Acid β-Glucosidase: Intrinsic Fluorescence and Conformational Changes Induced by Phospholipids and Saposin C†

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ABSTRACT: Acid β-glucosidase is a lysosomal membrane protein that cleaves the *O*-β-D-glucosidic linkage of glucosylceramide and aryl-β-glucosides. Full activity reconstitution of the pure enzyme requires phospholipids and saposin C, an 80 aa activator protein. The deficiency of the enzyme or activator leads to Gaucher disease. A conformational change of acid β-glucosidase is shown to accompany activity reconstitution by selected phospholipids or, particularly, phospholipid/saposin C complexes by intrinsic fluorescence spectral shifts, fluorescence quenching, and circular dichroism (CD). Negatively charged phospholipid (NCP) interfaces with unsaturated fatty acid acyl chains (UFAC) induced concordant blue-shifts in tryptophanyl fluorescence spectra and a loss of β-strand structure by CD. The enzyme required an unsaturated fatty acid acyl chain in proximity (10−11 Å) within liposomal membranes for activation, fluorescence blue-shifts, and changes in CD spectra. Activity enhancements were greatest when UFAC and the negatively charged headgroup were present on the same phospholipid. NCPs with UFAC protected the enzyme from fluorescence quenching by aqueous agents (I^-, Cs^+, acrylamide, TEMPO). Phosphatidylcholine with doxyl spin-labeled fatty acid acyl chains at carbons 7, 10, or 16 quenched enzyme fluorescence only when in NCP/PC liposomes. Saposin C (Trp-free) induced additional activity and fluorescence spectral changes in the enzyme only in the presence of NCP liposomes containing UFA. CD spectral changes indicated saposin C and acid β-glucosidase interaction only in the presence of NCPs with UFA. These studies show that acid β-glucosidase requires interfaces composed of NCPs, containing UFAC, for penetration into the outer leaflet of membranes. Furthermore, this interaction induces essential conformational changes for saposin C binding and further enhancement of acid β-glucosidase catalytic activity.

Acid β-glucosidase (*N*-acylsphingosyl-β-D-glucoside:glucohydrolase, EC 3.2.1.45) hydrolyzes the β-glucosidic linkage of glucosylceramide and synthetic β-glucosides. This glycoprotein enzyme has 497 amino acids (MW ∼55 400; unglycosylated protein) including 12 widely distributed tryptophanyl residues. The enzyme is present in lysosomes of all nucleated cells of mammals (*1*). Four of five Asn-linked glycosylation sites have attached oligosaccharides, and occupancy of the first site (Asn19) is necessary for enzyme activity (*2*). The enzyme normally is a lysosomal membrane protein that requires detergents for complete extraction. Although the enzyme has substantial hydrophobic properties, it is not a transmembrane protein and has neither a large cytoplasmic tail (*3*) nor computer-predicted transmembrane domains. Inherited defects of this enzyme lead to the various forms of Gaucher disease and the lysosomal accumulation of glucosylceramide (*4*).

The fully delipidated enzyme is essentially inactive, and a micellar or liposomal surface is needed for reconstitution of the enzyme function in vitro. To restore the hydrolytic activity of the pure enzyme toward glucosylceramide or arylglycosides, exogenous activators are needed, including phenoxyacyl detergents (e.g., Triton X-100), bile salts, or acidic phospholipids (phosphatidylserine, phosphatidylinositol) [reviewed in (*1*)]. Extensive kinetic and reconstitution studies of acid β-glucosidase have evaluated the effects of acidic phospholipids on the enhancement of enzyme activity [see (*1*) for detailed review]. Anionic phospholipids with a single net negative charge are effective in reconstituting partially purified, delipidated enzyme in the following order: phosphatidylserine (PS)[1] > phosphatidylinositol ≫ phosphatidic acid (*5*). Neutral phospholipids, i.e., phosphatidylcholine (PC) or phosphatidylethanolamine, are ineffective (*6*). These studies showed that the acidic headgroup is essential for activation and that an interface is required for enzyme interaction.

Less well studied are the effects of the fatty acid acyl chains of acidic phospholipids. Although natural PS and phosphatidylglycerol, containing unsaturated fatty acid acyl

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[1] Abbreviations: PS, phosphatidylserine; PC, phosphatidylcholine; PG, phosphatidylglycerol; EPC, egg PC; BPS, brain PS; HCA, heart cardiolipin; LPI, liver phosphatidylinositol; SPI, soybean phosphatidylinositol; SLPC, 1-palmitoyl-2-stearoyl(*n*-doxyl)-*sn*-glycero-3-phosphocholine; TEMPO, 2,2,6,6-tetramethylpiperidine-*N*-oxyl; 4MU-Glc, 4-methylumbelliferyl-β-D-glucopyranoside; CD, circular dichroism.

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chains, reconstituted rat liver β-glucosidase activity, phosphatidylglycerols with saturated acyl chains [i.e., PG (18:0,0)] were ineffective in stimulating the enzyme's activity (*7*). In comparison, the PS with saturated short acyl chains [PS (12:0,0)] reconstituted enzyme activity whereas those with ≤10 carbon bonds did not (*6*). This is likely due to the inability of PSs with acyl chains ≤10 carbon bonds to form stable interfaces (*8*). These findings suggested that interaction between an interface and acid β-glucosidase may require penetration to a depth of about 12 carbon bond lengths and that other physical effects may impede enzyme function in liposomes composed of longer saturated acyl chains. The results of these kinetic studies suggested that the acidic phospholipid interface interacts with acid β-glucosidase to conform the enzyme into an active structure (*1*, *9*).

In addition to the acidic phospholipids, saposin C is essential for significant in vivo activity (*10*, *11*). This 80 amino acid protein is tryptophan free and very heat stable. Saposin C enhances acid β-glucosidase in vitro activity in the presence of acidic phospholipids [e.g., (*11*)]. Functional studies of saposin C indicate that enzymatic activation requires the C-terminal half of the protein (*11*). Saposin C has been proposed to enhance acid β-glucosidase activity by several mechanisms including (a) direct binding to the enzyme (*12*), (b) interaction with enzyme sites that are exposed (altered) following PS−enzyme association (*13*), and (c) destabilization of phospholipid membranes at acidic pH that promotes acid β-glucosidase binding to the membrane (*14*). Each of these mechanisms includes postulated conformational changes in acid β-glucosidase that lead to increased hydrolysis of substrates. However, the physical effects of the lipid membrane/enzyme and enzyme/saposin C interactions and structural changes induced by these interaction remain unknown.

Unraveling of these basic interactions is essential to further understanding the relationship between the molecular enzymatic effects of the Gaucher disease mutations on the phenotype and, potentially, to the improvement in enzyme therapy for this disease. For example, acidic phospholipids and bile acids were found to differentially activate specific mutant forms of the enzyme (*15*−*17*). The enzyme derived from the most common mutation, N370S, has increased degrees of activation by PS compared to the normal enzyme whereas activation of the L444P mutant enzyme was much less [reviewed in (*4*)]. The N370S and L444P mutants are highly associated with the nonneuronopathic (type 1) and neuronopathic (types 2 or 3) phenotypes, respectively (*4*). For enzyme therapy, purified natural or recombinant enzyme is administered intravenously and is taken up into cells by mannose receptor-mediated endocytosis (*18*, *19*). For function, the administered enzyme must attach to the inner lysosomal membrane, presumably to lipids. Studies in normal mice indicate that about 50% of the administered enzyme is inactive in the cells (*19*) and may not be attached to the lysosomal membrane (*20*). Thus, understanding of the enzyme/phospholipid interactions may provide a better appreciation of the factors that could modulate the Gaucher disease phenotypes and potentially lead to therapeutic approaches that improve the function of endogenous or administered acid β-glucosidase.

In this paper, the interaction of pure human acid β-glucosidase with phospholipids and saposin C is examined directly by intrinsic tryptophanyl fluorescence of the enzyme and CD spectroscopy. These analyses and fluorescence quenching studies show the essential requirements of fatty acid acyl and polar headgroup composition of phospholipids for conformational changes leading to enhancement of activity. These results provide direct support for a model of acid β-glucosidase/phospholipid/saposin C interaction that requires several steps, including interfacial attachment, and membrane insertion of the enzyme and activator followed by their binding and resultant conformational change.

EXPERIMENTAL PROCEDURES

Materials. The following were from commercial sources: potassium iodide (KI) and TEMPO (2,2,6,6-tetramethylpiperidine-*N*-oxyl) (Fischer, Pittsburgh, PA); cesium chloride (CsCl) and acrylamide (Life Technologies, Gaithersburg, MD); 4-methylumbelliferyl-β-D-glucopyranoside (4MU-Glc) and Ceredase (alglucerase for injection) (Genzyme Corp., Cambridge, MA); egg phosphatidylcholine (EPC), brain phosphatidylserine (BPS), heart cardiolipin (HCA), liver phosphatidylinositol (LPI), soybean phosphatidylinositol (SPI), palmitoyl-2-stearoyl(*n*-doxyl)-*sn*-glycero-3-phosphocholine (SLPC, $n = 7$, 10, 16), and all synthetic phospholipids (Avanti Polar Lipids, Alabaster, AL); oleic acid (Sigma, St. Louis, MO); anionic lipids were sodium salts; anti-human albumin Ig fraction (Boehringer-Mannheim, Indianapolis, IN); sodium taurocholate (Calbiochem, Los Angeles, CA). All other reagents were reagent grade or better.

Enzyme Purification. Alglucerase containing α-mannosyl-terminated acid β-glucosidase from human placentas and human serum albumin (HSA) was used as the source to purify acid β-glucosidase. The enzyme was purified to homogeneity by FPLC (Pharmacia Biotech Inc., Piscataway, NJ) using sequential Mono Q, Octyl Sepharose, and Q Sepharose chromatography. Commercial alglucerase was applied to a Mono Q column equilibrated with 0.02 M Bis-Tris, pH 5.8. The enzyme eluted in the wash whereas the bulk of the human serum albumin bound to the column. The enzyme was then applied to an Octyl Sepharose column, and the enzyme was eluted in a linear gradient (0−90%) of ethylene glycol. This fraction contained small amounts of residual human serum albumin detectable with anti-human serum albumin antibodies on Western blots. These trace amounts of human serum albumin were removed by Q Sepharose chromatography according to the Mono Q protocol above. The purified protein had a single band on SDS−PAGE and had no detectable human serum albumin on overloaded Western blots using anti-human serum albumin antibodies. Also, acid β-glucosidase and human serum albumin were resolved on 20% SDS−PAGE (PhastSystem, Pharmacia Biotech Inc.), and human serum albumin was absent from overloaded Coomassie-stained gels. Pure human acid β-glucosidase, free of human serum albumin, was used for all studies. The enzyme was estimated to be >99% pure by SDS−PAGE and Coomassie staining, and immunoblotting studies (Figure 1).

The pure enzyme solution was adjusted to 7−17 μM with 0.02 M Bis-Tris, pH 5.8, and stored at 4 °C. Enzyme

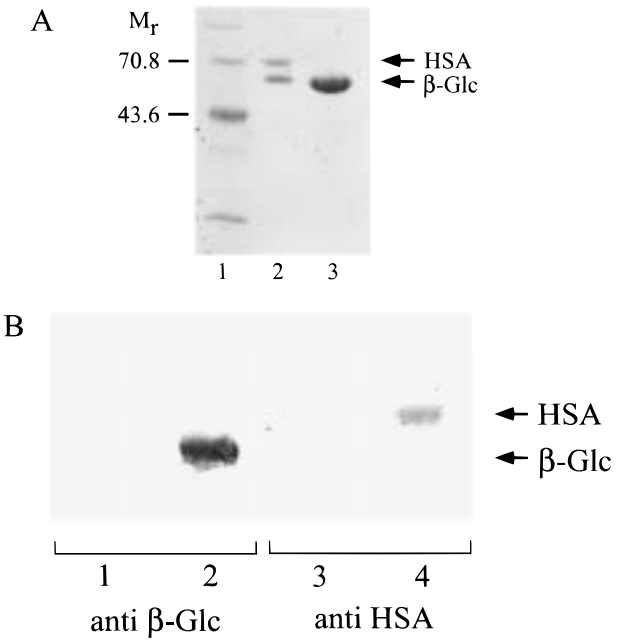

FIGURE 1: Analytical SDS−PAGE (A) and Western blots (B) of acid β-glucosidase preparations. (A) Coomassie blue stained gels of a 1:1 (w/w) mixture of human serum albumin and acid β-glucosidase (β-Glc) (1.0 μg, lane 2), pure acid β-glucosidase (1.5 μg, lane 3), and protein standards (lane 1). (B) Western blots with anti-acid β-glucosidase or anti-human serum albumin were conducted with pure acid β-glucosidase (lanes 2 and 3) and pure human serum albumin (lanes 1 and 4). 20% SDS−PAGE was used to resolve human serum albumin and acid β-glucosidase. Pure acid β-glucosidase, free of human serum albumin, was used in all studies.

activities were determined in this buffer unless otherwise indicated. The enzyme activity, emission fluorescence spectra, and CD spectra were unchanged over 2−3 months of storage. Immunoblotting studies using anti-acid β-glucosidase and anti-human serum albumin were as described (*21*).

Extinction Coefficient for Acid β-Glucosidase. The FPLC-purified acid β-glucosidase was dialyzed exhaustively against H_2O, and aliquots of protein were dried directly in vials with a centrifuge/vacuum system for amino acid composition analysis (Biotechnology Resource Laboratory at Yale University). The amounts of each amino acid were normalized by dividing the total representation of each residue in 1 nmol of acid β-glucosidase as predicted from the cDNA sequence. The concentration of protein in each hydrolyzed sample was determined by averaging the normalized values of 10 acid-stable amino acids. The molar extinction coefficient was calculated by correlating the concentration of the protein with the absorbance at 280 nm in H_2O. The value of 1.01×10^5 M^{-1} cm^{-1} [range = $(0.97-1.05) \times 10^5$] ($E^{1\%} = 18.2$) is the average from three hydrolysates. This value was used to determine the concentration of acid β-glucosidase for all the experiments.

Preparation of Saposins. The coding regions for the human saposins A and C individually were cloned into the pET vector, overexpressed in *E. coli*, and purified on nickel columns as described (*22*). After elution from the His•Bind resin, the saposin eluates were dialyzed and lyophilized. The dried proteins were dissolved in 0.1% trifluoroacetic acid and applied to a HPLC C_4 reverse phase column. The column was washed with 0.1% trifluoroacetic acid for 10 min, and then a linear (0−100%) gradient of acetonitrile was established over 60 min. The major protein peak was collected and lyophilized. The protein concentrations were determined by the Lowry method (*23*) using bovine serum albumin as the standard.

Enzyme Assays. The enzyme activities were estimated fluorometrically (Sequoia-Turner Model 450 fluorometer) with 4MU-Glc as substrate. Assays were conducted in detergent-free systems with or without phospholipids (*6*, *22*). Assays contained lipid-to-protein ratios as indicated; most were at 20:1 (mol/mol).

Preparation of Liposomes. Unilamellar vesicles were prepared as follows: Phospholipids (10−300 nmol) were dissolved in chloroform; the solvent was evaporated under N_2 and then under vacuum (30 min). The films were suspended by vigorous agitation into the selected buffer at room temperature. The suspension was sealed and sonicated until clarified (15−30 min). In some cases, ice was added into bath water to avoid overheating. The temperature during preparation steps was at least 5 °C above the T_c of the lipid mixtures.

Fluorescence Spectra and Quenching Studies. The intrinsic fluorescence of acid β-glucosidase is mainly contributed from its 12 tryptophanyl residues. The tyrosines and phenylalanines contribute ≤10% of the total fluorescence at $\lambda_{Tyr} = 303$ nm and $\lambda_{Phe} = 282$ nm. Fluorescence intensity was monitored in an SLM-Aminco Bowman Series 2 luminescence spectrometer (Urbana, IL), using 1.0 × 0.4 cm matched quartz cuvettes at room temperature. Unless otherwise indicated, $\lambda_{ex} = 280$ nm, and 4 nm spectral bandwidths were used for the excitation and emission monochromators. Emission spectra were acquired by scanning from 300 to 400 nm. The liposome suspensions had no detectable fluorescence in this wavelength range. The enzyme (0.15 or 0.5 μM) was added into liposomal dispersions in lipid-to-protein ratios of 200:1 or 20:1 (mol/mol) in 0.02 M Bis-Tris, pH 5.8. Emission scanning was initiated (<2 min) after enzyme addition. Over a period of several hours, the fluorescence spectra were stable. For fluorescence quenching measurements, the enzyme/liposome mixtures in 0.02 M Bis-Tris, pH 5.8, with or without 0.1 M NaCl, and at lipid-to-protein ratios of ≥100 (mol/mol), were incubated at room temperature for at least 60 min prior to addition of an aqueous quencher. With the quenchers, acrylamide and CsCl, stock solutions were 5 M. KI solution was freshly prepared in 0.1 mM $Na_2(S_2O_3)$ to prevent I_3^- formation (*24*). TEMPO was dissolved in 50% ethanol/0.02 M Bis-Tris, pH 5.8, to a concentration of 1 M. A stock solution of 1 mM was obtained by dilution with buffer. Quenching experiments were conducted for 15 min, and evaluated at $\lambda_{ex} = 280$ nm and $\lambda_{em} = 335$ nm. The individual experimental conditions are presented in the figure legends. The inner-filter effects were minimized because the protein concentrations were kept low. F_{obs} was used in Stern−Volmer plots (*25*, *26*) (eq 2) since no significant intensity differences were observed following correction with eq 1 (*25*):

$$F_c = F_{obs} \text{ antilog } [(A_{ex} + A_{em})/2] \quad (1)$$

$$F_0/F_1 = 1 + K_{sv}[Q] \quad (2)$$

where A_{ex} and A_{em} are the absorbance at the excitation (ex)

and emission (em) wavelengths, respectively. F_{obs} and F_c are the observed and corrected fluorescence intensity, respectively. F_0 and F_1 are the observed fluorescence intensities in the absence and presence of the quencher (Q), and K_{sv} is the Stern−Volmer quenching constant. This can be written as

$$F_0/F_1 = 1 + K_{app}[Q] \quad (3)$$

where

$$K_{app} = (K_D + K_S) + K_D K_S[Q] \quad (4)$$

The apparent quenching constant, K_{app}, includes dynamic (K_D) and static (K_S) quenching components.

Circular Dichroism (CD) Spectra. CD spectra were recorded on a Jasco J-710 spectropolarimeter (Jasco Inc., Easton, MD), using a 0.1 cm sample cell at room temperature. The instrument was interfaced with a computer that was used for deconvolution calculations. To reduce noise at $\lambda < 200$ nm, the experiments were done in 10 mM sodium phosphate, pH 5.8, or 0.005 M citrate/0.01 M phosphate, pH 5.8, rather than in activity assay buffers (0.02 M Bis-Tris, pH 5.8, or 0.1 M sodium acetate, pH 4.7). The use of phosphate buffer rather than activity assay buffer did not produce any CD spectral changes or in the effects of lipids on the enzyme. The CD spectra of human acid β-glucosidase were used to calculate secondary structure with reference to an average of seven proteins with known X-ray structure, i.e., myoglobin, lysozyme, ribonuclease A, papain, cytochrome *c*, hemoglobin, and α-chymotrypsin A. These analyses provided calculations of α-helix (% α), β-strand (% β), β-turn (% T), and random-coiled or other structures (% R) present, respectively. The CD spectra of acid β-glucosidase and saposin A or C mixtures in the absence or presence of phospholipids were acquired under the same conditions as used for the individual spectra. The major purpose of these experiments was to monitor changes in CD spectra, and therefore protein conformation, under different experimental conditions and not to attempt to portray these spectra and calculations as true reflections of secondary structures. Such calculation may require several different protein reference sets. The deconvolution programs were from Jasco, Inc. The addition CD spectra of acid β-glucosidase and saposin C were mathematically generated using analysis programs provided by Jasco.

RESULTS

Activation of Acid β-Glucosidase by Anionic, Unsaturated Phospholipids (Table 1). EPC, containing a mixture of saturated (18:0,0) and unsaturated (18:1,0) fatty acid acyl chains, does not enhance acid β-glucosidase activity. Pure PC with one double bond in each fatty acid acyl chain (18:1,1) also did not activate the enzyme. Brain phosphatidylserine [BPS containing a mixture of PS (18:1,0) and PS (18:0,0)] is known to activate acid β-glucosidase, and the negative charge on its headgroup is critical to this effect (*6*). To investigate the effects of fatty acid acyl chain saturation on acid β-glucosidase activity, several pure phospholipids (PLs) containing either saturated or unsaturated chains were tested. PLs with saturated acyl chains [PS (18:0,0); PA (18:0,0)] did not alter acid β-glucosidase activity (Table 1). In comparison, those negatively charged PLs containing fatty acid acyl chains (Figure 2) with one or two double bonds enhanced the enzyme activity by ~ 20−30-fold, including PS (18:1,1), PS (18:2,2), phosphatidylinositols from liver [LPI (mixture of 18:0,0 to 20:4)] or soybean [SPI (mixture of 16:0,0 to 18:2,2)], or heart cardiolipin (HCA; ~90% of 18:2,2,2). The same degrees of activation were observed with these lipids at pH 5.8 or 4.7, but maximal activity was greater at pH 5.8. BPS produced acid β-glucosidase activation levels similar to pure PS (18:1,1) or PS (18:2,2) (Table 1). These data showed that anionic PLs with at least one unsaturated fatty acid acyl chain are required to be in proximity to the enzyme for enhancement of activity.

Table 1: Effects of Phospholipid Fatty Acid Acyl Chain on Acid β-Glucosidase Activity and Emission Spectrum

phospholipid[a]	activation (*x*-fold)	fluorescence emission blue-shift
none	1.0	−
neutral phospholipids		
egg phosphatidylcholine (18:1,0/18:0,0)	1.0	no
phosphatidylcholine (18:1,1)	1.0	no
phosphatidylethanolamine (18:0,0)	1.0	no
phosphatidylethanolamine (18:1,1)	1.1	no
anionic phospholipids		
phosphatidylserine (18:0,0)	1.3	no
brain phosphatidylserine (18:1,0)/(18:0,0)	24.8	yes
phosphatidylserine (18:1,1)	23.1	yes
phosphatidylserine (18:2,2)	23.0	yes
phosphatidic acid (18:0,0)	1.0	no
phosphatidic acid (18:1,1)	10.7	yes
liver phosphatidylinositol	23.0	yes
soybean phosphatidylinositol	30.6	yes
heart cardiolipin	7.1	yes
phosphatidyl ethylene glycol (18:1,1)	5.7	yes
phosphatidylglycerol (18:0,0)	1.1	no
phosphatidylglycerol (18:1,1)	13.7	yes

[a] The total phospholipid concentration in all instances was 7.5 μM diluted in 0.02 M Bis-Tris, pH 5.8.

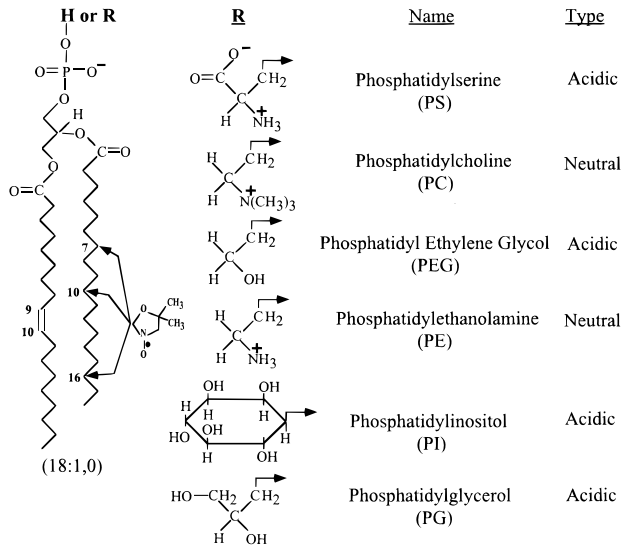

FIGURE 2: Schematic diagrams of various phospholipids: The backbone, phosphatidic acid, is shown on the left with a single unsaturated bond at position 9 which is the usual physiologic fatty acid acyl chain. The R column represents various polar headgroups, and the arrows show the attachment site to the phosphatidic headgroup. The doxyl groups shown by the arrows near the acyl chain indicate the position (carbons 7, 10, or 16) of substitution in PC giving rise to SLPC. For SLPC, the other acyl chain is saturated.

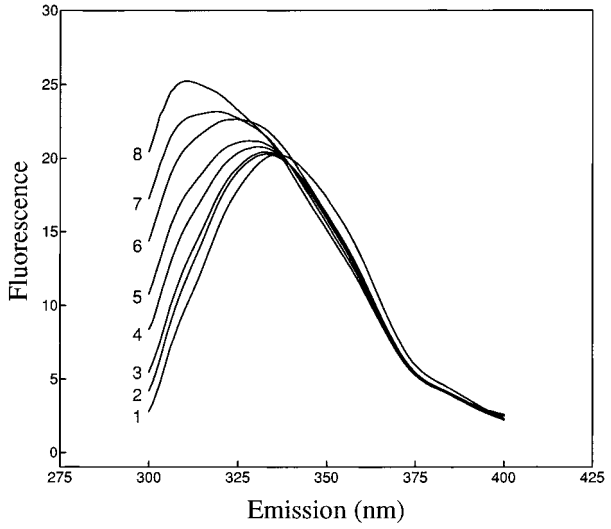

FIGURE 3: Intrinsic fluorescence spectra of acid β-glucosidase in the presence or absence of BPS and/or saposin C. Emission spectra were acquired at $\lambda_{ex} = 280$ nm in 0.005 M sodium citrate/0.01 M sodium phosphate, pH 4.7, at room temperature. Curve 1 is for acid β-glucosidase alone (0.15 μM); curves 2−8 had mixtures of BPS (30 μM), acid β-glucosidase (0.15 μM), and saposin C at the following concentrations: (2) 0 μM; (3) 0.15 μM; (4) 0.9 μM; (5) 1.5 μM; (6) 2.1 μM; (7) 3.0 μM; and (8) 4.1 μM.

Fluorescence Emission Spectra. The fluorescence emission spectrum of acid β-glucosidase in Bis-Tris, pH 5.8, was determined at $\lambda_{ex} = 280$ nm (Figure 3). A maximum occurred at 335−336 nm with a half-bandwidth, $\Delta\lambda_{1/2}$, of 50−53 nm. No alteration in the shape of the emission spectrum occurred with varying λ_{ex} from 280 to 295 nm. The absence of a shoulder at $\lambda_{em} = 308-310$ nm indicates a minimal contribution from tyrosine residues and that the major intrinsic fluorescence of acid β-glucosidase derived from tryptophanyl residues.

Emission spectra of proteins change when the tryptophanyl environments shift toward lesser polarity (*25*). For example, blue-shifts in the emission spectra of proteins have been observed as a consequence of protein/lipid complex formation. The fluorescence spectra of acid β-glucosidase, obtained upon addition of BPS liposomes (200:1; mol/mol, lipid-to-enzyme), showed a 2 nm blue-shift from 335 to 333 nm (Figure 3). Although this blue-shift was small, it was reproducible with little variation in experiments conducted over 1 year. The same result was obtained with a lipid-to-protein ratio of 20:1 (mol/mol). Essentially, identical shifts were observed in the presence of 0.25% taurocholate. Addition of saposin C, that contains no tryptophan, into the enzyme−BPS mixture is accompanied by significant changes in the acid β-glucosidase emission spectrum (Figure 3). The quantum yield increases, and the emission maxima progressively blue-shift to shorter wavelengths as the concentration of saposin C increases. The greatest blue-shift in the emission maximum was about 24 nm, i.e., a shift from 335 to 311 nm, in the presence of a 27-fold molar excess of saposin C over enzyme concentration.

In separate experiments, several phospholipids were evaluated for their ability to induce acid β-glucosidase fluorescence spectral shifts. The 2 nm blue-shift in the emission spectra of acid β-glucosidase was observed only with liposomes composed of anionic PLs that had unsaturated fatty acid acyl chains, i.e., PS (18:1,0), PS (18:1,1), PS (18:2,2), LPI, SPI, and HCA (Table 1). These were the lipids that provided substantial enhancements of acid β-glucosidase activity. With the lipids that had no activation effects on enzyme activity, the fluorescence spectra remained unchanged. Thus, a direct correlation exists between the fluorescence emission spectrum blue-shifts and the ability of the specific lipid to enhance acid β-glucosidase activity. The double bonds in the fatty acid acyl chains of the lipids did not, by themselves, produce a blue-shift in the acid β-glucosidase emission spectrum. This was evident from the lack of a blue-shift of the enzyme's fluorescence emission spectrum in the presence of the unsaturated PC (18:1,1) (Table 1).

Fluorescence Quenching Studies of Acid β-Glucosidase. Nonpolar and polar quenching compounds were used to evaluate the accessibility of the tryptophanyl residues in acid β-glucosidase to solvent in the absence or presence of PS or PC liposomes. Acrylamide is an efficient neutral quencher (*27*−*29*). In the absence of liposomes, the intrinsic fluorescence of acid β-glucosidase was largely quenched by 0.3 M acrylamide (Figure 4A). In the presence of EPC liposomes, 0.5 M acrylamide was needed to achieve the same level of quenching as observed in the absence of liposomes (Figure 4A). This indicates that EPC and the enzyme weakly interact. The Stern−Volmer plots (eq 3) in the presence or absence of EPC liposomes also showed a substantial upward curvature, indicating combined dynamic and static quenching for the tryptophanyl residues in acid β-glucosidase (*25*). In the presence of BPS liposomes, 0.5 M acrylamide produced only ~10% quenching of the intrinsic fluorescence of acid β-glucosidase ($K_{sv} = 8.4$ M^{-1}), and the curve was linear. TEMPO, a more hydrophobic (but aqueous-soluble) spin-labeled quencher, showed similar quenching patterns of acid β-glucosidase fluorescence to the corresponding profiles with acrylamide in the presence of EPC or BPS liposomes (Figure 4B). Liposomes composed of BPS protected the enzyme's fluorescence very effectively from quenching by TEMPO. Essentially none of the tryptophanyl residues in acid β-glucosidase was accessible to TEMPO; $K_{sv} = 6.6 \times 10^{-3}$ M^{-1}. Increasing concentrations of EPC and BPS provided greater protection from TEMPO fluorescence quenching, but BPS alone was always more effective (Figure 5).

Similar experiments were conducted with charged quenchers, I^- and Cs^+, to determine the accessibility of the tryptophanyl residues in acid β-glucosidase to the aqueous environment. For experiments with increasing KI concentration, the ionic strength was maintained constant with KCI as the counterion ($\lambda_{ex} = 295$ nm). In the absence of liposomes, these charged compounds (0.3 M) quenched the intrinsic fluorescence of acid β-glucosidase with an efficiency of only ~30% of that achieved with acrylamide (Figure 4C,D). In the presence of BPS liposomes, both of these charged compounds produced much smaller quenching effects on acid β-glucosidase fluorescence compared to that achieved in the presence of EPC liposomes (Figure 4C,D); $K_{sv} = 3.0$ and 2.6 M^{-1} for KI and CsCl, respectively. The greater quenching effect on acid β-glucosidase fluorescence by uncharged (acrylamide and TEMPO) than by charged quenchers indicates that many of the tryptophanyl residues are not readily accessible to the aqueous solvent. BPS liposomes shielded the intrinsic fluorescence of acid β-glu-

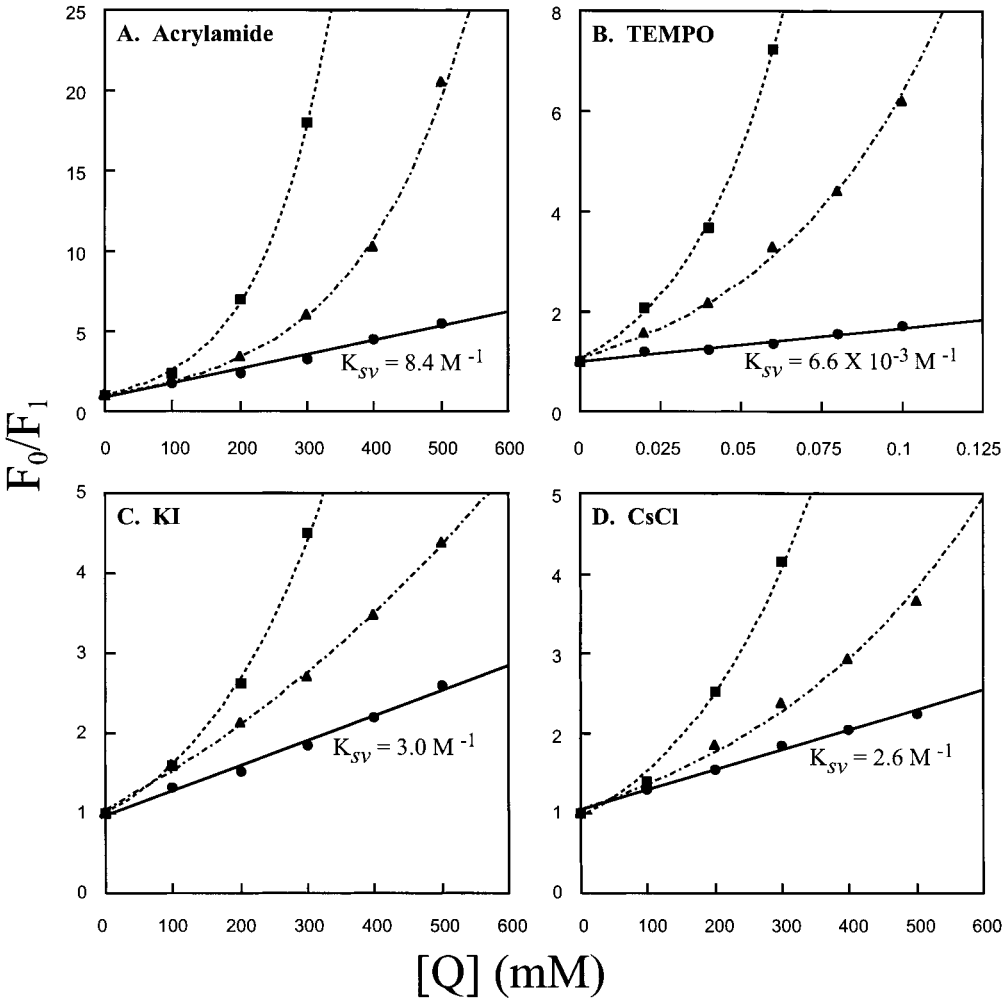

FIGURE 4: Stern−Volmer plots for quenching of intrinsic fluorescence of acid β-glucosidase by water-soluble agents. Quenching was done in the absence (- - -), or presence of phosphatidylcholine (− · −) or phosphatidylserine [BPS] (—) liposomes. [Q] is the concentration of the quenchers indicated in each panel. F_0/F_1 is the fluorescence intensity in the absence (F_0) or presence (F_1) of the quenching agent. $[E]_t$ = 0.2 μM. The phospholipid concentrations were 50 μM in all experiments. Conditions as in Figure 5.

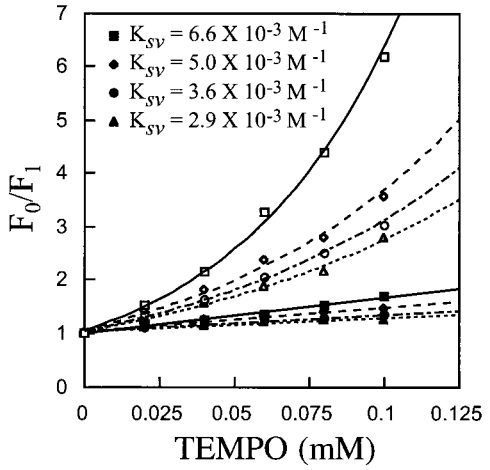

FIGURE 5: Stern−Volmer plots of TEMPO quenching of the intrinsic fluorescence of acid β-glucosidase at various phospholipid concentrations. Open and filled symbols indicate different concentrations of EPC and BPS, respectively. Squares, diamonds, circles, and triangles are 0.05, 0.1, 0.15, and 0.3 mM of each lipid, respectively. The different line styles are presented for clarity. F_0/F_1 has the same meaning as in Figure 4. $[E]_t$ = 0.2 μM. The studies were done in 0.02 M Bis-Tris, pH 5.8, at room temperature.

cosidase from the bulk solvent due to formation of the lipid−enzyme complex. This was not observed with EPC liposomes. The ability of specific lipids to protect acid β-glucosidase from fluorescence quenching by these aqueous compounds correlated well with the lipid-induced blue-shift of acid β-glucosidase fluorescence emission spectra and enzyme activation.

To determine whether acid β-glucosidase associated with BPS liposomes by surface interaction and/or penetration, spin-labeled phosphatidylcholine (SLPC), a fluorescence quencher, was incorporated into EPC or BPS liposomes. SLPCs were used with the doxyl groups located at different carbons (n) in the acyl chain: SLPC7 (n = 7), SLPC10 (n = 10), and SLPC16 (n = 16) (Figure 2). The BPS/SLPC liposomes activated acid β-glucosidase to a similar degree as BPS/EPC liposomes composed of the same mole fractions (data not shown). Increasing mole percentages (0−25%) of SLPC were incorporated into liposomes composed of EPC or BPS. After addition of acid β-glucosidase, the enzyme/liposome mixture was incubated at room temperature for 0.5, 1, 3, 5, and 16−18 h, and, then, the fluorescence intensity changes were determined. Similar results were obtained at all time points, and 1 h of incubation was routinely used. Some quenching effects (~15%) were observed in the EPC/SLPC liposomes (Figure 6). In comparison, the intrinsic fluorescence of acid β-glucosidase was quenched by ~50%

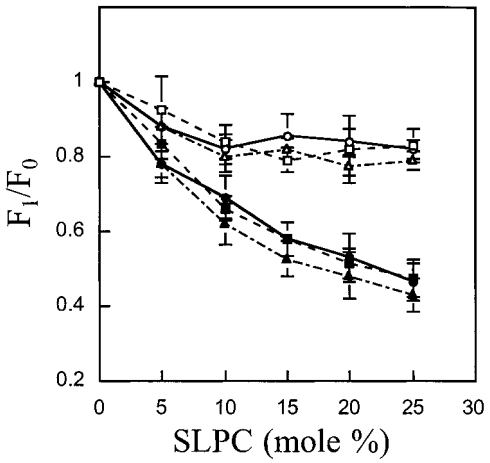

FIGURE 6: Inverse Stern−Volmer plots of spin-labeled phosphatidylcholine (SLPC) quenching of acid β-glucosidase intrinsic fluorescence. Hydrophobic quenching of acid β-glucosidase (0.1 μM) intrinsic fluorescence was in the presence of egg phosphatidylcholine EPC/SLPC (open symbols) or brain phosphatidylserine BPS/SPLC (closed symbols) liposomes. Squares, triangles, and circles represent 7-SLPC, 10-SLPC, or 16-SLPC, respectively, where 7-, 10-, and 16- represent the position of the spin-label in the fatty acid acyl chain (Figure 2). The bulk phospholipid concentration was 20 μM, and the SLPC concentration is given by mole percent of the total. These analyses were in 0.02 M Bis-Tris, pH 5.8, with 0.1 M NaCl at room temperature after 1 h of incubation.

in the presence of BPS/SLPC (3:1; mol/mol). This was not dependent upon the location in the acyl chain of the doxyl group. Apparently some of tryptophanyl residues in acid β-glucosidase were accessible to doxyl groups on SLPC, and these fluorophores resided at various depths in the membrane. This finding suggests that part of acid β-glucosidase resides in the BPS lipid bilayer at depths of <7 to $\sim$16 carbons bond lengths.

The necessity of an unsaturated fatty acid acyl chain attached to an anionic phospholipid for enhancement of acid β-glucosidase activity and induction of a fluorescence spectral blue-shift was shown above. The fluorescence quenching studies with SLPC suggested proximity of the fluorescence quencher and tryptophanyl residues on acid β-glucosidase. Since the doxyl group in SLPC has some double bond characteristics (*30, 31*), these results suggest that the unsaturated fatty acid acyl chain need not reside on the phospholipid with the negatively charged headgroup, but could be present in a neighboring lipid. To test this, a series of experiments were conducted using various phospholipids with saturated or unsaturated fatty acid acyl chains, or oleic acid, in mixed liposomes. These results (Table 2) show that particular combinations of phospholipids with saturated and unsaturated fatty acid acyl chains, or phospholipids with saturated acyl chains and oleic acid (18:1) produced small to large activation enhancements of acid β-glucosidase activity. Low-level activation (2.2−2.5-fold) was achieved with anionic phospholipids containing saturated fatty acid acyl chains in liposomes with EPC, SLPC, or PC (18:1,1). A similar result was obtained with PC (18:0,0)/oleic acid (18:1) liposomes. A blue-shift in the fluorescence emission spectra was not reproducibly obtained in these reaction mixtures (Table 2). Moderate degrees of enhancement of acid β-glucosidase activity were obtained with anionic phospholipids with saturated fatty acid acyl chains in the presence of oleic acid or phosphatidylethanolamine (18:1,1). Such mixed liposomes produced 6−16-fold enhancements of acid β-glucosidase activity, or $\sim$30−90% of the fold activation achieved with the corresponding anionic phospholipid containing at least one unsaturated fatty acid acyl chain. These data also indicate that compared to PE, generally lower degrees of activation were obtained when PC contained the unsaturated the fatty acyl chain. These results implicate the nature of the headgroup as a significant factor influencing the degree of activation. For those liposomes that resulted in moderate to substantial degrees of enzyme activation, the blue-shift in the intrinsic fluorescence of acid β-glucosidase also was detected (Table 2).

Effects of Phospholipids or Phospholipids/Saposin C on Acid β-Glucosidase Conformation. The above studies showed a blue-shift in the intrinsic acid β-glucosidase fluorescence and some degree of penetration of the enzyme into membranes containing anionic phospholipids and selected other lipids with unsaturated fatty acid acyl chains. To evaluate concordant conformational changes, circular dichroism spectroscopy (CD) was conducted with the enzyme or enzyme−lipid complexes. Several phospholipids were used for CD studies, and the secondary structural changes in acid β-glucosidase were determined by deconvolution of the spectra in the absence and presence of the phospholipids at pH 5.8 (Table 3). These results were similar to those for the enzyme in the presence of phospholipids that exerted no activation effects on acid β-glucosidase, i.e., EPC, PC (18:1,1), and PS (18:0,0) (data not shown). These changes in CD spectra indicated detectable conformational alteration of

Table 2: Activation of Acid β-Glucosidase by Liposomes of Mixed Lipids

added lipid[a]	mole ratio	activation (*x*-fold)	fluorescence emission blue-shift
none		1.0	−
phosphatidylserine (12:0,0)	−	24.9	yes
egg phosphatidylcholine (18:1,0/18:0,0)	−	1.0	no
phosphatidylcholine (18:0,0)	−	1.0	no
phosphatidylcholine (18:1,1)	−	1.1	no
phosphatidylserine (18:0,0)/egg phosphatidylcholine	1:1	2.4	?
phosphatidylserine (18:0,0)/SLPC(n=10)	1:1	2.2	?
phosphatidylserine (18:0,0)/oleic acid (18:1)	1:1	7.4	yes
phosphatidylcholine (18:0,0)/oleic acid (18:1)	1:1	3.0	yes
phosphatidic acid (18:0,0)/phosphatidylcholine (18:1,1)	1:1	2.5	?
phosphatidic acid (18:0,0)/phosphatidylethanolamine (18:1,1)	1:1	15.7	yes
phosphatidylglycerol (18:0,0)/phosphatidylethanolamine (18:1,1)	1:1	6.3	yes

[a] Bulk lipid concentration was 7.5 μM in all assays.

Table 3: Circular Dichroism Analyses of Acid β-Glucosidase with Various Phospholipids[a]

	deconvolution calculations			
phospholipids	% α	% β	% T	% R
none (195−250 nm)	43.3	11.2	22.5	22.9
egg phosphatidylcholine (18:1,0)	39.3	10.5	25.7	24.5
phosphatidylcholine (18:1,1)	43.8	8.8	24.0	23.5
phosphatidylserine (18:0,0)	44.3	7.4	23.7	24.6
phosphatidylserine (18:1,1)	59.7	0.0	22.6	17.8
phosphatidylserine (18:2,2)	44.2	0.0	30.4	25.5
none (200−250 nm)	32.0	11.0	25.9	31.0
taurocholate (0.02%)	76.6	0.0	14.5	8.9

[a] Acquired in 0.01 M sodium phosphate, pH 5.8.

the enzyme due to the formation of a protein−negatively charged phospholipid complex.

The spectral changes were pH dependent and were greater at pH 5.8 than pH 4.7. Thus, we calculated the above secondary structural changes from data acquired at pH 5.8 since they provided larger and more reproducible changes compared to the smaller effects at pH 4.7. These experiments were done only to obtain relative, not absolute, changes in structure. The deconvolved secondary structure of acid β-glucosidase at pH 5.8 in the presence of several other activating phospholipids suggested similar changes in secondary structure. The most prominent of these was a decrease in β-sheet content to 0%. The α-helical content changes varied with the degree of unsaturation of the PS. For example, an increase in α-helix of ∼15% was found with PS (18:1,1) liposomes, while no alteration of α-helical content was detected with PS (18:2,2) liposomes (Table 3). Taurocholate, an anionic detergent, is known as a potent activator of acid β-glucosidase (*4*). With 0.02% taurocholate, the α-helical content was increased ∼30%, and the β-sheet content was decreased to 0%. To avoid the increasing noise below 200 nm, the CD spectrum of acid β-glucosidase with taurocholate was acquired between 200 and 250 nm. These results show that the phospholipid-induced CD spectral changes correlated with the ability of the phospholipid to enhance acid β-glucosidase activity.

Saposin C has its greatest effects at pH ∼4.7. Thus, CD spectra for assessing the effects of saposin C in the absence of phospholipids were acquired at pH 4.7 (Figures 7 and 8). Mixtures of acid β-glucosidase and saposin C were evaluated by CD spectroscopy to determine the interaction of these proteins in the absence (Figure 7) and presence (Figure 8) of phospholipids. The CD spectra (195−250 nm) of acid β-glucosidase (1.5 μM) and saposin C (8.4 μM) are in Figure 7. The individual spectra of the enzyme and saposin C are shown as curves A and B. The mathematical sum of these spectra is curve C. The spectrum of a physical mixture of the enzyme and saposin C is curve D. The identity of the calculated sum and the actual CD spectrum implies a lack of conformational change of either the enzyme or saposin C and, therefore, a lack of interaction. The results of similar studies in the presence of PS (18;1,1) are in Figure 8. The CD spectrum of acid β-glucosidase in the presence of PS (18:1,1) differed subtly, but reproducibly, from that in the absence of PS (Figure 8A). These analyses show that the mathematical sum of the individual spectra for the enzyme and saposin C in the presence of PS (18;1,1) is quite different from that obtained from the actual mixture of these proteins

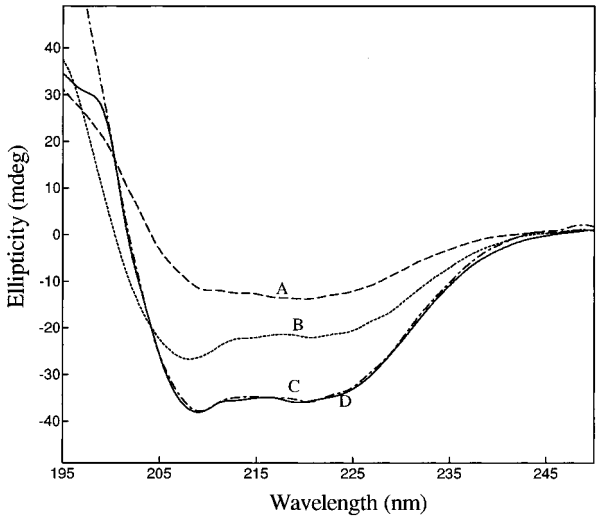

FIGURE 7: CD spectra of acid β-glucosidase and saposin C in the absence of phospholipids. (A) Acid β-glucosidase (1.5 μM); (B) saposin C (8.4 μM); (C) the mathematical sum of (A) and (B); (D) acid β-glucosidase and saposin C mixture at the same concentrations as in (A) and (B). Spectra were acquired in 0.005 sodium citrate/0.01 M sodium phosphate, pH 4.7, at room temperature.

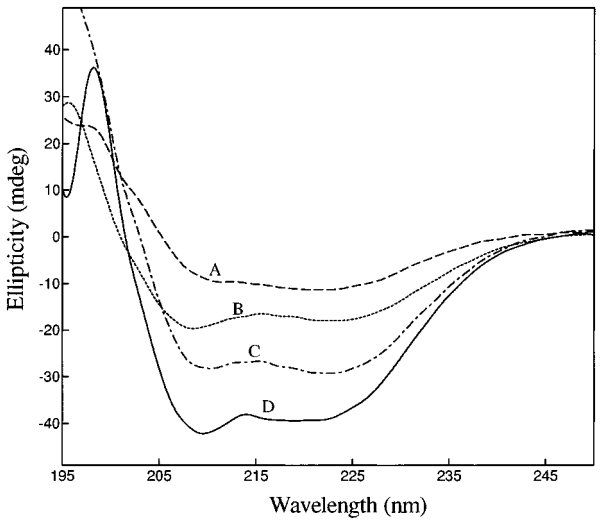

FIGURE 8: CD spectra of acid β-glucosidase and saposin C in the presence of phosphatidylserine. (A) Acid β-glucosidase (1.5 μM); (B) saposin C (8.4 μM); (C) the mathematical sum of (A) and (B); (D) acid β-glucosidase and saposin C mixture at the same concentrations as in (A) and (B). Spectra were acquired in 0.005 sodium citrate/0.01 M sodium phosphate, pH 4.7, at room temperature in the presence of 22.5 μM PS (18:1,1).

in the presence of the phospholipid. Essentially identical results were obtained using BPS instead of PS (18:1,1). Control experiments included using saposin A instead of saposin C, and EPC or PS (18:0,0) instead of PS (18:1,1). The CD spectra of any such mixtures were, within experimental error, identical to the mathematical sum of the individual spectra. These results indicate that, in the presence of PLs that activate acid β-glucosidase, mixture of the enzyme and saposin C leads to additional conformational changes. These additional, nonadditive, changes correlate with enhancement of enzyme activity. These results suggest a direct interaction of the enzyme and saposin C occurring only in the presence of specific lipids and that saposin A does not interact significantly with acid β-glucosidase.

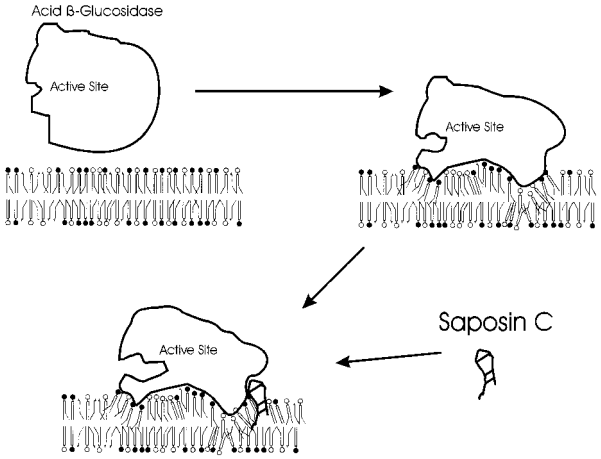

FIGURE 9: Proposed interaction of acid β-glucosidase, negatively charged phospholipid membranes, and saposin C. The diagram proposes that acid β-glucosidase interacts with a membrane containing negatively charged phospholipids with at least one unsaturated fatty acid acyl chain. This is shown as bent lines attached to open (neutral charge) or closed (negatively charged) circles. The single bent lines represent unsaturated free fatty acids (i.e., oleate) that may substitute for the fatty acid acyl chain on saturated (straight lines) phospholipids to facilitate binding of the enzyme. Acid β-glucosidase attaches to regions enriched in negatively charged phospholipids and undergoes a conformational change to allow enhanced active site function and development of a binding site for saposin C. The enzyme is not transmembrane, potentially penetrates into the membrane to the equivalent of about 7−16 carbon bonds, and is probably a monotopic membrane protein whose binding is determined by electrostatic and hydrophobic forces. The quantitative contribution of either is not implied by the diagram. Saposin C in solution attaches to the membrane and moves in the membrane to collide with a site on acid β-glucosidase that was exposed by the enzyme's interaction with the membrane. The binding of saposin C induces an additional conformational change in the enzyme and, possibly, in saposin C to maximize the catalytic activity of the enzyme. The active site remains near or at the surface since the enzyme has activity toward water-soluble aryl-*O*-β-glucosides and lipoidal substrates, glucosylceramides. Although the diagram suggests that the major interaction is electrostatic, there is clearly interaction of the enzyme with the hydrophobic layers of the membrane.

DISCUSSION

In the present studies, we analyzed acid β-glucosidase's conformational changes, following interaction with phospholipids and saposin C, by monitoring intrinsic fluorescence and overall secondary structure by CD spectroscopy. These studies show that an enzyme conformational change occurs upon interaction with negatively charged phospholipid membranes of particular fatty acid acyl structure. Indeed, the data support a model in which acid β-glucosidase first interacts, with some specificity, with the negatively charged phospholipid polar headgroup at the interface prior to insertion into the membrane. Once in the membrane, the enzyme can interact with surrounding hydrophobic acyl chains containing particular unsaturated composition, preferentially with those on the same negatively charged phospholipid(s), or less well with those on closely associated/neighboring phospholipids or fatty acids (Figure 9). This interaction conforms the enzyme into an active state. By inference, the catalytic residues in the active site must be near (at or above) the membrane surface to allow interaction with and cleavage of *O*-β-glucosidic bonds of substrate headgroups (Figure 9). The steps for interaction at the surface of the membrane were indicated by the need for a net negative charge, or specific nature, of the PL headgroup for optimal activation. Without the appropriate headgroup, interaction with the membrane is greatly diminished. Penetration of the enzyme into the membrane, as measured by protection from fluorescence quenching, was influenced by the presence of a double bond in the fatty acid acyl moiety in close proximity to the enzyme. The fluorescence quenching of the enzyme by SLPC indicates that penetration into the membrane could be to a depth of 7−16 carbons. However, due to the 12 tryptophans in acid β-glucosidase and their unknown 3-space distributions, and the potential for the 10 Å quenching distance effect of SLPC, the depth of membrane penetration cannot be assessed accurately. Acid β-glucosidase does not contain sequences with characteristics of transmembrane domains, and no significant cytoplasmic domains have been detected by in vitro translation analyses with dog pancreatic microsomal membranes (*3*). Such findings support the notion that acid β-glucosidase binds with membranes initially via electrostatic interactions and, then, enters the membrane to some degree. This insertional phase is modulated by the unsaturation of the phospholipid's fatty acid acyl chain or the presence of unsaturated free fatty acid moieties in the membrane.

Aqueous soluble quenchers at high concentrations were able to quench some of the intrinsic fluorescence of acid β-glucosidase in the presence of PC liposomes. Some interaction did occur since the Stern−Volmer curves were shifted to the right in the presence of the liposomes. These curves were complex, and represent static and dynamic quenching effects of the 12 tryptophanyl groups on the enzyme that cannot be resolved by the current analyses. The right shift in fluorescence quenching could be mediated through shielding by PC liposomes due to the large molar excess of lipid over enzyme or could be by direct interaction through weak binding of the enzyme to the phosphatidylcholine liposomes. Also, liposomes could interact with the quencher itself to reduce the efficiency of quenching, but these effects would be expected to be similar with PS and PC. This was not observed. Irrespective of the mechanism, the interaction between pure PC liposomes and the enzyme is insufficient to enhance enzymatic activity or induce conformational changes large enough to be detected by fluorescence emission or CD spectroscopy.

PSs with unsaturated acyl chains formed liposomes that nearly completely protected the enzyme's intrinsic fluorescence from quenching by the aqueous agents. This shows directly that acid β-glucosidase interacts with anionic phospholipids by sequestering parts of the enzyme in nonaqueous environments. PS afforded nearly complete protection from fluorescence quenching by aqueous agents, demonstrating that all or at least most of the tryptophan residues are protected by a PS-induced conformational change and/or by the membranous environment. This was supported by the blue-shift in fluorescence emission spectra observed with acid β-glucosidase in the presence of PS containing unsaturated acyl chains.

Only PSs with relatively long fatty acid acyl chains (i.e., $\geq$ diC_{12}) or those containing at least one unsaturated acyl chain at Δ9 conformed the enzyme into an active state. Indeed, PS (18:0,0) did not enhance enzymatic activity, result in a blue-shift in fluorescence emission spectra, or produce

changes in CD spectra. These results show that the negative charge on the headgroup is necessary, but not sufficient, for conformational changes that result in acid β-glucosidase activity. It should be noted that the spectral blue-shift, induced by enzyme−membrane interaction, was small (2 nm), but very reproducible. Because of the presence of 12 tryptophanyl residues with unknown distribution in 3-space, particularly the partitioning into aqueous accessible and inaccessible environments, the contribution of each of these to the overall fluorescence spectrum is unknown. These are likely complex phenomena whose effective sum produces a smaller than expected shift in the fluorescence emission spectrum. A way to resolve this difficulty would be to mutagenize acid β-glucosidase to contain one or only a few tryptophanyl residues in specific locations. However, three considerations exclude this approach from consideration: (1) A primary objective of this research is to understand the requirements for enzymatic activity. (2) There are no conservative substitutions for tryptophanyl residues. (3) Identification and functional characterization of conservative (i.e., Val to Leu) or nonconservative substitutions in acid β-glucosidase that predispose to Gaucher disease, as well as random mutagenesis, indicate a significant difficulty in selecting substitutions that do not adversely affect catalytic activity (*32*).

A dissociation of the enzyme's interaction with the negatively charged surface and the fatty acid acyl chain was obtained in mixed liposomal preparations. Liposomes composed of PS (18:0,0) and oleic acid (a Δ9 unsaturated fatty acid) led to the activation of acid β-glucosidase, a blue-shift in the fluorescence spectrum essentially identical to those observed with PS (18:1,0). These results and the varying degrees of enhancement of enzyme activity by liposomes of various compositions of a neutral or anionic phospholipids and/or anionic fatty acids indicate that the proximity to an unsaturated fatty acid acyl chain at a depth of 9−10 carbon bonds was sufficient to result in partial reconstitution of enzyme activity. This also was supported by the results with mixed liposomes containing BPS and SLPC. With these liposomes, quenching of the intrinsic fluorescence of acid β-glucosidase could be explained only by the close proximity of the spin-label at various depths in the membrane and tryptophans of the enzyme. The spin-labeled acyl chains have similar properties to those of unsaturated acyl chains and, thus, mimic those structural effects (*30, 31*). Since the spin-label quenches only in relative proximity (∼10−11 Å) (*33*) to tryptophan residues, these results implicate a juxtaposition of some tryptophanyl residues within the three-dimensional hydrophobic bilayer containing the spin-label of SLPC. Consequently, penetration of acid β-glucosidase into a PS membrane is accompanied by a conformational change that leads to exposure of tryptophanyl residues to the surrounding lipid milieu with resultant quenching by SLPC in proximity. Since PS (18:0,0) liposomes do not reconstitute acid β-glucosidase activity, this implies that the proximity of unsaturated fatty acid acyl chains in a variety of lipids within the membrane could effect conformational changes in acid β-glucosidase, resulting in partial reconstitution in enzymatic activity. This would be true even if the unsaturated acyl chain was attached to a different molecule than that having the negatively charged polar headgroup, i.e., oleic acid or PC, PE, etc. Thus, it is possible that acid β-glucosidase in the inner lysosomal membrane could be modulated by varying the composition of the fatty acid acyl chains in lipids of these membranes. These studies do not address the question of whether the maintenance of the negatively charged headgroup interaction with acid β-glucosidase is essential after the enzyme is in or on the membrane.

As an aside, attempts by us and a commercial company to synthesize spin-labeled PS by transacylation using phospholipid D were unsuccessful. Although small amounts of spin-labeled PS were obtained using crude preparations of phospholipase D, no product was obtained with various purified enzyme preparations. It appears that the spin-labeled fatty acid is a poor substrate for this reaction and crude phospholipase D may contain additional activities.

For over a decade it has been known that the Δ9 unsaturation in the sphingosyl chain of glucosylceramide is essential for optimal hydrolytic activity of acid β-glucosidase toward this substrate (*7*). The coincidence of the positioning of the unsaturated bond in the phospholipids and that in the sphingosyl moiety of a substrate cannot be overlooked. The fluorescence quenching data by SLPC suggest that the interaction with the enzyme extends into the membrane to a depth near the Δ9 bond. The activation of the enzyme by diC_{12}-PS indicates that fatty acid acyl chains longer than 9 or 16 carbon have relatively little, if any, direct interaction with the acid β-glucosidase active site or enzyme. The fact that inhibitors such as *N*-acyldeoxynojirmycins or *N*-acyl-β-glucosylamines have maximal potency at acyl chains lengths of 12 carbons reinforces the regional depth of these interaction domains (*34*). It cannot be answered from the current studies whether the interaction domains with phospholipid acyl chains and substrate acyl chains represent the same physical structures.

Previous kinetic analyses indicated interaction of saposin C and acid β-glucosidase in the presence of negatively charged PLs [reviewed in (*1*)]. Other studies used synthetic peptides in the presence or absence of such PLs to suggest the location of an activation domain of saposin C in its COOH-terminal half (*11*). The present studies show by direct measurement that a conformational change in the enzyme and/or saposin C occurs only in the presence of activating PLs. Previous kinetic experiments support this contention (*12, 13*), but evidence for direct contact between saposin C and acid β-glucosidase is still required. Furthermore, under the conditions used here, this interaction is specific, but, as suggested by Figure 3, relatively weak. Previously, we (*22*) and others (*35*) showed that high concentrations of saposin A could activate acid β-glucosidase. We concluded that the development of a Gaucher-like disease in patients genetically deficient only in saposin C excluded a physiologic role for saposin A in the catabolism of glucosylceramide (*22*). The current studies support this conclusion and further indicate that the in vitro interactions of acid β-glucosidase and saposin A are not specific. The availability of a specific, direct assay system for the dissection of the interaction of acid β-glucosidase and saposin C, kinetically and structurally, may facilitate development of cost-effective enhancements to enzyme or gene therapy for Gaucher disease and a greater understanding of the effects of disease-associated mutations on this enzyme's catalytic activity.

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