BBA 66585

THE TRYPSIN-CATALYSED HYDROLYSIS OF D-LYSINE AND D-ARGININE ETHYL ESTERS

JOCELYN E. PURDIE, RUTH E. DEMAYO, JOHN H. SEELY AND N. LEO BENOITON
Department of Biochemistry, University of Ottawa, Ottawa K1N 6N5 (Canada)

(Received November 30th, 1971)

SUMMARY

Steady-state parameters have been obtained for the tryptic hydrolysis of the ethyl esters of D-arginine and D-lysine. At acid pH the rates of hydrolysis are substantial. The stereospecificity ratio $(k_{\mathrm{cat}}/K_m)_{\mathrm{L}}$ to $(k_{\mathrm{cat}}/K_m)_{\mathrm{D}}$ is six times greater for the arginine ester than for the lysine ester. The stereospecificity ratios for the α-N-unsubstituted arginine and lysine esters are smaller by one to two orders of magnitude than those for the corresponding α-N-acylated derivatives. This decrease is due to a reduction in k_{cat}/K_m for the L-isomer together with a proportionate increase in k_{cat}/K_m for the D-isomer.

INTRODUCTION

Phenylalanine and its ring-substituted homologues can be resolved by the action of chymotrypsin (EC 3.4.4.5) on their ethyl esters[1,2]. During the course of studies on the specificity of trypsin[3] (EC 3.4.4.4), an attempt was made to resolve homolysine in a similar manner using trypsin[4]. It was found, however, that the amino acid isolated from a digest of DL-homolysine ethyl ester by trypsin at pH 5 was actually a mixture of both the D- and L-isomers, in the proportion of 1:3, and that the unhydrolysed ester also was not the expected pure D-isomer. It was concluded that the D-homolysine ethyl ester had been hydrolysed by the trypsin[4]. This paper describes some kinetic studies on the action of trypsin on the D- and L-isomers of lysine and arginine ethyl esters. The statement, unsupported by experimental detail, that D-lysine ethyl ester is a substrate for trypsin has appeared in the literature[5].

MATERIALS AND METHODS

D-Lysine·HCl and D-arginine·HCl were purchased from Schwarz/Mann, L-arginine from Nutritional Biochemical Corp. Esters were prepared from these by the

HCl/ethanol procedure*. The esterification was carried out twice in each case. L-Lysine ethyl ether·2HCl was purchased from Sigma Chemical Co. and re-esterified once since it contained an appreciable amount of unesterified amino acid. The D-lysine·HCl was shown to contain less than 0.5% of the L-isomer by a modification of the method of Manning and Moore[6]. A sample was converted to the ε-*N*-acetyl derivative by stirring in the presence of excess phenyl acetate at pH 11[7], evaporating the solution, and reacting the residue with L-alanine *N*-carboxyanhydride[8]. The dipeptides formed were determined on the long column (50 cm; AA-15 resin) of a Beckman amino acid analyser eluted with 0.2 M sodium citrate, pH 4.25, at a flow rate of 68 ml/h. The homolysine alluded to in the Introduction had been analysed by the same method. Elution times: L-Ala–D-Lys(Ac), 49; L-Ala–L-Lys(Ac), 56; L-Ala–D-Hly(Ac), 71; L-Ala–D-Hly(Ac), 79 min.

Trypsin (bovine pancreas) was a Worthington product, Code TRL, lot OEA. Solutions of trypsin in 0.001 M HCl containing 5 mM $CaCl_2$ were prepared daily and kept at 0 °C. The concentration of enzyme in terms of active sites was determined by titration with *p*-nitrophenyl-*p*'-guanidinobenzoate·HCl[9]. Fresh solutions of the esters were made up for each experiment. Since the arginine esters were hygroscopic, they were dried over P_2O_5 in an evacuated desiccator before use, and the concentration of each solution was established (to within ±2%) by analysis of an aliquot for arginine with the analyser after saponification.

The steady-state kinetic parameters V and K_m were calculated from hydrolysis rates obtained titrimetrically in thermostatted reaction vessels using a Radiometer pH-stat type PHM 26c/TT11a/SBUI/SBR2/TTA3 as described previously[10]. The electrode system (glass, G202B; calomel, K401) was standardised with the primary standard buffers, borate (pH 9.18 at 25 °C) and phthalate (pH 4.01 at 25 °C), using the *iso* pH adjustment. The observed rates were corrected for ionisation of the α-amino group of the substrate as previously described[10] using pK' values of 7.33 for lysine ethyl ester and 7.26 for arginine ethyl ester, the pK' values being obtained by potentiometric titration in the reaction medium (0.1 M NaCl, 5 mM $CaCl_2$) at 25 °C.

RESULTS AND DISCUSSION

The results confirm that trypsin catalyses the hydrolysis of esters of the D-forms of basic amino acids at moderate rates; the derived steady-state parameters for the ethyl esters of the D- and L-isomers of lysine and arginine are given in Table 1. The data for the arginine esters are less precise than those for the lysine esters. This was partly due to the fact that the concentrations had to be determined using the amino acid analyser because of the deliquescent nature of the ester hydrochlorides. Anomalous time-progress curves with initial convex curvature were observed at all concentrations for the hydrolysis of the D-arginine ester. At the lower concentrations the rate decreased to a steady value after a few minutes and the reaction then obeyed Michaelis-Menten kinetics up to 25 mM ester. At higher ester concentrations, depar-

---

* It has been suggested that esterification might induce slight racemization. We contend that it is already recognised that esterification by the classical HCl/alcohol procedure does not racemize an amino acid. We have demonstrated this in the case of a racemization-sensitive amino acid, phenylalanine[10]. Moreover, the use of ethyl and methyl esters in the azide coupling procedure in peptide synthesis rests on this point.

TABLE I

STEADY-STATE CONSTANTS FOR THE TRYPTIC HYDROLYSIS OF ARGININE AND LYSINE ETHYL ESTERS

The hydrolyses were carried out in 0.1 M NaCl containing 5 mM $CaCl_2$ at pH 6.00 and 25 °C. Concentration ranges were: 0.9–10.0 mM L-lysine ethyl ester, 0.2–2.7 mM L-arginine ethyl ester, 0.038–0.045 µM trypsin; 2–45 mM D-lysine ethyl ester, 2–42 mM D-arginine ethyl ester, 0.8–1.1 µM trypsin. The values given are the mean and standard deviation of three determinations.

Ethyl ester of	k_{cat} (s^{-1})	K_m (mM)	$10^{-3} \times k_{cat}/K_m$ ($M^{-1} \cdot s^{-1}$)	$\frac{(k_{cat}/K_m)_L}{(k_{cat}/K_m)_D}$
L-Lysine	36.4 ± 3.0	5.9 ± 0.4	6.15 ± 0.16	108
D-Lysine	1.6 ± 0.1	28.1 ± 3.5	0.057 ± 0.0035	
L-Arginine	15.6 ± 1.4	0.69 ± 0.13	23.4 ± 2.8	632
D-Arginine	0.52	14.2	0.037	

ture from linearity occurred in the $[S]$ *vs* $[S]/v$ plots in the direction of a relative increase in v. This indicates that the D-arginine ester was contaminated with L-isomer which caused the higher velocity initially observed and which had not been adequately corrected for at the higher ester concentrations.

The stereospecificity ratio $(k_{cat}/K_m)_L/(k_{cat}/K_m)_D$ (ref. 11) for lysine ethyl ester is more than an order of magnitude lower than the value $5.8 \cdot 10^3$ (pH 7.0, 0.1 M NaCl, 25.0 °C) calculated for the N-acetylated analogue[12]. This underlines the positive contribution of the acylamino moiety to the stereospecificity of the reaction. The decrease in stereospecificity ratio on the removal of this *N*-acyl function in esters is due both to an increase in $(k_{cat}/K_m)_D$ as well as to a decrease in $(k_{cat}/K_m)_L$. The value of k_{cat}/K_m for *N*-acetyl-D-lysine ethyl ester[12] is given as 36 $M^{-1} \cdot s^{-1}$ (pH 7.0, 0.1 M KCl, 25.0 °C). By analogy with the variation of k_{cat}/K_m with pH for L-lysine methyl ester[13], our value of 57 $M^{-1} \cdot s^{-1}$ for D-lysine ethyl ester at pH 6.00 would correspond to about 180 $M^{-1} \cdot s^{-1}$ at pH 7.0. In addition, the k_{cat} for D-lysine ethyl ester at pH 6.00 (1.6 s^{-1}) is significantly higher than that for the *N*-acetyl analogue, which is 0.07 s^{-1} at pH 7.

The specificity constants[14] k_{cat}/K_m for arginine compounds are generally higher than those for the corresponding lysine derivatives. From the data of Seydoux and Yon[15], the stereospecificity ratio for *N*-benzoylarginine ethyl ester (pH 8, 0.025 M $CaCl_2$, 25.0 °C) is $76.7 \cdot 10^3$. No data are available for *N*-acetylarginine derivatives but for the lysine esters[13] replacement of *N*-acetyl by *N*-benzoyl causes an increase in k_{cat}/K_m of less than an order of magnitude. This, with our tentative value of 632 for the stereospecificity ratio for arginine ethyl ester, would mean that trypsin exhibits greater stereospecificity towards arginine esters than towards lysine esters. On the other hand, a stereospecificity ratio of $1.7 \cdot 10^3$ (pH 8.0, 0.2 M KCl, 0.05 M $CaCl_2$, 25 °C) is obtained from the data of Trowbridge *et al.*[5] for tosylarginine methyl ester (for which substrate activation was observed). It should be noted, however, that in all of the above cases, the optical integrities of the D-arginine esters were not established.

It has been proposed[5,12] that trypsin contains two main binding sites, a primary site for arginine and lysine substrates and a secondary site for neutral substrates and modifiers. It is not known whether the 'neutral' site or yet another site is involved in substrate activation phenomena. It has been suggested[12] that the productive binding mode for *N*-acetyl-D-lysine ethyl ester places the lysine side-chain in the 'neutral' site. The steady-state parameters for this ester are of the same order of magnitude as those for the neutral esters[12], α-*N*-benzoyl-L-citrulline methyl ester and *N*-acetyl-L-heptyline methyl ester whereas the parameters for the esters of the *N*-acyl derivatives of L-arginine and L-lysine are 3–4 orders of magnitude greater. It is possible that the productive binding modes of D-arginine and D-lysine ethyl esters also place the amino acid side-chains in the 'neutral' site. On the other hand, unless charge interactions involving the α-$^{+}NH_3$ are unfavourable, the absence of the acylamino function with its associated binding restraints may permit these D-esters to bind productively at the primary site. The efficiency of this process would necessarily be less than that for the L-isomer but it could still be greater than that arising from binding at the 'neutral' site.

It is interesting that trypsin displays a considerably lower degree of stereospecificity towards esters of *N*-acylamino acids and N-unsubstituted amino acids than does α-chymotrypsin. No hydrolysis of the ethyl or *p*-nitrobenzyl esters of D-phenylalanine could be detected using the pH-stat technique, and these esters acted as competitive inhibitors of the hydrolysis of the L-isomers[10]. The stereospecificity ratio for the amino acid ethyl esters (pH 7.0, 0.1 M NaCl, 25.0 °C) is $\geqslant 3 \cdot 10^4$. Ingles and Knowles[11] quote a value of $\geqslant 63 \cdot 10^4$ for the *N*-acetylphenylalanine ethyl esters (pH 7.0, 0.1 M NaCl, 25.0 °C). α-Chymotrypsin appears to have a much lesser ability to accommodate productive binding modes for esters of D-amino acid derivatives, *i.e.* any secondary binding site compatible with the *N*-acylamino group or benzyl side-chain cannot be very closely associated with the catalytic groups. Also, although the cationic forms of the ethyl esters of L- and D-phenylalanine are more loosely bound than those of the *N*-acetyl analogues[10] there is little, if any, relaxation of the steric requirements for the subsequent catalytic steps.

ACKNOWLEDGEMENTS

This work was financially supported by the Medical Research Council of Canada. N.L.B. is an Associate of the M.R.C.C.

REFERENCES

1 C. Petitclerc, A. D'Iorio and N. L. Benoiton, *J. Labelled Compounds*, 5 (1969) 265.
2 J. H. Tong, C. Petitclerc, A. D'Iorio and N. L. Benoiton, *Can. J. Biochem.*, 49 (1971) 877.
3 J. H. Seely and N. L. Benoiton, *Can. J. Biochem.*, 48 (1970) 1122.
4 J. H. Seely, Ph. D. Thesis, University of Ottawa, Ottawa, Canada, 1969.
5 C. G. Trowbridge, A. Krehbiel and M. Laskowski, *Biochemistry*, 2 (1963) 843.
6 J. M. Manning and S. Moore, *J. Biol. Chem.*, 243 (1968) 5591.
7 J. Leclerc and L. Benoiton, *Can. J. Biochem.*, 46 (1968) 1047.
8 J. R. Coggins and N. L. Benoiton, *J. Chromatogr.*, 52 (1970) 251.
9 T. Chase and E. Shaw, *Biochem. Biophys. Res. Commun.*, 29 (1967) 508.
10 J. E. Purdie and N. L. Benoiton, *Can. J. Biochem.*, 48 (1970) 1058.
11 D. W. Ingles and J. R. Knowles, *Biochem. J.*, 108 (1968) 561.
12 B. M. Sanborn and G. E. Hein, *Biochemistry*, 7 (1968) 3616.
13 F. Seydoux, *Eur. J. Biochem.*, 17 (1970) 209.
14 F. E. Brot and M. L. Bender, *J. Am. Chem. Soc.*, 91 (1969) 7187.
15 F. Seydoux and J. Yon, *Biochem. Biophys. Res. Commun.*, 44 (1971) 745.