Vol. 96, No. 2, 1980
September 30, 1980

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 762-769

MAPPING OF THE SUBSTRATE - BINDING SITE OF THE HUMAN GRANULOCYTE ELASTASE BY THE AID OF TRIPEPTIDYL-p-NITROANILIDE SUBSTRATES

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Received July 3,1980

SUMMARY: The kinetic properties of the human granulocyte elastase /EC 3.4.21.11/ were investigated with 24 tripeptidyl-pNA substrates. By the regression analysis of the kinetic data obtained with 15 substrates a relatively hydrophobic compound, Boc-D-Phe-Ala-Nle-pNA, was predicted as the optimal substrate sequence. The compound was synthesized, assayed and the predicted K_m = 4.2 μM was confirmed experimentally. The substrate-binding site of granulocyte elastase appeared to be hydrophobic and very much similar to that of the pancreatic enzyme at the S_2-S_4 subsites, but the S_1 subsite, which determines the primary specificity, could accomodate bulkier residues and it was less selective than that in the pancreatic enzyme.

INTRODUCTION

The substrate specificity of the human granulocyte elastase has been studied with several peptide substrates /1,2/ and peptidyl-chloromethyl ketones /3/, and comparison with the pancreatic elastase has revealed slight differences at the P_1 position /notation of Schechter and Berger, 4/. The regression analysis of kinetic parameters obtained with peptidyl-pNA substrates has appeared to be a useful tool for mapping the substrate-binding site of pancreatic elastase /5/. Therefore, it seemed to be reasonable to study a similar set of substrates consisting of 24 compounds with the granulocyte enzyme too, to compare its specificity to that of the pancreatic elastase. In contrast to the set

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Abbreviations: pNA, p-nitroanilide; Nle, norleucine; Suc, succinyl-; Bz, benzoyl-; Boc, tert-butoxycarbonyl-

0006-291X/80/180762-08$01.00/0

of substrates tested in other laboratories /1,2/ the present one contained at least four substituents at each subsite, and thus, more detailed information could be expected about the properties of the binding site.

MATERIALS AND METHODS

Tripeptidyl-pNA substrates were synthesized by stepwise method /6/. Amino acids and p-nitroaniline were coupled by the phosphazo method /7/. The purity of the synthesized compounds was chechked by thin layer chromatography, by amino acid analysis as well as by elementary analysis. Only compounds having at least 95% purity were assayed. Substrates No. 6, 12, 20 and 21 were kindly gifted by Dr. S. Bajusz /Institute for Pharmaceutical Research, Budapest, Hungary/, and substrate No. 8 was generously supplied by Dr. R. Simonsson /AB KABI Peptide Research, Mölndal, Sweden/.

Granulocyte elastase was isolated on a Gordox*-Sepharose column according to Baugh and Travis /8/, and about a sixfold purification was achieved, compared to the specific activity of the enzyme in the granules. An absorption coefficient of $A^{1\%}_{280\ nm} = 9.85$ and a molecular weight of 30 000 were accepted for the calculations /8/. The amount of active elastase was determined by active site titration /9/ with the protease inhibitor isolated from the granulocyte cytosol /10/. Before assay the enzyme was pretreated with a 500-fold molar excess of tosyl-phenylalanyl-chloromethyl ketone /Serva, Heidelberg, FRG/ to extinguish probable chymotrypsin-like contamination.

Enzyme assays were carried out at 37°C in 50 mM Tris-HCl buffer, pH 8.0, containing 50 mM sodium chloride and 7% dimethylsulfoxide / v/v /. The concentration of the enzymes varied between 50 and 500 nM, and that of the substrates between 1 and 300 μM. The amount of p-nitroaniline cleaved during hydrolysis was calculated from the increase in absorption at 405 nm /$\varepsilon_{405} = 10\ 600\ M^{-1}cm^{-1}$/. K_m, the Michaelis constant, and V_{max}, the maximum velocity, were calculated from the initial velocities according to Lineweaver and Burk.

RESULTS

Kinetic constants determined with tripeptidyl-pNA substrates.

The kinetic constants are summarized in Table 1. In compliance with the requirements of regression analysis the reciprocal value of the Michaelis constant, $1/K_m$, is presented. These data indicate

* Trade name of basic pancreatic trypsin inhibitor preparation, which was generously gifted by Dr. A. Kelemen /G. Richter Ltd., Budapest, Hungary/.

Table 1. Kinetic Constants of the Human Granulocyte Elastase with Tripeptidyl-p-Nitroanilide Substrates

	P_4	P_3	P_2	P_1	$1/K_m$ mM^{-1}	k_{cat} s^{-1}	k_{cat}/K_m M^{-1} s^{-1}
1.	Boc	D-Phe	Pro	Ala	123.15	1.209	148 890
2.	Z	D-Phe	Pro	Ala	108.23	1.140	123 380
3.	Z	Gly	Val	Val	40.65	0.609	24 760
4.	Z	Arg	Nle	Nle	22.88	0.021	480
5.	Suc	Gly	Val	Val	18.90	0.082	1 550
6.	Z	Asp	Pro	Leu	12.48	0.029	362
7.	Suc	Gly	Ala	Leu	9.09	0.005	45
8.	Bz	D-Phe	Pro	Arg	6.06	0.011	67
9.	Z	Arg	Val	Leu	5.21	0.029	151
10.	Z	Gly	Val	Ala	5.15	0.792	4 079
11.	Z	Gly	Ala	Leu	4.52	0.037	167
12.	Z	D-Phe	Pro	Arg	2.58	0.042	108
13.	Suc	D-Phe	Pro	Ala	2.17	0.145	314
14.	Z	Ala	Ala	Ala	2.00	1.473	2 957
15.	Suc	Ala	Ala	Ala	1.79	0.180	322
16.		D-Phe	Pro	Ala	10.00	2.000	20 000
17.		Ala	Ala	Ala	4.65	0.130	605
18.	Suc	Gly	Val	Gly		n.d.	
19.	Z	Gly	Val	Gly		n.d.	
20.	Z	D-Phe	Gly	Arg		n.d.	
21.	Z	Phe	Pro	Arg		n.d.	
22.		Gly	Val	Ala		n.d.	
23.		Gly	Val	Gly		n.d.	
24.		Gly	Val	Val		n.d.	

Determined at 37°C in 50 mM Tris-HCl buffer, pH 8.0, containing 50 mM sodium chloride and 7% dimethylsulfoxide. n.d. = hydrolysis is not detectable

that the kinetic parameters of N-protected substrates in most instances are superior to those having a free amino terminal group. This difference may be brought about by the fact that granulocyte elastase prefers larger substrates than the tripeptides. With the substrates containing Gly at the P_1 or P_2 posi-

tion /e.g., No. 18, 20 and 23 in Table 1/ no hydrolysis could be detected under the conditions applied in the present experiments. Furthermore, substrates containing a P_3 Gly were cleaved only in that case when the N-terminal amino group was blocked, cf. compounds No. 3 and 24. The substitution of L-Phe for D-Phe at P_3 also prevented effective enzyme action /compounds No. 12 and 21/.

Regression analysis of the kinetic parameters. The kinetic parameters measured with substrates No. 1 to 15 in Table 1 were analyzed according to Free and Wilson /11/ as described earlier /12/. The results provided by this method permit us to give a quantitative evaluation of the influence of individual side-chains at P_1-P_4 subsites on the kinetic parameters relative to μ, the overall effect of the substrate molecule. From the contribution values listed in Table 2 we are able, in principle, to compute the kinetic parameters of any one out of 400 possible sequence combinations simply by summing up the contribution values of four selected side-chains and adding the value of μ to it. The correlation coefficients at the bottom of Table 2 were obtained by the comparison of calculated and measured kinetic data, and the values indicate a satisfactory correspondence.

In addition, from the greatest individual subsite contributions we can predict both the sequence and the kinetic values of the "best" substrate which can be built up from the investigated set of residues. Thus, for example, Boc-D-Phe-Ala-Nle-pNA was predicted as an optimal compound, since its calculated $1/K_m$ was 241 mM^{-1} /K_m = 4.2 uM/, two times greater than 123.15 mM^{-1}, that of substrate No. 1 in Table 1, the best one applied for the previous kinetic investigations. The compound was synthesized. However, due to its hydrophobic nature, its assay could

Table 2. Contribution of Tripeptidyl-p-Nitroanilide Substrates to the Kinetic Parameters of the Human Granulocyte Elastase /Substrates No. 1-15/

Subsite	Substituent	Contribution $1/K_m$ mM^{-1}	Contribution k_{cat} s^{-1}	Contribution k_{cat}/K_m $M^{-1}\ s^{-1}$
P_4	Boc	57.02	0.388	72 137.3
	Bz	7.98	0.146	4 337.4
	Z	4.49	0.178	6 226.5
	Suc	- 26.37	-0.534	- 33 128.3
P_3	D-Phe	33.40	0.218	31 419.6
	Arg	- 10.56	-0.686	4 599.8
	Gly	- 15.03	-0.059	- 13 818.1
	Asp	- 21.96	-0.673	- 27 949.0
	Ala	- 24.39	0.624	- 34 629.3
P_2	Ala	16.16	-0.093	44 033.0
	Pro	11.66	0.129	38 738.3
	Val	- 6.30	0.183	5 986.5
	Nle	-109.37	-1.135	-432 507.7
P_1	Nle	110.09	1.315	401 645.9
	Val	37.34	-0.0001	13 883.1
	Ala	- 2.72	0.091	- 14 831.0
	Leu	- 6.73	-0.035	- 37 172.0
	Arg	- 70.77	-0.866	- 95 869.4
Overall contribution /μ/		24.32	0.387	20 517.6
Correlation coefficient /r/		0.8996	0.9365	0.9163

be performed only at a relatively high solvent concentration, i.e. in the presence of 25% dimethylsulfoxide. The assays provided a $1/K_m$ value of 500 mM^{-1} /K_m = 2.0 μM/ indicating a relatively good agreement with that predicted by calculations. The high solvent concentration did not alter significantly the K_m of compound No. 1, either. By the removal of the Boc group the solubility could be raised and, consequently, the k_{cat} was also increased from 0.0025 s^{-1} to 0.025 s^{-1}.

DISCUSSION

Properties of the substrate-binding site of the enzyme. From the data in Table 2 we may infere which residues exert favourable or unfavourable influence on the kinetic parameters. This, in turn, enables us to draw conclusions concerning the structure of the binding site.

It follows from the results that the granulocyte elastase requires uniformly hydrophobic moieties at the S_1-S_4 segment of the binding site. The extremely hydrophobic nature of the substrate-binding site of the granulocyte elastase prevented us from designing new peptide sequences for the improvement of the catalytic efficiency of the substrates. A charged group, e.g. Suc, at P_4 diminished the rate of hydrolysis, while hydrophobic protective groups, particularly Boc, proved to be good. This finding suggests that the S_4 subsite is at least of medium size.

At the S_3 subsite only Ala and Pro have been applied in earlier studies /2,3/. In the present experiments we investigated the influence of four additional amino acid residues. Similarly to other serine proteases /Pozsgay et al., in preparation/ D-Phe was the most effective and had the highest contribution, as $1/K_m$ is concerned. When the rate of hydrolysis, k_{cat}, is taken into account, Ala seems to be favourable, but D-Phe is nearly as good as Ala. These data suggest that S_3 is a shallow cavity. The aromatic ring of D-Phe is assumed to point outwards of the binding crevice.

In agreement with Tuhy and Powers /3/ it was found that at the S_2 subsite medium size hydrophobic side chains /Ala, Pro, Val/ can be accomodated, suggesting that this crevice is of medium size. Nle is too large, while Gly is too small to permit the formation of a productive enzyme-substrate complex.

At S_1, the primary specificity site, aliphatic side-chains of various size were equally accepted, although Nle proved to be outstanding particularly in respect of binding. Arg binds to the S_1 subsite in those cases only, when the interaction of the rest of the substrate molecule is extremely favourable /compare, e.g., compounds No. 8 and 12 vs 20 and 21/. Therefore, the S_1 subsite appears to be the largest one in the S_1-S_4 segment.

Comparison of human granulocyte and porcine pancreatic elastases. Although the two elastases are similar, since both bind, most strongly, hydrophobic peptide segments adjacent to the bond to be cleaved, some differences could certainly be demonstrated. First, as compared with the pancreatic enzyme /5/, the granulocyte elastase preferred substrates which were more hydrophobic. The above conclusions were supported by the structure of the substrates found to be optimal regarding either $1/K_m$ /Suc-D-Phe--Pro-Ala- for the pancreatic and Boc-D-Phe-Ala-Nle- for the granulocyte enzyme/ or k_{cat} /Z-Ala-Ala-Ala- and Boc-Ala-Val-Nle-, respectively/. Secondly, in the S_1 site of the granulocyte enzyme side-chains of larger steric requirement can fit in and this site seems to be less selective than that in the pancreatic enzyme. Comparison of the two enzymes by the aid of peptidyl substrates /1,2/ and sulfonyl fluorides /13/, as well as by studying the proteolytic cleavage of the B-chain of insulin /14,15/ has also pointed to slight differences, particularly at the S_1 subsite. Complete correspondence in the properties of the two elastases cannot obviously be expected, since their origin, structure and biological role are entirely different. In spite of this, it was surprising that the granulocyte elastase was almost a thousand-fold as effective in the inactivation of blood clotting factor VIII as the pancreatic enzyme /16/.

ACKNOWLEDGEMENTS: The excellent technical assistance of Miss K. Faragó and Miss S. Merész is gratefully acknowledged. This work was supported by a grant from the Hungarian Ministry of Health.

REFERENCES

1. Zimmerman, M. and Ashe, B.M. /1977/ Biochim. Biophys. Acta 480, 241-245.
2. Nakajima, K., Powers, J.C., Ashe, B.M. and Zimmerman, M. /1979/ J. Biol. Chem. 254, 4027-4032.
3. Tuhy, P.M. and Powers, J.C. /1975/ FEBS Lett. 50, 359-361.
4. Schechter, I. and Berger, A. /1967/ Biochem. Biophys. Res. Commun. 27, 157-162.
5. Cs. Szabó, G., Pozsgay, M., Gáspár, R., Jr. and Elődi, P. /1980/ Acta Biochim. Biophys. Acad. Sci. Hung. 15, in the press.
6. Houben-Weyl /1974/ Methoden der organischen Chemie Band 15, Teil 1, 2 Synthese von Peptiden. Wünsch, E. Ed. Georg Thieme, Stuttgart.
7. Goldschmidt, S. and Rosculet, G. /1960/ Chem. Ber. 93, 2387-2394.
8. Baugh, R.J. and Travis, J. /1976/ J. Biochemistry 15, 836-841.
9. Winninger, C., Lestienne, P., Dimicoli, J.L. and Bieth, J. /1978/ Biochim. Biophys. Acta 526, 227-234.
10. Marossy, K., Hauck, M. and Elődi, P. /1980/ Biochim. Biophys. Acta, in the press.
11. Free, S.M.,Jr. and Wilson, J.W. /1964/ J. Med. Chem. 7, 395-399.
12. Pozsgay, M., Gáspár, R.,Jr., Bajusz, S. and Elődi, P. /1979/ Eur. J. Biochem. 95, 115-119.
13. Lively, M.O. and Powers, J.C. /1978/ Biochim. Biophys. Acta 525, 171-179.
14. Blow, A.M.J. and Barrett, A.J. /1977/ Biochem. J. 167, 17-19.
15. Ardelt, W., Tomczak, Z. and Dudek-Wojciechowska, G. /1979/ Acta Biochim. Polon. 26, 267-273.
16. Váradi, K., Marossy, K., Asbóth, G., Elődi, S. and Elődi, P. /1980/ Thromb. Haemost. 43, 45-48.