Biochimica et Biophysica Acta, 302 (1973) 406–410

BBA 66863

COMPETITIVE INHIBITION OF PANCREATIC ELASTASE

L. DZIALOSZYNSKI* AND T. HOFMANN
Department of Biochemistry, University of Toronto, Toronto M5S 1A8, Ontario (Canada)

(Received October 9th, 1972)

SUMMARY

N-Acetyl and *N*-carbobenzoxy derivatives of dipeptides of alanine, valine, leucine and isoleucine were found to be effective competitive inhibitors of the esterolytic activity of pancreatic elastase (EC 3.4.4.7) with K_i values between 1 and 10 mM. Poorest binding was obtained with the derivatives of alanylalanine, best binding with the derivatives of valylvaline. Little difference was found between the acetyl and carbobenzoxy peptides. No hydrolysis of the dipeptides was observed. The results show that effective side-chain binding occurs close to the active site. Acetylvalylvaline binds to elastase in the crystalline form and is potentially useful for studying the binding site by X-ray crystallography.

INTRODUCTION

Recent studies on the action and specificity of elastase (EC 3.4.4.7)[1–6] have shown that among small substrates, derivatives of alanine show the highest "specificity" constants (as defined by the value k_{cat}/K_m[7]) and that for efficient hydrolysis a chain length of at least five amino acids (in Positions P_1–P_4 and P_1', as defined by Schechter and Berger[8] are required[5,6]. It has been suggested that the large $K_{m(app)}$ of the small alanine substrates results from poor substrate binding at the binding site S_1 (ref. 5), the site in elastase which corresponds to the specificity cavity in α-chymotrypsin[9]. In elastase, the mouth of this hydrophobic pocket is partially covered by the side-chain of valine-216. Since, however, K_m values are not necessarily identical with or similar to true binding constants, especially with ester substrates, a better indication of binding can be obtained from the measurement of inhibition constants. The experiments described in this paper were undertaken in order to (a) characterize further the nature of the binding site of elastase and (b) find a competitive inhibitor with a sufficiently high binding constant to form a specific enzyme–inhibitor complex suitable for X-ray analysis[9,10].

* Present address: Department of Biochemistry, Kopernikus University, Torun, Poland.

Abbreviations: Ac, acetyl; Cbz, carbobenzoxy; Cbz-Gly-ONp, carbobenzoxyglycine *p*-nitrophenyl ester; Ac-Ala_3-OMe, acetylalanylalanylalanine methyl ester.

MATERIALS

Pancreatic elastase, free of tryptic and chymotryptic activity (< 0.1%) was prepared as described by Gertler and Hofmann[11]. Carbobenzoxyglycine *p*-nitrophenyl ester (Cbz-Gly-ONp) was from Sigma Chemical Corp. and acetylalanylalanylalanine methyl ester (Ac-Ala_3-OMe) from Cyclo Chemical Corp. Unsubstituted and *N*-carbobenzoxy(Cbz)-dipeptides of L-amino acids were from Cyclo Chemical Corp. or from Mann Research Laboratories, acetylated dipeptides were prepared by treating the free dipeptides in glacial acetic acid with an excess of acetic anhydride, removing the solvents in vacuo and recrystallizing the products from water.

METHODS

All enzyme assays were at 25 °C. In most experiments at pH 8.2 elastase activity was measured with Cbz-Gly-ONp as substrate as described by Rao and Hofmann[12] except that Cbz-Gly-ONp was dissolved in acetonitrile. Inhibition experiments were also carried out at pH 5.5 in 0.1 M sodium acetate buffer. Because the concentration of the nitrophenolate ion at this pH is very low the assays were carried out at 320 nm where the difference spectrum between Cbz-Gly-ONp and *p*-nitrophenol has a maximum. The assays were carried out as follows: to 2 ml buffer containing the inhibitor was added 10 μl of 10^{-2} M Cbz-Gly-ONp in acetonitrile. The reaction was started by the addition of 20 μl of elastase (10 mg/ml). In some cases the activity was also determined with $AcAla_3OMe$ as described by Gertler and Hofmann[1] but at pH 8.2.

Inhibitor constants

These were determined from Dixon plots[13]; an example is shown in Fig. 1.

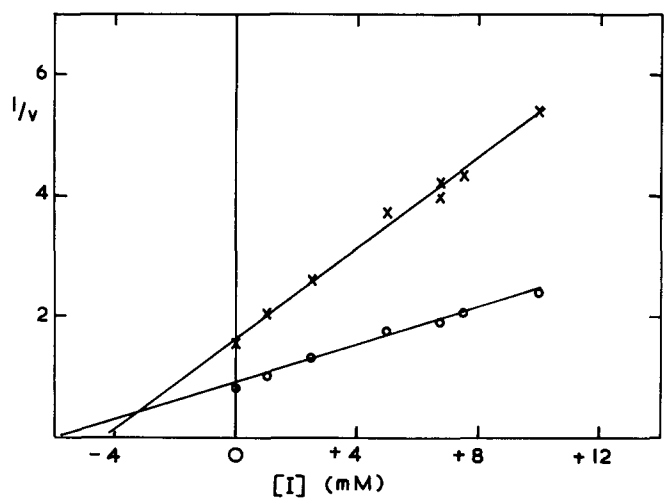

Fig. 1. Dixon plot of inhibition of pancreatic elastase by Cbz-Leu–Val. Conditions: 0.05 M Tris–HCl (pH 8.2), 0.5% acetonitrile; Cbz-Gly-ON_p concentration $1.25 \cdot 10^{-5}$ M (×—×) and $2.5 \cdot 10^{-5}$ M (○—○); elastase concentration, 25 μg/ml; the curves are regression lines calculated by a least square method.

A computer programma (kindly written for us by Mr K. Siren) was used to calculate K_i (and its standard deviation) from the intercept of two regression lines calculated by a least squares method. This programme written in Fortran IV is available from T.H. upon request. Where more than two substrate concentrations were used K_i was calculated from all possible pairs and the final value averaged.

Since this method of calculating K_i is applicable to both competitive and non-competitive inhibition, the nature of the inhibition was ascertained by plotting the calculated regression lines. Competitive inhibition was observed in all cases.

RESULTS AND DISCUSSION

A variety of dipeptides and two tripeptides were examined for their inhibitory action on pancreatic elastase at pH 8.2 and 5.5. The latter was used because this

TABLE I

INHIBITION CONSTANTS OF ELASTASE INHIBITORS

Values were determined with Cbz-Gly-ONp as substrate, with the exceptions given below*.

Peptide	*K_i at pH 8.2 (mM)*	*K_i at pH 5.5 (mM)*
Cbz-Val–Val	2.5 ± 0.2*	1.23 ± 0.19**
Cbz-Val–Ile	5.0 ± 0.6	3.5 ± 1.0
Cbz-Val–Leu	7.0 ± 1.1	3.1 ± 0.4
Cbz-Ala–Val	4.8 ± 1.0	n.d.
Cbz-Ile–Val	4.6 ± 0.6	2.4 ± 0.6
Cbz-Leu–Val	5.7 ± 0.8	2.5 ± 0.2
Cbz-Ala–Ala	10.0 ± 0.7*	4.05 ± 0.4**
Cbz-Gly–Ala	33.0 ± 2.0	
Ac-Val–Val	2.9 ± 0.1	2.5 ± 0.2
Ac-Ile–Val	4.8 ± 0.28	4.2 ± 0.3
Ac-Leu–Val	4.5 ± 0.1	4.2 ± 0.4
Ac-Ala–Ala	8.6 ± 0.1*	10.0 ± 0.9
Ala-Ala–Ala	9.0 ± 1.5	45.0 ± 5.1
Ac-Ala	32 ± 2.5	40 ± 2.5

* Values obtained with $AcAla_3OMe$ were in agreement with those from Cbz-Gly-ON_p and are included in the average.

** Similar values were obtained at pH 5.0.

study was undertaken partly in order to find a substrate-like inhibitor which would be suitable for the X-ray analysis of an enzyme–inhibitor complex*.

Inhibition constants for peptides which showed inhibition at concentrations up to approx. $2 \cdot 10^{-2}$ M are listed in Table I. Some of the peptides which did not inhibit at this concentration are listed in Table II. Lack of inhibition (less than 10%) at this concentration implies that the K_i is greater than 0.1 M.

The results show that N-substituted dipeptides composed of two aliphatic side chains are effective inhibitors with K_i in the millimolar range. The lowest values were

* pH 5.5 was used because the initial X-ray studies in this laboratory were carried out at this pH[10]. The structure of elastase was determined, however, at pH 5.0 by Shotton and Watson[9]. K_i values for two peptides were determined therefore at pH 5.0 as well and were the same as those at pH 5.5.

TABLE II

PEPTIDES WHICH DID NOT INHIBIT ($K_i > 0.1$ M)

Cbz-Ala–Gly
Cbz-Gly–Phe
Cbz-Phe–Ser
Cbz-Gly–Glu
Cbz-Glu–Gly
Leu–Gly–Gly
Ala–Ala-NH_2
and twelve dipeptides with free amino and carboxyl groups composed of a variety of amino acids (glycine, alanine, valine, leucine, threonine, histidine and phenylalanine)

obtained with the divaline derivatives, the highest with the dialanine derivatives and the trialanine with a free N-terminal.

There was no significant difference between the carbobenzoxy and the acetyl peptides, at pH 8.2. At pH 5.5 the K_i values of the carbobenzoxy peptides are lower than those of the acetyl peptides. The carbobenzoxy compounds also bind better at pH 5.5 than at pH 8.2. There is, however, no pH difference amongst the acetyl compounds. This latter fact agrees well with the findings of Shotton *et al.*[14] that there is a major binding site at the active centre of elastase to which substrate analogues bind efficiently both at pH 4.2 and at pH 8.5.

The higher K_i value for Ala–Ala–Ala at pH 5.5 compared to pH 8.2 can probably be attributed to the charge on the amino group.

Thompson and Blout[5] have measured inhibitor constants for N-acetylated alanine peptide amides. For Ac-Ala–Ala-NH_2 K_i was 50 mM (at pH 9.0, 37 °C) compared to our value of 8.6 mM (a value closer to that of 3 mM obtained for Ac-Ala–Ala–Ala-NH_2 (ref. 5)). There are two likely reasons for this difference. Either binding at the higher pH and temperature is less efficient or there is a contribution to the binding by the negative charge on the free carboxyl group of our inhibitors.

Although for efficient catalysis binding in several subsites is required[5,6] the weak inhibition by acetylalanine shows that some binding at least occurs with small compounds. The failure of dipeptides with free amino and carboxyl groups to inhibit can probably be ascribed to the presence of the positive charge on the amino group.

Binding site

It is tempting to assume that the N-substituted dipeptides bind in Subsites S_1 and S_2 with the carboxyl group pointing towards the catalytic site. This assumption is supported by the fact that the methyl ester of one of the inhibitors Ac-Ala–Ala is a good substrate; also, in its action on the chain of insulin elastase preferentially cleaves bonds with aliphatic amino acids in P_1[2] and short alkyl chains can bind in Subsite S_1 as Brown and Wold[15] have shown. However, X-ray analysis of an Ac-Ala–Ala complex of elastase shows that the alanine residues of this inhibitor preferentially occupy Subsites S_2 and S_3 (ref. 14) with only limited binding of the C-terminal alanine in Subsite S_1. This discrepancy can be explained by assuming that the constraints imposed by the incorporation of the enzyme molecules in the crystal lattice causes a shift in binding and that in solution preferential binding occurs with the C-terminal alanine in Subsite S_1. The following argument supports this. As Shotton *et al.*[14] show

the alanine side-chain in Subsite S_2 points into solution, while the side-chains of Residues P_3 and P_1 make contact with the enzyme surface. The failure of Cbz-Ala–Gly to inhibit suggests that the side chain of the C-terminal residue contributes significantly to the binding and thus is less likely to occupy Position S_2 than S_1 (or S_3). A similar conclusion can be drawn from the fact that Cbz-Ala–Val binds better than Cbz-Ala–Ala. Conversely, Cbz-Gly–Ala is a reasonably effective inhibitor (K_i 33 mM) when compared to Cbz-Ala–Ala (K_i 10 mM) while no inhibition was observed with Cbz-Ala–Gly ($K_i > 100$ mM).

It is of interest to note that Cbz-Phe–Ser and Cbz-Gly–Phe do not inhibit although Atlas *et al.*[6] have shown that when a phenylalanine residue in P_2 replaces an alanine K_m decreases. The significance of this observation is not clear at present.

In summary, our results show that a number of small peptide derivatives of aliphatic amino acids bind efficiently to the active site of elastase. In preliminary experiments it was shown that Ac-Val-Val like Ac-Ala–Ala [14] formed a complex with elastase crystals. Ac-Val–Val has two advantages over Ac-Ala–Ala, its K_i is considerably lower and the valyl side-chains provide more distinctive features of the electron density map. The other good inhibitor Cbz-Val–Val can probably not be used since crystals soaked in solutions of this peptide cracked and slowly disintegrated.

ACKNOWLEDGEMENTS

The authors are grateful to Mr K. Siren for writing the computer programme and to Miss E. Fischer, Mr G. Mains and Mr S. R. Jones for their help.

This work was supported by a Medical Research Council of Canada Grant (No. MT–1982)

REFERENCES

1 Gertler, A. and Hofmann, T. (1970) *Can. J. Biochem.* 48, 384–386
2 Sampath-Narayan, A. and Anwar, R. A. (1969) *Biochem. J.* 114, 11–17
3 Geneste, P. and Bender, M. L. (1969) *Proc. Natl. Acad. Sci. U.S.* 64, 683–689
4 Kaplan, H., Symonds, V. G., Dugas, H. and Whitaker, D. R. (1970) *Can. J. Biochem.* 48, 649–658
5 Thompson, R. C. and Blout, E. R. (1970) *Proc. Natl. Acad. Sci. U.S.* 67, 1734–1740
6 Atlas, D., Levit, S., Schechter, I. and Berger, A. (1970) *FEBS Lett.* 11, 281–283
7 Brot, F. E. and Bender, M. L. (1969) *J. Am. Chem. Soc.* 91, 7187–7191
8 Schechter, I. and Berger, A. (1967) *Biochem. Biophys. Res. Commun.* 27, 157–162
9 Shotton, D. M. and Watson, H. C. (1970) *Nature* 225, 811–816
10 Shotton, D. M., Hartley, B. S., Camerman, N., Hofmann, T., Nyburg, S. C. and Rao, L. (1968) *J. Mol. Biol.* 32, 155–157
11 Gertler, A. and Hofmann, T. (1967) *J. Biol. Chem.* 242, 2522–2527
12 Rao, L. and Hofmann, T. (1970) *Can. J. Biochem.* 48, 1249–1259
13 Dixon, M. (1953) *Biochem. J.* 55, 17c–171
14 Shotton, D. M., White, N. J. and Watson, H. C. (1971) *Cold Spring Harbor Symp. Quant. Biol.* 36, 91–105
15 Brown, W. E. and Wold, F. (1971) *Science* 174, 608–610