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Enzymatic assays for studying intramembrane proteolysis

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Abstract

Proteolysis within the membrane is catalyzed by a diverse family of proteases immersed within the hydrophobic environment of cellular membranes. These ubiquitous intramembrane-cleaving proteases (I-CLiPs) hydrolyze the transmembrane domains of a large variety of membrane-embedded proteins to facilitate signaling events essential to normal biological functions found in all forms of life. The importance of this unique class of enzyme is highlighted by its central involvement in a variety of human pathologies, including Alzheimer's disease, Parkinson's disease, cancer and the virulence of a number of viral, bacterial and fungal pathogens. I-CLiPs therefore represent promising targets for the therapeutic treatment of numerous diseases. The key to understanding the normal biological function of I-CLiPs and capitalizing on their therapeutic potential is through a thorough understanding of the complex catalytic mechanisms that govern this unusual class of enzyme. This is an intrinsically difficult endeavor, given that these enzymes and their substrates reside within lipid membranes, making any *in vitro* assay technically challenging to design and execute. Here we describe several *in vitro* enzymatic assays for the study of the Alzheimer's disease associated γ -secretase protease, which have aided the development of potent γ -secretase-targeting compounds as candidate therapeutics. These assays have also been applied in various forms for the study of other I-CLiPs, providing valuable mechanistic insights into some of the functional similarities and differences between several members of this fascinating family of proteases.

1. Introduction

Intramembrane-cleaving proteases (I-CLiPs) are ubiquitous membrane proteins found in all forms of life (Urban, 2013). They catalyze the cleavage of the transmembrane domain of many membrane-embedded proteins to facilitate important cellular signaling events. The first I-CLiP to be discovered, site-2 protease (S2P), was uncovered while investigating the cellular signaling events that regulate cholesterol homeostasis (Rawson et al., 1997). This zinc metalloprotease is also involved in the ER unfolded protein response (Ye et al., 2000). Shortly thereafter, an aspartyl protease named presenilin was found to be responsible for γ -secretase activity (Wolfe et al., 1999). This unique protease is responsible for the intramembrane cleavage of both notch receptors (De Strooper et al., 1999) and amyloid precursor protein (APP) (Li et al., 2000a; Wolfe et al., 1999), putting the enzyme at the center of both metazoan developmental biology and Alzheimer's disease (AD). Shortly

thereafter, a family of serine intramembrane proteases named rhomboid were found to regulate important signaling events driving *Drosophila* development (Urban et al., 2001). Conspicuously absent, an intramembrane protease utilizing a catalytic cysteine has yet to be identified. Together, the discovery of these three families of intramembrane proteases founded a new field, termed regulated intramembrane proteolysis (RIP) (Brown et al., 2000).

Of the many I-CLiPs distributed ubiquitously throughout nature, the presenilin/ γ -secretase complex is arguably the most well-studied intramembrane protease. Its involvement in notch signaling and the generation of potentially pathogenic amyloid beta (A β) peptides via cleavage of APP in Alzheimer's disease has thrust this unusual protease into the spotlight. γ -secretase is a multicomponent complex comprised of the catalytic presenilin, Pen-2, Aph-1 and nicastrin (Edbauer et al., 2003; Kimberly et al., 2003; Takasugi et al., 2003). Pen-2 and Aph-1 are thought play a scaffolding role within the complex (LaVoie et al., 2003; Takasugi et al., 2003), while nicastrin functions to sterically occlude non-substrates from interacting with the protease (Bolduc et al., 2016). All four components are required for complex assembly and full activity. Presenilin's requirement of cofactors for activity is unique among other members of the aspartyl intramembrane protease family. Other aspartyl I-CLiPs such as signal peptide peptidase (SPP) and SPP-like proteases are thought to function alone, without the need for other protein cofactors or subunits (Voss et al., 2013).

Initially, the notion that proteolysis could occur within the hydrophobic environment of cellular membranes was met with skepticism. How could the catalytic water required for hydrolysis enter the lipid bilayer where water is normally excluded? The purification of recombinant intramembrane proteases and the subsequent development of *in vitro* enzymatic assays directly demonstrated that these I-CLiPs were indeed catalyzing hydrolysis within the transmembrane domain of their substrates. Later, high-resolution structures of rhomboid (Wang et al., 2006), an archeal S2P homolog (Feng et al., 2007) and more recently γ -secretase (Bai et al., 2015) revealed that the catalytic amino acids of each of these enzymes reside within their membrane-immersed transmembrane domains.

In this chapter we describe three *in vitro* enzymatic assays regularly used in our labs for the study of γ -secretase catalysis. We and others have utilized these assays and variations thereof to dissect the intricate catalytic mechanisms that govern these truly unique enzymes.

2. Detergent-Solubilized Assay

Detergent-solubilized assays require the solubilization of both the intramembrane protease and its substrate in a detergent system that allows for catalytic activity. In the case of γ -secretase, a relatively weak zwitterionic detergent CHAPSO was chosen, as this detergent allowed for the γ -secretase complex to remain intact, whereas most other detergents dissociated the complex (Li et al., 2000b). These assays have been described for at least one member of each class of intramembrane-cleaving proteases and were the first assays developed for studying I-CLiPs *in vitro* (Li et al., 2000b; Urban and Wolfe, 2005; Weihofen et al., 2002). The detergents useful for studying these I-CLiPs in a soluble state have been determined empirically. From a technical standpoint, detergent-solubilized assays are easier to design and implement than more complex proteoliposome assays. However, because of

their more artificial nature—removing enzyme and substrate from physiologic lipid membranes—detergent-solubilized assays sometimes fail to fully recapitulate key mechanistic features imparted by the intact lipid bilayer.

Although the detergents are used to solubilize enzyme and substrate, the addition of lipids to the reaction mixture has been shown to be necessary for presenilin/ γ -secretase activity. Furthermore, certain lipid compositions modulate not only the total activity of the enzyme but also the relative amounts of A β species produced from the cleavage of the APP based substrate C100-FLAG (Holmes et al., 2012; Osenkowski et al., 2008). Below is the description of a CHAPSO-solubilized assay for the study of γ -secretase.

2.1 Methods

1. DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine) (Avanti Polar Lipids, catalog number: 850375) and DOPE (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine) (Avanti Polar Lipids, catalog number: 850725) lipids stored in chloroform are first dried down under a stream of nitrogen in a glass test tube until all of the chloroform is removed and only a film of lipid remains on the bottom of the tube. The lipids are then hydrated in 50 mM HEPES pH 7.0, 150 mM NaCl and 1% CHAPSO (Sigma Aldrich) detergent at 37°C for 30 min. In order to completely dissolve the lipids, the glass test tube must be vortexed vigorously for up to 5 min after the 30 min incubation. The solution will be completely clear. Once the lipids are dissolved, the solution is diluted in 50 mM HEPES pH 7.0, 150 mM NaCl such that the final concentrations of DOPC, DOPE and CHAPSO in the reactions are 0.1%, 0.025% and 0.25%, respectively.
2. Purified γ -secretase complexes (Fraering et al., 2004) are added to the dissolved lipid solution at the desired concentration (typically 5 – 30 nM final concentration of enzyme in the reaction), and the solution is pre-incubated for 30 min at 37°C in 20 μ L reaction volumes. We find that this pre-incubation step increases γ -secretase activity. After 30 min, the proteolytic reaction is then initiated with purified recombinant substrate (Bolduc et al., 2016) and incubated at 37°C for the desired length of time. We typically use C100-FLAG (a recombinant C-terminally FLAG-tagged substrate mimicking the β -secretase-generated APP product, C99) or N100-FLAG (a recombinant C-terminally truncated and FLAG-tagged notch based substrate), but other γ -secretase substrates have been utilized in *in vitro* detergent assays as well (e.g., Chávez-Gutiérrez et al., 2012). Because these hydrophobic substrates must be solubilized in NP40 or TritonX-100 detergent (two detergents which dissociate the γ -secretase complex over time), care must be taken to limit the amount of such detergents added into the enzymatic reaction (i.e., use concentrated substrate; we typically store substrate at 30 μ M concentrations). The final concentration of substrate used ranges from 0.05 – 2 μ M, as the K_m for most γ -secretase substrates in the detergent-solubilized state is in the mid-nM range (Bolduc et al., 2016; Chávez-Gutiérrez et al., 2012; Fernandez et al., 2014). We attempt to keep the final concentration of NP40 or TritonX-100 detergent below 0.02%. Regardless, the progress of the reaction must be checked over time to verify that

product is formed linearly with time. We find that under these conditions, the generation of product is linear for the first ~60 min of the reaction.

3. For measuring the cleaved intracellular domain product (AICD from C100-FLAG or NICD from N100-FLAG), reactions are quenched with SDS gel-loading dye and then run on SDS/PAGE. The cleavage products are visualized by western blot using an antibody to a C-terminal tag (FLAG) or if using notch as a substrate, with an NICD neoepitope-specific antibody (anti-V1744, Cell Signaling). In order to quantify the amount of intracellular domain produced, a standard curve of recombinant purified AICD or NICD must be run concurrently on each western blot being analyzed. Each band is quantified by densitometry.
4. If the γ -secretase product being measured is A β from the cleavage of recombinant APP C100 substrate, then the reactions must be quenched by flash freezing. The reactions are then thawed on ice and centrifuged $10,000 \times g$ for 5 min at 4°C prior to measuring A β peptides in the supernatant by ELISA (e.g., using A β 40 or A β 42 ELISA kits from Invitrogen, catalog number: KHB3481 and KHB3441).

3. Proteoliposome assay

An enzyme-incorporated proteoliposome assay involves incorporating an intramembrane protease into a lipid vesicle to generate a proteoliposome and subsequently adding detergent-solubilized substrate to initiate the reaction. Immersing an intramembrane protease into the lipid bilayer of a proteoliposome has been shown to more accurately recapitulate key enzyme-substrate interactions compared to the detergent-solubilized assay. For example, we find that γ -secretase processing of the APP-based substrate C100-FLAG produces an A β 42/40 ratio closer to the physiologic ratio of ~0.2 when γ -secretase is incorporated into proteoliposomes (Holmes et al., 2012); γ -secretase cleavage of C100-FLAG in the CHAPSO-solubilized assay (above) produces an artificially elevated ratio. Similarly, rhomboid cleavage site-specificity of Spitz substrate is maintained only in the context of the lipid bilayer (Moin and Urban, 2012).

Although the enzyme-incorporated proteoliposome assay allows for the more accurate study of enzyme-substrate-lipid interactions than the detergent-solubilized assay, it still fails to allow for the precise determination of the kinetic variables k_{cat} and K_m due to the fact that the substrate is added to the reaction mixture in the detergent-solubilized state (see below). Nevertheless, as described below for γ -secretase, this assay has proved to be a facile and robust means to study I-CLiPs while recapitulating some of the key regulatory features of lipid membranes on catalytic activity.

3.1 Methods

1. Whole brain lipid extract (Avanti Polar Lipids, catalog number: 131101) is dried under a stream of nitrogen until only a film of lipid remains. The lipid is then hydrated in the presence of 50 mM HEPES pH 7.0, 150 mM NaCl, 1% CHAPSO at 37°C for 30 min prior to being vigorously vortexed to resuspend the lipid. The detergent/lipid solution will remain cloudy. The solution is then diluted in 50

mM HEPES pH 7.0, 150 mM NaCl to a final concentration of CHAPSO of 0.25%. The final concentration of whole brain lipid extract is 3 mM.

2. SM-2 biobeads (Bio-Rad, catalog number: 1523920) are used to remove detergent from the detergent/lipid mixture to form proteoliposomes. 62.5 mg of SM-2 biobeads are used per mL of lipid:detergent (3 mM:0.25%) mixture. The biobeads are prepared by washing once with 0.5 mL MeOH, then twice with 0.5 mL H₂O. Purified γ -secretase (final concentration is typically 5–30 nM) is mixed with the detergent/lipid solution from step 1 on ice prior to the addition of biobeads. The proteoliposomes are formed when the mixture is incubated at 4°C for 2 h while rotating. After the 2 h incubation, the newly formed proteoliposomes are removed from the biobeads and placed in a new tube.
3. The proteoliposomes are pre-warmed at 37°C for 15 min prior to the addition of various amounts of purified detergent-solubilized recombinant substrate (typically 0.05 – 2 μ M final concentration) to initiate the reaction in 50 μ L volumes. The reactions are quenched after the desired period of time by SDS loading dye for western blot analysis of intracellular domain cleavage products or by flash freezing for ELISA analysis of A β as described above. Again it must be verified that product is being produced linearly with respect to time for accurate kinetic measurements.

4. Inducible Proteoliposome Assay

Both the detergent-solubilized assay and enzyme-incorporated proteoliposome assay outlined above have provided valuable insights into the function and regulation of γ -secretase and other I-CLiPs. However, both assays are artificial in that normally γ -secretase enzyme and its substrates are both confined to a lipid bilayer under physiologic conditions rather than substrate (and also enzyme in the case of the CHAPSO assay) being solubilized in detergent. Studying γ -secretase (or other I-CLiPs) exclusively in artificial detergent-solubilized conditions runs the risk of measuring artifacts and/or missing out on key features of the enzyme's catalytic mechanism present only when both enzyme and substrate are oriented in a 2-dimensional lipid bilayer.

When studying the kinetic parameters of any enzyme, an *in vitro* assay must be designed with defined start and stop times for initiating and terminating enzymatic activity. Accomplishing this with an intramembrane protease having both enzyme and substrate incorporated into a proteoliposome is inherently difficult, given that any procedure for generating proteoliposomes is laborious and time-consuming, during which period the enzyme will have free access to substrate. Thus, an inducible proteoliposome assay was needed to study I-CLiPs with a mechanism in place to initiate cleavage only when desired. To this end, Urban and colleagues recently developed an innovative and elegant, yet conceptually simple, inducible proteoliposome assay to study rhomboid intramembrane kinetics (Dickey et al., 2013). As outlined below (Fig. 1), we have adapted this assay to study γ -secretase cleavage of a notch-based substrate (Bolduc et al., 2016).

For the serine protease GlpG rhomboid, Urban and coworkers found that the nucleophilic serine rotated to an inactive orientation within the enzyme active site when the catalytic histidine was protonated at low pH (Dickey et al., 2013). They therefore formed proteoliposomes containing both rhomboid and TatA substrate at low pH to prevent premature substrate cleavage and subsequently raised the pH to initiate the reaction after the proteoliposomes were formed. We took a similar approach with the aspartyl protease γ -secretase, forming the proteoliposomes at a basic pH before initiating the reaction by returning the reaction to neutral pH (details below). Presumably, basic pH conditions deprotonate both catalytic aspartates of γ -secretase, rendering it inactive (Quintero-Monzon et al., 2011). Although this method has only been applied to rhomboid and γ -secretase to date, a similar approach should work for other intramembrane proteases, assuming any pH-induced conformational changes in enzyme are rapidly reversible upon pH neutralization.

Initially, we attempted to use the same N100-FLAG notch based substrate for this inducible proteoliposome assay as we previously used for the detergent-solubilized assays discussed above. However, we encountered two technical hurdles that forced us to use an alternative substrate: 1) the recombinant notch substrate was inefficiently incorporated into vesicles by detergent dilution; 2) the substrate oriented primarily in one direction when inserted into the membrane (N-terminus inside the vesicle). We hypothesized that this may be due the presence of the large and highly charged intracellular domain of the recombinant N100-FLAG substrate. We therefore designed a synthetic substrate in which the intracellular domain of notch is replaced with fluorescein (N43, see below) to allow us to monitor the cleavage reaction. As discussed below, this substrate allowed for efficient proteoliposome incorporation as well as roughly equal N-terminal orientations upon insertion into the lipid bilayer. Urban and colleagues similarly used a short synthetic TatA peptide tagged with fluorescein in their assay. Together, this may argue that future inducible proteoliposome assays developed to study other I-CLiPs should attempt to utilize short synthetic peptides as substrates as well, as they appear to be better behaved in this assay system than are large recombinant substrates.

In contrast to the detergent-solubilized assays, the inducible proteoliposome assay allows for the precise calculation of physiologically meaningful k_{cat} and K_m values. In both the detergent-solubilized assay and the γ -secretase-incorporated proteoliposome assay, the substrate is solubilized in detergent prior to being added to the reaction mixture. K_m values for substrate must therefore be calculated in terms of molarity here, which makes no physiologic sense, as substrate and enzyme are normally confined to the 2-dimensional lipid bilayer within cells. For the inducible proteoliposome assay, intramembrane K_m values are calculated and expressed in terms of mole percent with respect to total lipid concentration. γ -secretase and rhomboid have been found to have vastly different intramembrane K_m values using this assay, demonstrating these two I-CLiPs have evolved very different mechanisms for substrate recognition (Bolduc et al., 2016; Dickey et al., 2013).

k_{cat} values calculated in the detergent-solubilized or enzyme-incorporated proteoliposome assay can potentially be misleading. Although intramembrane proteolysis by I-CLiPs appears to be an intrinsically slow process (Dickey et al., 2013; Kamp et al., 2015), the detergent-solubilized substrate assays require substrate to partition between detergent and/or

detergent-lipid states, which could potentially be rate-limiting in the enzymatic reaction. The inducible proteoliposome assay eliminates the requirement of detergent, therefore allowing for a more accurate calculation of k_{cat} and intramembrane K_m values. The description of an inducible proteoliposome assay below was adapted by us from Urban and colleagues for the study of γ -secretase.

4.1 Methods

Synthetic notch-based substrates for utilization in the inducible proteoliposome assay:

N43: VKSEPVEPPLPSQLHLMYVAAAAFVLLFFVGCGVLLSRRRAAK(5-FAM)-amide

N41: CEPVEPPLPSQLHLMYVAAAAFVLLFFVGCGVLLSRRRAAK(5-FAM)-amide

1. DOPC and DOPE (Avanti Polar Lipids) are dried under a stream of nitrogen in a glass test tube. The resulting lipid film is then hydrated at 50°C in the presence of 50 mM Bicine pH 8.5, 150 mM NaCl for 20 min. At this step, the total lipid concentration is 1 mM (90% DOPC, 10% DOPE). The lipids are then briefly vortexed to form large multilamellar vesicles (LMVs). Small unilamellar vesicles (SUVs) of uniform diameter are then formed by extrusion of the LMVs through a polycarbonate extrusion filter (Avanti Polar Lipids) with a pore size of 200 nm.
2. Both enzyme (γ -secretase) and substrate (N43) are incorporated into the vesicles formed in step 1 by a detergent dilution method. Here the detergents used to solubilize γ -secretase (digitonin) and N43 (NP40) are diluted below their critical micelle concentration (CMC) to allow for enzyme and substrate insertion into the vesicles and proteoliposome formation. After mixing the desired amount of γ -secretase (1 – 20 ng) and/or N43 (final concentration ranging from 0.0001 – 0.01 mol percent) with 75 μ L extruded vesicles for 20 min at room temperature, the mixture is then diluted (final volume 750 μ L) with 10 mM Bicine pH 8.5, 10 mM NaCl. The proteoliposomes are then pelleted and concentrated by centrifugation at 100,000 $\times$ g for 25 min.
3. The reaction is initiated when the pelleted proteoliposomes are resuspended in 50 mM HEPES pH 7.0, 150 mM NaCl by gentle pipetting (Figure 1). The reactions proceed linearly at 37°C for > 4 h, though typically time points are taken at 2 h. The reactions are quenched with SDS loading dye, and cleavage products are separated on a 16.5% tris-tricine gel. The fluorescein-tagged substrate and cleavage product are visualized with a fluorescence scanner (Typhoon or Storm imager from GE). The bands are analyzed by densitometry and quantified against a standard curve of substrate run concurrently on each gel.

4.2 Verification of enzyme and substrate proteoliposome incorporation

1. Proper incorporation of substrate should be measured by fluorescence quenching of the fluorophore, circular dichroism, selective cysteine labeling of substrate

and by quantifying the amount of substrate that pellets with the vesicles during the ultracentrifugation step.

- a. Fluorescence quenching – Vesicle incorporation of the fluorescein-labeled substrate will cause quenching of the fluorophore (Bolduc et al., 2016; Dickey et al., 2013). After the ultracentrifugation of proteoliposomes from step 2 above, the pelleted proteoliposomes are resuspended in either 50 mM HEPES pH 7.0, 150 mM NaCl or 50 mM HEPES pH 7.0, 150 mM NaCl containing 0.25% NP40 to break apart the proteoliposomes. The solutions are then excited at 485 nm and the emission spectrum measured from 510 – 700 nm for the fluorescein labeled N43 substrate on a Synergy H4 plate reader (BioTek). The NP40-containing sample of dissolved proteoliposomes should have a much higher fluorescence intensity compared to the quenched, proteoliposome-incorporated substrate.
- b. Circular dichroism (CD) – The substrate, comprised mostly of the transmembrane domain of notch, yields a CD spectrum consistent with an α -helical secondary structure. Proteoliposomes are made similarly to the above protocol with the only exception being they are made in the presence of 10 mM Tris base pH 7.0 rather than the HEPES- and NaCl-containing buffer. The final concentration of N43 peptide is 0.2 mg/mL. CD spectra can be obtained on a standard spectropolarimeter (e.g. Jasco J-815), scanning from 190 – 270 nm.
- c. Cysteine labeling to determine substrate orientation – Given that substrate can incorporate into the proteoliposome in two possible orientations (N-terminus inside or N-terminus outside of the proteoliposome), the relative abundance of each orientation must be experimentally measured. This allows for the determination of the amount of substrate that is available for cleavage of substrate by γ -secretase. This is accomplished for N-terminal Cys-containing N41 substrate with a membrane-impermeable Cys reactive dye (800cw maleimide, Licor, product number: 929-80020). 800cw is added to the formed proteoliposomes at a concentration of 1 μ M in the presence or absence of 1 mg/mL melittin peptide (Sigma Aldrich, product number: M2272) to permeabilize the proteoliposomes and allow passage of the dye through the membrane. The labeling reaction proceeds for 2 h at room temperature. Unreacted dye is quenched with 50 mM DTT for 30 min before the labeled peptide is run on a 16.5% tris-tricine gel and visualized with an Odyssey Licor scanner. In this way, the fraction of substrate labeled on the outside of the lipid bilayer can be measured. Fortuitously, the ratio happens to be approximately 50:50 for N41 substrate. This same experiment was carried out with FITC-TatA for rhomboid using detergent instead of melittin to permeabilize the membrane to measure total labeling (Dickey et al., 2013).

- d. Total substrate incorporation – Even after diluting the reaction mixture below the detergent CMC, not all of the substrate will be incorporated into the proteoliposome. This can be measured by residual fluorescence remaining in the supernatant after ultracentrifugation. To determine the amount of N43 substrate incorporated, a small aliquot of reaction mixture is taken before and after ultracentrifugation and quantified by densitometry after running on a 16.5% tris-tricine gel. The percent of substrate in the pellet is assumed to be incorporated into the proteoliposome. The percent of substrate incorporation into the proteoliposome should not vary significantly with the amount of substrate added.
2. The concentration of active γ -secretase enzyme in the proteoliposome after detergent dilution, ultracentrifugation and subsequent resuspension in a buffer of neutral pH is determined by titrating active enzyme with a tight-binding γ -secretase transition-state analog inhibitor. LY411,575 (Sigma Aldrich, product number: SML0506), a very potent γ -secretase inhibitor, is added to the resuspension buffer at varying concentrations, and the reaction is allowed to proceed for 2 h at 37°C. The reactions are then quenched and product visualized and quantified as described above. Unlike with N43 substrate, the orientation of γ -secretase after being incorporated into the proteoliposome doesn't need to be determined. Here, only the enzyme molecules that are oriented such that their active sites are exposed to the pH change will be active. The concentration of active enzyme molecules is determined by inhibitor titration.

5. Conclusions

Intramembrane cleaving proteases represent a unique class of enzyme at the forefront of biology and medicine. A thorough understanding of the intramembrane protease catalytic mechanism will be required to fully understand how these proteases govern important biological processes as well as allow researchers to capitalize on the therapeutic potential of these enzymes. This can only be accomplished with robust and facile methods by which to study the activity of these enzymes *in vitro*. Although the mysteries of the intramembrane protease cleavage mechanism are only now beginning to be unraveled, the enzymatic assays outlined here have proven highly valuable for interrogating the function of γ -secretase and other members of the I-CLiP family.

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Figure 1.

Flow chart for the inducible proteoliposome assay. Detergent-solubilized purified γ -secretase and synthetic N43 are mixed with extruded vesicles at pH 8.5 in BICINE buffer to prevent hydrolysis of the N43 substrate. Proteoliposomes are formed following dilution of the reaction mixture below the CMC of the solubilizing detergents. The newly formed proteoliposomes are then concentrated by ultracentrifugation at 100,000 g. The reaction is initiated when the pelleted proteoliposomes are resuspended at neutral pH.