THE INFLUENCE OF VARIOUS α-ALKYL AND α-HYDROXYALKYL SUBSTITUENTS ON THE STABILITY OF DIPEPTIDES IN DILUTE ALKALI

C. J. Jarboe[+], B. W. Noll and L. F. Hass

Department of Biological Chemistry
The Milton S. Hershey Medical Center of the
Pennsylvania State University

Received April 19, 1971

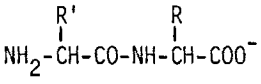
SUMMARY: The stability of alkyl dipeptides in dilute alkali is a function of the size of the substituents attached to the α-carbon atoms. When the various substituents are listed according to their stabilizing effects, the following order is obtained: $-CH_2CH(CH_3)_2 \geq -CH(CH_3)_2 > -CH_2CH_3 > -CH_3 > -H$. The relative alkaline stability of serine and threonine dipeptides depends upon the position of the $-CH(OH)R''$ group in $NH_2CH(R')CONHCH(R)COO^-$. In the R-position, the hydroxyalkyl group facilitates hydrolysis, while in the R'-position, hydrolysis is impeded. In dilute alkali, L,L-seryl dipeptides of alkyl-substituted amino acids have been found to generate D,L-diastereomers.

Previous reports from this laboratory indicated that proteins are selectively cleaved at a restricted number of peptidyl glycine, serine, and threonine bonds when exposed to alkaline borate buffer (pH 12.5) over the temperature range 0-30° (1-3). Cursory analyses of the resulting carboxyl-terminal amino acids gave no evidence that the selectivity is imparted by a specific residue in sequence with the hydrolytically formed amino-terminal residues.

In an effort to gain some understanding of the aforementioned phenomenon, we have investigated the influence of various α-alkyl and α-hydroxyalkyl substituents on the stability of several model dipeptides in dilute alkali. Previously, Levene et al. (4) had determined the hydrolysis constants of a number of alkyl dipeptides in 0.5 N NaOH at 25° (see Table I) and concluded that the stability of the amide bond in these compounds is a function of the bulkiness of the substituents (R and R' in compound I) attached to the α-carbon atoms. Under slightly different conditions (potassium borate, pH 12.6, 30°), we have

---

[+] Postdoctoral Fellow of the National Institutes of Health.



(I)

extended and confirmed the observations of Levene et al. We have also observed an alkali-induced labilization of the amide bond in dipeptides which contain either serine or threonine as the COOH-terminal amino acid. Moreover, it has been found that when the serine or threonine residue occurs in the NH_2-terminal position, the peptide bond becomes stabilized.

MATERIALS AND METHODS

Materials - Glycylglycine was obtained from Calbiochem (Los Angeles, California). L-Seryl-L-alanine and L-seryl-L-leucine were obtained from Miles Laboratories, Inc. (Elkhart, Indiana). All other peptides, except L-alanyl-L-serine and L-leucyl-L-serine, were purchased from Schwarz-Mann (Orangeburg, New York). Chloromethylated polystyrene resin (1.2 mmoles Cl/g), t-BOC-O-benzyl-L-serine[1], t-BOC-L-alanine, and t-BOC-L-leucine were also obtained from Schwarz-Mann.

L-Alanyl-L-serine and L-leucyl-L-serine were synthesized by the solid phase method of Merrifield (5), utilizing the dicyclohexylcarbodiimide coupling procedures described by Stewart and Young (6). After cleavage of the peptides from the resin with HBr in CF_3CO_2H, the solvents were removed by rotary evaporation, water was added, and the samples were lyophilized (6). The peptides ($\sim$ 1.0 g) were purified by ion exchange chromatography on a 100-200 mesh Dowex 50 column (2 X 65 cm) which had been previously equilibrated with 0.1 M pyridine--0.5 M acetic acid, pH 4.0 (7). The column effluent was monitored for ninhydrin-positive material and appropriate fractions were combined and lyophilized. The lyophilized residues were crystallized from aqueous ethanol.

The purities of all peptides were checked (both before and after total hydrolysis) on the Spinco Model 120C automatic amino acid analyzer (8).

---

[1] Abbreviation: t-BOC, N^{α}-t-butyloxycarbonyl.

Kinetic Procedures - Hydrolyses were initiated by the addition of a measured volume of 0.03 M potassium borate buffer, pH 12.6, to a weighed amount of dipeptide to give a final dipeptide concentration of 1-2 mM. All reactions were conducted at 30° in tightly stoppered, nitrogen-flushed vessels. At selected intervals, aliquots were withdrawn from the reaction mixture and were neutralized to pH 7.0 with 0.1 M KH_2PO_4, pH 5.5. The neutralized samples were usually stored at -20°. For analysis, the samples were thawed, the pH was lowered to 2.2 with 1 N HCl, and an appropriate aliquot was assayed on the amino acid analyzer (8). The pseudo first-order hydrolysis constants were obtained from the slope of a least squares plot of the log of the substrate concentration vs. time.

During continuous operation of the amino acid analyzer, a sample often remained at pH 2.2 and 23° for as long as 3 days; consequently, control experiments were performed to insure that all of the peptides used were sufficiently stable under the conditions cited.

RESULTS AND DISCUSSION

The pseudo first-order hydrolysis constants obtained in our studies are summarized in Table I along with the previously obtained results of Levene et al. (4). Comparison of these rate constants reveals that the stability of the alkyl-substituted dipeptides is a function of the size of the substituents (R and R' in compound I) attached to the α-carbon atoms. Thus, at pH 12.6 and 30° (Column a, Table I), glycyl-L-leucine ($k \cdot 10^4 = 2.1$) is approximately 13 times more resistant to alkaline hydrolysis than diglycine ($k \cdot 10^4 = 28.0$). When the various R-substituents are listed according to their stabilizing effects, the following order is obtained: $-CH_2CH(CH_3)_2 \geq -CH(CH_3)_2 > -CH_2CH_3 > -CH_3 > -H$. It should be pointed out, however, that Levene's data (Column c, Table I) indicate that the α,α-dimethyl group, $(-CH_3)_2$, may have the same stabilizing effect as the isobutyl and/or the isopropyl group. Comparison of the rate constants for diglycine, L-alanylglycine, and L-leucylglycine demonstrates that the order of stabilization by alkyl groups remains

Table I. The Influence of Various α-Substituents on the Rates of Alkaline Hydrolysis of Dipeptides

Substrate	Hydrolysis Constants, k X 10^4 hrs^{-1}			Substituents[d]	
	(a)	(b)	(c)	R'	R
Glycylglycine	28.0	22	63	H	H
Glycyl-L-alanine	8.3	--	15	H	CH_3
L-Alanylglycine	9.7	--	13	CH_3	H
Glycyl-L-leucine	2.1	--	3.9	H	$CH_2CH(CH_3)_2$
L-Leucylglycine	2.0	--	3.0	$CH_2CH(CH_3)_2$	H
Glycyl-L-valine	2.8	--	3.1	H	$CH(CH_3)_2$
L-Valylglycine	--	--	0	$CH(CH_3)_2$	H
Glycyl-L-α-amino-butyric acid	5.5	--	--	H	CH_2CH_3
Glycyl-L-α-aminoiso-butyric acid	--	--	0	H	$(CH_3)_2$
Glycyl-L-serine	29.0	30	--	H	CH_2OH
L-Serylglycine	7.6	--	--	CH_2OH	H
Glycyl-L-threonine	9.2	7.2	--	H	$CH(OH)CH_3$
L-Alanyl-L-serine	11.8	--	--	CH_3	CH_2OH
L-Seryl-L-alanine[e]	2.7	--	--	CH_2OH	CH_3
L-Leucyl-L-serine	3.8	--	--	$CH_2CH(CH_3)_2$	CH_2OH
L-Seryl-L-leucine[e]	1.9	--	--	CH_2OH	$CH_2CH(CH_3)_2$

[a] Incubations performed at 30° in potassium borate buffer, pH 12.6.

[b] Incubations performed at 30° and pH 12.6 in KOH.

[c] Values obtained by Levene et al. (4) after incubation of 0.1 M dipeptide (containing D,L-amino acids) in 0.5 N NaOH at 25°.

[d] R and R' refer to substituents in the α and α' positions, respectively.

[e] Dipeptides which give D,L-diastereomers in addition to the constituent amino acids. k is the initial rate constant for the disappearance of dipeptide.

unchanged when the substituents are in the R'- rather than the R-position. Moreover, the rates are quantitatively similar regardless of which α-carbon atom is occupied.

At this point it is appropriate to indicate that the highly alkali-labile sequence, glycylglycine, has been shown to occur in many proteins (9). Thus, a possible explanation is provided for the formation of NH_2-terminal glycyl peptides when proteins are exposed to dilute alkali (1-3). To the best of our

knowledge, other alkali-sensitive peptidyl glycine bonds have not been demonstrated.

Comparison of the hydrolytic constants of dipeptides containing a hydroxyalkyl (-CH(OH)R", R" = H or CH_3) substituent is of particular significance, since a group of this type can exert either a stabilizing or a labilizing effect on the peptide bond depending on whether it is present as R or R'. For example, the rate of hydrolysis of L-serylglycine ($k \cdot 10^4 = 7.6$) is about 4 times slower than that of glycylglycine ($k \cdot 10^4 = 28.0$) and about as fast as that of L-alanylglycine ($k \cdot 10^4 = 9.7$). Glycyl-L-serine ($k \cdot 10^4 = 29.0$), on the other hand, is hydrolyzed as fast as glycylglycine even though the R-substituent ($-CH_2OH$) is bulkier than that of glycyl-L-alanine ($k \cdot 10^4 = 8.3$). Glycyl-D,L-α-aminobutyrate, the methyl analog of glycyl-L-serine, is hydrolyzed at only one-fifth the rate ($k \cdot 10^4 = 5.5$) of the latter. Similarly, glycyl-L-threonine ($k \cdot 10^4 = 9.2$) is hydrolyzed about 3 times more rapidly than its methyl analog, glycyl-L-valine ($k \cdot 10^4 = 2.8$). The observed threefold difference in rates between glycyl-L-serine and glycyl-L-threonine is expected from differences in the reactivity of primary and secondary hydroxyl groups.

Of additional significance is the finding that the insertion of bulky substituents into the R'-position of serine dipeptides creates an impediment to hydrolysis which overrides the catalytic influence of the hydroxyalkyl group. Thus, L-alanyl-L-serine and L-leucyl-L-serine are 2-7 times more resistant to hydrolysis than glycyl-L-serine. The sequential isomers of the two former compounds (i.e., L-seryl-L-alanine and L-seryl-L-leucine), in addition to undergoing simple hydrolysis, selectively epimerize at the R'-position to form D,L-diastereomers[2] at relatively substantial rates. The values listed in Table I for the L,L-compounds, therefore, are not hydrolysis constants, but complex constants for the initial rate of disappearance of the

---

[2] We have isolated and characterized the D,L-epimer of L-seryl-L-leucine. Due to the influence of the carboxylate group, the L,D-epimer was not expected to be formed and, in fact, was not found.

parent substrate ($k = k_{H_2O} + k_e$, where k_{H_2O} is the hydrolysis constant and k_e is the epimerization constant). For both L-seryl-L-alanine and L-seryl-L-leucine, $k_e \cdot 10^4 = 1.3$ hrs^{-1}.

In a previous communication (2), it was postulated that polyanions (e.g., borate, phosphate, etc.) may act as essential catalysts in the selective cleavage of peptidyl serine and threonine bonds in proteins. However, since Table I shows that the hydrolytic rates of dipeptides are only slightly influenced by the borate anion (compare Columns a and b), the above proposal probably can be ruled out. Instead, it appears that certain sequences (e.g., glycyl-L-serine, glycyl-L-threonine, and L-alanyl-L-serine) are of themselves sufficiently labile to account for the preponderance of the NH_2-terminal hydroxyamino acids formed when proteins are exposed to dilute alkali (1-3). At this point, the latter concept is still conjecture and must await corroboration through additional experimentation.

REFERENCES

1. H. E. Sine and L. F. Hass, J. Am. Chem. Soc., 89, 1749 (1967).
2. H. E. Sine and L. F. Hass, J. Biol. Chem., 244, 430 (1969).
3. L. F. Hass, H. E. Sine, and J. A. Morganti, Fed. Proc., 27, 771 (1968).
4. P. A. Levene, R. E. Steiger, and A. Rothen, J. Biol. Chem., 97, 717 (1932).
5. R. B. Merrifield, Adv. in Enzymology, 32, 221 (1969).
6. J. M. Stewart and J. D. Young, Solid Phase Peptide Synthesis, W. H. Freeman and Co., San Francisco, 1969.
7. W. A. Schroeder, in C. W. H. Hirs (Editor), Methods of Enzymology, Vol. XI, Academic Press, New York, 1967, p. 351.
8. S. Moore, D. H. Spackman, and W. H. Stein, Anal. Chem., 30, 1185 (1958).
9. M. O. Dayhoff (Editor), Atlas of Protein Structure, Vol. 4, The National Biomedical Research Foundation, Silver Spring, Maryland, 1969.