RING CLEAVAGE OF 2-IMINOTHIAZOLIDINE-4-CARBOXYLATES BY CATALYTIC REDUCTION. A POTENTIAL METHOD FOR UNBLOCKING PEPTIDES FORMED BY SPECIFIC CHEMICAL CLEAVAGE AT HALF-CYSTINE RESIDUES *

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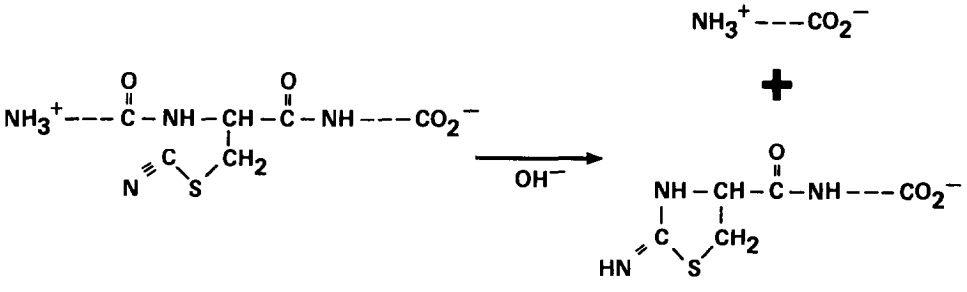
SUMMARY

Specific chemical cleavage of proteins at S-cyanocysteine residues leads to formation of iminothiazolidinecarboxylyl peptides which, because their amino termini are blocked, are not susceptible to Edman degradation. A catalyst prepared from $NiCl_2$ and $NaBH_4$ converts 2-iminothiazolidine-4-carboxylate to alanine and iminothiazolidine carboxylylglycine to Ala-Gly in good yield. Unmodified proteins treated with this catalyst in 8M guanidinium chloride are recovered in good yield, with quantitative conversion of methionine to aminobutyrate and half-cystine to alanine by desulfuration. The catalyst also induces cleavage of a small subclass of peptide bonds, probably Phe-Thr and Phe-Ser sequences, producing discrete fragments.

Jacobson et al. (1) have shown that S-cyanocysteine residues in proteins are cleaved in excellent yield under mildly alkaline conditions in a reaction originally discovered by Catsimpoolas and Wood (2).



Degani and Patchornik (3) have recently explored optimum conditions for introducing the cyano group. After cleavage, all peptides except the amino-terminal one are resistant to sequential Edman degradation. Despite the

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Table I. Reduction of 2-Iminothiazolidine-4-carboxylic Acid as a Function of Time and Temperature.

Hours of incubation	Temperature	Alanine recovered (%)
3	60°	40
5	55°	32
6	60°	52
12	50°	32
12	55°	54
15	60°	64
15	60°	67

Iminothiazolidine carboxylate (prepared by Jacobson et al. (1)) (0.1 to 20 μmoles) and catalyst (0.5 to 1.5 g) in 3.5 ml of 0.2 M sodium phosphate buffer, pH 7.0 were incubated for 12 hr with shaking. The relative amounts of catalyst and substrate had little effect on the yield in these ranges. The catalyst was prepared according to Paz et al. (5), with minor modifications. A solution of 7 g $NaBH_4$ in 100 ml water was placed into a resin reaction kettle maintained at 0 to 2° and was stirred magnetically. Tritiated or deuterated borohydride may be used. A solution of 12 g $NiCl_2 \cdot 6H_2O$ in 100 ml water was then added dropwise and slowly with an addition funnel. The kettle was closed with a check valve which released automatically at 6 to 10 psi so that the reaction could be left unattended. Three hrs were allowed for completion of the reaction and then excess borohydride was destroyed by titration of the reaction mixture to pH 4 with 12 M HCl; 200 ml of water were added and precipitated catalyst was separated by decantation, washed 3 times with water by centrifugation and stored in a closed bottle under a little water at 4°. The catalyst was used within 12 hrs. Its activity was checked by reduction of lanthionine to alanine in 3 hrs at 50°, as described by Paz et al. (5); recovery (70 to 80%) was best when the catalyst was washed well with boiling water after the reduction had been completed. Alanine itself was recovered in only 80 to 90% yield. Although all batches of catalyst worked well in the reduction of lanthionine, there was some variation in activity toward iminothiazolidinecarboxylate from batch to batch. It may be helpful to use highly purified borohydride.

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similarity of iminothiazolidinecarboxylyl and prolyl residues, we were not able to remove the former with phenylisothiocyanate even under drastic conditions (1). In order to extend the utility of the cystine cleavage method, we have explored the use of a reduced nickel catalyst (4) for unblocking. Paz et al. (5) had shown previously that this catalyst causes quantitative desulfuration of simple model peptides, with excellent recovery of products. We now report that iminothiazolidine carboxylic acid and iminothiazolidinecarboxylylglycine can be converted by the catalyst directly to alanine and Ala-Gly, respectively, in good

Table II. Reduction of 2-Iminothiazolidine-4-carboxylic Acid as a Function of pH.

pH	Buffer	Alanine recovered (%)
5.5	0.2 M succinate	22
7.0	0.2 M phosphate	31
8.5	0.2 M glycylglycine	60

Reactions were carried out in solutions 8 M in guanidinium chloride at 60° for 14 hrs with 6 μmoles of substrate and 0.8 g of catalyst. The pH values are uncorrected readings made at room temperature and in the presence of the guanidinium chloride.

yield. The reaction has not been extended to larger iminothiazolidine carboxylates. Unmodified proteins can be recovered from the catalyst in good yield, but new specific cleavage reactions occur. We report these findings in a preliminary way now in the hope that other workers may be interested in studying them further, since we do not intend to.

Effect of the catalyst on model compounds. The effects of varying time, temperature and ratio of catalyst to substrate on the yield of alanine obtained from 2-iminothiazolidine-4-carboxylic acid are shown in Table I. The optimum conditions are 15 hrs at 60°, which gives yields roughly comparable to those obtained from lanthionine ((5) and Table I). The pH-dependence of the reaction was studied in buffers containing 8M guanidinium chloride, for comparison with the pH-dependence of peptide bond cleavage (see below). The results (Table II) indicate that, in the presence of the denaturant, reduction of the ring occurs best at an apparent pH of 8.5. No increase in the amount of alanine was seen upon acid hydrolysis of products and no new peaks on the amino acid analyzer were seen upon oxidation with performic acid, showing that both reductive desulfuration and deformylation occur with the catalyst. It should be noted that iminothiazolidine carboxylic acid is extremely resistant to acid hydrolysis (6). A simple peptide derivative, 2-iminothiazolidine-4-carboxylylglycine, was prepared by cleavage of S-cyanoglutathione (prepared by

Jacobson et al. (1)). The derivative was purified as was the parent compound[1]; 121 nmoles were incubated with 1 g of catalyst in 3 ml of 0.012 M phosphate buffer, pH 7, for 16 hrs at 60°. The product gave no peak on the 55 cm column of the amino acid analyzer before hydrolysis; after hydrolysis, the recovery of alanine was 70% and that of glycine was 101%. Hydrolysis without prior treatment with catalyst gave only glycine.

Effect of the catalyst on proteins. Several unmodified proteins were treated with the catalyst in 8M guanidinium chloride. In all cases, recovery of the protein was good and essentially quantitative conversion of methionine to aminobutyrate and half-cystine to alanine was observed by amino acid analysis. We did not check the proteins for loss of amides, but extensive deamination of glutamine and asparagine residues is likely under the conditions employed. All but one of the unmodified proteins showed evidence of limited specific peptide bond cleavage after treatment with the catalyst.

One-mg portions of the catalytic subunit of Escherichia coli aspartate transcarbamylase were treated with catalyst for 1, 7 and 17 hrs as described in Figure 1. As shown in Figure 1A, the molecular weight of a majority of the protein changed from 33,000 (8) to 26,500. After 7 hrs, the band at 26,500 had disappeared and several discrete bands were apparent in a sodium dodecyl sulfate-urea gel (Figure 1D). Based on amino acid analysis, the recovery of protein was 73% after 7 hrs and about 80% of the methionine was converted to aminobutyrate. Based on an ultraviolet absorption spectrum in alkali, tryptophan and tyrosine were unchanged. The recovery after 17 hrs was 83% and the gel pattern was very similar to that of the 7 hr sample. A single amino terminal determination on the 17 hr products according to the method of Stark

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[1] Iminothiazolidinecarboxylylglycine (up to 1 μmole) gave no peak on the amino acid analyzer before hydrolysis. A quantitative amount of glycine was liberated after overnight hydrolysis at 110° in 6 M HCl. Descending paper chromatography in butanol : acetic acid : water (4 : 1.5 : 5) showed no ninhydrin positive spot for the unhydrolyzed material and only one spot was revealed with the $PtCl_3$ spray of Winegrad and Toennies (7), which is positive with iminothiazolidine carboxylate.

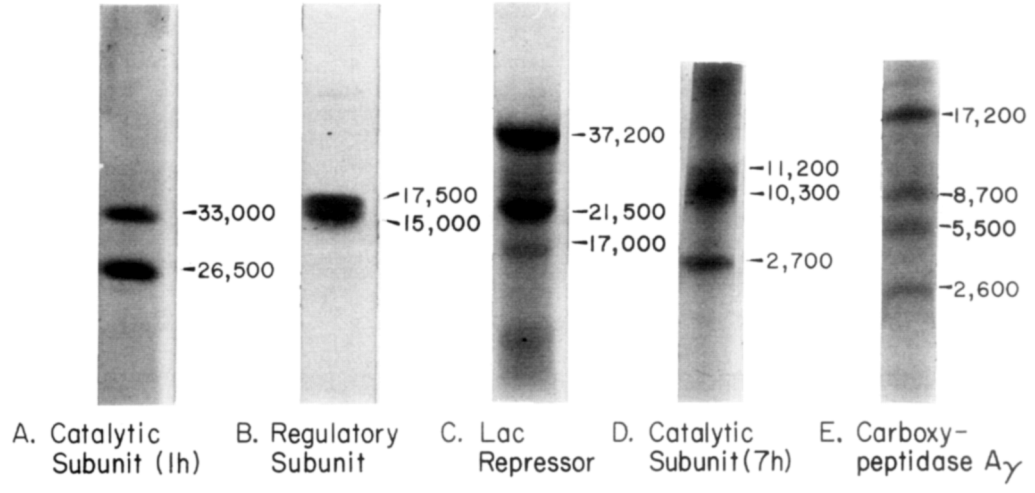


Figure 1. Cleavage patterns of proteins after treatment with catalyst. Proteins (1 to 10 mg) were treated at 60° for 16 or 17 hrs with 2.5 g of catalyst in 1 to 2 ml of 0.2 M phosphate buffer, 8 M in guanidinium chloride. The apparent pH was 7.0. Before gels were run, the samples were desalted on columns of Sephadex G-10 in 50% acetic acid without substantial loss of protein as judged by amino acid analysis, except that occasionally the yield of peptides from regulatory subunit was low. In each case, untreated protein and protein carried through the procedure without catalytst gave a single major band at the expected molecular weight (data not shown). Gels, 10% (A) or 13.5% in acrylamide (B and C) were run in the presence of sodium dodecyl sulfate as described by Davies and Stark (9). In D and E, the sodium dodecyl sulfate-urea system of Swank and Munkres (10) was used, except that the gels were presoaked in 5% trichloroacetic acid and 5% salicylic acid before staining. Assignments of molecular weight were made with the aid of standards run concurrently in each experiment.

and Smyth (11) showed that the predominant new end groups were serine and threonine, but this result should be regarded with caution because of the large correction factors for these amino acids. The amino acid sequence of the catalytic subunit is not yet known.

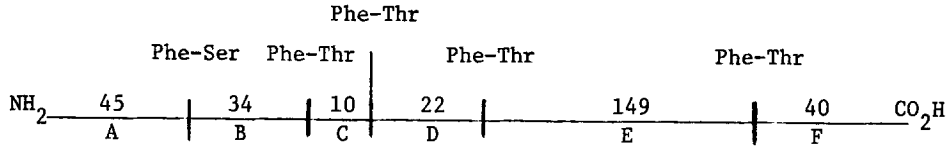
The pH dependence of peptide bond cleavage was investigated with the regulatory subunit of aspartate transcarbamylase, which was treated as described in Figure 1 at pH 5.5 (0.2 M succinate buffer), pH 6.5, and pH 7.0 and pH 7.5

(0.2 M phosphate buffers). Complete conversion of methionine to aminobutyrate was observed in all cases. Varying the pH had very little effect on the gel pattern; a typical analysis is shown in Figure 1B. Partial conversion of regulatory subunit (molecular weight 17,500 (8)) to a form with molecular weight 15,000 was observed, as shown in Figure 1B.

Considering the amino acid sequences of several proteins tested with the method, it was possible to obtain a good prediction of the sizes of peptide fragments by assuming that cleavage to produce amino-terminal serine or threonine occurs most rapidly at Phe-Thr and more slowly at Phe-Ser sequences. Regulatory subunit has only one such sequence, Phe-Ser at residues 144-145 (8) and cleavage at this point would be expected to yield the large fragment observed. Hen egg white lysozyme, which has no Phe-Thr or Phe-Ser sequence (12) is uncleaved by the catalyst under the conditions of Figure 1, as judged by its unchanged pattern in a sodium dodecyl sulfate gel and by its unchanged gel filtration profile on Sephadex G-100 in 50% acetic acid. Both halves of the excluded peak had an amino acid composition after hydrolysis indistinguishable from that of untreated lysozyme, except for the expected conversion of methionine and half-cystine to aminobutyrate and alanine, and no amino acids were found upon hydrolysis and analysis of the salt peak. Catalytic subunit, treated in parallel with the same batch of catalyst, was cleaved as usual.

Lac repressor (a generous gift from Dr. James Houston) gives incomplete cleavage into smaller fragments, with complete conversion of methionine to aminobutyrate. The gel pattern (Figure 1C) reveals, in addition to the uncleaved protein (molecular weight 37,200 (13)), a major band at 21,500 and a fainter band at 17,000. These correspond reasonably well to cleavage at the only Phe-Ser sequence, (residues 150-151) (13), which would result in fragments of 197 and 150 residues. There is no Phe-Thr sequence in lac repressor. There are additional faint fast-running bands in this gel which may result from impurities (the starting protein was somewhat less pure on a gel than the other proteins employed) or possibly from an additional cleavage

Table III. Fragments produced upon specific cleavage of carboxypeptidase A



Fragment and number of residues	Assignment to a band in Fig. IE
AB, 79	8,700
C, 10	Too small to see on the gel
D, 22	2,600
E, 149	17,200
A, 45; B, 34; F, 40	5,500

within the 17,000 m.w. fragment.

DFP-treated carboxypeptidase Aγ (Sigma) gives the pattern shown in Figure IE. All the methionine was converted to aminobutyrate. Carboxypeptidase Aγ contains 4 Phe-Thr sequences at positions 86-87, 96-97, 118-119, 267-268 and a Phe-Ser sequence at position 52-53 (14). (The numbering is for carboxypeptidase A. Aγ is missing the amino-terminal 7 residues. Its molecular weight is 34,491). The gel bands can be matched well with the expected sizes of the fragments if the Phe-Ser sequence is incompletely cleaved, as might be expected from the results with regulatory subunit and lac repressor. The results are summarized in Table III.

Conclusions. Catalytic reduction may be a practical means of removing iminothiazolidinecarboxylyl groups from peptides produced by specific chemical cleavage at half-cystine residues. There is a need for information with larger peptides. Although the limited number of additional specific cleavages introduced by the desulfuration catalyst itself is a complication, many iminothiazolidinecarboxylyl peptides should not be cleaved further. More work is cer-

tainly necessary to confirm our inferences about the sequences cleaved by the catalyst. Cleavage may begin with an N to O acyl migration, catalyzed by protons brought into correct juxtaposition by adsorption to the catalyst of the aromatic ring of the neighboring phenylalanine residues. The resulting ester might be reductively cleaved, or simply hydrolyzed.

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