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INVESTIGATION OF THE SUBSTRATE-BINDING SITE OF HUMAN PLASMIN USING TRIPEPTIDYL-p-NITROANILIDE SUBSTRATES

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ABSTRACT

The hydrolysis of tripeptidyl-p-nitroanilides by human plasmin /EC 3.4.21.7./ was studied and the kinetic parameters were determined. The individual contributions of the amino acid side chains at the P_1-P_4 subsites to the kinetic parameters were calculated by regression analysis. The highest contributions yielded the structure of an "optimum" substrate, D-Ile-Phe-Lys-pNA. Its predicted kinetic parameters, $K_m = 9.6 \times 10^{-6}$ M and $k_{cat}/K_m = 284\,789\ M^{-1}s^{-1}$, appeared to be about 40 times as good as those of H-D-Val-Leu-Lys-pNA /S-2251/ applied for the determination of the plasminogen and plasmin content of blood in various laboratories. At the S_2-S_4 segment in the binding site, which interacts with the P_2-P_4 moieties of the substrate, plasmin favoured uniformly hydrophobic substituents.

INTRODUCTION

Plasmin /EC 3.4.21.7./, the key enzyme of fibrinolysis splits peptide bonds specifically at basic amino acid side

Key words: plasmin, chromogenic substrates, substrate-binding site

chains, similarly as thrombin and trypsin do. Its substrate specificity has been studied, mainly in comparison with proteases of similar specificity, with Arg- and Lys-esters /1, 2/ and with tripeptidyl-p-nitroanilides /-pNA/ /3-8/. On the basis of structure-activity relationship data the sequence H-D-Val-Leu-Lys-pNA has been suggested by Claeson et al. /3/ as specific for the determination of plasmin activity in plasma. The structure of the binding site in plasmin is not yet known. Therefore, it would be hard to predict which amino acid sequence will meet better the requirements of the formation of a productive enzyme-substrate interaction. In our previous studies the treatment of kinetic data by regression analysis seemed to be a useful tool for characterizing the substrate-binding site of various proteinases. In addition, the data of the analysis permitted us to design better fitting substrates than the already examined ones for subtilisin /9/, trypsin /10, 11/, pancreatic elastase /12/ and thrombin /13/.

In the present paper we describe investigations of the substrate-binding site of plasmin, which were carried out with 22 tripeptidyl-pNA substrates and suggest new peptide sequences which may be appropriate /convenient/ for the determination of plasmin activity.

MATERIALS AND METHODS

Tripeptidyl-p-nitroanilide substrates having benzyloxycarbonyl- /Z-/ protecting groups were synthesized in the Institute for Drug Research /Budapest, Hungary/, those having benzoyl- /Bz-/ groups and those unprotected were prepared in the laboratories of KABI Peptide Research /Mölndal, Sweden/. Homogeneity of the substrates was checked by thin layer chromatography /10/ before assay.

Human plasmin was obtained from KABI Peptide Research /Mölndal, Sweden, No 77. 50 325, activity 15 CU/mg/ as freeze-dried powder, activated by matrix-bound Kabi-kinase.

Kinetic measurements were performed in 50 mM Tris-HCl buffer, pH 7.4, at 37°C. The concentration of plasmin varied between 20 and 80 nM and that of the substrates between 16 and 540 μM depending on the rate of hydrolysis. The absorption of p-nitroaniline liberated from the substrates was recorded at 405 nm for 3 min. /absorption coefficient, ε_{405}=10 600 $M^{-1}cm^{-1}$/.

A molecular weight of 76 000 was used for plasmin /14/ in the calculations.

Regression analysis of the kinetic parameters was carried out according to Free and Wilson /15/ as described earlier /9/.

RESULTS

Kinetic constants of the enzymatic hydrolysis of tripeptidyl-pNA substrates. The kinetic data of hydrolysis are shown in Table I. Due to practical considerations the reciprocal value of the Michaelis constant, $1/K_m$, is presented in

Table I, since it is required by the regression analysis that the best substrate should have the greatest value. Although substrates of identical sequence with or without a protecting group at the N-terminus were not available, the data in Table I show that if P_3 /notation of Schechter and Berger, 16/ contains a D-amino acid residue, the unprotected derivatives have better kinetic properties than the protected ones. Substrates with norleucine /Nle/, Leu and Ala side-chain at the P_1 subsite are not hydrolyzed by plasmin.

Regression analysis of the kinetic data. The application of regression analysis to a set of substrates renders possible to characterize quantitatively the contribution of each side chain at substrate subsites P_1-P_4 to the kinetic constants with respect to the overall contribution, μ. The value of μ characteristic of the analyzed set of compounds comprises undescribed properties of the substrates, such as the effect of peptide backbone, that of -pNA moiety, etc. Regression analysis was carried out separately both for substrate series A and B /Table II/. In contrast to our previous findings /9-12/ the k_{cat} values of unprotected substrates /Series B/ were similar to each other, their regression analysis was, therefore, meaningless. By adding up the appropriate contributions of individual subsites and the overall contribution, μ, listed in Table II the kinetic parameters of 160 /Series A/ and 30 /Series B/ substrates having various sequences can be computed, respectively.

The correlation coefficients, r, characterizing the accordance of the measured and computed kinetic constants are shown at the bottom of Table II.

Design of "better" substrates. From the amino acids which have the greatest values of subsite contribution to the kinetic constants, tripeptides can be designed which would be optimal substrates for the enzyme, in respect of $1/K_m$, k_{cat} or k_{cat}/K_m. This optimality applies always only to one given kinetic constant. Very often, a substrate with a great $1/K_m$ may have a poor k_{cat} or vica versa /12/. As seen in Table III, the predicted kinetic parameters of the designed substrates are substantially greater than any of those measured for the substrates within the original series, particularly in the case of series A. For k_{cat}/K_m, that indicates the efficiency of the substrate rather than any other kinetic parameter, D-Ile-Phe-Lys-pNA is the best sequence. Its calculated proteolytic constant, k_{cat}/K_m, was 284 789 $M^{-1}s^{-1}$, about 40-fold greater than that of D-Val-Leu-Lys-pNA /S-2251/, the one used for the assay of the plasminogen and plasmin content of blood.

DISCUSSION

The substrate-binding site of plasmin. Our recent studies of a large series of substrates /11, 13/ have revealed that the side chain interactions which may take place within the sub-

Table I

Kinetic constants of tripeptidyl-p-nitroanilide substrates /Measured in 50 mM Tris-HCl buffer, pH 7.4, at 37°C/

No.	Substrate subsites				$1/K_m$	k_{cat}	k_{cat}/K_m
	P_4	P_3	P_2	P_1	mM^{-1}	s^{-1}	$M^{-1}\ s^{-1}$
A. Protected substrates							
1.	Z	D-Val	Pro	Arg	30.00	2.35	70 334
2.	Z	Nle	Pro	Arg	10.00	3.52	35 222
3.	Z	D-Phe	Gly	Arg	8.30	1.82	15 204
4.	Z	Phe	Val	Arg	7.30	1.23	8 935
5.	Bz	Lys	Nle	Arg	6.00	2.07	12 390
6.	Bz	Lys	Pro	Arg	5.90	0.50	2 969
7.	Z	Lys	Nle	Arg	4.75	4.31	20 428
8.	Z	Phe	Pro	Arg	4.50	4.66	20 969
9.	Bz	Phe	Val	Lys	4.00	0.88	3 526
10.	Bz	Arg	Pro	Arg	3.10	1.03	3 804
11.	Bz	Ile	Leu	Lys	2.00	12.30	24 631
12.	Z	Pro	Gly	Arg	1.90	0.27	520
13.	Bz	D-Phe	Pro	Arg	1.67	1.64	2 740
14.	Bz	Phe	Val	Arg	1.60	1.45	2 321
B. Unprotected substrates							
15.		H-D-Pro	Phe	Lys	97.00	2.01	195 131
16.		H-D-Val	Phe	Lys	90.00	2.95	265 832
17.		H-D-Ala	Phe	Lys	45.50	1.18	53 851
18.		H-D-Phe	Phe	Arg	23.50	4.69	110 251
19.		H-D-Ile	Leu	Lys	17.00	1.54	26 170
20.		H-D-Phe	Pip	Arg	6.30	0.85	5 346
21.		H-D-Val	Leu	Lys	3.00	2.40	7 212
22.		H-D-Val	Pip	Arg	1.90	1.07	5 629

strate, do in fact not influence substantially the kinetic parameters. Hence, the participation of the amino acids at individual subsites in shaping the kinetic parameters can be characterized by regression analysis /Table II/, and in turn, from the properties of the most effective residues the topography of the substrate-binding site can be inferred.

The share of the P_4 protecting group in the action of plasmin seems to be of little value. At this position a Z-group, which is comparable to a Phe residue, proved to be more favourable than a Bz-group. In aggreement with other observations /3, 8/, our results also show that with P_3 residues of D-configuration the kinetic constants of substrates having an unprotected N-terminus are better than those of protected compounds.

At the P_3 position non-polar amino acids /Ile, D-Val, D-Ile/ have the greatest values of contribution in respect of $1/K_m$,

Table II

Contribution of the side chains of tripeptidyl-p-nitroanilide substrates to the kinetic parameters

Subsite	Residue	Contribution to $1/K_m$ mM^{-1}	k_{cat} s^{-1}	k_{cat}/K_m $M^{-1}\ s^{-1}$
A. Protected substrates				
P_4	Z	1.11	0.51	3 663
	Bz	- 1.11	-0.51	- 3 663
P_3	Ile	38.00	0.20	-83 000
	D-Val	20.00	-0.54	57 800
	Nle	- 0.70	0.55	30 900
	Lys	- 1.90	-1.39	- 2 400
	Arg	- 4.70	-0.85	- 1 500
	Phe	- 5.50	1.77	8 400
	D-Phe	- 6.20	-0.24	- 2 500
	Pro	-12.50	-1.79	-17 200
P_2	Gly	6.90	-1.15	- 1 800
	Val	3.70	-3.13	-18 600
	Pro	2.60	-0.32	- 6 800
	Nle	0.90	1.86	3 000
	Leu	-42.30	9.89	94 200
P_1	Lys	0.57	0.04	1 338
	Arg	- 0.09	-0.006	- 223
Overall contribution /μ/		6.50	2.72	15 999
Correlation coefficient /r/		0.9828	0.9930	0.9941
B. Unprotected substrates				
P_3	D-Ile	15.84		51 994
	D-Pro	8.84		- 37 665
	D-Phe	6.24		32 753
	D-Val	1.84		33 036
	D-Ala	-42.68		-178 943
P_2	Phe	26.05		90 881
	Pip*	8.85		- 14 023
	Leu	-60.95		-167 739
P_1	Lys	26.55		58 164
	Arg	-44.34		- 97 134
Overall contribution /μ/		35.56		83 750
Correlation coefficient /r/		0.9999		0.9999

* pipecolate

Table III

Predicted kinetic constants of the designed "best" substrates

Kinetic constant	Substrate sequence	Value of the kinetic constant
$1/K_m$, mM^{-1}	A. Z-Ile-Gly-Lys-pNA	53.08
	B. D-Ile-Phe-Lys-pNA	104.00
k_{cat}, s^{-1}	A. Z-Phe-Leu-Lys-pNA	14.33
k_{cat}/K_m, $M^{-1}s^{-1}$	A. Z-D-Val-Leu-Lys-pNA	173 001.00
	B. D-Ile-Phe-Lys-pNA	284 789.00

whereas the contribution of charged amino acids is smaller than the average. This result correlates with the observation of Claeson et al. /3/. For the effectivness of catalysis large hydrophobic side chains are favourable. In case of protected substrates the residues either of D-configuration or carrying positive charge decreased the rate of hydrolysis. Consequently, the S_3 segment of the substrate-binding site in the enzyme is capable of accomodating non-polar side chains of large steric requirement.

S_2-P_2 interaction. Similarly to other serine proteases, the S_2 crevice of plasmin forms a hydrophobic interaction with the P_2 side chain. As the value of $1/K_m$ is concerned, the non-polar amino acids are similar in behaviour, with the exception of Leu that had a considerably great negative value of contribution to $1/K_m$. It is noteworthy, however, that the contribution of Leu to k_{cat} was found optimal. A similar inverse phenomenon has been observed with plasmin as with other proteinases /11-13/ that the side chain being the most effective in respect of k_{cat} is, in respect of $1/K_m$, the poorest one. It follows, that the side chain which increases the strength of binding of a substrate, may concomittantly decrease the leaving of the product from the binding pocket of the enzyme, consequently it may reduce the overall rate of catalysis.

The S_1 binding site: primary specificity. The peptide bond is specifically split by plasmin at the positively charged side chains; hydrolysis is not catalyzed by plasmin near the non-polar side chains. As is shown by the contribution values, the S_1 subsite exhibits a distinct preference for Lys. This observation is in aggreement with the results of Troll et al. /2/ and Scheraga et al. /1/ obtained with lysine- and arginine-esters, as well as with those of others /4, 5, 7/ obtained with p-nitroanilides.

The above data reflect the non-polar nature of the S_2-S_4 binding domain of plasmin. Additional evidence for this has been provided by studies carried out with natural substrates /17/. Hence it is known that plasmin splits the peptide bond

of both fibrinogen and fibrin most rapidly at the C-terminus of the sequences ...Leu-Leu-Lys /β-chain/ and ...Leu-Ile-Lys /γ-chain/. The next bonds to be split are those adjacent to that lysine residue which is preceded by either Tyr-Gln /α-chain/, Thr-Gln /β-chain/ or Gln-Thr /γ-chain/. Concerning the effect of the latter side chains, in the case of tripeptidyl substrates we cannot as yet rely on any experimental evidence. Nevertheless, we assume that the above observations do not contradict our conclusions, since the carbon skeleton of Tyr and Gln is capable of entering into interactions. In addition, as compared to tripeptides, a natural substrate of high molecular weight forms more interactions with the enzyme. Therefore, it may possibly be split by the enzyme at less favourable sections.

Comparison of plasmin with other serine proteases. The structural similarity in the vicinity of the active serine of thrombin, trypsin, elastase, chymotrypsin and plasmin /18/ also manifests itself in the kinetic behaviour of these enzymes. Although the specificity of plasmin is similar to that of thrombin, plasmin is less "sharp", as thrombin cleaves peptide bonds almost only at Arg. While trypsin prefers Arg-p-nitroanilides, plasmin favours Lys-substrates. Of the interactions formed between the subsites of proteases and the side chains of tripeptidyl-p-NAs examined in our laboratory /9-13/ the S_3-P_3 one was found to be pre-dominant. The same holds true for the Michaelis constant of plasmin, too. The inverse phenomenon occurring at P_2 as well as the non-polar feature of the S_2-S_4 substrate-binding sites also appear to be common properties of the serine proteases studied so far.

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