ANOMALOUS CLEAVAGE OF ASPARTYL-PROLINE PEPTIDE BONDS DURING AMINO ACID SEQUENCE DETERMINATIONS*

Dennis Piszkiewicz, Michael Landon, and Emil L. Smith

Department of Biological Chemistry, UCLA School of Medicine, and Molecular Biology Institute, University of California, Los Angeles California 90024

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SUMMARY: Aspartyl-proline peptide bonds have been found to be hydrolyzed during exposure to low pH values under conditions where other aspartyl bonds are stable. The mechanism of this hydrolytic reaction is concluded to proceed via intramolecular catalysis by carboxylate anion displacement of the protonated nitrogen of the peptide bond. The enhanced rate with proline as compared to other amino acids is undoubtedly due to the greater basicity of the proline nitrogen.

The purpose of this communication is to call attention to the unusual lability of the peptide bond linking aspartic acid and proline at acid pH since anomalous cleavage at this bond is likely to occur in studies of polypeptides and proteins where this linkage occurs. We would like also to suggest the possible reasons for the greater sensitivity of this linkage as compared to other peptide bonds.

During studies of the amino acid sequence of bovine liver glutamate dehydrogenase (1) we observed that the two Asp-Pro bonds which occur in this protein were generally almost completely hydrolyzed in the tryptic, peptic, and other digests. A cursory inspection of the literature showed that similar lability of Asp-Pro bonds has been reported by others during studies of the amino acid sequences of various proteins; thus, the phenomenon appears to be a general one.

A summary of the Asp-Pro bonds of various proteins which we have found in the literature reported to hydrolyze during proteolytic or chemical fragmentation is given in Table I. Although a wide range of methods was employed, the common condition in all of these studies was that the enzymic digests were either performed or handled during purification of peptides at low pH values

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and relatively low temperatures (40° and below). During our studies on the sequence of glutamate dehydrogenase (1), partial hydrolysis at aspartyl bonds other than those listed in Table I was found to have occurred only to a minor extent during cleavage by CNBr (T. J. Langley and E. L. Smith, unpublished studies). The conditions used for this reaction (70% aqueous formic acid at 25° for 24 hours followed by rotary evaporation at 40° to remove solvent and excess reagent) were significantly more vigorous than those generally used for routine handling and purification procedures of various enzymic digests (e.g., 30% acetic acid, pyridine-acetic acid buffer at pH 2.8 or above).

Aspartyl-peptide bonds are selectively hydrolyzed under mildly acidic conditions for prolonged periods of time (4 to 24 hours) at relatively high temperature (6). The mechanism of hydrolysis undoubtedly involves intramolecular catalysis by a carboxyl group of the aspartyl residue. Hydrolysis of the structurally analogous compounds, phthalamic acid (7,8) and succinanilic acid (9), has been studied in the acid pH range, and reported to proceed at rates apparently proportional to the mole fraction of the undissociated carboxyl forms (7,8,9). An anhydride was proposed as a product in the first reaction (7,8), and the anhydride was demonstrated to be the product of hydrolysis of succinanilic acid (9). A mechanism of neighboring carboxyl group catalysis in the hydrolysis of amide bonds which accommodates both the kinetic data and product identification is nucleophilic displacement of the protonated amide by carboxylate anion (Equation 1, Fig. 1).

The ε-amino groups of lysyl residues in polypeptides have been acylated by reaction with maleic anhydride and then unblocked on standing at low pH (10). The mechanism of demaleylation is clearly analogous to Equation 1.

Under acidic conditions aspartyl-peptide bonds undergo a reversible isomerization of the α-amide to the β-amide bond via the cyclic α,β-imide intermediate (11,12) (Equation 2). Thus, the relative lability of the aspartyl-peptide bond could be the result of either intramolecular catalysis by carboxylate displacement of the protonated α-amide bond (k_β, Equation 2) or by α-carboxylate dis-

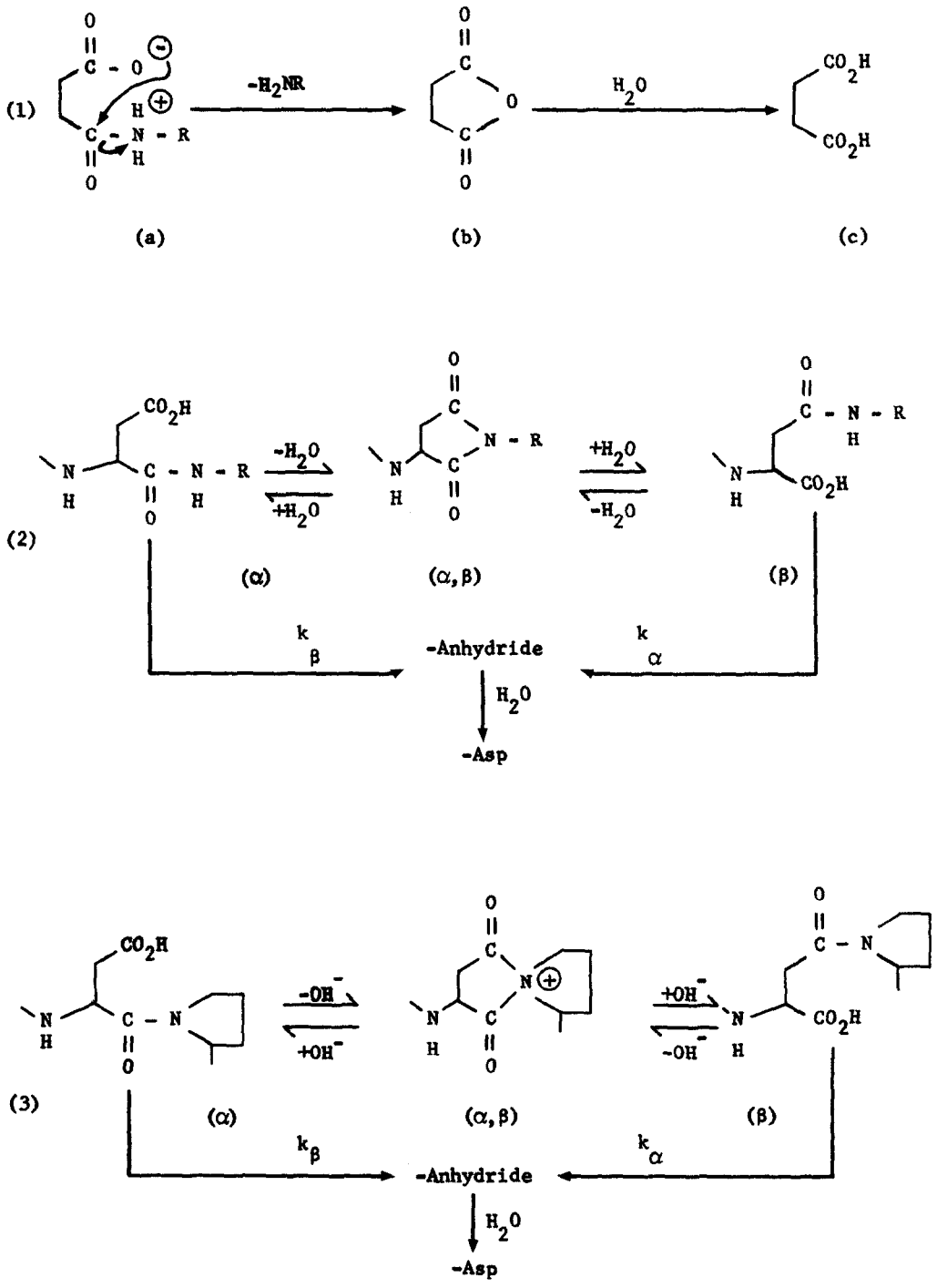

Fig. 1. Equation (1) shows the mechanism of hydrolysis postulated for succinanilic acid. Equation (2) illustrates the α to β interconversion of aspartyl residues via the cyclic imide and the two possible pathways for formation of the anhydride by the mechanism of equation (1). Equation (3) shows the mechanism of equation (2) for the very labile aspartyl-prolyl peptide bond.

TABLE I

ASPARTYL-PROLINE BONDS FOUND TO UNDERGO ANOMALOUS CLEAVAGE

Protein	Residues	Type of digest	Reference
Bovine liver glutamate dehydrogenase [a]	6-7	tryptic	(1)
		CNBr	
	264-265 [b]	tryptic	(1)
		peptic	
Rhodospirillum rubrum cytochrome c_2	84-85	CNBr	(2)
(Asn-Pro) [c]	73-74	CNBr	(2)
Golfingia gouldii hemerythrin	6-7 [d]	tryptic	(3,4)
Human γ-globulin Eu heavy chain [e]	270-271	tryptic	(5)

[a] Residue numbers for this protein are tentative.

[b] This bond was also hydrolyzed during fractionation of a chymotryptic and tryptic digest of a large peptide containing this bond (Brattin and Smith, unpublished).

[c] Deamidation of asparagine probably occurred prior to hydrolysis.

[d] Amino acid residues were numbered in ref. (4).

[e] We are grateful to Dr. G. M. Edelman for this information.

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placement of the protonated β-amide bond (k_α, Equation 2). Since the β-carboxyl group has a pK_a value from 0.8 to 1.7 units higher than the α-carboxyl group in an unfolded polypeptide chain (13), the former would be expected to be a stronger nucleophilic catalyst (14); however under the conditions where aspartyl-peptide cleavage is observed, approximately pH 2.5 to 3.5, the α-carboxyl group is almost completely ionized while the β-carboxyl group is not. Therefore, α-carboxyl participation (via k_α, Equation 2) could also be the pathway of aspartyl-peptide bond cleavage by virtue of a greater concentration of reagent in the properly ionized form (as (a), Equation 1). The available data are not sufficient

to allow an estimate of the relative rates by the two pathways (k_β or k_α, Equation 2).

Although aspartyl participation in the rapid cleavage of Asp-Pro bonds is evident, the properties of proline must also be considered since other aspartyl peptide bonds are much less labile. One of the most significant differences between the secondary amine nitrogen of proline and the primary amine group of α-amino acids is that the pK_a of the former is 10.6 (15) whereas the pK_a's of the latter group range from 9.1 to 9.7 (15). The proline nitrogen in a peptide linkage would have a correspondingly greater basicity than those of other amino acids. Since protonation of the leaving group is of primary importance in enhancing the rate of the final step in the hydrolysis of anilides (16), it is likely to be important in the acid catalyzed hydrolysis of acyl-proline bonds. Indeed the specific cleavage of acyl proline bonds under acidic conditions (12 M HCl, 30 min, 37°) has been observed recently (17). One factor in the increased lability of Asp-Pro bonds could involve an increased concentration of the reactive ionized form ((a), Equation 1).

An additional factor might involve an enhanced rate of the α-β isomerization for prolyl residues linked to aspartyl residues since they would pass through a quaternary, positively charged intermediate rather than an uncharged α-β aspartyl imide (equation 3). An enhanced rate of isomerization would be important in explaining the increased lability of the Asp-Pro bond only if the rate of cleavage of the β-peptide linkages (k_α, Equation 3) was significantly faster than that of the α-peptide linkages (k_β, Equation 3) and the α to β isomerization was the rate determining step. In the absence of further work the effect of the formation of this quaternary intermediate on the two possible mechanisms of carbonyl group catalysis (either k_β or k_α of Equation 3) is uncertain.

The possibility of recognizing other factors influencing the lability of Asp-Pro bonds and reaching a full understanding of the mechanism of hydrolysis requires further study. Nevertheless, the lability of this bond should be rec-

ognized as a possible point of anomalous cleavage in chemical and enzymic digests of proteins or peptides which are exposed to low pH during hydrolysis or purification of the resulting peptides. Furthermore, hydrolysis at other proline bonds has been observed in this laboratory after peptic hydrolysis at acid pH, e.g., -Thr-Pro- in papain (18) and at two sites in glutamate dehydrogenase (Piszkiewicz, Landon, and Smith, unpublished observations). Inasmuch as there is no convincing evidence that pepsin can hydrolyze at the peptide nitrogen of proline in peptide linkage, it seems possible that such lability may be due to the properties of the prolyl bonds at acid pH values.

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