Biochimica et Biophysica Acta, 484 (1977) 103—108

BBA 68223

THE INHIBITION OF RAT LIVER CHROMATIN PROTEASE BY CONGENERS OF THE PHENYLBORONIC ACIDS

DONALD B. CARTER [a], DAVID A. ROSS [a], KHALID S. ISHAQ [b], GUADALUPE M. SUAREZ [b] and CHI-BOM CHAE [a]

[a] *Department of Biochemistry and* [b] *the School of Pharmacy, University of North Carolina, Chapel Hill, N.C. 27514 (U.S.A.)*

(Received March 2nd, 1977)

Summary

A group of arylalkylboronic acids were synthesized in order to investigate the inhibitory potential of these compounds for rat liver chromatin protease (EC 3.4.—.—). The effect of side chain length, side chain substitution and aromatic substitution on proteolytic activity in chromatin dissociated in salt and urea was assayed. It was determined that a side chain length two carbons long provided the greatest inhibitory effect with complete inhibition attainable at 20 mM concentration of phenylethylboronic acid. Aryl substitution in the ortho position proved to be the most potent structural change with complete inhibition attained by 1 mM concentration of *o*-methylphenylethylboronic acid. The binding of these two inhibitors proved to be reversible.

Introduction

Chromatin from a number of tissues [1—4] has been shown to contain a proteolytic activity which degrades histones as well as non-histone chromosomal proteins. Chae and Carter [5] have shown the existence of a protease (EC 3.4.—.—) in rat liver chromatin which is active in 2 M NaCl/5 M urea (pH 6—8) a commonly used solvent for dissociation of chromatin. A number of inhibitors of the rat liver protease have been described by Carter and Chae [6]. Congeners of the phenylboronic acids have been reported to inhibit chymotrypsin [7,8]. Several of the phenylboronic acid derivatives were tested and found effective in the inhibition of the chromosomal protease endogenous to rat liver chromatin dissociated in 2 M NaCl/5 M urea (pH 8). This report investigates the structure-inhibitory effectiveness of six arylalkylboronic acids in chromatin dissociated in 2 M NaCl/5 M urea and 5 M urea alone.

Methods

Arylalkylboronic acids. The syntheses of benzylboronic acid (I), phenylethylboronic acid (II), phenylpropylboronic acid (III), phenylisopropylboronic acid (IV), *p*-methylphenylethylboronic acid (V), and *o*-methylphenylethylboronic acid (VI) were performed essentially according to the procedure described by Bean and Johnson [9].

Micro thin-layer chromatography on silica gel G using different solvent systems (chloroform/ether (3:2, v/v), chloroform/methanol (9:1, v/v), benzene/ether (3:1 and 2:1, v/v)) was utilized to determine the purity of the synthesized phenylalkylboronic acids. Data from NMR was consistent with the structures of compounds (I—VI).

Elemental analyses for the phenylethyl-, phenylpropyl-, *p*-methylphenylethyl- and *o*-methylphenylethylboronic acids agreed with theoretical values within ±0.4%. The benzylboronic acid could not be analyzed since it undergoes slow decomposition giving off benzaldehyde [10]. We were unable to obtain the correct elemental analysis for the 2-phenylpropylboronic acid probably due to its instability. Both of these compounds, however, were recrystallized and their purity was determined by thin-layer chromatography before biological testing.

Chromatin isolation. Chromatin was isolated from purified rat liver nuclei by the method of Huang and Huang [11]. Briefly, nuclei were prepared by centrifugation through 2.3 M sucrose and subsequent washings in 1% Triton X-100 containing 0.25 M sucrose/3 mM $MgCl_2$/10 mM potassium phosphate (pH 6.5). The nuclei washed with 0.075 M NaCl/0.024 M EDTA were washed successively (twice each) with three volumes of 50 mM Tris (pH 7.9) and three volumes of 10 mM Tris (pH 7.9). The final pellet was suspended in cold water and sheared for 1 min at 20 V in a Sorvall Omnimixer Model 17105.

SDS gel electrophoresis. Sodium dodecyl sulfate (SDS) gel electrophoresis on 7.5% acrylamide gels was carried out as described before [4]. Modified Laemmli [12] gels were 10% polyarylamide/0.27% bisacrylamide/0.5 M urea/0.1% SDS/0.66 M Tris · HCl (pH 9.0). Tray buffers and stacking were as described by Laemmli.

Labeled substrate. A mixture of F1 and F2B (1:1) was labeled with tritium-labeled acetic anhydride as previously described [6].

Proteolytic assay with labeled substrate. Chromatin having a concentration of 2 mg/ml of DNA was diluted to 0.8 mg/ml of DNA in the desired reaction mixture. The ratio of [^{3}H]histones (F_1 + F_{2B}) added to the reaction mixture was 0.1 mg/mg of chromatin DNA. To determine the acid-soluble radioactive peptides released during an incubation, an equal volume of cold 50% trichloroacetic acid was added to the reaction mixture and after 15 min in ice, the acid-insoluble material was removed by centrifugation at 15 000 × *g*. An aliquot of the supernatant was counted for radioactivity.

Inhibition studies. Inhibition of chromatin-bound protease in salt and urea or urea alone was carried out in aqueous conditions for the inhibitors *p*-methylphenylethylboronic acid (stock solution 50 mM in 10 mM Tris, pH 8), benzylboronic acid (stock solution 0.1 M in 10 mM Tris, pH 8), *o*-methylphenylethylboronic acid (stock solution 50 mM in 10 mM Tris, pH 8), phenyl-

ethylboronic acid (stock solution 0.1 M in 10 mM Tris, pH 8.0) and phenylisopropylboronic acid (stock solution 50 mM in 10 mM Tris, pH 8). The inhibitor phenylpropylboronic acid was suspended in 10 mM Tris, pH 8.0 at a concentration of 0.1 M. The inhibitors were added to chromatin in water and incubated for 20 min at 25° C. The reacted chromatin was subsequently made 2 M NaCl/5 M urea/10 mM Tris (pH 8.0) by addition of 3.6 M NaCl/9 M urea/18 mM Tris (pH 8.0) and incubated 16 h prior to assay for inhibition.

Reversibility. To sheared rat liver chromatin *o*-methylphenylethylboronic acid or phenylethylboronic acid was added to give a final concentration after subsequent additions of 1 and 20 mM, respectively. Chromatin was preincubated for 1.5 h at room temperature before solid NaCl and urea was added to make 2 M NaCl/5 M urea/10 mM Tris, pH 7.9. The final concentration of chromatin was 0.4 mg/ml. The incubation mixture was then dialyzed against 10 volumes of 2 M NaCl/5 M urea/10 mM Tris, pH 7.9 at 4° C. Dialysis was carried out for at least 8 h before a change of diffusate. Aliquots were taken at each dialysis change and prepared for electrophoresis as previously described.

Results

Rat liver chromatin dissociated in 2 M NaCl/5 M urea/10 mM Tris (pH 8) can be protected from proteolysis by congeners of the phenylboronic acids. The phenylboronic acid derivatives which have been tested as inhibitors for the rat liver chromatin protease active in NaCl and urea are shown in Table I with the attendant relative capacity of each compound to inhibit proteolytic activity. Assay of inhibition was done by quantitating the amount of acid-soluble peptides released from ^{3}H-labeled (F_1 + F_{2B}) substrate after a 16 h incubation of chromatin in 2 M NaCl/5 M urea/10 mM Tris (pH 8). The most effective inhibitor is *o*-methylphenylethylboronic acid (VI) requiring at most 1 mM concentration for complete inhibition. Phenylethylboronic acid (II) is required at 20 mM concentration to reach complete inhibition as is benzylboronic acid (I). Further evidence of the inhibitory capability of II and VI was shown by sodium dodecyl sulfate polyacrylamide gel electrophoresis of chromatin.

TABLE I

PERCENT INHIBITION OF RAT LIVER CHROMATIN PROTEASE INCUBATED IN 2 M NaCl/5 M UREA WITH THE INDICATED CONCENTRATIONS OF CONGENERS OF PHENYLBORONIC ACIDS

Percent inhibition was determined by calculating the ratio of acid-soluble counts released in the test incubations to the acid-soluble counts released by unprotected chromatin during a co-incubation and subtracting the ratio from 1.0.

Boronic Acid	Concentration			
	1 mM	5 mM	10 mM	20 mM
Benzyl (I)	33	50	75	100
Phenylethyl (II)	60	80	90	100
Phenylpropyl (III)	0	38	62	
Phenylisopropyl (IV)	0	17	64	
p-Methylphenylethyl (V)	14	15	17	20
o-Methylphenylethyl (VI)	100	100	100	100

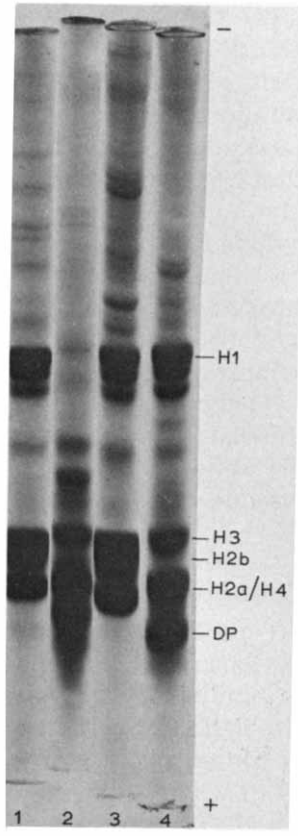

Fig. 1. Inhibition of chromatin protease by *o*-methylphenylethylboronic acid (VI). Chromatin incubated for 16 h at 4°C (1) in 2 M NaCl/5 M urea with VI; (2) in 5 M urea with VI; (3) in 1% sodium dodecyl sulfate (non-degraded control); (4) in 2 M NaCl/5 M urea without any inhibitor. Chromosomal proteins were fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis as described in Methods. Proteins migrated from top (—) to bottom (+). Histone bands are indicated in the figure, and DP represents the degradation product.

It is interesting that the phenylboronic acid derivatives show inhibitory activity in 2 M NaCl/5 M urea but not in 5 M urea or in NaCl alone. An example is shown in Fig. 1. It appears possible from this result that chromatin protease may show different substrate specificity under different environment.

The inhibition of chromatin protease in 2 M NaCl/5 M urea by phenylboronic acid derivatives is reversible. This was shown by subsequent dialysis of inhibited chromatin against 2 M NaCl/5 M urea/10 mM Tris · HCL (pH 7.9). The dialysed chromatins were prepared and run on SDS polyacrylamide gels as described.

Discussion

Various *n*-alkyl-, aryl- and arylalkylboronic acids have been prepared by several investigators as inhibitors of chymotrypsin [7,8,13,14]. Accordingly, we synthesized the arylalkylboronic acids (I—VI) to assess the inhibitory activity of these compounds on the chromatin-bound protease in rat liver. The

arylalkylboronic acids were prepared in order to determine the effect of chain length, (I—III), side chain substitution, (IV), and aromatic substitution, (V—VI), on proteolytic activity. Since it has been demonstrated that the proteolytic activity of rat liver, and other chromatins [6] resides in a protease sensitive to serine active site -directed reagents such as diisopropylfluorophosphate and phenylmethylsulfonylfluoride, it is anticipated that the phenylboronic acid derivatives may also competitively inhibit the chromatin protease. The results of this study indicate that the phenylboronic acid derivatives investigated are competitive inhibitors of rat liver chromatin protease activity in 2 M NaCl/5 M urea.

The effect of side chain length may be assessed by comparison of the capacity of compounds (I—III) to inhibit proteolytic activity at 5 mM concentration. From Table I a two carbon side chain provides the greatest inhibitory activity. The effect of side chain substitution, (IV), does not enhance the inhibitory potential of the parent compound, phenylethylboronic acid. The effect of aromatic substitution is rather dramatic. The methyl group substituted in the para position (V) decreases substantially the inhibitory potential of the parent compound (I). On the other hand aromatic substitution in the ortho position (VI) substantially increases the inhibitory potential of the parent compound (II).

That no inhibition is achieved by compound VI when chromatin is dissociated in 5 M urea alone suggests that the conformation of the protease has changed enough to prevent binding of the inhibitor. However, the protease remains very active in 5 M urea. When chromatin is incubated in 0.3 M NaCl, the inhibitors II or VI are not effective as inhibitors and only partial inhibition is achieved when chromatin is dissolved in 2 M NaCl. These results suggest that the enzyme is sensitive to the conditions of ionic strength or strength of denaturing solutions used to dissociate chromatin and that potential for inhibition by any of the compounds tested is determined by the solvent system used. The phenylboronic acid derivatives do not appear to react with urea.

We found that different histones are degraded at different ionic strength and in 5 M urea and 2 M NaCl/5 urea [5]. The variable inhibition of the proteolytic degradation of chromosomal proteins under different conditions suggest the possible changes of substrate specificty of the chromatin-bound protease due to changes of structure of the enzyme.

We are currently synthesizing derivatives of phenylethylboronic acid in order to probe the sensitivity of protease activity to ring substitution by various alkyl groups in the ortho, meta and para positions.

The fact that the chromatin-bound protease is inhibited by phenylboronic acids suggests that the enzyme is chymotrypsin-like. We have shown earlier that the protease activity is inhibited by carbobenzoxyphenylalanine chloromethylketone but not by tosyllysine chloromethylketone or tosylphenylalaninchloromethylketone [6]. These results show that the chromatin protease is at least not trypsin-like.

Acknowledgements

This research was supported by grant (GM 21846) from the Institute of General Medical Sciences, N.I.H., a grant from the Public Health Service (P.H.S.

1-RO1-CA20047-01), and a University of North Carolina School of Pharmacy General Research Support Grant.

References

1 Bartley, J. and Chalkley, R. (1970) J. Biol. Chem. 246, 4286—4292
2 Kurechi, T. and Toczko, K. (1972) Bull. Pol. Sci. 20, 543—546
3 Furlan, M. and Jericijo, M. (1967) Biochim. Biophys. Acta 147, 145—153
4 Garrels, J.I., Elgin, S.C.R. and Bonner, J. (1972) Biochem. Biophys. Res. Commun. 46, 545—551
5 Chae, C-B. and Carter, D.B. (1974) Biochem. Biophys. Res. Commun. 57, 740—745
6 Carter, D.B. and Chae, C-B. (1976) Biochemistry 15, 180—185
7 Koehler, K.A. and Lienhard, G.E. (1971) Biochemistry 10, 2477—2483
8 Rawn, J.D. and Lienhard, G.E. (1974) Biochemistry 13, 3124—3130
9 Bean, F.R. and Johnson, J.R. (1932) J. Am. Chem. Soc. 54, 4415—4418
10 Yabroff, D.L., Branch, G.E.K. and Bettman, B. (1934) J. Am. Chem. Soc. 56, 1850—1855
11 Huang, R.C.C. and Huang, P.C. (1969) J. Mol. Biol. 39, 365—378
12 Laemmli, U.K. (1970) Nature 227, 680—685
13 Antonov, V.K., Ivanina, T.V. and Ivanova, A.G. (1972) FEBS Lett. 20, 37—41
14 Philipp, M. and Bender, L. (1971) Proc. Natl. Acad. Sci. U.S. 68, 478—481