Modifications of Cysteine Residues in the Solution and Membrane-Associated Conformations of Phosphatidylinositol Transfer Protein Have Differential Effects on Lipid Transfer Activity†

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ABSTRACT: The α isoforms of mammalian phosphatidylinositol transfer protein (PITP) contain four conserved Cys residues. In this investigation, a series of thiol-modifying reagents, both alkylating and mixed disulfide-forming, was employed to define the accessibility of these residues and to evaluate their role in protein-mediated intermembrane phospholipid transport. Isolation and analysis of chemically modified peptides and site-directed mutagenesis of each Cys residue to Ala were also performed. Soluble, membrane-associated, and denatured preparations of wild-type and mutant rat PITPs were studied. Under denaturing conditions, all four Cys residues could be detected spectrophotometrically by chemical reaction with 4,4′-dipyridyl disulfide or 5,5′-dithiobis(2-nitrobenzoate). In the native protein, two of the four Cys residues were sensitive to some but not all thiol-modifying reagents, with discrimination based on the charge and hydrophobicity of the reagent and the conformation of the protein. With the soluble conformation of PITP, achieved in the absence of phospholipid vesicles, the surface-exposed Cys^{188} was chemically modified without consequence to lipid transfer activity. Cys^{188} exhibited an apparent pK_a of 7.6. The buried Cys^{95}, which constitutes part of the phospholipid substrate binding site, was covalently modified upon transient association of PITP with a membrane surface. The Cys-to-Ala mutations showed that neither Cys^{95} nor Cys^{188} was essential for lipid transfer activity. However, chemical modification of Cys^{95} resulted in the loss of lipid transfer activity. These results demonstrate that the Cys residues of PITP can be assigned to several different classes of chemical reactivity. Of particular interest is Cys^{95}, whose sulfhydryl group becomes exposed to modification in the membrane-associated conformation of PITP. Furthermore, the inhibition of PITP activity by thiol-modifying reagents is a result of steric hindrance of phospholipid substrate binding.

PITP[1] is a soluble eukaryotic protein that binds and transports phospholipids and participates in signal transduction and vesicular trafficking pathways (*1*). Within metazoan animal species, PITP is represented by the PITP/rdgB family of protein isoforms whose structure and function are highly conserved (*1*, *2*). The functionally related, although structurally dissimilar, sec14/PITP protein family has been described in yeasts and plants (*3*). The mammalian isoforms PITPα and PITPβ interact reversibly with a single phospholipid molecule, preferably PtdIns and PtdCho. The determination of substrate specificity has been accomplished by monitoring the ability of PITP to transport phospholipids between distinct membrane populations in vitro. Recent evidence suggests that PITP functions in the presentation of PtdIns to phosphoinositide kinases that, in turn, generate lipid cofactors associated with multiprotein complexes that participate in intracellular membrane trafficking, exocytosis, and receptor-initiated signal transduction (reviewed in refs *4*−*6*). The intra- and intermembrane transport capacity of PITP and its ability to produce a facilitated PtdIns flux are, in all likelihood, directly related to, if not solely responsible for, its support of membrane-bound lipid phosphorylation and hydrolysis (*7*).

Despite this spectrum of cellular activities, there is limited information about the structure−function relationships of PITP. Our laboratory showed that limited digestion of PITPα with trypsin generated 12- and 18-amino acid cleavages from the C-terminus (*8*). Both truncations resulted in decreases in phospholipid transfer activity that could be attributed to altered protein conformation and increased membrane affinity (*9*, *10*). Interestingly, each truncated PITP species retained the molecule of noncovalently bound phospholipid. More recently, confirmation of the importance of the C-terminus of PITP to cellular functions was demonstrated with a series of 5-, 10-, and 20-amino acid deletions, all of which displayed altered phospholipid transfer activity and the ability to restore phosphoinositide-mediated signal transduction in permeabilized cells (*11*). Several investigations have focused

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[1] Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoate); DTT, dithiothreitol; GdnCl, guanidinium chloride; IAA, iodoacetate; IAM, iodoacetamide; LacCer, lactosylceramide; ME, 2-mercaptoethanol; MMS, methyl methanethiosulfonate; NEM, *N*-ethylmaleimide; PDS, 4,4′-dipyridyl disulfide; PITP, phosphatidylinositol transfer protein; PtdCho, phosphatidylcholine; PtdIns, phosphatidylinositol; PtdOH, phosphatidate.

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on specific amino acid residues in mammalian PITP. The screening of randomly generated rat PITPα mutants for their failure to rescue a temperature-sensitive PITP mutant yeast strain led to the identification of one group of amino acids that appear to be critical to PtdIns binding but not PtdCho binding. These mutations, Ser25Phe, Thr59Ile, Pro78Leu, and Glu248Lys, supported more than 70% of normal PtdCho transport in vitro, yet they were incapable of facilitating an intermembrane movement of PtdIns (*12*). For human PITPα, two mutations in the C-terminal region of the protein, Lys264Ile and Thr267Ala/Val, resulted in no change in PtdIns transfer in vitro or in the reconstitution of cellular phosphoinositide hydrolysis (*11*).

With the extraordinary level of sequence conservation among mammalian PITPs (>98% amino acid identity) and the strong sequence similarities among widely divergent species (*2*), it has been very difficult to predict critical amino acid residues. Some years earlier it had been shown that bovine PITP has at least two classes of amino acid residues that are sensitive to *N*-ethylmaleimide: one that reacted in the absence of membranes and had little effect on activity and another that reacted in the presence of membranes and caused the complete inhibition of phospholipid transfer (*13*). We set out in this study to examine in detail the status of the four conserved Cys residues and their requirement for phospholipid transport. We employed a series of thiol-modifying reagents, both alkylating and mixed disulfide-forming, with soluble, membrane-associated, and denatured preparations of wild-type and mutant rat PITPα. Quantitation of Cys reactivity and analysis of lipid transfer activity were performed and correlated. Identification of specifically modified peptides and Cys residues was established by chromatographic and amino acid compositional analyses. The results not only confirm the preliminary data (*13*) but also extend our understanding of the roles that Cys residues in PITPα play in catalytic activity. Moreover, the recent elucidation of the structure of rat PITPα complexed with PtdCho (*2*) presents an opportunity to compare our chemical and mutagenesis results with the location and potential function of specific Cys residues in PITP.

EXPERIMENTAL PROCEDURES

Materials. *N*-[*ethyl*-1,2-^{3}H]Ethylmaleimide (60 Ci mmol^{-1}) and cholesteryl [1-^{14}C]oleate (57 Ci mmol^{-1}) were purchased from NEN Life Science Products (Boston, MA). [1-^{14}C]-Iodoacetamide (55 mCi mmol^{-1}) was purchased from American Radiolabeled Chemicals (St. Louis, MO). 1-Acyl-2-[9,10-^{3}H]oleoyl-PtdCho and 1,2-diacyl-Ptd[2-^{3}H]Ins were synthesized as described previously (*14*). PtdCho was purified from crude egg yolk lipids (Sigma Chemical, St. Louis, MO) by column chromatography on silicic acid (*15*). *N*-Decylmaleimide was synthesized according to a published procedure (*16*). *N*-Pyrenylmaleimide was purchased from Molecular Probes (Eugene, OR). Bovine liver PtdIns and 1,2-dioleoyl-PtdOH were purchased from Avanti Polar Lipids (Alabaster, AL). GdnCl (>99% purity) was obtained from Fisher Scientific (Pittsburgh, PA). *N*-Tosyl-L-phenylalanine chloromethyl ketone-treated trypsin (T-8642) and other thiol-modifying reagents of the highest available quality were purchased from Sigma Chemical. Curve fitting and plots were generated with SigmaPlot software (SPSS, Inc., San Rafael, CA).

Phospholipid Vesicles and PITP Transfer Activity. Small unilamellar vesicles were prepared by either extensive bath sonication under N_2 or rapid injection of ethanol/dimethyl sulfoxide (4:1, vol %) solutions of mixed lipids into buffer warmed to 40 °C. The extent of phospholipid transfer was measured between two populations of vesicles (*14*). The donor vesicles contained PtdIns, LacCer, and PtdCho (5:8:87, mol %), with either phospholipid radiolabeled; the acceptor vesicles contained PtdIns, PtdCho, and cholesteryl oleate (5:95:0.3, mol %). Unless otherwise specified, assays were performed in 10 mM HEPES-Na, 50 mM NaCl, and 1 mM EDTA (pH 7.4) (assay buffer) at 37 °C for 30 min with PITP, 1.2 μM bovine plasma albumin, 0.25 mM donor vesicles, and 0.75 mM acceptor vesicles. Control assays lacking PITP were performed for all procedures.

Tryptic Digestion and Chromatographic Analysis of Peptides. Portions of PITP (~0.3 mg, native or modified) were precipitated with TCA (10%, w/v) and washed three times with 90% ice-cold ethanol. After drying in air, the protein was resuspended in 80 μL of 0.01 N NaOH to which were added HEPES and $CaCl_2$ at final concentrations of 100 and 10 mM, respectively, at pH 8 in a total volume of 100 μL. Trypsin (6 μg in assay buffer) was added with the protein and the mixture incubated overnight in a 37 °C water bath. The following morning, an additional aliquot of trypsin (1.5 μg) was added and incubation continued for 1−4 h. Digests were stored at −80 °C until analysis. Peptides (~0.15 mg) were separated initially by HPLC (Bio-Rad HRLC, Hercules, CA) using a C8 reversed-phase column (Vydac 208TP54, The Separations Group, Hesperia, CA) eluted with a linear gradient from 2 mM HCl to 100% acetonitrile. Detection was based on the absorption (215 nm) and fluorescence (λ_{ex} = 290 nm; λ_{em} = 340 nm) properties of the peptides. When appropriate, fractions were further analyzed for radioactivity by liquid scintillation spectrometry. Selected fractions were pooled and subjected to further HPLC analysis on a C18 reversed-phase column (Vydac 218TP54); elution consisted of a linear gradient from 2 mM HCl to 100% acetonitrile. Isolated peptides were analyzed for amino acid composition by acid hydrolysis, reversed-phase HPLC, and ninhydrin detection; these procedures were performed by the University of Kansas Medical Center Biotechnology Support Facility.

Chemical Modification and Spectrophotometric Analysis of Thiol Groups. Protein to be subjected to thiol modification was dialyzed extensively overnight after treatment with a large molar excess of DTT. Buffer systems consisted of either 25 mM sodium phosphate and 0.1 mM EDTA-Na or 25 mM sodium borate and 0.1 mM EDTA-Na, adjusted to the indicated pH. Stock solutions of the modifying reagents were prepared in ethanol and stored at 4 °C; dilutions into buffer were made as needed. PITP was unfolded by addition of protein solutions to crystalline GdnCl (final concentration, 6 M). The mixture was kept at 25 °C for 1.5−2 h before further treatment or analysis. In some cases, 10 μM DTT was added to the denatured protein to maintain the reduced status of the Cys residues. When indicated, excess thiol-modifying reagent was quenched and removed by addition of 2-ME (final concentration, 20 mM) and overnight dialysis. Kinetic analysis of Cys reactivity was performed at 25 °C using DTNB or PDS; absorption measurements were recorded at 10 s intervals for 5−20 min on an Aminco-3000

array spectrophotometer. All spectral data were corrected for contributions of equivalent concentrations of reagents. Quantitation of reactive sulfhydryl groups was based on the following liberated products and their absorption maxima and extinction coefficients: 5-thio-2-nitrobenzoate, 412 nm and 14 150 M^{-1} cm^{-1} in buffer alone and 412 nm and 13 700 M^{-1} cm^{-1} in 6 M GdnCl (*17*); and pyridine-4-thione, 324 nm and 19 800 M^{-1} cm^{-1} in buffer alone (*18*) and 329 nm and 24 800 M^{-1} cm^{-1} in 6 M GdnCl (G. Helmkamp, unpublished).

Oligonucleotide-Directed Mutagenesis. Each of the four Cys residues in PITP was mutated to Ala using the QuikChange (Stratagene, La Jolla, CA) polymerase chain reaction protocol and reagents. Pairs of complementary primers (39 bases in length) were designed to replace the wild-type Cys encoded by TGC and TGT with Ala encoded by GCG in the pET-11c vector (Novagen, Madison, WI) containing the rat PITPα cDNA. Following transformation into *Escherichia coli* strain XL1-Blue (Stratagene), plasmid DNA was purified (Qiagen, Valencia, CA) and characterized spectroscopically. Comparison of results obtained from sequencing of the open reading frames in both directions confirmed that four single-site mutations were generated: Cys95Ala, Cys188Ala, Cys192Ala, and Cys231Ala. Oligonucleotide primer synthesis and DNA sequence analysis were carried out by the University of Kansas Medical Center Biotechnology Support Facility.

Protein Expression and Purification. Expression of native and mutant proteins was achieved by transformation of *E. coli* strain BL21(DE3) (Novagen) that had been previously transformed with a plasmid encoding the *gro*ELS chaperone system (*8*). Induction of both PITP and the chaperone expression was regulated through the *lac* operon. Controlled growth at 20 °C yielded sufficient protein in the bacterial lysate supernatants for purification by molecular sieve (Sephadex G-100) and anion exchange (Q Sepharose Fast Flow) chromatography. All proteins were homogeneous and intact, as revealed by polyacrylamide gel electrophoretic and immunoblot analyses (*8*). All proteins contained 1 mol of phosphatidylglycerol (mol of protein)$^{-1}$, based on a protein extinction coefficient of 79 700 M^{-1} cm^{-1} at 280 nm (*9*). Native and mutant PITP preparations were stored at 4 or −80 °C.

RESULTS

Thiol Modification of Native and Denatured PITP. Using two common colorimetric reagents, PDS and DTNB, we determined the reactivity of Cys residues by spectrophotometric titration under native and denaturing conditions. Of the four Cys residues in PITP, only one was readily modified in the native structure: 1.06 ± 0.10 mol mol^{-1} [mean ± standard deviation (SD)] with PDS and 1.17 ± 0.13 mol mol^{-1} with DTNB. The other Cys residues were not exposed to either reagent unless the protein was unfolded in 6 M GdnCl, in which case all four reacted: 4.08 ± 0.08 mol mol^{-1} with PDS and 4.29 ± 0.08 mol mol^{-1} with DTNB. These data suggest that all Cys residues in PITP not only are reduced but, more interestingly, also belong to at least two classes of reactivity. It should be pointed out that these determinations were performed on a variety of PITP preparations, including protein freshly isolated from the bacterial host or protein stored for several months at −80 °C.

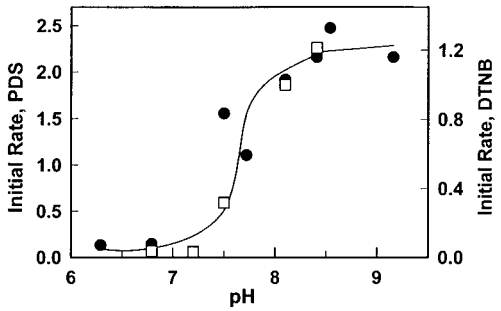

FIGURE 1: pH dependence of chemical modification of Cys residues in native PITPα. PITPα (3.7 μM) was treated at 25 °C with thiol-modifying reagents, 15 μM PDS (●) or 25 μM DTNB (□). Absorbance measurements were recorded at 10 s intervals for 2−10 min. Initial rates (nanomoles per minute) were determined at various pH values using the buffer systems 25 mM sodium phosphate or 25 mM sodium borate, both of which contained 0.1 mM EDTA-Na. Values represent the average of two or three separate experiments.

Table 1: Titratable Cysteine Residues and Catalytic Activity following Treatment with Thiol-Modifying Reagents[a]

thiol-modifying reagent	titratable cysteine residues (mol/mol of protein) native	6 M GdnCl	transfer activity (% of control)
none	1.0	4.2	100
N-ethylmaleimide	0.3	3.1	104 ± 3
N-decylmaleimide	0.2	nd[b]	83 ± 5
N-pyrenylmaleimide	0.1	nd[b]	94 ± 7
iodoacetate	1.1	4.3	107 ± 8
iodoacetamide	0.2	3.3	108 ± 5
methyl methanethiosulfonate	0.1	nd[b]	117 ± 9
dipyridyl disulfide	0.2	nd[b]	98 ± 4

[a] Protein (12 or 21 μM) was treated in 25 mM sodium phosphate and 0.1 mM EDTA (pH 8.2) with 0.8 or 2.5 mM reagent in the dark for 2 h at 25 °C. ME (20 mM) was then added to quench the alkylating reagents, after which all samples were extensively dialyzed. The levels of titratable sulfhydryl groups were determined spectrophotometrically using 60 μM DTNB under native or denaturing conditions. Intervesicular transfer of PtdIns was assessed with 0.4−0.5 μg aliquots of the native protein (unmodified or modified). Results represent the average of two to four experiments. [b] Not determined.

Kinetics and pH Dependence of Thiol Modification of Native PITP. Spectrophotometric titrations permitted us to monitor the initial rate of Cys modification. At pH 8.2, reaction with PDS was complete after 5 min and maximal at concentrations of >30 μM. To define the environment of the single reactive Cys residue, initial rates were determined over a broad pH range. Superimposable sigmoidal pH−rate profiles were observed with both PDS and DTNB (Figure 1). The titration midpoint of 7.6 is consistent with a Cys sulfhydryl group that is solvent-exposed but with an apparent pK_a somewhat perturbed by its environment. The nearly 2-fold more rapid reactivity with PDS than with DTNB, however, suggests some steric and/or electrostatic constraints to the thiol−disulfide exchange reaction.

Sensitivity of PITP to Chemically Different Thiol-Modifying Reagents. The common thiol-modifying reagents act through different chemical mechanisms, including alkylation, Michael addition, and mixed disulfide formation. Protein aliquots were initially treated, dialyzed extensively to remove excess reagent, and then analyzed for titratable Cys and lipid transfer activity (Table 1). Nearly all reagents appeared to modify up to one residue of Cys in the native protein, as

Table 2: Inhibition of Transfer Activity following Treatment with Thiol-Modifying Reagents under Assay Conditions in the Absence and Presence of Phospholipid Vesicles[a]

thiol-modifying reagent	with vesicles [b]	with ME	transfer activity (% of control)
none	−/+	−/+	100
N-ethylmaleimide	−	+	97
	+	+	5
iodoacetamide	−	+	95
	+	+	106
iodoacetate	−	+	101
	+	+	102
methyl methanethiosulfonate	+	−	10
dipyridyl disulfide	+	−	4
dithiobis(nitrobenzoate)	+	−	94

[a] Protein (25 or 37 nM) was treated in assay buffer with 1.0 mM thiol-modifying reagent and 1.5 mM vesicles, as indicated, for 15 min at 37 °C. To quench the alkylating reagents, 20 mM ME, as indicated, was added. The remaining assay components were added (albumin, donor and acceptor vesicles, and buffer to 400 μL), and the incubation was continued for 30 min at 37 °C. Results represent the average of two to four experiments. [b] Sonicated, small unilamellar phospholipid vesicles were composed of PtdOH and PtdCho (2:98, mol %).

indicated by the loss of the spectrophotometrically titratable sulfhydryl group. Both *N*-alkyl- and *N*-arylmaleimide derivatives, which act through Michael adducts, and the alkylating agent IAM could access and modify a single Cys residue. In contrast, the negatively charged IAA failed to alkylate the native protein. The surprising difference between IAA, on one hand, and NEM and IAM, on the other, was further illustrated by quantitating the reactive sulfhydryl groups under denaturing conditions. As expected, only three Cys residues could be titrated following modification with NEM or IAM, yet all four Cys residues remained reactive after an initial treatment with IAA. Two thiol−disulfide exchange reagents, MMS and PDS, were equivalent in their reaction stoichiometry. Despite the chemical modification of the exposed Cys residues by a variety of reagents, no significant change in phospholipid transfer activity was detected, leading to the conclusion that the exposed Cys is not essential to the in vitro function of PITP.

Effect of Phospholipid Vesicles and Thiol-Modifying Reagents on Transfer Activity. In the course of protein-mediated phospholipid transport, PITP must spend significant time associated with membrane surfaces. We have already documented conformational changes between the solution and membrane-bound forms of PITP (*8*, *9*, *19*). Accordingly, PITP was exposed to a variety of thiol-modifying reagents under phospholipid transfer assay conditions: low protein concentration, acceptor vesicles, 37 °C, and limited time. Following the initial treatment of PITP, an excess of ME was added to quench further alkylation or Michael addition. The results (Table 2) clearly separate the modifying reagents into two categories. In the presence of vesicles, NEM was able to reduce PITP catalytic activity by >90%; in the absence of vesicles, NEM failed to inhibit transfer activity. The half-maximal concentration for NEM inhibition was 20 μM (data not shown). Under similar conditions, neither IAM nor IAA had any effect on PITP activity. Among the chemical reagents that act by forming mixed disulfides, MMS and PDS were capable of inhibiting transfer activity, while DTNB was not. For this class of reagent, quenching with ME would have reversed any covalent protein modifications.

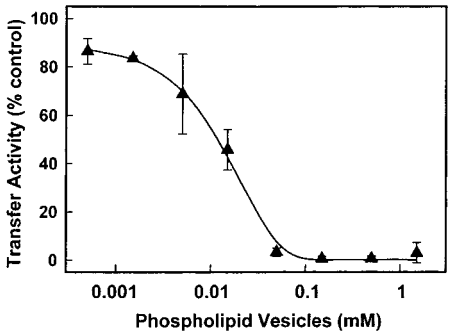

FIGURE 2: Dependence of NEM inhibition on phospholipid vesicles. PITPα (50 nM) in assay buffer was treated at 37 °C for 15 min with 1 mM NEM. Small unilamellar vesicles composed of PtdOH and PtdCho (2:98, mol %) were added to the indicated concentration. Excess reagent was quenched by addition of 20 mM ME. After 5 min, the remaining assay components were added, and the extent of transfer of radiolabeled PtdIns was determined (37 °C for 30 min) from 0.25 mM donor vesicles to 0.75 mM acceptor vesicles. Data points and error bars represent the means ± SD of two to four determinations.

These results further underscore differences among the thiol-modifying reagents that are small and relatively nonpolar and those that are larger and more polar: the former readily inhibit PITP transfer activity while the latter do not.

Vesicle Dependence of Cys Reactivity. Our results thus far confirm and extend the early observation that partially purified bovine heart PITP could be inhibited by NEM only when phospholipid vesicles or mitochondria were present (*13*). We investigated in greater detail this membrane requirement. The dependence of NEM inhibition on vesicle concentration is described by a sigmoid curve (Figure 2) whose midpoint is ~0.01 mM. The reaction isotherm most likely reflects the transient association of PITP with membranes during phospholipid exchange. For comparison, we had calculated a kinetic equivalent of the apparent K_m with PtdCho vesicles containing 2 mol % PtdOH or PtdIns of 0.5 or 0.1 mM, respectively (*20*, *21*). We reported previously that PITP does not form a stable complex (i.e., capable of separation from free protein) with PtdCho vesicles containing 2 mol % PtdOH (*10*). Taking advantage of its spectral properties, we used PDS to modify PITP in the absence and presence of phospholipid vesicles. Analysis of the difference spectra (Figure 3) indicates modification of a single Cys residue (1.2 mol mol^{-1}) in the solution conformation of PITP (absence of vesicles) and two Cys residues (2.4 mol mol^{-1}) in the membrane-associated conformation (presence of vesicles). Comparable stoichiometries were obtained from difference spectra of DTNB-modified PITP (data not shown).

Identification of the Exposed Cys Residue in the Native Protein. To identify the Cys residue that is generally accessible to thiol-modifying reagents in the solution conformation, PITP was covalently modified with [1-^{14}C]IAM and subjected to proteolytic digestion. Peptides were separated by reversed-phase HPLC; fractions were monitored for ultraviolet absorption (215 nm) and radioactivity (Figure 4). The prominent radioactive peptide was subjected to amino acid analysis; the four most abundant amino acids were unambiguously consistent with just one region of the primary sequence (Asp-Cys^{188}-Pro-Tyr). Radioactivity was coincident, as expected, with *S*-carboxymethyl-Cys. Interestingly, this peptide has a Tyr residue at its C-terminus. We interpret this to indicate a contamination of trypsin by a chymotrypsin-

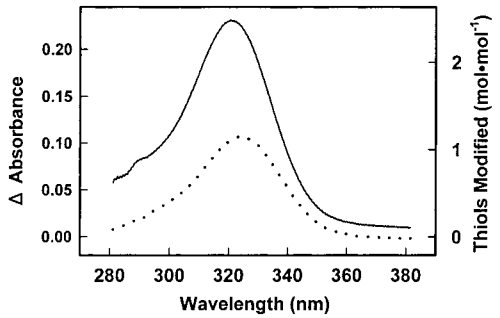

FIGURE 3: Reaction of PITPα with PDS in the absence and presence of phospholipid vesicles. PITPα (5.0 μM) in assay buffer was treated at 25 °C for 15 min with 0.5 mM PDS. Difference spectra were recorded using tandem cuvettes, in which all components were present in various mixtures in the two compartments. Reactions were performed in the absence (—) or the presence (•••) of small unilamellar vesicles, composed of PtdOH and PtdCho (2:98, mol %) and added to a final concentration of 1.5 mM. The extent of chemical modification was calculated from the appropriate protein and reagent extinction coefficients described in Experimental Procedures.

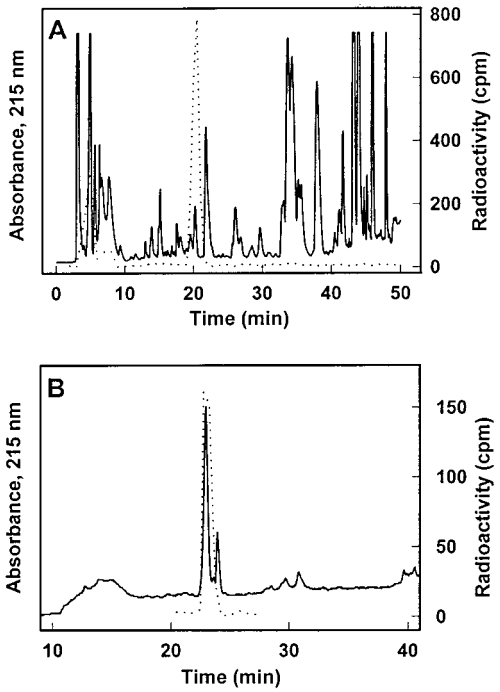

FIGURE 4: Isolation and characterization of a chemically modified peptide. PITPα (1 mg) in 25 mM sodium phosphate and 0.1 mM EDTA-Na (pH 8.25) was treated in the dark at 25 °C for 3 h with [1-^{14}C]IAM. Excess reagent was quenched by addition of 20 mM ME. Peptides were generated and resolved by reversed-phase HPLC, as described in Experimental Procedures. Fractions were monitored for ultraviolet absorption (—) and radioactivity (•••). For the initial separation, a C8 analytical column was used (A). The prominent region of radioactive product eluting between 18 and 22 min was further separated using a C18 analytical column (B) to yield the profile that is illustrated; the amino acid composition of the larger, leading peptide was determined.

like activity. Using *N*-[*ethyl*-1,2-^{3}H]ethylmaleimide in the absence and presence of phospholipid vesicles, our attempts to identify the Cys residue(s) responsible for transfer activity losses were unsuccessful.

Titratable Cysteine Residues in Mutant Proteins. Four single-site Cys-to-Ala mutant PITPs were expressed, purified, and evaluated for reactivity toward sulfhydryl reagents in the absence and presence of 6 M GdnCl. As summarized in Table 3, the total number of reactive Cys residues under denaturing conditions in each mutant was reduced from four to three. However, only the Cys188Ala mutation resulted in the loss of the titratable Cys when the reaction with DTNB was performed without exposure to denaturants. This result strongly supports the designation of Cys^{188} as the sole Cys residue that is susceptible to chemical modification in the solution conformation of PITP.

Table 3: Titratable Cysteine Residues following Mutation of Cysteine Residues to Alanine[a]

	titratable cysteine residues (mol/mol of protein)	
	native	6 M GdnCl
native protein	1.0	4.1
Cys95Ala	1.0	3.1
Cys188Ala	0.03	3.0
Cys192Ala	1.0	3.0
Cys231Ala	1.1	3.0

[a] Protein (1−8 μM), in 25 mM sodium phosphate and 0.1 mM EDTA (pH 7.0), was treated with 60 μM DTNB at 25 °C and analyzed spectrophotometrically after 15 and 60 min to ensure completion. Denaturation was achieved by exposing the protein to 6 M GdnCl for 2 h prior to sulfhydryl analysis. Results represent the average of two experiments.

Table 4: Transfer Activity of Cysteine Mutants and Inhibition by Thiol-Modifying Reagents[a]

	thiol-modifying reagent	transfer activity[b] (% native)	
		PtdIns	PtdCho
native protein	none	100	100
	N-ethylmaleimide	4 ± 7	3 ± 3
	iodoacetamide	102 ± 14	nd[c]
Cys95Ala	none	126 ± 7	97 ± 3
	N-ethylmaleimide	32 ± 6	53 ± 10
	iodoacetamide	120 ± 43	nd[c]
Cys188Ala	none	91 ± 18	105 ± 5
	N-ethylmaleimide	12 ± 11	4 ± 3
	iodoacetamide	74 ± 7	nd[c]
Cys192Ala	none	80 ± 2	105 ± 3
	N-ethylmaleimide	0 ± 8	5 ± 5
	iodoacetamide	68 ± 3	nd[c]
Cys231Ala	none	91 ± 6	115 ± 9
	N-ethylmaleimide	0 ± 12	12 ± 5
	iodoacetamide	68 ± 14	nd[c]

[a] Protein was treated in assay buffer with 1.0 mM thiol-modifying reagent and 0.6 mM acceptor vesicles, as indicated, for 15 min at 37 °C. The remaining assay components were added (plasma albumin, donor and acceptor vesicles, and buffer to 400 μL), and the incubation was continued for 30 min. Results represent the average of three experiments. [b] For PtdIns transfer, donor and acceptor vesicles were composed of PtdIns, LacCer, and PtdCho (10:10:80, mol %) and PtdIns and PtdCho (10:90, mol %), respectively; the amount of protein assayed was 3.9 pmol. For PtdCho transfer, donor and acceptor vesicles were composed of PtdOH, LacCer, and PtdCho (5:10:85, mol %) and PtdOH and PtdCho (5:95, mol %), respectively; the amount of protein assayed was 6.3 pmol. [c] Not determined.

Transfer Activity and Thiol Sensitivity of Mutant Proteins. Mutation of the Cys residues to Ala had a very modest impact on the protein-catalyzed transfer of phospholipids between membranes (Table 4). Compared to that of the native protein, transfer of PtdCho was essentially unchanged for each of the four mutant PITPs. Similarly, for two of the mutants, Cys188Ala and Cys231Ala, the transfer of PtdIns was not different from that of native PITP. On the other hand, the Cys95Ala protein was slightly more active toward PtdIns and the Cys192Ala protein somewhat less active. Proteins

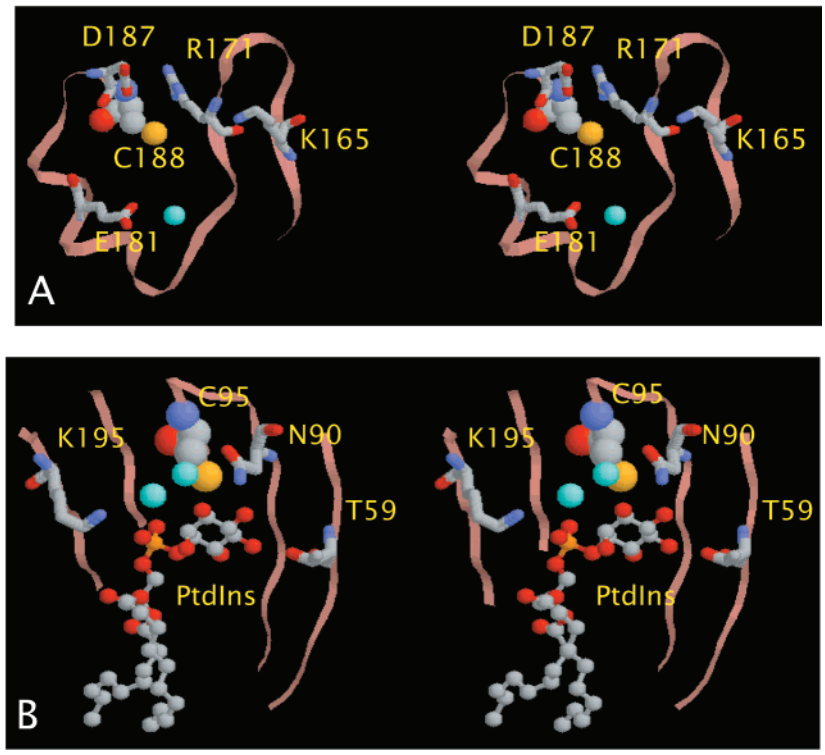

FIGURE 5: Stereo projections of the chemically reactive Cys residues in PITPα. (A) In the protein's solution conformation, Cys[188] is readily modified. The solvent-accessible Cys[188] (space-filling diagram) is shown within a surface depression that is formed, in part, by the side chains of Lys[165], Arg[171], Glu[181], and Asp[187] (thick wire frame). One water oxygen is shown in bright blue. The polypeptide backbone for residues 163−189 is depicted as a flesh-colored ribbon. (B) When the protein is adsorbed to a membrane surface, Cys[95] (space-filling diagram) can be modified. A portion of the β-sheet surface of the lipid-binding cavity around the polar headgroup of PtdIns (ball-and-stick representation) is shown. The side chains of Thr[59], Asn[90], and Lys[195] (thick wire frame) are within noncovalent bonding distance of the phosphorylinositol function. Two water oxygens are shown in bright blue. The polypeptide backbone for a portion of the β-sheet is depicted as a flesh-colored ribbon.

were then treated with either NEM or IAM in the presence of acceptor vesicles to ascertain their sensitivity or resistance to chemical modification. Using phospholipid transfer activity to monitor the effects of sulfhydryl modification, native PITP, as shown in other experiments, was completely sensitive to NEM and fully resistant to IAM. The ability to transfer both PtdIns and PtdCho was virtually eliminated following reaction with NEM. Similar results were obtained with the Cys188Ala, Cys192Ala, and Cys231Ala mutant protein species. In contrast, significant residual transfer activity toward both phospholipids was measured for the Cys95Ala protein. These data are consistent with the identification of Cys[95] as one of the sites of covalent sulfhydryl modification and subsequent catalytic inhibition in the membrane-associated conformation of PITP.

DISCUSSION

Cysteine residues provide excellent targets for the study of the structure, conformational transitions, and function of proteins (*22*−*24*). Observed differences in side chain reactivity, coupled with a wide spectrum of reversible and irreversible thiol-modifying reagents, can provide useful information about the location, environment, and functional significance of specific Cys residues. During the course of these investigations of Cys residues in PITP, we and our colleagues completed the three-dimensional structure of the rat PITPα isoform complexed with PtdCho (*2*). Knowledge of the protein's tertiary structure permits a more meaningful and insightful interpretation of our thiol modification, lipid transfer activity, and mutation data. The most striking feature of the protein−phospholipid complex is an enclosed cavity, the lipid-binding core, that consists of a seven-stranded β-sheet that is crossed diagonally by two long α-helices. This cavity is large enough to sequester completely from the bulk solvent a single, noncovalently bound phospholipid molecule.

Exposed Cys[188] May Be Modified without Consequence to Transfer Activity. Examination of the exterior surface of PITP reveals only one exposed sulfhydryl group, that belonging to Cys[188] (*2*). The exposed Cys[188] is one of the regulatory loop residues of the PITP molecule; it is conserved in the soluble mammalian α and β isoforms. We have proposed that this loop may be involved in the interactions of PITP with lipid and protein kinases and would play no direct role in substrate phospholipid or membrane surface binding. The observation that neither chemical modification nor mutation of Cys[188] alters intermembrane lipid transfer activity is consistent with this function.

Our survey of thiol-modifying reagents indicated that Cys[188] displayed differential reactivity based, to some extent, on the charge of the reagent. The relatively small anionic reagent IAA was ineffective, while uncharged and more hydrophobic reagents readily modified the protein. Cys[188] is located in a small depression on the surface of PITPα (Figure 5A). On the perimeter of this depression are the side chains of Glu[181] and Asp[187], the charges of which could impede penetration of anionic molecules. Such charge

repulsion must be more influential than any attractive forces that could derive from the nearby side chains of Lys^{165} and Arg^{171}. Distances between the Cys^{188} sulfur and the carboxylate oxygens of Glu^{181} and Asp^{187} are in the range 0.49−0.99 nm. Ironically, these cationic amino acids probably facilitate the association between the more reactive DTNB and PITP. In an extensive investigation of the reactivity of β-globin Cys^{93} reactivity in adult human hemoglobin (*25*), it was shown that modification by anionic DTNB was sensitive to nearby His, Lys, Asp, and Glu residues. Natural mutations to these residues significantly altered the rates and pH dependence. In contrast, reaction of the normal and mutant hemoglobins with the uncharged 2,2′-dipyridyl disulfide was less sensitive to nearby charged amino acid side chains. Like PITP, the hemoglobin β-globin Cys^{93} exhibited little reactivity toward IAA and other negatively charged aliphatic thiol-modifying reagents (*26*).

The chemical environment around Cys^{188} is, in part, revealed by the apparent pK_a of 7.6. The more acidic character of Cys^{188}, compared to the typical unperturbed sulfhydryl group with a pK_a of 8.5−8.8 (*27*−*29*), may be attributed to the proximity of both Lys^{165} and Arg^{171}. Distances between the Cys^{188} sulfur and electronegative nitrogens of Lys^{165} and Arg^{171} are in the range 0.50−0.87 nm. While these distances are long for energetically favorable noncovalent bond formation (*30*, *31*), the strategic placement of solvent molecules could make weak hydrogen bonds more likely. The X-ray crystallographic data do, in fact, show one water molecule in this surface depression, but it appears to be associated with the more buried Glu^{181} (Figure 5A). A dramatic perturbation of sulfur nucleophilicity is seen for the pair of active site Cys residues in thiol−disulfide oxidoreductases (*32*−*35*). Using chemical modification, site-specific mutagenesis, Raman spectroscopy, and nuclear magnetic resonance spectroscopy, there is general agreement on an apparent pK_a of 7.0−7.5 for the more solvent-exposed Cys and a preponderance of evidence for a slightly less acidic pK_a for the less exposed Cys. From these and other investigations have emerged several possible explanations for changes in the thiol−thiolate equilibria: hydrogen bonds with backbone amide groups, electrostatic interaction with His or Lys side chains, and electrostatic interaction with the dipole moment of the α-helix containing the Cys. We can eliminate the last possibility, as Cys^{188} of PITPα is located *between* a short α-helix and a β-strand.

Buried Cys^{95} May Be Modified Only When Protein Is Membrane-Bound, Leading to Loss of Transfer Activity. A different class of Cys residue in PITP was identified in our studies of protein sulfhydryl reactivity under conditions that promote intermembrane phospholipid transfer. When unilamellar phospholipid vesicles were present at the same time with certain thiol-modifying reagents, lipid transfer catalytic activity could be inhibited, or even eliminated. As for the exposed Cys^{188}, a differential reactivity among thiol-modifying reagents was observed for the buried Cys^{95} residue. The more hydrophobic, uncharged reagents were inhibitory; the polar or charged reagents were not. It is noteworthy that the covalent modification of hemoglobin β-globin Cys^{93} in intact erythrocytes could readily be achieved using reagents that were membrane-permeable; compounds that were charged did not react (*26*). Our observations confirm and extend the observation reported some years ago that the PtdCho transfer activity of partially purified bovine heart PITP was more than 90% inhibited by NEM only in the presence of lipid vesicles (*13*). Interestingly, these investigators also provided evidence for the second class of sulfhydryl groups whose modification did not compromise transfer activity. More recently, PITP was identified as one of the NEM-sensitive proteins that are essential to normal vesicle budding from the trans-Golgi network of cultured mammalian kidney cells (*36*).

To characterize more fully the buried Cys residue in PITPα, we took advantage of site-directed mutagenesis to generate four Cys-to-Ala replacements. Only the Cys95Ala mutation gave rise to a protein that was now resistant to NEM inhibition and retained significant lipid transfer activity. We would suggest that the loss of some activity in the NEM-treated Cys95Ala mutant could be the result of more widespread NEM reactivity. Although we have carried out our thiol modifications at low reagent concentrations, near-neutral pH, and short times, it is possible that NEM could have reacted with His, Lys, and N-terminal residues (*37*, *38*). The precise nature of the critical, though obviously nonessential, role of Cys^{95} in the native protein became clear from X-ray crystallographic data. Within the cavity formed by the lipid-binding core of PITPα, one molecule of PtdCho is oriented along the long axis. Accommodating the polar head region of the lipid through electrostatic and hydrogen bonds are the side chains of Gln^{22}, Thr^{59}, Glu^{86}, Asn^{90}, Cys^{95}, Thr^{97}, Thr^{114}, and Lys^{195}, and a number of tightly bound water molecules (*2*). Cys^{95} is potentially capable of hydrogen bonding, through one or two waters, to the quaternary ammonium and phosphoryl ester functions of PtdCho. With a myoinositol function modeled into the protein−phospholipid structure, Cys^{95} is seen to be situated ideally to participate in the binding of the phospholipid substrate (Figure 5B). Pertinent *interatomic* distances are 0.38 nm for the Cys $\underline{S}H-\underline{O}H_2$ distance and 0.25 nm for the inositol-5-$\underline{O}H-\underline{O}H_2$ distance for one bond and 0.29 nm for the Cys $\underline{S}H-\underline{O}H_2$ distance and 0.29 nm for the phosphoryl$=\underline{O}-\underline{O}H_2$ distance for another. Sequence alignment of 18 proteins in the PITP/rdgB family shows that Cys is found at position 95 (or its equivalent) in 11 of 12 soluble PITPs and is replaced by Thr in the others, including all of the membrane-associated rdgB proteins (*2*). Either Cys or Thr could function as hydrogen bond donors to the protein-bound phospholipid's polar headgroup.

Our data suggest that there would be at least two consequences of chemical modification of Cys^{95}: one being the disruption of noncovalent bonds that stabilize the substrate phospholipid−polypeptide interaction and the other being steric hindrance (and even altered conformation) within the internal cavity of the lipid-binding core. Either alone would be sufficient to compromise substrate phospholipid binding and further transfer activity. Adsorption of PITP to a membrane surface appears to be a necessary prerequisite to these events. A reasonable scenario involves (1) association of a soluble phospholipid−PITP complex with a vesicle to form a transient ternary complex, (2) diffusion of the protein-bound lipid into the vesicle membrane's outer monolayer, and (3) chemical susceptibility of Cys^{95} to those thiol-modifying reagents that are lipophilic enough to penetrate the interface between the vacated lipid-binding core of PITP and the membrane. An alternative mechanism by

which the buried Cys of PITP could be modified would involve the diffusion of the reagent through structural discontinuities in the folded polypeptide generated during membrane adsorption or desorption. Interestingly, the fate of covalently modified, inactive PITP could be either dissociation from the membrane as a lipid-free apoprotein or stabilized association with the membrane due to the failure to acquire membrane-bound PtdIns or PtdCho. Experiments are currently underway to distinguish between these possibilities.

In conclusion, we have used a combination of chemical modification, site-specific mutagenesis, and activity measurements to characterize the four Cys residues of mammalian PITP. In the native protein, two nonessential Cys residues were sensitive to some but not all thiol-modifying reagents, with discrimination based on charge and hydrophobicity. We demonstrated that the surface-exposed Cys in the soluble conformation of the protein could be modified or mutated without consequence to lipid transfer activity. We further showed that a buried Cys that constituted part of the substrate binding site could undergo covalent modification only upon association of the protein with a membrane and that such modification resulted in the loss of lipid transfer activity. These results provide a framework for more detailed investigations of different PITP conformational states and a possible means of generating stable intermediates in the lipid transfer process.

REFERENCES

1. Rogers, D. P., and Bankaitis, V. A. (2000) *Int. Rev. Cytol. 197*, 35−81.
2. Yoder, M. D., Thomas, L. M., Tremblay, J. M., Oliver, R. L., Yarbrough, L. R., and Helmkamp, G. M., Jr. (2001) *J. Biol. Chem. 276*, 9246−9252.
3. Li, X., Xie, Z., and Bankaitis, V. A. (2000) *Biochim. Biophys. Acta 1486*, 55−71.
4. Wirtz, K. W. A. (1997) *Biochem. J. 324*, 353−360.
5. Cockcroft, S. (1999) *Chem. Phys. Lipids 98*, 23−33.
6. Klenchin, V. A., and Martin, T. F. J. (2000) *Biochimie 82*, 399−407.
7. Currie, R. A., MacLeod, B. M. G., and Downes, C. P. (1997) *Curr. Biol. 7*, 184−190.
8. Tremblay, J. M., Helmkamp, G. M., Jr., and Yarbrough, L. R. (1996) *J. Biol. Chem. 271*, 21075−21080.
9. Voziyan, P. A., Tremblay, J. M., Yarbrough, L. R., and Helmkamp, G. M., Jr. (1996) *Biochemistry 35*, 12526−12531.
10. Tremblay, J. M., Voziyan, P. A., Helmkamp, G. M., Jr., and Yarbrough, L. R. (1998) *Biochim. Biophys. Acta 1389*, 91−100.
11. Hara, S., Swigert, P., Jones, D., and Cockcroft, S. (1997) *J. Biol. Chem. 272*, 14908−14913.
12. Alb, J. G., Jr., Gedvilaite, A., Cartee, R. T., Skinner, H. B., and Bankaitis, V. A. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 8826−8830.
13. DiCorleto, P. E., Warach, J. B., and Zilversmit, D. B. (1979) *J. Biol. Chem. 254*, 7795−7802.
14. Kasper, A. M., and Helmkamp, G. M., Jr. (1981) *Biochim. Biophys. Acta 664*, 22−32.
15. Welti, R., and Helmkamp, G. M., Jr. (1984) *J. Biol. Chem. 259*, 6937−6941.
16. Trommer, W., and Hendrick, M. (1973) *Synthesis 8*, 484−485.
17. Riddles, P. W., Blakeley, R. L., and Zerner, B. (1983) *Methods Enzymol. 91*, 49−60.
18. Grassetti, D. R., and Murray, J. F. (1967) *Arch. Biochem. Biophys. 119*, 41−49.
19. Voziyan, P. A., Tremblay, J. M., Yarbrough, L. R., and Helmkamp, G. M., Jr. (1997) *Biochemistry 36*, 10082−10088.
20. Helmkamp, G. M., Jr., Wirtz, K. W. A., and van Deenen, L. L. M. (1975) *Arch. Biochem. Biophys. 174*, 592−602.
21. Helmkamp, G. M., Jr. (1980) *Biochim. Biophys. Acta 595*, 222−234.
22. Riggs, A. (1961) *J. Biol. Chem. 236*, 1948−1954.
23. Callaci, S., Heyduk, E., and Heyduk, T. (1998) *J. Biol. Chem. 273*, 32995−33001.
24. Ramachandran, S., Rami, B. R., and Udgaonkar, J. B. (2000) *J. Mol. Biol. 297*, 733−745.
25. Hallaway, B. E., Hedlund, B. E., and Benson, E. S. (1980) *Arch. Biochem. Biophys. 203*, 332−342.
26. Garel, M. C., Beuzard, Y., Thillet, J., Domenget, C., Martin, J., Galacteros, F., and Rosa, J. (1982) *Eur. J. Biochem. 123*, 513−519.
27. Antosiewicz, J., McCammon, J. A., and Gilson, M. K. (1994) *J. Mol. Biol. 238*, 415−436.
28. Darby, N. J., and Creighton, T. E. (1993) *J. Mol. Biol. 232*, 873−896.
29. Antosiewicz, J., McCammon, J. A., and Gilson, M. K. (1996) *Biochemistry 35*, 7819−7833.
30. Liu, T.-Y. (1977) in *The Proteins* (Neurath, H., and Hill, R. L., Eds.) 3rd ed., pp 239−402, Academic Press, New York.
31. Jeffrey, G. A., and Saenger, W. (1991) *Hydrogen Bonding in Biological Structures*, Springer-Verlag, Berlin.
32. Dyson, H. J., Jeng, M. F., Tennant, L. L., Slaby, I., Lindell, M., Cui, D. S., Kuprin, S., and Holmgren, A. (1997) *Biochemistry 36*, 2622−2636.
33. Nordstrand, K., Åslund, F., Meunier, S., Holmgren, A., Otting, G., and Berndt, K. D. (1999) *FEBS Lett. 449*, 196−200.
34. Vohník, S., Hanson, C., Tuma, R., Fuchs, J. A., Woodward, C., and Thomas, G. J., Jr. (1998) *Protein Sci. 7*, 193−200.
35. Mössner, E., Iwai, H., and Glockshuber, R. (2000) *FEBS Lett. 477*, 21−26.
36. Simon, J.-P., Morimoto, T., Bankaitis, V. A., Gottlieb, T. A., Ivanov, I. E., Adesnik, M., and Sabatini, D. D. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 11181−11186.
37. Brewer, C. F., and Riehm, J. P. (1967) *Anal. Biochem. 18*, 248−255.
38. Jocelyn, P. C. (1987) *Methods Enzymol. 143*, 44−67.

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