Membrane Permeable Fluorogenic Rhodamine Substrates for Selective Determination of Cathepsin L

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Summary

The dipeptidyl rhodamine diamide substrates (Z-Phe-Arg)$_2$-R110 and (Z-Arg-Arg)$_2$-R110 are 820- and 360-fold more selective for cathepsin L than for cathepsin B allowing a sensitive determination of cathepsin L activity in the presence of high activity of cathepsin B. The results obtained with cell lysates suggest that the cysteine proteinase activity of vital macrophages detected by flow cytometry with these substrates is mainly due to cathepsin L.

Introduction

Rhodamine-based fluorogenic dipeptide substrates (Fig. 1) have been synthesized by Leytus et al. [1,2] and have been used in cuvette assays of trypsin, plasmin and thrombin [2]. The non-fluorescent substrates penetrate readily into living cells where they release strongly fluorescent products that are entrapped within the cells due to their positive charge (G. Rothe et al., [3]). Using these substrates in flow cytometry of vital cells, substantial protease activity was detected in human monocytes and rat macrophages which was completely inhibited by specific inactivators of cysteine proteinases like E-64 and Z-Phe-Ala-CHN$_2$. Similar results have been obtained with rhodol-based peptidyl monoamide substrates that have been synthesized recently by one of us (S. Klingel).

In order to provide a basis for the interpretation of the flow cytometric results we investigated the specificity of rhodamine-based substrates for the lysosomal cysteine proteinases cathepsin B and cathepsin L in cuvette experiments. Enzymatic studies were performed with the isolated human cathepsins and with lysates of phagocytic cells using the peptide coumaryl amide substrates Z-Phe-Arg-NMec and Z-Arg-Arg-NMec [4] for comparison.


Materials and Methods

Enzymes

Human cathepsins B and L were from Medor (D-8036 Herrsching). The active concentrations of the enzymes were determined by titration with E-64 (Sigma) according to [4].

Substrates

(Z-Phe-Arg)$_2$-rhodamine was synthesized as described by Leytus et al. [1,2]. Synthesis of (Z-Arg-Arg)$_2$-rhodamine will be published elsewhere (S. Klingel). The substrates were dissolved in DMF (20 mM) and diluted with DMSO to 1-10 mM prior to the assay. Z-Phe-Arg-NMec and Z-Arg-Arg-NMec were purchased from Bachem, aminomethylcoumarin from Serva.
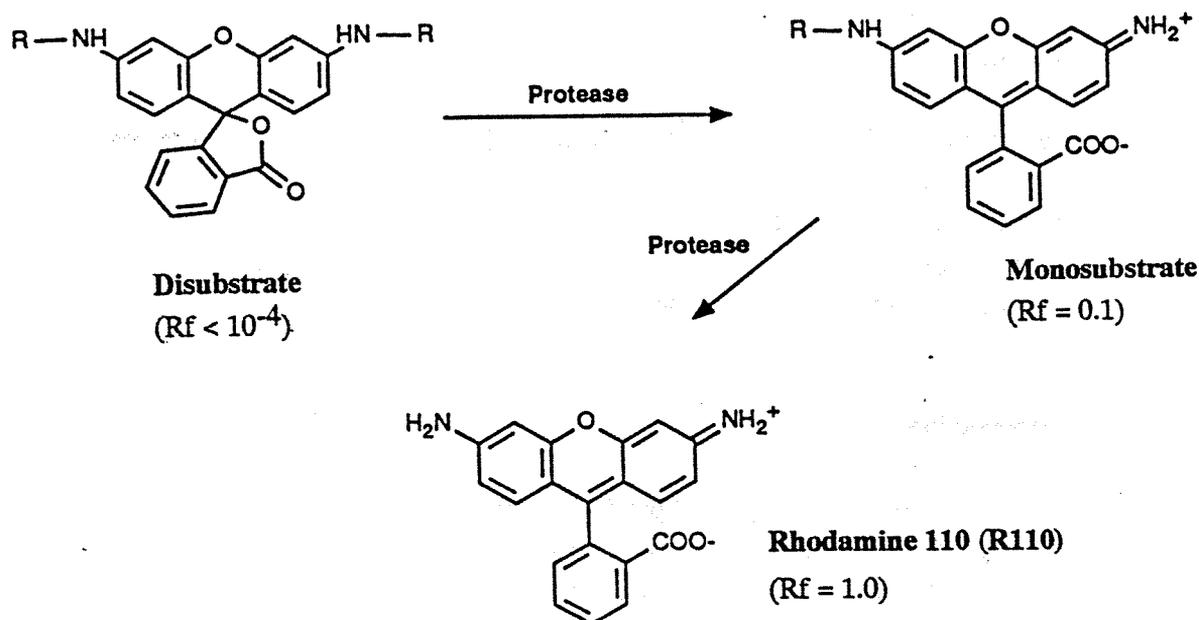
Fig. 1. Cleavage of rhodamine-based disubstrates. R = Z-Phe-Arg or Z-Arg-Arg; Rf = relative fluorescence.

Preparation and lysis of cells

Human peripheral blood monocytes (containing lymphocytes) and PMN granulocytes were isolated as described [5]. Peritoneal macrophages were obtained from Wistar rats through peritoneal lavage with 10 ml HBS-EDTA as either resident or elicited cells 4 days after 3 ml thioglycolate injection. Brain macrophages were prepared following the cultivation of mechanically dissociated brain of newborn rats for 2-4 weeks in Dulbecco's modified Eagle medium substituted with 20% heat-inactivated fetal calf serum. The microglia growing on top of a confluent astrocyte layer was removed, sedimented and resuspended in lysis buffer (R. Banati et al., [6]).
All cells were lysed in 50 mM sodium actetate buffer pH 5.5 containing 0.5% Triton X-100, 0.3 mM EDTA and 0.01% Brij 35.

Instrumentation

A spectrofluorometer Kontron SFM 25 equipped with a 4 cell auto-cell changer is operating on-line to an IBM-compatible personal computer. Self-made programs perform collection and storage of digital

data from the four cells. Real-time product concentration and slope curves are presented on screen. The collected data were exported to commercial nonlinear regression and graphic programs like Enzfitter and FigP (Biosoft, Cambridge). Alternatively, off-line evaluation of data (interactive calculation of slopes by linear regression, documentation etc.) was performed on a personal computer by self-made software (W. Machleidt et al., unpublished).

Assays

Enzymatic activity of isolated cysteine proteinases and of cell lysates was determined at pH 5.5, 30 °C, in continuous assays. The assay buffer was 0.3 M sodium acetate pH 5.5, containing 2 mM EDTA and 0.015% Brij 35. DMSO 9% (with $(Z\text{-}Phe\text{-}Arg)_2 R110$) or Pluronic 0.1% [7] (with $(Z\text{-}Arg\text{-}Arg)_2\text{-}R110$) was added for solubilization of substrates. Emission of the fluorescent products was measured at 523 nm using 492 nm for excitation. Aminomethylcoumarin (excitation: 380 nm; emission: 460 nm) was used for calibration in all experiments. In all assays of cell lysates, E-64 (12.5 μM) was added to verify cysteine proteinase activity specifically [8].

Results

Activity of isolated cathepsins B and L

Both $(Z\text{-}Phe\text{-}Arg)_2\text{-}R110$ and $(Z\text{-}Arg\text{-}Arg)_2\text{-}R110$ were found to be sensitive substrates for cathepsin L, but 820- and 360-fold less sensitive substrates for cathepsin B (Table 1). Activity was linear with substrate concentration below 20 μM (Fig. 2). Due to limited solubility of the substrates, determination of K_m values was not feasible. Therefore a substrate concentration of 10 μM was chosen in all experiments and "apparent" k_{cat} values were calculated for this concentration. The corresponding values determined with the coumarin substrates aggree reasonably well with published data for the human and the rat enzymes indicating that species differences seem negligible [9,10].

Table 1. Apparent k_{cat} for rhodamine and coumarin substrates (10 μM)

Enzymes	k_{cat} [s^{-1}] for 10 μM substrate			
	$(Z\text{-}Phe\text{-}Arg)_2\text{-}R110$	Z-Phe-Arg-NMec	$(Z\text{-}Arg\text{-}Arg)_2\text{-}R110$	Z-Arg-Arg-NMec
Cathepsin L	4.9	20.2	12.6	< 0.002
Cathepsin B	0.006	6.1	0.035	1.9
Cath. L/Cath. B	817	3.3	360	< 0.001
Cath. B/Cath. L	0.001	0.303	0.003	> 1000

All values are based on calibration with aminomethylcoumarin.

The rhodamine-based diamides are double-headed substrates. The final reaction product, rhodamine, is about ten times more fluorescent than the peptidyl monoamide intermediate (see Fig. 1). We observed a linear increase of reaction rate with enzyme concentration in the case of cathepsin B with both rhodamine substrates, but in the case of cathepsin L only with (Z-Arg-Arg)$_2$-R110 (Fig. 3). When cathepsin L was assayed with (Z-Phe-Arg)$_2$-R110, the initial reaction rate (after 10 min activation) raised nonlinearly with the enzyme concentration, though the overall substrate consumption was less than 3 % within this time.
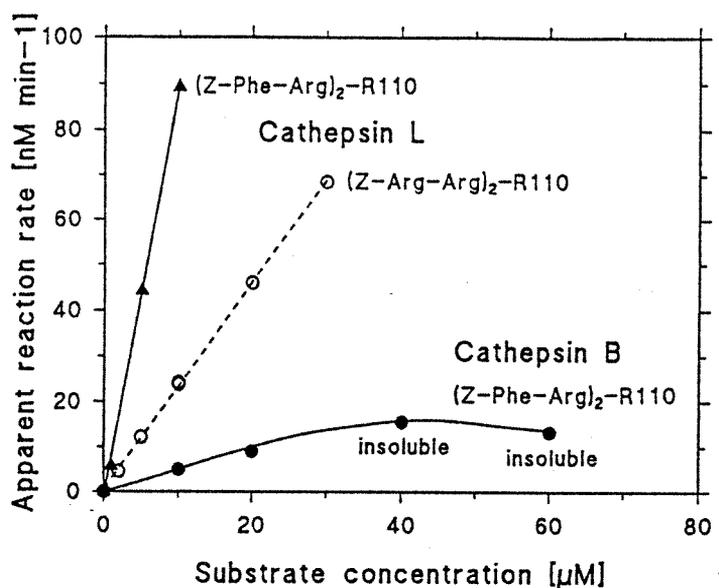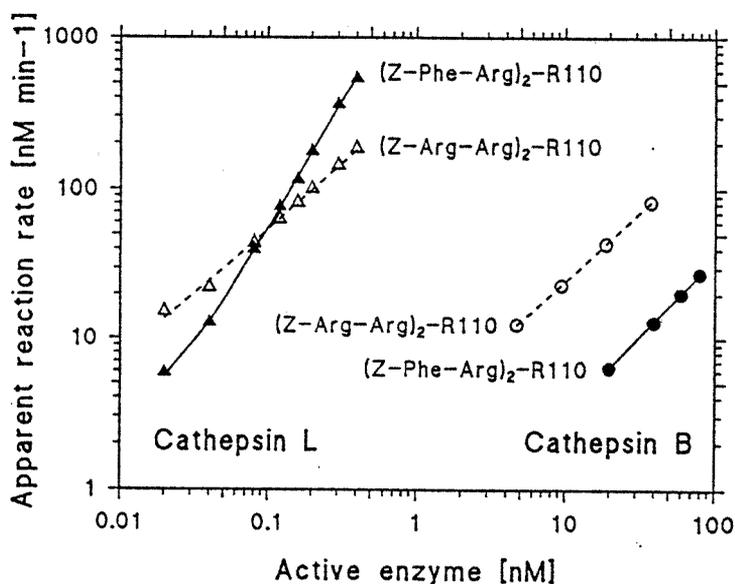

Fig. 2. Effect of substrate concentration

Fig. 3. Effect of enzyme concentration at 10 μM substrate.

Enzyme activity of cell lysates

The enzymatic activity of acid (pH 5.5) cell lysates (human granulocytes and monocytes, rat resident and elicited peritoneal macrophages and microglia cells from rat brain) measured with both rhodamine substrates (Fig. 4) in the presence of EDTA was completely abolished by the addition of E-64, a specific inhibitor of lysosomal cysteine proteinases and calpain (not shown). The activity with the two rhodamine substrates roughly paralleled the activity obtained with the coumarin substrate Z-Arg-Arg-NMec which is highly specific for cathepsin B under these conditions [4]. However, as shown above with the isolated proteinases, the rhodamine substrates should be selective for cathepsin L even in the presence of high amounts of cathepsin B. When the expected contribution of cathepsin B (calculated from Z-Arg-Arg-NMec activity using the apparent k_{cat} values from Table 1) was subtracted, the (Z-Arg-Arg)$_2$-R110 activity of the lysates of macrophages and microglia cells was only slightly diminished suggesting that this activity is mainly due to cathepsin L. (see Fig. 4). In the case of human granulocytes and monocytes, the measured (Z-Arg-Arg)$_2$-R110 activities of cell lysates were at the lower limit of detection rendering it doubtful whether the applied calculation is relevant for these cell types. As Reilly et al. [11] have not detected any cathepsin L mRNA in monocytes, the (Z-Arg-Arg)$_2$-R110 activity of these cells may be due to cathepsin B.
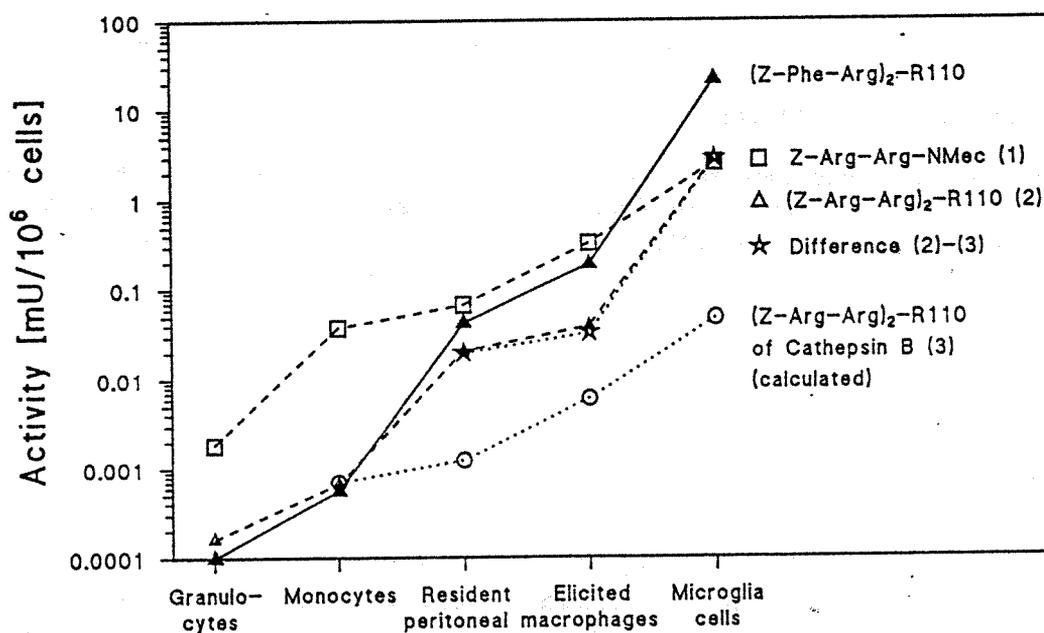
Fig. 4. Activity of cell lysates. Lysates were prepared from human peripheral granulocytes and monocytes, rat resident and thioglycolate-elicited peritoneal macrophages and rat microglia cells as described in Methods. All substrates were 10 μM.

The differences between (Z-Phe-Arg)$_2$-R110 and (Z-Arg-Arg)$_2$-R110 activity may be explained by the observed nonlinearity of cathepsin L activity with (Z-Phe-Arg)$_2$-R110 (see Fig. 3). Therefore (Z-Arg-Arg)$_2$-R110 was selected for an estimation of the active cathepsin L amounts in cell lysates

(Table 2). Because only small differences in the kinetic properties of human and rat cathepsins B and L have been reported [9,10], the k_{cat} determined with human enzymes were applied for the rat cell lysates.

Table 2. Active cathepsins B and L in lysates of phagocytic cells

Cells	Cathepsin B [ng/10^6 cells]	Cathepsin L [ng/10^6 cells]
Granulocytes (human)	0.45	-*
Monocytes (human)	9.3	-*
Peritoneal macrophages (rat)		
resident	16.5	0.73
elicited	80.6	1.2
Microglia cells (rat)	644	105

* below the limit of detection

The enzyme contents were calculated from the activities determined with the substrates Z-Arg-Arg-NMec (cathepsin B) and (Z-Arg-Arg)$_2$-R110 (cathepsin L) after subtraction of the contribution of cathepsin B to the (Z-Arg-Arg)$_2$-R110 activity (see Fig. 4) using the apparent k_{cat} values of the human cathepsins presented in Table 1 and a molecular mass of 28,000 for both enzymes.

Discussion

Substrate specificity

The coumarin substrate Z-Arg-Arg-NMec is highly selective for cathepsin B whereas Z-Phe-Arg-NMec is slightly more sensitive for cathepsin L than for cathepsin B at 10 μM substrate concentration (see Table 1). Therefore the high selectivity of both corresponding rhodamine substrates, (Z-Arg-Arg)$_2$-R110 and (Z-Phe-Arg)$_2$-R110, for cathepsin L was unexpected, but may be explained on the basis of the three-dimensional structure of cathepsin B obtained recently [12]. Access of large molecules like the rhodamine diamide substrates to the active site cleft of cathepsin B is restricted by an "occluding loop" which is not present in papain, and, probably, in cathepsin L that is more closely related to papain according to the amino acid sequences, the proteolytic specificities [13], and the characteristics of inhibition by protein inhibitors [14].

Within the range of reaction rates determined with cell lysates, the rate of hydrolysis of (Z-Arg-Arg)$_2$-R110 was linear with the concentration of both cathepsin B and L, whereas (Z-Phe-Arg)$_2$-R110 activity was linear only with cathepsin B but not with cathepsin L concentration (see Fig. 3). It may

be assumed that only one peptidyl amide bond is cleaved in (Z-Arg-Arg)$_2$-R110, but significant additional cleavage of the second peptidyl amide bond by cathepsin L occurs in (Z-Phe-Arg)$_2$-R110 resulting in the formation of rhodamine which is approx. 10-times more fluorescent than the monoamide intermediate [1].

The nonlinearity due to a two-step reaction could be prevented by the use of peptidyl monoamides of O-acylated rhodol as substrates. These substrates have been synthesized (S. Klingel, unpublished). Preliminary results indicate that substrates of this type are also much more sensitive for cathepsin L than for cathepsin B but are about 30-500-fold slower hydrolysed than the corresponding rhodamine diamide substrates.

The specificity of the rhodamine substrates for cathepsin S, which has been characterized in detail recently [10], remains to be investigated.

Cathepsin B activity of cell lysates

In contrast to cathepsin B which is relatively stable at neutral pH and is "stored" in form of readily dissociating complexes with its endogeneous protein inhibitors, cathepsin L is much more unstable at neutral pH and is very tightly, quasi-irreversibly, complexed by its protein inhibitors [5,8]. Therefore the detection of cathepsin L activity in biological samples in the presence of cathepsin B seems very difficult. The selectivity of the peptidyldiazomethane inhibitor Z-Phe-Phe-CHN$_2$ for cathepsin L has been used for discrimination of the two enzymes [15], but this approach is not accurate when cathepsin L activity is low compared to cathepsin B activity.

The rhodamine derivatives are the first substrates that enable a direct sensitive assay of cathepsin L activity -without use of discriminating inhibitors- even in the presence of an excess of cathepsin B activity. Cathepsin B activity can be determined specifically with the coumarin substrate Z-Arg-Arg-NMec and its contribution to the (Z-Arg-Arg)$_2$-R110 activity can be subtracted before calculation of cathepsin L activity from hydrolysis of the latter substrate.

The obtained results with cell lysates suggest that the rhodamine substrates detect cathepsin L selectively when used in flow cytometry of phagocytic cells. Obviously these substrates are able to penetrate into the lysosomes where they meet high concentrations of active cathepsin L separated from its cytosolic protein inhibitors. But even in cell lysates, where the inhibitors are present, significant amounts of cathepsin L activity can be detected with these selective substrates. It remains open whether part of the activity observed with rhodamine substrates is due to cathepsin S which shares some properties with cathepsin L as well as with cathepsin B and has been found in lung macrophages [16].

Cathepsin B as well as cathepsin L have been implemented as markers of differentiation and activation of cells of the monocyte/macrophage system [11,17]. Cathepsin B immunoreactivity has been found in functionally activated glia cells [18]. In local or systemic inflammation, cathepsin B activity of inflammatory secretions and blood plasma correlates with the severity of organ dysfunctions [5,19]. Cathepsin L, due to its limited pH stability and its high affinity for endogeneous

protein inhibitors, should be extremely short-lived after discharge from the lysosomes and is therefore not detectable as active enzyme in blood plasma or secretions. Therefore determination of cathepsin L activity in vital phagocytes by flow cytometry is expected to provide further insight to the role of lysosomal cysteine proteinases in inflammation.

Acknowledgements

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