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Functional reconstitution of the lysosomal sialic acid carrier into proteoliposomes

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ABSTRACT **The lysosomal carrier for the acidic monosaccharides sialic acid and glucuronic acid was solubilized from rat liver lysosomal membranes and reconstituted into phospholipid vesicles. Membrane proteins were extracted from lysosomal membranes with Triton X-100. Upon removal of the detergent by absorption on Amberlite XAD-2 beads, the solubilized proteins were incorporated in egg yolk phospholipids. The reconstituted proteoliposomes show proton-driven carrier-mediated uptake of acidic monosaccharides. The reconstituted carrier was compared in several characteristics with the transporter as present in the native lysosomal membrane. Transporter substrate affinity (K_t for glucuronic acid = 0.4 mM) and specificity for acidic monosaccharides are completely retained. The proteoliposomes also demonstrate trans-stimulation properties with both substrates sialic acid and glucuronic acid. The transporter is inhibited, both in its native and in the reconstituted state, by the sulfhydryl-modifying agents *p*-chloromercuribenzoic acid, *N*-ethylmaleimide, and phenyl isothiocyanate. In native membrane vesicles, arginine and histidine modifiers phenylglyoxal and diethyl pyrocarbonate inactivated transport in a substrate-protectable manner. In reconstituted proteoliposomes, similar inhibition was observed. However, protection by substrates was achieved only after treatment with phenylglyoxal. These data suggest that arginine or histidine residues or both are present at or near the substrate binding site of the carrier. Possibly, other essential histidines become exposed in the reconstituted state. The successful functional reconstitution of the lysosomal sialic acid carrier represents an important step towards its purification and its detailed molecular characterization.**

In lysosomes several membrane transport systems have been characterized for amino acids, nucleosides, monosaccharides, vitamins, and miscellaneous compounds (1–5). In general, these carriers permit the efflux of catabolites from the organelle to the cytosol, allowing their (re)utilization. So far none of these carriers has been identified, and very little is known of their molecular properties. Recently, we have developed a sensitive transport assay making use of vesiculated highly purified lysosomal membranes. This technique allowed us to demonstrate the existence of a proton cotransport system for anionic monosaccharides, with the most important sialic acid, *N*-acetylneuraminic acid (AcNeu), and glucuronic acid (GlcUA) as important physiological substrates (3). In sialic acid-storage diseases, a heterogeneous group of inherited disorders characterized by lysosomal storage and excessive excretion of sialic acids (6–8), a specific transport defect of the lysosomal acidic monosaccharide carrier has been found (9). Sialic acids and GlcUA are produced by intralysosomal degradation of, respectively, sialylglycoconjugates and glycosaminoglycans. Considering the importance of this lysosomal carrier in the metabolism of normal and diseased states, we try to extend our knowledge about the function of this transporter at the molecular level. The first approach to the identification of transport proteins in general is the development of a functional reconstitution system by which the membrane proteins are first solubilized and then incorporated into artificial phospholipid membranes. This system provides the functional assay to follow the fractionation and purification of the solubilized transporters. Several methods have been described in the literature for the reconstitution of transport proteins from various sources. However, none of them has been successfully reported for lysosomal transporters except the proton pump (10).

In the present paper, we report the successful solubilization and functional reconstitution of the lysosomal carrier for acidic monosaccharides, using the nonionic detergent Triton X-100 and egg yolk phospholipids. The active reconstituted transporter is compared with the carrier in its native state with respect to several kinetic parameters, substrate specificity, and some structural characteristics.

MATERIALS AND METHODS

Solubilization and Reconstitution. Highly purified lysosomal membrane vesicles from rat liver were prepared as described (3, 11). Extraction of membrane proteins took place in ice by mixing 150 μl of membrane vesicles [in 20 mM Hepes sodium salt (NaHepes, pH 7.4; about 1.3 mg of protein)] with 150 μl of 6% (wt/vol) Triton X-100/200 mM KCl/2 mM dithiothreitol/20 mM NaHepes, pH 7.4. After 10 min, unextracted material was pelleted by centrifugation at 4°C at 150,000 × *g* in a Beckmann SW 60 rotor for 20 min, and the supernatant was collected and kept in ice. This extract usually contained 50–60% of the original protein. Crude phospholipids were prepared from fresh egg yolk as described and were stored under N_2 at −20°C (12, 13). To prepare liposomes, 300 mg of phospholipids were dried under N_2 and resuspended in 3 ml of 20 mM NaHepes, pH 7.4/100 mM KCl/1 mM EDTA. The tube was sonicated in an ice bath with a Branson B15 sonifier (pulsed mode at 50% duty for 60 min). Reconstitution was done in a final volume of 680 μl containing 200 μl of the detergent extract, 60 μl of 10% Triton X-100, 100 μl of the sonicated liposomes, 320 μl of 24 mM NaHepes (pH 7.4), and 119 mM KCl. Proteoliposomes were formed by removing the detergent on an Amberlite XAD-2 (Fluka) column (0.5 × 3.6 cm) at room temperature and passing the sample 15 times through the same column, which was equilibrated with 20 mM NaHepes, pH 7.4/100 mM KCl. Proteins were determined by the procedure of Lowry *et al.* as

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Abbreviations: DEP, diethyl pyrocarbonate; EAI, ethyl acetimidate; GlcUA, D-glucuronic acid; *N*-BrSuc, *N*-bromosuccinimide; NEM, *N*-ethylmaleimide; AcNeu, *N*-acetylneuraminic acid, the most important sialic acid; *p*CMB, *p*-chloromercuribenzoic acid; PG, phenylglyoxal; PITC, phenyl isothiocyanate; TNBS, trinitrobenzensulfonic acid.

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modified by Peterson (14) after acetone precipitation to concentrate the samples and resuspension in 1% SDS.

Transport Assays. GlcUA transport was assayed in the presence of an inward-directed proton gradient, using D-[1-^{3}H]GlcUA (Amersham; specific activity, 1.8 Ci/mmol; 1 Ci = 37 GBq) as a substrate. Uptake was started by adding 25 μl of proteoliposomes to 5 μl of 240 mM Mes containing 2 μCi of D-[1-^{3}H]GlcUA (37 μM final concentration) and 60 μM valinomycin at 37°C. This titrated the extravesicular pH to 5.5. After the required incubation time, uptake was stopped by adding 70 μl of ice-cold incubation buffer. The sample was immediately applied to a Sephadex G-50 (fine; Pharmacia LKB) column (0.5 × 5 cm), run at 4°C at 0.3 ml/min, and eluted with 1 ml of the incubation buffer. The recovered radioactivity was assayed by liquid scintillation. Experiments without a pH gradient were performed at pH 7.4 in 20 mM NaHepes/100 mM KCl/10 μM valinomycin. For trans-stimulation experiments, a 60% proteoliposome solution (25 μl) was preequilibrated for 60 min at 37°C with 40 mM Mes/1 mM sodium salt of GlcUA or *N*-acetylneuraminic acid (AcNeu)/10 μM monensin (final concentrations) in an isoosmotic solution (pH 5.5). The assay was started by adding 75 μl of an equivalent buffer containing 2 μCi of radiolabeled GlcUA at 37°C. Control experiments were performed by preequilibration of the membranes with the same buffer without the sodium salt of either GlcUA or AcNeu, but the external final concentration was corrected as in the case of preloading (3). Assays were regularly performed in duplicate or triplicate. Kinetic parameters from the experimental results were calculated by the computer program ENZFITTER, from Elsevier-Biosoft, Cambridge, U.K.

Protein Side-Chain Modification. On the basis of the amino acid selectivity and of the tolerance of the carrier to the different assay conditions, a choice was made among different covalent protein modifiers. Native lysosomal membranes (50 μl) were incubated with (final concentrations in 250 μl) 1 mM *p*-chloromercuribenzoic acid (*p*CMB) for 15 min at 37°C in 50 mM NaHepes (pH 7.0), or with 1 mM *N*-ethylmaleimide (NEM) for 20 min at 20°C in 50 mM NaHepes (pH 7.4), or with 8 mM phenyl isothiocyanate (PITC) for 20 min at 22°C in 20 mM NaHepes (pH 7.4), or with 1 mM ethyl acetimidate (EAI) or trinitrobenzensulfonic acid (TNBS) for 30 min at 20°C in 20 mM NaHepes (pH 8.0), or with 4 mM *N*-bromosuccinimide (*N*-BrSuc) or 1 mM diethyl pyrocarbonate (DEP) for 30 min at 20°C in 30 mM Mes sodium salt (pH 6.0), or with 1 mM phenylglyoxal (PG) for 60 min at 37°C in 50 mM NaHepes (pH 7.4). All the incubations were performed in the dark. Alternatively, the same incubations were performed in the presence of 30 mM AcNeu or GlcUA. Afterwards the membranes were washed with 25 ml of 20 mM NaHepes (pH 7.4) and centrifuged at 50,000 × *g* in a Beckman SW 41 Ti rotor 30 min at 4°C. The pellet was resuspended in 20 mM NaHepes (pH 7.4) for direct assay of transport and protein content. Controls were run per each assay condition without modifiers in the presence and absence of excess substrate. None of these conditions significantly altered basal transport rates. The effect of 100 mM NH_2OH·HCl in Tris/Hepes (pH 7.4) for 1 hr at 20°C was tested on washed membranes, pretreated with DEP, before proceeding to the transport assay. Reconstituted proteoliposomes were incubated with protein modifiers under appropriate conditions as described for native lysosomal membranes. The only difference was that after the preincubations the extravesicular modifier and the eventual excess substrate were removed by filtration of 200-μl samples on small (2 ml) Sephadex G-50 (medium; Pharmacia LKB) columns equilibrated in 20 mM NaHepes (pH 7.4)/100 mM KCl and centrifuged at 100 × *g* for 2 min. The cycle was repeated twice. The resultant eluate was directly assayed for protein content and transport activity. All the chemicals were provided by Sigma except valinomycin (from Boehringer Mannheim) and NEM (from Merck).

RESULTS

Initial experiments focused on developing reconstitution conditions that yielded proteoliposomes sufficiently impermeable to protons. This should be necessary to prove the activity of the proton gradient-driven acidic monosaccharide (sialic acid) carrier after solubilization and reconstitution.

We have applied a rapid reconstitution procedure, which had been originally developed for several mitochondrial carriers (15). This method employs the low-critical-micelle-concentration nonionic detergent Triton X-100 for extraction. Mixtures of extracted lysosomal membrane proteins and egg yolk phospholipids are depleted of Triton X-100 by repetitive chromatography on small columns of Amberlite XAD-2. This procedure leads to proteoliposomes that are able to transport GlcUA (Fig. 1). Since AcNeu and GlcUA are transported by the same lysosomal carrier for acidic monosaccharides (3, 9), we have performed all studies using radiolabeled GlcUA, which was more readily available. After reconstitution we observed that uptake of GlcUA was significantly stimulated in the presence of an inward-directed proton gradient, leading to a transient overshoot. At 37°C, initial uptake rates were linear for about 1 min. Since the influence of membrane potentials was circumvented by the presence of valinomycin and K^+, these results prove that the pH gradient represents the driving force for a GlcUA–proton cotransport system.

We next determined if initial transport rates of proton-driven GlcUA uptake were saturable and displayed a similar affinity constant as in native lysosomal membranes. The results of this experiment confirm typical kinetics of carrier-mediated transport for the reconstituted system (Fig. 2). The proteoliposomes show only one mode of uptake with a transporter affinity constant K_t of 0.4 mM, similar to the affinity constant of the native carrier. No significant leakage (not carrier-mediated component) was present. Control liposomes, made without addition of protein, did not show significant proton-stimulated uptake. These results show that

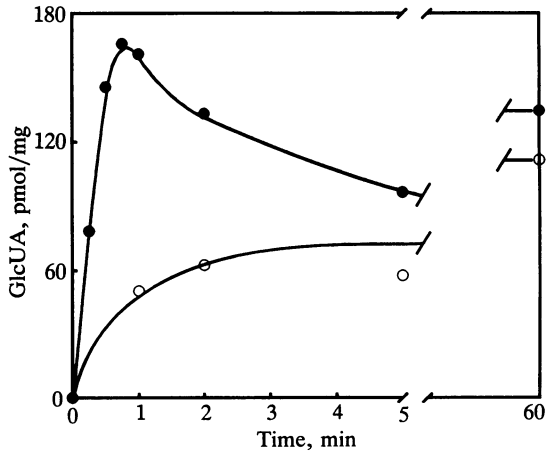

FIG. 1. Proton gradient-dependent uptake of the acidic monosaccharide GlcUA into reconstituted proteoliposomes. Lysosomal membrane proteins were solubilized from rat liver lysosomal membranes in the presence of 3% Triton X-100 and centrifuged at 150,000 × *g* for 20 min. The solubilized proteins were incorporated in liposomes and the transport of [^{3}H]GlcUA was determined at 37°C. Proteoliposomes prepared in 20 mM NaHepes/100 mM KCl, pH 7.4, were incubated with 37 μM [^{3}H]GlcUA and 10 μM valinomycin (no proton gradient, $pH_{in} = pH_{out} = 7.4$) (○) or in the same buffer titrated to pH 5.5 with 40 mM Mes free acid (with proton gradient $pH_{in} = 7.4 > pH_{out} = 5.5$) (●).

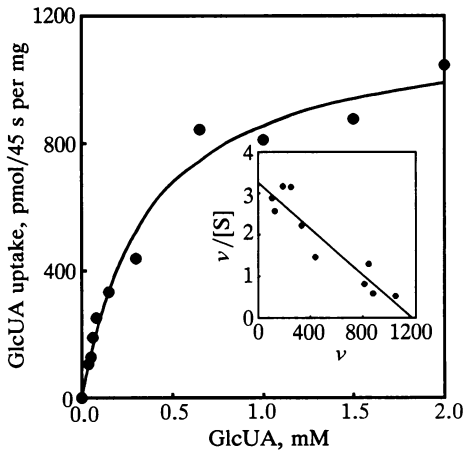

FIG. 2. Saturable uptake of GlcUA into proteoliposomes. Proteoliposomes were incubated in the presence of a proton gradient as described in the legend of Fig. 1. Initial uptake rates were measured for 45 sec with different GlcUA concentrations. (*Inset*) Scatchard transformation of the experimental data, where v is velocity in pmol/45 s per mg and [S] is the substrate millimolar concentration.

the sialic acid-carrier polypeptide(s) is stable under the conditions used for extraction and subsequent reconstitution into liposomes. Observation of the preparations at the electron microscope revealed that the reconstituted proteoliposomes are homogeneous in size and unilamellar (data not shown). As measured from the equilibration point, the internal volume is about 3 μl/mg of protein, slightly larger than the native membrane vesicles (3).

The carrier in its native membrane displays trans-stimulation properties. To investigate if the reconstituted carrier shows this same characteristic, proteoliposomes were preloaded with concentrations above the K_t value of either AcNeu or GlcUA; after a 1:3 dilution, uptake of trace amounts of [^{3}H]GlcUA was measured (Fig. 3). The presence of an outward-directed gradient of unlabeled AcNeu or GlcUA stimulates the time-dependent uptake of radiolabeled GlcUA. This effect disappears upon dissipation of the outward gradient. Apparently the reconstituted carrier retains its typical trans-stimulation property with both substrates.

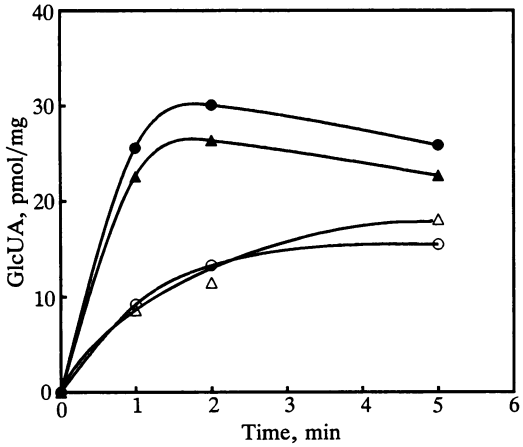

FIG. 3. Trans-stimulation of [^{3}H]GlcUA uptake after preloading proteoliposomes with the unlabeled acidic monosaccharides AcNeu or GlcUA. Proteoliposomes were preincubated 60 min at 37°C in the presence (●, ▲) or absence (○, △) of either 1 mM unlabeled AcNeu (circles) or GlcUA (triangles) in 20 mM NaHepes/100 mM KCl/40 mM Mes/10 μM monensin. The transport assay was started by a 1:3 dilution in pH 5.5 incubation buffer supplemented with 37 μM [^{3}H]GlcUA.

The ligand specificity was further investigated by cis-inhibition experiments. Uptake of radiolabeled GlcUA was measured in proteoliposomes in the presence of high concentrations of several potential ligands (inhibitors). The carrier retained the same substrate specificity pattern as in native membrane vesicles (3) (Table 1). Furthermore some interesting inhibitors are found like saccharic acid-1,4-lactone and hydroxycinnamate derivatives, both of which could be useful for developing further purification steps.

To get insight into the molecular characteristics of the carrier, we investigated the sensitivity of the transporter to protein modifiers. Amino acid modifiers have been selected among those reacting with amino acids like lysine, arginine, and histidine, which are often present at the active site (binding site) of proteins interacting with anionic ligands (16). Care was taken in choosing assay conditions for modification (temperature, pH, etc.), which did not inactivate *per se* the transporter. The results in Table 2 indicate that significant inactivation of transport in native lysosomal membrane vesicles was achieved by thiol covalent modifiers *p*CMB, NEM, and PITC; under the conditions used, the last two also can react with lysine residues. However, the lysine-specific reagent TNBS and EAI did not inhibit GlcUA transport. In separate experiments, the apparent K_i for inactivation was calculated to be about 0.8 mM for NEM and 6 mM for PITC. PG is an arginine modifier and, to a lesser extent, a histidine modifier, whereas DEP is considered to be a specific histidine modifier; 1 mM PG and DEP produced significant inactivation of glucuronic acid transport with respective apparent K_i values of 0.67 mM and 0.46 mM. *N*-BrSuc (4 mM), another histidine modifier, also inactivated the carrier. Inhibition by DEP and PG could be completely prevented by concomitant incubation with 30 mM GlcUA (Table 2) or AcNeu (not shown) with identical results. Lower ligand concentrations were progressively less effective (not shown). Although both PG and DEP can react with histidine residues, the assay conditions we chose would make the PG reaction exclusive for arginines (17, 18). In theory, the reactions of histidines with DEP, generating a carbethoxy group, can be reversed by treatment with hydroxylamine. We could not reverse the effect of DEP with NH_2OH, but control membranes, treated

Table 1. cis-inhibition of GlcUA transport

Inhibitor (7 mM)	Transport activity: Into proteoliposomes	Transport activity: In original membrane vesicles
None	100	100
GlcUA	16	4
AcNeu	17	5
GcNeu	18	10
Glucose	88	95
GlcNAc	95	100
Aspartic acid	105	85
Cyano-3-hydroxycinnamic acid	9	24
Cyano-4-hydroxycinnamic acid	10	19
D-Saccharic acid 1,4-lactone	11	ND
No proton gradient	31	10

Proteoliposomes were incubated 45 sec at 37°C with 37 μM [^{3}H]GlcUA as described in the legend of Fig. 1, in the presence of an inward-directed proton gradient and with 7 mM of the indicated compounds. Control uptake without inhibitor was 167 ± 7 pmol/45 sec per mg of protein. Original lysosomal membrane vesicles (25 μl) were incubated in the presence of a pH gradient as described in ref. 9, with 7 mM inhibitor for 30 sec at 20°C. Uptake in the presence of inhibitor is expressed as the percentage of the control uptake without inhibitor. Results are averages of two independent determinations. ND, not determined; GcNeu, *N*-glycolylneuraminic acid (a sialic acid).

Table 2. Effect of amino acid modifiers on transport activity of the sialic acid carrier in rat liver lysosomal membrane vesicles

Reagent	Transport activity, % of rate without modifier*	
	Without protection	With protection by substrate
No modifier	100	—
*p*CMB	0	0
NEM	20	0
PITC	10	18
EAI	100	ND
PG	30	100
DEP	20	95
NH_2OH + DEP	9	ND
NH_2OH	19	ND
TNBS	100	ND
N-BrSuc	0	ND

Lysosomal membrane vesicles were pretreated under the appropriate conditions for each amino acid modifier with or without 30 mM GlcUA. After preincubation, vesicles were centrifuged at 50,000 × *g* for 30 min, and the resultant pellets were suspended in 20 mM NaHepes (pH 7.4). Vesicles (50 μl) were assayed for [^{3}H]GlcUA uptake as described in text.

*The activities after treatment (without or with protection) are expressed as the percentage of the transport rate displayed by vesicles treated without modifier. ND, not determined.

with NH_2OH only, lost their transport activity completely (Table 2).

Interestingly, inactivation of transport was also achieved in reconstituted proteoliposomes by NEM, DEP, and PG (Table 3), revealing that the effect is likely directed to the transporter polypeptide(s). However, inhibition could be prevented by the contemporary presence of substrate in PG-treated but not in DEP-treated membranes.

DISCUSSION

Our recent investigations on transport of the acidic monosaccharides AcNeu and GlcUA have demonstrated the existence of a carrier in the lysosomal membrane with substrate specificity for these carboxylated sugars. This carrier was demonstrated to be defective in the sialic acid storage diseases (9). All of these studies are based on transport assays with vesiculated lysosomal membranes. We have now shown that the lysosomal sialic acid carrier can be extracted from the lysosomal membrane with the detergent Triton X-100 and later functionally reconstituted in liposomes from crude egg yolk phospholipids without loss of activity. The reconstitution procedure is fast and easy and can be performed on a small scale. The reconstitution assay opens the door, by using fractionated solubilized lysosomal membrane proteins, to purification and further molecular characterization of the transporter polypeptide(s).

Table 3. Effect of amino acid modifiers on transport activity of the reconstituted sialic acid carrier

Reagent	Transport activity, % of control uptake	
	After treatment	After treatment + protection
No modifier	100	100
NEM	0.2	1.3
DEP	0.0	2.3
PG	15.0	101

Proteoliposomes were preincubated at the appropriate condition for each modifier, with or without 30 mM GlcUA. Afterwards extravesicular modifier and excess substrate were removed by gel filtration. Vesicles (50 μl) were incubated with [^{3}H]GlcUA as described in text in the presence of a proton gradient. The transport activity is expressed as a percentage of proton gradient-dependent uptake in control proteoliposomes similarly preincubated without protein modifiers. Results are average values of two independent determinations.

The carrier functionally reconstituted in proteoliposomes was compared in several characteristics with the carrier in its native membrane. Uptake of GlcUA in proteoliposomes is also driven by an imposed proton gradient. The uptake process is saturable with an affinity constant similar to that measured in original membranes. Trans-stimulation and cis-inhibition characteristics of the carrier were also fully retained after reconstitution.

Some additional interesting inhibitors were found, which could be considered for further affinity chromatography purification steps. The first was saccharic acid 1,4-lactone, as a strong competitive inhibitor of β-glucuronidases (19), which has been used in its immobilized state for the purification of these GlcUA-recognizing proteins. The others are cinnamic acid derivatives, potent inhibitors of monocarboxylate transporters in mitochondria, which have been used for their purification (20, 21).

Several covalent side-chain protein modifiers were chosen to investigate which amino acid residues are determinant for the transport activity, in the native and reconstituted state and might be located at or nearby the substrate binding site. In particular, thiol modifiers (NEM, PITC and *p*CMB) were selected because preliminary observations had suggested the importance of thiol amino acids in the native conformational state (3). Cationic amino acids, and particularly arginines, have been observed often at the substrate binding site of enzymes reacting with anionic substrates (16). Preliminary observations with side-chain modifiers on native proteins have often been confirmed after cloning of the correspondent gene and expression of *in vitro* mutagenized residues involved in the formation of the active site (22, 23). Thus, PITC (cysteine- and lysine-specific); EAI and TNBS (lysine-specific); and *N*-BrSuc, DEP, and PG (arginine- and histidine-specific) were used. In conclusion, thiol modifiers inhibit GlcUA transport in a substrate-unprotectable manner. Although this does not exclude cysteine residues from being involved in the substrate-binding process and in the consequent conformational changes, certainly other cysteines must be kept in their native state to maintain activity. Lysines are apparently not essential for transport, since EAI and TNBS did not alter transport function. Instead, histidines and arginines are essential for the normal carrier function and apparently are present at or near the ligand-binding site. The same was observed in reconstituted proteoliposomes, where, however, protection could be obtained only for the PG treatment. We could not definitively demonstrate with NH_2OH that histidines were modified by DEP. On the other hand, the pH of the PG reaction should only select arginines for covalent modification. Taken together, the present results suggest that in the native state both arginines and histidines are involved in substrate recognition. Upon solubilization and reconstitution, it is possible that other essential histidines are exposed that are not involved in substrate binding. In membrane-spanning regions of integral membrane proteins, it seems that histidines can be "buried" with low-energy expenditure (24). We hypothesize that an unmasking mechanism is responsible for exposure of histidines upon solubilization and reconstitution of the lysosomal acidic sugar carrier.

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