fig. 3 (bottom right). 10% sodium dodecyl sulfate-polyacrylamide gels of 40-µg portions of C-chain-CN incubated in 6 M guanidinium chloride, 0.2 M Tris, pH 8.0, at 37°C. A, no incubation; B, 2 hours; C, 5 hours; D, 8 hours, E, 19½ hours.

Table II

<table>
<thead>
<tr>
<th>Amino acid composition of peak II (Fig. 5) from cleaved R-chain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino acid</td>
</tr>
<tr>
<td>------------</td>
</tr>
<tr>
<td>Lysine</td>
</tr>
<tr>
<td>Histidine</td>
</tr>
<tr>
<td>Arginine</td>
</tr>
<tr>
<td>Aspartic acid</td>
</tr>
<tr>
<td>Threonine</td>
</tr>
<tr>
<td>Serine</td>
</tr>
<tr>
<td>Glutamic acid</td>
</tr>
<tr>
<td>Proline</td>
</tr>
<tr>
<td>Glycine</td>
</tr>
<tr>
<td>Alanine</td>
</tr>
<tr>
<td>Valine</td>
</tr>
<tr>
<td>Isoleucine</td>
</tr>
<tr>
<td>Leucine</td>
</tr>
<tr>
<td>Tyrosine</td>
</tr>
<tr>
<td>Phenylalanine</td>
</tr>
</tbody>
</table>

* Assumed; values for other basic amino acids are based on this.
* Assumed; values for other neutral and acidic amino acids are based on this; the value is 1.86 if lysine = 1.00.

TABLE I

<table>
<thead>
<tr>
<th>Migration (cm)</th>
<th>Molecular Wt.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Worthington DFP-Carboxypeptidase A</td>
<td>33,000 mol wt</td>
</tr>
<tr>
<td>CPA Fragment 1</td>
<td>26,700 mol wt</td>
</tr>
<tr>
<td>CPA Fragment 2</td>
<td>17,500 mol wt</td>
</tr>
<tr>
<td>CPA Fragment 3</td>
<td>14,000 mol wt</td>
</tr>
<tr>
<td>Horse heart cytochrome c</td>
<td>12,700 mol wt</td>
</tr>
</tbody>
</table>

Fig. 6. A, carboxypeptidase A cysteinyl cleavage products: separation on 10% acrylamide-0.1% bisacrylamide. 1, carboxypeptidase control; 2, molecular weight standard containing Escherichia coli aspartate transcarbamylase, α-chymotrypsinogen A, bovine α-lactalbumin, and cytochrome c plus uncleaved carboxypeptidase A-CN: 3, cleaved carboxypeptidase A-CN: 4, molecular weight standards. B, molecular weight determinations. Standards. Aspartate transcarbamylase C-subunit, 33,000 mol wt; α-chymotrypsinogen A, 26,700 mol wt; aspartate transcarbamylase, R-subunit, 17,500 mol wt; bovine α-lactalbumin, 14,000 mol wt; horse heart cytochrome c, 12,700 mol wt.

Fig. 7. Absorption spectrum of cleaved [cyano-14C] R-chain-CN from a Sephadex G-25 column (100 x 1.1 cm) equilibrated with 50% acetic acid. Absorbance at 230 nm (••••••) and counts per min per 25 µl, (○--○). I, II, and III indicate the fractions pooled.
FIG. 7. Cleavage of +X174 spike protein (cistron G).

A, control; B, oxidized insulin B-chain; C, cleaved +X174 spike protein; D, molecular weight standards plus uncleaved spike protein. Standards as in Fig. 6A-2. Separation on 12.5% acrylamide-0.7% bisacrylamide.

observed correspond to molecular weights of 17,000 ± 1,000, 15,000 ± 1,000, and 14,000 ± 1,000. The probable assignment of the bands is as follows. The upper band is the larger overlap peptide, the middle band is the largest unique fragment (residues 161 to 307) plus the smaller overlap peptide, and the lower band is the unique fragment (residues 8 to 137); the smallest unique fragment (residues 138 to 160) probably runs with the tracking dye.

In other experiments using Procedure 1, the amount of the band assigned to the larger overlap fragment was much reduced relative to the lower bands, indicating more extensive cleavage than that shown in Fig. 6A-3. Further incubation of both preparations under cleavage conditions resulted in no change of the gel patterns; therefore, the cleavage reaction has probably gone to completion after 12 hours and the presence of overlap fragments is probably due to incomplete modification.

Cleavage of Major Spike Protein from +X174 (Cistron G Product)—The major spike protein from +X174 (mol wt 20,000) has 3 half-cystine residues, as determined by amino acid analysis of hydrolysates of reduced and carboxymethylated or oxidized protein. In order to position these cysteine residues in the polypeptide chain, analytical cleavage experiments were carried out using Procedure 2. The resulting gel patterns are shown in Fig. 7. It is striking that only a single major band is seen in the cleaved sample, with an apparent molecular weight of 4,700. This result indicates that the cysteine residues are evenly positioned along the chain to give the following structure:

\[
\text{SH} \quad \text{SH} \quad \text{SH} \\
\text{NH}_2 \quad +5,000 \quad +5,000 \quad +5,000 \quad +5,000 \quad \text{COOH.}
\]

This possibility is being confirmed by more detailed analysis. Such an even placement of sulphhydryl groups may indicate a gene duplication mechanism in the evolution of this protein.

Stability of Amide and Peptide Bonds—To establish whether the alkaline cleavage conditions affect asparagine or glutamine, 7 mg of lysozyme were incubated under the same conditions used to cleave R-chain-CN. For comparison, a like sample of lysozyme was incubated in parallel at 37°C for 12 hours in 0.2 M imidazole-HCl-6 M guanidinium chloride, pH 7. The samples were acidified and run through a Sephadex G-25 column equilibrated with 50% acetic acid. The protein peaks were located by their absorbances at 280 nm, and aliquots were taken from the peak tubes and hydrolyzed in acid. Amino acid analysis indicated that the ammonia contents of the proteins exposed to pH 9 and pH 7 were 25.1 and 23.4 residues per chain, respectively. Although both these values are appreciably above the expected value of 16 residues per chain (13), they are not significantly different from each other. Apparently there was some contamination, probably from ammonia in the acidic acetic acid, but since both samples were treated in exactly the same manner after incubation it seems safe to conclude that side chain amide bonds are reasonably stable under the conditions used for cleavage.

Peptide bonds are also stable under these conditions. No cleavage of underivatized R-chain was detected on sodium dodecyl sulfate-polyacrylamide gels after exposure to 6 M guanidinium chloride at pH 9 for 12 hours at 37°C. Furthermore, quantitative analysis of the fragments produced upon specific cleavage of R-chain-CN (Fig. 5) precludes the possibility that an appreciable fraction of the 14C counts or amino acids were lost from the large fragments by random unspecific cleavage.

Attempts to Remove Iminothiazolidinyl Residues—A drawback for the most extensive use of the cysteine cleavage method to produce large fragments for sequence analysis is the presence of an NH₂-terminal iminothiazolidinyl residue on all but the fragment which contains the original NH₂-terminal sequence. Preliminary experiments have been carried out to determine whether the iminothiazolidinyl residue might be specifically removed. Edman degradation was attempted on ITH-gly, using the technique described by Konigsberg (14). Phenylthio-carbamylamyle was attempted in 50% pyridine-2% triethylamine, and cyclization was attempted for 15 min at 40°C in anhydrous trifluoroacetic acid. No glycine was liberated, as judged by amino acid analysis. ITH-gly was also treated exhaustively with pyroglutamyl peptidase from Bacillus subtilis (a gift from Dr. Robert E. Fellows). Under conditions where the model substrate pyroglutamylcarboxylylalanine gave complete release of alanine, no release of glycine was observed. Studies on other possible ways of selectively removing the terminal iminothiazolidinyl residue using a Raney Nickel-type catalyst or the enzyme prolinase are in progress.

2 T. C. Vanaman, unpublished observation.
Mechanism of Cleavage Reaction

Deacylation—Solutions of AcITH have an absorption peak at 254.5 nm with molar extinction coefficients between 10³ and 10⁴, the precise value depending on the pH of the solution. ITH absorbs very weakly in this region, and therefore the rate of deacylation of AcITH as a function of pH was determined by following the absorbance at 254.5 nm. The rate constant varied between 10⁻² and 10⁻¹ h⁻¹, and the pH dependence was rather complex and very different from that for cleavage of C-chain-CN or R-chain-CN. For example, AcITH has a half-life of 12 hours in 0.2 M sodium acetate buffer at pH 4.0 and 37°, but shows no detectable deacylation (less than 5%) in 0.1 M guanidinium chloride-0.1 M sodium borate buffer at pH 9.0 in 24 hours. On the other hand, no detectable cleavage of an S-cyano-protein occurs at pH 4.0, whereas cleavage is complete after 12 hours at 37° in the guanidinium chloride-borate buffer at pH 9.0.

By analogy with AcITH, a cyclized but uncleaved S-cyano-protein would be expected to have an intense absorption band near 255 nm, and thus it should be possible to detect an appreciable amount of cyclized but uncleaved derivative by examining its ultraviolet spectrum. Therefore, the spectra of uncleaved C-chain- and R-chain-CN were compared with the spectra of the undecavatized proteins. If the derivative were fully cyclized and had the same extinction coefficient as AcITH it would contribute a peak one-third the size of the 280-nm peak of C-chain-CN (one sulfhydryl group per 33,000 mol wt) and three times the size of the 280-nm peak in R-chain-CN (four sulfhydryl groups per 17,000 mol wt). However, no differences were seen when the spectra of unmodified and modified proteins were compared. Furthermore, the absorbance of C-chain-CN at 254.5 nm was monitored continuously for 24 hours at 37° in 0.1 M guanidinium chloride-0.2 M Tris-acetate buffer, pH 8.2. Although sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed that nearly complete cleavage had occurred, the absorbance at this wavelength did not change during the course of the reaction. Failure to detect any change in the ultraviolet spectra of R-chain-CN or C-chain-CN indicates that cyclization without cleavage does not occur readily. Furthermore, failure to see a change at 254.5 nm during the cleavage of C-chain-CN suggests that no appreciable amount of acyliminothiazolidine intermediate accumulates during the reaction. This idea is supported by the great difference in pH dependence between the deacylation of AcITH and the cleavage reaction for a protein.

GSCN Cleavage—GSCN was incubated in 0.5 M sodium phosphate buffer, pH 7.0, at 50° for 48 hours. These conditions cause complete cleavage of C-chain-CN (see above) and, according to the results of Catsimpoolas and Wood (1), should have caused appreciable cleavage of GSCN. An aliquot of the cleavage mixture was chromatographed on the medium column of an amino acid analyzer, and, although the peak corresponding to starting material had disappeared, no free glutamic acid was observed. After acid hydrolysis, the expected amounts of glutamic acid and alanine were found. Catsimpoolas and Wood (1) reported finding 2-pyrrolidone-5-carboxylic acid as a product of GSCN cleavage and we conclude that, at least under our conditions, the cleavage reaction forms this product exclusively.

When the cleavage products of GSCN were separated on a column of Spinco 50-A resin (0.9 x 20 cm) in pyridine-acetic acid buffers, two major fractions were found. After acid hydrolysis they contained glutamic acid and glycine, respectively. When the fraction containing glutamic acid was analyzed without hydrolysis, no free amino acid was detected.

Cleavage Reaction—Hydroxide ion stimulates the cleavage reaction greatly, as shown by the influence of rising pH on the rate of cleavage of R-chain-CN. This stimulation is not due to general base catalysis, since 1 M imidazole has a negligible effect on the cleavage rate at pH 7.0. Therefore, specific hydroxide ion catalysis is implicated. A possible explanation of the role of OH⁻ is shown in Fig. 8. The amide nitrogen is apparently too weak a base to carry out the nucleophilic attack (the pKₐ of a protonated amide is -4.0 or less (15)), and the reaction probably proceeds only after attack of OH⁻ on the carbonyl carbon of the amide. This generates a much more basic nitrogen, which then can participate in concerted cyclization and cleavage, without formation of an acyliminothiazolidine intermediate.

In the case of glutathione, the terminal amino group might replace OH⁻ as the attacking base, since the intramolecular reaction would result in formation of a five membered ring. Thus, 2-pyrrolidone-5-carboxylic acid would be the product, as observed (see above).

GENERAL DISCUSSION

The amino neighbors of cysteine residues at which cleavage has been carried out are shown in Table III. Several classes of amino acids are represented, and in each case incubation at 37° and pH 9 probably causes very extensive cleavage.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Molecular weight</th>
<th>-SH groups per chain</th>
<th>Amino neighbors of cysteine</th>
<th>Extent of cleavage</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-chain of aspartate transcarbamylase</td>
<td>33,000</td>
<td>1</td>
<td>Unknown</td>
<td>&gt;90</td>
</tr>
<tr>
<td>R-chain of aspartate transcarbamylase</td>
<td>17,000</td>
<td>4</td>
<td>Val, Asn, Lys, Tyr</td>
<td>05</td>
</tr>
<tr>
<td>Carboxypeptidase A</td>
<td>39,000</td>
<td>2</td>
<td>Leu, Pro</td>
<td>*</td>
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<tr>
<td>Spike protein from φX174</td>
<td>20,000</td>
<td>3*</td>
<td>Ala, un, known, unknown</td>
<td>&gt;95</td>
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</table>

* Not determined.
* P. Shank, M. Edget, and T. C. Vanaman, unpublished observations.
The TNB-CN reagent is specific for cysteine (3), and it should be possible to modify and cleave selectively at free sulfhydryl groups proteins which contain disulfides as well, simply by eliminating the reduction step at the beginning of the protocol. Such results could then be compared with parallel experiments in which the protein has been fully reduced before cleavage. Since protein sulfhydryl groups often show marked differences in reactivity, modification with TNB-CN or DTNB plus KCN under nondenaturing conditions, followed by treatment with a blocking reagent in denaturant to modify the less reactive sulfhydryl groups, might allow specific cleavage at the more reactive groups. On the other hand, treatment with a sulfhydryl reagent such as iodoacetate first, followed by exposure to TNB-CN under denaturing conditions, could be used for selective cleavage at the unreactive sulfhydryl groups.

The marked pH dependence of the cleavage reaction and the ability of the reaction to be terminated instantaneously with 2-mercaptoethanol or a change in pH should allow one to isolate overlap fragments in high yield after the cleavage rate and gel pattern have been investigated for a particular protein. Such fragments would, of course, be useful in ordering the set of fragments obtained after complete cleavage of the cyano-protein in the primary sequence.

Preliminary results indicate that cleavage of cyano-proteins is also easily accomplished in 0.1% sodium dodecyl sulfate at pH 9. This solvent would make direct analysis of the course of cleavage much easier, since aliquots of a reaction mixture could be quenched with excess 2-mercaptoethanol and immediately subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis without prior removal of guanidinium chloride.

**General Protocol**

The modification is carried out in 6 M guanidinium chloride-0.2 M Tris-acetate buffer, pH 8. For nonselective modification the protein is treated with a slight excess of dithiothreitol at room temperature, when only cysteine is present, or with at least 0.01 M dithiothreitol (in large excess) at 37° for 5 hours when cystine is present. After the solution has been cooled to room temperature, a 5-fold excess of TNB-CN over total thiol is added, and the pH is rapidly readjusted to 8 with NaOH if necessary. After 15 min the reaction mixture is quickly acidified with glacial acetic acid to pH 4 or below and dialyzed into 50% acetic acid at 4°. Alternatively, the sample could be desalted on a Sephadex column with 50% acetic acid. In either case the extent of modification can be determined from the specific activity of the sample, but for this purpose desalting on a Sephadex column is preferable because extraneous radioactivity occasionally persists through dialysis. The sample may be stored indefinitely at -20° in 50% acetic acid.

For cleavage, the protein is taken to dryness, N₂ is blown over the residue for 5 min to drive off residual acetic acid, and the sample is taken up in 6 M guanidinium chloride-0.1 M sodium borate buffer, pH 9.0, and incubated for 12 hours at 37°. Aliquots may be taken for analysis during the course of the reaction, as described above. The steps involved in completely cleaving a polypeptide at cysteine, using either the two-step or the one-step modification method, are summarized in Fig. 9.

Note Added in Proof—Degani and Patchornik (17) have found that the extent of modification of small sulfhydryl peptides by 2-nitro-5-thiocyanobenzoic acid is substantially decreased at high concentrations of reactants, and recommend that the concentration of sulfhydryl groups not exceed 0.5 mM during modification. Although our results indicate that proteins in denaturants can be modified nearly quantitatively at very much higher concentrations, it may be wise to work in the concentration range suggested by Degani and Patchornik if difficulty in achieving quantitative modification is encountered.

![Fig. 9. Flow chart for the modification and cleavage reactions.](http://www.jbc.org/)}
REFERENCES

Specific Chemical Cleavage in High Yield at the Amino Peptide Bonds of Cysteine and Cystine Residues
Gary R. Jacobson, Martin H. Schaffer, George R. Stark and Thomas C. Vanaman


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