EVIDENCE FOR MUTUAL EXCLUSION IN THE TRYPTIC HYDROLYSIS OF TWO PEPTIDE BONDS IN MALEYLATED TRYPSINOGEN

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SUMMARY

Treatment of completely maleylated trypsinogen with trypsin resulted in a mutual exclusion of hydrolysis of the two arginyl peptide bonds wherein one half of the molecules of the modified zymogen were cleaved at the Arg^{55}-Leu^{56} bond and the other half of the molecules were cleaved at the Arg^{105}-Val^{106} bond and no molecules were cleaved at both arginyl bonds.

INTRODUCTION

It is common practice to use trypsin for the purpose of making partial hydrolysates of proteins prior to performing sequencing studies. Masking the ε-amino groups of lysine residues in the protein restricts tryptic hydrolysis specifically to arginyl residues in the polypeptide chain and reduces the number of peptide fragments to be separated and sequenced (1). One of the ways of masking the ε-amino groups of lysine residues is maleylation (2), which has the advantage of being reversible, and allows further tryptic cleavage of the separated, deblocked peptides. The usefulness of the procedure depends on a nearly quantitative hydrolysis of the susceptible bonds of the protein or modified protein. During studies examining the possibility of cleaving maleylated trypsinogen with trypsin at arginyl residues only, we observed an incidence of a mutual exclusion of the tryptic hydrolysis of the two arginyl peptide bonds in this modified zymogen, wherein one half of the molecules were cleaved at the Arg^{55}-Leu^{56} bond while the other half of the molecules were cleaved at the Arg^{105}-Val^{106} bond. The occurrence of this phenomenon during the preparation of partial

hydrolysates of unsequenced proteins prior to separation and sequencing of the peptides would lead to confusing results and reinforces the necessity of exact correlation of amino acid analysis and peptide mapping in sequence studies.

MATERIALS AND METHODS

Trypsin (3X crystallized) and trypsinogen was purchased from Worthington Biochemical Corporation. Maleic anhydride was obtained from Aldrich Chemical Company and was used without recrystallization.

Carboxypeptidase B (Worthington) was immobilized by the method of Glassmeyer and Ogle (3) for the preparation of insoluble trypsin.

Trypsinogen was completely maleylated by the method of Butler et al. (2) and checked for the absence of free amino groups with trinitrobenzene sulfonic acid (4). Deblocking was performed at pH 3 either in suspension or in the presence of 1% SDS or 8 M urea or 6 M quanidinium chloride for solubilization. Two μmoles of 100% maleylated trypsinogen were reacted under nitrogen flush at room temperature with 0.1 μmole of trypsin in 0.01 M Tris·HCl buffer and the pH was kept at 8.0 with 0.01 N NaOH. An aliquot of the reaction mixture was treated with insoluble carboxypeptidase B at 25° for 2 hours at pH 8.0 and filtered. The filtrate was concentrated and assayed on the Beckman amino acid analyzer. Another aliquot of the reaction mixture was analyzed for N-terminal amino acid residues by the cyanate method of Stark (5), employing leucylglycine as the control peptide for correcting for losses. Quantitation of newly formed N-terminal amino groups in the reaction mixture was done by the trinitrobenzene sulfonic acid method according to Habeeb (4). Aliquots of the reaction mixture were dialyzed against either 0.001 N HCl or 1% sodium dodecyl sulfate, followed by concentration, 6 N HCl hydrolysis at 110° for 20 hours and analysis on the Beckman amino acid analyzer.

RESULTS AND DISCUSSION

Completely maleylated trypsinogen contains only two potential places for tryptic cleavage, Arg^{55}-Leu^{56} and Arg^{105}-Val^{106}. Cleavage at both of these arginine residues would release a 50-residue fragment (MW 6,300) which contains no disulfide bridges and which isn't attached to the remaining modified trypsinogen fragment by disulfide bridges. The 50-residue fragment does not contain His 46, Ser 183, or Asp 177, residues implicated in the active center of trypsin but does contain Asp^{90} which may (6) or may not be required for a charge relay system homologous with that in chymotrypsin. Theoretically, activation of this cleaved, maleylated trypsinogen, before or after deblocking, could give rise to a modified species of trypsin with or without altered specificity. Maleyltrypsin (100% lysyl residues modified)

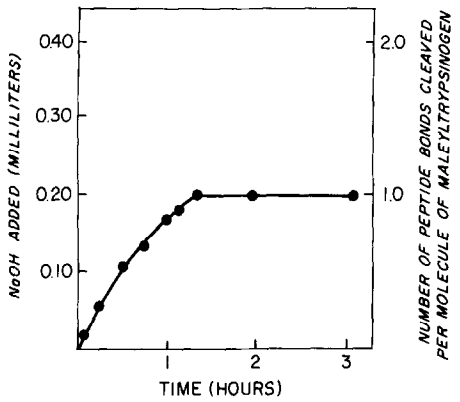

Figure 1. NaOH titration of the tryptic cleavage of maleyltrypsinogen (100% of lysyl residues blocked). Two μmoles of maleyltrypsinogen were reacted with 0.1 μmole of trypsin in 0.001 M Tris, pH 8, 0.02 M $CaCl_2$ 25°, under N_2 flush. Titrant was 0.01 N NaOH.

still possess enzymatic activity (7). This would be a further and drastic extension of the examples of different molecular species of trypsin which heretofore have been produced by autolysis of the protease (8,9). Desnuelle has reported an autolytic cleavage at Arg^{105}-Val^{106} of trypsin (9) but there is no report of a cleavage at the other arginyl bond.

The lysyl residues of trypsin were completely maleylated with excess maleic anhydride (2). The maleyltrypsinogen could be deblocked only to the extent of 50% by incubation at 37° and pH 3.5 for as long as 31 hours. The 50% maleyltrypsinogen could not be converted to an active form by treatment with trypsin, suggesting that probably Lys^6 at the normal activation site was still modified. Nor could the 50% or the 100% maleylated trypsinogen be converted to an active form by pronase (10) subtilisin, thermolysin, or chymotrypsin. Nevertheless, the 100% maleyltrypsinogen was reacted with trypsin to determine whether the two susceptible arginyl bonds could be cleaved. Two μmoles of 100% maleylated trypsinogen were reacted with 0.1 μmole of trypsin and the pH was kept at 8.0 with 0.01 N NaOH. The uptake of NaOH is shown in Figure 1 and amounted to only 2 μmoles instead of the expected 4 μmoles. This corresponds to an average of one peptide bond

cleaved per molecule of maleyltrypsinogen instead of the expected two bonds broken. Treatment of an aliquot of the reaction mixture with insoluble carboxypeptidase B yielded only 0.95 μmole of arginine per μmole of tryptic treated maleyltrypsinogen instead of the expected 2 μmoles. No lysine was found as would be expected. Another aliquot of the reaction mixture was analyzed for N-terminal amino acid residues which resulted in only 0.55 μmole of valine and 0.51 μmole of leucine (corrected values) per μmole of treated maleyltrypsinogen. The control maleyltrypsinogen yielded no N-terminal valine hence this residue was blocked. Quantitation of newly formed N-terminal amino groups of the tryptic treated maleyltrypsinogen yielded 1.1 μmoles of free amino groups per μmole of cleaved maleyltrypsinogen instead of the expected 2 μmoles. Dialysis of aliquots of the reaction mixture either against 0.001 N HCl or in the presence of 1% SDS followed by 6 N HCl hydrolysis of the dialysates at 110° for 20 hours and amino acid analysis gave no indication of the presence of any of the 50 amino acid residue fragment. Nor could any of the fragment be detected in the reaction mixture by SDS polyacrylamide gel electrophoresis with or without β-mercaptoethanol or by sedimentation equilibrium studies in the presence or absence of 8 M urea. Thus the data confirm that one half of the maleyltrypsinogen molecules were cleaved at the Arg^{55}-Leu^{56} bond while the other half of the molecules were cleaved at the Arg^{105}-Val^{106} bond and no fragment was released in any of the molecules. The possible failure of maleylation of the lysyl residues in Lys^{95}-Leu^{96}, Lys^{192}-Leu^{193} and Lys^{214}-Val^{215} and subsequent tryptic cleavage of these bonds could give rise to newly formed N-terminal valine and leucine. However, this hydrolysis would also produce C-terminal lysine which would be released by the carboxypeptidase B treatment of the reaction mixture. Since lysine was not detected the N-terminal valine and leucine arose from hydrolysis of arginyl peptide bonds containing these residues.

These observations indicate that when the maleyltrypsinogen was cleaved

by trypsin at either one of the arginyl bonds, the molecule underwent a conformational change such that the other arginyl bond was no longer accessible to trypsin. The failure to find any released 50-residue fragment indicates that trypsin did not hydrolyze both arginyl bonds of just 50% of the maleyltrypsinogen malecules. The data also indicate that two molecules of trypsin did not simultaneously bind one molecule of maleyltrypsinogen and hydrolyze both arginine bonds in the same molecule of the substrate. In addition, the results suggest that both arginyl peptide bonds in the intact modified zymogen are equally susceptible to tryptic cleavage. The phenomenon of mutual exclusion of amino acid residues to reaction has been observed in the chemical modification of the active site residues histidine^{12} and histidine^{119} in ribonuclease (11). As far as is known this is the first incidence of mutual exclusion in enzymatic bond cleavage and may well be significant in the interpretation of the results of proteolysis of proteins for the purpose of preparing partial hydrolysates for sequencing.

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