An Active Mechanism for Completion of the Final Stage of Protein Degradation in the Liver, Lysosomal Transport of Dipeptides*

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Lysosomes are major sites for protein degradation in the liver. Amino acids and dipeptides are the final products of proteolysis within this organelle (1, 2). These products must be exported into the cytosol if lysosomes are to retain their biological integrity. Failure to export may result in pathological consequences. This is evidenced by the genetic defect in the export of cysteine, which results in accumulation of high concentrations of this amino acid in the lysosomes causing cystinosis. Cystinosis is a progressive systemic disease with many pathological expressions (3).

Although as yet no genetic disease of dipeptide accumulation in lysosomes has been described, there is evidence that such an accumulation would also have pathological effects. This is evidenced by the osmotic protection experiments which determine the rates of release of lysosomal enzymes. Lloyd (4) showed that incubation of lysosomes isolated from rat liver with 0.25M solutions of dipeptides or their constituent amino acids increased the release of their enzyme contents. Furthermore, the release was greater with dipeptides than with their constituent amino acids. He suggested that amino acids and dipeptides entered the lysosomes, and the entry is more efficient for dipeptides than for amino acids. Recently, Bird and Lloyd (5) found that the rate of enzyme release was greater with dipeptides containing L-isomers than D-analogs, suggesting a stereospecificity in lysosomal permeation of dipeptides. However, as reviewed by Forster and Lloyd (6), the osmotic protection technique does not allow any conclusion regarding the mechanism which a solute uses for entry into the lysosomes. For example, the greater rate of enzyme release by dipeptides containing L-rather than D-amino acids could be explained by the hydrolysis of dipeptides within the lysosomes which is stereospecific (7). This is consistent with a more recent study of Bird and Lloyd (8) who found a positive correlation between ability of dipeptides to disrupt lysosomes and their susceptibility to hydrolysis by lysosomal enzymes.

To determine whether the liver lysosomal membrane indeed possesses a dipeptide transporter, in the present experiments we have used a membrane vesicle uptake technique which has been validated for the studies of solute transport by the liver lysosomes. Jonas and Jobe (9) showed that properties of rat liver lysosomal membrane, like the proton-pumping ATPase activity, are preserved in vesicle preparations. For the present experiment we used labeled glycylglutamine (Gly-Gln) as the probe because we have previously validated the use of this dipeptide for characterization of oligopeptide transport in membrane vesicles prepared from kidney and intestine (10, 11).

EXPERIMENTAL PROCEDURES

Materials—Adult male Harlan Sprague Dawley rats with body weights of 250–300 g were purchased from Zivic-Miller. Custom synthesized glutamine-3,4-3H-glycylglutamine (49 Ci/mmol) was obtained from DuPont NEN. Amino acids, oligopeptides, carbonyl cyanide m-chlorophenylhydrazone (CCCP); and acridine orange dye were purchased from Millipore Corp., Bedford, MA.

Preparation of Lysosomal Membrane Vesicles—Rats were killed and their livers were removed for preparation of lysosomal membrane vesicles (LMV). Vesicles were prepared as described by Symons and Jonas (12). Briefly, livers were homogenized in buffer 1 (250 mM sucrose, 20 mM Hepes, 1 mM EDTA, pH 7.0, with Tris base) containing 1 mg/ml leupeptin, using a Dounce homogenizer. The liver homogenate was suspended at an average protein concentration of 35 mg/ml in buffer 1 and centrifuged for 20 min at 750 g. The nuclear pellet was discarded and the centrifugation was repeated. The resulting supernatant was centrifuged for 10 min at 20,000 × g, and the pellet was resuspended in buffer 2 (250 mM sucrose, 20 mM Hepes, pH 7.0, with Tris base) and recentrifuged for 10 min at 20,000 × g. The final pellet was suspended in 9 ml of buffer 2 and mixed with 11 ml of isotonic Percoll (1 ml of 2.5 M sucrose, 200 mM Hepes/Tris, pH 7.0, mixed with 9 ml of Percoll). The resulting Percoll mixture was centrifuged for 90 min.

The abbreviations used are: CCCP, carbonyl cyanide m-chlorophenylhydrazone; LMV, lysosomal membrane vesicles; Mes, 4-morpholinethanesulfonic acid.

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at 40,000 × g. The dense brownish lysosomal band near the bottom of the gradient was removed and diluted with buffer 2 and centrifuged for 10 min at 20,000 × g. The resulting pellet containing lysosomes was incubated in freshly prepared solution containing 5 mM methionine methyl ester, 2 mg/ml bovine serum albumin, 2 mM magnesium chloride, 2 μg/ml leupeptin, and 1 μg/ml pepstatin for 15–20 min at 37 °C. Following this treatment, an equal volume of ice-cold isotonic Percoll was added. The mixture was centrifuged for 30 min at 35,000 × g. A dark brown band located at the top of the dense gradient containing purified LMV was removed and diluted in buffer 2 and centrifuged at 20,000 × g. The purified LMV pellet was resuspended in transport buffer sufficient to provide 3–5 mg/ml protein content. All of the above procedures were done at 4 °C. Protein content of the preparation was determined by a modified method.

### Table I

| Enzyme                        | Homogenate | Membrane vesicle |
|-------------------------------|------------|------------------|
| β-Hexosaminidase              | 47 ± 4.50  | 495 ± 24         |
| Cytochrome c oxidase          | 1.91 ± 0.29| 0.35 ± 0.14      |
| 5'-Nucleotidase               | 2.42 ± 0.09| 1.40 ± 0.14      |
| β-Glucosidase                 | 1.02 ± 0.07| 1.10 ± 0.12      |

An ice-cold solution was prepared containing 23 mM HEPES/Tris (pH 7.0), 23 mM NaCl, 6 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, and 0.1% Nonidet N-40. The solution was preincubated with 50 μM valinomycin and incubated with 180 mM sucrose for 60 min. The initial 2 min of incubation all of the uptake values were significantly greater when the outside pH was 5.0 than when both pH values were 7.0.

### Gly-Gln Uptake by LMV—Uptake studies by LMV were performed at 23 °C using a rapid filtration technique as described previously (10). Gly-Gln uptake was initiated by mixing 20 μl of membrane suspension (preloaded with 100 mM KCl, 100 mM sucrose, 20 mM HEPES/Tris at pH 7.0) preincubated with 50 μM valinomycin and incubated with 180 μM of transport buffer containing 0.1 mM Gly-3H-Gln. Composition of the transport buffer varied with different experiments and is described in the figure legends. Incubation of vesicles was terminated by injecting 20 μl of vesicle mixture into 2 ml of ice-cold stop solution (same composition of the transport buffer but without Gly-3H-Gln), followed by filtration. The filters were then washed with 5 ml of ice-cold stop solution. The radioactivity associated with filters was counted in a Beckman scintillation spectrometer. Nonspecific binding of the Gly-3H-Gln was determined by adding the transport solution and vesicles directly to the respective ice-cold stop solution, followed by filtering, washing, and counting.

### Calculation and Statistics—Kinetic constants of Gly-Gln transport were determined by applying a nonlinear regression method to the Michaelis-Menten kinetic equation using Graftit (Sigma): 
\[ V = \frac{V_{\text{max}} [S] \cdot K_{\text{m}} + [S]}{K_{\text{m}} + [S]} \]
where \( V \) is Gly-Gln uptake in picomoles per milligram of protein per 45 s, \( [S] \) is the external Gly-Gln concentration in millimolar, \( V_{\text{max}} \) is the maximal Gly-Gln uptake, and \( K_{\text{m}} \) is the concentration of [S] that yielded one-half \( V_{\text{max}} \). Each rate of Gly-Gln uptake, which was corrected for nonspecific binding, is given as mean ± S.E. of three replicates. Significant differences between values were determined by Student's t test.

### RESULTS

#### Purity of LMV—As shown in Table I, there was no enrichment of either cytochrome c oxidase (mitochondrial membrane) or 5'-nucleotidase (plasma membrane). In contrast, there was a more than 100-fold enrichment of a lysosomal membrane enzyme (β-glucosidase). Furthermore, there was only a modest enrichment of our preparation with a non-membrane-associated lysosomal enzyme (β-hexosaminidase).

#### Establishing Uptake as Transport—Preliminary experiments revealed that there was uptake of Gly-Gln by LMV. To determine whether the observed Gly-Gln uptake by LMV was indeed transport into vesicles, the following experiment was performed. The rates of Gly-Gly uptake were measured as a function of medium osmolality. Details of these experiments are presented in the legend to Fig. 1. The results showed that the rate of uptake decreased as a linear function of the reciprocal of medium osmolality. Extrapolation of the data to infinite osmolality showed no uptake, indicating the Gly-Gln was entirely or mostly transport into an osmotically reactive space. The data also indicate that the vesicles were properly released during preparation for uptake studies.

### Driving Force for Dipeptide Transport—To investigate the driving force for dipeptide transport we determined lysosomal uptake of Gly-Gln in the presence and absence of a sodium or a proton gradient. Details of these experiments are presented in the legend to Fig. 2. As shown in this figure, the imposition of an inwardly directed sodium gradient (Na⁺ concentration) had no effect, but the imposition of an inwardly directed proton gradient (pH) transiently stimulated Gly-Gln uptake by LMV. During the initial 2 min of incubation all of the uptake values were significantly (p < 0.01) greater when the outside pH was 5.0 and the inside pH was 7.0 than when both pH values were 7.0.

To investigate whether the above proton gradient indeed served as the driving force for Gly-Gln transport, the following experiment was performed. Prior to imposition of the proton gradient, the vesicles were preincubated with a proton ionophore (CCCP). As shown in Fig. 2, CCCP did not reduce Gly-Gln uptake by LMV, suggesting that the proton gradient was not the driving force in this case.

To investigate whether membrane potential influences transport of Gly-Gln by the liver LMV, the following experiment was performed. The effect of K⁺-generated diffusion potential (inside negative) in the presence and absence of an acidic pH (5.0) in the transport medium was determined. The membrane potential was generated by replacing the KCl in the transport medium with 100 mM choline chloride while maintaining the inside concentration of KCl at 100 mM. Results showed that imposition of membrane potential, when the pH of the transport medium was 7.0, transiently stimulated Gly-Gln uptake.
uptake by the liver LMV (Fig. 3). All of the uptakes during the initial 2 min of incubation were significantly (p < 0.01) greater in the presence than in the absence of membrane potential. The stimulatory effect of membrane potential was greatly enhanced by acidification of the transport medium, resulting in a near 2-fold overshoot in transport of Gly-Gln at 2 min. Therefore, the presence of a membrane potential and an acidic pH was necessary for observing uphill transport of Gly-Gln.

Characterization of Transport—To investigate the affinity and the number of transporters involved in the uptake of Gly-Gln by membrane vesicles we investigated the rate of uptake as a function of dipeptide concentration. The rates of uptake by the liver LMV were determined in the presence of both a membrane potential and an acidic pH. Before this experiment, it was necessary to determine for how long the rate of Gly-Gln transport was linear. The results showed that for at least 50 s the rate was linear (data not shown); therefore, 45 s was chosen as the incubation time for the experiment detailed in the legend to Fig. 4. Eadie-Hofstee plot of data (Fig. 4) showed the presence of a single system for transport of Gly-Gln. The calculation of kinetic constants by the Michaelis-Menten equation showed a low affinity transporter with a $K_m$ value of 4.67 ± 0.80 mM and a $V_{max}$ value of 880.38 ± 3.81 pmol/mg protein/45 s.

Relationship between Transport of Gly-Gln and $H^+$—We used the fluorescent dye acridine orange technique to determine whether there is a coupling between proton and Gly-Gln transport. The results are shown in Fig. 5. There was a less than 5% change in the fluorescence when the liver LMV were added to the medium containing no dipeptide. The fluorescence change became appreciably greater (more than 11%) when the medium contained Gly-Gln.

The results of the above experiment suggested that Gly-Gln and $H^+$ are cotransported into the vesicles. To investigate the kinetics of this interaction, we investigated the effect of a range of pH on Gly-Gln transport (Fig. 6). There was a steady decline in the rate of Gly-Gln uptake by the liver LMV when the pH of the transport medium was increased above 5.0 (Fig. 6, right inset). There was a hyperbolic relationship between the rates of Gly-Gln uptake and $H^+$ concentration (Fig. 6). The linearity of the Eadie-Hofstee transformation of the data (Fig. 6, left inset) showed a coupling ratio of 1 (1.036 ± 0.192). Calculation of kinetic constants by the Michaelis-Menten equation showed a $K_m$ value of 0.36 ± 0.02 mM ($pK_a = 6.4$) and $V_{max}$ of 57.01 ± 1.43 pmol/mg protein/45 s.

Substrate Specificity of the Transporter—To investigate the substrate specificity of the transporter in the liver LMV, we determined the inhibitory effect of representative groups of amino acids, dipeptides, and tripeptides on Gly-Gln uptake. The results showed (Table II) inhibition by dipeptides and tripeptides but not by amino acids. Due to poor solubility of tetrapeptides at 30 mM concentration, the effect of these oligopeptides on Gly-Gln uptake was not studied.
Composition of the incubation medium was as follows: 6 m
continuously stirred during the experiment.

Dipeptides and tripeptides but not amino acids, and it
lated by an acidic pH and membrane potential, its substrates
(16). For example, it is capable of uphill transport, it is stimu-
cated in the brush border membrane of kidney and intestine
functional similarities between this transporter and those lo-
lysosomes. The information obtained reveals that there are
terization of a peptide transporter in the membrane of liver

Before the addition, LMV were preloaded with 100 mM sucrose, 100 mM
orange, 100 mM sucrose, 100 mM KCl, and 20 mM Hepes/Tris (pH 6.5).

FIG. 5. Effect of Gly-Gln on H⁺ transport. Using an α scan fluo-
rescence spectrophotometer, the fluorescence of the incubation medium
containing either no (A) or 5 mM (B) Gly-Gln was monitored for 1.5 min
before and for 3.5 min after the addition of 40 µl of LMV to the cuvettes.

FIG. 6. Gly-Gln uptake as a function of H⁺ concentration. LMV
were preloaded with 100 mM KCl, 100 mM sucrose, and 20 mM Hepes/Tris
(pH 7.0) and preincubated for 30 min with 50 µM valinomycin.

LMV were preloaded with 100 mM KCl, 100 mM Hepes/Tris (pH 7.0) and preincubated for 30 min with 50 µM valinomycin.

The present results suggest that the driving force for uphill
transport is membrane potential which normally exists in ly-
sosomes (inside positive/outside negative). At the pH used in
the present experiment (4.5–7.0) Gly-Gln is in zwitterionic
form. Therefore, its uptake does not add any electrical charge
to the transporter. On the other hand, binding of a proton adds
a positive charge to the transporter, making it more available
for the influence of membrane potential.

The present results may provide an explanation for the dis-
parity among the results of previous studies looking for the
presence of an oligopeptide transporter in the liver. Lombardo
et al. (18), using functional analysis, did not find any evidence
for the presence of a dipeptide transporter in plasma mem-
brane of rat liver. On the other hand, Fei et al. (19), using
Northern analysis, found expression of Pept-1 mRNA in the
liver. In view of the present data, it is possible that the basis for
a positive Northern analysis in the liver (19) is the presence of
an oligopeptide transporter in lysosomes. This suggestion is
based on reports of a strong homology between genes encoding
different oligopeptide transporters (20).

DISCUSSION

The present study is the first step in the functional charac-
terization of a peptide transporter in the membrane of liver
lysosomes. The information obtained reveals that there are
functional similarities between this transporter and those lo-
cated in the brush border membrane of kidney and intestine
(16). For example, it is capable of uphill transport, it is stimu-
lated by an acidic pH and membrane potential, its substrates
include dipeptides and tripeptides but not amino acids, and it
shows a 1:1 stoichiometry between cotransport of Gly-Gln and H⁺.

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We think that the physiological function of the transporter
we have characterized in the lysosomal membrane is mainly to
export rather than to import dipeptides because (a) previous
studies have shown that the lysosomal carriers can be demon-
strated by either uptake or efflux studies (21); (b) as already
mentioned, the vesicles have both inside-out as well as outside-
out orientations; and (c) it is generally believed that the condi-
tions across lysosomal membranes are the determinant of sol-
ute flux. For example, the inside pH is highly acidic, while the
outside pH is above neutral. As we have shown in the present
study, these conditions greatly favor transport in the direction
of cytosol.

The Kₘ of lysosomal oligopeptide transporter (4.67 mM) is
considerably greater than the Kₘ of lysosomal amino acid
transporters, which ranges between 0.01 and 0.5 mM (6). Usu-
ally a greater Kₘ is associated with a greater transport capacity.
This may explain the efficiency of dipeptides over amino
acids in causing the release of enzymes when lysosomes were
incubated with these substrates in vitro (4).

Finally, the present finding leads to a new concept in protein
catabolism. Hydrolysis of proteins, whether in the gut lumen,
renal tubules, or in liver lysosomes may result in production of
colosiderable amounts of dipeptides (1, 2, 22, 23). However, the
hydrolyase activity against dipeptides as studied in the intestine
(24, 25) is either totally or mostly located in the cell cytoplasm.
Furthermore, rates of hydrolysis are much greater when dipep-
tides are incubated with liver homogenates than with liver
lysosomes (26). Therefore, oligopeptide transporters, whether
located in the brush border or lysosomal membranes, appear to
serve an important physiological function. They provide an

| Substrate       | % uptake |
|-----------------|----------|
| Control (0.1 mM)| 100      |
| Gly-Gln         | 100      |
| Glutamine       | 100      |
| Gly-Sar         | 16       |
| Gly-Pro         | 0        |
| Gly-Gly-Gly     | 29       |
| Val-Ala-Leu     | 0        |

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active mechanism for transporting dipeptides from a region of low to a region of high dipeptidase activity. In this manner, they participate in completion of the final stage of protein degradation.

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