USE OF AN INTRAMOLECULARLY QUENCHED FLUOROGENIC SUBSTRATE FOR STUDY OF A THIOL-DEPENDENT ACIDIC DIPEPTIDYL CARBOXYPEPTIDASE IN CELLULAR EXTRACTS AND IN LIVING CELLS

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1. Introduction

Intramolecularly quenched compounds have been successfully used as sensitive and specific substrates for proteolytic enzymes [1]. In an attempt to apply this type of substrate for the study of intracellular enzymes in living cells, we modified a previously developed substrate (specific for DCP) namely *o*-aminobenzoylglycyl–*p*-nitro–L-phenylalanyl–L-proline (ABz-Gly–Phe(NO_2)–Pro) [2]. The ABz fluorophore was replaced with the dansyl group which emits in the visually observable wavelength range, and is also intramolecularly quenched by the Phe(NO_2) residue. Using this derivative with mouse macrophages, intracellular hydrolysis caused the appearance of cell-associated fluorescence clearly seen under the fluorescent microscope. We propose that the emission depends on hydrolysis by a lysosomal DCP, the presence of which we demonstrate in the macrophage extract, and not by ACE, also present in the cells [3]. The lysosomal DCP present also in rat liver was investigated in more detail.

2. Materials and methods

Dns-Gly–Phe(NO_2)–Pro was prepared essentially as for the ABz-derivative [2], and used also for preparation of Dns-Gly–Phe(NO_2)–Pro–Gly. Details will be described elsewhere. Captopril (SQ 14225) was kindly supplied by Dr D. W. Cushman (Squibb Institute, Princeton NJ). Other chemicals were reagent grade from various commercial sources.

Thioglycollate activated adherent macrophages from C57/B1 2-month-old male mice were cultured for 1–7 days in DMEM containing 10% foetal calf serum, penicillin (100 units/ml), streptomycin (100 μg/ml) and fungizone (0.25 μg/ml). For visualization of intracellular DCP activity, the cells were washed in PBS and then incubated in the above medium containing 0.5 mM Dns-Gly–Phe(NO_2)–Pro at 40°C for 90 min. The PBS washed cells adhering to cover glasses were observed under a Zeiss fluorescence microscope equipped with an UG1 excitation filter ($\lambda_{trans} < 400$ nm), a chromatic splitter FT 510 and a barrier filter no. 50 ($\lambda_{trans} > 510$ nm). To prepare cell extracts, the adherent cells (6–7 × 10^6/0.5 ml, 70% viable by the trypan blue-exclusion test) were PBS washed, harvested with a rubber policeman, collected at 500 × *g* and resuspended in the assay buffer containing 0.1% Triton X-100. Debris was removed at 500 × *g*.

Lysosomal DCP was partially purified from rat liver as follows: Lysozome-enriched fractions [5] from homogenates of rat liver were homogenized in presence of 0.1% Triton X-100 in 0.5 M phosphate buffer (pH 6.5) containing EDTA (6.0 mM) and cysteine (3.0 mg/ml). Acetone precipitation (0.9 ml acetone/1.0 ml extract) resulted in an 18-fold increase in specific activity. ACE was prepared from calf lung [4].

For the assay of DCP in extracts of mouse macrophages and rat liver, the extract (20–100 μl) and 10 mM substrate (20 μl) in 50 mM Na-acetate (pH 4.0)

Abbreviations: ACE, angiotensin I converting enzyme (EC 3.4.15.1); ABz, *o*-aminobenzoyl; DCP, dipeptidyl carboxypeptidase (peptidyl dipeptide hydrolase); L-DCP, acidic thiol-dependent DCP; DMEM, Dulbecco's modified Eagles medium; Dns, dansyl (1-dimethylaminonaphtalene-5-sulphonyl); DTT, di-thiothreitol; EDTA, ethylenediaminetetraacetate (di-potassium salt); PBS, phosphate-buffered saline

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or 50 mM phosphate (pH 6.5) were placed in a fluorimetric cuvette and the volume was made up to 400 μl with the respective buffer. The fluorescence increase was continuously monitored at 40°C, using a Perkin-Elmer fluorescence spectrophotometer (MPF-32), at λ_{ex} = 360 nm, λ_{em} = 540 nm, or λ_{ex} = 360 nm and λ_{em} = 410 nm for the Dns- and ABz-compounds, respectively. All kinetic measurements were performed in the linear range up to 5% hydrolysis.

3. Results and discussion

3.1. *Fluorogenic substrate for dipeptidyl carboxypeptidases*

In contrast to ABz-Gly–Phe(NO_2)–Pro emitting at λ_{max} = 410 nm, the fluorescence of Dns-Gly–Phe(NO_2)–Pro is visually observable. Emission at 560 nm (λ_{ex} = 360 nm) of the dansyl derivative in 0.1 M Tris–HCl (pH 8.0) was on a molar basis 13-times lower than that of Dns-glycine.

Incubation of Dns-Gly–Phe(NO_2)–Pro with calf lung ACE resulted in cleavage of the Gly–Phe(NO_2) peptide bond and an enhancement of fluorescence to a maximum level of 13-fold, indicating complete hydrolysis of the substrate. Under the same conditions no hydrolysis was observed with the substrate analogue Dns-Gly–Phe(NO_2)–Pro–Gly. This was expected, since DCPs, such as ACE, do not cleave peptide bonds involving a prolyl nitrogen [6]; it was therefore used in control experiments, designed to exclude hydrophobic environment and action of other enzymes, as the cause of the appearing fluorescence.

3.2. *Lysosomal dipeptidyl carboxypeptidase in extracts of mouse peritoneal macrophages and rat liver*

The presence of ACE in macrophage extracts has been demonstrated and it was shown that the activity increases during cultivation of the cells and that the increase is markedly elevated in presence of dexamethasone [3]. We confirmed these findings with the above dansylpeptide as the substrate. In addition, we detected in the extracts an enzymatic activity which hydrolyzes this fluorogenic substrate in the presence of cysteine and EDTA at acidic pH-values. Since under such conditions ACE is not active [6], the hydrolysis was apparently caused by another enzyme, which we show to be an acid, thiol-dependent DCP (L-DCP). The hydrolysis of Dns-Gly–Phe(NO_2)–Pro (0.5 mM) by extracts of macrophages (derived from 2×10^6 cells, 0.4 ml) at pH 6.5 in presence of EDTA and cysteine was linear up to at least 8% conversion of substrate. Leupeptin [7] (5×10^{-8} M) inhibited this activity completely, and no activity was detected in absence of cysteine. The inhibitors of ACE, EDTA (10 mM) and captopril (SQ 14225) [8] (0.1 mM) had no effect. The activities of both enzymes increase with time and are clearly enhanced by dexamethasone (fig.1). No fluorescence increase occurred when Dns-Gly–Phe(NO_2)–Pro–Gly was incubated with the extract instead of the substrate. This indicates specific cleavage of the substrate by DCP activity present in the extract, probably of lysosomal origin. In order to obtain larger amounts of lysosomal DCP we isolated it from lysosome-enriched rat liver homogenates. Enzyme activity capable of cleaving dipeptides from the carboxyl end of polypeptide chains has been reported for preparations of cathepsin B_1 obtained from the same tissue [9]. We partially purified the enzyme by acetone precipitation. Like the macrophage enzyme, the rat liver DCP is thiol dependent (cysteine or DTT), acts in the acidic pH range (pH optimum =

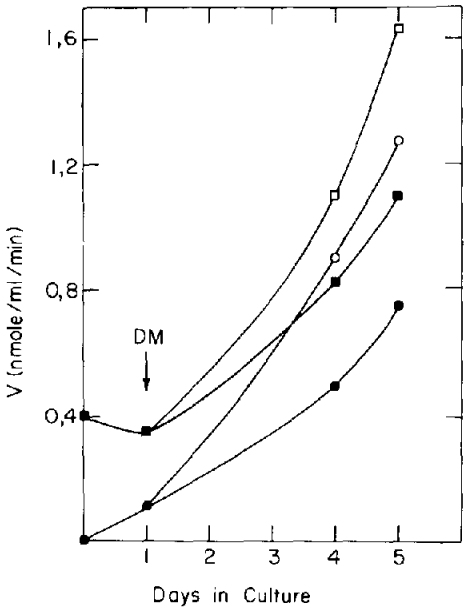

Fig.1. Dependence of ACE (circles) and L-DCP (squares) activities in extracts of mouse peritoneal macrophages on the time of cell cultivation in the presence (closed symbols) and absence (open symbols) of dexamethasone (0.5 μM). The arrow marks the time of adding dexamethasone to the mixture.

3.5), is completely inhibited by 5×10^{-8} M leupeptin and is not inhibited by EDTA (10 mM) or captopril (0.1 mM). The dependence of initial hydrolysis rates on the enzyme concentration was linear in a 10-fold range. The substrate analogue Dns-Gly–Phe(NO_2)–Pro–Gly was not hydrolyzed by the partially purified lysosomal DCP. Quantitative analysis of the products formed during hydrolysis of the substrate ABz-Gly–Phe(NO_2)–Pro by crude rat liver DCP showed that the amount of ABz-Gly formed at various time intervals was the same whether calculated from fluorescence measurements or determined by high-performance liquid chromatography. This demonstrates that the first bond cleaved is the Gly–Phe(NO_2) peptide bond. A much slower and equimolar appearance of Phe(NO_2) and proline was observed by amino acid analysis (fig.2). The Phe(NO_2)–Pro product was apparently hydrolysed by prolidase or aminopeptidase P. While our study strongly indicates that the only enzyme activity being measured by our substrate under the specified conditions in the crude rat liver extract is a lysosomal DCP, final proof will be provided only after obtaining the enzyme in pure form. This task is now being undertaken in our laboratory.

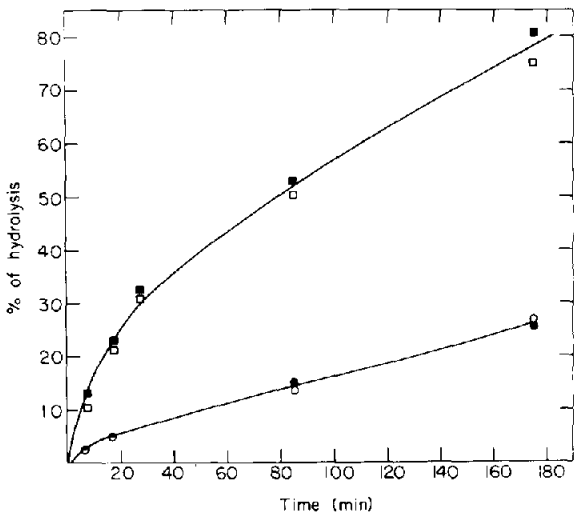

Fig.2. Specific cleavage of ABz-Gly–Phe(NO_2)–Pro by lysosome-enriched homogenates from rat liver. The substrate (5.0 mM) was incubated with the homogenate (100 μl, 17 mg protein/ml) at 40°C in 0.5 M phosphate buffer (pH 6.0) containing EDTA (6.0 mM) and cysteine (3.0 mg/ml). Quantitative formation of ABz-Gly was determined fluorimetrically (■) and by reversed-phase high performance liquid chromatography [μm Bondapak C_{18} column (30 cm × 3.9 mm i.d.), 0.2 M phosphate buffer (pH 2.1) isocratic elution] monitored by UV absorption (□). Proline (○) and Phe(NO_2) (●) were determined by quantitative amino acid analysis.

3.3. *Lysosomal dipeptidyl carboxypeptidase in living cells*

Having established that peritoneal mouse macrophages contain peptidyldipeptide hydrolase activity, we investigated whether this activity can be observed under the fluorescence microscope after internalization by cells of the fluorogenic substrate Dns-Gly–Phe(NO_2)–Pro.

Thiol-activated peritoneal mouse macrophages, cultivated for 2–5 days, turned fluorescent when incubated with the substrate for 90–150 min at 37°C. No fluorescence was observed in the external medium. After 3 h, the cell-associated fluorescence disappeared and the medium became fluorescent. Restaining of the same cells 2 days later proceeded as if the cells had not been pre-stained, thus no damage seemed to be caused to the cells. The use of Dns-Gly–Phe(NO_2)–Pro–Gly instead of the substrate did not cause appearance of fluorescent cells, indicating absence of an endopeptidase cleaving the Gly–Phe(NO_2) bond, of a carboxypeptidase followed by DCP, and of influence of hydrophobic environment on the emission of the dansyl group or/and on its interaction with the quencher.

That the substrate was hydrolyzed intracellularly is strongly indicated, since the fluorescence was not elevated in the cell-periphery. During the first 2 days in culture the cells did not appear fluorescent when incubated with the substrate. Marked fluorescence was observed at day 3, and higher levels were observed when the cells were tested on days 4 8 (fig.3). The level of fluorescence was higher when the cells were incubated in presence of 5×10^{-7} M dexamethasone.

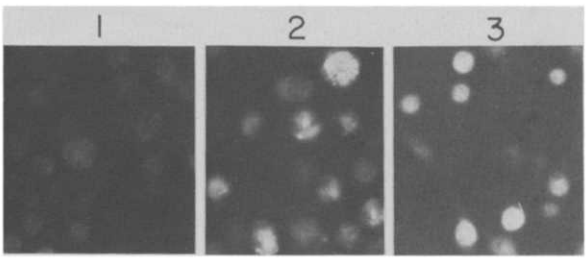

Fig.3. Fluorescence micrographs of thioglycollate-activated mouse macrophages stained with Dns-Gly–Phe(NO_2)–Pro. Cells were cultivated for 3 days (1) or 6 days (3) and stained for 90 min. Cells cultivated for 3 days were stained in presence of concanavalin A for 20 min [2].

No fluorescence appeared in absence of the substrate. When the incubation was performed in the presence of concanavalin A (50 μg/ml), known to enhance the internalization of the external medium by pinocytosis [10], the fluorescence of the cells was stronger and appeared much earlier (after 20 min). When cells were incubated with 0.5 mM leupeptin prior to incubation with the substrate, no cell-associated fluorescence appeared. Since under the same conditions leupeptin did not prevent cell-associated fluorescence occurring upon incubation with the fluorescent dansyl-glycine, it is very likely that leupeptin did not prevent the incorporation, but rather inhibited the intracellular hydrolysis by L-DCP. In contrast, incubation of the cells under the same conditions with 0.5 mM captopril, which is a potent inhibitor of ACE but not of lysosomal DCP, hardly affected the fluorescence produced by incubation with the fluorogenic substrate. Apparently a lysosome-localized DCP and not an ACE localized elsewhere, is responsible for the intracellular hydrolysis of the fluorogenic substrates.

Cell-sorting of macrophages according to their lysosomal DCP activity, as well as applications to other cells, are now under investigation.

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