Influence of the Carbohydrate Moiety on the Proteolytic Cleavage Sites in Ribonuclease B

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The influence of glycosylation on proteolytic degradation was studied by comparing cleavage sites in ribonuclease A (RNase A) and ribonuclease B (RNase B), which only differ by a carbohydrate chain attached to Asn34 in RNase B. Primary cleavage sites in RNase B were determined by identifying complementary fragments using matrix-assisted laser desorption/ionization mass spectrometry and compared with those in RNase A [Arnold et al. (1996), Eur. J. Biochem. 237, 862-869]. RNase B was cleaved by subtilisin even at 25°C at Ala20-Ser21 as known for RNase A. Under thermal unfolding, the peptide bonds Asn34-Leu35 and Thr45-Phe46 were identified as primary cleavage sites for thermolysin and Lys31-Ser32 for trypsin. These sites are widely identical with those in RNase A. Treatment of reduced and carbamidomethylated RNase A and RNase B with trypsin led to a fast degradation and revealed new primary cleavage sites. Therefore, the state of unfolding seems to determine the sequence of degradation steps more than steric hindrance by the carbohydrate moiety does.

KEY WORDS: Ribonuclease B; proteolysis; carbohydrate chain; trypsin; thermolysin.

1. INTRODUCTION

Glycosylation is the most extensively occurring natural modification of proteins. Its biological role, however, is not sufficiently understood. Such cellular functions as traffic marker, signal modifier, or regulator of biological activity are attributed to the carbohydrate content in glycoproteins (Rademacher et al., 1988; Lis and Sharon, 1993). The comparison of the molecular properties of glycosylated and nonglycosylated proteins revealed differences in their solubility (Jaenicke, 1991), thermal stability (Mer et al., 1996), and susceptibility toward proteolytic attack (Rudd et al., 1995), which might be of biological significance, too.

An ideal model system for studying effects induced by glycosylation has been provided by nature in the form of the couple ribonuclease A (RNase A)3 and ribonuclease B (RNase B). Both enzymes occurring in bovine pancreas have identical protein sequences consisting of 124 amino acid residues (Smyth et al., 1963; Plummer et al., 1968), which form a nearly identical tertiary structure (Berman et al., 1981; Williams et al., 1987). They only differ by an oligosaccharide chain N-linked to Asn34 in RNase B (Plummer et al., 1968). This carbohydrate chain is not uniform, but contains 5–9 mannose units (GlcNAc-Man5–9), resulting in an increase of the molecular mass from 13,683 Da (RNase A) to 14,899–

1 Abbreviations: CAM-RNase, reduced and carbamidomethylated RNase; CD, circular dichroism; DTE, 1,4-dithioerythritol; EDTA, ethylenediaminetetraacetic acid disodium salt; GdnHCl, guanidine hydrochloride; MALDI-MS, matrix-assisted laser desorption/ionization mass spectrometry; NMR, nuclear magnetic resonance; PMSF, phenylmethanesulfonyl fluoride; RNase, ribonuclease; RP-HPLC, reversed-phase high-performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; Tris, tris(hydroxymethyl)-aminomethane; UV, ultraviolet.

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and refolding of RNase A was investigated by isomerization on the thermally induced unfolding pathway of RNase A. Ooi et al. (1963), Rupley and Scheraga (1963), and Klee (1967) gave some information on the thermally induced unfolding pathway of RNase A. Results by Ooi et al. (1963), Rupley and Scheraga (1963), and Klee (1967) gave some information on the thermally induced unfolding pathway of RNase A. The role of proline isomerization in the unfolding and refolding of RNase A was investigated by isomer-specific proteolysis (Lin and Brandts, 1983, 1984), while the refolding of GdnHCl-denatured RNase A was followed by a trypsin-pulse method (Lang and Schmid, 1986). Recently, Arnold et al. (1996) localized the structural region first unfolded under thermal denaturation by limited proteolysis with trypsin and thermolysin. This region is modified by the carbohydrate chain in RNase B, so that dramatic changes in the unfolding pathway and the position of primary proteolytic cleavage sites were to be expected.

In the present paper, the primary cleavage sites in native and thermally denatured RNase B are located for subtilisin, trypsin, and thermolysin and are compared to those of RNase A. The studies have been completed by the analysis of proteolytic fragments in the degradation of the reduced and carbamidomethylated RNase A and RNase B (CAM-RNases). The results allow us to compare the structural flexibility and steric accessibility of the two enzymes in their native, thermally denatured, and reduced/carbamidomethylated states.

2. MATERIALS AND METHODS

RNase A from Serva and RNase B from Sigma were purified on an FPLC-column MONO S (Pharmacia) resulting in single bands in the SDS–PAGE. Thermolysin, subtilisin Carlsberg, angiotensin II (human), insulin (bovine), cytochrome c (horse heart), soybean trypsin inhibitor, bovine pancreas trypsin inhibitor from Sigma, and trypsin (treated with N-α-tosyl-L-phenylalanine chloromethyl ketone) from Serva were used without further purification.

Acrylamide, N,N,N′-methylenebisacrylamide, N,N,N′,N′-tetramethylethylenediamine, and ammonium persulfate were purchased from Pharmacia, tris(hydroxymethyl)aminomethane (Tris), N-tris(hydroxymethyl)methylglycine, and Coomassie brilliant blue G250 from Serva, calcium chloride and phenylmethanesulfonyl fluoride (PMSF) from Merck, acetonitrile (ultraviolet-grade) from Roth, ultrapure GdnHCl from Schwarz-Mann Biotech, and trifluoroacetic acid (TFA), 1,4-dithioerythritol (DTE), ethylenediaminetetraacetic acid disodium salt (EDTA), iodoacetamide, and SDS from Sigma. Sinapinic acid from Aldrich and α-cyano-4-hydroxycinnamic acid from Sigma were twice recrystallized from methanol. All other reagents were the purest ones commercially available.

2.1. Proteolysis

In a typical experiment 80 μl of 50 mM Tris-HCl buffer, pH 8.0, was preincubated in a thermostat RM 6