Tetramization of Glycosylphosphatidylinositol-specific Phospholipase C from Trypanosoma brucei

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Glycosylphosphatidylinositol-specific phospholipase C (GPI-PLC) is an integral membrane protein in the protozoan parasite Trypanosoma brucei. Enzyme activity appears to be suppressed in T. brucei, although the polypeptide is readily detectable. The basis for the apparent quiescence of GPI-PLC is not known. Protein oligomerization was investigated as a possible mechanism for post-translational regulation of GPI-PLC activity. An equilibrium between monomers, dimers, and tetramers of purified GPI-PLC was detected by molecular sieving and shown to be perturbed with specific detergents. Homotetramers dominated in Nonidet P-40, and dimers and monomers of GPI-PLC were the major species in 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate. The detergents were exploited as tools to study the effect of oligomerization on enzyme activity. Tetrameric GPI-PLC was 3.6–20-fold more active than the monomeric enzyme. Tetramer existence was confirmed by chemical cross-linking. In vivo cross-linking revealed the oligomeric state of GPI-PLC during latency and after enzyme activation. During quiescence, monomers were the predominant species in T. brucei. Assembly of tetrameric GPI-PLC occurred when parasites were subjected to conditions known to activate the enzyme. In Leishmania where heterologous expression of GPI-PLC causes a GPI deficiency, the enzyme existed as a tetramer. Hence, oligomerization of GPI-PLC is associated with high enzyme activity both in vivo and in vitro.

Trypanosoma brucei is the causative agent of sleeping sickness in humans. In the bloodstream of a vertebrate host, the plasma membrane of the parasite is covered with a variant surface glycoprotein (VSG), a glycosylphosphatidylinositol (GPI)-anchored molecule. These trypanosomes contain a GPI-specific phospholipase C (1–3) that can cleave the VSG GPI, although the physiological function of the enzyme remains unclear (4, 5). GPI-PLC is highly specific for GPIs (kcat, 2.92 × 103 min–1; Km, 360 nM) (1, 6). Turnover of the enzyme-substrate complex is regulated by thio-myristoylation and palmitoylation of the enzyme (7). Efficient substrate recognition by GPI-PLC requires a glucosaminylinositol moiety on the substrate (8).

GPI-PLC activity is detectable only in developmental stages of the parasite where VSG is present. As revealed by immunoelectron microscopic analysis, GPI-PLC is associated with vesicular structures on the cytoplasmic side of intracellular membranes (9, 10). Little or no cleaved VSG is released from healthy cells (11). While T. brucei divides every 6–8 h, the half-life of cell-associated VSG is 32–34 h (11–13). Clearly, VSG is not released actively by GPI-PLC. This situation is in sharp contrast to the phenotype of Leishmania major or Trypanosoma cruzi that have been stably transfected with a cDNA for the T. brucei GPI-PLC (14, 15). In Leishmania promastigotes (extracellular insect-stage form), gp63, the major GPI-anchored protein, is secreted constitutively into the culture medium due to a GPI deficiency (15). Replication of Leishmania amastigotes (the intracellular mammalian stage) is severely inhibited by GPI-PLC expression (14). A similar phenotype has been noted in T. cruzi where division of the cell nucleus is blocked as a result of a GPI deficiency (16), and differentiation of amastigotes to trypomastigotes is inhibited (17).

GPI intermediates (e.g. GlcN-PI and Man1,GlcN-PI) are found on the cytoplasmic side of the ER membrane (18). These compounds appear to co-localize with GPI-PLC, given the data from the in vivo transfection studies with Leishmania and T. cruzi. Why then in T. brucei are these GPs spared from cleavage by this potent membrane-bound phospholipase C? It is estimated that there are 2.4 × 104 molecules of glycolipid A (ethanolamine-phospho-Man3-GlcN-Ins-phospho-dimyristoylglycerol), the prefabricated GPI anchor (19), and 3.5 × 104 molecules of GPI-PLC (3); this represents an excess of the enzyme over the complete GPI anchor. With a turnover number (kcat) of 2920 min–1 (6), GPI-PLC could cleave all the glycolipid A inside T. brucei within seconds. Since the parasites remain capable of adding GPs to VSG, it seems clear that GPI-PLC is somehow prevented from depleting T. brucei of GPI anchors. The absence of the GPI-negative phenotype observed with transgenic T. cruzi and L. major expressing GPI-PLC is indirect evidence for regulation of the enzyme in T. brucei.

In lieu of efforts to understand factors that might control activity of GPI-PLC in T. brucei, we characterized the native state of the purified enzyme, and we explored possible contributions of self-association to enzyme activity both in vivo and in vitro. Our observations led us to propose a model for post-translational regulation of GPI-PLC activity in vivo where prevention of tetramerization might play an important role.

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‡ The abbreviations used are: VSG, variant surface glycoprotein; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate; MeSO3, dimethyl sulfoxide; DOC, deoxycholate; DSS, disuccinimidyl suberate; DSP, dithiobis-succinimidyl propionate; DTT, dithiothreitol; LDAO, lauryl dimethyl amine oxide; MBS, -maleimidobenzoyl-N-hydroxysuccinimide ester; PBS, phosphate-buffered saline; GPI-PLC, glycosylphosphatidylinositol-specific phospholipase C; TbGPI-PLC, Trypanosoma brucei GPI-PLC; BiP, binding protein; ER, endoplasmic reticulum; PAGE, polyacrylamide gel electrophoresis; Man, mannose; GlcN, glucosamine; PI, phosphatidylinositol.
Monomorphic T. brucei strain 427 bloodstream form was used in this work. Parasites were grown in rodents and harvested by chromatography on DE52 (20).

**Materials**

Superdex 75 HR10/30 column, Superdex 200 HR10/30 column, the fast protein liquid chromatography system, and [35S]methionine were from Amersham Pharmacia Biotech. Lauryl dimethyl amine oxide (LDAO, Nonidet P-40 grade), and transferrin were purchased from Calbiochem. Thesit and aprotinin were obtained from Roche Molecular Biochemicals. GelCode Blue, disuccinimidyl suberate (DSS), dithiobisuccinimidyl propionate (DSP), and m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS) were purchased from Pierce. Gel filtration standards were purchased from Bio-Rad. Sodium deoxycholate (DOC), CHAPS, and all other reagents were from Sigma.

**Native Gel Electrophoresis of GPI-PLC**

Purified recombinant GPI-PLC expressed in Escherichia coli (1–2 µg) was fractionated in 20–30 µl of 37.5 mM Tris-HCl, pH 9.3, 0.5% Nonidet P-40, containing one of these reagents as follows: 3–4 µl urea, 100 mM dithiothreitol (DTT), 1% Nonidet P-40, or 0.1–2% CHAPS at 27 °C for 5 min. Samples were resolved by continuous glycine non-denaturing polyacrylamide gel electrophoresis (pH 9.5; 5% minigel (Bio-Rad) (21). To keep track of protein migration, 2 µl of the detergent-solubilized extract. Proteins were visualized with GelCode Blue (Pierce). Standards used were a-lactalbumin (14.2 kDa), pl = 4.5), carbonic anhydrase (29 kDa, pl = 5.4–5.9), ovalbumin (43 kDa, pl = 4.6), bovine serum albumin (66-kDa monomer and 122-kDa dimer, pl = 4.7), and the recombinant hexamer, pl = 5.0 (Sigma). Seven micrograms of marker proteins were analyzed.

**Gel Filtration Analysis of GPI-PLC**

Molecular sieving was performed by fast protein liquid chromatography on either Superdex 75 HR10/30 at 5 °C or Superdex 200 HR10/30 at 27 °C. For all runs, a 100-µl sample was loaded, and 500-µl fractions were collected at a flow rate of 1 ml/min. The void volume of the columns was determined with blue dextran, using PBS (140 mM NaCl, 3 mM KCl, 10 mM NaH2PO4, 1.8 mM KH2PO4; pH 7.4) as running buffer. Prior to loading each sample, the columns were equilibrated with 3 column volumes of PBS containing the indicated amounts of detergent (see figure legend). For each running buffer, the columns were calibrated with thyroglobulin (670 kDa), IgG (160 kDa), transferrin (80 kDa), and the recombinant hexamer, pl = 5.0 (Sigma). Seven micrograms of marker proteins were analyzed.

**Recombinant GPI-PLC—Purified GPI-PLC (620 ng) in 5 µl of 75 mM Tris-HCl, pH 9.3, containing 1% Nonidet P-40 (6), was added to 95 µl of the indicated running buffer (see figure legends) and incubated at 27 °C for 5 min before loading onto the specified column. The elution profile of GPI-PLC was determined by assaying 5 µl of each fraction for enzyme activity (22).**

**Enzyme (TgGPI-PLC) from a Crude Lysate of Trypanosomes—T. brucei (5 × 10⁶ parasites) was lysed hypotonically in 1 ml of hypotonic lysis buffer (10 mM sodium phosphate, 1 mM EDTA, pH 8) containing a protease inhibitor mixture and centrifuged at 14,000 × g at 4 °C for 10 min (7). The membranous pellet obtained was solubilized with 500 µl of PBS containing either 1% Nonidet P-40 or 1% CHAPS. An aliquot of each sample (100 µl cell equivalents for Nonidet P-40 and 100 µl cell equivalents for CHAPS) was fractionated on the indicated gel filtration column.**

**Immunoprecipitation of GPI-PLC**

Immunoadsorption of GPI-PLC to a monoclonal antibody was performed as described previously (7). SDS-PAGE, Western Blotting, and Fluorography

**Recombinant GPI-PLC—A 40-µl aliquot of the cross-linked reaction mixture containing recombinant GPI-PLC (~120 ng) was analyzed by Western blotting (7).**

**Chemical Cross-linking**

**In Vivo Cross-linking of T. brucei—T. brucei (2 × 10⁶ cells) was metabolically labeled with [35S]methionine (7) and washed with PBS. Parasites were resuspended in 396 µl of PBS. To initiate cross-linking, 4 µl of 100 mM DSS, DSP, or MBS (m-maleimidobenzoyl-N-hydroxysuccinimide ester), all membrane-permeable reagents, was added. In control experiments, 4 µl of MeSO was introduced.**

**In Vivo Cross-linking of Leishmania—Leishmania (2.5 × 10⁶ trophozoites) were lysed hypotonically (2.5 × 10⁶ trophozoites) expressing GPI-PLC (15) was metabolically labeled with [35S]methionine (7) and washed with PBS. Parasites were resuspended in 990 µl of PBS, pH 7.4. Cross-linking was initiated by the addition of 10 µl of 100 mM MBS. Ten µl of MeSO was introduced in control experiments.**

**Immunoprecipitation of GPI-PLC**

Immunoadsorption of GPI-PLC to a monoclonal antibody was performed as described previously (7). SDS-PAGE, Western Blotting, and Fluorography

**Recombinant GPI-PLC—A 40-µl aliquot of the cross-linked reaction mixture containing recombinant GPI-PLC (~120 ng) was analyzed by Western blotting (7).**
TbGPI-PLC—[35S]Methionine-labeled GPI-PLC, from intact cells or lysates, was immunoadsorbed to protein A-Sepharose-mc2A6-6 beads (6) and eluted by heating at 90 °C for 2–3 min in 25 μl of 2.5× SDS-PAGE sample buffer. When preservation of DSP cross-links was required, a modified 2.5× SDS-PAGE sample buffer lacking Tris and β-mercaptoethanol was used. Such samples were only warmed at 37 °C for 5 min. Eluates were separated from the Sepharose beads by centrifugation at 14,000×g for 3 min at 27 °C. A 30-μl aliquot of each supernatant was subjected to SDS-PAGE (12% minigel) and processed for phosphorimaging or fluorographic detection with BioMax™ MR film (Eastman Kodak Co.).

RESULTS

CHAPS Alters Mobility of GPI-PLC during Native Gel Electrophoresis—GPI-PLC is a 39-kDa polypeptide (24). In native gel electrophoresis, it was detected as a diffuse band that barely entered a 5% minigel (without DTT or SDS) (Fig. 1A, lane 6). Absence of SDS from the sample and running buffers may explain the diffuse nature of protein bands in this experiment. Urea (up to 4 M) was added in an attempt to disrupt hydrogen bonds and dissociate what appeared to be a complex of unusual shape and/or size. The mobility of GPI-PLC did not change (Fig. 1A, lane 7; Fig. 1B, lane 3). Likewise, pretreatment with DTT (100 mM) to reduce disulfide bonds failed to alter mobility of GPI-PLC in the native gel (Fig. 1B, lane 2). When the enzyme was treated with both DTT (100 mM) and urea (4 M), a minor change occurred in its migration (Fig. 1B, lane 4). Treatment with 1% SDS caused GPI-PLC to run off the gel (data not presented), most likely due to denaturation and net negative charge introduced by the detergent.

CHAPS caused a significant change in the mobility of GPI-PLC, resolving it into two species (Fig. 1B, lane 5). A titration of the detergent revealed that 0.5% was sufficient for optimal migration of GPI-PLC (Fig. 1C, lanes 2–5). The major species of GPI-PLC detected during electrophoresis in CHAPS is labeled F (Fig. 1C).

Two conclusions can be drawn from these observations. First, the size, shape, or net charge of GPI-PLC can be modulated by CHAPS. (The detergent has no net charge (Fig. 8).) Second, the quaternary structure of GPI-PLC is not dependent on disulfide bonds.

Detergents Modulate the Size and/or Structure of GPI-PLC—One hypothesis to explain the altered mobility of GPI-PLC in the native gel after addition of CHAPS was that the detergent changed the quaternary structure of the enzyme. To examine this proposal, GPI-PLC was analyzed by gel filtration in a buffer containing different detergents, some with structures similar to CHAPS. A Superdex 75 HR10/30 column employed for this purpose had a void volume of 9 ml (fraction 18) and a bed volume of 24 ml (fraction 48). The experiment was performed at 5 °C. Proteins of 100 kDa and higher were excluded from the column.

FIG. 1. Native polyacrylamide gel analysis of GPI-PLC. Purified GPI-PLC was subjected to non-denaturing gel electrophoresis without SDS. A, short run, 7 μg each of protein standards was analyzed: lane 1, α-lactalbumin; lane 2, carbonic anhydrase; lane 3, ovalbumin; lane 4, bovine serum albumin; lane 5, urease. GPI-PLC (~1 μg) was preincubated with either Nonidet P-40 (NP-40) or urea prior to analysis: lane 6, 1% Nonidet P-40; lane 7, 1% Nonidet P-40 and 3 M urea. Electrophoresis was for 30 min. B, long run, CHAPS alters electrophoretic mobility of GPI-PLC. Lane 1, carbonic anhydrase; lanes 2–5, GPI-PLC (~1 μg) preincubated with several reagents: lane 2, 1% Nonidet P-40 and 100 mM DTT; lane 3, 4 M urea; lane 4, 1% Nonidet P-40, 100 mM DTT, and 4 M urea; lane 5, 1% CHAPS. Electrophoresis was for 5 h. C, optimization of CHAPS-induced changes in mobility of GPI-PLC. Two μg of GPI-PLC was incubated with detergent prior to electrophoresis. Lane 1, 1% Nonidet P-40; lane 2, 0.1% CHAPS; lane 3, 0.5% CHAPS; lane 4, 1% CHAPS; lane 5, 2% CHAPS. The gel was run for 4 h. Protein standards ran off the gel. Proteins were detected with GelCode blue. S, slow; F, fast; →.

Tetramerization of GPI-Phospholipase C
In 0.1% Nonidet P-40, the peak of GPI-PLC activity was in fraction 22, close to the elution position of an 80-kDa standard (Fig. 2A). The migration of enzyme activity is consistent with that of a dimer (78 kDa). Twelve percent of the enzyme activity was detected in the void volume (Fig. 2A), suggesting one of two possibilities. First, GPI-PLC forms complexes (e.g. trimers) larger than 100 kDa. Alternatively, GPI-PLC may be associated with micelles of the detergent that have a molecular mass of 90 kDa such that a monomer-micelle complex will have a mass of 130 kDa, causing it to be excluded from the column. In 1% Nonidet P-40, GPI-PLC was predominantly dimeric (data not shown).

To determine whether the hydrophilic head group or hydrophobic tail of Nonidet P-40 contributed to dimerization of GPI-PLC, Thesit (65–68 kDa) whose hydrophilic head is identical to that of Nonidet P-40 was tested. In 1% Thesit, most of GPI-PLC was a dimer and 29% of the enzyme eluted in the void volume (Fig. 2A). More importantly, detection of dimers suggest that the polymeric oxyethylene ((CH2-CH2-O)9–10) head (present in both Nonidet P-40 and Thesit) may be sufficient to cause oligomerization of purified GPI-PLC.

In CHAPS, the majority of the enzyme migrated with a molecular mass greater than the 39-kDa monomer but less than the 80-kDa dimer (Fig. 2B). This finding is consistent with the assignment of a 47-kDa molecular size to the enzyme, after adsorption to a 7-kDa CHAPS micelle (1). Only 23% of the total activity migrated as a dimer (Fig. 2B). About 13% of active enzyme eluted in fraction 19 (Fig. 2B), suggesting the presence of larger oligomers. Analysis of the enzyme in 2% LDAO produced similar results. The majority of GPI-PLC behaved as a tetramer in Nonidet P-40 but monomeric in CHAPS. Purified GPI-PLC was preincubated in the appropriate running buffer (see below) before loading onto the column. A, GPI-PLC (620 ng) was analyzed in PBS containing Nonidet P-40 (0.1%) or Thesit (1%) on Superdex 75 HR10/30 column at 5 °C. B, GPI-PLC (620 ng) was analyzed in PBS containing CHAPS (2%) on Superdex 75 HR10/30 column at 5 °C. C, GPI-PLC (124 ng) was analyzed in PBS containing Nonidet P-40 (NP40) (0.1%) on Superdex 200 HR10/30 column at 27 °C. D, GPI-PLC (620 ng) was analyzed in PBS containing CHAPS (2%) on Superdex 200 HR10/30 column at 27 °C. A 5-μl portion of each fraction (500 μl) was assayed for enzyme activity with 3H-membrane-form variant surface glycoprotein as substrate in buffer AB containing 1% Nonidet P-40 (“Experimental Procedures”). No activity was detected when CHAPS replaced Nonidet P-40 in the assay buffer. Migration position of the standards, thyroglobulin (670 kDa), IgG (160 kDa), transferrin (80 kDa), ovalbumin (43 kDa), and chymotrypsinogen A (25 kDa) are indicated.
FIG. 4. Native GPI-PLC can be cross-linked into oligomers. A, GPI-PLC forms oligomers. Purified recombinant GPI-PLC (~120 ng) was incubated at 27 °C for 10 min in the presence of either Me2SO (lane 1) or 250 μM DSS (lanes 2–8). Reactions were performed in the presence of detergents: lanes 1 and 2, Nonidet P-40 (NP-40) (1%); lane 3, Thesit (1%); lane 4, CHAPS (1%); lane 5, LDAO (1%); lane 6, DOC (1%); lane 7, SDS (1%); lane 8, SDS (2%). GPI-PLC was detected by immunoblotting after SDS-PAGE. B, dimers of GPI-PLC are detectable at lower temperatures. GPI-PLC (120 ng) was incubated in 1% of the indicated detergent for 40 min at 2°C (lanes 1–3) or 15°C (lanes 4 and 5) in the presence of either Me2SO (lane 1) or 250 μM DSS (lanes 2–5). GPI-PLC was detected by Western blotting. C, dimers are the major product of cross-linking at 27°C in excess Tris-HCl. GPI-PLC (1.24 μg) was diluted 10-fold into 50 mM Tris-HCl, pH 8, 5 mM EDTA. Ten microliters of the diluted solution was made up to 30 μl with deionized water (i.e. final concentrations of 16.7 mM Tris-HCl, pH 8, 1.7 mM EDTA) and 1% Nonidet P-40. After addition of DSS or MBS (to a final concentration of 1 mM), the mixture was incubated at 27°C for 30 min, quenched, and analyzed (“Experimental Procedures”). Lane 1, Me2SO; lane 2, DSS, lane 3, Me2SO; and lane 4, MBS.

GPI-PLC Is Most Active in Its Tetrameric Form—Capitalizing on the ability of detergents and temperature to modulate self-association of GPI-PLC, we investigated whether different oligomers of the enzyme possessed varying activity. When an enzyme assay was performed in Nonidet P-40 at 5°C where GPI-PLC is predominantly dimeric (Fig. 2A), the activity was 1.7-fold higher than the monomer (Fig. 3) found in CHAPS (Fig. 2B). This implies that dimeric GPI-PLC may be slightly more active than the monomer.

To compare the activity of dimeric GPI-PLC with that of the tetramer, enzyme was assayed in Nonidet P-40 at 27°C and compared with its activity in CHAPS at 27°C (Fig. 2D). Tetrameric GPI-PLC was 3.6-fold more active than dimers and monomers (Fig. 3). An attempt to isolate tetramers generated by cross-linking for the purposes of activity determination was not feasible, since DSS, DSP, and MBS were all found to inhibit the activity of the enzyme (data not presented). Replacement of Nonidet P-40 with Thesit or LDAO reduced GPI-PLC activity 2–4-fold at 5°C (Fig. 3). Deoxycholate (1%) completely inhibited GPI-PLC activity at both 5 and 27°C (Fig. 3). When the assay was performed at 37°C in 1% of detergent, GPI-PLC was 20-fold more active in Nonidet P-40 than in CHAPS, Thesit, LDAO, or DOC (Fig. 3).

These observations indicate that GPI-PLC activity is increased under conditions where tetramer formation or stabilization is enhanced.

Cross-linking of GPI-PLC—Additional evidence for self-association of GPI-PLC was obtained by chemical cross-linking.
Purified GPI-PLC migrates normally as a 39-kDa protein after SDS-polyacrylamide gel electrophoresis (Fig. 4A, lane 1). Following cross-linking with DSS, the major product formed was a doublet, most likely tetramers and possibly pentamers (Fig. 4A, lanes 2–6). Interestingly, denaturation of GPI-PLC with SDS inhibits cross-linking by DSS, leaving the monomer as the predominant species (Fig. 4A, lanes 7 and 8). Aberrant (possibly intramolecular) cross-linking might also have occurred in the presence of SDS, since a ladder of bands with apparent molecular masses of 80 kDa and larger is visible (Fig. 4A, lanes 7 and 8).

To test whether intermediates between monomers and tetramers could be detected, cross-linking was performed (i) at lower temperatures, or (ii) in the presence of Tris (16.7 mM). At 2 °C, dimers of GPI-PLC were detected in both Nonidet P-40 and CHAPS (Fig. 4B, lanes 2 and 3). In addition, a small proportion of trimers was detected. The species of GPI-PLC marked with an asterisk in Fig. 4B (lanes 2 and 3) could arise from intramolecular monomer cross-linking that produced a knotted molecule that migrates faster than a monomer. Some dimers were detected at 15 °C, but the major product was the tetramer (Fig. 4B, lane 4). As observed previously, tetramerization was inhibited by SDS (Fig. 4B, lane 5). Finally, a minor product that failed to enter the stacking gel was observed in all cases where tetramers were the major product (Fig. 4A and B).

Dimers were the major product when Tris was present during cross-linking at 27 °C (Fig. 4C). Both DSS (Fig. 4C, lane 2) and MBS (Fig. 4C, lane 4) gave similar results. Since approximately 50% of GPI-PLC remained as a monomer, the accumulation of dimers may have resulted from reduced efficiency of cross-linking.

These observations have three implications. First, monomers of purified GPI-PLC can form tetramers that are detectable by chemical cross-linking. Dimers accumulate only when cross-linking is ineffective. Second, effective oligomerization requires the native conformation of GPI-PLC, since SDS, which inhibits enzyme activity (8), blocks tetramerization. Finally, Nonidet P-40, Thesit, CHAPS, LDAO, and DOC are unlikely to denature GPI-PLC, even if they cause tetramers to dissociate. Unlike SDS the detergents did not suppress tetramerization.

The oligomerization state of native GPI-PLC from T. brucei was determined. GPI-PLC from detergent extracts of T. brucei membranes. T. brucei membranes from a hypotonic lysate were solubilized in PBS containing 1% of either Nonidet P-40 (NP-40) or CHAPS. An aliquot was analyzed on Superdex 75 HR10/30 column at 5 °C in PBS containing the solubilizing detergent. A 5-μl portion of each eluate was assayed for GPI-PLC activity as described in the legend to Fig. 2.

### Fig. 5. Oligomeric properties of GPI-PLC solubilized from T. brucei membranes.

T. brucei membranes were solubilized in PBS containing 1% of either Nonidet P-40 (NP-40) or CHAPS. An aliquot was analyzed on Superdex 75 HR10/30 column at 5 °C in PBS containing the solubilizing detergent. A 5-μl portion of each eluate was assayed for GPI-PLC activity as described in the legend to Fig. 2.

### Fig. 6. Oligomers of GPI-PLC are not detected in living parasites.

A, cross-linking of GPI-PLC in intact T. brucei. [35S]Methionine-labeled T. brucei (2 × 10^9 intact parasites) were treated with MeSO (DMSO, lane 1), DSS (lane 2), or DSP (lane 3). Parasites were lysed hypotonically and solubilized, and GPI-PLC was immunoadsorbed. GPI-PLC was detected by SDS-PAGE and phosphorimaging. B, an aliquot of the membrane fraction of T. brucei used in A was analyzed by Western blotting with anti-BiP antibodies. DSP cross-links were either left intact (lane 3) or reversed by heating in β-mercaptoethanol (β-ME) (lane 4). C, cross-linking of recombinant GPI-PLC in Leishmania. [35S]Methionine-labeled Leishmania (2.5 × 10^6 cells) were treated with MeSO (lane 1) or MBS (lane 2). As a control, [35S]methionine-labeled intact T. brucei were treated with MBS (lane 3) or DSS (lane 4). GPI-PLC was then analyzed as described for A.
lysates (TbGPI-PLC) migrated during gel filtration at 5 °C on Superdex 75 HR10/30 column in Nonidet P-40 as a dimer; in CHAPS, it was predominantly a monomer (Fig. 5). These results are in agreement with data on the purified enzyme (Fig. 2). When the effect of self-association on activity of TbGPI-PLC was determined at 27° C, a 2-fold drop in activity occurred in CHAPS compared with Nonidet P-40 (data not presented). This result suggests that oligomeric TbGPI-PLC is more active than the monomer. We conclude that GPI-PLC produced in T. brucei can have its properties altered by detergents in a manner similar to that obtained for E. coli-expressed GPI-PLC (22). Recombinant GPI-PLC is not modified by lipid, whereas the enzyme expressed in T. brucei is thio-myristoylated and palmitylated (7). Hence, lipid modification is unlikely to play a role in regulating oligomerization of GPI-PLC.

Quaternary Structure of GPI-PLC in Vivo—Although GPI-PLC polypeptide is detectable in bloodstream form T. brucei, the enzyme appears to be largely inactive in vivo (see Introduction). This fact raises the possibility that activation of the enzyme involves post-translational mechanisms (e.g. covalent modifications or protein-protein interactions). This idea was pursued by an investigation of the oligomeric state of GPI-PLC in vivo. T. brucei were incubated with membrane-permeable reagents to cross-link GPI-PLC to its nearest neighbors. Products were immunoadsorbed to an anti-GPI-PLC monoclonal antibody and analyzed (Fig. 6). In intact T. brucei the majority 80% (by PhosphorImager quantitation) of GPI-PLC was monomeric (Fig. 6, lanes 1–3). DSS (Fig. 6A, lane 2) and DSP (Fig. 6A, lane 3) produced a high molecular weight complex, amounting to 5 and 11%, respectively, of the total GPI-PLC (Fig. 7B), which did not enter the stacking gel.

As a control for the in vivo cross-linking experiments, the lysates analyzed in Fig. 6A were examined by Western blotting with antibody against the ER chaperone BiP (Fig. 6B). In the absence of cross-linker, a single 80-kDa band corresponding to the size of monomeric BiP was detected (Fig. 6B, lane 1). Higher molecular weight complexes containing BiP appeared as a result of cross-linking with either DSS (Fig. 6B, lane 2) or DSP (Fig. 6B, lane 3). Since BiP binds to many different proteins in the lumen of the ER in its role as a chaperone, one does not expect to produce discrete bands from cross-linking of the protein to its binding partners. The DSP-dependent complexes were cleaved by β-mercaptoethanol (Fig. 6B, lane 4), as expected. These data demonstrate that DSP traverses the cytosol and enters the ER lumen in T. brucei to cross-link full-length BiP. Therefore, if oligomers of GPI-PLC existed on the cytosolic side of ER membranes in vivo, they would not have been missed. Further evidence for this last assertion comes by way of

![Diagram](https://example.com/diagram.png)

**Fig. 7. Tetramers of GPI-PLC are detected in a hypotonic lysate of T. brucei.** A, cross-linking of TbGPI-PLC in membranes from a hypotonic lysate of T. brucei. Parasites (2.5 x 10^6 [35S]methionine-labeled) were lysed hypotonically. The lysate was treated with MeSO (DMSO, lane 1), DSS (lane 2), or DSP (lanes 3 and 4). DSP cross-links were reversed with β-mercaptoethanol (β-ME) (lane 4). GPI-PLC was adsorbed from the solubilized membranes, resolved by SDS-PAGE, and the gel exposed to BioMax MR film at –80 °C for 2–3 days. B, quantitation of tetramers and GPI-PLC complex (from A). C, components of GPI-PLC complex and tetramers. DSP cross-links were performed and analyzed as described for A (except that 3 x 10^9 cell equivalents of lysate were used). The bands corresponding to GPI-PLC complex or tetramers were excised from an SDS-polyacrylamide gel, cut into pieces (100 µl total), and an equal volume of 2.5× SDS-PAGE sample buffer containing 12.5% β-mercaptoethanol added. The polyacrylamide pieces were heated at 90 °C for 10 min. Radioactivity present in the sample buffer was analyzed by SDS-PAGE (12% minigel) phosphorimaging. Lane 1, gel-purified tetramers; and lane 2, gel-purified GPI-PLC complex.
Tetramerization of GPI-Phospholipase C

| DETERGENT       | STRUCTURE                                      |
|-----------------|-----------------------------------------------|
| NP-40           | ![structure](image) n = 9-10                  |
| Thesit          | ![structure](image) n = 9-10                  |
| LDAO            | ![structure](image)                            |
| CHAPS           | ![structure](image)                            |
| Sodium deoxycholate | ![structure](image)                         |

**Fig. 8. Structures of detergents used.** NP-40, Nonidet P-40.

Studies of the enzyme heterologously expressed in *Leishmania* (15).

*Leishmania* lack a GPI-PLC-like activity. Interestingly, the parasites become GPI-deficient when *T. brucei* GPI-PLC is expressed in them (14, 15). Based on these observations, it was hypothesized that GPI-PLC could be constitutively activated in that parasite. To test whether assembly of GPI-PLC into oligomers, possibly a contributory factor to activation of the enzyme, occurred in *Leishmania*, the organization of GPI-PLC was determined. Cross-linking of GPI-PLC was attempted in intact *Leishmania*. MBS proved to be the best reagent in this trypanosomatid. Tetramers and the so-called “GPI-PLC complex” comprised most of the enzyme in *Leishmania* (Fig. 6C, compare lane 2 to lane 1). By using MBS in *T. brucei* monomers were the major species detected (Fig. 6C, lane 3; also see Fig. 6A). As compared with the monomers detected with DSS (Fig. 6C, lane 4), the MBS monomers migrated at a position suggesting of a smaller size (compare lanes 3 and 4). We presume that the lower molecular weight species of GPI-PLC arises from intramolecular cross-linking. Although this latter species is detectable in *Leishmania*, it is efficiently cross-linked into oligomers in that cell.

Two conclusions may be drawn from the work with *Leishmania*. First, the chemical probes are able to detect oligomers of GPI-PLC in cells where multimers of the enzyme exist. That is, had there been a significant proportion of tetramers in *T. brucei*, we would have detected them. Second, in *Leishmania* where GPI-PLC is constitutively active against GPIs in vivo (14, 15), tetramers and the GPI-PLC complex are the predominant form of the enzyme.

**Activation of GPI-PLC Is Accompanied by Increased Oligomerization**—Although GPI-PLC may be largely inactive against GPIs in *T. brucei* (see Introduction), it cleaves VSG after hypotonic lysis of *T. brucei* (1), implying that the enzyme can be activated under such conditions (2, 3). The oligomerization status of activated GPI-PLC was therefore examined by cross-linking after hypotonic lysis (Fig. 7). By using DSS, a significant proportion of GPI-PLC (33% of the total GPI-PLC, from densitometric analysis) was detected as an aggregate with mobility corresponding to a tetrameric complex (Fig. 7A, lanes 1 and 2). Residual monomers and a larger complex (the “GPI-PLC complex”) were also observed (Fig. 7A, lane 2). A proportion of protein, possibly derived from intramolecular cross-linking and migrating faster than the monomer, was detectable. In the presence of DSP, the major product was the GPI-PLC complex (Fig. 7A, lane 3).

The relative proportion of enzyme in tetramers and in the “GPI-PLC complex” from intact parasites was determined. Similar quantitation was performed for enzyme present in the hypotonic lysates, and ratios of the two were obtained. In DSS cross-linking the quantity of tetrameric GPI-PLC increased 2.3-fold upon activation of the enzyme. The fraction in the GPI-PLC complex rose 3-fold (Fig. 7B). When DSP was used, an 8-fold increase in the amount of the GPI-PLC complex was observed after activation (Fig. 7B). Concurrently, the proportion of GPI-PLC in tetramers doubled (Fig. 7B). Similar proportions of products were obtained when cross-linking was performed at 2 °C (data not presented).

Constituents of the GPI-PLC complex and the “tetramer” (Fig. 7A) were examined (Fig. 7C). For this purpose, the two complexes were generated with DSP, resolved by and obtained from a polyacrylamide gel. Cross-links were cleaved in-gel with β-mercaptoethanol, and the components of each complex were examined after SDS-PAGE and phosphorimaging (Fig. 7C). From the tetramer, monomeric GPI-PLC was the major protein released (Fig. 7C, lane 1). The GPI-PLC complex contained three major polypeptides of 160, 117, and 55 kDa (marked with asterisks) (Fig. 7C, lane 2) in addition to GPI-PLC. Since these polypeptides are not associated with GPI-PLC prior to activation.
tion (Fig. 6A), we hypothesize that they are recruited into proximity of GPI-PLC during or after activation of the phospholipase C.

In summary, monomers of GPI-PLC predominate in "quiescent" *T. brucei*. Following activation of the enzyme, GPI-PLC is found either in a tetrameric complex or in association with other proteins in a very large complex. Thus, activation of GPI-PLC both in vitro and in vivo entails increased protein-protein interactions of which tetramerization is a central part.

**DISCUSSION**

**Monomer-Oligomer Equilibrium of GPI-PLC May Be Modulated by Detergents**—GPI-PLC is an integral membrane protein (1, 22). Consequently, in most biochemical studies a detergent is needed to keep the enzyme solubilized. Although widely used to study membrane proteins, secondary effects of detergents frequently go unrecognized. In this work, we show that several "mild" detergents can perturb the equilibrium of an integral membrane protein between different oligomeric forms, presumably by inserting between dimerization interfaces of the subunits. This observation has been exploited to study aspects of GPI-PLC biochemistry that were not amenable to investigation otherwise.

By gel filtration analysis, GPI-PLC can be fractionated into various oligomeric species in a detergent- and temperature-dependent manner (Fig. 2). GPI-PLC forms tetramers at 27 °C but exists as dimers and monomers at 5 °C (Fig. 2). These data suggest that hydrophobic interactions play a role in oligomerization of GPI-PLC, since hydrophobic interactions are facilitated by increased temperature. Activity of the enzyme was highest when tetramers were the predominant form of the enzyme (Figs. 2 and 3). Loss of GPI-PLC activity after chemical cross-linking (in a failed bid to obtain active tetramers) may have been due to reaction of the reagents with residues in or close to the active site of the enzyme. Recent experiments indicated that a modification of Cys-80 and Gln-81 inhibit the major effect of the detergents is allosteric. Instead, production and/or stabilization of monomers may suffice to inhibit GPI-PLC activity.

As monitored by chemical cross-linking, only native GPI-PLC forms tetramers. The protein loses its ability for efficient tetramerization when denatured with SDS (Fig. 4B). Loss of GPI-PLC activity after chemical cross-linking (in a failed bid to obtain active tetramers) may have been due to reaction of the reagents with residues in or close to the active site of the enzyme. Recent experiments indicated that a modification of Cys-80 and Gln-81 inhibit GPI-PLC biochemistry that were not amenable to investigation otherwise.

All detergents that converted GPI-PLC into monomers (e.g., CHAPS, LDAO, and DOC) (Fig. 2) strongly inhibited enzyme activity (Fig. 3). This observation rules out the contention that hydrophobic interactions play a role in oligomerization of GPI-PLC, since hydrophobic interactions are facilitated by increased temperature. Activity of the enzyme was highest when tetramers were the predominant form of the enzyme (Figs. 2 and 3). Loss of GPI-PLC activity after chemical cross-linking (in a failed bid to obtain active tetramers) may have been due to reaction of the reagents with residues in or close to the active site of the enzyme. Recent experiments

As monitored by chemical cross-linking, only native GPI-PLC forms tetramers. The protein loses its ability for efficient tetramerization when denatured with SDS (Fig. 4B), dimers were found when cross-linking was performed at 2 °C (Fig. 4B) in both CHAPS and Nonidet P-40. Detection of dimers at 2 °C (Fig. 4B) but not at 27 °C (Fig. 4A) could be due to (i) a slower rate of cross-linking and/or (ii) stabilization of GPI-PLC dimers at the lower temperature. The former possibility is supported by the demonstration of dimers in the presence of Tris (Fig. 4C), whereas the latter is substantiated by the stabilization of dimers in Nonidet P-40 at 5 °C (Fig. 2A) compared with tetramers at 27 °C (Fig. 2C).

CHAPS increased the migration of GPI-PLC in a native gel (Fig. 1, B and C). Two explanations may account for this phenomenon. First, CHAPS might alter the quaternary structure by interacting with the oligomerization interfaces. Second, a positive charge on CHAPS (see Fig. 8) may form a salt bridge with an acidic residue on the enzyme. This would leave an extra negative charge on the enzyme. Thus, activation of GPI-PLC, either in a tetrameric complex or in association with other proteins in a very large complex. Thus, activation of GPI-PLC both in vitro and in vivo entails increased protein-protein interactions of which tetramerization is a central part.

**Oligomerization May Trigger Activation of GPI-PLC in Vivo**—Most GPI-PLC molecules in intact trypanosomes remain monomeric (Fig. 6A), unlike the purified enzyme that is predominantly tetrameric (Figs. 2C and 4A). Since monomers of purified GPI-PLC are less active than oligomers (Fig. 3), we speculate that prevention of tetramerization in *T. brucei* is one of the mechanisms by which the activity of GPI-PLC is held in check (Fig. 9). This action would avert excessive cleavage of GPIs in *T. brucei*. In line with our hypothesis, activation of GPI-PLC is associated with assembly of the enzyme into tetramers and a larger complex in *T. brucei* (Fig. 7C). In *Leishmania* where heterologously expressed GPI-PLC is constitutively active, the enzyme exists predominantly as an oligomer (Fig. 6C). Thus, both in vivo and in vitro, the quaternary structure of GPI-PLC has a striking effect on enzyme activity (Fig. 9).

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