J. Enzyme Inhibition, 1991, Vol. 4, pp. 227–232
Reprints available directly from the publisher
Photocopying permitted by license only

INHIBITION OF CHYMOTRYPSIN AND PANCREATIC ELASTASE BY 4*H*-3, 1-BENZOXAZIN-4-ONES

U. NEUMANN†, J. STÜRZEBECHER, S. LEISTNER‡, and H. VIEWEG‡

Institute of Pharmacology and Toxicology, Medical Academy Erfurt, DDR-5010 Erfurt, G.D.R. and ‡Department of Pharmaceutical Chemistry, Section of Biosciences, Karl Marx University Leipzig, DDR-7010, G.D.R.

(Received 4 July 1990)

Eight 3,1-Benzoxazin-4-ones have been used to inactivate chymotrypsin and pancreatic elastase. Whereas 6,7-dimethoxy substitution only slightly decreased the acylation rate constant, the deacylation reaction was nearly unaffected. Bulky alkoxy groups in position 2 of the heterocyclic moiety were shown to increase enormously the acylation rate of chymotrypsin, but not that of elastase.

KEY WORDS: Chymotrypsin, Elastase, Acyl-enzyme, Benzoxazinone

INTRODUCTION

Serine proteases catalyze the hydrolysis of amide and ester bonds by transferring the acyl part of the substrate to a serine hydroxyl within the active site, resulting in an acyl-enzyme intermediate. The free enzyme is regenerated by hydrolysis by external water, thereby deacylating the acyl-enzyme.[1] Taking advantage of this mechanism, efficient serine protease inhibition can be achieved by compounds that form stable acyl-enzyme intermediates. A series of heterocyclic compounds including isatoic acid anhydride,[2] *N*-acyl saccharines[3] and 6-chloro-2-pyrones[4] were found to rapidly acylate the enzyme. 3,1-Benzoxazin-4-ones were first described as competitive inhibitors of chymotrypsin,[5] but it was demonstrated by Hedstrom[6] that they act due to their ability to form stabilized anthranoyl-enzymes. On the basis of this concept Krantz and coworkers[7–9] developed highly potent inactivators of human leukocyte elastase. Recently the X-ray structure of complexes of porcine pancreatic elastase and two valine-derived benzoxazinones has appeared.[10] It was demonstrated by Stein[11] that the "slow-binding" behaviour is due to the simultaneous action of a good (peptidyl-4-nitroanilide) and a poor (acylating inhibitor) substrate towards the enzyme.

In this paper we report structure-activity relationships of the inhibitory action of substituted 3,1-benzoxazin-4-ones towards chymotrypsin and pancreatic elastase.

MATERIALS AND METHODS

Materials

Chymotrypsin was purchased from Spofa (Prague, Czechoslovakia) and pancreatic

†Correspondence.

elastase (120 U/mg) from Serva (Heidelberg, F.R.G.). The enzyme concentrations were determined spectrophotometrically with $\varepsilon = 5 \times 10^4$ (chymotrypsin)[12] and 5.23×10^4 (pancreatic elastase).[13] The substrates Suc-Ala-Ala-Phe-pNA and Suc-$(Ala)_3$-pNA were kindly provided by Dr. S. Fittkau, Halle, G.D.R. The inhibitors were synthesized as described previously.[14,15] Enzymes were dissolved in 1 mmol/l HCl (stock solutions 0.01 mg/ml for chymotrypsin and 0.05 mg/ml for elastase) and stored at 4°C. The substrates were dissolved in the working buffer (0.05 mol/l Tris-HCl, pH 8.0) and inhibitor stock solutions were prepared in acetonitrile.

Inhibition Studies

Reaction progress was measured by monitoring the release of 4-nitroaniline at 405 nm and 25°C. For inhibiton of chymotrypsin a cuvette containing 1.68 ml buffer and 0.2 ml Suc-Ala-Ala-Phe-pNA (0.94 mmol/l) and 0.02 ml inhibitor solution was brought to thermal equilibrium in a Specol 221 spectrophotometer (Carl Zeiss Jena, G.D.R.). The reaction was started by application of 0.1 ml enzyme solution, and the increase in absorbance was followed over 10–30 min. In elastase studies, 0.2 ml of 0.68 mmol/l Suc-$(Ala)_3$-pNA was used.

Experiments for determination of competitive inhibition were carried out at three different substrate and two different inhibitor concentrations and K_i was determined graphically according to Dixon.[16]

Deacylation rates were estimated by following the liberation of hydrolysis products by UV-spectrophotometry during enzyme-catalyzed turnover of the benzoxaziones.

Data Analysis

Inhibition of serine proteases by benzoxazinones (as well as by other acylating

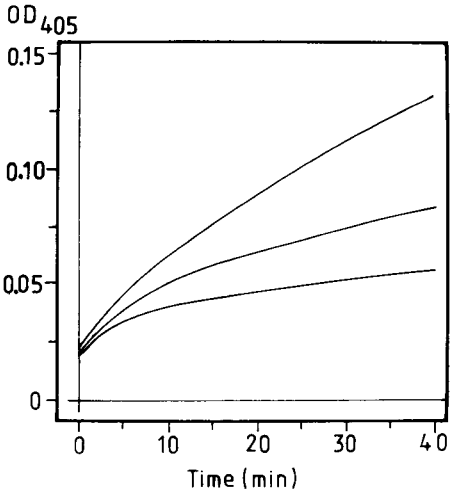

FIGURE 1 Progress curves of the inhibition of pancreatic elastase by 2-methoxy-6,7-dimethoxy benzoxazinone **3**. Benzoxazinone concentration 12.5 μM (bottom), 6.25 μM (middle) and 2.5 μM (top); Suc-Ala_z-pNA concentration 68 μM.

reagents) can be described by kinetic models developed for slow-binding inhibition.[17] In both cases, the active enzyme concentration is decreased in a steady-state time scale due to the reaction with the inhibitor until it reaches a constant value (Figure 1). Progress curves (30–70 absorbance-time pairs) were fitted to Equation (1) where $[P]$ is product concentration, v_o and v_s are initial and final velocity, k is pseudo-first-order rate constant for the approach of steady-state and d is displacement from zero at $t = 0$. Equation (1) holds true as long as the concentration of the acylating inhibitor as well as of the chromogenic substrate remains practically constant.

$$[P] = v_a t + (v_o - v_a)[1 - \exp(-kt)]/k + d \quad (1)$$

After fitting a set of progress curves obtained at different inhibitior concentrations to Equation (1), two cases can be distinguished: (a) v_o remains constant at different inhibitor concentrations and no inhibition is observed at the initial stage of the reaction and $K_i >> [I]$ or, (b) v_o decreases while $[I]$ is increased. The compound behaves like a competitive inhibitor before acylation occurs. In case (a), a linear dependence of k on inhibitor concentration is observed. Further analysis includes extrapolation of the pseudo-first-order rate constants obtained at different inhibitor concentrations to $[I] = 0$ according to Equation (2):

$$k = k_{on}[I]/(1 + [S]/K_M) + k_{off} \quad (2)$$

A plot of $[I]$ vs. k yields a straight line with the acylation rate constant $k_{on}/(1 + [S]/K_M)$ and the deacylation rate constant k_{off} as slope and intercept, respectively (Figure 2).

RESULTS

The compounds investigated were – with one exception – acylating inhibitors of

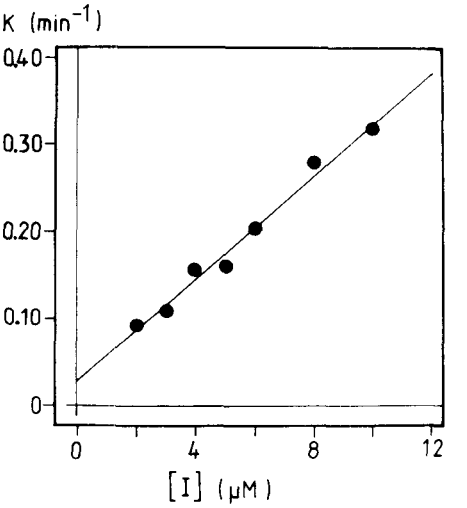

FIGURE 2 Kinetics of the inhibition of pancreatic elastase by **3**. The line was drawn from the best fit parameters $k_{on} = 387\ M^{-1}s^{-1}$ and $k_{off} = 0.001\ s^{-1}$ according to Equation (2).

TABLE I
Kinetic constants of inactivation of chymotrypsin by 3,1-benzoxazin-4-ones at pH 8.0 and 25°C

Compound	R_2	R_8	R_7	k_{on} ($lmol^{-1}s^{-1}$)	k_{off} (s^{-1})
1	H	OCH_3	OCH_3	260	0.0038
2	CH_3	OCH_3	OCH_3	52	0.013
3	OCH_3	OCH_3	OCH_3	1050	0.0012
4	OC_2H_5	OCH_3	OCH_3	12700	0.0019
5	$OCH_2CH(CH_3)_2$	OCH_3	OCH_3	69000	0.001
6	OC_2H_5	H	H	16000	0.0008
7	$OCH_2CH(CH_2)_2$	H	H	143000	0.0007
8	OC_2H_5	CH_3	H	106000	0.0015

TABLE II
Kinetic constants of inactivation of porcine pancreatic elastase by 3,1-benzoxazin-4-ones at pH 8.0 and 25°C

Compound	R_2	R_8	R_7	k_{on} ($lmol^{-1}s^{-1}$)	k_{off} (s^{-1})
1	H	OCH_3	OCH_3	slow acylation	
2	CH_3	OCH_3	OCH_3	no acylation	
3	OCH_3	OCH_3	OCH_3	387	0.001
4	OC_2H_5	OCH_3	OCH_3	4250	0.0011
5	$OCH_2CH(CH_3)_2$	OCH_3	OCH_3	1740	0.0022
6	OC_2H_5	H	H	4200	0.006
7	$OCH_2CH(CH_3)_2$	H	H	6500	0.0023
8	OC_2H_5	CH_3	H	not determined	

TABLE III
First-order rate constants of nonenzymatic breakdown of the 3,1-benzoaxazin-4-ones in borate buffer at pH 9.18 and 37°C

Compound	R_2	R_8	R_7	k (s^{-1})
1	H	OCH_3	OCH_3	0.0008
2	CH_3	OCH_3	OCH_3	0.0003
3	OCH_3	OCH_3	OCH_3	0.0007
4	OC_2H_5	OCH_3	OCH_3	0.0007
5	$OCH_2CH(CH_3)_2$	OCH_3	OCH_3	0.0005
6	OC_2H_5	H	H	0.0044
7	$OCH_2CH(CH_3)_2$	H	H	0.0041
8	OC_2H_5	CH_3	H	0.0031

chymotrypsin and pancreatic elastcase, as shown by the following experiments: Addition of the enzymes to a solution of the 3,1-benzoxazinone caused rapid formation of hydrolysis products with spectra identical with those of alkaline hydrolysis. Following the reaction at a fixed wave length a plateau was reached after a short period of time, the height of which was linearly dependent on the 3,1-benzoxazinone concentration and corresponded well to the expected absorbance difference calculated on the basis of the absorption coefficient. Thus, kinetic constants describing the acylation process (k_{on}) and the deacylation step (k_{off}) could be determined.

Compound **2** was an exception in that it did not acylate pancreatic elastase. It proved to be a weak competitive inhibitor with K_i = 0.4 mmol/l. Compound **1**

showed slow-binding behaviour. However, inhibition was too slow to maintain constant reaction conditions necessary for accurate estimation of rate constants.

Results for chymotrypsin and elastase are presented in Tables I and II; Table III gives values for alkaline hydrolysis of the 3,1-benzoxazinones for comparison.

DISCUSSION

The potential of a protease inhibitor working in a substrate-like manner by accumulation of an acyl-enzyme intermediate is mainly expressed as the velocity of acylation, which should be high and the velocity of deacylation, which should be as low as possible. Specific protease substrates show both high acylation and deacylation rates. The less specific nonpeptide substrates in general cannot activate the whole catalytic machinery of the enzyme so leading to higher energy barriers and to significantly reduced reaction rates for both acylation and deacylation. For example, the specific chymotrypsin substrate acetyl-tryptophan ethyl ester[1] has an acylation rate of $4.45 \times 10^5\,\mathrm{lmol^{-1}s^{-1}}$ and a deacylation rate constant of $46.5\,\mathrm{s^{-1}}$. In contrast, chymotrypsin is only slowly acylated by benzoylimidazole ($k_{on} = 970\,\mathrm{lmol^{-1}}$) or nitrophenylbenzoate ($k_{on} = 1440\,\mathrm{lmol^{-1}s^{-1}}$).[18,19] The two compounds give identical acyl enzymes deacylating with a rate constant of $2.3 \times 10^{-4}\,\mathrm{s^{-1}}$. Inactivators with acylation rate constants comparable to those of good substrates can be obtained by the use of activated lactones.[2,5]

Our results clearly indicate structure-activity relationships for the inactivation of chymotrypsin by 4*H*-3,1-benzoxazinones being very different from those of human leukocyte elastase.[7,9]

The 2-methyl substituted compound **2** is only slowly attacked by nucleophiles, as demonstrated by low rates of alkaline hydrolysis and acylation. The unusual high deacylation rate is assumed to be due to recyclisation to give intact benzoxazinone.[11] When the methyl group is substituted for alkoxy groups in position-2, no kinetic evidence for recyclisation is obtained.

Substitution in position-2 by bulky alkoxy groups enormously increase the acylation rate, while alkaline hydrolysis rates are not influenced (see compounds **2**, **3**, **4** in Tables I and III). The deacylation reaction is nearly unaffected. However, the rates are somewhat higher than those reported for benzoyl-chymotrypsin.[20] This might be indicative of acyl-enzyme structures with the bulky substituent in position-2 filling the specifity pocket of the chymotrypsin active site. The fact that 6-methyl or 6,7-dimethoxy substitution does not have any effect on the deacylation rate suggests that deacylation is mainly controlled by steric factors. The reaction of pancreatic elastase with 3,1-benzoxainones is more puzzling. It is characterized by relatively low acylation rates (lower than $10^4\,\mathrm{lmol^{-1}s^{-1}}$). Bulky alkoxy substituents in position-2 do not generally give more active compounds. It seems that there are some unfavourable interactions between benzoxazinone and enzyme if the alkoxy groups in position-6 and-7 and bulky alkoxy groups in position-2 are attached to the benzoxazinone (cf. compounds **4**, **5** and **7**). The 6,7-dimethoxy benzoxazinones **2**, **3** and **4** have low deacylation rates, but the increased value for 2-ethoxy benzoxazinone **6** cannot yet be explained. There is no evidence for electronic contribution of the *N*-acyl substituent in the deacylation of the anthranoyl-enzyme. Presumably, the differently substituted anthranoyl-enzymes formed by reaction of pancreatic elastase with substituted 3,1-benzoxazinones have different structures.

References

1. Bender, M.L. and Kezdy, F.J. (1964) *J. Am. Chem. Soc.*, **86,** 3714–3721.
2. Moorman, A.R. and Abeles, R.H. (1982) *J. Am. Chem. Soc.*, **104,** 6785–6786.
3. Zimmerman, M., Morman, H., Mulvey, D., Jones, H., Frankshun, R. and Ashe, B.M. (1980) *J. Biol. Chem.*, **255,** 9848–9851.
4. Gelb, M.M. and Abeles, R.H. (1984) *Biochemistry,* **23,** 6596–6604.
5. Teshima, T., Griffin, J.C. and Powers, J.C. (1982) *J. Biol. Chem.*, **257,** 5085–5091.
6. Hedstrom, L., Moorman, A.R., Dobbs, and Abeles, R.H. (1984) *Biochemistry,* **23,** 1753–1759.
7. Krantz, A., Tam, T.F. and Spencer, R.W. (1985) Eur. Pat. Appl. EP 147211.
8. Spencer, R.W., Copp, L., Bonaventura, B., Tam, T.F., Liak, T.J., Billedeau, R. and Krantz, A. (1986) *Biochem. Biophys. Res. Comm.*, **140,** 928–933.
9. Krantz, A., Spencer, R.W., Tam, T.F., Thomas, E. and Copp, L.J. (1987) *J. Med. Chem.*, **30,** 591–597.
10. Radhakrishnan, R., Presta, L.G., Meyer, E.F. and Wildonger, R.A. (1987) *J. Mol. Biol.*, **198,** 417–424.
11. Stein, R.L., Strimpler, A.M., Viscarello, B.R., Wildonger, R.A., Mauger, R.C. and Trainor D.A. (1987) *Biochemistry,* **26,** 4126–4130.
12. Wilcox, P.E. (1970) *Meth. Enzymol.*, **XIX,** 64–108.
13. Shotton, D. (1970) *Meth. Enzymol.*, **XIX,** 113–140.
14. Leistner, S., Simon, R., Wagner, G. and Stürzebecher J. (1987) *Pharmazie,* **42,** 694–695.
15. Synthesis of **1**: Vieweg, H. unpublished results. Synthesis of **2**: Simon, R. Doctoral Thesis, Leipzig, 1987.
16. Dixon, M. (1953) *Biochem. J.*, **55,** 170–171.
17. Morrison, J.F. (1982) *Trends Biochem. Sci.*, **7,** 102–105.
18. Kogan, R.L. and Fife, T.H. (1984) *Biochemistry,* **23,** 2983–2989.
19. Hubbard, C.D. and Kirsch, J.F. (1972) *Biochemistry,* **11,** 2483–2493.
20. Wang, C.C. and Shaw, E. (1972) *Archs. Biochem. Biophys.*, **150,** 259–269.