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Evidence for a dipeptide porter in the lysosome membrane

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Small neutral dipeptides such as Gly-Gly are known to cross the lysosome membrane rapidly. The mode of dipeptide translocation was studied, using an osmotic-protection method. Results with dipeptide analogues, such as ω-amino aliphatic acids and taurine, indicated that dipeptides do not cross the rat liver lysosome membrane by unassisted diffusion. Using seven pairs of dipeptide stereoisomers, the penetration of the L-isomer was always found to be much more rapid than that of the D-analogue. It is concluded that the lysosome membrane contains a porter that recognizes and transports L-dipeptides.

Introduction

The mammalian lysosome membrane contains several distinct porters for amino acids, each porter specific for a different range of substrates (for a review, see Ref. 1). These porters are presumably required for the efficient delivery to the cytosol of amino acids generated by intralysosomal proteolysis, although direct evidence for a physiological role is available only for the cystine porter, whose absence, in the human recessively inherited disease cystinosis, leads to the accumulation of cystine in the lysosomes.

A well-established method for determining whether any solute can cross the lysosome membrane measures its ability to afford osmotic protection. Lysosomes themselves contain osmotically active material and rapidly swell and break if suspended in hypotonic solutions. Lysosomes suspended in an iso-osmotic solution of a non-permeant solute are in osmotic balance and are consequently stable. If suspended in an iso-osmotic solution of a permeant solute, lysosomes are initially stable, but swell and break as the solute crosses the lysosome membrane and produces progressive osmotic imbalance. This technique has recently [2] been used to study the passive diffusion of non-electrolytes across the lysosome membrane, and specifically to identify the molecular parameters that determine the rate of diffusion. The advantages and the limitations of the method are discussed fully in Ref. 2; one advantage that is particularly pertinent to the present paper is the opportunity the method affords to study the passage across the lysosome membrane of substances that are not readily available in radiolabelled form.

In an early investigation [3] the osmotic-protection method was applied to amino acids. The results indicated that glycine and L-alanine penetrated very slowly, indeed not significantly more rapidly than molecules accepted as non-penetrants, such as mannitol and sucrose. In contrast to glycine and L-alanine, dipeptides such as Gly-Gly and Gly-L-Ala appeared to traverse the membrane rapidly [3], a finding confirmed and extended by Goldman [4].

In 1971 there was no reason to postulate metabolite porters in the lysosome membrane, and the slow penetration of amino acids was explicable in terms of the poor diffusion of charged molecules across biological membranes. The rapid penetration of dipeptides was more puzzling, as these too are charged, and it was suggested [3] that two features of dipeptides not possessed by amino acids might be responsible. First, dipeptides can exist as *cis*-rotamers, producing a degree of charge-neutralization and thus a less polar species. Secondly, the pK_a (NH_2) of dipeptides is much lower than that of amino acids, so that a significant proportion of the unprotonated species exists at pH 7.

The recent discovery of membrane porters for L-alanine and other neutral amino acids has led us to re-investigate the earlier observations on dipeptides. We have done this by comparing the osmotic protection provided by dipeptides with that afforded by some non-physiological analogues. It was argued that the

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latter are intrinsically unlikely to be substrates for metabolite porters, and that their entry into lysosomes would in most cases be by unassisted diffusion.

Materials and Methods

Materials

Glycine and all ω-amino aliphatic acids except 5-aminopentanoic acid were obtained from BDH Chemicals, Poole, Dorset, U.K.; 5-aminopentanoic acid and all cyclic amides were from Aldrich Chemical Co., Gillingham, Dorset, U.K.; taurine and the dipeptides were from Sigma Chemical Co., Poole, Dorset, U.K. All were of the highest grade available. Solutions (0.25 M) in water were adjusted to pH 7.0 with 1 M NaOH, but not otherwise buffered.

Preparation and incubation of lysosomes

A lysosome-rich fraction of rat liver was prepared, as previously described [2], resuspended in the 0.25 M test solution, and incubated at 25°C. At 0, 30 and 60 min duplicate samples were withdrawn and assayed at 25°C for free and total β-hexosaminidase [2].

Results

As a preliminary test of the charge-neutralization hypothesis, which had been advanced to explain the rapid penetration of Gly-Gly (see Ref. 3 and Introduction), the osmotic protection afforded to rat liver lysosomes by some cyclic amides similar to the *cis*-rotameric form of Gly-Gly was first measured. Table I shows that, in contrast to sucrose and even Gly-Gly, these compounds fail to provide even transient osmotic protection, implying that they cross the lysosome membrane extremely rapidly, presumably by passive diffusion. An alternative explanation of the rapid rupture of lysosomes resuspended in 0.25 M solutions of the cyclic amides, viz. that these substances exert a detergent-like effect, was negated by the finding that lysosomes were stable when suspended in a solution 0.25 M in both sucrose and cyclic amide (Table I). These results with the cyclic amides suggest that the charge-neutralization hypothesis for the rapid entry of dipeptides was worth further investigation.

Table II shows the results of incubating lysosomes at 25°C in 0.25 M solutions of Gly-Gly and of a series of ω-amino-aliphatic acids. Like Gly-Gly, these acids all possess terminal amino and carboxylate (or sulphonate) groups but, in striking contrast to Gly-Gly, they all provided quite prolonged osmotic protection, implying poor penetration rates into the lysosomes. This result effectively refutes the charge-neutralization hypothesis,

TABLE I

Free N-acetyl-β-D-glucosaminidase activity of rat liver lysosomes pre-incubated in 0.25 M solutions of cyclic amides

A lysosome-rich fraction from rat liver was resuspended in the indicated solution, pH 7.0 at 4°C to give a 1:40 (w/v) dilution based on the original liver pulp weight. The resuspended fraction was then incubated at 25°C and samples were removed for assay of *N*-acetyl-β-D-glucosaminidase at the indicated times. Free enzyme activity is expressed as a percentage of the total activity measured in the presence of Triton X-100 (0.2%). Values shown are the mean ± S.D., for the number of experiments given in parentheses.

Solute (s) (0.25 M)	Mol. wt.	Free hexosaminidase (% of total activity)		
		0 min	30 min	60 min
Sucrose	342	9±2 (7)	8±1 (7)	11±2 (7)
Glycylglycine	132	14±2 (3)	79±5 (3)	94±2 (3)
δ-Pentano-lactam	99	85±4 (3)	84±4 (3)	87±1 (3)
δ-Pentano-lactam and sucrose		3±1 (3)	6±0 (3)	13±2 (3)
2-Pyridone	95	83±2 (3)	84±1 (3)	85±1 (3)
2-Pyridone and sucrose		5±0 (3)	8 (2)	11±1 (3)
4-Pyrimidone	96	88 (2)	88 (2)	96 (2)
4-Pyrimidone and sucrose		5±2 (3)	7±2 (3)	9±4 (3)

TABLE II

Free N-acetyl-β-D-glucosaminidase activity of rat liver lysosomes pre-incubated in 0.25 M solutions of ω-amino-aliphatic acids

A lysosome-rich fraction was resuspended, as described in Table I, in 0.25 M dipeptide, pH 7.0, at 4°C, prior to incubation at 25°C. *N*-Acetyl-β-D-glucosaminidase activities were assayed at the indicated times. Free enzyme activity is expressed as a percent of total activity measured in the presence of Triton X-100 (0.2%). Values shown are the mean ± S.D., for the number of experiments given in parentheses.

Solute (0.25 M)	Mol. wt.	pK_a (NH_2)	Free hexosaminidase (% of total activity)		
			0 min	30 min	60 min
Glycylglycine	132	8.2	14±2 (3)	79±5 (3)	94±2 (3)
2-Aminoacetic acid (glycine)	75	9.8	9±2 (4)	16±2 (4)	35±3 (4)
3-Aminopropionic acid (β-alanine)	89	10.2	8±2 (5)	11±2 (5)	23±1 (5)
4-Aminobutyric acid	103	10.6	7±1 (4)	11±1 (4)	19±1 (4)
5-Aminopentanoic acid	117	10.8	10±2 (5)	11±2 (5)	14±3 (5)
6-Aminohexanoic acid	131	10.8	9±1 (30)	13±1 (3)	25±1 (3)
2-Aminoethane sulphonic acid (taurine)	125	8.7	15±1 (3)	18±1 (3)	27±2 (3)

TABLE III

Free N-acetyl-β-D-glucosaminidase activity of rat liver lysosomes pre-incubated in 0.25 M solutions of dipeptide stereoisomers

A lysosome-rich fraction was resuspended, as described in Table I, in 0.25 M dipeptide, pH 7.0, at 4°C, prior to incubation at 25 °C. *N*-Acetyl-β-D-glucosaminidase activities were assayed at the indicated times. Free enzyme activity is expressed as a percent of total activity measured in the presence of Triton X-100 (0.2%). Values shown are the mean ± S.D. for three experiments, except values for D-Ala-D-Ala which are means of two experiments.

Dipeptide (0.25 M)	Mol. wt.	Free hexosaminidase (% of total activity)		
		0 min	30 min	60 min
Gly-Gly	132	14±2	79±5	94±2
Gly-L-Ala	146	10±2	53±5	71±4
Gly-D-Ala	146	11±4	18±5	32±6
L-Ala-Gly	146	10±2	80±4	87±2
D-Ala-Gly	146	8±1	26±7	56±8
L-Ala-L-Ala	160	13±6	85±4	86±6
D-Ala-D-Ala	160	8	8	10
Gly-L-Ser	162	11±3	62±9	77±6
Gly-D-Ser	162	9±2	14±2	18±3
Gly-L-Val	174	12±1	21±7	47±9
Gly-D-Val	174	12±3	13±1	16±2
Gly-L-Thr	176	13±1	52±8	74±0
Gly-D-Thr	176	12±2	16±3	24±1
Gly-L-Asn	189	14±4	29±6	43±3
Gly-D-Asn	189	11±3	15±1	18±1

since 5-aminopentanoic acid should be as capable as Gly-Gly of adopting a *cis*-conformation.

There is a significant difference between Gly-Gly and 5-aminopentanoic acid, however: the pK_a (NH_2) value of the latter is 10.8, 2.6 units higher than that of the former (see Table II). The much more rapid entry of Gly-Gly could therefore be due to the presence of significant amounts of the unprotonated form at pH 7.0 (Ref. 3 and Introduction). However, the prolonged osmotic protection provided by taurine (Table II), whose pK_a (NH_2) is only 0.5 units above that of Gly-Gly, but 1.5 below that of its carboxylate-analogue β-alanine, makes it impossible to sustain this alternative argument that the rapid influx of dipeptides is due to the penetration of the unprotonated species.

Table III shows the osmotic protection provided by a series of L- and D-dipeptides. The D-isomers consistently afford much better protection: at 30 min of incubation, the free activities for the D-isomers were between 8 and 18%, whereas those for the L-isomers were between 29 and 95%. Within the D-series, the osmotic protection seen with some dipeptides, such as D-Ala-D-Ala, was prolonged and as good as that seen with sucrose, whereas with others, notably D-Ala-Gly, the free activity rose between 30 and 60 min. In the L-series, patterns of loss of latency fell into three broad categories: the slow (Gly-Asn, Gly-Val), the medium (Gly-Ala, Gly-Leu, Gly-Ser, Gly-Thr), and the rapid (Ala-Ala, Ala-Gly, Gly-Gly).

Discussion

We have shown that many of the non-physiological dipeptide analogues investigated here provide quite prolonged osmotic protection to isolated rat liver lysosomes. The implication is that such substances, which include D-Ala-D-Ala, Gly-D-Val and 5-aminopentanoic acid, have a very limited capacity to enter the lysosomes by passive diffusion. It has recently been shown, with a large series of non-electrolytes, that the rate of passage of a substance across the lysosome membrane correlates inversely with the molecule's hydrogen-bonding capacity [2]. The hydrogen-bonding capacity of the dipeptide analogues is estimated as at least 11 [2], a value at the extreme top end of the range of penetrant molecules. The present results for the dipeptide analogues are in close agreement with those for non-electrolytes of similar hydrogen-bonding capacity.

Although the rate of loss of latency varied from one dipeptide to another, within both the L-series and the D-series, the rate was in every case much higher for an L-dipeptide than for its D-analogue. This result clearly implicates some mechanism capable of stereochemical differentiation. There are two possibilities. The simplest is that the lysosome membrane contains a porter or porters capable of transporting L-dipeptides. The second explanation is that no porter exists, but that the L-dipeptides on entering the lysosome by passive diffusion are hydrolysed by a dipeptidase, yielding amino acids incapable of leaving the lysosome as rapidly as they are generated, and thus leading to osmotic imbalance. This latter mechanism was proposed by Goldman [4] to explain the rupture of lysosomes incubated in 0.25 M sucrose containing 10 mM L-Ala-L-Ala; rupture was not seen, however, with 10 mM D-Ala-D-Ala.

We have concluded that it is not possible to explain our results, nor indeed those of Goldman [4], without postulating that L-dipeptides enter lysosomes on a porter.

Let us first propose that no such porter exists. L-Dipeptides must then cross the lysosome membrane solely by passive diffusion. The rate at which this occurs in osmotic-protection experiments is indicated by the rate of latency loss seen with a dipeptide's D-analogue. In all cases this rate was insufficient to generate significant latency loss within the first 30 min of incubation. Even if hydrolysis of L-dipeptide within the lysosome was as rapid as its diffusion into the lysosome, the rate of accumulation of the amino acid products could never exceed twice the rate of entry of the dipeptide. It would be less if any of the amino acid products left the lysosome by diffusion or on any of the amino acid carriers now known to exist. This maximum rate of accumulation appears totally insufficient to explain the rapid lysis of lysosomes observed in our experiments with L-dipeptides. Similarly, in Goldman's [4] experi-

ment, it is inconceivable that 10 mM L-Ala-L-Ala could generate sufficient L-alanine in the lysosome if its entry was solely by passive diffusion. The rate of entry by passive diffusion would be only 4% of that from a 0.25 M solution of D-Ala-D-Ala.

It therefore seems inescapable that there is a porter in the lysosome membrane capable of mediating the rapid entry of L-dipeptides. The different rates of lysosome rupture seen with different dipeptides must reflect different affinities or maximum velocities, plus in some cases, for example L-Ala-L-Ala, the added effect of intralysosomal hydrolysis to amino acids. The lysosome rupture seen with a few D-dipeptides, such as D-Ala-Gly, presumably indicates that these are carried by the porter.

As previously mentioned, the lysosome membrane contains several porters for amino acids. It is therefore at first sight surprising that amino acids such as L-alanine provide such good osmotic protection [3]. A satisfactory explanation can be found in the K_m values reported for these porters. These are all in the micromolar range and, as pointed out [5] in the context of glucose transport across the lysosome membrane, such porters will be ineffective in osmotic-protection experiments, where the external concentration of solute is 250 mM. This is because, within a few seconds, the concentration of solute both outside and inside the lysosome will grossly exceed the K_m, resulting in a net transport rate of effectively zero. The putative dipeptide porter is clearly effective at pH 7 at mediating net transport in osmotic-protection experiments. We deduce from this that its K_m for dipeptides must lie well into the millimolar range.

This paper presents the first major study of the rates of transfer across the lysosome membrane of a wide range of doubly charged molecules whose net charge at neutral pH is close to zero. Although the method provides an indirect, rather than a direct, measure of transfer rates (see Ref. 2 for further discussion), it is the only practicable experimental approach. We believe that the results provide sufficiently strong evidence of a dipeptide porter to justify the custom synthesis of a few radiolabelled dipeptides, so that the characteristics of the porter or porters can be identified. This work is in hand in our laboratory.

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