Importance of Phospholipid in the Folding and Conformation of Phosphatidylinositol Transfer Protein: Comparison of Apo and Holo Species†

Paul A. Voziyan, Jacqueline M. Tremblay, Lynwood R. Yarbrough, and George M. Helmkamp, Jr.*

Department of Biochemistry and Molecular Biology, University of Kansas Medical Center, Kansas City, Kansas 66160-7421

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ABSTRACT: The significance of noncovalently bound phospholipid as a structural component of phosphatidylinositol transfer protein (PITP) and its role in acquisition and maintenance of the native conformation of the protein have been addressed by studying the refolding of PITP after exposure to 6 M guanidinium chloride (GdnCl). Protein conformations were characterized by (1) the intrinsic tryptophan fluorescence, circular dichroism, and absorbance spectroscopy, (2) the degree of binding of the fluorescent probe 1,8-ANS, and (3) limited proteolytic digestion. When the GdnCl concentration was reduced 100-fold by rapid dilution at 25 °C, practically all of the native transfer activity was regained within 20 min. Endogenous phospholipid demonstrated a strong interaction with the native PITP. Separation of the phospholipid from the protein by chromatography on a lipophilic matrix was achieved only under denaturing conditions and resulted in spontaneous oxidation of the apo-protein, accompanied by almost complete loss of recoverable transfer activity. Under reducing conditions, however, apo-PITP recovered more than 80% of the native transfer activity and was similar to holo-PITP in the kinetics of phospholipid transfer. Renatured apo-PITP demonstrated a significant relaxation of the tertiary structure, compared to native and renatured holo-PITP. Incubation of apo-PITP with phospholipid vesicles resulted in a more compact protein conformation. We conclude that the polypeptide can spontaneously fold to a native-like conformation, sufficient for interaction with a lipid membrane and acquisition of a phospholipid ligand. Binding of a phospholipid ligand brings about the final adjustments of protein conformation to the more compact native structure.

Phosphatidylinositol transfer protein (PITP[1]) catalyzes the transfer of phospholipids between membranes. While multiple isoforms of PITP have now been identified in several eukaryotic species (Tanaka & Hosaka, 1994; Tanaka *et al.*, 1995), we have confined our investigation to α-PITP. This isoform of PITP is expressed in organisms from yeast to human, and its activity has been found in all mammalian tissues examined [for review, see Helmkamp (1990)]. Although the specific physiological function of PITP is not fully understood, recent observations describe its involvement in several important cellular processes. Thomas *et al.* (1993) and Kauffmann-Zeh *et al*. (1995) have demonstrated a requirement in transmembrane signaling and postulated a specific role of PITP in the PtdIns 4-kinase- and phospholipase C-mediated synthesis of intracellular messengers. Hay and Martin (1993) and Ohashi *et al*. (1995) have suggested a participation in vesicular trafficking, presumably as a priming factor for regulated fusion of secretory granules with the plasma membrane and as a stimulator of the formation of secretory vesicles.

Each molecule of PITP, purified either from mammalian tissues or from recombinant bacterial systems, always contains one molecule of noncovalently bound phospholipid (Helmkamp, 1990; Geijtenbeek *et al*., 1994; Tremblay *et al*., 1996). This endogenous phospholipid can be readily exchanged with other lipids during the process of intermembrane transport. While PtdIns is preferentially bound and transported, PtdCho and, with lower efficiency, PtdGro also interact with PITP (George & Helmkamp, 1985). Hydrophobic forces have been shown to play an important role in accommodating the *sn*-1 and *sn*-2 glycerol fatty acid chains in a lipid binding site (Karusinen *et al.*, 1990). However, it is unclear how the protein distinguishes among structurally diverse phospholipid polar moieties. The binding of one substrate molecule excludes the simultaneous binding of another phospholipid, and the distribution of PITP species separable by isoelectrofocusing is a direct reflection of bound phospholipid and the phospholipid composition of the tissue from which the proteins are isolated (Van Paridon *et al.*, 1987; Venuti & Helmkamp, 1988).

The fact that purified native PITP always contains a bound phospholipid molecule raises a question about the significance of this ligand as a structural component of the protein. In the present study, we employed denaturation techniques, *in vitro* transfer activity measurements, and a number of biophysical approaches to characterize phospholipid−polypeptide interactions and describe conformations of holo- and apo-PITP. Our data demonstrate the importance of phospholipid in the acquisition and maintenance of a native conformation of PITP.

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* Author to whom correspondence should be addressed (telephone, 913-588-6958; fax, 913-588-7440; e-mail, ghelmkam@kumc.edu).

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[1] Abbreviations: PITP, phosphatidylinositol transfer protein; PtdIns, phosphatidylinositol; PtdCho, phosphatidylcholine; PtdGro, phosphatidylglycerol; GdnCl, guanidinium chloride; HAPD, (hydroxyalkoxypropyl)dextran; CD, circular dichroism; λ_{em}max, fluorescence emission maximum wavelength; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); Py-PtdCho, 1-hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphocholine; 1,8-ANS, 1-anilinonaphthalene-8-sulfonic acid.

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MATERIALS AND METHODS

Materials. 1-Acyl-2-[9,10-^{3}H]oleoyl-PtdCho (4.8 Ci/mmol) was synthesized as described earlier (Kasper & Helmkamp, 1981a); cholesteryl[1-^{14}C]oleate (56.6 Ci/mol) was purchased from New England Nuclear (Boston, MA). PtdCho was purified from crude egg PtdCho (Sigma, St. Louis, MO) by column chromatography on Silica Gel G (Welti & Silbert, 1982). GdnCl, >99% pure, was purchased from Fisher Scientific (Pittsburgh, PA). 1,8-ANS and Py-PtdCho were obtained from Molecular Probes (Eugene, OR); DTNB was purchased from Sigma. All other chemicals were the highest quality available.

The α-isoform of rat PITP was expressed in *E. coli* and purified from the bacterial lysate by gel-filtration and ion-exchange chromatography (Tremblay *et al*., 1996). Immunoreactivity and ligand specificity of the recombinant PITP were identical to those of proteins purified from mammalian tissues. Purified recombinant PITP contained an equimolar amount of PtdGro.

Unfolding and Refolding Conditions. To ensure equilibrium conditions, PITP was unfolded by incubation with specific concentrations of GdnCl in 10 mM HEPES, 50 mM NaCl, and 1 mM EDTA, pH 7.4 (buffer A), until λ_{em}max no longer changed (16 h at 4 °C or 2 h at room temperature). Refolding was initiated by a rapid, 100-fold dilution, accomplished by direct mixing of denatured PITP and buffer A; in some instances, 5 mM 2-mercaptoethanol was added to buffer A as a reducing agent. The mixing was followed by incubation at either 4 °C, 25 °C, or 37 °C for various times. In selected experiments, refolded proteins were concentrated in an Amicon apparatus using a PM membrane with a molecular mass cut-off of 10 000 Da.

Determination of PITP Transfer Activity. The transfer activity of PITP was determined by either measuring the rate of ^{3}H-labeled PtdCho transfer from donor vesicles to acceptor vesicles, as described previously (Kasper & Helmkamp, 1981a), or monitoring the intermembrane transfer of the fluorescently labeled phospholipid Py-PtdCho (Van Paridon *et al.*, 1988).

Determination of Lipid:Protein Molar Ratio. The protein content of the samples was determined using a molar extinction coefficient of 79 700 M^{-1} cm^{-1} at 280 nm (Voziyan *et al.*, 1996). Lipid was extracted from the samples into chloroform−methanol according to Bligh and Dyer (1959) and quantified by estimation of the phosphorus content as described by Bartlett (1959).

Column Chromatography, Gel Electrophoresis, and Immunoblotting. Protein samples were chromatographed on a (hydroxyalkoxypropyl)dextran (HAPD) lipophilic matrix (Sigma) that contained alkyl chains 18 carbons in length or on a hydroxylapatite Bio-Gel HT matrix (Bio-Rad, Hercules, CA). For chromatography on hydroxylapatite, samples were eluted with a linear gradient of 10−280 mM sodium phosphate buffer (pH 6.8) containing 0.1 mM EDTA. For unfolded PITP, the HAPD column was equilibrated and eluted with buffer A containing 6 M GdnCl. Elution of the proteins was monitored by measuring the absorbance at 280 nm.

For the experiments on limited proteolysis, protein samples were digested with trypsin at 37 °C for 5 min; the ratio of protease to PITP was 1:26 (w/w). Digestion was stopped by the addition of sample buffer and heating for 10 min at 90 °C. Samples were fractionated by SDS−PAGE and visualized by staining with Coomassie Brilliant Blue. Proteins blotted to nitrocellulose were incubated with polyclonal antibodies either to the central region (residues 95−162) or to the C-terminal region (residues 260−271) of PITP. Protein bands were visualized using a secondary antibody conjugated with alkaline phosphatase as described by Venuti and Helmkamp (1988).

Absorbance Measurements. Second derivatives of absorbance spectra were recorded with an Aminco-3000 array spectrophotometer at 25 °C. Tyrosine solvation changes were documented as described earlier (Voziyan *et al.*, 1996) using the technique developed by Ragone *et al*. (1984).

Reactivity of Sulfhydryl Groups. The reactivity of sulfhydryl groups of PITP species was measured using DTNB (Ellman, 1959) as described earlier (Voziyan *et al.*, 1996). Protein solutions (3.0 μM) were prepared in 10 mM sodium phosphate buffer, pH 7.6. Aqueous DTNB was added to the samples to a final concentration of 480 μM.

Measurements of Fluorescence and Scattered Light. Fluorescence measurements were performed with a Hitachi F-3010 fluorescence spectrophotometer at 25 °C. All spectra were recorded in the range of 300−400 nm with an excitation wavelength of 295 nm. For titration with 1,8-ANS, excitation and emission wavelengths were 350 nm and 465 nm, respectively. Each titration point was separately corrected for the fluorescence of appropriate blanks, dilution, and inner filter effects; dissociation constants were calculated by replotting the data in double-reciprocal coordinates. To estimate the aggregation of the protein, the intensity of the light scattered by the samples was determined using the fluorescent spectrophotometer; for these experiments, excitation and emission monochromators were set at 340 nm with bandpaths of 3 nm.

Circular Dichroism Spectra. CD spectra were recorded in 10 mM sodium phosphate, 50 mM NaCl (pH 7.4) at 25 °C on a computer-driven Jasco J-500A spectropolarimeter. Spectra were recorded from 200 to 250 nm in a 0.2 cm path length cuvette, with subtraction of appropriate blanks.

RESULTS

Effect of GdnCl Concentration on Unfolding and Refolding of Holo-PITP. The unfolding of PITP was studied by incubating the protein with up to 6 M GdnCl. An increase in GdnCl concentration from 0 to 3 M was accompanied by a significant shift in the λ_{em}max from 341 to 351 nm, consistent with increased solvent exposure of tryptophan residues; above 3 M GdnCl, no further changes in λ_{em}max or fluorescence intensity were observed. Denaturation curves had a midpoint at 2.2 ± 0.2 M GdnCl (data not shown). Native PITP exhibited a strong CD spectrum in the range of 200−240 nm; after the protein was treated with 6 M GdnCl, no ordered α-helical or β-sheet structure was detected (data not shown).

Within the narrow range of denaturant concentrations, 1.5−2.5 M GdnCl, PITP failed to regain an active conformation after a rapid, 100-fold dilution at 25 °C (final protein concentration, 320 nM), as shown in Figure 1; these samples exhibited a significant intensity of scattered light at 340 nm (not shown), indicative of protein aggregation. Transfer activity could only be recovered in those samples subjected to higher concentrations of denaturant, with a maximum

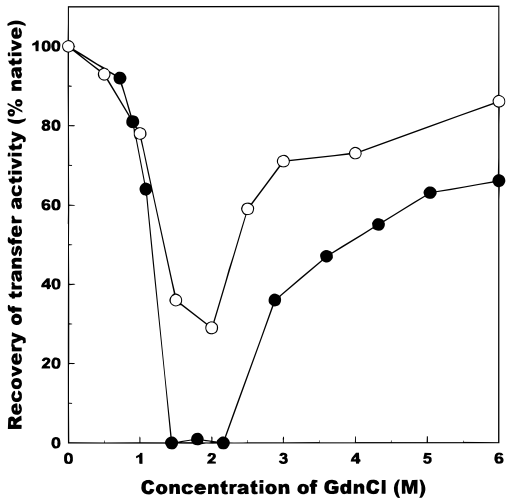

FIGURE 1: Refolding of PITP after denaturation in different concentrations of GdnCl. Samples were incubated with the indicated concentrations of GdnCl at 4 °C for 16 h and were then rapidly diluted 100-fold into 10 mM HEPES, 1 mM EDTA (pH 7.4) to the final protein concentrations of 80 nM (○) or 320 nM (●). After 1 h at 25 °C, lipid transfer activity was determined in a vesicle−vesicle transfer assay using 1-acyl-2-[9,10-^{3}H]oleoyl-PtdCho as a substrate. Each point represents the average of three independent measurements; SD did not exceed 15% of the mean.

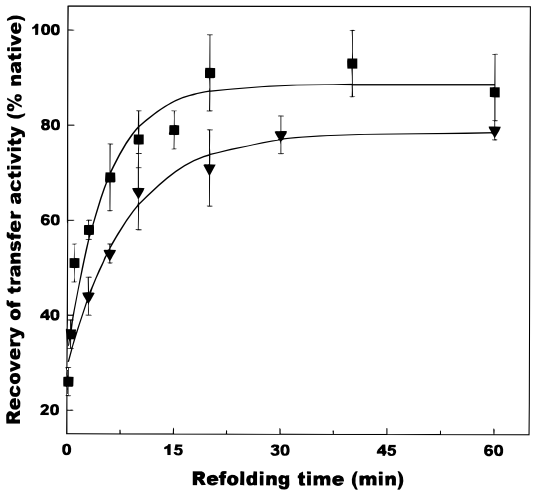

FIGURE 2: Kinetics of refolding of holo- and apo-PITP. Samples of holo-PITP (■) or apo-PITP (▼) denatured in 6 M GdnCl were rapidly diluted into 10 mM HEPES (pH 7.4), 1 mM EDTA, and 5 mM 2-mercaptoethanol to the final protein concentration of 80 nM. Samples were incubated at 25 °C for the indicated times, and their transfer activity toward PtdCho was determined in a vesicle−vesicle transfer assay. Each bar represents the mean ± SD of three incubations.

recovery after treatment with 6 M GdnCl (Figure 1). Under these conditions, sample light scattering was significantly lower (not shown). By decreasing the final PITP concentration to 80 nM during the refolding stage, a dramatically increased yield of active protein was obtained. However, the shape of the refolding profile appears to be independent of the final protein concentration (Figure 1).

Temperature, like denaturant concentration, was also a critical variable in the refolding of denatured PITP. Protein unfolded in 6 M GdnCl regained full transfer activity after approximately 20 min of incubation in refolding buffer at 25 °C (Figure 2). At 4 °C, the kinetics of refolding were significantly slower, although full activity was recovered after about 16 h (data not shown). When refolding was performed at 37 °C, the maximum recovery of activity did not exceed 40%, even after 16 h (not shown). The decrease in recoverable activity at 37 °C most likely is caused by increased hydrophobic interactions and aggregation of (partially) folded protein molecules (Rudolph *et al*., 1979).

Table 1: Phospholipid Content of Native and Denatured PITP [a]

sample	phospholipid/protein (mol/mol)
native PITP	
before chromatography	0.95 ± 0.10
after chromatography on lipophilic matrix	0.76 ± 0.06
denatured PITP	
before chromatography	0.92 ± 0.08
after chromatography on lipophilic matrix	0.04 ± 0.01

[a] Native protein or protein denatured in 6 M GdnCl was subjected to chromatography on a lipophilic matrix. For the native protein, the elution buffer was 10 mM HEPES, 1 mM EDTA (pH 7.4); for the unfolded protein, the buffer contained 6 M GdnCl. Phospholipid and protein content of the samples was determined as described under Materials and Methods. Data in this and other tables represent the mean ± SD of three determinations.

Phospholipid−Protein Interactions and Formation of Apo-PITP. Having described the denaturation and renaturation of PITP, we wished to define the interactions between the polypeptide and the noncovalently bound phospholipid in those conformational states. Accordingly, we employed column chromatography on lipophilic and hydroxyapatite matrices. The lipid:protein molar ratio of native protein (holo-PITP) equaled unity. After chromatography on a lipophilic matrix (Table 1), there was a loss of approximately 25% of the endogenous phospholipid. Chromatography of the unfolded protein on the same matrix removed virtually all the lipid, thereby yielding apo-PITP (Table 1). These data posed the question of whether phospholipid and polypeptide have dissociated under denaturing conditions, prior to their separation by chromatography. In order to address this question, we had, in preliminary experiments, established conditions of chromatography on hydroxylapatite that were sufficient to remove completely up to a 70-fold molar excess of exogenous phospholipid (PtdCho vesicles) from native PITP preparations without removing the protein-bound endogenous lipid. These conditions were then applied to the experiments with the purified holo-protein. It is noteworthy that the concentration of endogenous phospholipid after refolding in these experiments (80 nM) was approximately 100 times higher than its critical micelle concentration (Tanford, 1973). Therefore, phospholipid molecules that dissociate from denatured protein would most likely form vesicles upon dilution into refolding buffer, and these vesicles would then be removed by hydroxylapatite chromatography. Holo-PITP was denatured in 6 M GdnCl for 16 h, refolded, and subjected to hydroxylapatite chromatography. After chromatography, the lipid:protein molar ratio for refolded holo-PITP equaled 0.94 ± 0.14, the value identical to native protein. Thus, denaturation alone did not cause any loss in PITP-bound endogenous phospholipid.

Cysteine Oxidation in Denatured Holo- and Apo-PITP and Recovery of Transfer Activity upon Refolding. All four Cys residues in holo-PITP, following denaturation in 6 M GdnCl, were resistant to oxidation when incubated at 4 °C for up to 72 h (Table 2). No significant decrease in the number of Cys residues reactive with DTNB was detected under these conditions, even after 10 days of incubation at 4 °C (not

Table 2: Cysteine Oxidation and Recovery of Transfer Activity in Holo- and Apo-PITP[a]

incubation time (h)	no. of reactive cysteine residues		additions to refolding buffer	recovery of transfer activity upon refolding (% native)	
	holo-PITP	apo-PITP		holo-PITP	apo-PITP
1.5	4.3	3.5	−	106 ± 1	86 ± 5
115	4.3	1.8	−	86 ± 2	3 ± 1
115	−	−	2-mercaptoethanol	85 ± 6	86 ± 9

[a] PITP was denatured or denatured and delipidated as described under Materials and Methods. Holo-PITP and apo-PITP were incubated in 6 M GdnCl at 4 °C for the indicated times. Incubation started immediately after elution of apo-PITP from the HAPD column. At the end of each incubation time, aliquots were taken to determine the number of cysteine residues reactive with DTNB as described under Materials and Methods. Other aliquots were refolded by rapid, 100-fold dilution of the samples into either 10 mM HEPES buffer or 10 mM HEPES buffer containing 5 mM 2-mercaptoethanol, followed by incubation at 25 °C for 60 min. Transfer activity was measured in a vesicle−vesicle transfer assay.

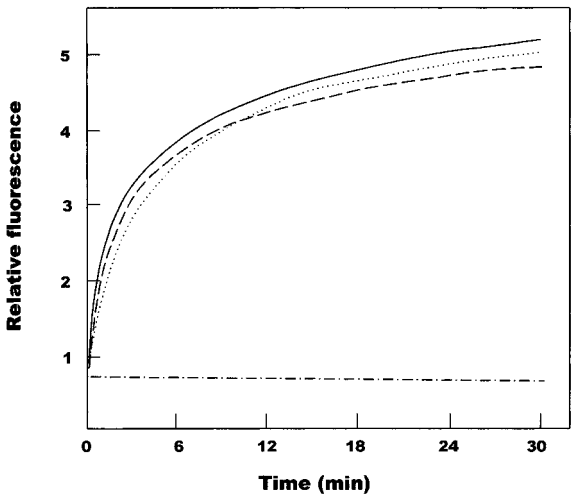

FIGURE 3: Transfer of fluorescent phospholipid mediated by native or renatured PITP. Kinetics of PITP-mediated lipid transfer from 0.5 μM Py-PtdCho donor vesicles to 500 μM PtdCho acceptor vesicles were measured at 25 °C. Donor and acceptor vesicles were mixed in a fluorescent cuvette, containing 10 mM HEPES, 50 mM NaCl, pH 7.4. After recording of the base line (dot−dash line), transfer was initiated by addition of 1 μg of either native PITP (solid line), renatured holo-PITP (dashed line), or renatured apo-PITP (dotted line). Exitation and emission wavelengths were 342 nm and 378 nm, respectively.

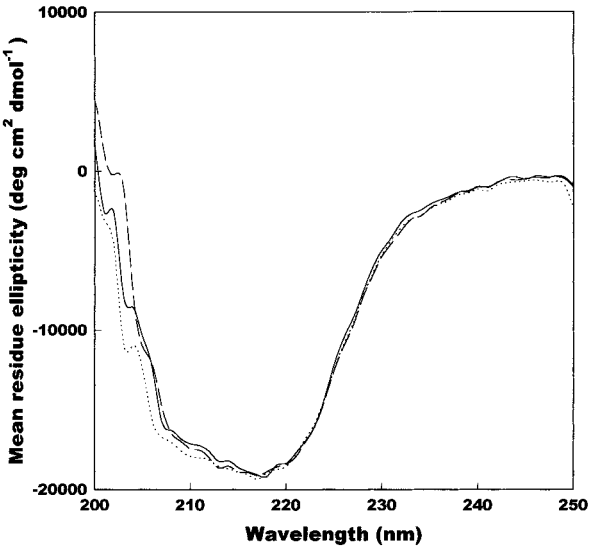

FIGURE 4: CD spectra of different PITP species. The CD spectra of native PITP (solid line), renatured holo-PITP (dashed line), and renatured apo-PITP (dotted line) were measured at a concentration of 0.1 mg/mL in 10 mM sodium phosphate, pH 7.5, in a 0.2-cm light path cell at 25 °C. Each spectrum represents an average of five scans.

shown). In contrast, denatured apo-PITP demonstrated relatively rapid formation of cystine; in 72 h, half the Cys residues were no longer titratable with DTNB. This partially oxidized preparation exhibited no recoverable transfer activity upon refolding (Table 2). That there was some formation of both intra- and intermolecular disulfide bonds was demonstrated by SDS−PAGE under nonreducing conditions (data not shown); a ladder of monomers, dimers, trimers, and higher multimers was observed. When refolding of oxidized apo-PITP was performed under disulfide reducing conditions, transfer activity was fully restored (Table 2). The refolding kinetics were similar to those of holo-PITP (Figure 2). SDS−PAGE analysis of this sample indicated a single protein band, corresponding to the molecular size of native PITP (not shown).

Transfer Activity and Conformational Analysis of Renatured Holo- and Apo-PITP. In order to characterize conformational changes in PITP brought about by the removal of endogenous phospholipid, holo-PITP and apo-PITP were refolded under reducing conditions and concentrated to the amounts sufficient for functional and structural studies. The kinetics of Py-PtdCho transfer by native and renatured holo- and apo-PITP were similar (Figure 3). These data indicate that the capability of apo-PITP to interact with the phospholipid membrane and to acquire and transfer phospholipid ligand is comparable to that of native and renatured holo-PITP.

CD spectra of both renatured holo- and apo-PITP species in the spectral region of 200−250 nm were similar and did not differ from the CD spectrum of native PITP, indicating the absence of detectable differences in their secondary structures (Figure 4).

Binding of 1,8-ANS, a fluorescent probe that interacts noncovalently with both polar and nonpolar regions of the proteins (Lakowicz, 1983), was increased in renatured protein species compared to native protein, with apo-PITP demonstrating the highest binding capacity for the probe (Figure 5). Analysis of the binding of 1,8-ANS by native PITP yielded a K_d of 36 μM; interaction of the probe with the refolded protein species had lower affinities, 53 μM and 98 μM for holo-PITP and apo-PITP, respectively.

We also characterized the tertiary structures of PITP species more specifically by studying the solvent exposure of Tyr residues. Solvation changes were determined from the second derivatives of the protein absorbance spectra (Ragone *et al.*, 1984). Renatured apo-PITP had a noticeably higher exposure of Tyr residues: 7−8 out of total of 13 tyrosines were accessible to solvent compared to only 5−6 Tyr residues in both native and renatured holo-PITP (Table 3). Interestingly, for apo-PITP in the presence of phospholipid vesicles, this number decreased and shifted toward the values determined for the protein species with bound

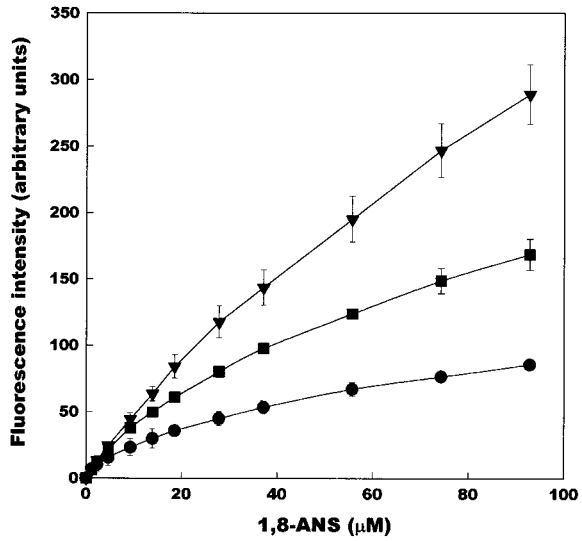

FIGURE 5: Fluorescence titration of PITP species with 1,8-ANS. Titrations of the proteins with the indicated amounts of 1,8-ANS were performed in 10 mM sodium phosphate, pH 7.5 at 25 °C. Samples were excited at 350 nm, and emission was measured at 465 nm. Protein concentration was 3.0 μM. Each point represents the mean ±SD of three experiments. (●) Native PITP; (■) renatured holo-PITP; (▼) renatured apo-PITP.

Table 3: Solvent Exposure of Tyrosine in PITP Species[a]

samples	no. of Tyr residues exposed to solvent	
	without vesicles	with 0.5 mM PtdCho vesicles
native PITP	5.09 ± 0.02	5.18 ± 0.03
renatured holo-PITP	5.62 ± 0.14	5.78 ± 0.06
renatured apo-PITP	7.61 ± 0.09	6.83±0.04

[a] Protein samples (3 μM) were incubated either in the absence of vesicles or in the presence of 0.5 mM PtdCho vesicles for 5 min at 25 °C. Number of Tyr residues exposed to solvent was determined from second-derivative spectra as described under Materials and Methods. Data represent an average of at least four measurements.

phospholipid (Table 3).

As a further characterization of holo- and apo-PITP, we examined the accessibility of Lys and/or Arg residues to tryptic digestion. Under our experimental conditions, both native and renatured holo-PITP underwent very little digestion, and the addition of PtdCho vesicles to the digestion system only slightly increased the extent of proteolysis (Figure 6A, lanes 1−6). In contrast, a majority of renatured apo-PITP was cleaved by trypsin even in the absence of vesicles, generating a lower molecular weight digestion product (Figure 6A, lanes 7 and 8). To determine the approximate location of the trypsin cleavage site(s), undigested and digested apo-PITPs were blotted to nitrocellulose and incubated with antibodies to the central region (residues 95−162) of PITP (Figure 6B) or with antibodies to the C-terminal region (residues 260−171) of PITP (Figure 6C). No reaction of the digestion product with the antibodies to the C-terminus was detected. The molecular size of the digestion product and its differential reactivity with antibodies suggest that the trypsin cleavage sites are most likely located at Arg-259 and Arg-253 (Tremblay *et al*., 1996). In the presence of PtdCho vesicles, digestion of apo-PITP was noticeably reduced (Figure 6A, lane 9). In a control experiment (not shown), vesicles had no effect on the activity of the protease itself. Moreover, irreversible binding of a significant fraction of apo-PITP to phospholipid vesicles can

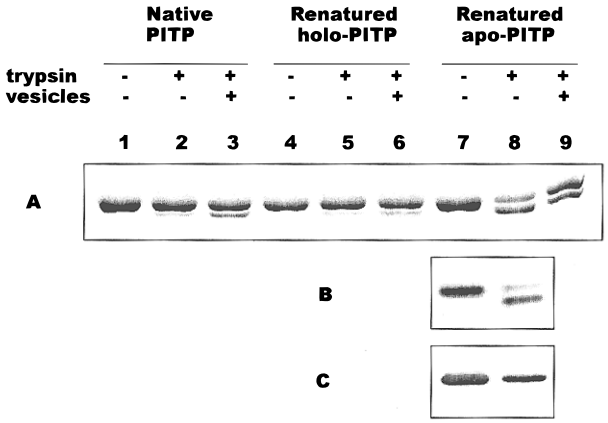

FIGURE 6: Digestion of PITP species by trypsin. PITP was digested at 37 °C for 5 min with trypsin (26:1, w/w); in some instances, prior to digestion, samples were incubated in the presence of 0.5 mM PtdCho vesicles for 5 min. Digestion was stopped by the addition of sample buffer and heating for 10 min at 90 °C. Samples were fractionated on 10% SDS−PAGE. (A) Coomassie Brilliant Blue staining. Digestion conditions for native PITP, renatured holo-PITP, and renatured apo-PITP, respectively, were as follows: lanes 1, 4, and 7, undigested controls; lanes 2, 5, and 8, trypsin digestion without vesicles; lanes 3, 6, and 9, trypsin digestion in the presence of 0.5 mM PtdCho vesicles. (B) Immunoblot with antibody to the central region of PITP of the samples treated similarly to the samples in lanes 7 and 8. (C) Immunoblot of the same samples with antibody to the C-terminus of PITP.

be excluded because the kinetics of lipid transfer were no different from those of holo-PITP (Figure 3). We conclude, therefore, that the observed decrease in digestion of apo-PITP in the presence of vesicles represents an enhanced resistance of the protein to proteolysis after it has acquired a phospholipid molecule and achieved a more compact conformation.

DISCUSSION

Phospholipid−Protein Interactions of Native and Denatured PITP. The association between endogenous phospholipid ligand and PITP has been investigated using denaturation techniques and physical and chemical characterization. That the endogenous phospholipid could not be readily removed from native PITP, at least by chromatography on lipophilic matrix, was not an unexpected result. Indeed, the hydrophobic portion of the lipid binding site should be well sequestered from the surface of the cytosolic protein (Karusinen *et al.*, 1990), thereby preventing phospholipid acyl chains from interaction with a lipophilic matrix. More surprising, however, was our finding suggesting that the lipid−protein complex did not dissociate after unfolding of the polypeptide. It is supported, first of all, by the very efficient refolding of PITP denatured in 6 M GdnCl, with the full retention of endogenous phospholipid. If dissociated from the protein, phospholipid would have to reassociate with almost 100% efficiency during refolding in order to achieve the observed lipid−protein stoichiometry. We consider this an unlikely event at equimolar concentrations. That the phospholipid remained bound to the unfolded protein is more persuasively supported by the observation that the rate of oxidation of protein sulfhydryl groups under denaturing conditions was much greater for apo-PITP than for holo-PITP. These findings reveal a paradoxical relationship between phospholipid and protein. As we and many other groups have demonstrated, PITP interacts with a variety of

membrane surfaces and readily exchanges its polypeptide-bound (endogenous) phospholipid ligand with a membrane phospholipid. That this one-for-one exchange is the mechanism of PITP-facilitated intermembrane transfer was shown in earlier experiments (Kasper & Helmkamp, 1981b). Yet it is this same endogenous phospholipid ligand that is strongly bound to native PITP and remains associated with the unfolded protein, even at high concentration of denaturant. The specific nature of lipid−polypeptide interactions in both native and unfolded PITP remains to be elucidated. Interestingly, nonrandom local interactions that involve only a few amino acid residues have been described for a number of unfolded proteins (Neri *et al.*, 1992; Fersht, 1995). These interactions exist at high concentrations of denaturants and most likely involve alkyl and aromatic side chains of amino acids (Kemmink & Creighton, 1995). In the case of holo-PITP, phospholipid acyl chains may participate in and even stabilize such interactions.

Folding and Conformation of Apo- and Holo-PITP. Holo-PITP denatured in 6 M GdnCl was capable of spontaneous, nonassisted refolding to an active conformation, a process that was sensitive to temperature and protein concentration. The completion of the unfolding transition was also a critical parameter for efficient refolding. It appears that the polypeptide chain of PITP requires a certain degree of conformational freedom to enter a folding pathway and will otherwise misfold and aggregate if this freedom is limited by partially denatured states. This denaturant-dependent formation of aggregates in the range of unfolding transition has been observed for a number of proteins (Jaenicke, 1978). The tertiary structure of refolded holo-PITP was similar, but not identical, to that of native PITP. Very little change was observed in Tyr solvent exposure, and no differences in tryptic digestion were detectable between native and refolded holo-PITP. On the other hand, refolded holo-PITP exhibited a notable increase in hydrophobic and/or charged regions capable of binding 1,8-ANS compared to the native protein. The cause of the observed differences in the structures of native and renatured holo-PITP is unclear. One possible explanation for this phenomenon is that the refolding, at least under our experimental conditions, is a multistep process and may require additional factors (for example, interaction with the membrane) for completion. Alternatively, holo-PITP may acquire multiple, thermodynamically stable conformations upon refolding, each of which possesses comparable transfer activity. Our data suggest that the recovery of the catalytic activity, a criterion that is often used in studies of polypeptide folding, may not always be sufficient to characterize the completion of the refolding process.

Under reducing conditions, the refolding of apo-PITP to an active conformation was essentially as efficient as that of the holo-protein, indicating that this process does not require prior association of the polypeptide with a phospholipid ligand. Although apo-PITP regained practically all native secondary structure upon refolding, its tertiary structure was substantially more relaxed than those of native and renatured holo-proteins. This conformational relaxation has been specifically characterized by the solvent exposure of two additional Tyr residues and by the increased exposure and/or flexibility of the C-terminal region of the protein. Further refinement of the conformation to yield a more compact tertiary fold occurred only upon binding of the phospholipid. There have been other reports of lipid-induced conformational relaxation in protein structure. An increased proteolytic digestion in the absence of ligand was reported for cellular retinoid binding proteins (Jamison *et al.*, 1994). X-ray crystallographic analysis of cellular retinoic acid binding apo-protein demonstrated a more opened backbone conformation compared to the holo-protein (Thompson *et al*., 1995). More recently, an increased backbone disorder in the absence of bound ligand, as determined by using triple-resonance 3-D NMR spectroscopy, was reported for intestinal fatty acid binding protein (Hodsdon & Cistola, 1997).

Membrane−Protein Interactions of Apo- and Holo-PITP. Interaction of PITP with a phospholipid membrane is a critical, and possibly, rate-limiting step in protein-facilitated lipid transfer (Somerharju *et al.*, 1981). Structural modifications to the protein result in often dramatic changes in membrane affinity and phospholipid transfer. Our data suggest that interaction of native PITP with a membrane induces an efficient release and exchange of an endogenous phospholipid molecule that otherwise is strongly bound to the protein. Small increases in Tyr exposure and proteolytic digestion in native and renatured holo-PITP in the presence of phospholipid vesicles (Table 3 and Figure 6A, lanes 3 and 6) may be indicative of protein structural relaxation during the membrane binding and lipid exchange events. Interaction of apo- and holo-proteins with a membrane appears to be very similar, at least by the criterion of the kinetics of phospholipid transfer. A productive interaction of apo-PITP with a membrane has a special significance, since it is likely that *in vivo* the nascent polypeptide binds to a membrane and acquires a phospholipid molecule early after synthesis and initial folding in order to achieve a compact, protease-resistant native conformation. On this point, it is noteworthy that recombinant PITP, expressed in the bacterium *E. coli*, acquires PtdGro, a poorly transferred phospholipid (George & Helmkamp, 1985) but the only potential ligand available in this organism (Geijtenbeek *et al*., 1994; Tremblay *et al*., 1996). Association of this ligand with a fully active protein provides an additional illustration of the important role of phospholipid in the overall folding and structure of PITP.

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