# TRYPTIC CLEAVAGE AT CYSTEINYL PEPTIDE BONDS*

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Received February 14, 1963

Cysteine may be converted to its S-β-aminoethyl derivative (AEC) by reaction with bromoethylamine in alkaline solution (pH 13) at 70° for 10 minutes (Cavallini et al., 1955). Lindley (1956) suggested that peptide bonds involving the carboxyl group of AEC would be subject to hydrolysis by trypsin, and indeed he found that a polymer of AEC was digested by trypsin. He also showed that a protein (reduced wool) contained some AEC after reaction with bromoethylamine at pH 11. Unfortunately, no quantitative data were given with which to evaluate the rate or extent of substitution by bromoethylamine or digestion by trypsin. Tietze et al. (1957) followed the procedure of Lindley in preparing an aminoethylated insulin. Digestion of this derivative with trypsin and carboxypeptidase-B yielded AEC, but again no quantitative data were given.

Since convincing evidence for the quantitative conversion of cysteinyl residues to AEC residues is still lacking, and because the reaction conditions used above were rather stringent for peptides and proteins, it seemed worthwhile to search for a reagent which would effect the rapid, quantitative conversion of cysteinyl residues to aminoethyl cysteine residues at conditions near to physiological. The reaction of bromoethylamine with sulfhydryl groups is slow under mild conditions. The reaction occurs in two steps, first the conversion of bromoethylamine to the cyclic ethylenimine (Freudlich et al., 1914,

*Supported by USPHS grant AM 06482-01.

1926), and second the opening of the ring by the attacking sulfhydryl group. In the following report it is shown that the first step is rate limiting and so ethylenimine has been used to convert cysteinyl residues to S-(β-aminoethyl) cysteine residues. The usefulness of this conversion has been evaluated with respect to the completeness of both the conversion reaction and tryptic digestion.

## Experimental

Preparation of aminoethylated insulin B-chain. The thiosulfonate of insulin B-chain was prepared by the method of Bailey and Cole (1959), and converted to the free sulfhydryl form by treatment with mercaptoethanol (40 moles per mole of peptide chain) in 8 M urea at pH 8.6 for 2 hours at room temperature. The resulting reduced insulin B-chain was then treated with ethylenimine (10 moles per mole mercaptoethanol) after addition to the reduction mixture of 5.0 M 1-amino-2-methyl-1,3-propane diol, pH 8.6 (25 moles per mole of mercaptoethanol). The reaction was allowed to proceed for 30 minutes, at which time the solution was found to give a negative nitroprusside test. The mixture was diluted with approximately an equal volume of water which resulted in precipitation of most of the peptide. The suspension was then dialyzed for 10 hours against several changes of distilled water. The dialyzed precipitate was centrifuged, washed with ethanol and acetone, dissolved in dilute acetic acid and lyophilized. In several experiments 60-75% of the B-chain thiosulfonate were recovered as aminoethylated derivative.

Enzymic digestion. Tryptic digestions were carried out in a Radiometer Autotitrator using Worthington 2x crystallized enzyme. Digestions were performed at 37° C using an enzyme-substrate ratio of 1:20. Carboxypeptidase-B (Worthington Chemical Corporation) digestions were carried out in 0.2 M "Tris" buffer at pH 7.4 and 37° C for 8 hours, using an enzyme-substrate ratio of 1:20. Before addition of carboxypeptidase-B

to tryptic digests, the trypsin was inactivated by addition of a slight molar excess of diisopropylfluorophosphate.

Amino acid analysis. Amino acid analyses of enzymic digests and acid hydrolysates (6 N HCl, 110° C, in vacuo, 20 hours) of aminoethylated insulin were performed using a Beckman Spinco Amino Acid Analyzer, Model 120. S-(β-aminoethyl) cysteine was eluted between the positions of lysine and histidine under the standard conditions of Spackman, Stein and Moore (1958).

## Results and Discussion

That ethylenimine (EI) reacts much more rapidly than does bromoethylamine (BEA) with sulfhydryl groups was established by estimating their rates of reaction with glutathione (pH 8.6, room temperature, using a 5-fold molar excess of reagent to peptide). In the case of EI, the reaction mixture was no longer nitroprusside positive after 5-7 minutes in contrast to the case of BEA where the reaction mixture was still strongly positive to nitroprusside after 5 hours. This difference in rates of reaction was also clear in the finding that AEC could be produced in 95-100% yield (estimated by use of an amino acid analyser) after the reaction of cysteine with EI (1.5 equivalents) in bicarbonate solution at room temperature for 30 minutes, while a yield of only 25-30% was obtained from the reaction of BEA (1.5 equivalents) and cysteine in bicarbonate solution at 70° C for 3 hours.

The extent to which cysteinyl residues of proteins may be converted to AEC residues was examined using the B-chain of insulin as a model. The B-chain was isolated from insulin as the thiosulfonate derivative and then converted to the aminoethylated derivative as described in the experimental section. The amino acid analysis of a hydrolysate of such a preparation is shown in Table I. Of the 2 cysteinyl residues 91% was recovered as AEC. The analysis also indicates that none of the other amino acids were modified by the reagent. Further

evidence of this was obtained by treating a standard mixture of amino acids with a 100-fold molar excess of EI at room temperature for 2 hours. All the amino acids were recovered quantitatively on chromatographing the treated mixture on an amino acid analyzer and no extraneous peaks were observed. Clearly, then, the reaction is both quantitative and specific for sulfhydryl groups under the conditions used.

Table I

Amino-Acid Composition of Aminoethylated Insulin B-Chain

| Amino Acid | Residues Found (molar ratios) | Theoretical Composition |
|---|---|---|
| Asp. | 0.98 | 1 |
| Thr. | 0.95# | 1 |
| Ser. | 0.99# | 1 |
| Glu. | 3.00+ | 3 |
| Pro. | 0.98 | 1 |
| Gly. | 2.94 | 3 |
| Ala. | 1.92 | 2 |
| 1/2 Cys. | - | - |
| Val. | 2.52** | 3 |
| Met. | - | - |
| Ileu. | - | - |
| Leu. | 3.72** | 4 |
| Tyr. | 1.90 | 2 |
| Phe. | 2.65 | 3 |
| Lys. | 0.93 | 1 |
| His. | 2.01 | 2 |
| Arg. | 0.99 | 1 |
| AEC* | 1.81# | 2 |
| $NH_3$ | 3.8 § | 2 |

*S-(β-aminoethyl) cysteine

+Taken as the base for the calculation of molar ratios.

#Not corrected for hydrolytic destruction.

**The release of these amino acids is not complete on 20-hour hydrolysis.

§Since the preparation was not completely salt free a trace of urea probably makes this value high.

The effectiveness of S-(β-aminoethyl) cysteinyl peptide bonds as substrates for trypsin was demonstrated by digestion of aminoethylated insulin B-chain successively with trypsin and carboxypeptidase-B. The uptake of alkali during tryptic digestion of the aminoethylated chain at pH 9.4 reached the theoretical[+] amount of 3.5 moles per mole of chain corresponding to the rupture of four peptide bonds. (As a control insulin was digested in a parallel experiment in which the theoretical uptake of 1.5 moles of alkali per mole of peptide was obtained.) After poisoning of the trypsin in the digestion mixture by addition of 1.1 mole of diisopropylfluorophosphate per mole of trypsin and treatment with carboxypeptidase-B, the lysine, arginine and S-(β-aminoethyl) cysteine released were estimated. The results of this analysis showed that 95, 100 and 80% of the amounts of lysine, arginine and S-(β-aminoethyl) cysteine known to be present in the aminoethylated chain were released. The near-quantitative values for lysine and arginine do not represent true values since there is some contribution to these figures from the action of carboxypeptidase-B upon the autolytic products of trypsin. It would appear therefore that the rate of release of S-(β-aminoethyl) cysteine is essentially equal to those of lysine and arginine.

Since it is now established that it is possible to get quantitative conversion of cysteinyl residues to AEC residues and complete cleavage of the corresponding peptide bonds by trypsin, a working method is available for selective cleavage of peptide chains at cysteinyl residues. This technique in addition to being useful for establishing peptide overlaps may provide a way for the controlled degradation of hitherto resistant tryptic cores. Another application would be the labelling of unusually reactive sulfhydryl groups in enzymes and the direct exposure of such sites to determination of their immediate environment.

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[+] Tryptic digestion yields alanine and four peptides. At 37° C, $pK_2$ for alanine = 9.5 (Smith et al., 1937), and $pK_2$ for peptides < 8.

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