ROLE OF AMINO ACID RESIDUES IN CHROMOGENIC SUBSTRATES CLEAVED BY PANCREATIC ELASTASE

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Anionic chromogenic substrates, 3-carboxypropionyl tripeptide *p*-nitroanilides modified with glycine, β-alanine, alanine, leucine, and proline in positions P_1, P_2, and P_3 were synthesized. The substrates were digested with pancratic elastase and the values of K_m, k_{cat}, and k_{cat}/K_m were determined. Alanine plays a decisive role in position P_1, substrates with glycine or β-alanine in this position are not cleaved. The substitution in P_2 is dominant for proline; next follow alanine, leucine, and glycine. The substitution in P_3 is the least specific one.

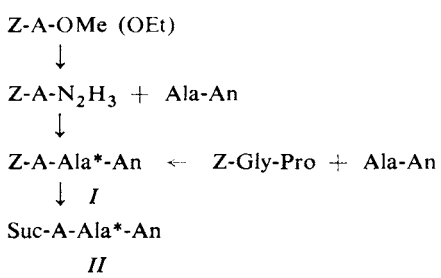
The anionic chromogenic substrates of pancreatic elastase (PE), first introduced by us and synthesized to contain the residues of maleic, succinic, and glutaric acids established a basis for the design of novel substrates[1]. The aim of these experiments has been an investigation of the relations and mechanism of binding existing between elastase and the substrate. 3-Carboxypropionyl tripeptide *p*-nitroanilides* containing various amino acid residues in P_1 and having a different peptide chain length[2] were synthesized for this purpose. We were able to show that alanine is optimal for position P_1 of the anionic substrate and that the optimal chain length of an elastolytic substrate (N-part) is 3 to 4 amino acid residues; substrates containing one or two alanine residues were not cleaved by PE.

The aim of the present work was to prepare additional anionic substrates, 3-carboxypropionyl tripeptide *p*-nitroanilides with a changed sequence in P_2 and P_3 and to examine their binding interactions with PE. The residues of glycine, alanine, leucine and, in one case, of proline were used for the replacement. Since the alanine residue is of key importance for the elastolytic cleavage, our series of substrates also included three combined substrates containing β-alanine in position P_1, P_2, and P_3.

* The symbols and names of amino acids and peptides follow the suggestion of the IUPAC-IUB Commission on Biochemical Nomenclature[3,4]. All amino acids (with the exception of glycine and β-alanine) are of L-configuration. Suc = succinyl, *i.e.* 3-carboxypropionyl-, An = 4-nitroaniline.

We synthesized the *p*-nitroanilides of 3-carboxypropionylglycyl-glycyl-alanine (*IIa*), 3-carboxypropionylglycyl-alanyl-alanine (*IIb*), 3-carboxypropionylalanyl-glycyl-alanine (*IIc*), 3-carboxypropionylalanyl-leucyl-alanine (*IId*), 3-carboxypropionylleucyl--alanyl-alanine (*IIe*), 3-carboxypropionylleucyl-leucyl-alanine (*IIf*), 3-carboxypropionylglycyl-prolyl-alanine (*IIg*), 3-carboxypropionylalanyl-β-alanyl-alanine (*IIi*), 3-carboxypropionyl-β-alanyl-alanyl-alanine (*IIj*), and 3-carboxypropionyl-β-alanyl--β-alanyl-alanine (*IIk*).

The anionic substrates were synthesized by the standard procedure shown in Scheme 1. Protected benzyloxycarbonyltripeptide *p*-nitroanilides *Ia*–*Ik*, except for



In formulae *I* a *II*:

a, A = Gly-Gly
b, A = Gly-Ala
c, A = Ala-Gly
d, A = Ala-Leu
e, A = Leu-Ala
f, A = Leu-Leu
g, A = Gly-Pro
h, A = Ala-Ala
i, A = Ala-β-Aly
j, A = β-Ala-Ala
k, A = β-Ala-β-Ala

SCHEME 1

Ig, were prepared by the azide method from the corresponding hydrazides of N--terminal benzyloxycarbonyl dipeptides by condensation with alanine or β-alanine *p*-nitroanilides[5]. The corresponding protected dipeptide esters, benzyloxycarbonylglycyl-alanine methyl esters[6], benzyloxycarbonylalanyl-glycine methyl esters[7], benzyloxycarbonylleucyl-alanine methyl esters[8] and benzyloxycarbonylalanyl-leucine hydrazides[9], benzyloxycarbonylleucyl-alanine hydrazides[10], and benzyloxycarbonylglycyl-alanine hydrazides[11] were prepared according to recorded data. The remaining protected dipeptide esters were prepared by the carbodiimide method; the corresponding hydrazides were prepared by hydrazinolysis in 80% hydrazine hydrate. *Ig* was prepared by the carbodiimide method from benzyloxycarbonylglycyl-proline[12] and alanine *p*-nitroanilide. β-Alanine *p*-nitroanilide was obtained by the phosphoazo method[13] from benzyloxycarbonyl-β-alanine. The protected tripeptide *p*-nitroanilides

* For *h* = β-Ala; An = 4-nitroaniline.

were decarbobenzoxylated by the standard procedure in 4 mol l^{-1} HBr in glacial acetic acid, deionized on Zerolit G (in OH-form) in methanol and the free tripeptides were acylated by succinic anhydride in dimethylformamide[2].

The yields and the remaining analytical data on benzyloxycarbonyl dipeptides and their hydrazides are given in Table I, on the protected tripeptides and final products in Table II; the values of K_m, k_{cat}, and C (k_{cat}/K_m) are shown in Table III.

The value of the specificity constant C is almost identical, *i.e.* 600 for substrates *IIa*, *IIb*, *IIc*, and *IIj* which belong to the group in which the combinations of glycine, alanine, β-alanine, leucine, and proline in positions P_2 and P_3 were examined; in this population of substrates at least one or two amino acids are achiral and cannot interact *via* their side chains (with the exception of hydrogen bonds). In contrast,

TABLE I
Substituted peptide esters and hydrazides

Compound Method (yield, %)	m.p., °C $[\alpha]_D^{20}$ [a]	Formula (mol. wt.)	Calculated/found		
			% C	% H	% N
Z-β-Ala-Ala-OMe	77–78	$C_{15}H_{20}N_2O_5$	58·43	6·54	9·09
— (91)	−28·2°	(308·3)	59·07	7·03	9·46
Z-Ala-β-Ala-OEt	78–79	$C_{16}H_{22}N_2O_5$	59·61	6·88	8·69
— (59)	−19·9°	(322·4)	60·29	7·24	9·03
Z-β-Ala-β-Ala-OEt	83–84	$C_{16}H_{22}N_2O_5$	59·61	6·88	8·69
— (93)	—	(322·4)	59·43	7·12	8·90
Z-Gly-Ala-N_2H_3	128–130 [c]	$C_{13}H_{18}N_4O_4.1/2\ H_2O$	51·48	6·31	18·47
A (93)	−2·6° [b]	(303·3)	51·25	6·10	19·31
Z-Ala-Gly-N_2H_3	148–149	$C_{13}H_{18}N_4O_4$	53·05	6·16	19·04
B (59)	+3·6° [b]	(294·3)	53·21	6·25	19·51
Z-Leu-Leu-N_2H_3	148–150	$C_{20}H_{32}N_4O_4$	61·20	8·22	14·27
B (48)	−19·9° [b]	(392·5)	61·54	8·60	14·35
Z-β-Ala-Ala-N_2H_3	152–153	$C_{14}H_{20}N_4O_4$	54·53	6·54	18·17
B (88)	−3·2° [b]	(308·3)	54·30	6·68	18·40
Z-Ala-β-Ala-N_2H_3	181–182	$C_{14}H_{20}N_4O_4$	54·53	6·54	18·17
B (84)	+3·2° [b]	(308·3)	54·90	6·50	18·49
Z-β-Ala-β-Ala-N_2H_3	181–182	$C_{14}H_{20}N_4O_4$	54·53	6·54	18·17
B (67)	—	(308·3)	54·20	6·57	17·95

[a] In methanol; [b] in dimethylformamide; [c] $[\alpha]_D^{20}$ −32·6° in methanol, ref.[11] m.p. 134°C; $[\alpha]_D^{25}$ −30·0° (c = 1, methanol).

TABLE II
p-Nitroanilides of tripeptides

Compound Solvent[a]	m.p., °C (yield, %)	$[\alpha]_D^{20}$[b]	Formula (mol. wt.)	Calculated/found		
				% C	% H	% N
Ia	174—176	−10·3°	$C_{21}H_{23}N_5O_7$	55·15	5·07	15·31
A	(61)		(457·4)	54·67	5·04	15·55
Ib	207—209	−12·9°	$C_{22}H_{25}N_5O_7$	56·05	5·35	14·86
B	(55)		(471·4)	56·19	5·35	14·62
Ic	112—115	−20·2°	$C_{22}H_{25}N_5O_7$	56·05	5·35	14·86
C	(63)		(471·4)	55·83	5·38	15·08
Id	218—221	−20·4°	$C_{26}H_{33}N_5O_7$	59·20	6·30	13·27
B	(54)		(527·5)	59·12	6·51	13·34
Ie	213—215	−20·2°	$C_{26}H_{33}N_5O_7$	59·20	6·30	13·27
B	(52)		(527·5)	59·27	6·44	13·61
If	210—213	−23·3°	$C_{29}H_{39}N_5O_7$	61·15	6·90	12·30
B	(79)		(569·6)	61·98	7·33	12·56
Ig	135—138	−11·2°	$C_{24}H_{27}N_5O_7$	57·95	5·47	14·08
D	(57)		(497·5)	58·30	5·87	14·04
Ih	180—182	−5·9°	$C_{23}H_{27}N_5O_7$	56·91	5·61	14·43
B	(47)		(485·4)	56·84	5·68	14·72
Ii	198—199	−1·9°	$C_{23}H_{27}N_5O_7$	56·91	5·61	14·43
B	(78)		(485·4)	57·25	5·72	14·44
Ik	187—190	−2·4°	$C_{23}H_{27}N_5O_7$	56·91	5·61	14·43
B	(53)		(485·4)	57·49	6·01	13·92
IIa	207—210	−54·9°[c]	$C_{17}H_{21}N_5O_8$	48·24	5·00	16·54
E	(41)		(423·3)	48·24	5·06	16·72
IIb	118—121	−39·9°[c]	$C_{18}H_{23}N_5O_8.H_2O$	47·40	5·52	15·35
E	(31)		(456·1)	47·40	5·54	15·38
IIc	116—118	−55·4°[c]	$C_{18}H_{23}N_5O_8$	49·43	5·30	16·01
F	(45)		(437·4)	49·12	5·65	15·42
IId	218—221	−76·9[c]	$C_{22}H_{31}N_5O_8$	53·55	6·33	14·19
G	(43)		(493·5)	53·63	6·22	13·99
IIe	189—192	−52·6°[c]	$C_{22}H_{31}N_5O_8.1/2\ H_2O$	52·59	6·42	13·94
G	(41)		(502·5)	52·77	6·40	13·92
IIf	187—189	−75·6°[c]	$C_{25}H_{37}N_5O_8$	56·07	6·96	13·08
H	(46)		(535·5)	55·29	6·98	13·00
IIg	228—231	−65·2°[c]	$C_{20}H_{25}N_5O_8$	51·84	5·44	15·11
G	(39)		(463·4)	52·16	5·67	15·38

TABLE II
(*Continued*)

Compound Solvent[a]	m.p., °C (yield, %)	$[\alpha]_D^{20}$[b]	Formula (mol. wt.)	Calculated/found		
				% C	% H	% N
IIh	224–226	−16·5°[c]	$C_{19}H_{25}N_5O_8$	50·56	5·58	15·52
H	(42)		(451·4)	50·40	5·68	15·35
IIi	199–202	−49·2°[c]	$C_{19}H_{25}N_5O_8$	50·56	5·58	15·52
F	(46)		(451·4)	50·66	5·77	15·22
IIj	159–161	−105·3°[c]	$C_{19}H_{25}N_5O_8$	50·56	5·58	15·52
E	(32)		(451·4)	50·64	5·92	15·36
IIk	158–160	−39·6°[c]	$C_{19}H_{25}N_5O_8.H_2O$	48·62	5·80	14·92
F	(42)		(469·4)	48·63	5·70	14·87

[a] A 2-propanol–light petroleum, B 2-propanol–water, C N,N-dimethylformamide–2-propanol, Didichlormethane–light petroleum, E water, F acetic acid–ether, G 2-propanol–water, H methanol–water; [b] in N,N-dimethylformamide; [c] in methanol.

if both amino acids are chiral, the value of *C* significantly increases to 3·240 for the alanyl-leucyl derivative *IId* and to 5·740 for the leucyl-alanyl derivative *IIe*. This increase is limited by steric effects and its minimal after placing the more demanding

TABLE III
Kinetic constants of substrates cleaved by pancreatic elastase

Substrate	$K_m \cdot 10^4$, mol l^{-1}	k_{cat}, s^{-1}	k_{cat}/K_m, $mol^{-1}\,ls^{-1}$
IIa	1·18	0·71	601
IIb	1·64	1·09	664
IIc	1·64	1·09	664
IId	0·46	1·49	3 240
IIe	1·33	7·64	5 740
IIf	5·00	6·72	1 344
IIg	3·57	3·09	865
IIh	[a]	—	—
IIi	[a]	—	—
IIj	1·75	1·01	577
IIk	[a]	—	—

[a] Not cleaved.

leucine into both positions; thus, *e.g.* it is 1·344 for leucyl-leucyl derivative *IIf*. The kinetic data on substrate *IIg* (C = 865), *i.e.* the sequence glycyl-prolyl, confirmed that a proline residue[14] is optimal for position P_2, compared to *IIb*, 3-carboxypropionylglycyl-alanyl-alanine *p*-nitroanilide, for which C = 664. Position P_3 favors a hydrophobic residue[15]; supporting evidence is provided by our data showing that the replacement of hydrophobic alanine *IId* or leucine *IIe*, *IIf* by glycine *IIa* results in a decrease in C (k_{cat}/K_m).

The combination of alanine with β-alanine was investigated with the second group of substrates. This population yielded data indicating the key position of the alanine residue in P_1 in the immediate neighbourhood of the scissile bond. The importance of this position decreases in direction to position P_2 and is almost negligible for position P_3. Hence, whereas *IIh* (β-Ala in P_1) or *IIi* and *IIk* (β-Ala in P_2 in both cases) are not cleaved by elastase, substrate *IIj* (β-Ala in P_3) is relatively well hydrolyzed by elastase.

EXPERIMENTAL

Pig pancreatic elastase (Serva) (EC 3·4·21·36) was dissolved in 1 mmol l^{-1} acetic acid; the enzyme concentration was 15·5 nmol l^{-1}. The incubation medium contained 2·7 ml of 0·1 mol . l^{-1} Tris buffer; pH 8·0, 0·1 ml of elastase solution, and 0·2 ml of substrate (in dimethylsulfoxide). The enzymatic activity was assayed kinetically (Unicam SP 800B) by continuous measurement of 4-nitroaniline liberated (25°C, 410 nm). The values of the Michaelis constant K_m were determined over a range of substrate concentrations 0·025 mmol l^{-1}—1 mmol l^{-1}) by the Lineweaver–Burk plot.

The melting points were determined in a Kofler block and are not corrected. The samples for analysis were dried *in vacuo* at 70 Pa over phosphorus pentoxide at 105°C. Compounds melting below 120°C were dried at room temperature. The optical rotations were measured in the Perkin–Elmer photoelectric polarimeter; the concentration of the solutions was 0·2—0·3. The solvents were evaporated in a rotary evaporator under reduced pressure. The standard procedure represents the following treatment: dissolving of the compound in ethyl acetate, stepwise extraction with 1 mol l^{-1} hydrochloric acid, water, 5% sodium bicarbonate, water, drying over anhydrous sodium sulfate and evaporation. Thin layer chromatography was carried out on silica gel layers (Kieselgel G, Merck) in the solvent systems 1-butanol–acetic acid–water, 4 : 1 : 1 (S_1) and 1-butanol–acetic acid–pyridine–water, 15 : 3 : 10 : 6 (S_2).

Benzyloxycarbonyl-β-alanyl-alanine Methyl Ester

N,N′-Dicyclohexylcarbodiimide (4·4 g) was added to a solution of benzyloxycarbonyl-β-alanine (2·90 g; 20 mmol) and alanine methyl ester released from the corresponding hydrochloride (2·8 g; 20 mmol) by the addition of N-ethylpiperidine (2·8 ml) in dichloromethane (60 ml) cooled down to −5°C. After the mixture had been stirred 2 h at 0°C the suspension was set aside for 12 h at room temperature; N,N′-dicyclohexylurea which had separated was filtered off, the filtrate was taken to dryness and treated according to the standard procedure. Crystallization from ethyl acetate and light petroleum afforded 5·6 g (91%) of a product melting at 75—77°C. The sample for analysis was crystallized in an analogous manner. The remaining β-alanine dipeptides were prepared by the same procedure.

Benzyloxycarbonylglycyl-alanine Hydrazide (Procedure *A*)

A solution of benzyloxycarbonylglycyl-alanine methyl ester (11·8 g; 40 mmol) in methanol (100 ml) and 80% hydrazine hydrate (10 ml) was refluxed for 2 h. The solution was then taken to dryness and the residue was crystallized from water (20 ml). The yield of the product was 11·3 g (93%); the sample for analysis was crystallized in an analogous manner.

Benzyloxycarbonylalanyl-glycine Hydrazide (Procedure *B*)

A solution of benzyloxycarbonylalanyl-glycine ethyl ester (6·1 g; 20 mmol) in methanol (25 ml) and 80% hydrazine hydrate (5 ml) was allowed to stand 1 day at room temperature. The solution was then taken to dryness and the residue was crystallized from boiling ethanol (25 ml). The yield was 4·8 g (59%) of a product melting at 148–149°C; the sample for analysis was crystallized in an analogous manner.

Benzyloxycarbonyl-β-alanine *p*-nitroanilide

A cooled solution (−10°C) of PCl_3 (4·6 ml) in pyridine (20 ml) was added to a solution of *p*-nitroaniline (14·0 g; 100 mmol) in pyridine precooled to −20°C. After 30 min of standing at −20°C and 30 min at room temperature the suspension was treated with benzyloxycarbonyl-β-alanine (23·3 g; 100 mmol) and the mixture was refluxed for 3 h. It was evaporated afterwards and the residue was treated in the standard manner. Crystallization from ethyl acetate (150 ml) and light petroleum afforded 27·5 g (80%) of a product melting at 152°C; the sample for analysis was crystallized in an analogous manner, the m.p. remained uncharged. For $C_{17}H_{17}N_3O_5$ (343·3) calculated: 59·48% C, 4·99% H, 12·24% N; found: 59·33% C, 5·15% H, 12·22% N.

β-Alanine *p*-Nitroanilide

A solution of benzyloxycarbonyl-β-alanine *p*-nitroanilide (10·5 g; 30 mmol) in glacial acetic acid (30 ml) was treated with 36% hydrogen bromide in glacial acetic acid (30 ml). The crystalline hydrobromide had separated from the solution after 10 min of standing. Ether (80 ml) was added to the suspension after 1 h of standing at room temperature, the hydrobromide was filtered off, washed with ether and dried over phosphorus pentoxide and sodium hydroxide. The sample for analysis was crystallized from methanol, m.p. 263–264°C. For $C_9H_{11}N_3O_5.HBr$ (289·1) calculated: 37·40% C, 4·18% H, 14·54% N; found: 37·41% C, 4·14% H, 14·59% N.

The crude hydrobromide was aded to a 5% solution of sodium bicarbonate (75 ml), the mixture was stirred 4 h at room temperature and the free base was then filtered off and washed three times with water (10 ml). Crystallization from water (60 ml) afforded 3·8 g (61%) of the product. The sample for analysis was crystallized in an analogous manner, m.p. 145–147°C. For $C_9H_{11}N_3O_3.1/2\ H_2O$ (218·2) calculated: 49·55% C, 5·54% H, 19·26% N; found: 49·61% C, 5·23% H, 19·46% N. R_F 0·29 (S_1); 0·60 (S_2).

Benzyloxycarbonylalanyl-β-alanyl-alanine *p*-Nitroanilide (*Ii*)

A solution of sodium nitrite (208 mg) in water (0·84 ml) was added to a solution of benzyloxycarbonyl-β-alanine hydrazide (925 mg; 3 mmol) in tetrahydrofuran (40 ml) and 20% hydrochloric acid (1·2 ml) and water (0·37 ml) precooled to −12°C. Precooled (−15°C) ethyl acetate was added to the mixture after 8 min of stirring and cooling (−10°C). The organic layer was extracted after 2 min of stirring with precooled saturated solution of sodium bicarbonate in 16·8% NaCl, dried by anhydrous sodium sulfate and added to a solution of alanine *p*-nitroanilide (630 mg; 3 mmol) in dimethylformamide (15 ml). The solution was taken to dryness after 12 h

of standing at +3°C and the residue was treated with water (about 25 ml). The product which had separated was filtered off after 2 h and was crystallized from a mixture of methanol (15 ml) and water (15 ml). The yield was 1·15 g (79%) of a product melting at 183—188°C. All *p*-nitroanilides of benzyloxycarbonyltripeptides *Ia—Ik* (with the exception of *Ig*) were prepared by a similar procedure; the yields and m.p.'s are shown in Table II.

Benzyloxycarbonylglycyl-prolyl-alanine *p*-Nitroanilide (*Ig*)

N,N'-Dicyclohexylcarbodiimide (1·1 g) was added to a solution of benzyloxycarbonylglycyl--proline (1·53 g; 5 mmol) and alanine *p*-nitroanilide (1·1 g; 5 mmol) in dimethylformamide (25 ml) precooled to 0°C. The suspension was stirred 2 h at 0°C and then set aside for 12 h at room temperature; N,N'-dicyclohexylurea which had separated was filtered off, the filtrate was taken to dryness and the residue was treated in the standard manner. Crystallization from dichloromethane (20 ml) and light petroleum (50 ml) afforded 1·4 g (57%) of a product melting at 135—138°C. The sample for analysis was crystallized in an analogous manner; the m.p. was unchanged.

3-Carboxypropionylalanyl-leucyl-alanine *p*-Nitroanilide (*IId*)

Hydrogen bromide (36%) in glacial acetic acid (5 ml) was added to a solution of *Id* (830 mg; 1·57 mmol) in glacial acetic acid. The solution was allowed to stand 1 h at room temperature and ether (1 000 ml) was then added. The hydrobromide which had separated was washed with ether (2 × 50 ml by settling), dried 2 h over phosphorus pentoxide and sodium hydroxide. The hydrobromide was then deionized on Zerolit G (in OH-form) in methanol, the methanolic effluents were evaporated to dryness, the residue was dried by distillation with benzene (2-times about 30 ml) and finally dissolved in dimethylformamide (20 ml). The solution was treated with succinic anhydride (300 mg) and subsequently heated 30 min at 80°C, then evaporated and the residue triturated with 1% citric acid (5 ml). The precipitate formed was filtered off and washed with water. Crystallization from methanol (5 ml) and water (10 ml) afforded 360 mg (46%) of a product of m.p. 217—219°C. All *p*-nitroanilides *IIa—IIh* were prepared by a similar procedure; the yield, m.p.'s and analytical data are given in Table II.

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