The Hydrolysis of Diastereoisomers of Alanine Peptides by Carboxypeptidase A and Leucine Aminopeptidase*

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ABSTRACT: The enzymatic hydrolysis of thirty-nine peptides of alanine (di- to hexapeptides composed of L and D residues at known positions) by leucine aminopeptidase (LAP) and carboxypeptidase A (CP-A) was investigated. From the quantitative analysis of the products obtained it was concluded that LAP and CP-A rapidly hydrolyze the N- and C-terminal peptide bonds, respectively, provided that in each case the two residues forming the bond are of the L configuration. Both enzymes hydrolyzed at much slower rates bonds in which one of the residues was of the D configuration. In these cases it was observed that the action of the enzymes is determined not only by the stereochemical configuration of the two residues adjacent to the bond to be split, but also by residues which are further removed.

The stereospecificity of exopeptidases has been investigated mainly with the aid of small peptides (Neurath, 1960; Smith and Hill, 1960). It was hoped that the use of substrates of increasing size, in which systematic changes in the optical configuration of residues had been introduced, would lead to more detailed information on the mode of action of these enzymes. This study deals with the action of two exopeptidases, leucine aminopeptidase[1] and carboxypeptidase A, on peptides of alanine. Peptides (39) of different lengths (di- to hexapeptide) containing L and D residues in different positions (Schechter and Berger, 1966) were investigated. From the results obtained it was possible to deduce the general stereospecificity of these enzymes toward the above substrates. It was found that the action of the enzymes is determined not only by the stereochemical configuration of the two residues adjacent to the bond to be split, but also by residues which are further removed. The data presented are mainly of a stoichiometric nature. Kinetic data are included only when necessary to prove that enzymatic reactions observed are not due to contamination of the substrates by stereoisomers.

Materials and Methods

Enzyme Solutions. Carboxypeptidase A (bovine pancreas), DFP treated, three times crystallized (aqueous suspension, lot 6125, 35 units/mg), was purchased from Worthington Biochemical Corp., N. J. The enzyme was dissolved at room temperature in 2 M NH_4HCO_3 and the concentration was adjusted to 4 mg/ml using the value of $\epsilon_{278} = 1.94$ ml mg^{-1} cm^{-1}. This solution was kept at 4° and used within 2 days. Before use it was diluted with water (1:5) and then mixed with an equal volume of an aqueous solution of the substrate (0.030 M). A volatile buffer, NH_4HCO_3, was used in order to avoid tailing of the spots on the electrophorogram.

Leucine aminopeptidase (hog kidney) was obtained as a lyophilyzed powder from Sigma Chemical Co., St. Louis (type II, C_1 = approx 10, lot 14B-0300). Stock solutions of the enzyme (1 mg/ml in 0.05 M Veronal buffer, pH 8.0, and 0.01 M $MnSO_4$) were prepared. No significant change in activity occurred at 4° within 2 weeks. Before use the stock solution was diluted to 0.050 mg/ml of enzyme with the above buffer-$MnSO_4$ solution, and then mixed with an equal volume of an aqueous solution of the substrate (0.030 M).

Alanine peptides were synthesized stepwise by coupling the mixed anhydride of benzyloxycarbonyl-L- (or D-) alanine to the amino group of the *p*-nitrobenzyl ester of the appropriate peptide. The free peptides were obtained by catalytic hydrogenation of the blocked peptides. Details of the synthesis and analytical data for 35 peptides were published previously (Schechter and Berger, 1966). Additional four peptides were prepared by the same procedure and the analytical data for these are given in Tables I and II.

Quantitative High-Voltage Paper Electrophoresis. The separation procedure for alanine peptides by paper electrophoresis at pH 1.4 (Schechter and Berger, 1966) was adapted for quantitative work as follows. Up to nine aliquots (5–15 μl) of the reaction mixtures and two standard samples containing 75 mμmoles each of alanine and di- to hexaalanine were applied to the anodic side of a Whatman No. 1 sheet (46 × 100 cm). Electrophoresis was carried out in pH 1.4 buffer (23 ml of 90% formic acid–water to 1 l.–6 N hydrochloric acid, about 5 ml, to bring the pH to 1.4)

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[1] Abbreviations used: LAP, leucine aminopeptidase; CP-A, carboxypeptidase A; DFP, diisopropylphosphorofluoridate; LLL, tri-L-alanine peptide.

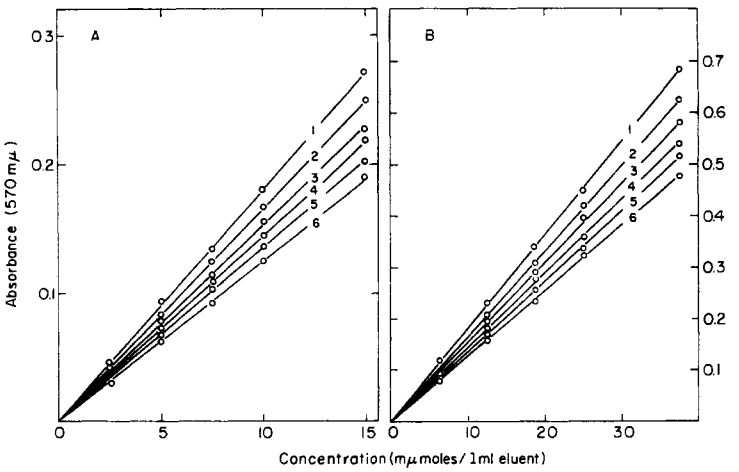

FIGURE 1: Calibration curves showing the proportionality between ninhydrin color and amount of peptide applied to one electrophoresis sheet, for alanine (1) and di- to hexaalanine (2 to 6) eluted with 2 ml of solvent (A); 4 ml of solvent (B).

TABLE I: Analytical Data of Benzyloxycarbonylalanine Peptide *p*-Nitrobenzyl Esters.

Peptide	Formula	Mol Wt	Mp, °C (cor)	Calcd (%) C	Calcd (%) H	Calcd (%) N	Found (%) C	Found (%) H	Found (%) N
Z[a]·Ala_3·Y(DLD)	$C_{24}H_{28}N_4O_8$	500	147[b]	57.6	5.6	11.2	57.6	5.8	11.2
Z·Ala_4·Y (LDDL)	$C_{27}H_{33}N_5O_9$	571	198[b]	56.7	5.8	12.2	56.9	5.9	12.2
Z·Ala_4·Y (LDDD)	$C_{27}H_{33}N_5O_9$	571	182[b]	56.7	5.8	12.2	56.8	5.8	12.1
Z·Ala_5·Y (DDLLL)	$C_{30}H_{38}N_6O_{10}$	642	246[c]	56.1	5.9	13.1	55.9	5.9	13.2

[a] Z = $C_7H_7CO_2$; Y = $OC_7H_6NO_2$. [b] Crystallized from ethanol. [c] Crystallized from acetic acid.

TABLE II: Analytical Data and Specific Rotations (in Degrees) of Alanine Peptides.

Peptide[a]	N_{total}[b] / $n\,N_{amino}$	c (%)	$[\alpha]_\lambda^{27}$ (0.2 N HCl)[c] λ_{589}	λ_{546}
H·Ala_3·OH(DLD)	1.01	1.4	−36.8	−43.6
H·Ala_4·OH(LDDL)	1.00	0.6	+83.5	+98.2
H·Ala_4·OH(LDDD)	1.00	0.6	+150	+176
H·Ala_5·OH(DDLLL)	1.03	0.9	−78.6	−92.9

[a] All peptides gave only one spot on high-voltage electrophoresis (0.5-μmole load). [b] The ratio of total nitrogen (micro-Kjeldahl) to n times (n = 3–5) amino nitrogen (Van Slyke) should equal to unity. [c] Measured with a Rudolph photoelectric spectropolarimeter. Concentrations are based on nitrogen analyses of the solutions.

for 4.5 hr at 30 v/cm. The paper sheet was dried in a well-ventilated oven at 60° for 20 min, dipped in a ninhydrin solution (ninhydrin, 2.5 g–acetone, 500 ml–water, 25 ml–pyridine, 2 ml), and heated as above. The colored spots were cut out, transferred into test tubes, and eluted for 2 hr in the dark, with occasional shaking. The eluting solution contained: ethanol (75 ml)–water (25 ml)–pyridine (0.4 ml). Quantities of 5–30 mμmoles were eluted with 2 ml of solvent, and quantities of 30–150 mμmoles with 4 ml of solvent. After centrifugation at 3000 rpm for 10 min the absorbance of the clear supernatant was measured at 570 mμ (in a Zeiss spectrophotometer) against an eluate of an appropriate blank section of the developed paper. The amounts of peptides in the unknown were calculated, assuming proportionality, on the basis of the color obtained for each peptide in the standard mixtures on the same sheet.

The proportionality between ninhydrin color and amount of peptide applied is shown in Figure 1. The slope of the straight line obtained for each peptide depends on its size and varies slightly from run to run. In each size group the color yield is independent of sterical configuration. In evaluating the molar ratio of products to substrate, errors due to sample size were avoided by calculating the equivalent of substrate, present originally in the sample, from the amounts of

TABLE III: Hydrolysis of Alanine Peptides by Exopeptidases.

		Products (moles/mole of substrate)[a]											
		Leucine Aminopeptidase[b]						Carboxypeptidase A[c]					
	Peptide	Ala_1	Ala_2	Ala_3	Ala_4	Ala_5	Ala_6	Ala_1	Ala_2	Ala_3	Ala_4	Ala_5	Ala_6
1	H·Ala_2·OH(LL)	2.0						0.09	*1.0*[d]				
2	(LD)	0.06	*1.0*						*1.0*				
3	(DL)		*1.0*						*1.0*				
4	(DD)		*1.0*						*1.0*				
5	H·Ala_3·OH(LLL)	3.0						1.0	1.0				
6	(LLD)	1.0	1.0							*1.0*			
7	(LDL)	0.01	0.01	*1.0*						*1.0*			
8	(LDD)	0.6	0.6	*0.4*						*1.0*			
9	(DLL)	0.06		*1.0*				1.0	1.0				
10	(DLD)			*1.0*						*1.0*			
11	(DDL)			*1.0*						*1.0*			
12	(DDD)			*1.0*						*1.0*			
13	H·Ala_4·OH(LLLL)	4.0						2.0	1.0				
14	(LLLD)	2.0	1.0					0.08	0.04		*1.0*		
15	(LLDL)	1.0		1.0							*1.0*		
16	(LLDD)	1.3	0.3	0.7							*1.0*		
17	(LDLL)				*1.0*			1.0		1.0			
18	(LDDL)	0.35		0.35	*0.65*						*1.0*		
19	(LDDD)	0.35		0.35	*0.65*						*1.0*		
20	(DLLL)	0.04			*1.0*			2.0	1.0				
21	(DLLD)	0.02	0.01		*1.0*			0.06	0.03		*1.0*		
22	(DDLL)				*1.0*			1.0		1.0			
23	(DDDD)				*1.0*						*1.0*		
24	H·Ala_5·OH(LLLLL)	5.0						3.0	1.0				
25	(LLLLD)	3.0	1.0					0.09	0.03			*1.0*	
26	(LLLDL)	2.0		1.0								*1.0*	
27	(LLDLL)	1.0			1.0			1.0			1.0		
28	(LDLLL)					*1.0*		2.0		1.0			
29	(DLLLL)	0.05				*1.0*		3.0	1.0				
30	(DLLLD)					*1.0*		0.03	0.01			*1.0*	
31	(DDLLL)					*1.0*		2.0		1.0			
32	(DDDDD)					*1.0*						*1.0*	
33	H·Ala_6·OH(LLLLLL)	6.0[e]						4.0	1.0				
34	(LLLLLD)	4.0	1.0					0.04	0.01				*1.0*
35	(LLDLLL)	1.0				1.0		2.0			1.0		
36	(LDLLLL)						*1.0*	3.0		1.0			
37	(DLLLLL)	0.06					*1.0*	4.0	1.0				
38	(DLLLLD)						*1.0*	0.12	0.03				*1.0*
39	(DDLLLL)						*1.0*	3.0		1.0			

[a] Estimated by quantitative high-voltage paper electrophoresis. [b] Reaction mixture: LAP, 0.025 mg/ml; substrate, 0.015 M; Veronal buffer, pH 8.0, 0.025 M; $MnSO_4$, 0.005 M. Hydrolysis was performed at 36° for 2 hr. [c] Reaction mixture: CP-A, 0.4 mg/ml (except for peptides **1, 5, 13, 24, 33,** where 0.2 mg/ml of enzyme was used); substrate, 0.015 M; NH_4HCO_3, 0.20 M (pH 8.4). Hydrolysis was performed at 36° for 4 hr. [d] Italics indicate the unchanged substrate. [e] Determined after 6 hr; at 2 hr hydrolysis was incomplete due to insolubility of the substrate.

all peptides found in the hydrolysate.

Results

The relative amounts of the products obtained on enzymatic digestion of the alanine peptides by CP-A and LAP under the conditions specified are given in Table III.

Leucine Aminopeptidase. This enzyme is seen to hydrolyze rapidly N-terminal peptide bonds involving two L residues (LL). Bonds of the DD type are not hydrolyzed. Bonds of the DL and LD type are either not

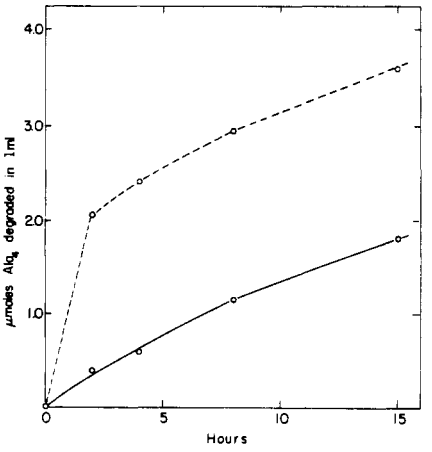

FIGURE 2: Course of degradation of tetraalanines by CP-A (0.4 mg/ml) in 0.2 M NH_4HCO_3 at 36°. Substrates: solid line, LLLD (0.0150 M); broken line, LLLD (0.0150 M) + LLLL (0.0018 M). The amount of Ala_4 degraded was calculated from the amounts of alanine and dialanine formed.

hydrolyzed at all or are split extremely slowly. An exception to this generalization is observed in three peptides, *viz.*, LDD (**8**), LDDL (**18**), and LDDD (**19**) which were digested to the extent of about 60, 35, and 35%, respectively. On prolonged digestion or with higher enzyme concentration, these peptides were completely hydrolyzed by LAP. However, the fastest reacting LD bond (**8**) is still about five times slower than the slowest LL bond (**1**). The trace digestions recorded in Table III (which are two to three orders of magnitude slower than for LL bonds) are not due to trace racemization of D residues; this was concluded from the observation that progressive slow digestion takes place on prolonged incubation, and that at higher enzyme concentrations some of these peptides (**2** and **9**) were degraded completely. These findings will be the subject of a future communication.

Carboxypeptidase A. Carboxyl-terminal bonds of the LL type are hydrolyzed rapidly by this enzyme. L-Alanyl-L-alanine (**1**) however is digested very slowly (more than a hundred times slower than LLL). Bonds of the types DL and DD were found to be resistant to the action of CP-A under the conditions employed. A number of peptides with terminal LD-type bonds were not attacked by the enzyme. The remainder (**14, 21, 25, 30, 34,** and **38**) were digested very slowly. In order to show that these degradations are true LD bond cleavages and not due to trace racemization at the terminal D residue, some of these peptides were digested for longer periods and the course of the reaction was followed. With LLLD degradation progressed steadily with time, reaching 12% after 15 hr (see Figure 2). A more detailed study of the splitting of LD-type bonds will be published separately.

Discussion

The general rule governing the hydrolysis of a large number of alanine peptides by the exopeptidases LAP and CP-A can be stated as follows. Both enzymes effect rapid hydrolysis of bonds between L residues (an exception is the very slow digestion of the dipeptide by CP-A). In other words, pure L peptides are degraded to L-alanine by LAP (n moles/mole of an n-peptide), while CP-A degrades them to ($n - 2$) moles of L-alanine and 1 mole of di-L-alanine. In peptides containing D residues both enzymes will progressively remove L residues from the appropriate end but will not detach the last L residue from its D neighbor. However, in some peptides these rules do not hold rigorously; slow enzymatic attack can be observed at "forbidden" bonds, hydrolysis rates being two to three orders of magntiude lower than at "allowed" bonds. The reaction conditions (enzyme concentration and reaction time) used in Table III were deliberately chosen so as to emphasize the differences in reactivity.

It was stated in an earlier publication (Schechter and Berger, 1966) that the optical purity of the residues in a large number of positions in the alanine peptides synthesized can be proven with the aid of exopeptidases. That this can indeed be done is shown by the following arguments. In all cases where the reaction products are obtained in integral numbers of moles per mole of substrate, the interpretation is straightforward, as described. In cases where products are formed either in traces or in fractions of moles, a kinetic experiment will decide whether the pattern of degradation observed is due to racemization or due to one of the "slow" reactions. Figure 2 shows the course of degradation by CP-A of Ala_4 (LLLD), which is digested at a slow rate (solid curve). The fact that the peptide being digested is not Ala_4 (LLLL) is seen from the dotted line which represents the digestion of a mixture containing 1 mole of Ala_4 (LLLD) and 0.12 mole of Ala_4 (LLLL). The added L peptide is digested rapidly and the remaining LLLD peptide at its own slow rate. A different problem arises in the case of a nondigestible peptide (such as LDLL with LAP). A possible contamination by the digestible isomer LLLL might be obscured by inhibition of LAP by the main component. That this is not the case is demonstrated as follows. The L peptide, when added to the LDLL compound (0.005 mole/mole) is completely degraded under the conditions specified.

The rules of action of exopeptidases described here may be of importance in peptide chemistry. When exopeptidases are employed to detect D-amino acid residues, either as components in naturally occurring peptides or as products of racemization during a synthesis, it should be borne in mind that the terminal residue of the peptide fragment obtained is probably an L residue and only its next neighbour is the D residue being sought.

The fact that one D residue participating in a peptide bond (irrespective of which side) renders it resistant (or nearly resistant) to hydrolysis by both LAP and CP-A is interesting. It shows that these enzymes "recog-

nize" both residues forming the bond. Moreover, when different peptides containing the same terminal bonds are compared, large differences in reactivity are observed. Thus, with LAP, Ala_4 (LDLL) is not digested under our conditions, Ala_3 (LDL) is digested in trace amounts only, and Ala_4 (LDDL) is digested at an appreciable rate. Yet all three peptides contain the N-terminal LD-type bond. Additional examples of this kind can be found in Table III. This is an indication that not only the nature of the bond hydrolyzed determines its susceptibility to cleavage, but that residues further removed also exhibit an influence on the rate of enzymatic attack. Similar findings with respect to the action of CP-A on LL bonds have been described in a preliminary communication (Schechter *et al.*, 1965). It was shown by comparing kinetic rate constants that the enzyme "recognizes" at least four residues in the substrate. This was deduced from the observation that the C-terminal bonds of the three tetrapeptides LLLL, DLLL, and LDLL are split at relative rates of about 100:10:1. Other proteolytic enzymes seem to behave in a similar manner. Papain was shown to interact simultaneously with five residues in the substrate (Schechter *et al.*, 1965). Results obtained recently in this laboratory indicate that trypsin and aspergillopeptidase B (Subramanian and Kalnitsky, 1964) recognize a sequence of five and seven residues, respectively, in a peptide chain.

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The Interaction of Silver Ion with Guanosine, Guanosine Monophosphate, and Related Compounds. Determination of Possible Sites of Complexing*

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ABSTRACT: Complex formation of guanosine, guanosine 3′(2′)-monophosphate, inosine, inosine 5′-monophosphate, theophylline, caffeine, uridine, uridine 3′(2′)-monophosphate, and ribose with silver ion at neutral pH was studied. It was found that guanosine, guanosine 3′(2′)-monophosphate, inosine, inosine 5′-monophosphate, and theophylline combined with silver ion in equimolar ratio. An equivalent amount of hydrogen ion was released from each compound on complexing. There was considerable hypochromicity at the wavelength of maximum ultraviolet absorption on complexing.

Little change in the ultraviolet spectra was observed for the compounds that did not combine with silver. The infrared absorption spectra indicated that the carbonyl stretching bands disappeared after complexing with the exception of theophylline. Possible sites for silver in the complexes are proposed.

The ability of nucleosides (Edelman *et al.*, 1960; Eichhorn *et al.*, 1966; Ropars and Viovy, 1965a), nucleotides (Eichhorn and Clark, 1965; Rich, 1959), and nucleic acids (Trim, 1959; Yamane and Davidson, 1962; Fuse *et al.*, 1965; Coates *et al.*, 1965) to complex with certain metals has been reported by various investigators. Occurrence of some metals in nucleic acids (Jungner, 1951; Holden and Pirie, 1955; Wacker and Vallee, 1959; Tu, 1961) and tobacco mosaic virus ribonucleic acid (TMV-RNA) (Loring *et al.*, 1958) and viruses (Hoagland *et al.*, 1941; Racker and Krimisky, 1945; Loring *et al.*, 1959) has also been reported. The significance of these trace metals present in such important biological macromolecules cannot be fully understood unless the nature of the metal binding with the nucleotide unit is clarified. Most of the metal–nucleic acid complexes are insoluble materials. Trim (1959) used this property as a means for isolating

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