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β-Aspartyl Peptide Formation from an Amino Acid Sequence in Ribonuclease*

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ABSTRACT: Exhaustive enzymic hydrolysates of bovine ribonuclease have a much higher β-aspartyl peptide content than digests of other proteins of known structure previously studied. β-Aspartyl di-, tri-, and tetrapeptides are shown to derive from 22 to 34% of a certain amino acid sequence beginning with asparagine at residue 67 of ribonuclease. Evidence is presented that the β-aspartyl linkage does not preexist in the protein but forms spontaneously and nonenzymatically in fragments liberated during the digestion.

Previous experiments (Haley *et al.*, 1966; Pisano *et al.*, 1966), in which β-aspartyl peptides were found in enzymic digests of several proteins, failed to demonstrate conclusively that these linkages originated from the natural amino acid sequence of the molecules. In proteins of known sequence molar yields of Asp(Gly),[1] the most abundant peptide, were always below 4% of the theoretical, a value too low to exclude with certainty the occasional presence of a side-chain glycine attached *in vivo* by transamidation (Loewy *et al.*, 1966). Our results indicated but did not prove that β-aspartyl bonds formed during the digestions and did not preexist.

Because the nonenzymic conversion of Asn-Gly to Asp(Gly) was found to proceed at approximately ten times the rate of the conversion of Asp-Gly to the β isomer it was felt that bovine ribonuclease, which has one -Asn-Gly- and no -Asp-Gly- sequences (Hirs *et al.*, 1960; Smyth *et al.*, 1962, 1963), might yield more Asp(Gly) than hemoglobin or lysozyme, which have -Asp-Gly- but no -Asn-Gly- sequences (Konigsberg *et al.*, 1962, 1963; Canfield, 1963). Digestions of native as well as performic acid oxidized ribonuclease by methods we previously used with other proteins resulted in molar yields of Asp(Gly) not much different from those obtained with hemoglobin and lysozyme, but in total yields of β-aspartyl di-, tri-, and tetrapeptides as high as 34% of theory and matching a known sequence in the protein. Isolation and study of the 19 residue peptide, *O*-Trp 2,[2] and the heptapeptide (*O*-Trp 2)-Chy 3[3] (Hirs *et al.*, 1956), which contain the sequence, give convincing evidence that the β-aspartyl peptides arise from the sequence. The results point strongly to the nonenzymic conversion of an N-terminal asparaginyl to a β-aspartyl linkage during the digestions.

Experimental Section

Materials. Bovine ribonuclease and chymotrypsin, both three times crystallized, were purchased from Worthington; ribonuclease A, 100 Kunitz units/mg, from Sigma; and trypsin, free of chymotrypsin, from Calbiochem. Asp(Gly-Gln) was prepared as described elsewhere (Dorer *et al.*, 1967). Other hydrolytic enzymes and aspartyl peptides were described previously (Haley *et al.*, 1966).

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[1] Abbreviations used: Asp-Gly, α-L-aspartylglycine; Asp(Gly), β-L-aspartylglycine; Asp(Gly-Gln), β-L-aspartylglycyl-L-glutamine, etc.; VK, Viokase; PRN, Pronase; LAP, leucine aminopeptidase; CA, carboxypeptidase A; CB, carboxypeptidase B; TR, trypsin; Chy, chymotrypsin; PRL, prolidase.

[2] *O*-Trp 2 is Asn-Gly-Gln-Thr-Asn-$CySO_3H$-Tyr-Gln-Ser-Tyr-Ser-Thr-$MetSO_2$-Ser-Ile-Thr-Asp-$CySO_3H$-Arg.

[3] (*O*-Trp 2)Chy 3 is Asn-Gly-Gln-Thr-Asn-$CySO_3H$-Tyr.

TABLE I: Enzymic Hydrolyses of Ribonuclease.

Expt	Protein (g)	Enzyme Addn[a] (mg)	Cumulative Digestion Time (hr)	Hydrolysis[b] (%)	Total ^{14}C Recov (%)
1	RNase, 0.5	VK, 12.3, 12.3	120	57	77
		PRN, 5.1	166	74	
		LAP, 0.5; PRL, 5.1	210	87	
		CA, 2.5; CB, 0.3	280	94	
2	RNase, 0.2	VK, 6.4, 5.9	91	70	71
		PRN, 2.6	139	75	
		LAP, 0.3; PRL, 2.5	186	80	
		CA, 1.3; CB, 0.2	258	95	
3	Oxidized RNase, 0.2	VK, 6.2, 6.1	87	57	66
		PRN, 2.5	136	57	
		LAP, 0.3; PRL, 2.5	207	67	
		CA, 1.3; CB, 0.2	352	*c*	
4	Oxidized RNase, 0.2	VK, 6.7, 6.1	96	55	46
		PRN, 2.6, 2.5	167	63	
		LAP, 0.3; PRL, 2.6	240	75	
		CA, 1.3; CB, 0.2	260	78	

[a] LAP added was 100–150 units/g of substrate; CB, 45–60 units/g. [b] Followed with ninhydrin reaction (AutoAnalyzer). Complete hydrolysis taken as that obtained with 6 N HCl at 120° for 18 hr. [c] No analytical value.

Methods. Ribonuclease was oxidized with performic acid at 0° (Hirs, 1956). Procedures for sequential enzymic digestions of ribonuclease (Table I), and separations on ion-exchange resins and thin layer and analytical chromatography were as previously described for hemoglobin (Haley *et al.*, 1966) except that Asp(Gly-Gln) and Asp(Gly-Gln-Thr), which failed to separate on the Dowex 2 (acetate) column, were rechromatographed on a 0.6 × 137 cm Dowex 1-X4 (~25-μ beads, formate) column eluted with 0.1 M formic acid at room temperature. Asp(Gly-Glu) and Asp(Gly-Glu-Thr) were similarly separated. The concentrations of the four tri- and tetrapeptides were determined by acid hydrolysis and amino acid analysis of the separated compounds or sometimes in the case of Asp(Gly-Gln) by direct comparison with the synthetic compound. Asp(Gly) and Asp(Thr) were determined directly on a Dowex 50 column (Dorer *et al.*, 1967). Enzyme blank determinations were carried out to match the sequence, timing, and concentrations of all reagents except that no protein substrate was present. None of the peptides reported except Asp(Gly) was found in these digests.

To isolate the peptide, *O*-Trp 2, oxidized ribonuclease was digested with trypsin (Hirs *et al.*, 1956) for 5 hr. HCl (2 N) was added to pH 2.2 and the digest was applied to a 2.0 × 143 cm column of AG50W-X2 (200–400 mesh) equilibrated at 38° with 0.1 M pyridine–formic acid buffer (pH 2.6). The column was pumped with the buffer at 0.7 ml/min, and the stream divided so that 94% was collected as 5-min fractions and the remainder was run through the analyzer. Alkaline hydrolyses and ninhydrin analyses were performed on column fractions of the leading and trailing edges of the peaks to define the boundaries more exactly. Fractions containing the most acidic peptides were combined and samples were then hydrolyzed for amino acid analyses. After isolation *O*-Trp 2 was digested with chymotrypsin (Hirs, 1960) and the acidified digest was applied to the AG50-X2 column described above. Elution was the same as for the tryptic digest, and the most acidic peptides were collected as before.

Results

β-Aspartyl Peptides Matching Sequence 67–70 of Ribonuclease. After enzymic treatment of native and oxidized ribonuclease under the conditions of Table I there was up to 95% hydrolysis. Recoveries of labeled Asp(Gly), added prior to the digestions, were lower than those found previously with other proteins (Haley *et al.*, 1966). Because only the yield of Asp(Gly) could legitimately be corrected for recovery of radioactivity the yields of the other β-aspartyl peptides are minimal values.

When the digests were subjected to the separation procedure described in the previous report (Haley *et al.*, 1966) and in the Methods section of this paper, five compounds with the native protein and six with the oxidized material, all with the typical blue ninhydrin color of β-aspartyl peptides (Buchanan *et al.*, 1966), were detected by thin layer chromatography of the Dowex 2 fractions. Peptides subsequently proven to be

Asp(Gly-Gln-Thr), Asp(Gly-Gln), and Asp(Thr), the latter only with the oxidized ribonuclease, preceded aspartic acid but separations were incomplete. Asp(Gly) was separate but Asp(Gly-Glu-Thr) and Asp(Gly-Glu), brought off the column with 1 M formic acid, were mixed. Fractions were combined so that the first pooled fraction contained all of the Asp(Gly-Gln-Thr) and part of the Asp(Gly-Gln); the second contained the remainder of the tripeptide, Asp(Thr) (when present), and some of the aspartic acid; the third contained only Asp(Gly); and the final pooled fraction contained tri- and tetrapeptides with glutamic acid instead of glutamine.

Separations and analyses were performed on aliquots of these to give the results of Table II. The tripeptide of the first two fractions was identical with synthetic Asp(Gly-Gln) on analytical columns of Dowex 1 and Dowex 50 (Dorer *et al.*, 1967) and with thin layer chromatography. This compound and the tetrapeptide of the first combined fraction gave satisfactory amino acid and ammonia analyses after acid hydrolysis. The tetrapeptide when treated with carboxypeptidase A liberated 1 residue of threonine and 0.1 residue of glutamine. The predominant residual peptide was identical with synthetic Asp(Gly-Gln) on an analytical column of Dowex 1 (formate) and by thin layer chromatography. The less abundant tri- and tetrapeptides of the final fraction were presumed to be the glutamic acid counterparts of the peptides in the first fractions because of their amino acid analyses, the absence of ammonia after acid hydrolysis, their higher acidity as evidenced by their behavior on Dowex 1 (formate), and their blue ninhydrin color.

Of the five β-aspartyl peptides matching the ribonuclease sequence Asp(Gly-Gln) was the most abundant and the tetrapeptide containing glutamine was next. The least abundant compounds were the two peptides that contained glutamic acid. These were presumed to have arisen by deamidation of their analogs during digestion or isolation. The high total yield of the group of peptides offered hope of isolating the β-aspartyl analog of a larger peptide fragment.

TABLE II: β-Aspartyl Peptide Content[a] of the Hydrolysates.

Peptide	Per Cent of Theory[b]		
	Native RNase	Oxidized	
Asp(Gly)	2	2	2
Asp (Gly-Gln)	20	15	15
Asp(Gly-Gln-Thr)	9	3	6
Asp(Gly-Glu)	2	0.9	0
Asp(Gly-Glu-Thr)	1	0.8	0
Total[c]	34	22	23
Asp(Thr)	0	1	2

[a] All analytical values corrected for sampling aliquots. Asp(Gly) values were corrected also to complete recovery of the added radioactivity, for added labeled peptide, and for the enzyme blank. Values for other peptides are uncorrected. [b] Moles/mole of protein × 100. Based on one -Asn-Gly- and one -Asn-Thr- sequence per molecule of ribonuclease. [c] Conversion at residue 67.

Isolation and Properties of Peptides O-Trp 2 and (O-Trp 2)Chy 3 and Their β-Aspartyl Analogs. Two tryptic digests of 0.23 (17 μmoles) and 0.44 g (33 μmoles) of oxidized ribonuclease A were separated. Amino acid analyses (Table III) of the content of peaks that eluted between 790 and 1040 ml in each separation were consistent with Hirs' *O*-Trp 2 peptide in yields of 6 and 11 μmoles (33 and 35% of theory). Preceding this peak in the larger digest was a small peak that gave an amino acid analysis consistent with approximately 0.2 μmole (0.6% of theory) of the β-aspartyl analog of the *O*-Trp 2 peptide but with some contamination.

Two portions of the material identified as the *O*-Trp 2 peptide were digested with chymotrypsin (Hirs, 1960), acidified, and chromatographed. From both digests peptides emerged from the column between 284 and 348 ml and at 580–700 ml. In both runs the first-mentioned peptide gave the characteristic blue ninhydrin color of a β-aspartyl peptide and an amino acid analysis (Table III) for the heptapeptide, (*O*-Trp 2) Chy 3, less 1 equiv of ammonia. In the two runs yields of this compound were 11 and 13% of theory. Digestion of 0.34 μmole of this peptide with Pronase followed by carboxypeptidase A yielded 0.25 μmole of Asp(Gly-Gln) by Dowex 1 (formate) and thin layer chromatography.

The second compound to emerge in both runs gave the correct amino acid analysis for the unaltered (*O*-Trp 2)Chy 3 peptide. Yields were 60 and 73% of theory. The peptide proved to be excessively labile. Separation from the β-aspartyl analog was easily achieved on an analytical column (0.6 × 137 cm) of AG50W-X2 (200–400 mesh) pumped at 0.5 ml/min with the same buffer used for the preparative separation but on immediate evaporation to dryness *in vacuo* at 37°, storage overnight at −20°, and rechromatography noticeable quantities of the β-aspartyl compound were evident. Incubation at 37° in 0.05 M phosphate buffer (pH 7.4) for 11 days (for sterility control *cf.* Haley *et al.*, 1966) resulted in complete conversion to the β-aspartyl peptide.

Evidence for Nonenzymic Conversion of the Asparaginyl to the β-Aspartyl Linkage. The formation of a β-aspartyl linkage six residues removed from the site of attack of chymotrypsin on the peptide, *O*-Trp 2, and the ease of nonenzymic conversion of the asparaginyl peptide (*O*-Trp 2)Chy 3 to its β-aspartyl analog suggested that the conversion was not catalyzed by enzymes.

Three digestions, each of 2.0 μmoles, of oxidized ribonuclease A were performed. In the first digestion enzyme concentrations and timing were approximately those used previously. In the second, enzyme concentrations were threefold greater and the incubation times were reduced to one-third. In the third, the concentra-

TABLE III: Amino Acid Composition of Tryptic and Chymotryptic Peptides of Oxidized Ribonuclease.[a]

Amino Acid	*O*-Trp 2		(*O*-Trp 2)Chy 3		β-Aspartyl-(*O*-Trp 2)Chy 3	
Cysteic acid	1.8	1.9	0.9	0.9	1.1	1.0
Aspartic acid			2.0	2.0	2.0	2.0
Aspartic acid + methionine sulfone[b]	3.9	3.9				
Threonine	3.2	3.0	1.0	1.0	0.9	1.0
Serine	2.9	2.8				
Glutamic acid	2.1	2.0	1.1	1.0	1.1	1.1
Glycine	1.2	1.1	1.0	1.0	1.0	1.0
Isoleucine	0.9	1.0				
Tyrosine[c]	1.4	1.5	0.7	0.7	0.6	0.7
Arginine	1.0	1.0				
Ammonia (from amide)	4.8	4.2	3.0	3.0	2.0	2.4

[a] Composition expressed as residues per molecule of peptide for two preparations of each peptide. [b] Aspartic acid and methionine sulfone formed a symmetrical peak. The color yield used in the calculation was a weighted mean for the individual amino acids. [c] Low yield of carboxyl-terminal tyrosine was reported also by Hirs (1960).

tions were tenfold and the times one-tenth. The protein was digested sequentially with trypsin, chymotrypsin, Pronase, leucine aminopeptidase, and carboxypeptidase A (Table IV), a combination of enzymes considered suitable for the liberation and degradation of the *O*-Trp 2 peptide. Analyses of the digests were carried out only for the two more abundant β-aspartyl peptides Asp-(Gly-Gln) and Asp(Gly-Gln-Thr). Combined yields of the two compounds were 28, 14, and 5% of theory, respectively (Table IV). The 28% yield lay within the range of values in Table II. Amino acid analyses of the digests indicated that the total hydrolyses of the samples were approximately equal when enzyme blank analyses were subtracted. A precise analysis of the results was not possible because of the uncertainty of comparing "enzyme blanks" in the presence and absence of substrate.

Discussion

The finding of β-aspartyl peptides with two, three, four, and seven residues and the probable presence of one with 19 residues, all matching a known sequence of bovine ribonuclease, leaves little doubt that the peptides originated from the sequence and were not the result of an adventitious attachment of a few glycine and glycine peptide molecules to a β-carboxyl or amide group of aspartic acid or asparagine. The quantities found lend further strength to the thesis. The Asp(Thr) found could have been derived from the -Asn-Thr- sequence at residue 44–45. The lability of the N-terminal asparaginyl residue toward conversion to a β-aspartyl residue has been noted by Ambler (1963) who reported loss of the amide group under the mild conditions of electrophoresis and elution from paper. The resulting peptides possessed slightly acidic mobilities indicating aspartic acid residues. Similarly, Groskopf *et al.* (1966) attributed the occurrence of an anomalous N-terminal aspartyl peptide from tryptic digests of *G. gouldii* hemerythrin to deamidation of the asparaginyl peptide. The conversion did not result from acid catalysis which facilitates α- to β-aspartyl transformations.

TABLE IV: Enzymic Hydrolyses of Oxidized Ribonuclease and the Asp(Gly-Gln) and Asp(Gly-Gln-Thr) Content of the Hydrolysates. Effects of Incubation Time and Enzyme Concentration.

Enzyme Addn (mg)	Cumulative Digestion Time (hr)	Peptide Content (moles/mole of protein) Asp(Gly-Gln)	Asp(Gly-Gln-Thr)
TR[a] (0.12)	5	0.100	0.180
Chy (0.18)	25		
PRN (0.40)	49		
LAP (0.03)	73		
CA (0.25)	97		
TR (0.36)	1.7	0.053	0.086
Chy (0.54)	8.3		
PRN (1.2)	16.3		
LAP (0.09)	24.3		
CA (0.75)	32.3		
TR (1.2)	0.5	0.017	0.037
Chy (1.8)	2.5		
PRN (4.0)	4.9		
LAP (0.3)	7.3		
CA (2.5)	9.7		

[a] Incubation with trypsin and chymotrypsin was at 25° and with the other enzymes at 37°.

Because of the facility of spontaneous conversion of amino-terminal asparaginyl to β-aspartyl bonds under mild conditions it is not necessary to postulate enzymic catalysis to account for β-aspartyl peptides in protein digests or *in vivo* (Buchanan *et al.*, 1962; Haley *et al.*, 1966). It is, however, difficult to prove that none of the observed conversion is enzymic. The present experiments seem to show that the bulk of it is not. Shortening the time required to produce a given degree of hydrolysis by a compensating increase in enzyme concentration strikingly reduced the recovery of β-aspartyl peptides. If most asparaginyl to β-aspartyl conversions were catalytic, one would expect the yield of β-aspartyl peptides to be approximately equal in the three digests. This result would also be expected if the linkages existed in the intact protein.

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