Effect of glycyl-L-phenylalanine 2-naphthylamide on invertase endocytosed by rat liver

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1. The release by glycyl-L-phenylalanine 2-naphthylamide (Gly-L-Phe-2-NNap) of endocytosed invertase associated with the MLP fraction {sum of the M, L and P fractions [de Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955) *Biochem. J.* 63, 604–617]} of rat liver was investigated and compared with the release of cathepsin C. 2. The percentage of invertase released increases with time after the enzyme injection, whereas the release of cathepsin C is not influenced by this treatment and corresponds to 85–90% of the total activity of the enzyme. It takes about 2 h to attain a similar release of both enzymes. 3. The quantity of invertase releasable or not by Gly-L-Phe-2-NNap was plotted against the time after the injection. Results agree well with the hypothesis that unreleasable invertase is associated with a pre-lysosomal compartment, whereas releasable invertase is present in lysosomes. A kinetic analysis indicates that invertase enters the pre-lysosomal compartment with a zero-order rate constant of 0.48 unit/min per g fresh wt., and leaves this compartment with a first-order rate constant of 0.042 min⁻¹.

Numerous substances taken up by endocytosis reach the lysosomes after passing through a pre-lysosomal compartment: the endosomes. The kinetic analysis of the flux of compounds through the endosomal and lysosomal compartments offers some difficulties. Indeed it requires the knowledge of the amount of the endocytosed substance associated with each compartment and at different times after the compound has penetrated into the cell. To obtain such information, it is a prerequisite that the different organelles are well separated. Frequently, however, the behaviour on centrifugation of the structures of which the pre-lysosomal and lysosomal compartments are composed, although distinct enough for analytical-centrifugation purposes, does not differ sufficiently to allow a net separation of the two particle populations. As a result, the quantitative estimation of the proportion of the endocytosed substance distributed between the two compartments can be markedly vitiated. Moreover, such a methodology needs repeated high-speed centrifugation experiments. A simpler means to approach the problem would be to measure the proportion of an endocytosed sub-

stance that can be released from the organelles, by a procedure that selectively disrupts the lysosomal membrane without affecting the other organelles. As we have previously proposed (Jadot *et al.*, 1984), a choice method would be to determine the susceptibility to Gly-L-Phe-2-NNap of the structures associated with the endocytosed molecule. Indeed, the loss of lysosome integrity caused by this substance requires its intralysosomal hydrolysis by cathepsin C, and it is therefore highly specific for lysosomes. A non-lysosomal organelle that does not contain this hydrolase will not be affected by Gly-L-Phe-2-NNap.

In the work reported here, such a methodology has been used in the study of invertase endocytosis by rat liver. It should be noted that, according to several authors (Stahl *et al.*, 1976; Madnick *et al.*, 1978; Rodman *et al.*, 1978), invertase taken up by the liver is almost exclusively located in the non-parenchymal cells. Therefore our results mainly concern endocytosis performed by these cells.

Experimental

Tissue fractionation

Experiments were performed with male Wistar rats weighing 200–300 g. Yeast invertase (cata-

Abbreviation used: Gly-L-Phe-2-NNap, glycyl-L-phenylalanine 2-naphthylamide.

logue no. I-4504; Sigma, St. Louis, MO, U.S.A.) was injected intravenously (0.9 mg/100 g body wt.). The animals were killed at various times after injection; the liver was perfused with cold 0.15M-NaCl, removed, and homogenized in ice-cold 0.25M-sucrose. The homogenate was fractionated to give a nuclear fraction, N, and a cytoplasmic extract, E, as described by de Duve *et al.* (1955). The cytoplasmic extract was further fractionated to give a total mitochondrial fraction corresponding to the sum of the heavy (M) and the light (L) mitochondrial fractions, a microsomal fraction, P, and in unsedimentable fraction, S (de Duve *et al.*, 1955). For the experiments of activation by Gly-L-Phe-2-NNap, the fractions M, L and P were mixed ('MLP' fraction).

Activation experiments

The MLP fraction was incubated at 37°C in a total volume of 2 ml containing 0.25 mM-Gly-L-Phe-2-NNap, 0.05 M-sodium acetate buffer, pH 6, 0.25 M-sucrose, 5 mM-NaCl, with or without 0.05% Triton X-100. After 10 min, the mixture was chilled by adding 2 ml of ice-cold unbuffered 0.25 M-sucrose and keeping the tube in ice for 10 min. Then it was centrifuged at 25000 rev./min (45000 $g_{av.}$) for 25 min in a Spinco 40.3 rotor, and cathepsin C and invertase released in the supernatant were measured.

Enzyme assays

Cathepsin C was determined as described previously (Jadot *et al.*, 1984); invertase was measured as described by Brown & Segal (1977).

Results

Release of cathepsin C and invertase by different concentrations of Gly-L-Phe-2-NNap

Fig. 1 illustrates the release of cathepsin C and invertase from a MLP fraction by increasing concentrations of Gly-L-Phe-2-NNap. The granules were isolated 10 and 30 min after invertase injection. Maximal release of the lysosomal peptidase occurs when the Gly-L-Phe-2-NNap concentration reaches 0.25 mM. This is also the case for invertase; however, the proportion of released cathepsin C (approx. 90% of the total activity) is much greater than the proportion of released invertase. Moreover, the extent to which invertase is liberated depends on the amount of time after the injection. It should be noted that invertase not released by Gly-L-Phe-2-NNap can be released either by a hypo-osmotic treatment or by freezing and thawing, as exemplified by the results of Table 1.

As maximal liberation of cathepsin C and invertase took place in the presence of 0.25 mM-Gly-

Fig. 1. Release of cathepsin C and invertase by Gly-L-Phe-2-NNap

An MLP fraction originating from the liver of rats killed 10 or 30 min after invertase injection was incubated for 10 min at 37°C in the presence of various concentrations of Gly-L-Phe-2-NNap, 0.05 M-acetate buffer, pH 6, 0.25 M-sucrose and 5 mM-NaCl. The amounts of enzyme released from the granules were determined as described in the Experimental section, and are given as percentages of the amounts released under the same conditions in presence of 0.05% Triton X-100. Δ , Invertase released 10 min after injection; $\bullet$, invertase released 30 min after injection; $\circ$, cathepsin C released 10 min after injection; $\triangle$, cathepsin C released 30 min after injection.

Table 1. Release of cathepsin C and invertase by Gly-L-Phe-2-NNap, freezing and thawing and hypo-osmotic treatment

An MLP fraction originating from the liver of a rat killed 30 min after invertase injection was incubated for 10 min at 37°C in the presence of 0.25 mM-Gly-L-Phe-2-NNap/0.05 M acetate buffer (pH 6)/0.25 M-sucrose/5 mM-NaCl. The amounts of enzyme released from the granules after incubation were determined (a) without additional treatment, (b) after freezing and thawing three times in liquid N₂, and (c) after a 1:10 dilution of the preparation with distilled water. The amount of enzyme released are given as percentages of the amount released under the same conditions in presence of 0.05% Triton X-100.

Enzyme	Enzyme release (%)		
	A	B	C
Cathepsin C	91.0	88.0	89.2
Invertase	35.3	82.0	88.5

L-Phe-2-NNap, this concentration was used in the following experiments.

Release of invertase by Gly-L-Phe-2-NNap at increasing times after injection

In the presence of Gly-L-Phe-2-NNap, the lability of the structures with which cathepsin C

Fig. 2. Invertase activity present in liver, MLP fraction and plasma

Invertase was measured in homogenate (E + N) of rat liver, MLP fraction and plasma at increasing times after injection. The activities are given in units (μmol of glucose liberated/min) per g of liver or per ml of plasma. Means $\pm$ S.D. for three animals are presented. So as not to overload the presentation, the S.D. of experimental points for MLP fraction are not given.

and invertase are associated was determined at different times after invertase injection. In each experiment, the amount of enzyme present in the plasma, in the total liver and in the MLP fraction was measured. In agreement with the results of Jacques (1968) and Madnick *et al.* (1978), the invertase concentration in liver linearly increases until the enzyme has almost totally disappeared from the blood. After the maximal liver concentration has been reached at about 90 min, it is maintained up to at least 4 h (Fig. 2). On average, 75% of invertase is located in the MLP fraction.

The percentage of invertase that can be released by Gly-L-Phe-2-NNap progressively increases with time after the injection (Fig. 3), whereas the liberation of cathepsin C is not influenced by this treatment and always corresponds to 85–90% of the total enzyme activity; a period of 2 h is necessary to observe a similar release of both enzymes.

The amounts of invertase released or not released by Gly-L-Phe-2-NNap at different times have been calculated by using the curves in Figs. 2 and 3. The values are plotted as a function of time in Fig. 4; the curves were drawn as described in the Discussion section. The amount of invertase not releasable by Gly-L-Phe-2-NNap progressively increases with time until reaching a value, after 40 min, that remains practically constant during the next 50 min. After that, a slow decrease is observed. Invertase releasable by Gly-L-Phe-2-NNap

Fig. 3. Release of invertase by Gly-L-Phe-2-NNap: influence of the time after injection

Release of invertase in presence of 0.25 mM-Gly-L-Phe-2-NNap was determined as described in the legend of Fig. 1. MLP fractions were prepared from liver of rats killed at increasing times after injection. The values are given as percentages of the amounts of enzyme released in presence of 0.05% Triton X-100. Means $\pm$ S.D. for three animals are presented; 85–90% of the total cathepsin C activity is released by the treatment, whatever the time after injection.

Fig. 4. Invertase activity unreleasable and releasable by Gly-L-Phe-2-NNap

Invertase activity of MLP unreleasable (●) and releasable (○) by Gly-L-Phe-2-NNap is expressed in units/g fresh wt. Total MLP activity was inferred from the curve given in Fig. 2 and not by taking the individual experimental values. The percentages of release were those presented in Fig. 3. Curves were drawn as described in the Discussion section.

increases with time in a sigmoidal manner; a maximal value is obtained after about 2 h.

Discussion

Our results show that invertase endocytosed by the liver is associated with two types of structures, one that can be disrupted by Gly-L-Phe-2-NNap, and another that cannot. A first possibility is that such a distinction is an artefact and results from the fact that part of the invertase associated with the lysosomes cannot escape in the medium after granule disruption by Gly-L-Phe-2-NNap, because it remains adsorbed to the sedimentable components. There are two arguments against that inter-

pretation. First, when invertase is incubated for 10 min with a MLP fraction in the presence of Gly-L-Phe-2-NNap, only a slight proportion of the enzyme is adsorbed and becomes sedimentable. Secondly, if invertase not releasable by Gly-L-Phe-2-NNap was adsorbed to structures, it is likely that it could not easily be released by hypo-osmotic treatment or by freezing and thawing, as shown above. It seems probable, therefore, that the effect of Gly-L-Phe-2-NNap on invertase results from the presence of the enzyme in two distinct cellular organelles: a non-lysosomal one, A, and a lysosomal one, B.

The way in which the amount of invertase associated with each compartment changes with time agrees well with the hypothesis that A is a pre-lysosomal compartment, i.e. a compartment through which invertase has to travel before reaching the lysosomes. Indeed, the accumulation of the enzyme in A precedes its accumulation in B; later, a steady-state concentration is observed in A, whereas the concentration continues to increase in B. The decrease and finally the disappearance of the invertase in A is parallel with the occurrence of a constant concentration in B. Another kinetic behaviour could be expected if A and B concerned two independent compartments through which invertase has to go after endocytosis. On the other hand, the fact that the invertase concentration in the liver remains constant even after endocytosis has stopped and when all the enzyme is present in B supposes that no elimination or degradation of this molecule occurs for several hours. Accordingly, a simple compartmental model may be used for the kinetic analysis of our results:

As the accumulation of invertase in liver is, for most of the time, independent of the plasma concentration, k_1 is probably a zero-order rate constant. On the other hand, by referring to the period of invertase decrease in A, when endocytosis draws to an end, it may be inferred that k_2 is a first-order rate constant. Accordingly if [A] and [B] are the invertase concentrations in compartments A and B, during endocytosis the following relationships are applicable:

$$\frac{dA}{dt} = k_1 - k_2 [A] \quad (1)$$

$$[A] = \frac{k_1}{k_2} \left(1 - \frac{1}{e^{k_2 t}} \right) \quad (2)$$

$$\frac{dB}{dt} = k_2 [A] \quad (3)$$

$$[B] = k_1 t - \frac{k_1}{k_2} \left(1 - \frac{1}{e^{k_2 t}} \right) \quad (4)$$

When endocytosis is over, i.e. when invertase has been cleared from the blood, the relationships become:

$$\frac{dA}{dt} = -k_2 [A] \quad (5)$$

$$[A] = [A_m] e^{-k_2(t-t_m)} \quad (6)$$

$$[B] = [B_m] + [A_m] \left(1 - \frac{1}{e^{k_2(t-t_m)}} \right) \quad (7)$$

where $[A_m]$ and $[B_m]$ are the amounts found in non-lysosomal and lysosomal compartments respectively, after $t = t_m$, when endocytosis is over.

The values of k_1 and k_2 that best fit our experimental points were calculated with the aid of a computer by supposing that endocytosis was negligible after 90 min (t_m), $k_1 = 0.48$ unit/min per g fresh wt. and $k_2 = 0.042 \text{ min}^{-1}$. These parameters have been used to draw the curves in Fig. 4. According to these estimations, the half-life of the pre-lysosomal compartment through which invertase passes would be approx. 16 min.

Our results show that by monitoring the change of susceptibility to Gly-L-Phe-2-NNap of structures bearing endocytosed invertase as a function of time, a kinetic analysis of the intracellular flux of the compound may be performed. The method is easy and rapid, it is applicable to endocytosis taking place *in vivo*, and it does not require chase experiments. It can probably be used to study the endocytosis of a variety of molecules and of factors able to influence this process.

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