Inhibition of human liver cathepsin L by α_2 cysteine-proteinase inhibitor and the low- M_r cysteine proteinase inhibitor from human serum

Maurice PAGANO,* Frédéric ESNARD,† Robert ENGLER* and Francis GAUTHIER††

*Laboratoire des Protéines de la Réaction Inflammatoire, U.E.R. Biomédicale des Saints Pères, 45 Rue des Saints Pères, F-75270 Paris Cedex 06, France, and †Laboratoire de Biochimie, Faculté de Médecine, Université François Rabelais, 2 bis Boulevard Tonnellé, F-37032 Tours Cedex, France

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The inhibition of human liver cathepsin L by two specific proteinase inhibitors present in human serum, namely α_2 cysteine-proteinase inhibitor and the low- M_r cysteine-proteinase inhibitor, was studied. Kinetic parameters, including inhibition constants (K_i) and rate constants for association and dissociation (k_{+1} and k_{-1}), were determined. The values found are consistent with a possible physiological function of these inhibitors to control cathepsin L activity. Furthermore, a transfer of active proteinase from the complex with either cysteine-proteinase inhibitor species to α_2 -macroglobulin was demonstrated *in vitro*. Given the rate of dissociation of both cathepsin-L-cysteine-proteinase inhibitor complexes, a function of transitory inhibitor can therefore be hypothesized for these proteins and might then provide an explanation of the clearance of lysosomal proteinases.

Specific inhibitors of cysteine proteinases have been demonstrated in the plasma of several mammalian species (Järvinen, 1976; Minakata *et al.*, 1982; Christopher *et al.*, 1982; Iwata *et al.*, 1982). In human, two kinds of cysteine proteinase inhibitor (CPI) have been identified, one of large M_r (α_2 CPI, M_r 90000), which strongly inhibits papain, ficin and also lysosomal cysteine proteinases (Sasaki *et al.*, 1977; Ryley, 1979; Järvinen, 1979), and another of low M_r (<20000) (Lenney *et al.*, 1982), which is a potent inhibitor of cathepsin H (Gauthier *et al.*, 1983). The biological function of the main lysosomal cysteine proteinases, i.e. cathepsins, B, H and L, as well as the regulation of their activity, is not yet fully understood. Cathepsin L, however, has greater activity against protein substrates than other lysosomal cysteine proteinases and is implicated in collagen degradation (Kirschke *et al.*, 1982). This enzyme is inhibited by the bacterial inhibitor leupeptin (Barrett &

Kirschke, 1981) and also by the human serum inhibitor α_2 CPI (Pagano & Engler, 1982). Kinetic parameters of the interaction between this latter inhibitor and cathepsin L have not yet been determined, however. Neither is it known whether this enzyme is inhibited by the low- M_r CPI, so that the physiological effectiveness of these inhibitors for the modulation of cathepsin L activity remains to be defined.

In the present paper we report on the determination of the kinetic parameters of the interaction between human lysosomal cathepsin L and both the low- M_r CPI and α_2 CPI. The transfer of CPI-bound cathepsin L on to free α_2 M was also investigated, and the potentiality for such a mechanism to occur *in vivo* is discussed.

Materials and methods

Materials

Pleural fluid was obtained from patients of the Department of Medicine, the Cognacq-Jay Foundation, Paris, France. Z-Phe-Arg-NMec and compound E 64 were supplied by the Peptide Research Foundation (Osaka, Japan). CNBr-activated Sepharose, Blue Sepharose F 3GA, Sephacryl S-200 (superfine grade) and Sephadex G-200 were purchased from Pharmacia (Uppsala, Sweden). M Partigen plates for radial immunodiffu-

Abbreviations used: α_2 CPI, α_2 cysteine-proteinase inhibitor; low- M_r CPI, small- M_r cysteine-proteinase inhibitor; α_2 M, α_2 -macroglobulin; Arg-NNap, arginine 2-naphthylamide; Bz-Arg-NNap, *N*-benzoyl-DL-arginine 2-naphthylamide; Z-Phe-Arg-NMec, benzyloxycarbonyl-L-phenylalanyl-L-arginine 4-methyl-7-coumaryl-amide; compound E 64, L-3-carboxy-*trans*-2,3-epoxypropionyl-leucylamido-(4-guanidino)butane.

† To whom correspondence should be addressed.

sion were from Behring Diagnostics (Marburg, West Germany). Anti- α_2 CPI serum was raised in rabbits as described previously (Engler *et al.*, 1977) and was directed against both α_1 CPI and α_2 CPI components.

Papain, azocasein, Bz-Arg-NNap, ammonium sulphamate, NaNO_2 , *N*-(1-naphthyl)ethylenediamine and β -naphthylamine were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other reagents were of analytical grade.

Fluorescence measurements were carried out with a Perkin-Elmer 204A spectrofluorimeter ($\lambda_{\text{exc.}}$ 370 nm, $\lambda_{\text{em.}}$ 460 nm).

Purification of cysteine-proteinase inhibitors

Purification of α_2 CPI was carried out with pleural fluid as a starting material. A 20 ml volume of pleural fluid was fractionated with $(\text{NH}_4)_2\text{SO}_4$ and the precipitate formed between 30 and 55% of saturation was retained. The precipitate was extensively dialysed against 50 mM-Tris/HCl buffer, pH 7.4. This material was applied to a column (1.5 cm $\times$ 85 cm) of Blue Sepharose equilibrated in the same buffer. α_2 CPI was eluted with buffer containing 0.1 M-KCl and was located by its immunological and papain-inhibiting properties as described previously (Pagano & Engler, 1982). The other, probably dimeric, α_1 CPI component previously reported (Ryley, 1979; Järvinen, 1979; Pagano & Engler, 1982) was eluted with the equilibrium buffer. Though still able to inhibit papain, this component mainly appears during the course of purification and was not taken into account during the present study. α_2 CPI-containing fractions were concentrated and loaded on to a column (3 cm $\times$ 80 cm) of Sephacryl S-200 (superfine grade) equilibrated in 0.2 M-Tris/HCl buffer, pH 7.2, containing 0.5 M-NaCl. Papain-inhibiting fractions were pooled, concentrated and checked for their purity by electrofocusing. A single band of pI 4.85 was obtained, as shown previously (Pagano & Engler, 1982). Finally, the material was stored at -20°C until use.

Purification of the low- M_r CPI was carried out as described previously (Gauthier *et al.*, 1983).

Purification of α_2 M was carried out by the procedure of Virca *et al.* (1978) except for the final step, where Sephacryl S-200 was used as described above.

Purification of lysosomal cysteine proteinases

Cathepsin H and cathepsin L were isolated from human liver as previously described (Gauthier *et al.*, 1983; Pagano & Engler, 1982).

Immunochemical determination of α_2 CPI and α_2 M

The α CPI content of sera of known haptoglobin concentration (0.5–6.5 g/l) as well as of pleural

fluids was determined by electroimmunoassay (Laurell, 1972) with rabbit anti- α CPI serum. The concentration of α_2 M in sera and pleural fluids was determined by radial immunodiffusion (Mancini *et al.*, 1965).

Active-site titration of proteinases and inhibitors

Commercial papain before and after repurification on a thiol-Sepharose column (Brocklehurst *et al.*, 1973) as well as cathepsin L and cathepsin H were titrated with the irreversible inhibitor compound E 64 as described by Barrett *et al.* (1982) and with Bz-Arg-NNap, Z-Phe-Arg-NMec and Arg-NNap respectively as substrates.

Titration of papain was then used for the titration of α_2 CPI on the basis of a 1:1 molar interaction and also for the titration of α_2 M on the basis of a 2:1 enzyme/inhibitor stoichiometric ratio (Howell *et al.*, 1983). For both experiments papain (0.06 μM) was incubated for 10 min at 25°C with increasing amounts of either α_2 CPI or α_2 M in 0.1 M-sodium phosphate buffer, pH 6.8, containing 2 mM-EDTA and 1 mM-dithiothreitol (final volume 400 μl). A 10 μl volume of 22 mM-Bz-Arg-NNap was then added to the mixture containing α_2 CPI, and this mixture was then incubated at 40°C for another 10 min. The reaction was stopped with 500 μl of 40% (w/v) trichloroacetic acid, and the liberated 2-naphthylamine was assayed colorimetrically at 560 nm as described previously (Pagano *et al.*, 1982). Identical α_2 CPI concentrations were found whatever the papain solution used for the assay. This demonstrates that no significant binding of inactive papain had occurred under these experimental conditions.

Papain/ α_2 M mixtures were assayed with azocasein as a substrate. A 20 μl volume of 5% (w/v) azocasein was added to reaction mixtures, which were then incubated at 40°C for 30 min. Samples were then processed as described previously (Pagano *et al.*, 1982).

Titration of the low- M_r CPI was made as previously described, with cathepsin H (Gauthier *et al.*, 1983).

All proteinase and inhibitor concentrations given below in the present paper refer to active concentrations.

Interaction between cathepsin L and α_2 CPI or low- M_r CPI

Identical experiments were carried out for both inhibitors. Titrations were performed as follows. Cathepsin L (1 μM) was incubated for 30 min at 25°C with α_2 CPI concentrations varying from 0.23 to 1.38 μM or low- M_r CPI concentrations varying from 0.13 to 2.54 μM in phosphate buffer, pH 6.0, containing 2 mM-EDTA and 1 mM-dithiothreitol (final volume 50 μl) before 5 μl of 0.35 mM-Z-Phe-

Arg-NMec was added. The reaction was stopped after 15 min at 40°C by addition of 1 ml of 0.1 M-sodium monochloroacetate. A 1:100 dilution of samples in 0.1% Brij 35 was made before recording of the fluorescence of the free aminoethyl-coumarin. A reaction blank was made by adding the stopping reagent before the enzyme.

K_i determinations were carried out according to the same procedure but after lowering enzyme and inhibitor concentrations to obtain a non-linearity of dose-response curves. Thus cathepsin L was used at a concentration of 0.05 μM to react with $\alpha_2\text{CPI}$ varying from 0.011 to 0.168 μM and at a concentration of 0.1 μM to react with the low- M_r CPI at 0.18 to 1.1 μM . These experiments were carried out at four different substrate concentrations, namely 3.5, 7, 21 and 35 μM (final concentrations), and the results were analysed by the procedure of Krupka & Laidler (1959).

Determination of association rate constants (k_{+1})

k_{+1} values were determined by monitoring the time-dependence of the association under second-order conditions. Equimolar amounts of enzyme and inhibitor (0.05 μM in the final volume for $\alpha_2\text{CPI}$ and 0.2 μM for the low- M_r CPI) were mixed in a total volume of 100 μl of the same buffer as above at 25°C. Portions (10 μl) were removed at 0.1, 3, 5.8 and 10 min and assayed for their residual activity as described above, with 1 μl of 70 μM -Z-Phe-Arg-NMec.

Determination of dissociation rate constants (k_{-1})

Conditions for maximal association between cathepsin L and either inhibitor were achieved before the reaction was shifted towards dissociation by adding either $\alpha_2\text{M}$, which forms stable and enzymically active complexes with free enzyme, or excess of substrate, which also binds all the free enzyme (Bieth, 1980). Equimolar amounts (1 μM) of either inhibitor and cathepsin L were added to a slight excess (1.3 μM) of $\alpha_2\text{M}$ in the same buffer as before, and the mixture was divided into 100 μl portions. At various incubation times, 5 μl of 0.5 mM-Z-Phe-Arg-NMec was added to one of these portions and the enzyme activity was measured. The same experiment was carried out with removal of either $\alpha_2\text{M}$ or $\alpha_2\text{CPI}$ from the mixture as a control for checking the enzyme stability. Substrate-induced dissociation was monitored with identical enzyme-inhibitor complexes as before to which 4 μl of 9 mM-Z-Phe-Arg-NMec was added. Portions (10 μl) were removed after different times of incubation at 40°C and assayed for their enzymic activity.

Gel filtration of the CPI-cathepsin L complexes

The same equimolar CPI/cathepsin mixtures supplemented with $\alpha_2\text{M}$ as above were prepared

and incubated for 10, 30 and 120 min at 40°C. Portions of volume 100 μl were then removed and submitted to gel filtration on a Sephadex G-200 column (1.75 cm $\times$ 25 cm) equilibrated in 0.1 M-sodium phosphate buffer, pH 6.0, containing 2 mM-EDTA and 1 mM-dithiothreitol. Eluted fractions were analysed for their absorbance at 280 nm, cathepsin L activity and enzyme-inhibiting capacity, with papain and cathepsin H for α_2 CPI and the low- M_r CPI respectively.

Results

Titration

After active-site titration, crude commercial papain, repurified papain, cathepsin H and cathepsin L were found to be 24%, 80%, 50% and 45% active respectively. With cathepsin L at a final concentration of 1.0 μM ($[E]_0$) and increasing amounts of $\alpha_2\text{CPI}$ a linear decrease in activity is observed up to zero activity (Fig. 1a). Complete association between enzyme and inhibitor has been therefore achieved under these conditions. Since, in that case, the $[E]_0/K_i$ ratio must exceed 100 (Henderson, 1972), a rather low K_i value may be predicted for this reaction. Under identical conditions with the low- M_r CPI such a linear decrease in activity was not obtained on the addition of the inhibitor (Fig. 1b), which suggests a higher K_i value for this equilibrium.

Fig. 1. Titration of human serum cysteine-proteinase inhibitors

The fractional residual activity (v_i/v_0) of a 1.0 μM solution of human liver cathepsin L on Z-Phe-Arg-NMec was measured in the presence of increasing amounts of (a) $\alpha_2\text{CPI}$ varying from 0 to 1.38 μM or (b) low- M_r CPI varying from 0 to 2.54 μM . v_i and v_0 stand for the velocity with and without inhibitor respectively.

K_i determinations

Accurate K_i values may be determined by working at lower enzyme concentrations, which favour the dissociation of the complexes, and using the steady-state equation derived by Krupka & Laidler (1959). This may be linearized as follows (Henderson, 1972):

$$\frac{[I]_0}{1 - v_i/v_0} = K_i \left(1 + \frac{[S]_0}{K_m} \right) \frac{v_i}{v_0} + [E]_0 \quad (1)$$

where $[I]_0$, $[E]_0$ and $[S]_0$ are the initial concentration of inhibitor, enzyme and substrate respectively, v_0 is the velocity without inhibitor and v_i is the velocity in the presence of inhibitor. The plot of $[I]_0/(1 - v_i/v_0)$ against v_0/v_i is a straight line, the slope of which gives:

$$K_{i(\text{app.})} = K_i \left(1 + \frac{[S]_0}{K_m} \right) \quad (2)$$

As shown on the insets of Fig. 2, $K_{i(\text{app.})}$ increases as a function of the substrate concentration for both inhibitors. This is indicative of a competitive mechanism as described by Henderson (1972). The true K_i value may be obtained from a replot of $K_{i(\text{app.})}$ against $[S]_0$.

Values of $3.0 \times 10^{-9} \text{ M}$ and $1.3 \times 10^{-8} \text{ M}$ were found for the $\alpha_2\text{CPI}$ -cathepsin L equilibrium and the low- M_r CPI-cathepsin L equilibrium respectively.

Association rate constants

Association rate constants (k_{+1}) were calculated assuming that cathepsin L (E) and either inhibitor (I) react according to eqn. (3) and considering k_{-1} to be low enough to neglect the reverse reaction during the initial part of the process:

Thus, when initial concentrations of enzyme ($[E]_0$) and inhibitor ($[I]_0$) are identical, the integrated form of the equation giving the association rate becomes:

$$\frac{1}{[E]} = \frac{1}{[E]_0} + k_{+1}t \quad (4)$$

Concentrations in the 10^{-7} M range were convenient for both inhibitors to allow direct measurement of the time-dependence of the association. Data in Fig. 3 show the plots of $1/[E]$ versus time. The slope of the straight lines obtained gives k_{+1} . Experimental values are reported in Table 1.

Dissociation rate constants

On the basis of eqn. (3) the dissociation of enzyme-inhibitor complexes obeys first-order kinetics during the initial part of the reaction, i.e. when there is almost complete association. In this

Fig. 2. K_i determinations

Cathepsin L was used at a final concentration of $0.05 \mu\text{M}$ and $0.1 \mu\text{M}$ with increasing amounts of $\alpha_2\text{CPI}$ (0.011 to $0.168 \mu\text{M}$) and low- M_r CPI (0.18 to $1.1 \mu\text{M}$) respectively, and measurements of residual activity were made as described in the Materials and methods section. Four different substrate concentrations were used, i.e. $0.5 K_m$, $1 K_m$, $3 K_m$ and $5 K_m$, with $K_m = 7 \times 10^{-6} \text{ M}$ (Barrett & Kirschke, 1981). For the sake of clarity only the results obtained for $[S] = 5 K_m$ are shown for $\alpha_2\text{CPI}$ (a) and for $[S] = 3 K_m$ for the low- M_r CPI (b), in accordance with eqn. (1). The insets show a replot of experimental $K_{i(\text{app.})}$ values versus $[S]_0$. Intercepts on the ordinate give the true K_i .

Fig. 3. Determination of rate constants for association. Equimolar amounts of cathepsin L and α_2 CPI ($0.05\ \mu\text{M}$) or low- M_r CPI ($0.2\ \mu\text{M}$) were incubated, and residual activity (v_t) was measured as a function of time for α_2 CPI (a) and the low- M_r CPI (b). The fractional activity v_t/v_0 is plotted against time. Insets represent a plot of the data in accordance with eqn. (4).

Table 1. Kinetic constants for the interaction of cysteine-proteinase inhibitors with human liver cathepsin L

Inhibitor	K_i (M)	k_{+1} ($\text{M}^{-1}\cdot\text{s}^{-1}$)	k_{-1}^* (s^{-1})	Half-life of the complex (s)	$[I]_0/K_i^\dagger$
α_2 CPI	3.0×10^{-9}	8.0×10^4	(a) 7.0×10^{-4} (b) 5.5×10^{-4}	1.1×10^3	1800
Low- M_r CPI	1.3×10^{-8}	7.0×10^4	(a) 3.0×10^{-3} (b) 1.3×10^{-3}	0.32×10^3	76

* (a) α_2 M-induced dissociation; (b) substrate-induced dissociation.
† The $[I]_0/K_i$ ratio was estimated by assuming $[I]_0$ values of $5.5\ \mu\text{M}$ and $1\ \mu\text{M}$ for α_2 CPI and the low- M_r CPI respectively.

case the integrated form of the dissociation rate equation is given by:

$$\ln \left(\frac{[EI]}{[EI]_0} \right) = -k_{-1}t \tag{5}$$

from which k_{-1} and the half-life of complexes may be calculated by re-arranging as follows:

$$t_{0.5} = \frac{0.693}{k_{-1}}$$

These conditions are obtained by using high enzyme and inhibitor concentrations ($1\ \mu\text{M}$) to achieve maximal association between both partners. Free enzyme liberated from complexes was quantified by adding α_2 M, with which it forms stable and enzymically active complexes. Alternatively, rates of dissociation were measured with a substrate excess, since the low- M_r CPI, because of its small size, could readily react with α_2 M-bound cathepsin L to form ternary complexes and thus lead to erroneous results. Such a mechanism has

already been described with low- M_r inhibitors reacting with proteinases bound to α_2 M (Jacquot-Armand & Krebs, 1969; Bieth *et al.*, 1981). Fig. 4 shows a plot of the data in accordance with eqn. (3) for both inhibitors, with the use of α_2 M to favour dissociation. $[EI]/[EI]_0$ was measured as $(1 - v/v_\infty)$, with v being the rate at time t and v_∞ the rate of the cathepsin L + α_2 M control. When substrate excess was used to measure dissociation, the same treatment was carried out, with v_∞ corresponding to the cathepsin L control. Results are shown in Table 1. The k_{-1} values obtained for either cathepsin L-CPI equilibrium were found to be almost the same whichever the method, indicating that no significant formation of ternary complex between α_2 M-bound cathepsin L and free low- M_r CPI had occurred under these conditions. Further investigation of the transfer of cathepsin L from CPI to α_2 M was then carried out, since half-lives of cathepsin L-CPI complexes and particularly those formed with the low- M_r CPI are short enough (Table 1) to render the binding of free enzyme to an

Fig. 4. Determination of rate constants for dissociation

Cathepsin L-CPI complexes ($1 \mu\text{M}$) were preincubated for 30 min at 40°C before $\alpha_2\text{M}$ ($1.3 \mu\text{M}$) was added to the mixture. Appearance of cathepsin L activity was recorded as a function of time. Insets show plots of the data in accordance with eqn. (5) for α_2 CPI (a) and the low- M_r CPI (b).

irreversible inhibitor such as $\alpha_2\text{M}$ physiologically possible *in vivo*. The same cathepsin L-CPI + $\alpha_2\text{M}$ mixtures as before were incubated for different times at 40°C , then submitted to gel filtration as described in the Materials and methods section. Two peaks of cathepsin L activity were found in the eluted fractions (Fig. 5) whichever the inhibitor used to form the complex. The first peak was eluted with $\alpha_2\text{M}$, demonstrating the transfer of cathepsin L from CPI to this inhibitor, whereas the other was eluted in a volume consistent with the M_r of free enzyme. The remaining α_2 CPI-bound cathepsin L, since it forms enzymically inactive complexes, was undetectable under these conditions. Even after 2 h of preincubation total transfer of cathepsin L to $\alpha_2\text{M}$ was not obtained, judged by the recovery of free enzyme in the eluted fractions. This may possibly be due to further dissociation of the CPI-cathepsin complexes in response to the dilution that occurs during gel filtration. Maximal transfer was obtained after about 30 min of preincubation before chromatography when α_2 CPI-cathepsin L complexes were studied, whereas a preincubation time of 10 min was enough to

achieve maximal binding of cathepsin L with $\alpha_2\text{M}$ starting from the low- M_r CPI-cathepsin L complex. This agrees with the results reported above for the k_{-1} value of the equilibrium between cathepsin L and the low- M_r CPI. As shown in Fig. 5, superposable results were obtained for cathepsin L activity measurements in eluted fractions after 2 h of preincubation, which further demonstrates that no binding of free low- M_r CPI to $\alpha_2\text{M}$ -bound cathepsin L has occurred under these conditions. Nevertheless, a recovery of inhibitory activity was shown to be eluted in a volume consistent with that of the low- M_r CPI. This indicates the stability of the inhibitor as well as the reversibility of its association with cathepsin L. Such was not the case with α_2 CPI, since no inhibitory activity was recovered after $\alpha_2\text{M}$ -induced dissociation and gel filtration. This may be explained either by the inactivation of the inhibitor on gel filtration or by a more complex mechanism of interaction with proteinases including physicochemical modifications of the inhibitor, as has been already shown for α_1 proteinase inhibitor (Johnson & Travis, 1976; Beatty *et al.*, 1982).

Fig. 5. Transfer of cathepsin L from complexes with cysteine-proteinase inhibitors

Mixtures of α_2CPI -cathepsin L or low- M_r CPI-cathepsin L with α_2M were prepared as indicated in Fig. 4 legend, then applied after different incubation times at 40°C on a Sephadex G-200 column (1.75 cm $\times$ 25 cm) equilibrated in 0.1 M-phosphate buffer, pH 6.0, containing 2 mM-EDTA and 1 mM-dithiothreitol. Eluted fractions were analysed for their absorbance at 280 nm (—) and cathepsin L (CL) activity (-----) after 10 min (O) and 120 min (Δ) of pre-incubation.

Discussion

From the present study it appears that at least three different proteinase inhibitors present in human serum, namely α_2M , α_2CPI and the low- M_r CPI, are able to inhibit human liver cathepsin L. The determination of the kinetic parameters for the interaction between cathepsin L and either α_2CPI or the low- M_r CPI has shown, however, that

enzyme-inhibitor associations proceed at a rather low rate. Therefore, because of the presence of α_2M , these inhibitors would probably not bind this enzyme in plasma unless inactive ternary complexes of α_2M -bound cathepsin L and CPI have formed secondarily. Such a mechanism, which has been previously reported from analysis *in vitro* for the basic pancreatic inhibitor and the soya-bean

trypsin inhibitor interacting with α_2 M-bound trypsin (Jacquot-Armand & Krebs, 1969; Bieth *et al.*, 1981; Wang *et al.*, 1981), might occur because of the small size of the low- M_r CPI, which could still have access to the active site of the α_2 M-bound cathepsin L. As shown in the present paper from the gel-filtration experiments, the recovery of an enzymic activity due to α_2 M-cathepsin L complexes in the presence of freed active low- M_r CPI suggests that no such inactive ternary complex has formed. However, preliminary experiments have shown that α_2 M-cathepsin L complexes with free low- M_r CPI form a ternary complex with lower enzymic activity (M. Pagano, unpublished work).

Anyhow, speculation about a competition between proteinase inhibitors in plasma towards cathepsin L seems to be physiologically irrelevant because of the intracellular lysosomal origin of this enzyme, which prevents it from appearing in a free form in plasma. Cysteine proteinases are thought to act mainly within cells (Barrett, 1980) and in tissue spaces (Melloni *et al.*, 1981), and it is in these extravascular compartments that cysteine-proteinase inhibitors may be supposed to intervene to prevent tissue damage. Because of their lower M_r values than that of α_2 M, cysteine-proteinase inhibitors diffuse more easily from blood vessels and could therefore be the preferential inhibitors of these proteinases extravascularly. This is supported by the analysis of pleural fluid from which α_2 CPI was prepared. α_2 M is 5-fold less concentrated in this fluid than in plasma, whereas α_2 CPI is at the same concentration in both fluids. On the other hand, and unlike its homologue in rat serum, namely α_1 CPI (Esnard & Gauthier, 1983), human α_2 CPI was not found to be an acute-phase reactant. The presence of the low- M_r CPI in extravascular spaces and even intracellularly is all the more feasible as, in addition to its low- M_r , its site of synthesis is not yet known, and a tissue origin cannot be excluded. On the basis of an extravascular concentration ($[I]_0$) at least equal to that in plasma, the $[I]_0/K_i$ ratio for both inhibitors (Table 1) appears to be large enough to suggest a physiological efficiency of these inhibitors (Bieth, 1980).

It may therefore be hypothesized that, given the rate-constant values found for each cathepsin L-CPI equilibrium, both inhibitors could act as transitory inhibitors carrying cathepsin L or other cysteine proteinases from the extravascular to the intravascular space. Rapid transfer of proteinase on the irreversible inhibitor α_2 M would then occur, facilitated by the short half-life of cathepsin L-CPI complexes, and α_2 M-proteinase complexes would finally be eliminated via the reticuloendothelial system as previously described (Ohlsson & Laurell, 1976). This hypothetical function of transitory inhibition on the basis of a kinetic analysis could

also provide an explanation to the apparently inconsistent behaviour of α_2 CPI, which inhibits proteinases of plant origin, such as papain or ficin, much better than it does the homologous cysteine proteinases. Such a strong inhibition would prevent a rapid transfer of proteinases from one inhibitor to the other and therefore abolish the postulated physiological function. If such conclusions are valid, the efficiency of an inhibitor should not just be considered in terms of rates of association between enzyme and inhibitor, but it should also take into account the mechanism controlling its functioning *in vivo*.

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