Truncations of the C-Terminus Have Different Effects on the Conformation and Activity of Phosphatidylinositol Transfer Protein†

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ABSTRACT: Contributions of the C-terminus toward the conformation and activity of phosphatidylinositol transfer protein (PITP) were studied by comparing properties of the 271 amino acid, full-length protein, PITP(1−271), and two truncated species, PITP(1−259) and PITP(1−253). Using recombinant proteins and an *in vitro* phospholipid transfer assay with phosphatidylcholine vesicles, the activities of PITP(1−271) and PITP(1−259) were identical, while the activity of PITP(1−253) was almost totally abolished. By most physical and chemical criteria, however, PITP(1−259) and PITP(1−253) were virtually indistinguishable and differed significantly from the full-length protein. Results of second derivative analysis of absorbance spectra were consistent with an additional two Tyr residues being exposed to the solvent in PITP(1−259) and PITP(1−253) in comparison to PITP(1−271). Only one out of four Cys residues in PITP(1−271) reacted with dithiobisnitrobenzoic acid, while two Cys residues were accessible in both truncated species. Quenching of intrinsic Trp fluorescence by acrylamide demonstrated an increase in exposure of Trp residues in both PITP(1−259) and PITP(1−253); binding of the fluorescence probe 1,8-ANS to these proteins was also significantly higher compared to PITP(1−271). These results describe a more relaxed overall tertiary structure brought about by the C-terminal truncations. This altered structure did not affect the stability of the truncated proteins, as indicated by equilibrium unfolding in guanidinium chloride. Refolding of the denatured PITP(1−259), however, was considerably slower than that of full-length PITP. Our study suggests a critical role of the C-terminal residues 254−259 in transfer activity of PITP. Residues 260−271, on the other hand, appear to be more important for the rapid folding and maintenance of a compact native conformation of the protein.

Phosphatidylinositol transfer protein (PITP)[1] was first purified from bovine brain and characterized by its ability to transfer PtdIns and, to the lesser extent, PtdCho between lipid membranes *in vitro* (Helmkamp et al., 1974). Further studies have demonstrated that PITP is distributed in various species; in mammals its activity has been found in all tissues examined [for review, see Helmkamp (1990)]. The complete primary structures of mammalian PITPs consist of 270−271 amino acid residues (Dickeson et al., 1989, 1994; Geijtenbeek et al., 1994) and are highly conserved.

Several recent studies reveal important cellular functions of mammalian PITP. The protein appears to be required for inositol lipid signaling (Thomas et al., 1993; Kauffmann-Zeh et al., 1995) and vesicle-mediated secretion (Hay & Martin, 1993); a structural isoform of PITP, β-PITP, was recently identified in rat (Tanaka & Nosaka, 1994) and suggested to play a possible role in cellular metabolism of sphingomyelin (de Vries et al., 1995). Very little, however, is known about the protein's molecular structure. In the absence of X-ray crystallography or NMR data, only indirect information about PITP structure is currently available. Using fluorescently labeled phospholipids, Van Paridon et al. (1988) and Karusinen et al. (1990) have demonstrated that hydrophobic forces play an important role in the noncovalent binding of a single phospholipid ligand molecule to the protein and that *sn*-1 and *sn*-2 glycerol fatty acyl chains of bound ligand are accommodated in distinct microenvironments of protein structure. Utilizing random mutagenesis and activity screening in mutant yeast system, Alb et al. (1995) have shown that the Ser-25, Thr-59, Pro-78, and Glu-248 residues participate in the specificity of the rat protein toward PtdIns. With the exception of this fragmentary information, however, the structural organization of PITP and mechanism of its transfer activity remain unknown.

Recently we have observed that recombinant rat PITP, which is not easily cleaved by trypsin in solution, becomes susceptible to tryptic digestion at Arg-253 and Arg-259 when PITP is bound to anionic phospholipid vesicles (Tremblay et al., 1996). The current paper describes structural and functional properties of the full-length protein, PITP(1−271), and its truncated mutants, PITP(1−253) and PITP(1−259), and presents evidence that the C-terminal amino acid sequences, 254−259 and 260−271, have different effects on polypeptide conformation and transfer activity.

MATERIALS AND METHODS

Materials. [9,10-^{3}H-oleoyl]PtdCho (4.8 Ci/mmol) was synthesized as described earlier (Kasper & Helmkamp, 1981); 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

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[1] Abbreviations: PtdIns, phosphatidylinositol; PtdCho, phosphatidylcholine; GdnCl, guanidinium chloride; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); CD, circular dichroism; λ_{em}^{max}, fluorescence emission maximum wavelength; 1,8-ANS, 1-anilinonaphthalene-8-sulfonic acid.

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column chromatography on Silica Gel G (Welti & Silbert, 1982). GdnCl and acrylamide, both >99% pure, were purchased from Fisher Scientific (Pittsburgh, PA). 1,8-ANS was obtained from Molecular Probes (Eugene, OR); DTNB was purchased from Sigma. All other chemicals were the highest quality available.

Full-length rat PITP and PITP species truncated at Arg-259 or Arg-253 were expressed in *Escherichia coli* and purified from the bacterial lysate by gel-filtration and ion-exchange chromatography (Tremblay et al., 1996). Deletions of the DNA bases corresponding to residues 260−271 and 254−271 were verified by DNA sequencing using the dideoxy method.

Unfolding and Refolding Conditions. To ensure equilibrium conditions, PITP(1−271) and PITP(1−259) were unfolded by treatment with 6 M 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. This mixing was followed by incubation at 25 °C for various times.

Determination of PITP Transfer Activity. Transfer activity of full-length PITP and its truncated forms was determined by measuring the rate of ^{3}H-labeled PtdCho transfer from PtdCho donor unilamellar vesicles to PtdCho acceptor unilamellar vesicles, as described previously (Kasper & Helmkamp, 1981); assays were performed at 25 °C.

Determination of Lipid/Protein Molar Ratio. Determination of protein content of the samples was based on the measurements of absorbance at 280 nm. The molar extinction coefficient of 79 700 $M^{-1}cm^{-1}$ was calculated from the results of amino acid analysis of PITP(1−271). Molar extinctions of the full-length and both truncated proteins measured by Edelhoch method (Gill & von Hippel, 1989) were similar within experimental error, confirming our assumption that the molar extinction of PITP is not significantly altered by the truncations. PITP contains no aromatic residues in the sequence region 253−271. Lipid was extracted from protein samples into chloroform−methanol according to Bligh and Dyer (1959) and quantified by estimation of phosphorus content as described by Bartlett (1959).

Absorbance Measurements. Second derivatives of absorbance spectra were recorded with an Aminco-3000 Array spectrophotometer at 25 °C. Tyrosine solvation changes were documented using the technique described by Ragone et al. (1984).

Fluorescence Measurements. Steady-state fluorescence measurements were performed with an Hitachi F-3010 fluorescence spectrophotometer at 25 °C. For proteins unfolded in 6 M GdnCl and for titration with acrylamide or KI, spectra were recorded in the range of 300−400 nm with excitation wavelengths of 295 nm; both excitation and emission bandpaths were 5 nm. For titration with 1,8-ANS, excitation and emission wavelengths were 350 and 465 nm, respectively. Each titration point was separately corrected for the fluorescence of appropriate blanks, dilution, and inner filter effects. For titration with KI, the total halide concentration of the samples was adjusted to 200 mM with NaCl.

Kinetics of DTNB Reaction. The reactivity of sulfhydryl groups of PITP species was measured using DTNB (Ellman, 1959). Protein solutions (3.9 μM) were prepared in 10 mM sodium phosphate buffer, pH 7.6. Aqueous DTNB was added to the protein solution to a final concentration of 400 μM. The kinetics were followed by recording the absorbance at 412 nm every 10 s for 30 min at 25 °C. The data were corrected for the contribution of the same concentration of DTNB in buffer. The number of sulfhydryl groups was calculated from absorbance data using a molar extinction coefficient of 13 600 M^{-1} cm^{-1} for nitrothiobenzoate (Ellman, 1959).

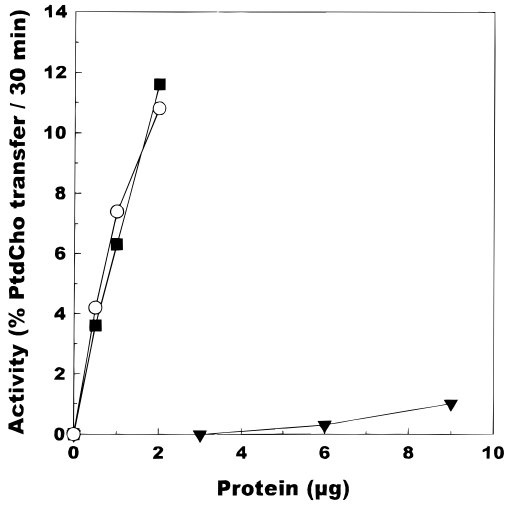

FIGURE 1: Transfer activity of full-length and truncated PITPs. Transfer activity of PITP(1−271) (○), PITP(1−259) (■), and PITP-(1−253) (▼) was determined by measuring protein-enhanced transfer of [^{3}H]PtdCho from donor to acceptor vesicles as described in Materials and Methods. Indicated amounts of proteins were incubated with vesicles at 25 °C for 30 min. Each point represents the average of three determinations.

Circular Dichroism Spectra. CD spectra were recorded in 10 mM sodium phosphate/150 mM NaCl (pH 7.5) at 25 °C on a computer-driven Jasco J-500A spectropolarimeter. Spectra were recorded from 190 to 250 nm in a 0.2 cm path length cuvette, with subtraction of appropriate blanks.

RESULTS

Lipid Transfer Activity of Native and Truncated PITP Species. Relative transfer activities of full-length and truncated proteins toward PtdCho were determined in vesicle−vesicle transfer assays. No difference in activity between PITP(1−271) and PITP(1−259) was detected after incubation for 30 min at 25 °C (Figure 1). Transfer activity of PITP(1−253), in contrast, was almost totally abolished.

Lipid/Protein Molar Ratio. The removal of up to 18 amino acid residues from the C-terminus did not affect the stoichiometry of phospholipid binding as determined by estimation of phosphorus content of the samples following lipid extraction. Lipid/protein molar ratios were close to unity: 0.98 ± 0.10 for the full-length PITP, 1.06 ± 0.16 for PITP(1−259), and 0.99 ± 0.19 for PITP(1−253).

Conformation and Folding of Full-Length and Truncated PITP Species. (*A) Secondary Structure.* CD spectra were measured to determine if the C-terminus truncation significantly altered the secondary structure of PITP. The CD spectra of PITP species in far-UV range reflected a significant content of ordered secondary structure (Figure 2). Comparison of the spectra suggests modest conformational differences between full-length protein and truncated species

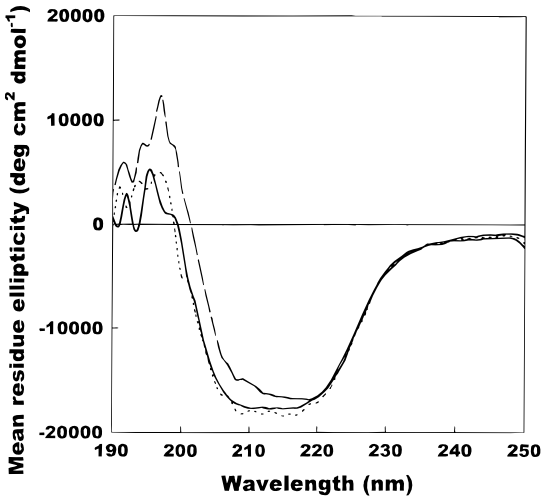

FIGURE 2: CD spectra of full-length and truncated PITPs. The CD spectra of native PITP (dashed line), PITP(1−259) (solid line), and PITP(1−253) (dotted line), were measured at a concentration of 0.13 mg/mL in 10 mM sodium phosphate, pH 7.5, in a 0.2-cm light path cell at 25 °C. Each spectrum was an average of three scans. The experiment was repeated with essentially the same results.

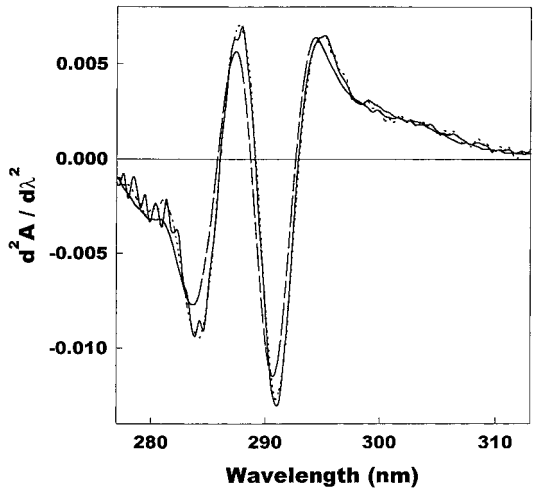

FIGURE 3: Second derivative spectra of full-length and truncated PITPs. The second derivative spectra of native PITP (dashed line), PITP(1−259) (solid line), and PITP (1−253), (dotted line) were measured at a concentration of 0.3 mg/mL in 10 mM sodium phosphate, pH 7.5, at 25 °C. At least three independent measurements were carried out for each sample; spectral parameters were determined and used for the calculation of tyrosine exposure (see Results for details). Typical scans in a single experiment are presented.

(Figure 2). The spectra of PITP(1−259) and PITP(1−253), on the other hand, were practically identical (Figure 2), indicating the absence of detectable changes in secondary structure between the two truncated protein species. Analysis of the CD spectra, according to Yang *et al.* (1986), yielded approximately 40% α-helix in PITP(1−271) and approximately 30% α-helix in both truncated proteins. Essentially no β-form was calculated for all three PITP species.

(*B) Exposure of Tyrosine Residues.* We employed second derivative analysis of the proteins' absorbance spectra to analyze relative solvent exposure of Tyr residues. While no spectral difference between PITP(1−259) and PITP(1−253) could be detected, a substantial difference was observed between the PITP(1−271) and the two truncated species (Figure 3). Ragone et al. (1984) have demonstrated that the ratio, *r*, of the two peak-to-trough second derivative distances in the spectral region of 280−295 nm is particularly sensitive to the degree of solvation of Tyr residues and can be used to calculate the number of the solvent-exposed Tyr residues. We estimated that six of the 13 Tyr residues in PITP(1−271) were accessible to the solvent. For both PITP(1−259) and PITP(1−253), an additional two Tyr residues became exposed (Table 1). PITP(1−271) denatured in 6 M GdnCl served as the standard for fully accessible Tyr residues. PITP contains no Tyr residues in the sequence region 253−271.

(*C) Reactivity of Cysteine Residues.* Full-length PITP and the truncated protein species contain four Cys residues at sequence positions 95, 188, 192, and 231. In PITP(1−271) denatured in 6 M GdnCl, four Cys residues reacted with DTNB (Table 1), indicating that all sulfhydryl groups of the protein were in reduced form and that no disulfide bonds were present in the native structure. In PITP(1−271) under nondenaturing conditions, only one Cys residue could be titrated with DTNB (Table 1), suggesting that the remaining Cys residues are buried in hydrophobic regions of the protein molecule and thus inaccessible to the water-soluble DTNB. In PITP(1−259) and PITP(1−253) the number of reactive sulfhydryl groups approached two, and the rates of DTNB reaction were distinctively faster compared to full-length protein (Table 1 and Figure 4). In these measurements, no significant difference between the truncated species could be established. As with full-length PITP, all four Cys residues in each of the truncated species reacted with DTNB after denaturation in 6 M GdnCl (data not shown).

Table 1: Solvent Exposure of Tyrosine and Cysteine Residues in Full-Length and Truncated PITPs

protein species	r[a]	number of Tyr exposed to solvent[b]	maximum absorbance at 412 nm[c]	number of Cys exposed to solvent[d]
PITP(1−271)	0.75 ± 0.01	6.0	0.048 ± 0.003	1.0
PITP(1−259)	0.85 ± 0.01	7.7	0.088 ± 0.012	1.8
PITP(1−253)	0.87 ± 0.01	7.9	0.081 ± 0.006	1.6
denatured PITP(1−271)	1.20 ± 0.02	(13)	0.206 ± 0.018	4.1

[a] *r* represents a ratio of peak-to-trough distances in second derivative spectra in Figure 3. [b] Calculated with the assumption that all 13 tyrosine residues in PITP(1−271) denatured in 6 M GdnCl are exposed to solvent. This assumption is based on our observation that denatured PITP(1−271) and a model mixture with the same Tyr/Trp molar ratio (13/8) demonstrated identical solvent exposure of tyrosine residues. [c] Calculated by extrapolation of the data in Figure 4 to infinite time. [d] Calculated from the maximum absorbance data using a molar extinction coefficient of 13 600 $M^{-1}cm^{-1}$.

(*D) Exposure of Tryptophan Residues.* Full-length PITP and both truncated species contain eight Trp residues. To determine if the truncations altered the solvent exposure of these residues, quenching of protein Trp fluorescence by acrylamide and KI was measured. In both truncated species, Trp residues were more accessible to acrylamide than in full-length PITP (Figure 5). Acrylamide quenching constants, characteristic of quenching efficiency, were calculated from the slopes of Stern−Volmer plots by linear regression analysis (Lakowicz, 1983); they were 5.6 M^{-1} for full-length protein and 6.7 and 7.3 M^{-1} for PITP(1−259) and PITP(1−253), respectively. Interestingly, for comparable experiments using the anionic iodide as the quenching agent, no difference was detected between full-length PITP and PITP(1−253); iodide quenching constants for these protein species were 3.0 and 2.9 M^{-1}, respectively. The similarity of these

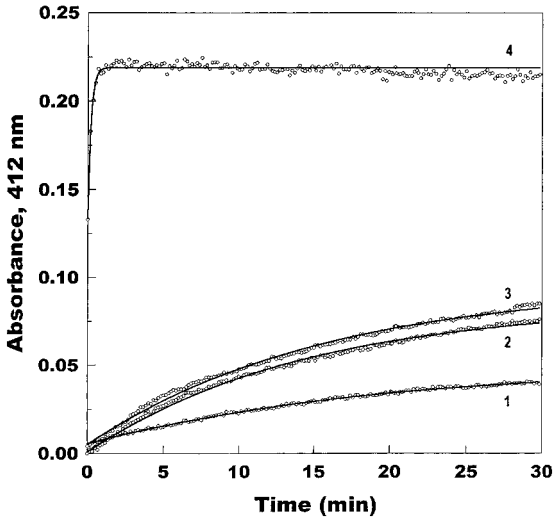

FIGURE 4: Kinetics of DTNB reaction with sulfhydryl groups of PITPs. Protein solutions (3.9 μM) were prepared in 10 mM sodium phosphate, pH 7.6; DTNB solution in the same buffer was added to a final concentration of 400 μM. Time course of the reaction was monitored at 25 °C for PITP(1−271) (1), PITP(1−253) (2), and PITP(1−259) (3) or for PITP(1−271) denatured in 6 M GdnCl (4). At least two independent measurements were carried out for each sample; results of a single experiment are presented.

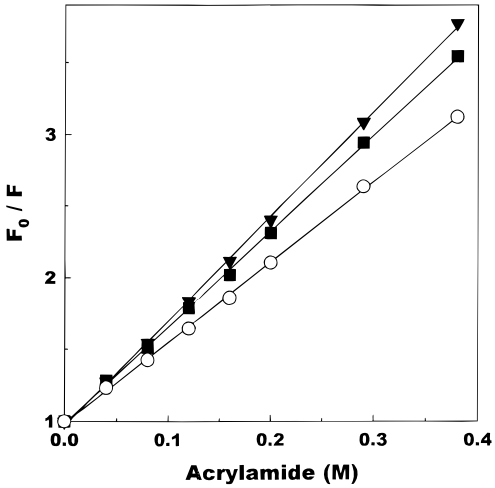

FIGURE 5: Stern−Volmer plots of acrylamide quenching for full-length and truncated PITPs. Tryptophan fluorescence was measured at emission maximum with excitation wavelength of 295 nm in 10 mM sodium phosphate, pH 7.5, at 25 °C. Protein concentration was 30 μg/mL. (○) PITP(1−271); (■) PITP(1−259); (▼) PITP(1−253).

quenching constants prompted us not to determine the value for PITP(1−259).

(*E) Binding of 1,8-ANS.* To study further the conformational characteristics of full-length and truncated PITP species, we measured the binding of the fluorescent probe 1,8-ANS. This probe has been shown to interact noncovalently with both polar and nonpolar regions of proteins (Lakowicz, 1983). Binding of 1,8-ANS to PITP(1−271) appears to approach saturation in the concentration range studied; analysis of these data yielded a K_d of 20−30 μM (Figure 6). In contrast, interaction of the probe with both truncated protein species had lower affinities (K_d > 200 μM) but significantly higher binding capacities (Figure 6).

(*F) Protein Stability.* Equilibrium unfolding of PITP in 0−6 M GdnCl was employed to analyze relative stability of full-length and truncated species. For these experiments,

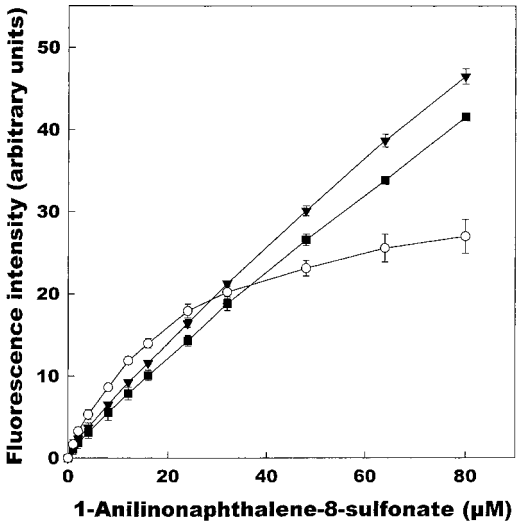

FIGURE 6: Fluorescence titration of full-length and truncated PITPs with 1,8-ANS. Titrations of the proteins with indicated amounts of 1,8-ANS were performed in 10 mM sodium phosphate, pH 7.5, at 25 °C. Samples were excited at 350 nm, and emission was measured at 465 nm. Protein concentration was 3.9 μM. Each point represents the mean ± SD of two experiments. (○) PITP(1−271); (■) PITP(1−259); (▼) PITP(1−253).

monitoring of λ_{em}^{max} of intrinsic Trp fluorescence was chosen because of its relative independence on variations in protein concentration. Denaturation curves of all three proteins, representing magnitude of λ_{em}^{max} as a function of GdnCl concentration, were consistent with a two-state transition. Calculated transition midpoints for all three proteins were in the range of 2.0−2.4 M and were not significantly different from one another (data not shown).

(*G) Refolding of Full-Length PITP and PITP(1−259).* We studied the refolding of denatured PITP species to define the effect of the C-terminal truncation on the ability of PITP to acquire an active conformation. After unfolding in 6 M GdnCl and rapid dilution into refolding buffer, PITP(1−271) recovered approximately 75% of its initial activity after 50 min of incubation (Figure 7). For PITP(1−259), the kinetics of refolding was significantly slower, and the corresponding recovery of activity continued to increase even after 5 h of incubation (Figure 7). An extremely low initial activity of PITP(1−253) precluded the study of its refolding.

DISCUSSION

In the present study we have utilized recombinant PITP species, structurally altered by truncations at Arg-259 and Arg-253, to investigate the contributions of C-terminal residues of the protein to its structural organization and transfer function. Since no single indirect method could give full and adequate information about structural changes in the protein, we employed a variety of biophysical approaches to measure different parameters of polypeptide macromolecular structure. Conformational differences involving both secondary and tertiary structures were studied by measuring solvent exposure of the specific amino acid residues. We employed nonionic (acrylamide) and ionic (iodide) quenchers of intrinsic Trp fluorescence; solvent accessibility of Tyr residues was assessed by second derivatives of the protein absorbance spectra, and a colorimetric reaction with DTNB was applied to quantitate the extent and rate of Cys modification. To characterize further conformational changes

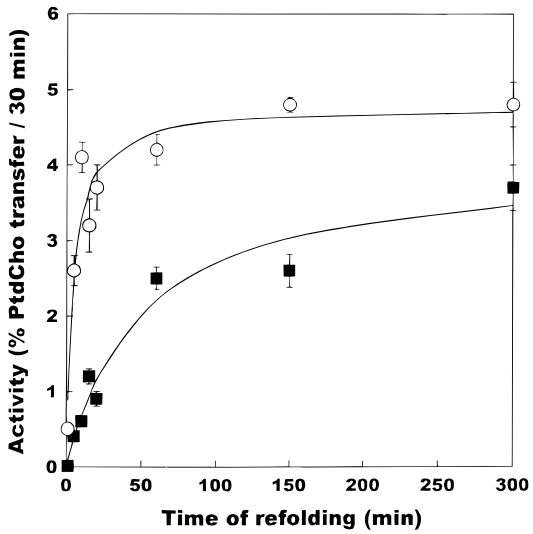

FIGURE 7: Refolding of full-length PITP and PITP(1−259) denatured in GdnCl. To study the kinetics of refolding, protein species were denatured by incubation with 6 M GdnCl for 16 h at 4 °C followed by rapid 100-fold dilution into 50 mM HEPES, pH 7.4, to the final protein concentration of 2.5 μg/mL. After dilution, PITP(1−271) (○) and PITP(1−259) (■) were incubated at 25 °C for indicated times. After these incubations, a 30-min transfer activity measurements were performed as described in Materials and Methods. Each point represents the average of two determinations.

brought about by the truncations, we studied the interaction of the fluorescence probe 1,8-ANS with hydrophobic and polar regions of the proteins. Changes in secondary structure were characterized using CD spectrometry.

Analysis of the CD spectra indicated that the ordered secondary structure of full-length PITP was approximately 40% α-helix. Predictions of the secondary structure based on several different algorithms (Chou & Fasman, 1974; Gibrat et al., 1987; Deleage & Roux, 1987; Levin et al., 1986; Rost & Sander, 1994; Geourjon & Deleage, 1994) projected a polypeptide with 40−65% α-helix, values which compared reasonably with our experimental data. In contrast, the predicted content of β-form (10−30%) was higher than our determined figure. It is interesting that several mammalian lipid-binding proteins with known three-dimensional structures, such as fatty acid binding protein (Sacchettini et al., 1989) and retinol binding protein (Newcomer et al., 1984) possess a predominantly β-sheet structure which forms a hydrophobic pocket for lipid binding; in several plant nonspecific lipid transfer proteins, however, formation of a hydrophobic cavity was achieved mainly by arrangement of amphipathic α-helices (Gincel et al., 1994; Heinemann et al., 1996).

Our data clearly show that PITP(1−259) has a more relaxed tertiary structure, compared to full-length protein. This conclusion is based upon the increased solvent exposure of Tyr and Cys residues and greater accessibility of Trp residues to acrylamide. In agreement with these findings was a significant change in the binding of 1,8-ANS by PITP-(1−259), which we interpret as increased solvent exposure of polypeptide regions that otherwise remain sequestered in the native structure. These results indicate that the 12-amino acid C-terminus sequence 260−271 is important to the more compact native tertiary conformation. The data further show that the removal of this sequence causes global conformational changes in the protein, changes that could account for indirect structural effects, such as an increased affinity of PITP(1−259) for negatively charged vesicles (L. R. Yarbrough, unpublished results). This same region of the protein also appears to be important for more rapid refolding of denatured PITP, as the truncation of the residues 260−271 significantly slowed the refolding kinetics.

On the other hand, the relatively small differences in the intensity and shape of CD spectra, absence of changes in protein stability (as measured by equilibrium unfolding in GdnCl), and unchanged accessibility of Trp residues to iodide suggest that the C-terminal truncations do not dramatically alter the overall protein structure. This is in agreement with the observation that PITP (1−271) and PITP(1−259) had similar catalytic activities toward the transport of PtdCho between unilamellar vesicles.

While PITP(1−259) and PITP(1−253) demonstrated virtually identical structural properties in all our experiments, their transfer activities differed dramatically. Removal of residues 254−259 almost totally eliminated the protein's catalytic activity, indicating their critical importance for the protein transfer function. Since the removal of the residues 254−259 did not appear to further perturb the overall protein structure, relative to PITP(1−259), we suggest that these residues may be directly involved in the mechanism of phospholipid transfer activity. A specific role of the residues 254−259 in phospholipid transfer is unclear. Alb et al. (1995) have recently shown that Glu-248 is critical for rat PITP transfer activity toward PtdIns and may participate in formation of the phospholipid binding site. Although the residues 254−259 are proximate to Glu-248 in the protein primary structure, in our study the stoichiometry of the phospholipid ligand binding was unchanged and did not correlate with the transfer activity upon the removal of these residues. It is noteworthy, however, that transfer activity of PITP does not depend solely on the ability of the protein to bind its phospholipid ligand. The mechanism of lipid transfer is a complex process which most likely includes association of the protein with the phospholipid membrane, exchange of ligands, and dissociation from the membrane. Which of these steps requires participation of the residues 254−259 remains to be determined.

In conclusion, our data suggest a critical role of amino acid residues 254−259 in transfer activity of PITP, while the C-terminal sequence 260−271 appears to be more important for the efficient folding and maintenance of a compact native conformation of the protein. Interestingly, the majority of the described earlier predictions of the secondary structure of full-length PITP assigned no ordered structure to the C-terminal sequence 259−271, while the immediately adjacent residues 254−259 were the part of an extended α-helical region. This may account for their dramatically different contributions to protein conformation and activity.

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