INHIBITION OF CATHEPSIN C BY PAPAIN INHIBITOR FROM CHICKEN EGG WHITE AND BY COMPLEX OF THIS INHIBITOR WITH CATHEPSIN B_1

H. KEILOVÁ and V. TOMÁŠEK

Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, 166 10 Prague 6

Received April 8th, 1974

We studied the effect of papain inhibitor, isolated from chicken egg white, on the enzymatic activity of cathepsin C and found that this enzyme is also inhibited. The type of the inhibition is pseudoirreversible and the inhibitor reacts even with cathepsin C whose thiol group of cysteine in the active center has been blocked by mercury. Cathepsin C, however, does not form a complex with the inhibitor in the absence of chloride ions. The enzymatic activity of cathepsin C is also inhibited by a complex of papain inhibitor with cathepsin B_1. This complex, however, does not inhibit the enzymatic activity of papain, ficin, or bromelain. These data show that there are two independent reactive sites in the inhibitor molecule, one for binding cathepsin C, the other one for binding cathepsin B_1, papain, ficin, and bromelain.

Avian egg white is a rich source of narurally occurring proteinase inhibitors. We found in our preceding study that dilute chicken egg white inhibits the enzymatic activity of cathepsin B_1 and cathepsin C (ref.[1]). A more detailed investigation showed that the inhibitor of both cathepsins is identical with the papain and ficin inhibitor described by Fossum and Whitaker[2]. These plant proteinases and cathepsin B_1 and C have one feature in common: they all belong to the group of thiol proteinases. It has been demonstrated[1,2], however, that the free SH-group of cysteine residue, essential for the catalytic function of these enzymes, is not necessary for the interaction with the inhibitor studied. Cathepsin B_1 considerably resembles in its molecular weight and enzymatic properties papain[3,4]; the finding that it is inhibited by the same naturally occurring inhibitor as papain shows that this similarity is even deeper. On the other hand, however, cathepsin C diametrically differs from cathepsin B_1 and the plant proteinases mentioned above. Cathepsin B_1 is an endopeptidase of molecular weight of 24000; cathepsin C is an exopeptidase, a dipeptidyl peptide hydrolase, with complicated subunit structure[5,6].

This study was intended to provide more detailed data on the interaction of cathepsin C with this inhibitor. Another aim was to determine whether during this interaction both cathepsins, B_1 and C, react with the same site of the inhibitor molecule or whether there are two independent reactive sites on the surface of the inhibitor molecule.

EXPERIMENTAL

Cathepsin B_1 (EC 3.4.4.-) was isolated from bovine spleen as described earlier[7]. Cathepsin C (EC 3.4.4.9.) was prepared from bovine spleen. The final purification of the enzyme was achieved by affinity chromatography on a mercurial Sepharose column. The lyophilized preparation was stored in inactive mercuri form[8]. Papain (EC 3.4.4.10), ficin (EC 3.4.4.12) and bromelain (EC 3.4.4.—) were commercial products of Calbiochem, U.S.A. Papain inhibitor* from chicken egg white was isolated as described earlier[1] and stored as a frozen solution at −20°C. The extinction coefficient $E^{1\%}_{280}$ of the solution was 8·241 (ref.[1]).

Preparation of complex of cathepsin B_1 *with* PI. Cathepsin B_1 (4 mg) was dissolved in 4 ml of 0.1M phosphate buffer at pH 6·0. This solution was mixed with a solution of PI (2·5 mg in 4 ml of 0·05M acetate buffer at pH 5). The reaction mixture was set aside for 5 min at room temperature. The excess of the inhibitor was separated from the complex by gel chromatography of the reaction mixture on a column of Sephadex G-75 (2·3 × 67 cm), equilibrated with 0·1M-KCl. Fractions of 3 ml/8 min were collected. The absorbance of the effluent was measured at 230 nm. A 100 µl aliquot of each fraction was taken for the determination of the activity of free cathepsin B_1, another 100 µl aliquot for the inhibition of cathepsin C. The high molecular weight fraction, which corresponded in its elution volume to the complex of cathepsin B_1 with PI and which inhibited cathepsin C, was concentrated by ultrafiltration through a UM-10 Amicon membrane. The concentrated solution of the complex was kept frozen at −20°C.

Preparation of complex of cathepsin C *with* PI. Lyophilized cathepsin C in mercuri form (5 mg) was added to a solution of 2·5 mg of PI in 4 ml of 0·05M acetate buffer at pH 5. (The buffer was 10 mM in KCl). After cathepsin C had dissolved, the solution was allowed to stand 10 min and then passed over a Sephadex G-200 column equilibrated with 0·1M-KCl. A 2·5 × 40 cm column and the upward flow arrangement were used. The flow rate was 2·5 ml/10 min. The hold-up volume was determined as the elution volume of Blue Dextran 2000 (Pharmacia Fine Chemicals). The column was calibrated by determination of elution volumes of hog immunoglobulin, ovalbumin, and bovine serum albumin (Institute of Sera and Vaccines, Prague). The absorbance at 230 nm was measured of fractions obtained by chromatography of the cathepsin C-PI complex. An aliquot of 100 µl was taken for the determination of enzymatic activity of cathepsin C, another 100 µl aliquot for the determination of inhibition of enzymatic activity of cathepsin C.

The enzymatic activity of cathepsin B_1, papain, ficin, and bromelain was determined in terms of hydrolysis of N_α-benzoyl-D,L-arginine-*p*-nitroanilide hydrochloride as substrate using the procedure described earlier[1]. The enzymatic activity of cathepsin C was determined in terms of liberation of *p*-nitroaniline from the substrate glycyl-L-phenylalanine-*p*-nitroanilide as described in the preceding paper[1]. The quantity of the enzyme which cleaved 1 µmol of the substrate in 1 min under the conditions described was taken for one unit (U).

Determination of inhibitory activity. The solution (100 µl) of the enzyme (containing 4 mU of enzyme) was mixed with 100 µl of the solution of PI or of the complex of cathepsin B_1-PI. The enzymatic activity was determined as described above after 5 min of preincubation at room temperature. The inhibition degree is expressed in per cent with respect to the control experiment carried out in the absence of the inhibitor.

The type of the inhibition of cathepsin C by PI was determined by the measurement of enzymatic activity at various concentrations of the enzyme and of the inhibitor[9].

Sedimentation constant of cathepsin C was determined in 0·1M phosphate buffer at pH 6·5 in a Spinco, model E centrifuge.

* Designated PI in the text.

RESULTS AND DISCUSSION

PI is the first naturally occuring inhibitor of cathepsin C ever reported. It was therefore necessary first of all to determine the type of inhibition. The plot of the dependence of the intensity of cleavage of glycyl-L-phenylalanine-*p*-nitroanilide by varying quantities of cathepsin C in the presence of different quantities of the inhibitor (Fig. 1) shows that all curves originate in one point. They diverge at low enzyme concentrations and are more or less parallel at higher enzyme concentrations. Such a profile of the dependence is typical of the so-called tight-binding inhibitors and of pseudoirreversible type of inhibition of enzymatic activity. The type of inhibition which occurs during the interaction of cathepsin C with PI is therefore the same as that involved in the interaction of this inhibitor with cathepsin B_1 (ref.[1]).

Cathepsin C requires for its full enzymatic activity both a free SH-group of the cysteine residue in the catalytic site and the presence of halogenide ions in the reaction medium[6]. We have shown in our preceding study[1] that cathepsin B_1 is – similarly to papain – capable of binding the inhibitor even if present in enzymatic form which is reversibly inactive, *i.e.* after its sulfhydryl group has been blocked by mercury. We found in this study that also cathepsin C in inactive mercuri form yet in the pre-

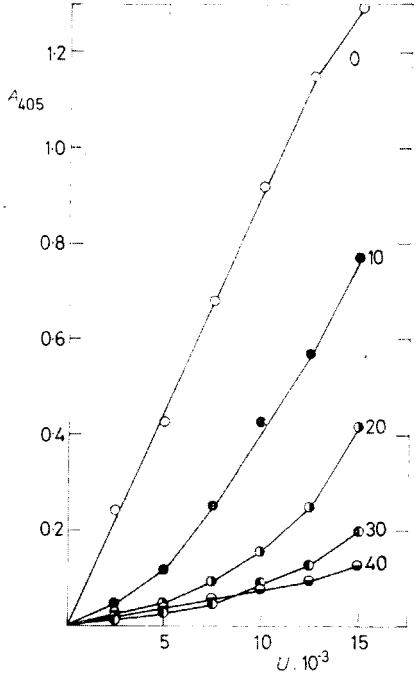

FIG. 1

Cleavage of Gly-L-Phe-*p*-nitroanilide by Various Concentrations of Cathepsin C in Presence of Different Concentrations of PI

A_{405} enzymatic activity, U quantity of cathepsin C added (in units, see text). The numerals on the individual curves stand for the quantity of solution of PI in μl (A_{280} = = 0·140). 0-curve, control in the absence of inhibitor. Time of hydrolysis 10 min at 40 C.

sence of chloride ions can form a complex with PI. This shows that the free SH-group in the catalytic site is not essential for the interaction with this inhibitor, as in the case of other thiol proteinases.

The course of gel chromatography on Sephadex G-200 of free cathepsin C is shown in Fig. 2*a*, of a mixture of cathepsin C and PI in Fig. 2*b*. Gel chromatography of mixture of cathepsin C and PI under identical conditions as in Fig. 2*b* yet in the absence of chloride ions in the reaction medium and column eluent, is shown in Fig. 2*c*. Cathepsin C in mercuri form was used in all these cases. The absorbance curve of cathepsin C during the chromatography shown in Fig. 2*a* corresponds to the enzymatic activity curve. The chromatography of a mixture of cathepsin C and PI in the presence of chloride ions (Fig. 2*b*) leads to separation of the excess of low molecular weight inhibitor and to a significant decrease of enzymatic activity of cathepsin C in the high molecular weight fraction. We assume that the interaction of cathepsin C, whose SH-group had been blocked by mercury, with PI took place and that the high molecular weight fraction, which almost lost the activity of cathepsin C, corresponds to a complex of mercuri–cathepsin C with PI. The fact that we still find residual cathepsin C activity in the high molecular weight fraction can be caused by the obviously complicated quaternary structure of the enzyme itself and perhaps also by

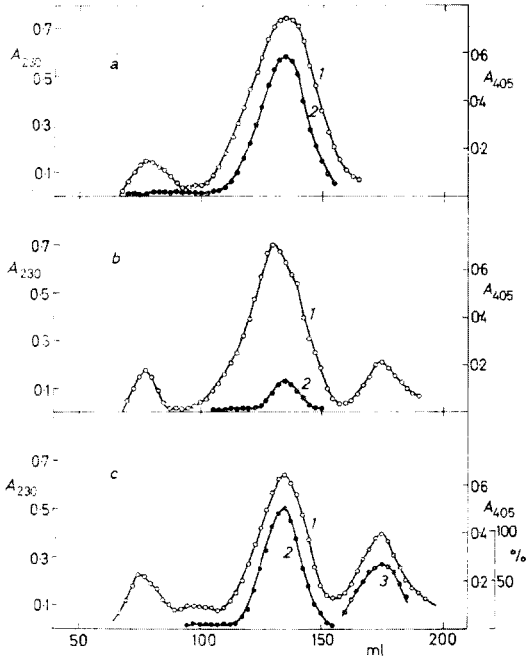

FIG. 2

Gel Filtration of Cathepsin C and its Complex with PI on Sephadex G-200

a Mercuri cathepsin C, *b* mercuri cathepsin C and PI in the presence of chloride ions, *c* mercuri cathepsin C and PI in the absence of chloride ions, ml elution volume, *1* absorbance at 230 nm, *2* enzymatic activity (A_{405nm}) of cathepsin C, *3* inhibition of enzymatic activity of cathepsin C, expressed in %. See text for details.

the type of inhibition. If the experiment is carried under identical conditions yet in the absence of chloride ions in the reaction mixture and in the column eluent, fully active cathepsin is separated from the free inhibitor and clearly there is no interaction between the two components and no formation of the enzyme–inhibitor complex (Fig. 2*c*). Chloride ions obviously play an important role in arrangement of the binding site, not only for the substrate but also for this inhibitor.

The molecular weight of cathepsin C was reported to be 200000 and its sedimentation coefficient 9·0S; hence, the molecule must consist of 8 subunits of molecular weight of 24000. It is known, however, that the molecule dissociates even in 1M guanidine hydrochloride to tetramers of sedimentation coeficient 6·0S which should correspond to a molecular weight of 100000 (ref.[5]). Our preparation of cathepsin C, used in all our experiments, shows a sedimentation coefficient of 6·1S in the ultracentrifuge; its elution volume on chromatography on Sephadex G-200 corresponds to a molecular weight of 100000 (Fig. 3). Hence, our preparation is obviously a tetramer.

When the complex of cathepsin C with PI is chromatographed on Sephadex G-200 (Fig. 2*b*), a slight shift of maximum of the protein peak toward low elution volumes with respect to the maximum of enzymatic activity of cathepsin C takes place. On

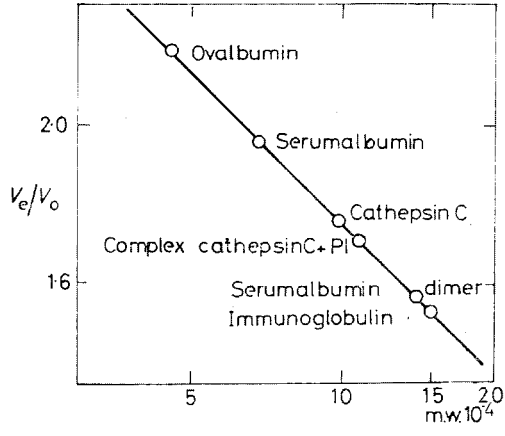

FIG. 3

Dependence of Elution Volumes on Molecular Weight in Gel Filtration on Sephadex G-200

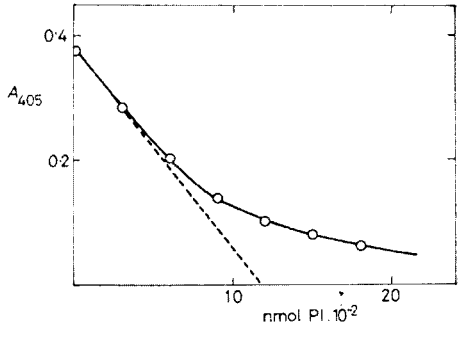

FIG. 4

Inhibition of Enzymatic Activity of Cathepsin C by Various Concentrations of PI

A_{405} enzymatic activity. The quantity of cathepsin C hydrolyzed was $10 . 10^{-2}$ nmol. The cleavage was allowed to proceed 10 min at 40°C.

condition that the molecular weight of the cathepsin C tetramer is 100000 and the molecular weight of the inhibitor 13700, this slight shift of the elution volume toward lower values permits us to eliminate the possibility that PI reacts with all four subunits of cathepsin C in the process of complex formation. If this were the case, the molecular weight of the cathepsin C-PI complex would be c. 152000 and the elution volume of the complex would then correspond approximately to the elution volume of immunoglobulin.

The extrapolation of the results of inhibition of cathepsin C by varying quantities of the inhibitor indicates that the cathepsin C tetramer reacts with PI at a ratio of 1 : 1 (Fig. 4). This conclusion permits us to interpret also the slight shift in the elution volume of the complex on gel chromatography.

Since cathepsin C and B_1 are two enzymes which considerably differ in the structure of their molecules and in enzymatic properties, we were interested in finding whether they interact with PI at a common site of the surface of the PI molecule or whether two different reactive sites are involved. We therefore prepared the complex of cathepsin B_1 with PI, separated PI which had not reacted by gel filtration on Sephadex G-75, and inhibited the enzymatic activity of cathepsin C by this pure complex. Since the inhibitory power of the complex, similarly to the free inhibitor, is destroyed by lyophilization, we stored the complex solution frozen at $-20°C$. As shown in Fig. 5, cathepsin C only is inhibited by the cathepsin B_1–PI complex; none of the plant proteinases is inhibited. This means that there is a common binding site for papain, ficin, bromelain, and cathepsin B_1 on the molecule of PI; this finding indicates similarities in structure of thiol proteinases isolated from plants on the one hand and from animal cells on the other. Cathepsin C, which has entirely different enzymatic characteristics, binds to another site of the PI molecule. The molecule

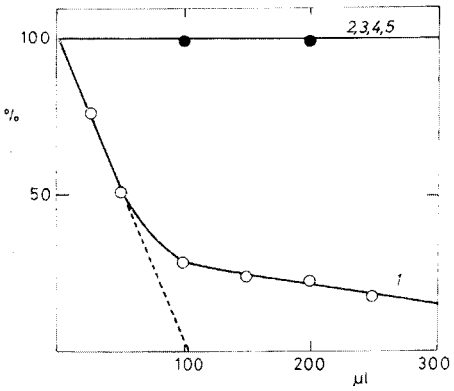

FIG. 5

Effect of Complex of Cathepsin B_1 and PI on Enzymatic Activity of Cathepsin C, Cathepsin B_1, Papain, Ficin, and Bromelain

% Enzymatic activity in % of original activity, µl quantity of cathepsin B_1-PI complex ($A_{280} = 0{\cdot}265$) added, *1* cathepsin C (Gly-L-Phe-*p*-nitroanilide as substrate), *2* cathepsin B_1, *3* papain, *4* ficin, *5* bromelain (N-α-benzoyl-D,L-arginine-*p*-nitroanilide as substrate). Enzymatic hydrolysis 10 min at 40°C.

of cathepsin B_1, attached to the inhibitor, obviously does not prevent, even sterically, the second reactive site on the PI molecule from interaction with cathepsin C.

The existence of independent reactive sites for various enzymes has been described with a great number of inhibitors. Rhodes and coworkers[10] proposed to call this type of inhibitors multiheaded inhibitors. These authors found independent nonoverlapping reactive sites on avian ovomucoids for trypsin and chymotrypsin. Similarly, chicken ovoinhibitor is capable to inhibit independently trypsin and chymotrypsin[11]. Likewise, the molecule of the proteinase inhibitor from dog submandibular glands is double headed. One reactive site binds chymotrypsin or subtilisin, the other one independently trypsin[12]. Proteinase inhibitor AA from soy bean or the inhibitor from Lima bean are also double headed[13,14] and bear independent reactive sites for trypsin and chymotrypsin.

Our results lead us to conclude that another inhibitor which falls into the group of various so-called multiheaded inhibitors is the papain inhibitor from chicken egg white. There are two independent nonoverlapping reactive sites on its molecule, one for the binding of thiol proteinases from plants and of cathepsin B_1, the other one for the binding of cathepsin C.

REFERENCES

1. Keilová H., Tomášek V.: Biochim. Biophys. Acta *334*, 179 (1974).
2. Fossum K., Whitaker J.: Arch. Biochem. Biophys. *125*, 367 (1968).
3. Keilová H., Keil B.: FEBS Letters *4*, 295 (1969).
4. Keilová H., Turková J.: FEBS Letters *11*, 287 (1970).
5. Metrione R. M., Okuda Y., Faircoulgh G. T.: Biochemistry *9*, 2427 (1970).
6. McDonald J. K., Callahan P. X., Ellis S., Smith R. E. in the book: *Tissue Proteinases* (A. J. Barrett, T. J. Dingle, Eds,) p. 69. North Holland Publishing Co, Amsterdam 1971.
7. Keilová H., Tomášek V.: FEBS Lett. *29*, 335 (1973).
8. Keilová H., Tomášek V.: Unpublished results.
9. Morrison J. F.: Biochim. Biophys. Acta *185*, 269 (1969).
10. Rhodes M. B., Bennett N., Fenney R. E.: J. Biol. Chem. *235*, 1686 (1960).
11. Tomimatsu Y., Clary J. J., Bartulovich J. J.: Arch. Biochem. Biophys. *115*, 536 (1966).
12. Fritz H., Jaumann E., Meister R., Pasquay P., Hochstrasser K., Fink E. in the book: *Proceeding of the International Research Conference on Proteinase Inhibitors* (H. Fritz, H. Tschesche, Eds) p. 257 Walter de Gruyter, Berlin 1971.
13. Birk Y., Gertler A.: ref. 12 p. 142 (1971).
14. Stevens F. C.: ref. 12., p. 149 (1971).

Translated by V. Kostka.