# Structural Properties of the Microsomal Triglyceride-Transfer Protein Complex†

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*Received October 25, 1990; Revised Manuscript Received February 2, 1991*

ABSTRACT: The microsomal triglyceride-transfer protein (MTP), which catalyzes the transport of triglyceride and cholesteryl ester between membranes, is a complex composed of two proteins having apparent molecular weights of 58 000 and 88 000. The 58 000 molecular weight component of MTP has been identified as the multifunctional protein, protein disulfide isomerase (PDI). The multisubunit nature of MTP as well as the presence of PDI as one of the subunits distinguishes this protein from previously characterized lipid-transfer proteins. In this study, we have more clearly defined structural elements of MTP that may play important functional roles. The molecular weight of the transfer protein complex was determined to be 150 000 by sedimentation equilibrium experiments performed at three different speeds, suggesting that MTP is a complex of one PDI and one 88 000 molecular weight polypeptide (88K). Following SDS–polyacrylamide gel electrophoresis, the Coomassie Blue staining intensity of PDI in a known amount of MTP was compared to that of known amounts of a PDI standard. A 1 to 0.98–1.30 ratio of PDI to 88K was determined, confirming the 1:1 stoichiometry of MTP. The sedimentation coefficient (5.85) determined by analytical ultracentrifugation and the Stokes radius (47 Å) determined by polyacrylamide gradient gel electrophoresis indicate that the 150 000 molecular weight MTP complex is asymmetric and/or has an unusually high water of hydration. PDI and 88K form a stable protein complex; there was no evidence of a dissociation–reassociation reaction occurring between the two components. Analysis of far-ultraviolet circular dichroism spectra revealed MTP has about 28% $\alpha$-helical and 28% $\beta$-structural content. PDI appeared to be relatively rich in $\alpha$-helical and random structure (31 and 34%, respectively), while 88K appeared rich in $\beta$ structure (35%). The stability of MTP and its components was investigated by guanidine hydrochloride denaturation as monitored by circular dichroism spectroscopy. MTP was resistant to denaturation up to 0.8 M guanidine hydrochloride. PDI structure appeared to be stabilized by interaction with 88K. The 88-kDa subunit appeared stable at 1.2 M guanidine hydrochloride, when it is dissociated from PDI. Both components of MTP had broad denaturation transitions, indicative of stable folding intermediates. Collectively, these data imply that PDI is an important integral part of the lipid-transfer protein.

The microsomal triglyceride-transfer protein complex (MTP)[1] catalyzes the transport of triglyceride (TG), cholesteryl ester (CE), and phosphatidylcholine (PC) between membranes. By utilization of synthetic membrane substrates, MTP expresses a distinct preference for neutral-lipid and, in particular, triglyceride transport, relative to polar-lipid transport (Wetterau & Zilversmit, 1984). MTP has a unique structure when compared to previously characterized lipid-transfer proteins in that it is a high molecular weight protein complex composed of two components having apparent molecular weights of 58 000 (58K) and 88 000 (88K) (Wetterau & Zilversmit, 1985; Wetterau et al., 1990). Intracellular lipid-transfer proteins, which typically catalyze the transport of polar lipids, are single polypeptides with molecular weights ranging from 8000 to 35 000 (Wirtz, 1982; Helmkamp, 1986). An extracellular protein that catalyzes the transport of neutral lipids, the cholesteryl ester transfer protein (CETP), is found in the plasma of several mammalian species, including humans. Human CETP is a glycoprotein with an apparent molecular weight of 74 000 (Hesler et al., 1987; Jarnagin et al., 1987) that contains a 53 000 molecular weight polypeptide backbone (Drayna et al., 1987).

The 58-kDa subunit of MTP was recently identified as protein disulfide isomerase (PDI) by amino-terminal sequence analysis, peptide mapping experiments, immunochemical characterization of MTP, and the expression of disulfide isomerase activity following the dissociation of the 58-kDa and 88-kDa subunits of MTP (Wetterau et al., 1990). PDI is believed to normally play a role in the proper folding of newly synthesized disulfide-bonded proteins within the lumen of the endoplasmic reticulum by catalyzing the correct formation of disulfide bonds (Bulleid & Freedman, 1988). PDI has also

† This work was supported in part by Grant HL 40993 from the National Institutes of Health and by a grant from the Institut National de la Santé et de la Recherche Médicale (INSERM, France).

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[1] Abbreviations: MTP, microsomal triglyceride-transfer protein or microsomal lipid-transfer protein; 58K, the subunit of the microsomal lipid-transfer protein that has a molecular weight of 58 000; 88K, the subunit of the microsomal lipid-transfer protein that has a molecular weight of 88 000; PDI, protein disulfide isomerase; CETP, plasma cholesteryl ester transfer protein; TG, triglyceride; CE, cholesteryl ester; PC, phosphatidylcholine; $R_s$, Stokes radius; CD, circular dichroism.

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been reported to be the $\beta$ subunit of prolyl 4-hydroxylase (Koivu et al., 1987) and to be highly similar to the glycosylation site binding protein, a component of oligosaccharyl transferase (Geetha-Habib et al., 1988).

The multisubunit nature of the microsomal TG-transfer protein complex as well as the presence of PDI in the transfer protein complex raises questions regarding the structure–function relationships in this unusual lipid-transfer protein. In this study, the physical–chemical characteristics of the microsomal transfer protein complex were examined as a prelude to further assessing the role of the individual components of MTP in the lipid-transport process. The stoichiometry of the protein complex and the secondary structure content of the complex and its individual components were determined. Insight into the tertiary structure was obtained through stability measurements and hydrodynamic characterization. The nature of the interaction between the components was also assessed.

## Materials and Methods

*Protein Isolation.* The microsomal triglyceride-transfer protein complex was isolated as described previously (Wetterau et al., 1990). Briefly, a bovine whole liver homogenate was prepared and the microsomal fraction was isolated. The microsomes were suspended in 1 mM Tris, pH 8.6, to release the soluble lumenal proteins. MTP was purified from the soluble proteins by DEAE-Sephacel, Sephadex G-200, DEAE-cellulose, Sephacryl S-300, and hydroxylapatite column chromatography. 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 described previously (Wetterau et al., 1990).

Protein disulfide isomerase was isolated from bovine liver by a modification of the procedure of Hillson et al. (1984) as previously described (Wetterau et al., 1990). Briefly, the lumenal proteins of bovine microsomes were isolated as described above for the isolation of MTP. PDI was further purified by ammonium sulfate precipitation, cation-exchange chromatography, and anion-exchange chromatography. Protein disulfide isomerase activity was measured by the ability of PDI to refold and activate reduced and denatured ribonuclease as described previously (Wetterau et al., 1990).

Free PDI has a more acidic isoelectric point than the MTP protein complex (Lambert & Freedman, 1983; Wetterau & Zilversmit, 1985). As a result, PDI and MTP are readily separated by DEAE-Sephacel ion-exchange chromatography. MTP elutes in 10 mM sodium phosphate, pH 7.0/175 mM NaCl, while PDI requires 225 mM NaCl for elution.

*Analytical Ultracentrifugation.* The ultracentrifugation studies were performed at 20 °C in an MSE Centriscan 75 (MSE, Crawley, Sussex, U.K.) analytical ultracentrifuge equipped with an ultraviolet absorption detector. Prior to the experiments, the samples were dialyzed to equilibrium against sample buffer. Three different MTP preparations were used: preparation A, 0.30 mg/mL in 20 mM sodium phosphate, pH 7.4/100 mM NaCl/1 mM ethylenediaminetetraacetate/0.02% $NaN_3$; preparation B, 0.62 mg/mL in 50 mM sodium phosphate, pH 7.5/0.02% $NaN_3$; preparation C, 0.43 mg/mL in 50 mM sodium phosphate, pH 7.5/0.02% $NaN_3$/0.5 mM dithiothreitol.

Meniscus-depletion sedimentation equilibrium (Yphantis, 1964) experiments were performed at 10000, 15000, and 20000 rpm. The optical density was recorded as a function of the distance from the center of rotation ($r$). Regression analysis was used to fit the logarithm of the protein concentration ($c$) against $r^2$. From the slope, the apparent molecular weight ($M_a$) was calculated from

$$M_a = \frac{2RT(2.303)}{(1 - \bar{v}\rho)\omega^2} \frac{d \log c}{dr^2} \quad (1)$$

Here, $\bar{v}$ is the partial specific volume of MTP, assumed to be 0.74 cm$^3$/g, $\rho$ is the density of the solution (for low concentrations this is approximately equal to that of the solvent), $R$ is the gas constant, $T$ is the absolute temperature, and $\omega$ is the angular rotor velocity.

Sedimentation velocity experiments were performed with MTP preparations A and B at 60000 rpm. The weight-average sedimentation coefficient was calculated from

$$\frac{d \ln r^*}{dt} = \omega^2 \bar{s} \quad (2)$$

where $r^*$ is the second-moment position of the sedimenting MTP complex. The $\bar{s}$ values were corrected for the density and the viscosity of the buffer relative to water at 20 °C to give $\bar{s}_{20,w}$ values.

The apparent molecular weight is related to the sedimentation coefficient in

$$M_a = \frac{Nf\bar{s}_{20,w}}{1 - \bar{v}\rho} \quad (3)$$

where $f$ is the frictional coefficient. The frictional coefficient equals $6\pi\eta R_s$, where $R_s$ is the Stokes radius and $\eta$ is the solvent viscosity.

The true Stokes radius and $R_{min}$, the minimum radius of the particle calculated from the molecular weight and the partial specific volume of the protein, can be used to calculate the frictional ratio, $f/f_0$, from

$$f/f_0 = R_s/R_{min} \quad (4)$$

The frictional ratio is a measure of the protein asymmetry.

*Circular Dichroism Spectra.* Circular dichroism (CD) spectra of samples in 1-mm circular cuvettes were recorded at room temperature on a Jasco J-500A Spectropolarimeter with a 2-nm slit width. The CD spectrum of air was subtracted from the CD spectrum of the sample after each scan. It was determined independently that the buffers made no contribution to the CD spectra. 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, using the reference data of Bolotina et al. (1980) and Yang et al. (1986).

*Additional Techniques.* Protein concentrations were determined by the method of Lowry et al. (1951) with use of bovine serum albumin as a standard. For the gel electrophoresis mass assay of PDI, aliquots of PDI or MTP were hydrolized for 20 h in 6 M hydrochloride at 110 °C and the protein concentrations were estimated by amino acid analysis with the Waters Pico-Tag system. Tryptophan and cysteine contents were not determined. The results agreed within 11% with those obtained by the method of Lowry et al. (1951).

$^{125}$I-PDI was prepared by the method of Bolton and Hunter (1973) with use of commercially available $^{125}$I-Bolton–Hunter reagent (DuPont, New England Nuclear Research Products). The 4–30% polyacrylamide gradient gels were obtained from Pharmacia LKB Biotechnology Inc. and run according to the manufacturer's instructions. Sodium dodecyl sulfate–polyacrylamide (8%) gel electrophoresis was performed by the

method of Laemmli (1970). Nondenaturing polyacrylamide (8%) gel electrophoresis was performed by the method of Davis (1964) except that stacking gels were omitted.

## Results

*Stoichiometry and Shape of the Microsomal Triglyceride-Transfer Protein Complex.* As compared to previously characterized lipid-transfer proteins, the microsomal triglyceride-transfer protein has a high molecular weight and an unusual multisubunit structure. To determine the molecular weight and the subunit composition of the transfer protein complex, meniscus-depletion sedimentation equilibrium experiments were performed at 10000, 15000, and 20000 rpm at initial MTP concentrations of 0.30–0.62 mg/mL. Three different MTP preparations were used for these determinations. At all speeds, a plot of the logarithm of the protein concentration versus the square of the distance from the center of rotation was linear. The calculated apparent molecular weight from eight sedimentation equilibrium measurements was 150000 ± 14000 (± SD). This suggests that MTP is formed of a 1:1 complex of PDI and 88K (with a calculated molecular weight of 146000).

The 1:1 stoichiometry of MTP was confirmed by an independent approach. MTP (0–1.0 μg) was electrophoresed on a dissociating SDS–polyacrylamide gel and stained with Coomassie Blue. Stained gels were scanned, and the area of the PDI and 88K peaks was determined by triangulation. Within MTP, the ratio of Coomassie Blue staining intensity for PDI and 88K was 1.0 to 1.6 ± 0.1 (±SD for four different MTP concentrations). If it is assumed that PDI and 88K have equal staining intensity per mass of protein, then these results suggest that PDI and 88K form a protein complex with a 1:1 mole ratio.

A mass assay for PDI was also developed and used to estimate the mass of PDI in MTP. Increasing concentrations of PDI or MTP (which had been dissociated into PDI and 88K by treatment with SDS) were electrophoresed on an SDS–polyacrylamide gel, stained with Coomassie Blue, and scanned. A linear standard curve of peak area versus PDI concentration (in the range of 0–1 μg of PDI) was established. The PDI mass in MTP was estimated by comparing the area of the PDI peak in a known mass of MTP to that of the standard PDI curve. The calculated mole ratio of PDI to 88K was 1.0 to 1.14 ± 0.16 (±range for two different experiments).

Having established the stoichiometry of the protein complex, we wanted to learn more about the shape of the complex. At initial MTP concentrations of 0.30 and 0.62 mg/mL, the sedimentation coefficients were 5.85 and 5.83 × $10^{-13}$ $s^{-1}$, respectively. This sedimentation coefficient is low for a protein of mass 150000 and implies that MTP has an unusually high water of hydration or asymmetry or both. This was confirmed by gradient gel electrophoresis. The limiting electrophoretic mobility of MTP was compared to that of standard proteins of known Stokes radii (see Figure 1). The apparent Stokes radius of MTP was 47.0 ± 0.3 Å. This Stokes radius is large for a protein of molecular weight 150000, again suggesting the presence of an unusually high water of hydration or asymmetry.

*Interaction between PDI and 88K.* Knowing that PDI and 88K form an asymmetric protein complex with a 1:1 stoichiometry, we wanted to develop an understanding of the interaction between the two components of MTP. PDI and 88K appear to be tightly associated with one another, which allows for their coisolation through a five-step column chromatography purification (two steps performed in the presence of low concentrations of denaturants). To investigate the strength of the subunit interaction, MTP was treated with increasing concentrations of guanidine hydrochloride, the guanidine hydrochloride was removed by dialysis, and the protein complex was characterized by nondenaturing polyacrylamide gel electrophoresis. At concentrations of guanidine hydrochloride above 0.4 M, progressively less protein with an electrophoretic mobility of MTP was recovered, suggesting that the transfer protein complex dissociated irreversibly (see Figure 2). At 1.2 M guanidine hydrochloride, native MTP was no longer apparent. In this gel, free PDI appears as a diffuse band with a mobility faster than that of MTP. Although not evident on the gel in Figure 2, 88K appears to aggregate following guanidine hydrochloride treatment and not enter the resolving gel. The 88K polypeptide from guanidine hydrochloride treated MTP does not elute from a Sephacryl S-300 gel permeation column, again suggesting that it had formed a protein aggregate.

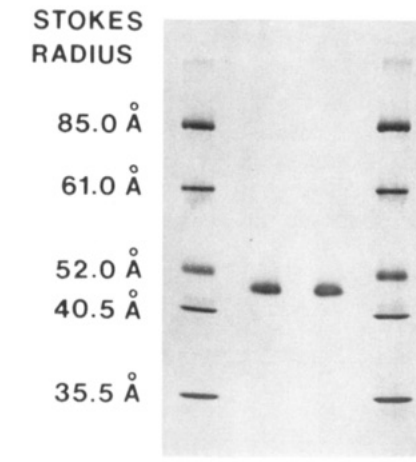


FIGURE 1: Gradient gel electrophoretogram of MTP and standard proteins. MTP was electrophoresed to its limiting migration within a 4–30% polyacrylamide gradient gel and visualized by Coomassie Blue staining. Thyroglobulin, ferritin, catalase, lactic dehydrogenase, and bovine serum albumin were used to determine the relationship between electrophoretic mobility and the Stokes radius (85.0, 61.0, 52.0, 40.5, and 35.5 Å, respectively, for the standard proteins). Standard proteins were run on the two outside lanes and MTP was run on the two center lanes.

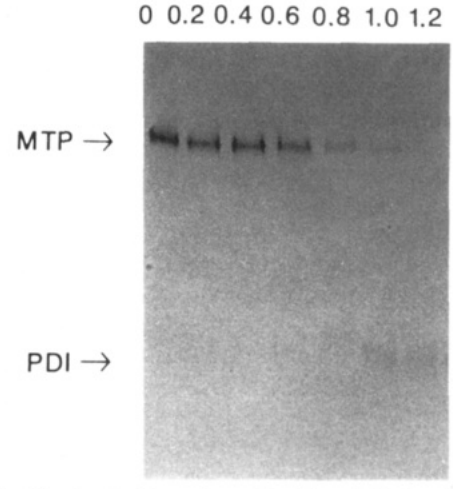


FIGURE 2: Nondenaturing polyacrylamide gel electrophoretogram of MTP following treatment with guanidine hydrochloride. MTP was adjusted to a concentration of 150 μg/mL in 50 mM sodium phosphate, pH 7.5, supplemented with 0, 0.2, 0.4, 0.6, 0.8, 1.0, or 1.2 M guanidine hydrochloride. The guanidine hydrochloride was removed by dialysis versus 50 mM sodium phosphate, pH 7.5, and an aliquot of the recovered protein was electrophoresed on a nondenaturing polyacrylamide gel. Protein was visualized by Coomassie Blue staining. The mobility of MTP and PDI is indicated in the left margin.

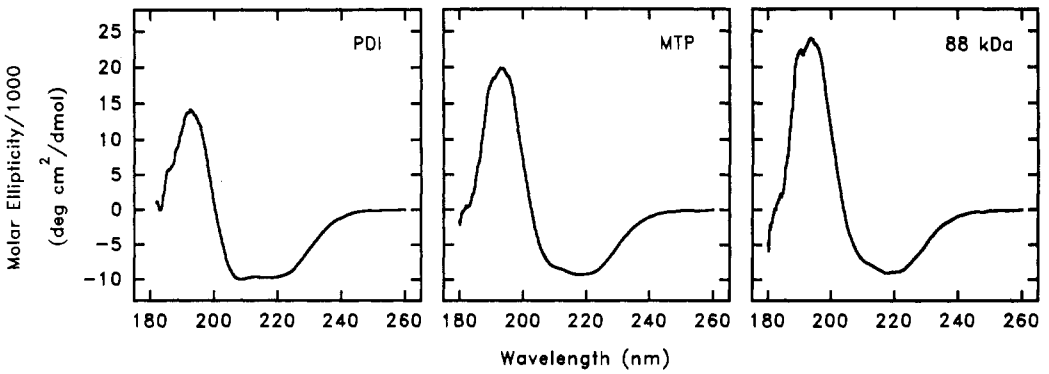


FIGURE 3: Circular dichroism spectra of protein disulfide isomerase, the microsomal triglyceride-transfer protein complex, and 88K. The molar ellipticity measured at 25 °C is plotted as a function of wavelength for PDI (left panel) and MTP (center panel). The protein concentrations were 0.114 and 0.100 mg/mL, respectively, in 50 mM sodium phosphate, pH 7.5. Assuming the secondary structure of PDI is the same in its free and 88K-associated state, the circular dichroism spectra for 88K was deduced from the spectra of PDI and MTP (right panel).

To investigate the dissociation–reassociation equilibrium of MTP in the absence of dissociating reagents, the rate of incorporation of exogenous $^{125}$I-PDI into the transfer protein complex was determined. This experimental approach is analogous to the hybridization technique used by Yang and Schachman (1987) to investigate similar dissociation–association reactions in aspartate transcarbamoylase. A 5-$\mu$g sample of MTP was incubated with 5 $\mu$g of $^{125}$I-PDI (40 000 cpm) for 1 to 7 days in 50 mM sodium phosphate, pH 7.5/0.02% $NaN_3$. At varying times, an aliquot of the incubation sample was fractionated by nondenaturing polyacrylamide gel electrophoresis and the $^{125}$I-PDI incorporated into the band corresponding to MTP was quantitated. If the PDI–88K protein complex has a measurable dissociation–reassociation rate, then a portion of the exogenous $^{125}$I-PDI should migrate with MTP. When MTP and free $^{125}$I-PDI were incubated at 25 or 4 °C for up to 7 days, less than 4% of the PDI in MTP was replaced by $^{125}$I-PDI. This suggests either that the transfer protein complex has a very slow rate of dissociation or that the dissociated complex cannot reassociate to the native structure. A variety of conditions were tried in an attempt to incorporate exogenous $^{125}$I-PDI into MTP. Experiments were performed in which low concentrations of a chaotropic agent (0.25 M KSCN), a denaturant (0.25 M guanidine hydrochloride), or small unilamellar vesicles, a substrate in a lipid transfer reaction, were included in the incubations. Still, no significant replacement of the PDI component of MTP was observed.

*Guanidine Hydrochloride Denaturation of MTP.* Since the two components of MTP appear to be tightly associated, we attempted to examine in more detail this interaction by denaturation experiments with guanidine hydrochloride. As a first step, the secondary structure of MTP and its two components was investigated by far-ultraviolet circular dichroism spectroscopy. The shapes of the circular dichroism spectra for PDI and MTP (Figure 3) are very similar. One notable exception is the region around 200 nm where the spectra change from negative to positive values. This region is highly sensitive to the $\beta$ structure of the protein (Yang et al., 1986). The higher molar ellipticity in this region of the MTP spectra suggests that it is enriched in $\beta$ structure relative to PDI. With the knowledge that the transfer protein complex has one PDI and one 88K per protein complexes, the CD spectra of 88K could be calculated (Figure 3). This assumes that the structure of PDI in its free state is identical with its structure in the transfer protein complex. In this case, the MTP spectra would be a linear combination of the contributions of PDI and 88K.

The secondary structure of MTP and its two components was estimated by comparing their CD spectra to spectra of proteins of known structures according to the method of McLean and Hagaman (1989). MTP has about equal $\alpha$- and $\beta$-structure content, with slightly less turn and random structure (see Table I). PDI is relatively rich in $\alpha$-helical structure (31.3 ± 1.8%) while 88K is relatively rich in $\beta$ structure (35.0 ± 2.7%).

Table I: Predicted Secondary Structure Content of MTP, PDI, and 88K from Circular Dichroism Analysis

| | $\alpha$ | $\beta$ | turn | random |
|---|---|---|---|---|
| MTP[a] | 27.7 ± 1.3[b] | 28.2 ± 1.5 | 19.5 ± 0.7 | 24.6 ± 0.9 |
| PDI[a] | 31.3 ± 1.8 | 18.0 ± 0.8 | 17.2 ± 0.8 | 33.5 ± 3.0 |
| 88 K[c] | 25.4 ± 1.2 | 35.0 ± 2.7 | 21.0 ± 1.7 | 18.7 ± 3.3 |

[a] The secondary structure content of MTP and PDI was determined from the circular dichroism spectra. [b] Average ± SEM for three different MTP and PDI preparations. The protein concentrations for the three samples approximated 0.1 mg/mL in 50 mM sodium phosphate, pH 7.5, and 0, 1, or 2 mM dithiothreitol. [c] The secondary structure content of 88K was determined from the deduced 88K spectra (see legend to Figure 3 for details).

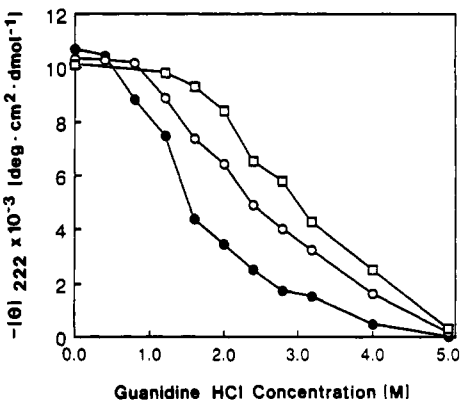


FIGURE 4: Relationship between the molar ellipticity, [$\theta$], at 222 nm of MTP, PDI, and 88K and the guanidine hydrochloride concentration. The molar ellipticity at 222 nm of MTP (○) or PDI (●) at concentrations of 0.090 and 0.100 mg/mL, respectively, in 50 mM sodium phosphate, pH 7.5, 0.02% $NaN_3$, 2 mM dithiothreitol, and increasing concentrations of guanidine hydrochloride was determined at 25 °C. Assuming that the denaturation of MTP-derived PDI is the same as free PDI at guanidine hydrochloride concentrations of 1.2 M or greater (when the two components of MTP are dissociated), the relationship between molar ellipticity and guanidine hydrochloride concentration for 88K (□) was deduced.

The structural stabilities of MTP and its individual components were investigated by following the increase in their molar ellipticities at 222 nm as a function of the guanidine hydrochloride concentration of the solution (see Figure 4). An increase in molar ellipticity is indicative of a loss in secondary structure. No change in the structure of MTP was detectable

at concentrations of guanidine hydrochloride up to 0.8 M. At higher concentrations, there was a broad transition from an ordered structure to a random structure (molar ellipticity approximates zero) at 5.0 M guanidine hydrochloride. The transition for PDI denaturation started at guanidine hydrochloride concentrations greater than 0.4 M. PDI was more sensitive to denaturation than MTP. At 1.6 M guanidine hydrochloride, the absolute value of the molar ellipticity of PDI was approximately half its initial value. For comparison, the midpoint for the denaturation transition of MTP was 2.4 M guanidine hydrochloride.

At guanidine hydrochloride concentrations greater than 1.0 M, the two components of MTP are dissociated (see Figure 2) and each component would denature independently of subunit interactions (see Figure 4). From the same assumptions as those used to calculate the CD curve of the 88-kDa subunit, the molar ellipticity of 88K as a function of guanidine hydrochloride concentration can be determined. The 88-kDa component of MTP appears more resistant to denaturation than PDI. The molar ellipticity of 88K is approximately half its initial value at 3 M guanidine hydrochloride. At 1.2 M guanidine hydrochloride, when the two subunits of MTP are dissociated, there is little evidence of 88K denaturation. In contrast to PDI, which regains its activity following denaturation, the denaturation of 88K and MTP is not reversible because 88K aggregates upon the removal of guanidine hydrochloride, rather than reassociating with PDI to form intact, functional MTP.

Both PDI and 88K have broad denaturation transitions, indicative of weakly cooperative transitions. The denaturation of 88K occurs over a 4 M guanidine hydrochloride concentration range. These broad transitions suggest the presence of independently folded domains within each polypeptide.

Almost no loss of MTP structure is detectable at 0.8 M guanidine hydrochloride, in contrast to PDI, which had a 17% decrease in the absolute value of the molar ellipticity. This reveals that the interfacial interaction between PDI and 88K stabilizes PDI secondary structure. In contrast, the interaction between PDI and 88K had no apparent effect on the stability of 88K. The molar ellipticity of 88K in MTP in the absence of guanidine hydrochloride (Figure 4) was almost identical with that of free 88K in 1.2 M guanidine hydrochloride (−9800 compared to −10100).

## Discussion

The microsomal triglyceride-transfer protein has an unusual structure when compared to previously characterized lipid-transfer proteins. Most notably, it is a complex of two proteins of molecular weights 58000 and 88000. The 58000 molecular weight component has been indentified as protein disulfide isomerase. Isolated PDI has no lipid-transfer activity (Wetterau et al., 1990), implying that 88K is the lipid-transfer protein or that this protein, when in a complex with PDI, confers transfer activity to the protein complex.

As an initial step toward understanding the structure–function relationships in this unusual lipid-transfer protein, we wanted to further our understanding of MTP structure. Sedimentation equilibrium experiments and PDI mass analysis established that MTP contains one PDI and one 88K per protein complex. The protein complex has a molecular weight of 150000. The hydrodynamic properties of MTP were further examined by polyacrylamide gradient gel electrophoresis and sedimentation velocity. The Stokes radius of 47 Å as determined by gradient gel electrophoresis is somewhat large for a protein of molecular weight 150000. If 0.3 g of $H_2O$/g of protein hydration is assumed for a spherical protein complex having this measured Stokes radius, the calculated molecular weight is 253000. In contrast, the sedimentation coefficient of $5.8 \times 10^{-13}$ s$^{-1}$ is more consistent with a protein having a molecular weight smaller than the 150000 determined by sedimentation equilibrium. For example, yeast enolase, a 90000 molecular weight protein, has a sedimentation coefficient of 5.90. Lactic dehydrogenase (molecular weight 136000), alcohol dehydrogenase (141000), glyceraldehyde 3-phosphate dehydrogenase (145000), and aldolase (156000) have sedimentation coefficients of 7.45, 7.61, 7.60, and 7.35, respectively (Brewer et al., 1974).

The apparent contradiction between the sedimentation velocity and polyacrylamide gradient gel electrophoresis experiments can best be explained if MTP has an abnormally high water of hydration or is asymmetric. The sedimentation coefficient is inversely proportional to the Stokes radius. An increased water of hydration or asymmetry would increase the Stokes radius, and the resultant sedimentation coefficient would be lower than expected. Assuming no water of hydration, an $f/f_0$ of 1.6 can be calculated from the sedimentation coefficient and the molecular weight. This would correspond to an ellipsoid axial ratio ($a/b$) of around 11–14 to 1. Alternatively, if it is assumed that MTP is spherical, then the calculated maximum water of hydration is 2.4 g of $H_2O$/g of protein.

In a polyacrylamide gradient electrophoresis gel, MTP migrates until its smallest dimension prevents further migration through the gel pores (Felgenhauer, 1974). Although the Stokes radius determined by this method is not a true hydrodynamic parameter, it does suggest that the Stokes radius is higher than expected for a protein of molecular weight 150000. These results are consistent with our previous estimate of 220000 for the molecular weight of MTP as determined by its elution position on a calibrated Sephadex G-200 gel permeation column (Wetterau & Zilversmit, 1985). This previous analysis assumed that MTP had the same globular shape as that of the proteins used to calibrate the column. Interestingly, a similar discrepancy was reported for the structural characterization of bovine liver protein disulfide isomerase. Gel filtration studies indicated that PDI was a homodimer with an apparent molecular weight of 107000 while the sedimentation coefficient of 3.55 was more consistent with monomer PDI (Lambert & Freedman, 1983). Electron microscopy of PDI isolated from chick embryo prolyl 4-hydroxylase revealed PDI to be rod-shaped with a diameter of 3.3 nm and a length of 7.0 nm (Olsen et al., 1973).

Protein disulfide isomerase has a transient association with numerous proteins as it catalyzes their folding. We wanted to investigate whether the association of PDI with 88K was also transient or if these two proteins formed a stable, tightly associated protein complex. To determine if PDI–88K undergoes an association–dissociation reaction, the hybridization technique of Yang and Schachman (1987) was employed. Appreciable exogenous PDI could not be exchanged into the lipid-transfer protein complex within a seven-day exchange reaction. This could be explained by assuming that the two components of MTP have a high affinity for each other or that once the two components are dissociated, they cannot reassociate. In the latter case, one would expect a dramatic decrease in the transfer protein complex with time. This has not been observed, suggesting that PDI–88K forms a highly stable protein complex. The absence of $^{125}$I-PDI exchange, even in the presence of lipid vesicles, also suggests that the intact PDI–88K protein complex is the catalytically active form of the transfer protein. If the two components transiently dis-

sociated during lipid transport, one would expect PDI exchange to occur in the presence of lipid vesicles. This was not observed. PDI can readily be dissociated from 88K with low concentrations (0.6–1.2 M) of guanidine hydrochloride (Figure 2). Previously, it has been demonstrated that PDI and 88K are not covalently linked (Wetterau & Zilversmit, 1985).

Prolyl 4-hydroxylase, which catalyzes the formation of 4-hydroxylproline in collagens, is another enzyme complex that contains PDI as a subunit. It is a tetramer consisting of two $\alpha$ subunits and two molecules of PDI (referred to as $\beta$ subunits) with an apparent molecular weight of 240 000 [for review see Kivirikko et al. (1989)]. The four subunits can be dissociated into inactive free monomers by exposing prolyl 4-hydroxylase to low ionic strength buffers or 1 mM dithiothreitol (Berg & Prockop, 1973). In isolated chick embryo tendon cells, the subunits of prolyl 4-hydroxylase are dissociated by treating the cells with 0.45 M dithiothreitol and the catalytic activity is lost. Following removal of the dithiothreitol, the $\alpha$ subunit and PDI reassociate and the catalytic activity is recovered (Tuderman et al., 1977). This suggests that the interaction between PDI and the $\alpha$ subunit of prolyl 4-hydroxylase is dependent upon the conformation of the two subunits. The conformation required to maintain the association appears to be preserved by an intramolecular disulfide bond (or bonds) in PDI or in the $\alpha$ subunit. In the case of MTP, neither low ionic strength buffers (which were used in the purification of MTP) nor dithiothreitol (which was used in some sedimentation equilibrium experiments) dissociates PDI from 88K.

The assembly of prolyl 4-hydroxylase in vivo occurs by the addition of the $\alpha$ subunit to the preexisting pool of excess free PDI in the lumen of the rough endoplasmic reticulum. On the basis of pulse–chase studies, there does not appear to be an association–dissociation reaction occurring between the components of prolyl 4-hydroxylase, as no equilibration of preexisting PDI with newly synthesized PDI occurs in prolyl 4-hydroxylase (Berg et al., 1980). The purification factors necessary to purify PDI and MTP to apparent homogeneity [7–19- and approximately 100-fold for PDI and MTP, respectively (Wetterau et al., 1990)] indicate that free PDI is also present in excess over MTP in the endoplasmic reticulum of bovine liver. The finding that PDI in MTP does not exchange with exogenous $^{125}$I-PDI suggests that the PDI in MTP also may not equilibrate with free PDI in vivo and that 88K may associate irreversibly with PDI immediately following the synthesis of 88K.

The tight association of 88K with PDI has implications regarding the intracellular transport of 88K. The carboxy-terminal sequence of PDI, Lys-Asp-Glu-Leu, is thought to play a role in the retention of proteins within the lumen of the endoplasmic reticulum (Munro & Pelham, 1987). A receptor for proteins with this carboxy-terminal retention sequence has recently been identified (Vaux et al., 1990). It is proposed that this receptor within a salvage compartment binds proteins with the retention sequence and returns them to the endoplasmic reticulum. The tight association of 88K with PDI may play a role in targeting 88K for retention within endoplasmic reticulum.

MTP has approximately 55% $\alpha$ and $\beta$ structure. To examine the structure of the individual components of MTP, we have assumed that native PDI and PDI incorporated into the transfer protein complex have similar secondary structures. The 88K secondary structure was then deduced from analysis of MTP and PDI. This approach seems justified in that frequently proteins fold to "structured monomers" prior to oligomerization (Jaenicke, 1987). In addition, there are numerous examples of oligomeric proteins that can be dissociated into their individual subunits without drastic perturbations of native secondary structure. Dissociation of the two components of MTP with 1.2 M guanidine hydrochloride appeared to result in only minor structural changes in MTP (see Figure 4). This analysis indicated PDI has 49% $\alpha$ and $\beta$ structure and is enriched in $\alpha$ structure (31%). The 88-kDa component had 60% $\alpha$ and $\beta$ structure, but in contrast to PDI, 88K is enriched in $\beta$ structure (35%). An alternative approach for investigating the secondary structure of 88K would be to isolate it prior to analysis. However, it has not been possible to isolate 88K in a soluble form due to the strong tendency of 88K to aggregate following its dissociation from PDI.

The exact physiological function of MTP is not known. Due to its ability to transfer triglyceride between membranes and its presence within the lumen of the endoplasmic reticulum (Wetterau & Zilversmit, 1986) in the liver and intestinal mucosa, we have speculated that it may play a role in the biogenesis of the triglyceride-rich plasma lipoproteins, very low density lipoproteins in the liver, and chylomicrons in the intestine. Presumably, the unusual structural features of this lipid-transfer protein play an important role of this or any other physiological function it may have.

## ACKNOWLEDGMENTS

We thank Kelly A. Combs and Sara N. Spinner for their excellent technical assistance and Terri Berning for her help with the preparation of the manuscript. The amino acid analysis was performed by Cleris M. Gill in the Protein Chemistry Core Laboratory, Department of Pharmacology and Cell Biophysics at the University of Cincinnati.

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# Conformational Transition of Fructose-1,6-bisphosphatase: Structure Comparison between the AMP Complex (T form) and the Fructose 6-Phosphate Complex (R form)†

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*Received November 30, 1990; Revised Manuscript Received February 14, 1991*

ABSTRACT: A structure of the neutral form of fructose-1,6-bisphosphatase complexed with AMP has been determined by the molecular replacement method and refined at a 2.5-Å resolution to a crystallographic *R* factor of 0.169. The root-mean-square errors of the structure from standard geometry are 0.013 Å for bond lengths and 2.99° for bond angles. Comparison of the AMP complex with the F6P complex shows that dimer C3-C4 twists about 19° about a molecular 2-fold axis when dimers C1-C2 of the R and T forms of the enzyme are superimposed one another and that a slight shift of about 1 Å of the AMP domain partially compensates this twist. The R to T transition of the enzyme does not significantly change the conformation of the F6P-binding site. However, residues at the divalent metal site and the AMP site show significant positional shifts. If these results can be extended to substrate in place of F6P, they suggest that regulation of the enzyme by AMP may occur partly through effects on metal-ion affinity or position. AMP binds to the same sites of the T and R forms, but only half-occupancy was observed in the alkaline R form. Sequential binding of AMP, at least in pairs, is suggested as the unligated R form is converted to the T form. Two possible pathways are suggested for allosteric communication over about 28 Å between the AMP site and the active site: one via helices H1, H2, and H3 and another via the eight-stranded β-sheet. In the former case, the loop of residues 54-68, which joins helices H2 and H3, may play an important role in the allosteric inhibition, compatible with the kinetic observation that the AMP inhibition is decreased or completely lost by proteolytic cleavages in the loop. Careful inspection of the interchain interfaces reveals that a few residues at the interface between dimers may be critical to lock the enzyme in either a T or R form.

Fructose-1,6-bisphosphatase (D-fructose 1,6-bisphosphate 1-phosphohydrolase, EC 3.1.3.11, abbreviated as Fru-1,6-Pase),[1] a key regulatory enzyme in gluconeogenesis, catalyzes the hydrolysis of fructose 1,6-bisphosphate (Fru-1,6-$P_2$) to fructose 6-phosphate (F6P) and inorganic phosphate (Benkovic & deMaine, 1982; Tejwani, 1983). Fru-1,6-Pase isolated from various sources consists of four identical polypeptide chains that aggregate into a relatively flat tetramer (Figure 1). The activity of Fru-1,6-Pase is regulated in vivo by an allosteric inhibitor AMP and also by a substrate analogue fructose 2,6-bisphosphate (Fru-2,6-$P_2$). Binding of Fru-2,6-$P_2$ was contradictorily suggested at the active site (Pilkis et al., 1987), at an allosteric site (Van Schaftingen, 1987), or at both (Meek & Nimmo, 1983). Details of the three-dimensional structures of the unligated Fru-1,6-Pase and its complex with Fru-2,6-$P_2$ have located the 6-phosphate and furanose of Fru-2,6-$P_2$ at

†Supported by the National Institutes of Health Grant GM06920.
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[1] Abbreviations: Fru-1,6-Pase, fructose-1,6-bisphosphatase; Fru-2,6-$P_2$, fructose 2,6-bisphosphate; F6P, fructose 6-phosphate.

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