**Leishmania donovani** Peroxin 14 Undergoes a Marked Conformational Change following Association with Peroxin 5*

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The import of PTS1 proteins into the glycosome or peroxisome requires binding of a PTS1-laden PEX5 receptor to the membrane-associated protein PEX14 to facilitate translocation of PTS1 proteins into the lumen of these organelles. Quaternary structure analysis of protozoan parasite *Leishmania donovani* PEX14 (LdPEX14) revealed that this protein forms a homomeric complex with a size >670 kDa. Moreover, deletion mapping indicated that disruption of LdPEX14 oligomerization correlated with the elimination of the hydrophobic region and coiled-coil motif present in LdPEX14. Analysis of the LdPEX5-LdPEX14 interaction by isothermal titration calorimetry revealed a molar binding stoichiometry of 1:4 (LdPEX5:LdPEX14 interaction by isothermal titration calorimetry). Circular dichroism, intrinsic fluorescence, and proteolysis assays established that in the presence of LdPEX5, LdPEX14 became more susceptible to proteolytic degradation consistent with this protein interaction triggering a significant conformational change in the recombinant and native LdPEX14 structures. These structural changes provide essential clues to how LdPEX14 functions in the translocation of folded proteins across the glycosomal membrane.

*Leishmania* and *Trypanosoma* protozoan parasites represent organisms that branched off early from the eukaryotic cell lineage (1–3). Consequently, these organisms have retained a myriad of unique metabolic, biochemical, and structural features that are distinctive from other eukaryotic cells. Prominent among these features is the glycosome, an organelle that is distinctly related to the peroxisomes in mammalian, yeast, fungi, and plant cells (4–6). The glycosome compartmentalizes a multitude of indispensable metabolic and biosynthetic pathways that include glycolysis, purine salvage, pyrimidine and ether-lipid biosynthesis, and β-oxidation of fatty acids (6, 7). Glycosomal function is essential for parasite viability as mis-targeting of glycolytic enzymes to the cytosol or disruption of glycosome biogenesis leads to a lethal phenotype (8–12) making the glycosome and glycosomal biogenesis machinery attractive chemotherapeutic targets (13, 14).

Glycosomal and peroxisomal matrix proteins are post-translationally trafficked from cytosolic ribosomes to these microbodies by utilizing primarily one of two topogenic signals termed peroxisomal targeting signal 1 and 2 (PTS1 and PTS2) located at the C or N termini of proteins, respectively (15–20). In *Leishmania* newly synthesized proteins containing PTS1 or PTS2 signals are bound by the receptors peroxin 5 (LdPEX5)3 and peroxin 7 (LdPEX7), respectively, and these cargo-laden receptors traffic to the glycosome surface where they bind to the membrane-associated protein peroxin 14 (LdPEX14). This latter protein-protein interaction is paramount for the translocation of proteins across the glycosomal and peroxisomal membrane and for the biogenesis of these organelles. In Δpex14 yeast and mammalian mutant cell lines that lack a functional PEX14, the matrix PTS1 and PTS2 proteins are mis-targeted into the cytosol (21–24). In trypanosomes knockdown of PEX14 using RNA interference caused mis-targeting of glycosomal matrix proteins to the cytosol and resulted in a lethal phenotype when parasites were cultivated in media containing glucose or glycerol (9, 12, 25).

PEX14 in fungi and mammals interacts with PEX13 forming a subcomplex known as the importomer (26–31); whether a comparable importomer complex exists in the protozoa *Leishmania* is not clear because a PEX13 homolog has not yet been identified in this group of organisms. Numerous studies in phylogenetically diverse organisms have demonstrated that PEX14 is a membrane-associated protein; however, the nature of this interaction and the topology of PEX14 in the peroxisomal and glycosomal membranes, as assessed by physiochemical tech-
niques, seem to vary. In mammalian cells, *Hansenula polymorpha*, *Pichia pastoris*, and *Trypanosoma brucei* (24, 25, 32–36) PEX14 is reported to be an integral protein, whereas in *Saccharomyces cerevisiae* PEX14 association with the peroxisomal membrane is more plastic and has been reported to behave either as a peripheral or integral membrane protein (21, 37, 38). Using similar biochemical approaches, the *Leishmania* PEX14 has been shown to be a peripheral membrane protein that associates tightly with the cytosolic surface of the glycosomal membrane (39).

Interestingly, despite the fact that PEX14 proteins exhibit <10% sequence conservation across phylogeny (39), this family of proteins has retained three structural elements. These include an N-terminal 33-amino acid signature motif \(\text{AX}X_X\text{FLX}_X\text{P}X_X\text{FLXKGX}_X\text{A}\) that contains a PEX5-binding motif (19, 40), a hydrophobic region, and a coiled-coil motif (21, 36, 37, 39, 41). The hydrophobic region and the coiled-coil motif are believed to be involved in the formation of PEX14 homomeric structures (24, 35, 42). However, little is known about the structural changes induced in PEX14 upon binding its receptor PEX5. Here we reported the use of a number of biophysical techniques that include size exclusion chromatography, intrinsic fluorescence, CD, analytical ultracentrifugation, isothermal titration calorimetry, and limited proteolysis to examine the LdPEX14 structural and conformational changes triggered in the LdPEX14 complex following LdPEX5 binding.

**EXPERIMENTAL PROCEDURES**

**Material**—All restriction endonucleases and DNA-modifying enzymes were purchased from Invitrogen or New England Biolabs (Beverly, MA). Horseradish peroxidase (HRP)-conjugated secondary goat anti-rabbit IgG were purchased from Sigma. All other reagents were of the highest quality commercially available.

**Cell Culture**—*Leishmania donovani* promastigotes were cultured in Dulbecco’s modified Eagle’s-Leishmania medium supplemented with hemin, xanthine, and fetal bovine serum as described (43).

LdPEX5 and LdPEX14 Expression and Purification—The open reading frames encoding the full-length LdPEX14 or mutant proteins encompassing residues 1–321 (Ldpex14–(1–321)), 1–254 (Ldpex14–(1–254)), 1–200 (Ldpex14–(1–200)), 1–148 (Ldpex14–(1–148)), or 1–120 (Ldpex14–(1–120)) were amplified by PCR and cloned into the Ncol/BamHI sites of the pET15b(+) or pET30b(+) expression vectors. The Ldpex5-(203–391) and Ldpex5-(203–391) W3F were expressed in *Escherichia coli* using the pTYB12 expression vector (45).

**Site-directed Mutagenesis**—The three tryptophan residues (Trp-246, Trp-293, and Trp-361) in Ldpex5–(203–391) W3F excited at 295 nm showed no notable fluorescence emission between 305 and 400 nm, diagnostic of this protein being devoid of a tryptophan residue. Binding studies performed with Ldpex5–(203–391) W3F using either pulldown assays or ELISA (39, 44) showed that this fragment exhibited LdPEX14 binding characteristics similar to Ldpex5–(203–391) (data not shown).

*E. coli* ER2566 cells transformed with pTYB12-LdPEX5, pTYB12-Ldpe5–(203–391), or pTYB12-Ldpe5–(203–391) W3F (45) were grown to an \(A_{600}\) of 1.2 at 37 °C then shifted to 20 °C for protein expression. Protein expression was induced for 4 h with 0.5 mM isopropyl thiogalactoside. Cell pellets were resuspended in 20 ml of 40 mM Tris–HCl, pH 8.0, containing an EDTA-free protease inhibitor mixture (Roche Applied Science) and lysed by a French press. Lysates were clarified by centrifugation, and the supernatant was loaded onto a chitin column (1 × 3 cm), and the column was washed with 100 ml of 0.5 M NaCl in TB buffer. LdPEX5 and Ldpex5–(203–391) were cleaved by incubating the column matrix with 5 ml of 50 mM dithiothreitol in TB buffer for 40 h at 4 °C. Recombinant LdPEX14/ Ldpex14 proteins were overexpressed in *E. coli* ER2566 strain and purified as described previously (39).

For analytical centrifugation and tryptic digest experiments the LdPEX5-LdPEX14 complex was isolated from *E. coli* ER2566 cells co-transformed with the pTYB12-LdPEX5 and pET30b-His\(_6\)/S-LdPEX14. Clarified cells lysates were applied onto a Ni\(^{2+}\)–NTA column (1 × 5 cm), and the bound proteins were eluted with 250 mM imidazole in 50 mM phosphate, pH 7.5, 150 mM NaCl (PBS). The eluates were then applied to a chitin column (1 × 3 cm) to capture complexes containing the chitin–LdPEX5 fusion protein. The column was washed with 50 ml of 40 mM Tris–HCl, pH 8.0, 0.5 M NaCl, and the LdPEX5–LdPEX14 complex was eluted by incubating the column matrix with 5.0 ml of 50 mM dithiothreitol in 40 mM Tris–HCl, pH 8.0, for 40 h at 4 °C. All recombinant proteins were concentrated, and the buffer was exchanged for 40 mM Tris–HCl, pH 8.0, 150 mM NaCl using a Biomax 5K NMWL centrifugal filter unit (Millipore, Bedford, MA). Protein concentrations were determined spectrophotometrically (48).

**Tryptic Analysis of LdPEX14 and LdPEX4-LdPEX5 Complex**—LdPEX4 (~100 μg) or LdPEX5–LdPEX5 (125 μg) complex in 100 μl of 50 mM Tris–HCl, pH 7.5, 150 mM NaCl (TBS) was incubated with 0.3 μg of sequencing grade trypsin (Promega, Madison, WI) at 20 °C. Alternatively, purified glycosomes (50 μg of total protein) from wild type *L. donovani* promastigotes (43) were incubated with recombinant LdPEX5 or bovine serum albumin (15 μg) and LDXPRT (50 μg) for 20 min.
at 20 °C prior to the addition of 0.3 μg of sequencing trypsin in 150 μl of TBS. Aliquots (10 μl) were removed at 0, 2, 5, 10, 15, 60, and 180 min and mixed with 4 μl of protease inhibitor mixture (1 mini-tablet/ml distilled H2O) to inactivate the trypsin prior to addition SDS-PAGE sample buffer. Samples were resolved on a 10% SDS-PAGE, transferred to PVDF membrane, and stained with Coomassie Blue R-250. Membranes were blocked with 3% skimmed milk powder in PBS and then probed with anti-Ni2+-NTA-HRP in PBS (1:2,000, Qiagen, Mississauga, Ontario) and anti-LdPEX14 (1:16,000) and anti-rabbit IgG-HRP (1:10,000).

**Quaternary Structure Analysis—**Size exclusion chromatography (SEC) was performed on a Beckman-Coulter system Gold equipped with a Superdex 200 column (GE HealthCare) equilibrated with 50 mM phosphate, pH 7.5, 150 mM NaCl, 5 mM β-mercaptoethanol. Samples containing 50–200 μg of protein in buffer containing 5 mM β-mercaptoethanol were injected, and the column was developed at 0.5 ml/min while monitoring the column effluent at 280 nm. Fractions (0.5 ml) were collected and proteins precipitated with trichloroacetic acid for SDS-PAGE analysis. The Superdex 200 column was calibrated using thyroglobulin (670 kDa), bovine IgG (17 kDa), and vitamin B12 (1.3 kDa) (Bio-Rad).

**Isothermal Titration Calorimetry (ITC)—**ITC experiments were performed on a Microcal VP-ITC calorimeter (Microcal, Northampton, MA). For the forward reaction, a 292 μM solution of LdPEX14 was loaded into the syringe and titrated into an 8 μM solution of LdPEX5 present in the ITC cell (1.42 ml). Alternatively, the syringe was loaded with a 1.0 mM solution of ldpex14-(1–120), which was titrated into a 29 μM solution of ldpex5-(203–391) in the ITC cell. For the reverse titrations, the ITC syringe was loaded with a 149 μM LdPEX5 solution, and the ITC cell was charged with a 40 μM LdPEX14 solution. Alternatively, the syringe was loaded with 397 μM solution of ldpex5-(203–391) and was titrated into a 145 μM ldpex14-(1–120) solution in the ITC cell. All reactions were performed at a constant temperature of 303 K, and protein solutions were dialyzed against the same batch of 40 mM sodium phosphate, 120 mM NaCl, pH 7.5, 2 mM β-mercaptoethanol buffer to minimize heat of dilution effects. For all experiments, the contents of the cell were mixed at 300 rpm, and an equilibration time of 6 min between injections was used. The first injection used a 2-μl aliquot, and the 20–30 subsequent injections were performed using 10 μl volumes over a duration of 10 s. The experimental titration curves were corrected for the heat of dilution, and the initial 2-μl injections were typically omitted from the data set, and the curve fitting was performed using the Microcal Origin software 7.0 assuming a one-site model. The binding constants (Kd = 1/Ka) and the enthalpy (ΔH) and entropy (ΔS) were determined from the isotherm and the Gibbs free energy (ΔG), and entropy (ΔS) was calculated using the equation ΔG = ΔH − TΔS = −RTlnKd.

**Analytical Ultracentrifugation—**Sedimentation velocity experiments were performed in 40 mM Tris, pH 7.5, at 20 °C in a Beckman Optima XL-1 (Fullerton, CA) analytical ultracentrifuge using an An 55 AL aluminum rotor. Samples containing 250 μg/ml LdPEX14 or LdPEX5-LdPEX14 (molar ratio of 1:4) were loaded into double-sector cell with aluminum-filled Epon centerpieces. LdPEX14 and LdPEX5-LdPEX14 complex and ldpex14 deletion mutants were analyzed at rotor speeds of 26,000 and 30,000 rpm, respectively. UV scans were obtained at 230/280 nm and analyzed by the van Holde-Weischet method (49). The G/g(S) integral distribution attained with this method was determined using the XL-1 UltraScan II version 9.7 sedimentation data analysis software (B. Demeler, University of Texas Health Science Center, San Antonio, TX).

**Fluorescence Spectroscopy—**Fluorescent measurements were performed on a Varian Cary Eclipse spectrofluorometer (Palo Alto, CA) at 25 °C using an excitation wavelength of 295 nm. Emission spectra were recorded from 305 to 400 nm using a scan rate of 120 nm/min with excitation and emission slit widths of 5 nm. A solution of recombinant LdPEX14 (40 μM) in 40 mM Tris, pH 8.0, was titrated with ldpex5-(203–391) W3F to a final concentration of 0–40 μM. Dilution effects were corrected by titrating LdPEX14 with buffer alone. For the chemical denaturation experiment, 20 μM of LdPEX14 was diluted into a 4.5 M solution of guanidinium hydrochloride in 40 mM Tris, pH 8.0, and incubated for 18 h at 4 °C. Fluorescence spectra were recorded as indicated above. For quenching experiments, a 5 μM acrylamide solution was titrated into a mixture containing 40 μM LdPEX14 or LdPEX14-ldpex5-(203–391) W3F mixture (40:10 μM) in 75 mM sodium phosphate, pH 7.1.

**Circular Dichroism (CD)—**Purified proteins were exhaustively dialyzed at 4 °C against 10 mM phosphate buffer, pH 7.6, and the protein concentration was measured by the method of Pace et al. (48). CD measurements were performed on a Jasco 810 spectropolarimeter at 20 °C, using a cuvette with a 0.1-cm path length at a scan rate of 50 nm/min. Five spectra were collected and averaged per sample. For all samples, data were collected at wavelengths between 250 and 190 nm. LdPEX14 was diluted in dialysis buffer to a concentration of 3.1 μM, and ldpex5-(203–391) was added to a final concentration of 0.8 μM to obtain a 4:1 molar ratio of LdPEX14:ldpex5-(203–391).

**Cross-linking Studies—**L. donovani promastigotes (5 × 10⁸ cells/ml PBS) were permeabilized for 5 min at 20 °C with 15 μg/ml digitonin, and aliquots (100 μl) were incubated with increasing concentrations of glutaraldehyde (0–1.5 mM) for 20 min at 20 °C. Cells were washed three times with 1.0 ml of PBS to remove excess cross-linking agent, and the cell pellet was resuspended in 100 μl of 2× SDS-PAGE sample buffer containing 6 M urea. Purified recombinant LdPEX14 (2 μg/50 μl PBS) was subjected to glutaraldehyde cross-linking using the above conditions. The reaction was terminated by adding Tris-HCl to a final concentration of 50 mM, and the mixtures were resolved on a 5–10% gradient SDS-PAGE, and the proteins were transferred to a PVDF membrane. Western blots were probed with rabbit anti-LdPEX14 (1:10,000) (39) or rabbit anti-L. donovani adenine phosphoribosyltransferase (LdAPRT) (1:1,000) antibodies. For cross-linking reactions with ldpex5-(203–391) and ldpex14-(1–120), proteins were resolved for 3 h at 20 °C with 5 mM tris(2-carboxyethyl)phosphate to reduce potential disulfide bonds prior to cross-linking with glutaraldehyde (0–3.2 mM). Cross-linked complexes were characterized by Western blots probed with anti-LdPEX5 and anti-LdPEX14 antisera.
RESULTS

LdPEX14 Quaternary Structure—Native LdPEX14 extracted from glycosomes was previously demonstrated to migrate on a sucrose density gradient predominantly as a macromolecular structure of ~800 kDa. To further validate oligomeric structure of LdPEX14 on the glycosome surface, chemical cross-linking was used to trap these complexes. Western blot analysis of digitonin-permeabilized L. donovani promastigotes treated with glutaraldehyde showed a concentration-dependent accumulation of a cross-linked complex that SDS-PAGE was estimated to have an apparent mass of >250 kDa (Fig. 1). A comparable complex was also observed when the cross-linking reaction was performed with zero length cross-linking agent ethyl dimethyaminopropyl carbodiimide (data not shown). Western blots of glutaraldehyde cross-linked reactions probed with antisera against adenine phosphoribosyltransferase, a 26-kDa cytosolic protein (50, 51), revealed a single immunoreactive band indicating that nonspecific cross-linking was minimal (Fig. 1B). Similar complexes were also detected with purified glycosomes (39) or recombinant LdPEX14 treated with glutaraldehyde (Fig. 1C).

Analysis of the LdPEX14 primary sequence showed three structural motifs that are conserved among the PEX14 protein family. These include the PEX5 binding domain (residues 23–70), a hydrophobic region (residues 149–179), and a coiled-coil motif (residues 270–321) (21, 22, 37, 39, 52, 53). To elucidate the elements required for LdPEX14 oligomerization, a panel of ldpex14 truncation and internal deletion mutants was expressed in E. coli (Fig. 2A) and the quaternary structure examined by SEC. All of the ldpex14 proteins were readily purified from E. coli lysates using Ni2+-NTA affinity chromatography as soluble proteins. No precipitation was observed even after prolonged storage at 4°C or −80°C. Interestingly, SEC analysis of freshly purified full-length recombinant LdPEX14, ldpex14 (Δ1–63), ldpex14 (Δ270–321), ldpex14 (Δ149–179), ldpex14 (1–321), ldpex14 (1–254), and ldpex14 (1–200) revealed that these proteins all eluted in the void volume of a Superdex 200 column suggesting the formation of oligomeric complexes with a size of >670 kDa (Fig. 3A). Similar results were also observed when a 5–10-fold more dilute concentration of these proteins was injected onto the gel permeation column.

Analysis of several of these mutant proteins by analytical ultracentrifugation confirmed the formation of large complexes of differing sizes (Fig. 3D). In low ionic strength buffers LdPEX14 assembled into structures that varied in size from ~10 to 70 S, whereas ldpex14 (Δ149–179) appeared to form smaller structures ranging in size from 10 to 30 S (Fig. 3D). Surprisingly, in low ionic strength buffer ldpex14 (1–254) migrated as a relatively homogeneous complex of ~2–10 S. However, when the sedimentation analysis of this protein was performed in buffer containing 150 mM NaCl, a marked increase in oligomerization occurred resulting in structures with a size distribution of 2–90 S (data not shown). Similarly, sedimentation velocity analysis of ldpex14 (1–200) in low ionic strength buffers showed that this protein was also heterodimeric.
perse and sedimented with S values ranging from 10 to 170 S (Fig. 3).

In contrast, truncation mutants lacking the hydrophobic region, which include ldpex14-(1–148), ldpex14-(1–120), and ldpex14-(1–75), migrated on the Superdex 200 column with masses of \( \sim 50, 42, \) and 20 kDa, respectively, consistent with these proteins forming a dimeric structure (Fig. 2 and Fig. 3B). For ldpex14-(1–75), the dimer appears to be particularly stable as indicated by the significant population of the dimeric species (\( \sim 22 \) kDa) by SDS-PAGE (Fig. 3C). It should be noted that the wild type and mutant LdPEX14 proteins all migrated with anomalously higher molecular weight than theoretically predicted (39). Stabilization of the LdPEX14 homomeric structure probably involves multiple protein-protein contacts because the mutants ldpex14-(149–179) and ldpex14-(1270–321), which lack the hydrophobic domain or the coiled-coil motif, still formed large complexes that were not disrupted by singly deleting either of the elements (Fig. 3A and D).

**Isothermal Titration Calorimetry**—Isothermal titration calorimetry (ITC) was employed to determine the binding stoichiometry (N), binding affinity (\( K_d \)), enthalpy (\( \Delta H \)), entropy (\( T\Delta S \)), and the Gibbs free energy (\( \Delta G \)) for the LdPEX5-LdPEX14 and ldpex5-(203–391)-ldpex14-(1–120) interactions (54, 55). Fig. 4 illustrates the isothermograms for representative reactions. Titration of LdPEX14 into a solution of LdPEX5 showed that the LdPEX5-LdPEX14 interaction resulted in an exothermic heat release that diminished as the level of free LdPEX5 in the ITC cell decreased (Fig. 4A). Integration heat release for each injection was fit using the Microcal Origin 7.0 nonlinear regression software to a one-site model that had a binding stoichiometry of 4.2:1 (LdPEX14:LdPEX5). The \( K_d \) for the LdPEX5-LdPEX14 interaction was 512 nM (or 128 nM per LdPEX14 subunit). Similar titration experiments performed with ldpex14-(1–120) and ldpex5-(203–391), fragments known to a form stable interactions (44), gave a binding stoichiometry of 4.1:1 (ldpex14-(1–120):ldpex5-(203–391)) and a \( K_d \) of 625 nM (156 nM per ldpex14-(1–120) subunit) (Fig. 4B). The \( \Delta G \) \( (\sim -36 \text{ kJ/mol}) \) for the binding reaction was derived from favorable negative \( \Delta H \) and positive \( T\Delta S \) components (Table 1). In ligand binding reactions, hydrogen bonding and van der Waal interactions are proposed to be the major sources contributing to a negative \( \Delta H \), whereas desolvation of hydrophobic surfaces, an

![FIGURE 3. Quaternary structure of LdPEX14. A, quaternary structure of the full-length His\textsubscript{6}/S-LdPEX14, His\textsubscript{6}/S-ldpex14-(Δ1–63), His\textsubscript{6}/S ldpex14-(Δ149–179), His\textsubscript{6}/S ldpex14-(Δ1–321), and His\textsubscript{6}/S ldpex14-(Δ1–254), and His\textsubscript{6}/S ldpex14-(Δ1–200) was analyzed by injecting a 200-μl aliquot of a 5–10 mg/ml solution of freshly purified protein onto a Superdex 200 size exclusion chromatography column. The column was developed with 50 mM sodium phosphate, 150 mM NaCl, 5 mM β-mercaptoethanol, pH 7.6, at a flow rate of 0.5 ml/min, and proteins were detected by monitoring the column effluent at 280 nm to detect proteins. B, quaternary structure of His\textsubscript{6}/S-LdPEX14 (Δ1–148), His\textsubscript{6}/S-LdPEX14 (Δ1–120), and His\textsubscript{6}/S-LdPEX14 (Δ1–75). C, SDS-PAGE analysis of affinity purified LdPEX14 and ldpex14 proteins used for gel permeation analysis. D, to further examine the quaternary structures, His\textsubscript{6}/S-LdPEX14 and select group of the deletion mutants (His\textsubscript{6}/S-LdPEX14 (Δ149–179), His\textsubscript{6}/S LdPEX14 (Δ1–254), and His\textsubscript{6}/S LdPEX14 (Δ1–200)) were analyzed by sedimentation velocity on analytical ultracentrifugation at 20 °C in 40 mM Tris, pH 7.5, in a Beckman Optima XL-I ultracentrifuge at a rotor speed of 26,000 rpm.**
event that increases the randomness of the system, favors a positive $\Delta S$ (54, 56, 57). Collectively, this suggests that the LdPEX5-LdPEX14 interaction is stabilized by a combination of hydrogen bonding and hydrophobic interactions.

Reverse titration experiments in which LdPEX5 or ldpex5-(203–391) was titrated into a solution of LdPEX14 or ldpex14-(1–120), respectively, gave a binding stoichiometry of 0.25:1 (LdPEX5:LdPEX14 or ldpex5-(203–391):ldpex14-(1–120)) (Fig. 4, A and B; Table 1). These titrations, like the forward reaction, were exothermic throughout and had a $\Delta G$ of approximately $\sim 40 \text{kJ/mol}$ (Table 1). However, the favorable $\Delta G$ was primarily derived from a large negative $\Delta H$ term ($\sim 190$ to $\sim 299 \text{kJ/mol}$), which offset the negative entropy change ($T\Delta S$) (Table 1). The unfavorable $T\Delta S$ suggests that interaction of LdPEX5 or ldpex5-(203–391) with LdPEX14 or ldpex14-(1–120) likely leads to the solvation of a hydrophobic surface or a conformational change that decreases the degrees of freedom in the system (54, 56). Comparable $K_d$ values for the LdPEX5-LdPEX14 and ldpex5-(203–391)-ldpex14-(1–120) interactions were also obtained for these reactions (Table 1). The solution phase binding affinities for the LdPEX5-LdPEX14 interactions, measured by ITC, are somewhat higher than values previously reported using an ELISA-based assay (44). This discrepancy is likely because of the cooperative nature or avidity effects asso-

FIGURE 4. Isothermal titration microcalorimetry. The thermodynamic parameters, dissociation binding constants, and the binding stoichiometries for the LdPEX5-LdPEX14 interaction were determined by titrating. A, 292 $\mu M$ solution of LdPEX14 was titrated into an 8 $\mu M$ solution of LdPEX5. B, 1.0 $mM$ solution of ldpex14-(1–120) was titrated into a 29 $\mu M$ solution of ldpex5-(203–391). C, 149 $\mu M$ LdPEX5 solution was titrated into a 40 $\mu M$ LdPEX14. D, 397 $\mu M$ solution of ldpex5-(203–391) was titrated into 145 $\mu M$ ldpex14-(1–120). All reactions were performed at 30 °C in 40 mM sodium phosphate, 120 mM NaCl pH7.5, 2 mM $\beta$-mercaptoethanol buffer. In each panel the top portion of the figure represents the heat of reaction after each 10-$\mu l$ injection, and the lower portion represents the integrated heat of for each injection plotted as a function of the molar ratio of LdPEX14:LdPEX5, LdPEX5:LdPEX14, ldpex14-(1–120):ldpex5-(203–391), or ldpex5 203:ldpex14-(1–120). The solid line represents the best fit of the data assuming a one-site model.

TABLE 1

| Protein in the syringe | Protein in the cell | $K_d$ $\mu M$ | Stoichiometry | $\Delta H$ $\text{kJ/mol}$ | $T\Delta S$ $\text{kJ/mol}$ | $\Delta G$ $\text{kJ/mol}$ |
|------------------------|---------------------|--------------|----------------|--------------------------|--------------------------|--------------------------|
| LdPEX14                | LdPEX5              | 512          | 4.2:1          | $-17.6$                  | 26.4                     | $-36.4$                  |
| ldpex14-(1–120)        | ldpex5-(203–391)    | 625          | 4.1:1          | $-16.7$                  | 19.3                     | $-36.0$                  |
| LdPEX5                 | LdPEX14             | 74           | 0.23:1         | $-90.0$                  | 48.1                     | $-41.9$                  |
| ldpex5-(203–391)       | ldpex14-(1–120)     | 166          | 0.23:1         | $-99.6$                  | 60.3                     | $-39.3$                  |
SEC analysis of the ITC reaction mixtures revealed that the LdPEX5-LdPEX14 complex eluted in the column void volume suggesting that LdPEX5 was recruited to the LdPEX14 complex (Fig. 5A). The presence of LdPEX5 in the void volume was confirmed by SDS-PAGE (Fig. 5A). Previous studies demonstrated that LdPEX5 alone migrated on an SEC column with an apparent molecular mass of ~110 kDa (45), and indeed a small population of LdPEX5 alone was detected in the ITC reaction mixtures (fractions 31–36 (Fig. 5A)). SEC analysis of the ldpex5-(203–391)-ldpex14-(1–120) ITC reaction mixture revealed a peak eluting with a mass of ~95 kDa that by SDS-PAGE was found to contain both ldpex5-(203–391) and ldpex14-(1–120) (Fig. 5B) and is consistent with a hetero-oligomeric complex containing one ldpex5-(203–391) and four ldpex14-(1–120), as predicted by ITC.

To validate the structure of ldpex5-(203–391)-ldpex14-(1–120) complex, a glutaraldehyde cross-linking was performed. Treatment of ldpex14-(1–120) with increasing concentrations glutaraldehyde revealed a substantial accumulation of an ~50-kDa species that confirmed previous SEC analysis showing that the N-terminal region of LdPEX14 contained a dimerization domain (Fig. 3B and Fig. 5C, panel a). In contrast, only a monomeric species was detected for ldpex5-(203–391) (Fig. 5C, panel b). This was rather surprising, because by SEC ldpex5-(203–391) eluted with an apparent molecular mass of ~55 kDa suggesting that this protein behaved as a dimer (data not shown).

Western blot analysis of ldpex5-(203–391):ldpex14-(1–120) mixtures show that at low glutaraldehyde concentrations the ldpex14-(1–120) dimer is primarily detected. However, at a glutaraldehyde concentration of 0.8–3.2 mM, a ~100-kDa hetero-oligomeric complex is detected (Fig. 5C, panel c). The presence of the ~80- and 100-kDa doublets in the reactions containing 0.4 and 0.8 mM of glutaraldehyde suggests that the ldpex14-(1–120) dimers may bind sequentially to ldpex5-(203–391) (Fig. 5C, panel c).

Fluorescence Measurements—A striking feature of the LdPEX5-LdPEX14 ITC isotherm (Fig. 4C) was the large ΔH change that occurred with low levels of LdPEX5. The absence of this dramatic heat loss when LdPEX14 was added to LdPEX5 (Fig. 4A) suggested that this heat loss may be associated with a conformational change in the LdPEX14 oligomeric complex triggered by binding of LdPEX5. Secondary structure analysis of LdPEX14 using the HNN algorithm revealed that the single tryptophan residue (Trp-152) in LdPEX14 mapped to the N terminus of a putative α-helix formed by the hydrophobic domain (Fig. 2B). We exploited the intrinsic fluorescence of Trp-152 to follow structural changes induced in LdPEX14 on binding LdPEX5. To eliminate fluorescence contributions from LdPEX5, ldpex5-(203–391) W3F, a mutant fragment in which the three tryptophan residues found within this region were mutated to phenylalanines was used in these studies (59). Pull-down assays indicated that ldpex5-(203–391) and ldpex5-

![FIGURE 5. Analysis of the isothermal titration calorimetry complexes. Complexes formed in the ITC cell were analyzed by size exclusion chromatography on Superdex 200 column equilibrated with 50 mM phosphate, pH 7.5, 150 mM NaCl, 5 mM β-mercaptoethanol and developed at a flow rate of 0.5 ml/min. A 20-μl aliquot from the LdPEX5:LdPEX14 (A) or ldpex5-(203–391):ldpex14-(1–120) titration reactions (B) was injected onto the column, and 1.0-min fractions were collected and analyzed by Coomassie Blue- or Silver-stained SDS-PAGE (inset) to validate the protein composition of each peak. C, quaternary structure of the ldpex5-(203–391):ldpex14-(1–120) complex was confirmed using a cross-linking experiment. LdPEX14-(1–120) (panel a), ldpex5-(203–391) (panel b), or ldpex5-(203–391):ldpex14-(1–120) mixture (1:4 mole ratio) (panel c) was treated with 0–3.2 mM glutaraldehyde for 20 min at 20 °C. Brackets designate the monomer (m) and dimer (d) forms of ldpex14-(1–120) or the ldpex14-(1–120):ldpex5-(203–391) complex. Reaction mixtures were analyzed by Western blot using anti-LdPEX14 or anti-LdPEX5 antibodies.](http://www.jbc.org/content/283/46/31494.full)

Leishmania PEX14
W3F exhibited similar LdPEX14 binding characteristics. Excitation of LdPEX14 at 295 nm revealed that Trp-152 had an emission maximum ($\lambda_{\text{max}}$) at 332 nm, a wavelength diagnostic of a tryptophan located in a nonpolar environment (Fig. 6A). Addition of ldpex5-(203–391) W3F to LdPEX14, however, induced a concentration-dependent increase in the fluorescence intensity and a red-shift in the emission $\lambda_{\text{max}}$ to 341 nm, an alteration consistent with Trp-152 shifting to a more exposed polar environment (Fig. 6A). Correlating the wavelength change with the molar ratio of ldpex5-(203–391) W3F revealed a plateau in the shift of the Trp-152 emission $\lambda_{\text{max}}$ at an ldpex5-(203–391) W3F:LdPEX14 mole ratio of 0.25:1. This binding stoichiometry is in agreement with the results obtained by ITC. Denaturation of LdPEX14 with 4.5 M guanidinium hydrochloride resulted in a shift in the emission $\lambda_{\text{max}}$ to 360 nm, indicating that this tryptophan residue in native LdPEX14 was located in a nonpolar environment (Fig. 6B) (60).

Stern-Volmer plots revealed a linear response with Stern-Volmer constant of 5.4 M$^{-1}$ for LdPEX14-ldpex5-(203–391) W3F indicating that Trp-152 underwent dynamic quenching with acrylamide (Fig. 6C). A lower Stern-Volmer constant of 3.8 M$^{-1}$ was measured for LdPEX14 alone, indicating that in the absence of ldpex5-(203–391) W3F, Trp-152 was less accessible to the quenching agent (Fig. 6C) (60).

**CD Analysis**—The effect of ldpex5-(203–391)-LdPEX14 interaction on the secondary structure was examined by the method of Greenfield (61). The far-UV CD difference spectrum generated by subtracting the calculated spectra generated from the unmixed ldpex5-(203–391) and LdPEX14 spectra from the CD spectrum obtained for the LdPEX14:ldpex5-(203–391) complex (4:1) revealed a maxima at $\sim$215 nm consistent with an increase in the random coil content of the complex (Fig. 6D, inset). Interestingly, ldpex5-(203–391) did not show a prominent circular dichroism signal suggesting that this fragment has a relatively flexible conformation (Fig. 6D).

**Analysis of the LdPEX14 and LdPEX5-LdPEX14 Complex**—Sedimentation velocity analytical ultracentrifugation analysis of the LdPEX14 and LdPEX5-LdPEX14 macromolecular complexes by the method of van Holde-Weischet (49), which correlates the sedimentation coefficient of a protein species with its abundances at the moving boundary, gave rise to curves with a positive deflection (Fig. 7). This relationship is diagnostic of a
protein forming heterogeneous oligomeric structures (49). As demonstrated previously (Fig. 3D), LdPEX14 formed disperse structures with sedimentation coefficients ranging from 17 to 52 S. Under similar conditions, the LdPEX5-LdPEX14 complexes also exhibited a heterogeneous behavior, but the complexes had a more compact architecture with sedimentation coefficients ranging from −6 to 19 S. These results imply that binding LdPEX5 induced a conformational change in LdPEX14 leading to the formation of a more ordered LdPEX5-LdPEX14 hetero-oligomeric complex (Fig. 7) and support the ITC finding that docking of LdPEX5 to LdPEX14 induced a striking conformational change.

Limited Proteolysis—The LdPEX14 conformational changes were next examined using trypsin limited proteolysis. Treatment of recombinant LdPEX14 alone with trypsin resulted in cleavage of this protein to an N-terminal ∼40-kDa fragment that was more resistant to further proteolysis even after a prolonged incubation (3 h at 20 °C) (Fig. 8, A and B). Protease-resistant fragments have been reported for mammalian and S. cerevisiae PEX14; however, these fragments were associated with insertion of PEX14 into the peroxisomal membrane (21, 35, 36). Western blot analysis of the digests with Ni²⁺-NTA-conjugated horseradish peroxidase (Ni²⁺-NTA-HRP or anti-LdPEX14 antisera (44)) established that this ∼40-kDa proteolytic product corresponds to an N-terminal fragment (Fig. 8, B and C) exhibiting an electrophoretic mobility similar to ldpex14-(1–200) (Fig. 3C). Smaller N-terminal fragments ranging from ∼19 to 40 kDa were also observed within 15 min of digestion (Fig. 8C).

Formation of the LdPEX5-LdPEX14 (1:4 molar ratio) dramatically altered the susceptibility of LdPEX14 to proteolytic degradation, as shown by the Coomassie Blue-stained gels (Fig. 8A). Notably, no reactivity was detected with Ni²⁺-NTA-HRP, suggesting that the proximal N-terminal region of LdPEX14 became exposed resulting in rapid degradation of the hexahis-
**DISCUSSION**

The membrane-associated protein PEX14 is a crucial component required for the import of PTS1 and PTS2 proteins into glycosomes and peroxisomes (10, 27, 30, 39, 41, 44, 63, 64). In glycosome biogenesis, the importance of PEX14 has been underscored by genetic experiments demonstrating that knockdown of PEX14 in the kinetoplastid parasite *T. brucei* results in a lethal phenotype (9, 10, 25). Although a considerable amount of knowledge regarding the protein complement involved in the assembly of these microbody organelles has been amassed, far less is known about the molecular dynamics associated with the docking of the PEX5 and PEX7 receptors to PEX14 and the subsequent translocation of folded nascent polypeptides across the lipid bilayer membrane.

**In situ** cross-linking studies demonstrate that on the glycososomal surface, LdPEX14 forms a homo-oligomeric complex that on sucrose density gradients migrates with a density of ~800 kDa. Similar PEX14 oligomeric structures that constitute the importomer complex have also been reported in mammals and yeast (24, 27, 30, 53). Recombinant LdPEX14 expressed in *E. coli* also formed comparable homomeric complex and suggested that oligomerization did not appear to be contingent on accessory proteins or chaperones unique to *Leishmania*. However, it is unclear how these complexes participate in the import of folded proteins across the glycosomal membrane.

Functional domain mapping indicated that the hydrophobic domain, which is predicted to adopt an α-helix configuration (Fig. 2B), is important for stabilizing the LdPEX14 homomeric complex. In LdPEX14, the hydrophobic domain contains centrally located GXXA and SXXS motifs that by molecular modeling are predicted to be on the same face of the α-helix, an architecture that would promote helix-helix packing and oligomerization (63, 64). Indeed, mutagenesis of the analogous GXXXG and AXXXA motifs in the mammalian PEX14 caused disruption of oligomerization (53). In contrast to the mammalian PEX14, where deletion of this hydrophobic segment abrogated oligomerization and resulted in cytosolic targeting of the mutant protein, elimination of the LdPEX14 hydrophobic domain alone did not disrupt glycosomal targeting or homo-oligomerization, although ldpex14-(Δ149–179) was found to form smaller complexes. The association of ldpex14-(Δ149–179) with the glycosome, in contrast to the mammalian PEX14 mutants lacking the analogous hydrophobic domain (52), was not surprising because recent studies demonstrated that residues 1–23 of LdPEX14 are the crucial elements required for glycosomal membrane attachment. Whether ldpex14-(Δ149–179) is biologically functional and capable of mediating protein import into the glycosome is not clear however, because in kin-
etoplasts, PEX14 appears to be essential for parasite viability (9, 25) and generation of Leishmania donovani Δldpex14 mutant cell line required for these studies has been hampered by the absence of RNA interference machinery or tight regulatable expression systems (65). That the hydrophobic domain of LdPEX14 participates in a protein-protein or protein-membrane interaction may be inferred from the capacity of the Ldpex14-(1–200) to form large heterodisperse oligomeric structures that are dependent on residues 149–200. It should be noted that by analytical ultracentrifugation, Ldpex14-(149–170) assembled into structures that were smaller than LdAPRT antisera was generously provided by Dr. B. Ullman of the Oregon Health Sciences University. We thank Dr. Joanne Turnbull of Concordia University for providing access to the spectropolarimeter for the circular dichroism experiments.

Acknowledgments—A pivotal step in the import of PTS1 proteins into the glycosome involves the docking of LdPEX5 to LdPEX14. Thermodynamic studies indicate that this is not a simple bimolecular interaction as four molecules of LdPEX14 bind to one LdPEX5. Complexes isolated from peroxisomes appeared to have comparable PEX5:PEX14 (1:5) binding stoichiometries (46, 47). Because the N terminus of LdPEX14 forms dimers, it is unlikely that LdPEX14 monomers bind to LdPEX5 in a sequential fashion, but rather we argue that the initial association occurs at a LdPEX14 dimer that subsequently recruits a second LdPEX14 dimer to complete the interaction. This contention is supported by the glutaraldehyde cross-linking experiments showing 1:2 (~80 kDa) and 1:4 (~100 kDa) Ldpex5-(203–391): ldpex14-(1–120) species (Fig. 5C). However, the possibility that two LdPEX14 dimers associate to form a complete LdPEX5-binding site cannot be discounted. In contrast to PEX5-PEX14 complexes previously characterized from rat livers that had an apparent molecular mass of 250 kDa (46), the LdPEX5: LdPEX14 structures appear to be substantially larger with an apparent mass of >670 kDa.

Several lines of evidence suggest that LdPEX5 triggers marked conformational changes in LdPEX14. First, analytical ultracentrifugation revealed that binding of LdPEX5 caused a rearrangement of the LdPEX14 leading to the formation of more complex structures. Second, limited proteolysis experiments indicate that binding of LdPEX5 or LdPEX5-PTS1 dramatically impacted the quaternary and tertiary structures of both the soluble recombinant LdPEX14 and native LdPEX14 anchored to the glycosome, a consequence that rendered these proteins highly susceptible to proteolytic degradation. Third, intrinsic fluorescence measurement using the single tryptophan residue, located immediately adjacent to the hydrophobic domain of LdPEX14, confirmed that association with LdPEX5 caused a conformational perturbation that shifted this tryptophan from a nonpolar buried environment to a more polar and solvent-exposed environment. It should be emphasized that in the absence of an LdPEX5-PTS1 complex, LdPEX14 is attached to the glycosomal membrane via an N-terminal domain that requires the first 23 amino acids. Given these structural changes, it is tempting to suggest that docking of the PTS1 receptor leads to the exposure of the hydrophobic domain, which would interact and possibly insert into the glycosomal membrane to form a potential pore-like structure that would facilitate the import of PTS1 proteins into the glycosomal lumen. This speculation is supported by the finding that the hydrophobic domain of mammalian PEX14 is required for membrane insertion (52). Moreover, the proposed architecture LdPEX14 would be consistent with this protein being directly involved in the formation of a potential structure that is analogous to a transient pore that has been advanced for peroxisomal protein translocation (21, 28, 30, 31).

A. 179) assembled into structures that were smaller than LdAPRT antisera was generously provided by Dr. B. Ullman of the Oregon Health Sciences University. We thank Dr. Joanne Turnbull of Concordia University for providing access to the spectropolarimeter for the circular dichroism experiments.

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FIGURE 9. Ldpex14 interacting domains. The diagram illustrates the general architecture of His6/S-Ldpex14 (A), His6/S-Ldpex14(Δ149–170) (B), and His6/S-Ldpex14-(1–200) (C), and the potential interacting domains that mediate homo-oligomerization of this protein. The black arrow represents the N terminus and spans residues 1–23, a region that has been demonstrated to be important for the anchoring of Ldpex14 to the glycosome surface (see Footnote 4). The spheres represent residues 23–75, a region that contains the LdPEX5 binding domain and a dimerization domain. The gray cylinders denote the hydrophobic domain that forms an inter-helix interaction that is stabilized by LdPEX14 oligomerization. The stippled cylinders correspond to the coiled-coil motif that is known to form protein-protein interactions.
