for *Escherichia coli* RUMT should be applicable to the purification of other proteins which form stable Michael adducts, as well as identification of nucleophilic residues involved in covalent bond formation.

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Protein Disulfide Isomerase Appears Necessary To Maintain the Catalytically Active Structure of the Microsomal Triglyceride Transfer Protein†

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ABSTRACT: Protein disulfide isomerase (PDI) is a component of the microsomal triglyceride transfer protein (MTP) complex. This study was initiated to help elucidate the role of PDI in MTP. The 88-kDa polypeptide of MTP (88K) was dissociated from PDI by using chaotropic agents ($NaClO_4$ and KSCN), low concentrations of a denaturant (guanidine hydrochloride) or a nondenaturing detergent (octyl glucoside). As assessed by fluorescence and circular dichroism spectroscopy, these three different approaches appeared to dissociate the components of MTP under mild, nondenaturing conditions. The dissociating agents were diluted or removed by dialysis, and the free PDI and 88K were further characterized. In all cases, the dissociation coincided with the loss of triglyceride transfer activity. The free 88-kDa polypeptide readily aggregated, suggesting that it is a hydrophobic peptide. Even in the presence of chaotropic agents, when 88K was not aggregated, transfer activity was not expressed. These results suggest that the association of PDI with 88K is necessary to maintain the catalytically active form of the triglyceride transfer protein and prevent the aggregation of 88K.

The microsomal triglyceride transfer protein (MTP)[1] is found in the lumen of microsomes isolated from the liver or intestinal mucosa (Wetterau & Zilversmit, 1984, 1986). It facilitates the transfer of triglyceride (TG) between synthetic membranes or plasma lipoproteins (Wetterau & Zilversmit, 1985). MTP also catalyzes the transport of cholesteryl ester (CE) and to a lesser extent phosphatidylcholine (PC). The protein from bovine liver has been purified and characterized. It is a protein complex of molecular weight 150000, containing one 58000

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[1] Abbreviations: TG, triglyceride; CE, cholesteryl ester; PC, phosphatidylcholine; MTP, microsomal triglyceride transfer protein; 88K, subunit of the microsomal triglyceride transfer protein which has a molecular weight of 88000; PDI, protein disulfide isomerase; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; CD, circular dichroism.

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and one 88 000 molecular weight polypeptide (Wetterau et al., 1991). The 58 000 molecular weight component was identified as the multifunctional protein, protein disulfide isomerase (PDI) (Wetterau et al., 1990).

In vitro, PDI catalyzes the proper folding of reduced and denatured proteins by promoting the proper pairing of their cysteine residues into disulfide bonds (Goldberger et al., 1963, 1964; Steiner et al., 1965). In vivo, it is thought to catalyze the proper folding of newly synthesized disulfide-bonded proteins within the lumen of the endoplasmic reticulum (Bulleid & Freedman, 1988). PDI is also a subunit of the enzyme prolyl 4-hydroxylase (Koivu et al., 1987).

The heterodimer structure of the microsomal triglyceride transfer protein raises questions as to the role of the two subunits in the lipid transfer protein. Of particular interest is the role of the protein disulfide isomerase. Isolated PDI has no TG transfer activity (Wetterau et al., 1990). The simplest model for the transfer protein is one in which the 88-kDa component of MTP (88K) is the lipid transfer protein and that its association with PDI is transient or unnecessary. This model would be consistent with the structural features of previously characterized lipid transfer proteins which are single polypeptides. Alternatively, PDI may play a more integral role in the lipid transfer protein. It may contribute directly to the active site of the transfer protein, play a role in the folding of the transfer protein, or maintain it in an active conformation.

In this study, we have further defined the role of PDI in MTP by studying the two components of MTP following their dissociation. PDI and 88K were dissociated by treatment with either low concentrations of a denaturant (guanidine hydrochloride), chaotropic agents, or a nondenaturing detergent (octyl glucoside). Fluorescence and circular dichroism spectroscopy revealed that all three conditions were nondenaturing. Following removal or dilution of the dissociating reagents, triglyceride transfer activity was lost. In addition, the 88-kDa component of MTP formed a protein aggregate. These results suggest that PDI is an essential component of the transfer protein. At the least, it is necessary to maintain and stabilize the catalytically active structure of the transfer protein. A more direct role for PDI in the lipid transport process cannot be excluded.

Materials and Methods

Protein Isolation and Characterization. MTP was isolated as described previously (Wetterau et al., 1990), except as where noted below. Bovine liver was cut into small cubes and rinsed in 250 mM sucrose. One part bovine liver was mixed with two parts 50 mM Tris, pH 7.4, and 250 mM sucrose and homogenized in a blender. The homogenate was centrifuged at 10000*g* for 30 min, and the supernatant was retained. The yield of MTP appeared to be consistently greater when 10000*g* centrifugation was used as opposed to the previously reported 13000*g* centrifugation. Microsomes, isolated from the 10000*g* supernatant as previously described, were suspended in 1 mM Tris, pH 8.6, to release the soluble proteins from the lumen of the microsomes.

MTP was further purified by ammonium sulfate precipitation and sequential column chromatography on DEAE-Sephacel, Sephadex G-200, DEAE-cellulose, Sephacryl S-300, and hydroxylapatite columns. The columns were run as described previously except that 10 mM sodium phosphate, pH 7.4, 0.4 M guanidine hydrochloride, and 0.02% NaN_3 was used to elute MTP from the Sephacryl S-300 column and the hydroxylapatite matrix was acquired from Calbiochem. Triglyceride transfer activity was monitored by measuring the transfer of [^{14}C]triolein from donor egg phosphatidylcholine small unilamellar vesicles to acceptor small unilamellar vesicles as previously described (Wetterau et al., 1990).

Protein disulfide isomerase was purified by a modification of the method of Hillsen et al. (1984) as described by Wetterau et al. (1990). Protein disulfide isomerase activity was measured as the rate of PDI-mediated activation of reduced and denatured ribonuclease as describe previously (Wetterau et al., 1990).

Spectroscopic Analysis of MTP. Uncorrected intrinsic fluorescence spectra were recorded at 28 °C on a Perkin-Elmer 650-10S fluorescence spectrophotometer equipped with a Perkin-Elmer Hitachi 057 X-Y recorder. The instrument was operated in the ratio mode. Samples were excited at 280 nm, and the emission spectra were recorded from 320 to 380 nm. Excitation and emission slit widths were 6 nm.

Circular dichroism (CD) spectra of samples in 1-mm circular cuvettes were recorded at 25 °C on a Jasco J-500A spectropolarimeter with a 2-nm slit width. The scan rate was 2 nm/min, and the time constant was 8 s. Data were collected at 0.04-nm intervals and averaged over a 0.2-nm interval. Calculations of secondary structure were made as described previously (McLean & Hagaman, 1989) with parameters constrained to values >0 and <100% and summed to 100% structure. The reference data of Bolotina et al. (1980) and Yang et al. (1986) were used. As suggested by Wallace and Teeters (1987), this analysis can be used in the presence of spectral flattening which results from light scattering.

Fluorescent Assay To Measure Cholesteryl Ester Transfer Activity. The transfer of pyrene-labeled cholesteryl ester between small unilamellar vesicles was measured by using an assay similar to those used to measure the transfer of pyrene-labeled phospholipids between membranes (Roseman & Thompson, 1980; Correa-Freire et al., 1982; Pownall et al., 1982). The fluorescent emission properties of pyrene-labeled lipids incorporated into a phospholipid bilayer depend upon the concentration of the lipid within the bilayer. Following excitation at 340 nm, there is emission at 380 nm (monomer fluorescence) and 470 nm (eximer fluorescence). There is an increase in the eximer/monomer fluorescence ratio with increasing concentrations of pyrene-labeled lipids in the membrane. In a lipid transfer assay, the concentration of the fluorescent lipid within the bilayer is decreased by the transfer of the fluorescent lipid from the donor membranes rich in the fluorescent lipid to acceptor membranes which do not have the fluorescent lipid. This is detected by a decrease in the eximer/monomer ratio.

Donor small unilamellar vesicles were prepared by cosonication of a mixture of egg yolk phosphatidylcholine and 4 mol % cholesteryl 1-pyrenedecanoate (Molecular Probes, Eugene, OR) in a bath sonicator (Laboratory Supples Co., Inc., Hicksville, NY) at room temperature under a N_2 atmosphere. Acceptor egg yolk phosphatidylcholine vesicles with or without 1 mol % cholesteryl oleate were also prepared by bath sonication. Both the donor and acceptor vesicles contained 0.1% butylated hydroxytoluene and were prepared in 50 mM sodium phosphate, pH 7.5.

Donor vesicles, 12.5 nmol of PC, acceptor vesicles, 100 nmol of PC, and MTP were incubated for zero to 40 min at 28 °C in 1.5 mL of buffer. At varying times, the fluorescence emission of the pyrene-cholesteryl ester was characterized. The decrease in the eximer/monomer fluorescence emission intensity was used to detect lipid transfer activity.

Other Techniques. Protein concentrations were determined by the method of Lowry et al. (1951) using bovine serum

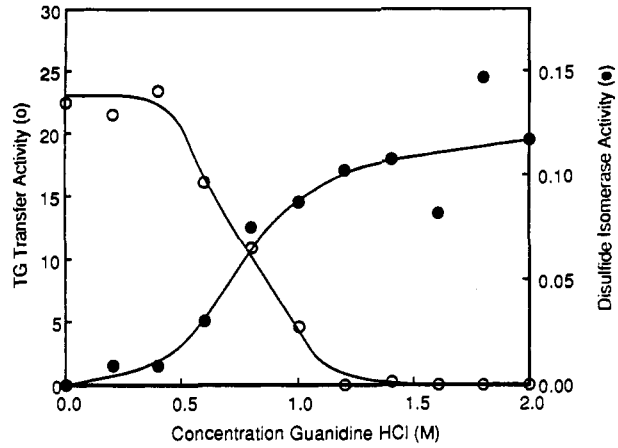

FIGURE 1: Effect of guanidine hydrochloride concentration on the TG transfer and disulfide isomerase activity expressed by MTP. MTP was incubated at a concentration of 35 μg/mL overnight at 4 °C in 50 mM sodium phosphate, pH 7.5, supplemented with increasing concentrations of guanidine hydrochloride. The samples were dialyzed into 50 mM sodium phosphate, and the TG transfer (open circles) and disulfide isomerase activities (solid circles) were measured. TG transfer activity is expressed as the percent donor TG transferred when 0.3 μg of MTP was assayed for 15 min. PDI activity represents the protein-catalyzed increase in ribonuclease activity following a 20-min incubation of 17.5 μg of treated MTP with reduced and denatured ribonuclease. Ribonuclease activity was quantitated by the digestion of RNA to acid-soluble nucleotides which were quantitated by OD_{260}. The PDI activity at each concentration of guanidine hydrochloride is expressed as the difference in OD_{260} of digested RNA observed at t = 20 and t = 0 min.

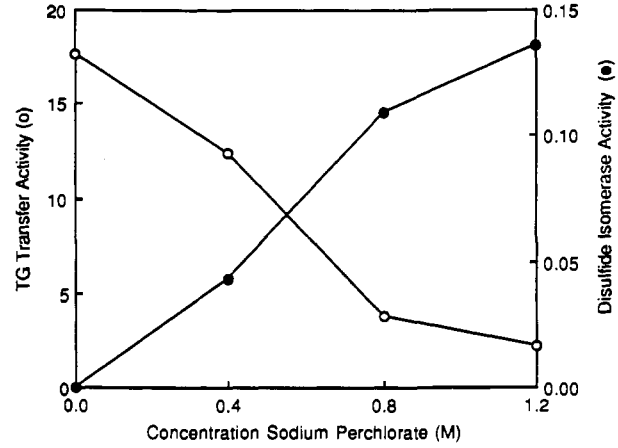

FIGURE 2: Effect of sodium perchlorate concentration on the TG transfer and disulfide isomerase activity expressed by MTP. MTP was incubated at a concentration of 100 μg/mL overnight at 4 °C in sodium phosphate, pH 7.5, supplemented with increasing concentrations of sodium perchlorate. The samples were dialyzed into 50 mM sodium phosphate, and the TG transfer (open circles) and disulfide isomerase activities (solid circles) were measured. Transfer activity is expressed as the percent donor TG transferred per 20-min assay with 0.1 μg of MTP. PDI activity represents the protein-catalyzed increase in ribonuclease activity following a 20-min incubation of 25 μg of treated MTP with reduced and denatured ribonuclease. Ribonuclease activity was quantitated by the digestion of RNA to acid-soluble nucleotides which were quantitated by OD_{260}. The PDI activity at each concentration of $NaClO_4$ is expressed as the difference in OD_{260} of digested RNA observed at t = 20 and t = 0 min.

albumin as a standard. ^{125}I-MTP was prepared by the method of Bolton and Hunter (1973) using commercially available ^{125}I-Bolton–Hunter reagent (DuPont, New England Nuclear Research Products). Sodium dodecyl sulfate (SDS)–polyacrylamide (8%) gel electrophoresis (PAGE) was performed by the method of Laemmli (1970). Nondenaturing polyacrylamide (8%) gel electrophoresis was performed by using the buffer system of Davis (1964). In some cases, the stacking gel was omitted. To dissociate the PDI and 88K subunits of MTP, aliquots of MTP were diluted to the desired final concentration with concentrated solutions of chaotropic agents, $NaClO_4$ (Aldrich Chemical Co.) or KSCN (Sigma); a denaturant, guanidine hydrochloride (Aldrich Chemical Co.); or nondenaturing detergents, octyl β-glucoside (Pierce Chemical Co.), CHAPS (3-[(cholamidopropyl)dimethylammonio]-1-propanesulfonate) (Pierce Chemical Co.), or $C_{12}E_8$ (octaethylene glycol mono-*n*-dodecyl ether) (Nikkol, Japan).

RESULTS

Effect of a Denaturant, Guanidine Hydrochloride, on the Microsomal Triglyceride Transfer Protein Complex. To dissociate the 88-kDa subunit of MTP from PDI, MTP was incubated in the presence of increasing concentrations of a denaturant, guanidine hydrochloride. MTP was then dialyzed into 50 mM sodium phosphate, pH 7.5, for further characterization. An irreversible loss of TG transfer activity was observed following guanidine hydrochloride treatment of MTP (Figure 1). This loss coincided with a dramatic increase in the expression of disulfide isomerase activity (Figure 1). The initial loss of TG transfer activity occurred at 0.6 M guanidine hydrochloride. Following treatment with 1.2 M guanidine hydrochloride, all the TG transfer activity had been lost. The 0.6–1.2 M guanidine hydrochloride concentration range corresponds to that which dissociates PDI from 88K (Wetterau et al., 1991), suggesting that dissociation of the two components of MTP results in the inactivation of the lipid transfer protein. Upon removal of the guanidine hydrochloride (as was done in these experiments prior to measurement of the catalytic activities), the 88-kDa component of MTP aggregates (Wetterau et al., 1991). This aggregation would explain the loss of TG transfer activity following guanidine hydrochloride treatment if 88K is the active component of the transfer protein of if both 88K and PDI are necessary to express lipid transfer activity.

Effect of Chaotropic Agents on the Microsomal Triglyceride Transfer Protein Complex. Chaotroptic agents are commonly used to reduce hydrophobic interactions and disrupt protein–protein interactions without denaturing the individual protein components (Livingston, 1974; Burns & Schachman, 1982). MTP was incubated in the presence of increasing concentrations of $NaClO_4$ to dissociate PDI from 88K under mild, nondenaturing conditions. The $NaClO_4$ was removed by dialysis, and the remaining TG transfer and disulfide isomerase activities were measured. There was a progressive loss of TG transfer activity and a corresponding increase in the expression of disulfide isomerase activity following treatment of MTP with increasing concentrations of $NaClO_4$ (Figure 2). The inactivation of MTP occurred within the first 5 min of MTP treatment (Figure 3). Nondenaturing PAGE of the treated MTP indicated that the loss of transfer activity coincided with the disruption of the native transfer protein complex (Figure 4). Following treatment of MTP with increasing concentrations of $NaClO_4$, there was a progressive loss of intact MTP and the appearance of a band with a mobility corresponding to that of PDI, and an aggregated protein, probably 88K (see below), at the top of the gel.

Iodinated MTP was used to further characterize MTP which was dissociated by treatment with chaotropic agents. ^{125}I-MTP which had been treated with 1.2 M NaCl (control), $NaClO_4$, or KSCN (another chaotropic agent which similarly inactivates MTP) was electrophoresed on a nondenaturing gel,

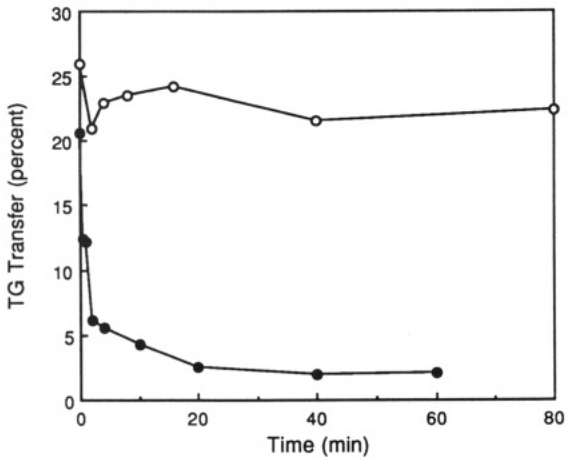

FIGURE 3: Inactivation of MTP by sodium perchlorate. MTP (235 μg/mL) in 50 mM sodium phosphate, pH 7.5, was adjusted to 1.2 or 0.3 M $NaClO_4$ by the addition of a 1.44 or 0.36 M solution of $NaClO_4$ (40 μL of MTP, 200 μL of $NaClO_4$). At varying times, 25 μL of the treated MTP was diluted to 1 mL with 15 mM Tris, pH 7.4, 35 mM NaCl, 1 mM ethylenediaminetetraacetate, and 0.02% NaN_3 supplemented with 1% BSA and stored at 4 °C. Diluted MTP, 0.07 μg, was assayed for TG transfer activity at 37 °C for 1 h. The results are expressed as the percent donor TG transferred as a function of time of incubation in 1.2 M $NaClO_4$ (solid circles) or 0.3 M $NaClO_4$ (open circles).

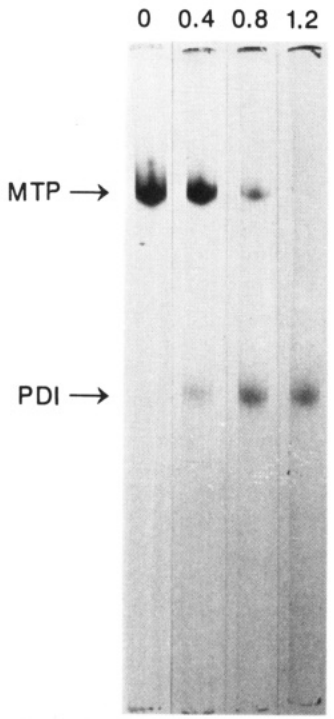

FIGURE 4: Effect of sodium perchlorate on the electrophoretic pattern of MTP. MTP was incubated at a concentration of 100 μg/mL overnight at 4 °C in sodium phosphate buffer, pH 7.5, supplemented with 0, 0.4, 0.8, or 1.2 M $NaClO_4$. The samples were dialyzed into 50 mM sodium phosphate. Sixteen micrograms of MTP was fractionated by electrophoresis on a nondenaturing polyacrylamide gel and visualized with Coomassie blue stain.

the gel was dried, and an autoradiogram was developed (Figure 5). Because 90% of the ^{125}I is incorporated into the 88-kDa subunit when MTP is iodinated by the method of Bolton and Hunter (1973), the mobility of the 88-kDa subunit can readily be visualized by autoradiography. Following treatment with 1.2 M NaCl, the 88-kDa subunit has an electrophoretic mobility identical with untreated MTP; however, following treatment with the chaotropic agents, most of the visible ^{125}I-protein appears at the interface of the stacking and resolving gel. In the KSCN-treated MTP, an additional band was visible on the top of the stacking gel. A weak, diffuse band with a mobility comparable to authentic PDI can also be seen in the treated samples. This band, apparently PDI, was more visible in Coomassie blue stained gels (see Figure 4). These results confirm that the transfer protein complex was dissociated by chaotropic agents and that the removal of these agents resulted in the aggregation of 88K.

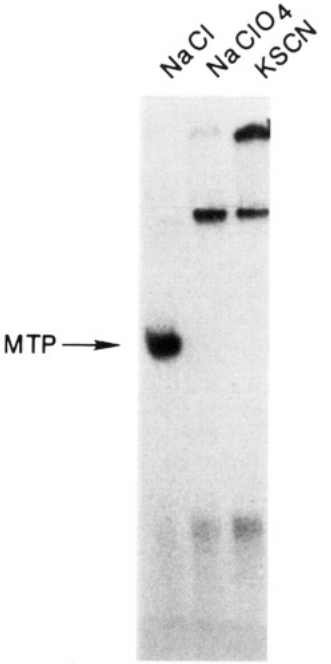

FIGURE 5: Effect of chaotropic agents on the electrophoretic pattern of MTP. MTP, at a concentration of 25 μg and 40000 cpm of ^{125}I-MTP/mL, was incubated at 25 °C for 2 h in 50 mM sodium phosphate, pH 7.5, and 1 mM DTT containing 1.2 M NaCl, $NaClO_4$, or KSCN. The protein was dialyzed at 4 °C into 50 mM sodium phosphate, pH 7.5, and 0.5 mM DTT. An aliquot was fractionated on a nondenaturing polyacrylamide gel, and the iodinated MTP was visualized by autoradiography. Approximately 90% of the ^{125}I incorporated into MTP labels the 88-kDa polypeptide.

Table I: Effect of Nondenaturing Detergents on the Triglyceride Transfer Activity Expressed by MTP[a]

	% TG transfer	% control
control	18.1	
$C_{12}E_8$	20.5	113
CHAPS	12.9	71
octyl glucoside	3.7	20

[a] MTP at 120 μg/mL in 50 mM sodium phosphate, pH 7.5, was adjusted to 5 mM $C_{12}E_8$, 20 mM CHAPS, or 30 mM octyl glucoside. The samples were incubated 1 h at 25 °C followed by 18 h at 4 °C. The protein was diluted 100-fold with 15 mM Tris, 35 mM NaCl, 1 mM ethylenediaminetetraacetate, and 0.02% NaN_3 supplemented with 1% BSA. MTP, 0.1 μg, was assayed for 1 h at 37 °C for TG transfer activity. (Control experiments in which MTP was diluted prior to the addition of equal masses of detergent were performed. No effect on the TG transfer activity was observed, demonstrating that the concentration of detergents employed did not interfere with the assay following the dilution step.) The values represent the average of duplicate assays.

Effect of Nondenaturing Detergents on the Triglyceride Transfer Protein Complex. MTP was treated with three nondenaturing detergents, $C_{12}E_8$, CHAPS, and octyl glucoside, at concentrations above the critical micellar concentrations of the respective detergents. $C_{12}E_8$ had little effect upon the TG transfer activity of MTP while CHAPS treatment resulted in a 25–30% loss of TG transfer activity (Table I). In contrast, treatment with octyl glucoside resulted in an 80% loss of TG transfer activity.

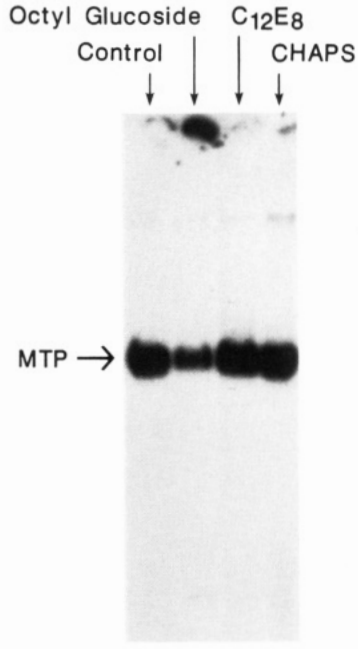

FIGURE 6: Effect of nondenaturing detergents on the electrophoretic pattern of MTP. MTP, 8.4 μg supplemented with 15000 cpm of ^{125}I-MTP, was incubated for 1 h at room temperature followed by 20 h at 4 °C in 60 μL of 25 mM sodium phosphate, pH 7.5, and 1 mM DTT. Three additional samples which contained 30 mM octyl glucoside, 5 mM $C_{12}E_8$, or 20 mM CHAPS were prepared and treated identically. The protein was fractionated by electrophoresis on a nondenaturing gel. The ^{125}I-MTP (with 90% of the label in 88K) was visualized by autoradiography.

Nondenaturing PAGE of ^{125}I-MTP following detergent treatment suggested $C_{12}E_8$ or CHAPS had little effect upon MTP (Figure 6). Some intact MTP was observed following octyl glucoside treatment; however, most of it appeared to aggregate and not enter the resolving gel. When the detergent to protein ratio was increased 6-fold (by decreasing the MTP concentration), the nondenaturing PAGE results were similar (data not shown).

PDI and 88K Which Are Dissociated, but Not Aggregated, Do Not Express Lipid Transfer Activity. Dissociation of the transfer protein complex, followed by removal of the perturbing reagents, results in the loss of TG transfer activity and aggregation of the 88-kDa polypeptide. To determine if free PDI and 88K which has not aggregated can express lipid transfer activity, the lipid transfer activity of MTP in the presence of chaotropic agents or guanidine hydrochloride was determined (see following section for the structural characterization of MTP in the presence of the dissociating reagents). The normal lipid transfer assay, which is sensitive to the ionic strength of the buffer, could not be used for this purpose. A fluorescent assay, based upon the transfer of pyrene-labeled cholesteryl ester between small unilamellar phospholipid vesicles, was developed to detect lipid transfer activity. Similar assays have been utilized to measure the transfer of pyrene-labeled phospholipids between membranes.

The fluorescent emission spectrum of pyrene-labeled lipids in a phospholipid bilayer is dependent upon their concentration within the membrane. At high concentrations, the ratio of the emission at 470 nm (eximer fluorescence) to the emission at 380 nm (monomer fluorescence) is relatively high. The transfer of the pyrene-labeled CE from donor vesicles rich in pyrene-CE to acceptor vesicles results in a dilution of pyrene-CE in the membrane and a decrease in the eximer/monomer fluorescence ratio. Protein-catalyzed pyrene-CE transport was not observed for MTP in the presence of the

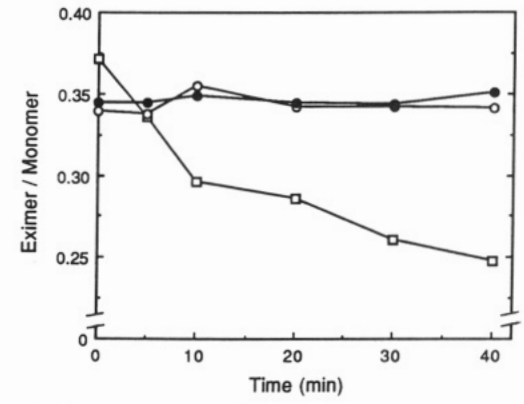

FIGURE 7: Fluorescent assay for MTP activity in the presence of chaotropic agents. Ten micrograms of MTP was incubated for 25 min at 28 °C in 50 mM sodium phosphate, pH 7.5, containing 1.2 M NaCl (squares), $NaClO_4$ (open circles), or KSCN (solid circles). Cholesteryl ester transfer activity was measured as described under Materials and Methods. A decrease in the eximer/monomer fluorescence ratio reflects the transfer of pyrene-labeled cholesteryl ester from the donor (12.5 nmol of PC, 4 mol % cholesteryl 1-pyrenedecanoate) to acceptor (100 nmol of PC) vesicles.

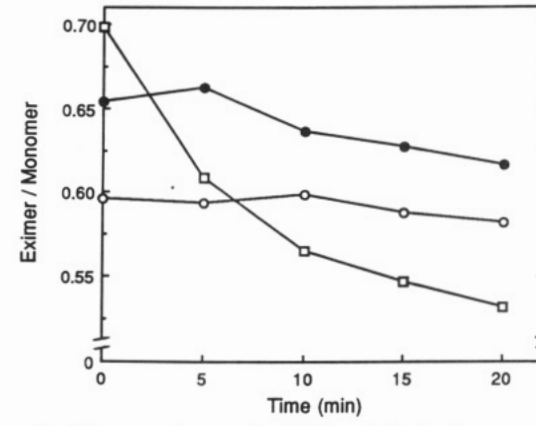

FIGURE 8: Fluorescent assay for MTP activity in the presence of guanidine hydrochloride. Ten micrograms of MTP was incubated for 25 min at 28 °C in 50 mM sodium phosphate, pH 7.5, containing 1.2 M NaCl (squares), 1.2 M guanidine hydrochloride (solid circles), or 1.2 M guanidine hydrochloride and 1 mM DTT (open circles). Cholesteryl ester transfer activity was measured as described under Materials and Methods. A decrease in the eximer/monomer fluorescence ratio reflects the transfer of pyrene-labeled cholesteryl ester from donor (12.5 nmol of PC, 4 mol % cholesteryl 1-pyrenedecanoate) to acceptor (100 nmol of PC, 1 mol % cholesteryl oleate) vesicles.

chaotropic agents $NaClO_4$ and KSCN (Figure 7). No time-dependent change in the eximer/monomer ratio was observed. As a control, MTP in the presence of 1.2 M NaCl had a time-dependent decrease in the eximer/monomer ratio.

Immediately following the addition of MTP to 1.2 M guanidine hydrochloride [the concentration at which PDI and 88K are dissociated (Wetterau et al., 1991)], some lipid transfer activity is expressed in the fluorescent assay (data not shown). Prolonged incubations in the presence of guanidine hydrochloride (25 min) result in a loss of transfer activity (Figure 8). Dithiothreitol was included in one assay to exclude the possibility that 88K had formed disulfide-linked aggregates. In some cases, disulfide bridges between 88-kDa subunits have been detected by nonreducing SDS–PAGE when 88K had been dissociated from PDI by guanidine hydrochloride treatment. However, even in the presence of 1 mM DTT, MTP in 1.2 M guanidine hydrochloride did not retain its lipid transfer activity. It is not clear if the transient expression of transfer activity immediately following the addition of guan-

Table II: Effect of Guanidine Hydrochloride on the Spectroscopic Properties of the Microsomal TG Transfer Protein and Protein Disulfide Isomerase[a]

	molar ellipticity (222 nm)			wavelength of maximum fluorescence (nm)	
	MTP	PDI	88K (deduced)	MTP	PDI
control	−10300	−10700	−10100	340	340
guanidine hydrochloride	−8870	−7460	−9800	348	348

[a] The molar ellipticity at 222 nm was recorded for MTP and PDI at 25 °C. MTP and PDI were at a concentration of 90 and 100 μg/mL in sodium phosphate, pH 7.5, and 2 mM DTT, respectively. The wavelength of maximum fluorecence was determined at MTP and PDI concentrations of 22.5 and 25 μg/mL, respectively. An identical analysis was performed, except 1.2 M guanidine hydrochloride was included in the buffer. The molar ellipticity for 88K was deduced from that of MTP and PDI by using the relationship $[\theta]_{222}$ for MTP = [58 000/(58 000 + 88 000)]($[\theta]_{222}$ for PDI) + [88 000/(58 000 + 88 000)]($[\theta]_{222}$ for 88K).

idine hydrochloride to MTP represents MTP which had not been dissociated, or dissociated MTP which transiently expresses lipid transfer activity but is unstable. Regardless, it is clear that MTP transfer activity is not maintained following its dissociation by guanidine hydrochloride.

Spectroscopic Analysis of MTP in the Presence of Dissociating Agents. Following the addition of dissociating agents to MTP, the TG transfer activity is lost. To investigate the possibility that the loss of TG transfer activity was the result of MTP denaturation, rather than dissociation, the structure of MTP in the presence of the dissociating agents was investigated by circular dichroism and fluorescence spectroscopy. The secondary structure of MTP in the presence of 1.2 M guanidine hydrochloride was monitored by its molar ellipticity at 222 nm. Guanidine hydrochloride interferes with the CD measurement at lower wavelengths, thus preventing analysis of a full spectrum. The molar ellipticities at 222 nm of both MTP and conventionally purified PDI in the presence of 1.2 M guanidine hydrochloride are increased relative to the native proteins (Table II), indicative of a loss of ordered structure. Assuming that the circular dichroism spectrum of MTP is the sum of the spectra of PDI and 88K (Wetterau et al., 1991), the molar ellipticity of 88K in the presence or absence of guanidine hydrochloride may be calculated (see legend to Table II). This analysis indicates that the molar ellipticity at 222 nm for 88K does not change substantially in the presence of guanidine hydrochloride, suggesting there is little or no change in 88K secondary structure.

Native MTP has an intrinsic fluorescence emission peak at 340 nm. The 8-nm red shift observed for the wavelength of maximum emission of MTP in the presence of 1.2 M guanidine hydrochloride suggests that although the components of MTP are not denatured, they are more loosely folded. The red shift indicates the tryptophan residues are more exposed to the solvent. For comparison, the emission maximum of MTP which is completely denatured in 5.0 M guanidine hydrochloride is 358 nm. Sephacryl S-300 gel permeation chromatography of ^{125}I-MTP supports the suggestion that 88K is more loosely folded in the presence of 1.2 M guanidine hydrochloride. The 88-kDa component of MTP in the presence of 1.2 M guanidine hydrochloride elutes before intact MTP in the absence of guanidine hydrochloride (data not shown).

Circular dichroism spectroscopic analysis indicated that MTP in the presence of $NaClO_4$ had not been extensively denatured (see Figure 9 and Table III). However, a decrease in β structure (6% of total) and an increase in random

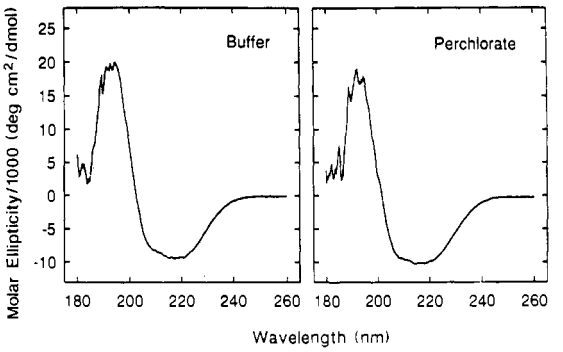

FIGURE 9: Circular dichroism spectra of MTP and MTP in the presence of sodium perchlorate. The molar ellipticity from 250 to 185 nm was recorded for MTP in 50 mM sodium phosphate, pH 7.5, and 1 mM DTT (left panel) or in 50 mM sodium phosphate, pH 7.5, 1 mM DTT, and 1.2 M sodium perchlorate (right panel). The MTP concentration was 100 μg/mL.

Table III: Spectroscopic Analysis of the Microsomal TG Transfer Protein and Protein Disulfide Isomerase in the Presence of Sodium Perchlorate or Octyl Glucoside[a]

	circular dichroism analysis				wavelength of maximum fluorescence (nm)
	α	β	turn	random	
MTP					
control	26.6	29.5	20.2	23.7	340
$NaClO_4$	28.1	23.5	17.5	31.0	343
octyl glucoside	19.0	27.2	22.6	31.2	336.5
PDI					
control	31.1	19.0	16.6	33.0	340.5
$NaClO_4$	31.4	18.1	16.4	34.1	341
octyl glucoside	32.2	18.3	16.0	33.4	341.5

[a] The circular dichroism spectra from 250 to 185 nm were recorded for MTP in 50 mM sodium phosphate, pH 7.5, and 1 mM DTT (control), or in 50 mM sodium phosphate, pH 7.5, 1 mM DTT, and 1.2 M $NaClO_4$ (see Figure 9), or in 50 mM sodium phosphate, pH 7.5, 1 mM DTT, and 30 mM octyl glucoside. The protein secondary structure contents were predicted by the method of McLean and Hagaman (1989). The fluorescence emission spectra were recorded, and the wavelengths of maximum emission were determined by visual inspection. The MTP concentrations were 100 and 20 μg/mL for the circular dichroism and fluorescence experiments, respectively. Identical analyses were performed with PDI.

structure (7.3%) were observed. For comparison, the structure of PDI in 1.2 M $NaClO_4$, as defined by CD analysis, was virtually identical with its structure in phosphate buffer. MTP in 1.2 M $NaClO_4$ has a fluorescence emission peak at 343 nm, indicating that the tryptophan residues reside in a slightly more polar environment than in native MTP. Sephacryl S-300 gel permeation column chromatography was used to confirm that the 88-kDa component of MTP in 1.2 M $NaClO_4$ is a free monomer. In the presence of 1.2 M $NaClO_4$, ^{125}I-88K elutes later than intact MTP in the absence of chaotropic agents (data not shown).

Spectroscopic characterization of the octyl glucoside treated MTP was complicated by the slight turbity which appeared in these samples. The 30% decrease in the absolute value of the molar ellipticity at 222 nm may in part reflect this turbidity. When the shape of the circular dichroism spectrum of MTP was used to predict the secondary structure content of MTP in the presence of octyl glucoside (see Materials and Methods), a decrease in α-helical structure (7.6%) and an increase in random structure (7.5%) were observed (Table III). This indicates that MTP had not been denatured by detergent treatment. No change in PDI structure was observed following an identical treatment with octyl glucoside. The fluorescence

emission spectrum of MTP was blue-shifted in the presence of octyl glucoside, indicating the tryptophan residues were in a more hydrophobic environment than in the native protein. This may reflect an aggregation of 88K in the presence of octyl glucoside (as is suggested by the sample turbidity).

Discussion

In this study, the role of PDI in the microsomal triglyceride transfer protein was addressed. Treatment of MTP with three different classes of dissociating reagents resulted in the dissociation of PDI from 88K and a coincident loss of TG transfer activity. In its free form, 88K readily aggregated, suggesting that it may be a very hydrophobic polypeptide. Even in the presence of chaotropic agents, when 88K is not aggregated, we were not able to detect lipid transfer activity. This latter experiment must be interpreted with caution as there is no positive control which demonstrated that the membranes were competent for TG transport in the presence of chaotropic agents. The chaotropic agents could be inhibiting lipid transport by their effect on the lipid bilayer, not the protein. Similar experiments were performed with guanidine hydrochloride. Although transfer activity could be detected immediately after the addition of MTP to 1.2 M guanidine hydrochloride, it was absent following prolonged incubations (25 min).

The loss of transfer activity following the dissociation of PDI from 88K could be due to (1) the loss of interfacial contact between PDI and 88K, (2) a change in 88K structure which results from its dissociation from PDI, or (3) a change in 88K structure which results from the agents used to dissociate the two components of MTP. Three different classes of dissociating agents (denaturants, chaotropic agents, and nondenaturing detergents) all produced similar results. This suggests that the dissociation, and not the agents themselves, was responsible for the loss of transfer activity and, ultimately, the aggregation of free 88K.

Our results indicate that PDI does have an important function in the lipid transfer protein. Clearly its interfacial contact with PDI is necessary to maintain the transfer protein in a nonaggregated and catalytically active form. Alternatively, PDI may have a more active role in the lipid transport process by contributing directly to the active site of the transfer protein. The possibility remains that following its dissociation from PDI, 88K can be stabilized in a form which expresses lipid transfer activity. However, before this could be accomplished, two problems have to be addressed: (1) the aggregation of free 88K must be prevented, and (2) minor conformational changes in 88K which accompany the dissociation of MTP components must be prevented. Although there appears to be some minor changes in 88K structure following its dissociation from PDI, the changes are not indicative of extensive polypeptide denaturation and may not account for the loss of transfer activity.

Protein disulfide isomerase is a multifunctional protein [for a review, see Freedman (1989)]. It would be tempting to speculate that it has a common role in the different protein complexes of which it is a part. The enzyme prolyl 4-hydroxylase is a tetramer consisting of two α and two β subunits [for a review, see Kivirikko et al. (1989)]. The β subunit appears identical with PDI. The exact role of PDI in prolyl 4-hydroxylase is not known; however, its presence is important for the expression of catalytic activity. Early evolutionary forms of prolyl 4-hydroxylase appear to have been monomers. The enzyme derived from algae is structurally related to the α subunit of the tetrameric enzyme (Kaska et al., 1988). By analogy, one could speculate that PDI has become associated with the 88-kDa protein which may have been a monomeric lipid transfer protein in an earlier evolutionary form. All previously characterized lipid transfer proteins have been identified as single polypeptides [for reviews, see Wirtz (1982) and Helmkamp (1986)].

There are some distinct differences between the association of PDI with 88K in MTP and the association of PDI with the α subunit of prolyl 4-hydroxylase. Although both enzymes are inactivated by chaotropic agents [data presented here and in Chen-Kiang et al. (1977)], prolyl 4-hydroxylase but not MTP is dissociated into inactive monomers in low ionic strength buffers or in 1 mM dithiothreitol (Berg & Prockop, 1973). Following its dissociation by dithiothreitol, prolyl 4-hydroxylase can be reassociated into an active tetramer by removing the reducing reagent (Tuderman et al., 1977). Unlike prolyl 4-hydroxylase, PDI and 88K apparently cannot reassociate following their dissociation [data presented here and in Wetterau et al. (1991)].

The inability of free PDI and 88K to reassociate (even in the presence of excess PDI, data not shown) implies that in vivo the processing of PDI and 88K may lead to their complex formation. Following the removal of $NaClO_4$, KSCN, or guanidine hydrochloride in vitro, the rate of 88K aggregation exceeds the rate of PDI–88K reassociation. The slight turbidity observed for MTP treated with octyl glucoside (but not PDI) suggests 88K may begin to aggregate even in the presence of detergent. In vivo, 88K may be added cotranslationally to the preexisting pool of free PDI present in the lumen of the endoplasmic reticulum. This would be analogous to the assembly of prolyl 4-hydroxylase which occurs by the addition of the α subunit to the preexisting pool of PDI (Berg et al., 1980). Alternatively, another component, perhaps a chaperone protein, may participate in the assembly of MTP. Given the strong tendency of free 88K to aggregate, it seems unlikely it would exist as a free precursor monomer prior to its association with PDI.

Protein disulfide isomerase can catalyze the proper folding of disulfide-bonded proteins. It is one of many proteins which are believed to play a role in promoting the proper folding of newly synthesized proteins. Within the lipid transfer protein, it also appears to play a vital structural role. In addition to the ability of PDI to help a protein aquire its proper structure, in the case of MTP and possibly other protein complexes, it appears that PDI may play a role in maintaining the active structure.

Recently, Morishima et al. (1990) reported that the 75-kDa subunit of yeast site-specific endonuclease *SceI* is a 70-kDa heat shock protein related polypeptide. To express full catalytic activity, both the 75- and 50-kDa subunits of this heterodimer enzyme are required. Heat shock proteins, like PDI, promote the proper folding or assembly of proteins in vivo [for a review, see Rothman (1989)]. This observation, in conjunction with the results presented here for PDI, suggests that proteins which play a role in the folding or assembly of proteins may have dual roles within a cell. They may also function as permanent components of heterooligomeric enzymes.

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Study of a Hammerhead Ribozyme Containing 2'-Modified Adenosine Residues†

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ABSTRACT: The improved synthesis of 2'-fluoro-2'-deoxyadenosine (2'-FA) starting from adenosine is described. This compound was converted to the phosphoramidite and incorporated into a hammerhead ribozyme RNA with the use of automated RNA synthesis techniques. Ribozymes containing 2'-deoxyadenosine (2'-dA) were prepared in a similar manner. A kinetic rate comparison of the unmodified ribozyme with two ribozymes that had every adenosine replaced with 2'-FA or 2'-dA revealed a large decrease in catalytic efficiency (k_{cat}/K_m) for the modified ribozymes resulting from a drop in k_{cat}. The kinetic analysis of a number of partially substituted 2'-FA or 2'-dA containing hammerheads revealed that the decrease in activity was not associated with any particular residue but was the result of the accumulation of modified nucleosides within the structure.

Several different satellite RNAs, associated with a number of plant viruses, can form a hammerhead ribozyme structure that undergoes self-catalyzed cleavage yielding an RNA with a 5'-OH and an RNA with a 2',3'-cyclic phosphate [for reviews see Bruening (1989) and Sheldon et al. (1990)]. The study of this cleavage reaction is of great interest for two reasons. First, the catalytic mechanism of this novel intramolecular RNA self-cleavage reaction is poorly understood. Second, these compounds are considered to be potential therapeutic agents targeted against viral diseases such as hepatitis B and AIDS (Cotten, 1990; Rossi & Sarver, 1990; Sarver et al., 1990).

Recent structure–function studies of hammerhead ribozymes have explored the effect of incorporation of phosphorothioate groups into the catalytic RNA (Buzayan et al., 1988, 1990; Ruffner & Uhlenbeck, 1990). This research has revealed that

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