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Evidence for an essential histidine residue located in the binding site of the cysteine-specific lysosomal transport protein

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Abstract

Previously, we observed that the activity of the cysteine-specific lysosomal transport system increases 7–10-fold between pH 6 and 7.3 to be maximally active in the neutral pH range. To understand what factors contribute to this pH dependence, different chemical modifying agents were used to probe the nature of amino acid residues residing in the transport protein binding site. Diethyl pyrocarbonate (1 mM) and *N*-ethylmaleimide (5 mM) each strongly inactivated lysosomal cysteine uptake $\geq$ 88%, whereas dicyclohexylcarbodiimide (2.5 mM), phenylisothiocyanate (2 mM), *N*-acetylimidazole (33 mM), and phenylglyoxal (2 mM) had a moderate to small effect. Maximal inactivation by DEPC occurs within 12–15 min upon exposure to DEPC concentrations $\geq$ 1 mM. DEPC inactivation is consistent with modification of a histidine residue, displaying no inactivation at pH $<$ 6, half-maximal inactivation at pH 6.6, and maximal inactivation at pH $\geq$ 7.3. The close correspondence of DEPC inactivation to the pH activity curve of cysteine uptake suggests the large increase in lysosomal cysteine transport activity between pH 6 and 7.3 reflects deprotonation of an essential histidine residue. The substrate, L-cysteine (4 mM), fully protects the transport protein from DEPC inactivation suggesting that this histidine residue is located in the carrier's substrate binding site. Finally, part of the pH dependence of the lysosomal cysteine carrier appears to be due to responsiveness to the lysosomal transmembrane proton gradient as indicated by lysosomal membrane vesicles which display a 1.5-fold greater rate of cysteine uptake when pH 7.4_{out} > pH 5.3_{in} than when pH 7.4_{out} = pH 7.4_{in}.

Keywords: Lysosome; Transport; Cysteine; Carrier protein; Histidine; Diethyl pyrocarbonate

1. Introduction

Lysosomes are a major intracellular site for the degradation of a wide variety of macromolecules including proteins, complex carbohydrates, nucleic acids, and glycolipids. A large repertoire of hydrolytic enzymes undertake this task of degrading macromolecules within the lysosomal compartment, yielding metabolites that are re-utilized for biosynthetic reactions in other cellular compartments. Recently, we described a cysteine-specific lysosomal transport system which displays high specificity for cysteine and is responsible for sequestering cysteine into the lysosomal compartment [1]. In this previous study, at least 50–60% of the total radioactivity taken up by human fibroblasts during a 2–5 min incubation with 20 μM L-[^{35}S]cysteine was found to be sequestered into the lysosomal compartment in the form of cysteine. This large percentage of cysteine accumulation into lysosomes is especially significant upon considering that lysosomes comprise only 4% of the intracellular volume of normal human fibroblasts [2]. The physiological importance of cysteine sequestration into lysosomes by the cysteine-specific lysosomal transport route may be to aid lysosomal proteolysis by: (1) maintaining the catalytic activity of thiol-dependent lysosomal enzymes and (2) disrupting protein disulfide bridges at susceptible linkages by sulfhydryl/disulfide exchange reactions, thereby allowing proteins to unfold, facilitating their degradation [1,3–5]. Since the description of this transport system, several studies have been published demonstrating the importance

Abbreviations: DEPC, diethyl pyrocarbonate; Mops, 3-(*N*-morpholino)propanesulfonic acid; PBS, phosphate-buffered saline; DTT, dithiothreitol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; hex, β-*N*-acetylhexosaminidase activity; Mes, 2-(*N*-morpholino)ethanesulfonic acid.

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of disulfide bond reduction and thiols in lysosomal function. Barrueco et al. [6] recently showed that degradation of methotrexate polyglutamates within the lysosomal compartment is highly dependent on the presence of reduced sulfhydryls and that cysteine ably fulfills this requirement. Collins, Unanue, and Harding [7] have demonstrated that reduction of disulfide bonds within lysosomes is a key step in antigen processing by macrophages suggesting that: (a) protein unfolding by reduction of disulfide bonds is a minimum processing requirement for at least some T cell epitopes, (b) disulfide bond reduction relaxes antigen structure allowing lysosomal proteinases to gain access to more potential cleavage sites, thus enhancing proteolysis and thereby producing a larger variety of potentially immunogenic peptides, and (c) T cell epitopes may contain cysteine residues that are disulfide bonded within native antigens; recognition of these epitopes may require disulfide bond reduction to produce linear peptides in which the free thiol of cysteine residues is maintained in its reduced state. This latter possibility has been demonstrated by Jensen who showed that presentation of the major immunogenic epitope of insulin has an absolute requirement for disulfide bond reduction and that one likely site for this reduction is the lysosomal compartment [8].

Further investigation of the cysteine-specific lysosomal transport system may increase our understanding of the role which this transport system plays in maintaining thiol levels within the lysosomal compartment within various tissues, and in health and disease. Understanding the pH dependence of this carrier is key to understanding its ability to sequester cysteine into the lysosomal compartment. In this study, chemical modifying agents have been used to probe the nature of the amino acid residues required for lysosomal cysteine transport as a means to understand the pH dependence of this carrier, and provide valuable information to guide future efforts in isolating the transport protein.

2. Materials and methods

2.1. Cell culture and preparation of percoll-purified lysosomes

Normal human fetal skin fibroblast cell lines (GM0010) were obtained from the Human Genetic and Mutant Cell Repository. Fibroblasts were grown and maintained in an atmosphere of 95% air/5% CO_2 in 100 mm tissue culture dishes or 850 cm^2 roller bottles in Coon's modification of Ham's F-12 medium (Sigma) supplemented with 10% fetal bovine serum. Confluent human fibroblast monolayers were routinely split 1:4 when passed using a CTC mixture consisting of 0.09% trypsin, 0.025% collagenase (Sigma, type IV,570 units/mg), and 2.2% heat-inactivated chicken serum in Ca^{2+}-, Mg^{2+}-free Hank's salt solution, pH 7.6 containing 2 mM Na_2EDTA [9]. Fibroblasts were not used beyond passage 19. Fibroblast lysosomes were purified on a 31.7% Percoll density gradient as described previously [10], and generally were resuspended in 0.25 M sucrose or as indicated. Typically, four to six confluent roller bottles of fibroblasts were required to provide a sufficient quantity of lysosomes for accurate measurement of lysosomal cysteine uptake.

2.2. Chemical modification studies

Percoll-purified lysosomes were suspended in different buffers for different time intervals depending on the chemical modifying agent used. These conditions were: 0.25 mM–2.0 mM diethyl pyrocarbonate (DEPC) in 50 mM potassium phosphate pH 7.3 buffer containing 0.25 M sucrose for 30 min at 30° C, or with 5 mM *N*-ethylmaleimide in 40 mM Mops/Tris pH 7.4 buffer containing 0.25 M sucrose for 35 min at 30° C, or with 2.5 mM dicyclohexylcarbodiimide in 50 mM potassium phosphate pH 7.3 buffer containing 0.25 M sucrose for 40 min at 30° C, or with 2 mM phenylglyoxal in 40 mM Mops/Tris pH 7.0 buffer containing 0.25 M sucrose for 30 min at 30° C, or with 2 mM phenylisothiocyanate in 40 mM Mops/Tris pH 7.0 buffer containing 0.25 M sucrose for 30 min at 25° C, or 33 mM *N*-acetylimidazole in 50 mM potassium phosphate pH 7.3 buffer containing 0.25 M sucrose for 40 min at 30° C. The above incubations were generally performed in a final volume of 0.15–0.2 ml and then terminated by diluting to 1.5 ml with wash buffer (20 mM Mops/Tris pH 7.0 buffer containing 0.275 M mannitol) followed by centrifugation for 15 min in an Eppendorf microcentrifuge at $15\,600 \times g$ at 4° C. The supernatant was discarded, lysosomal pellets were washed once more with 1.5 ml of ice-cold wash buffer, centrifuged as above. The final pellet was gently resuspended by up and down pipetting in 60–80 μl of ice-cold 40 mM Mops/Tris pH 7.0 buffer containing 0.25 M sucrose immediately before the 5 min cysteine uptake time-course assay. An exception to the above washing procedure is that 10 mM imidazole was included in the first wash for terminating DEPC incubations.

2.3. Five minute cysteine uptake time-course assay with isolated lysosomes

Lysosomes (30–60 μl) in 40 mM Mops/Tris pH 7.0 buffer containing 0.25 M sucrose were incubated at 37° C with 30–50 μl of 30–40 μM L-[^{35}S]cysteine in 20 mM Mops/Tris pH 7.0 buffer containing 0.25 M sucrose and 2 mM dithiothreitol. Aliquots of equal volume were removed from the incubation mixtures at time points of 0.5′, 1.5′, 2.5′, 3.5′, and 5′. These aliquots were mixed with 12 ml of ice-cold phosphate-buffered saline (PBS) and the lysosomes were collected by filtration through a 24 mm GF/A glass fiber filter (Whatman). The filters were washed twice with 12 ml portions of ice-cold PBS, mixed with 16 ml of

Cytoscint (ICN), vortexed, and counted for radioactivity. Blanks were prepared by substituting suspension buffer for lysosomes in the above incubation mixtures. Radioactivity for the blanks was subtracted from the radioactivity obtained for the lysosome-containing samples. Time-courses were linear for the first 4–5 min of cysteine uptake. The initial rate of cysteine uptake for each incubation condition then was determined by linear regression analysis of the data using the Kaleidagraph software program (Synergy Software) and the data is expressed as pmol of L-cysteine taken up per min per μg of lysosomal protein or per unit of β-hexosaminidase activity.

The L-[^{35}S]cysteine uptakes performed to assess the pH dependence of lysosomal cysteine uptake did not involve a 5 min time-course assay but rather uptakes were performed in duplicate for a 3.5 min incubation period at each indicated pH value.

2.4. L-[^{35}S]Cysteine uptake using lysosomal membrane vesicles

Percoll-purified lysosomes, obtained from 6–8 roller bottles of human fibroblasts, were hypotonically lysed by suspending the lysosomal pellet in 3.0 ml of 10 mM Mops/Tris pH 7.6 buffer containing 1 mM $MgSO_4$, 1 mM DTT, 25 μg/ml pepstatin, and 5 μg/ml of leupeptin, antipain, and aprotinin. The suspension was up and down pipetted five times, allowed to stand on ice for 15 min, mixed again, then centrifuged at 48 000 $\times$ g for 30 min at 4° C in a Sorvall SS-34 rotor. The lysosomal membrane pellet was resuspended in 0.1–0.2 ml of either 20 mM Mops/Tris pH 7.4 buffer or 20 mM Mes/Tris pH 5.3 buffer, resulting in the spontaneous formation of lysosomal membrane vesicles. L-[^{35}S]Cysteine uptakes were performed by mixing lysosomal membrane vesicle suspension with the radioactive substrate at 37° C as described in the figure legends. Uptakes at a given time point were terminated by placing an aliquot from the incubation mixture onto a cold Sephadex G-50 column (0.8 $\times$ 7.0 cm, in a KONTES Disposaflex column) equilibrated with 20 mM Na-Hepes pH 7.4 buffer at 4° C. Columns were washed with 1.5 ml of ice-cold column buffer; the eluant from this wash was collected directly into scintillation vials, mixed with 16 ml of Cytoscint, and counted for radioactivity in a Beckman LS-100 scintillation counter. This chromatography method allows lysosomal membrane vesicles to be separated from extravesicular radioactivity yielding low background radioactivity as described by Mancini et al. [11].

2.5. β-N-Acetylhexosaminidase and protein assay

The integrity of individual lysosomal preparations was generally found to be 80–90% intact as judged by the latency of β-N-acetylhexosaminidase activity, which was calculated as the difference in hexosaminidase activity in the presence or absence of 0.1% Triton. β-N-Acetylhexosaminidase activity (hex) was determined as described previously [12].

Lysosomal protein was quantified using bicinchoninic acid as described in the precipitation assay of Brown et al. [13].

2.6. Miscellaneous

L-[^{35}S]Cystine (400–500 Ci/mmol, 1 mCi/ml) was obtained from Dupont/NEN and was purified either as described previously by chromatography on Dowex 50W-X8 cation exchange resin (1) and/or by high voltage electrophoresis. High voltage electrophoresis was performed by streaking 0.1–0.2 ml of concentrated radioactive material onto the origin of electrophoresis paper immediately followed by electrophoresis for 25 min at 3500 V in 6% formic acid using a CAMAG high voltage electrophoresis apparatus [14]. Cystine standards bordering the radioactive material were visualized by separately spraying these lanes of the electrophoretogram with a ninhydrin spray. Using the standards as markers, the radioactive cystine lane was then cut out, eluted from the paper with deionized H_2O, concentrated under nitrogen gas, filtered through a 0.22 μm Spin-X filter (Costar), stored at -20° C, and used within 3 weeks of purification. L-[^{35}S]Cysteine was prepared from the purified [^{35}S]cystine by the addition of DTT as indicated in the various experiments. Percoll was purchased from Pharmacia LKB Biotechnology, N-ethylmaleimide was from Calbiochem-Behring; diethyl pyrocarbonate, dicyclohexylcarbodiimide, phenylglyoxal, phenylisothiocyanate, N-acetylimidazole, imidazole, and hydroxylamine were obtained from Sigma.

3. Results

The interest for the present investigation stemmed from our previous observation that the activity of the cysteine-specific lysosomal transport system increases 7–10-fold between pH 6 and 7.3 to be maximally active in the neutral pH range (Fig. 1). If this pH dependence of the cysteine-specific lysosomal transport system exists on both sides of the lysosomal membrane, then once cysteine is transported into lysosomes from the cytosol, the acidic intralysosomal pH would not be favorable for exodus of cysteine from lysosomes thereby resulting in net lysosomal cysteine accumulation. The question we have addressed in the present investigation is what factors contribute to the pH dependence of the cysteine-specific lysosomal transport system. This pH dependence could be due to titration of amino acid residues of the transport protein essential for transport activity or also could reflect a dependence of the transporter on a transmembrane proton gradient. The best candidate amino acid side chains, titratable in this pH range, are the imidazole group of a histidine residue or the

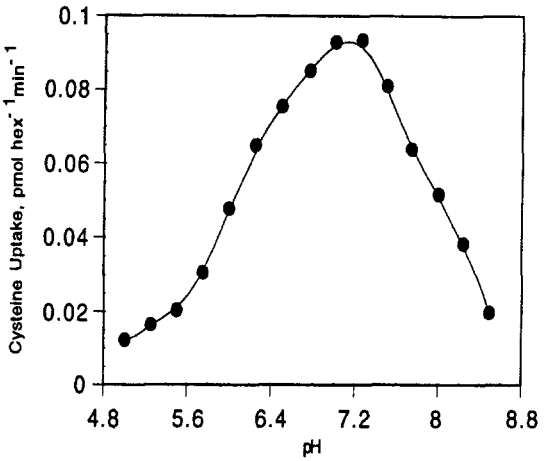

Fig. 1. pH dependence of 0.0131 mM L-[^{35}S]cysteine uptake by human fibroblast lysosomes. Fibroblast lysosomes (12 μl) suspended in 0.25 M sucrose were incubated with 24 μl of 0.0196 mM L-[^{35}S]cysteine in either 15 mM Mes or 15 mM Mops buffers containing 0.25 M sucrose and 1 mM DTT and which had been titrated to the indicated pH with Tris-free base. Incubations were performed at 37° C for 3.5 min at which time 28 μl aliquots were removed from incubation mixtures, lysosomes were collected and washed on GF/A filters, and the filters were mixed with 16 ml of Cytoscint for measurement of radioactivity.

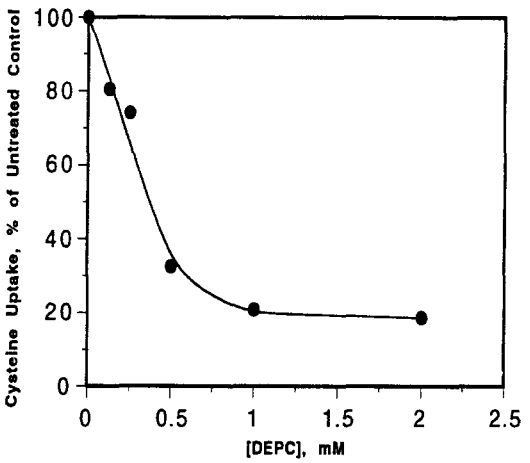

Fig. 2. Concentration dependence of DEPC inactivation of lysosomal cysteine uptake. Lysosomes were incubated with the indicated concentration of DEPC for 30 min at 30° C in 50 mM potassium phosphate pH 7.3 buffer containing 0.25 M sucrose. Lysosomes were washed by dilution/centrifugation as described in Materials and methods, and the resultant pellets were resuspended in 75 μl of ice-cold 40 mM Mops/Tris pH 7.0 buffer containing 0.25 M sucrose. The rate of L-[^{35}S]cysteine uptake for each lysosomal suspension then was measured by incubating 60 μl aliquots of a given lysosomal suspension with 40 μl of 31 μM L-[^{35}S]cysteine and performing a 5 min time-course assay as described in Materials and methods. Linear regression analysis was performed for each time-course assay with correlation coefficients ranging from $r = 0.95$ to 0.999. Data are expressed as a percent of two controls not exposed to DEPC (ave. = 0.082 ± 0.008 pmol L-cysteine taken up per min per μg lysosomal protein).

carboxyl side chain of an aspartyl or glutamyl residue residing in a hydrophobic pocket of the transport protein. In order to probe the nature of some of the amino acid residues required for transport activity of the cysteine-specific lysosomal transport system, we tested the ability of a variety of chemical modifying agents to inactivate lysosomal cysteine uptake in the neutral pH range. The results shown in Table 1 indicate that 1 mM diethyl pyrocarbonate (DEPC) and 5 mM *N*-ethylmaleimide were each able to strongly inactivate lysosomal cysteine uptake by 88–96%. DEPC has been shown to react with histidine residues or exposed α-amino groups, although some reactivity with thiols, serine, and tyrosine has been reported [15–18]. The strong inactivation caused by *N*-ethylmaleimide suggests that a thiol may be important for the function of this transport system. Because NEM could react with the radiolabelled substrate in these experiments, care was taken to wash NEM-treated lysosomes extensively so that residual NEM levels would be low enough that reaction with the L-[^{35}S]cysteine substrate used in the time-course assay would be negligible. The other chemical modifying agents that were tested, (1) dicyclohexylcarbodiimide (reactive with carboxyl groups), (2) phenylisothiocyanate (reactive with amino groups), (3) *N*-acetylimidazole (reactive with tyrosine residues), and (4) phenylglyoxal (reactive with arginine residues), had a moderate to very little effect on lysosomal cysteine transport activity.

Table 1
Inactivation of the cysteine-specific lysosomal transport system by various chemical modifying agents

	Transport activity (% of untreated control)
N-Ethylmaleimide (5 mM)	4
Diethyl pyrocarbonate (1 mM)	12
Dicyclohexylcarbodiimide (2.5 mM)	99
Phenylglyoxal (2 mM)	93
Phenylisothiocyanate (2 mM)	85
N-Acetylimidazole (33 mM)	78

Human fibroblast lysosomes were incubated for 30–40 min at 30° C with the indicated chemical modifying agent in an isotonic buffer containing 0.25 M sucrose at a pH of 7.0–7.5 depending on the chemical modifying agent as indicated in Materials and methods. Lysosomes then were centrifuged in ice-cold wash buffer, the pellets were re-suspended in ice-cold wash buffer and re-centrifuged at $15600 \times g$. The final lysosomal pellets were resuspended in 40 mM Mops/Tris pH 7.0 buffer containing 0.25 M sucrose and assayed for cysteine uptake in a 5 min time-course assay. Results are expressed as a percent of untreated controls which were carried through the same procedure but were not exposed to chemical modifying agents. Linear regression analysis was performed for each time-course with correlation coefficients ranging from $r = 0.95$ to 0.999.

Further investigation of DEPC inactivation of lysosomal cysteine transport revealed that maximal inactivation occurs with DEPC concentrations ≥ 1 mM (Fig. 2). DEPC inactivation of cysteine transport activity occurs rapidly with most of the inactivation occurring within the first 3 min of exposure to DEPC and maximal levels of inactivation being achieved after a 15 min exposure period (Fig. 3). Maximal inactivation consistently resulted in an 85–90% loss of lysosomal cysteine transport activity. The

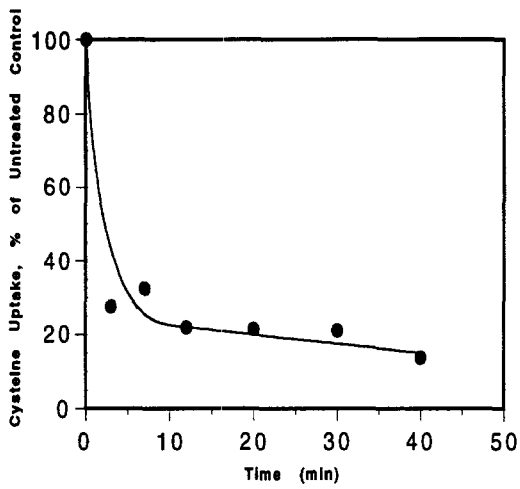

Fig. 3. Inactivation of lysosomal cysteine uptake by 1 mM DEPC as a function of time. Lysosomes were incubated for the indicated amount of time with 1 mM DEPC at 30° C in 50 mM potassium phosphate pH 7.3 buffer containing 0.25 M sucrose. Lysosomes were washed by dilution/centrifugation as described in Materials and methods, and the resultant pellets were resuspended in 75 μl of ice-cold 40 mM Mops/Tris pH 7.0 buffer containing 0.25 M sucrose. The rate of L-[^{35}S]cysteine uptake for each lysosomal suspension then was measured by incubating 60 μl aliquots of a given lysosomal suspension with 40 μl of 31 μM L-[^{35}S]cysteine and performing a 5 min time-course assay as described in Materials and methods. Linear regression analysis was performed for each time-course assay with correlation coefficients ranging from $r = 0.96$ to 0.999. Data are expressed as a percent of two controls not exposed to DEPC (ave. = 0.042 ± 0.002 pmol L-cysteine taken up per min per μg lysosomal protein).

10–15% of lysosomal cysteine uptake that remains following DEPC inactivation may be due to: (1) an ability of fibroblast lysosomes to transport cysteine by a minor, alternate route that is not sensitive to DEPC inactivation, (2) failure to achieve or maintain 100% modification of a transport protein amino acid residue essential for transport activity, or (3) an ability of the carrier to transport cysteine at a low transport rate despite DEPC modification. The present studies have not been able to distinguish among these possibilities.

The substrate, L-cysteine, was assayed for its ability to protect the transport protein from inactivation by DEPC. In these studies, lysosomes were incubated with 1 mM DEPC at 30° C and pH 7.3 in the presence or absence of 10 mM L-cysteine. Lysosomes then were diluted into an ice-cold wash buffer containing 10 mM imidazole to inactivate any residual DEPC, pelleted by centrifugation, washed once more in wash buffer without imidazole, and resuspended for the measurement of L-[^{35}S]cysteine uptake. The results shown in Fig. 4 demonstrate that 10 mM L-cysteine was able to completely protect the transport protein from inactivation suggesting that the reactive amino acid essential for cysteine transport activity is located in the carrier's substrate binding site. DEPC is known to react with imidazole to form *N*-carbethoxyimidazole which can be monitored by the increase in UV absorbance at 230 nm due to the formation of this product ($\Delta\epsilon = 3.0 \cdot 10^3$ cm^{-1} M^{-1}) [15]. Using this as a spectrophotometric assay to quantitate [DEPC], cysteine was found not to diminish DEPC's reactivity with imidazole throughout a 20 min incubation at 30° C in a 50 mM potassium phosphate pH 7.3 buffer containing 0.25 M sucrose (data not shown). These results indicate that cysteine does not react with DEPC under these reaction conditions. Therefore, in the substrate protection experiment described above, protection by cysteine is not due to cysteine reacting with DEPC but indicates that occupation of the carrier's substrate binding site by cysteine protects essential amino acid residues of the carrier protein from DEPC modification. Evidence for the specificity of protection was substantiated in a separate experiment (Table 2), demonstrating that L-alanine, which is not recognized by the cysteine-specific lysosomal transport system (1), does not protect this transport route from inactivation by 1 mM DEPC. It may be noteworthy to mention that the cysteine uptakes shown in Fig. 4 and Table 2 indicate that a greater rate of L-[^{35}S]cysteine uptake was observed for those incubation mixtures in which L-cysteine was present in the pre-incubations at 30° C as compared to the control in which cysteine was absent. This result may be an indication that a trans-stimulation property is associated with this transport system such that when lysosomes are pre-loaded with unlabelled cysteine, a greater rate of L-[^{35}S]cysteine uptake is observed subsequently.

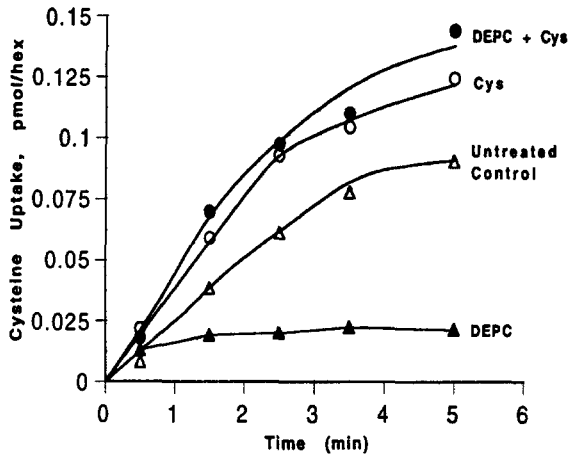

Fig. 4. Ability of 10 mM L-cysteine to protect the cysteine-specific lysosomal transport system from inactivation by 1 mM DEPC at pH 7.3. Lysosomes were incubated at 30° C for 30 min in 50 mM potassium phosphate/0.25 M sucrose pH 7.3 buffer containing either 1 mM DEPC (▲), 1 mM DEPC plus 10 mM L-cysteine (●), 10 mM L-cysteine (○), or no further addition (untreated control (△)). Lysosomes were washed by dilution/centrifugation as described in Materials and methods, and the resultant pellets were resuspended to 65 μl with ice-cold 40 mM Mops/Tris pH 7.0 buffer containing 0.25 M sucrose. The rate of L-[^{35}S]cysteine uptake for each lysosomal suspension then was measured by incubating 50 μl aliquots of a given lysosomal suspension with 30 μl of 30 μM L-[^{35}S]cysteine and performing a 5 min time-course assay as described in Materials and methods.

Table 2
Comparison of the ability of 4 mM L-alanine and 4 mM L-cysteine to protect the cysteine-specific lysosomal transport system from inactivation by 1 mM DEPC at pH 7.4

Incubation condition	Cysteine transport activity (pmol/ μg protein)
Buffer only (untreated control)	0.020 ±0.0005
Buffer + 1 mM DEPC	0.0001 ± 0.00001
4 mM L-cysteine	0.0248 ± 0.0008
4 mM L-cysteine + 1 mM DEPC	0.0245 ± 0.0006
4 mM L-alanine	0.0229 ± 0.0011
4 mM L-alanine + 1 mM DEPC	0.0006 ± 0.00001

Lysosomes were incubated at 30° C for 30 min in 50 mM potassium phosphate/0.25 M sucrose pH 7.3 buffer containing either: (i) 4 mM L-Ala, (ii) 4 mM L-Ala + 1 mM DEPC, (iii) 4 mM L-cysteine, (iv) 4 mM L-cysteine + 1 mM DEPC, (v) 1 mM DEPC, or (vi) no further addition (untreated control). Lysosomes were washed twice by dilution/centrifugation as described in Materials and methods, and the resultant pellets were resuspended to 50 μl with ice-cold 40 mM Mops/Tris pH 7.0 buffer containing 0.25 M sucrose. The rate of L-[^{35}S]cysteine uptake for each lysosomal suspension then was measured by incubating 10 μl aliquots of a given lysosomal suspension with 6 μl of 30 μM L-[^{35}S]cysteine for 4 min at 37° C. At the end of the uptake period, 12 μl aliquots were removed from the incubation mixtures and passed through a Sephadex G-50 column as described in Materials and methods. Material eluting in the void volume peak was collected and counted for radioactivity. The results given below are an average of quadruplicate determinations and the standard error associated with these measurements.

The pH dependence for inactivating lysosomal cysteine transport by DEPC was studied by incubating lysosomes at 30° C with 1 mM DEPC at various pH values over the range from pH 5 to 8. Lysosomes then were washed in an imidazole containing buffer at 4° C, and the rate of L-[^{35}S]cysteine uptake at pH 7.0 and 37° C was measured in a 5 min time-course assay. The results shown in Fig. 5 indicate that the pH dependence for DEPC inactivation of lysosomal cysteine uptake closely resembles the pH activity curve for lysosomal cysteine uptake. Half-maximal inactivation occurs near pH 6.6 which is consistent with titration of the imidazole group of a histidine residue [16]. This close correspondence suggests that the pH activity curve of the cysteine-specific lysosomal transport system reflects titration of an essential histidine residue of the transport protein.

For some enzymes in which an essential histidine residue has been modified with DEPC, it has been possible to reactivate the enzyme by exposure to hydroxylamine which results in decarbethoxylation of the modified histidine residue(s). The hydroxylamine concentration and the amount of time required for reactivation can vary widely for different proteins from 20 mM hydroxylamine for 30 min to 750 mM hydroxylamine for 20 h [15]. To determine if the DEPC modified cysteine transporter could be reactivated by hydroxylamine during a short incubation period, lysosomes were incubated with 1 mM DEPC for 30 min at pH 7.3 and 30° C, then exposed to 50 mM hydroxylamine for 30 min at pH 7.3. Lysosomes then were washed by dilution/centrifugation and assayed for cysteine transport activity. No significant reactivation of cysteine transport activity was observed for DEPC-treated lysosomes that were subsequently incubated with hydroxylamine (Data not shown). In addition, controls which were not treated with DEPC showed a 40% loss in cysteine transport activity when exposed to 50 mM hydroxylamine at pH 7.3 for 30 min at 30° C. Mancini et al. have noted a similar effect of hydroxylamine in chemical modification studies of the lysosomal sialic acid carrier [19].

Even though the preceding data suggest that titration of a histidine residue in the transport protein binding site may be responsible for the pH dependence of the cysteine-specific lysosomal transport system, it does not exclude the possibility that the large increase in lysosomal transport activity between pH 6.0 and 7.3 could also reflect a dependence of the transporter on a transmembrane proton gradient. We have shown previously that the initial rate of lysosomal cysteine uptake is diminished by 60% when uptakes are performed at pH 7.0 in the presence of either 15 μM carbonyl *m*-chlorophenylhydrazone or 25 μM monensin containing 75 mM NaCl [1]. These ionophores are known to dissipate the transmembrane proton gradient across the lysosomal membrane [20–22]. One concern in using ionophores is that a decrease in transport activity could also be due to the ionophore interacting with mem-

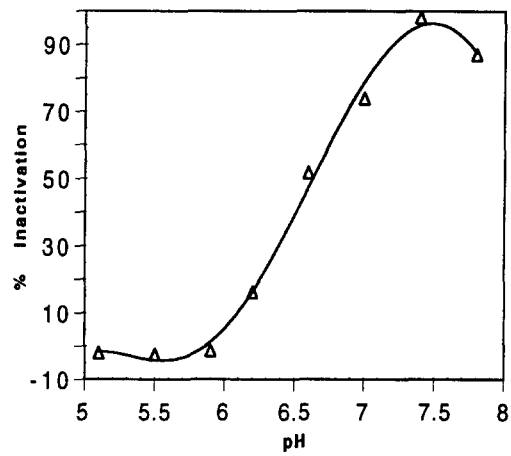

Fig. 5. pH dependence for inactivation of lysosomal cysteine uptake by 1 mM DEPC. Lysosomes, prepared from 12 confluent roller bottles of fibroblasts, were suspended in 360 μl of 0.25 M sucrose; 30 μl aliquots of the lysosomal suspension were mixed with 90 μl of 80 mM sodium phosphate buffer of the indicated pH containing 1.33 mM DEPC. Incubations were performed for 30 min at 30° C and then terminated by washing the lysosomes by dilution/centrifugation as described in Materials and methods. Lysosomal pellets were resuspended with 75 μl of ice-cold 40 mM Mops/Tris pH 7.0 buffer containing 0.25 M sucrose. The rate of L-[^{35}S]cysteine uptake for each lysosomal suspension then was measured by incubating 50 μl aliquots of a given lysosomal suspension with 50 μl of 40 μM L-[^{35}S]cysteine and performing a 5 min time-course assay as described in Materials and methods. In addition to the DEPC-containing incubations described above, control incubations were carried out in sodium phosphate pH 5.1 and pH 6.6 buffers in the absence of DEPC. The average of the untreated controls was 0.0289 ± 0.0002 pmol L-cysteine taken up per min per μg lysosomal protein.

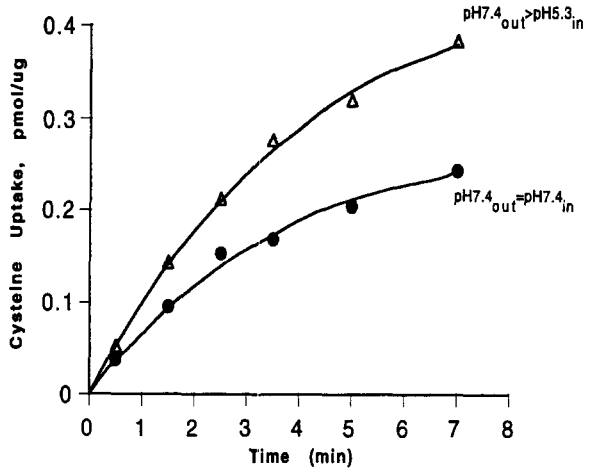

Fig. 6. Cysteine uptake by lysosomal membrane vesicles in the presence or absence of a proton gradient. Lysosomal membrane vesicles were prepared by hypotonic lysis of isolated lysosomes in either 20 mM Mops/Tris pH 7.4 buffer or 20 mM Mes/Tris pH 5.3 buffer containing protease inhibitors as described in Materials and methods. Following centrifugation, lysosomal membrane vesicles were resuspended in 100 μl of the same buffer used for hypotonic lysis but without protease inhibitors. Aliquots (30 μl) of each vesicle suspension were mixed with an equal volume of 30 μM L-[^{35}S]cysteine prepared so that the final pH of the incubation mixture was pH 7.4. Incubation mixtures were placed at 37° C, and at the indicated times, 9 μl aliquots were removed, and applied to Sephadex G-50 columns. Material eluting in the void volume was mixed with 16 ml scintillation fluid and counted for radioactivity.

brane domains affecting transport activity. Thus, an ionophore could cause a decrease in transport activity without the carrier actually being responsive to changes in the transmembrane proton gradient. To assess the response of the cysteine-specific lysosomal transporter to an imposed transmembrane proton gradient in which ionophores are not used, we hypotonically lysed Percoll-purified lysosomes to form lysosomal membrane vesicles having an intravesicular pH of either 5.3 or 7.4. L-[^{35}S]Cysteine uptake into these vesicles then was measured with the extravesicular pH adjusted to pH 7.4. The results shown in Fig. 6 demonstrate that the rate of cysteine uptake is ~ 1.5-fold greater when pH_{out} 7.4 > pH_{in} 5.3 as compared to uptakes performed when $pH_{in} = pH_{out} = 7.4$. These results provide additional evidence that part of the pH dependence of the cysteine-specific lysosomal transport system is due to a responsiveness to the lysosomal proton gradient which accelerates the rate of cysteine entry into the lysosomal compartment.

4. Discussion

The cysteine-specific lysosomal transport system is distinct from other amino acid transport systems that recognize cysteine by displaying high specificity for cysteine while poorly recognizing relatively similar amino acids such as homocysteine, serine, alanine, and threonine [1]. Our previous observation that significant amounts of cysteine are sequestered into the lysosomal compartment via this transport route and that the pH activity curve of the cysteine carrier is aptly designed for net delivery of cysteine into lysosomes, has led us in the present investigation to examine what factors account for the pH dependence of the cysteine-specific lysosomal transport system. Previously, we provided evidence that agents which are known to dissipate the lysosomal transmembrane proton gradient result in a decrease in lysosomal cysteine uptake [1]. In the present study, we further substantiated this finding by demonstrating a greater rate of cysteine uptake into human fibroblast lysosomal membrane vesicles when a transmembrane proton gradient was present (pH_{out} 7.4 > pH_i 5.3) than when absent (pH_{out} 7.4 = pH_i 7.4). Thus, part of the pH dependence of the cysteine-specific lysosomal transport system is due to an increase in cysteine uptake in response to the proton gradient ($pH_{out} > pH_{in}$) across the lysosomal membrane of intact lysosomes.

Our analysis of the effects of various chemical modifying agents revealed that lysosomal cysteine transport activity is strongly inactivated by diethyl pyrocarbonate and *N*-ethylmaleimide. The effect of NEM suggests that a thiol may play an important role in the operation of the lysosomal cysteine transporter. However, since the only known substrate for the cysteine-specific lysosomal transport system is a thiol, we were unable to perform substrate protection experiments to determine if NEM inactivation involved reaction with a thiol located in the transport protein binding site. It may be noteworthy that lysosomal cysteine uptake steadily declines with increasing pH above pH 7.3 such that only a small amount of cysteine transport is observed by pH 8.5 (Fig. 1). This response to pH may reflect deprotonation of a thiol important for the transport activity of the cysteine-specific lysosomal carrier or reflect deprotonation of the substrate, cysteine.

Inactivation by diethyl pyrocarbonate is highly consistent with modification of a histidine residue. This finding is based on the pH dependence for DEPC inactivation of lysosomal cysteine transport which exhibits minimal inactivation at pH values less than 6, half-maximal inactivation at ~ pH 6.6, and maximal inactivation at pH ≥ 7.3, a profile characteristic of DEPC reaction with histidine [16]. The substrate, cysteine, completely protects the transport protein from DEPC inactivation suggesting location of this essential histidine residue in the carrier's substrate binding site whereas the non-substrate, alanine, fails to protect the cysteine carrier from DEPC inactivation. The substrate protection experiments, however, do not exclude the possibility that an essential histidine could lie outside the amino acid binding site and upon substrate binding to the transporter, conformational changes within the carrier could block accessibility of the essential histidine residue to DEPC.

We did not obtain any evidence for other amino acids, which could have titratable groups in the pH range from

pH 6–7.5, being required for transport activity. The carboxyl-reactive agent, DCCD, and the amino reactive agent, PITC, had little effect on lysosomal cysteine transport. Overall, our results indicate that the imidazole side chain of the essential histidine residue must be deprotonated to allow for transport by the cysteine-specific lysosomal transport protein. Consequently, the cysteine-specific lysosomal transport system would not be expected to significantly participate in the export of cysteine from the lysosomal compartment since, at the human fibroblast intralysosomal pH of 5.3, the essential histidine residue(s) would be protonated to a large degree. In view of the pH dependence of the cysteine-specific lysosomal transport system, it may be insightful to examine in future investigations whether intralysosomal thiol levels are significantly lowered upon alkalinization of the lysosomal interior. For instance, as the intralysosomal pH is raised from pH 5.3 to 6.3, the cysteine-specific lysosomal transport system would be expected to significantly contribute to the export of cysteine from the lysosomal compartment with the possibility of thereby lowering steady state intralysosomal cysteine levels. Thiol has been shown to be important for a variety of lysosomal hydrolytic activities [3–7], and thus substantial decreases in intralysosomal cysteine levels may diminish the activity of some lysosomal hydrolases. This possibility may be particularly relevant to ras transformation of human fibroblasts which Jiang et al have shown to cause alkalinization of the lysosomal interior from pH 5.0 to pH 6.1 [23]. Recently, Sameni et al. [24] have shown that the rate of intralysosomal processing of cathepsin B is delayed in ras transformed human breast epithelial cells. The extent to which diminished lysosomal hydrolytic activity as a result of lysosomal alkalinization is due to a given enzymatic activity operating at a sub-optimal pH or due to the effect of possibly lowered thiol levels remains to be determined.

Despite the present knowledge of approx. 20 different lysosomal transport systems and three different genetic disorders due to defects in lysosomal transport [25], neither a gene or a protein has been isolated for any of the lysosomal transport systems. Identification of amino acid residues essential for transport function is an important step in understanding the lysosomal carriers at the molecular level. Future experiments can grow upon this base of knowledge and ultimately lead to the isolation and further molecular characterization of these proteins. The results of the present study, demonstrating substrate protection of the cysteine-specific lysosomal transport system from DEPC inactivation, suggest the feasibility of specifically labelling this transport protein with radiolabelled DEPC after modifying other lysosomal protein histidine residues with unlabelled DEPC in the presence of substrate.

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