A bovine liver protein which catalyzes the transfer of triglyceride between membranes has previously been isolated from the lumen of the microsomal fraction. When further purified about 100-fold, two polypeptides of molecular mass 58,000 and 88,000 were identified (Wetterau, J. R., and Zilversmit, D. B. (1985) Chem. Phys. Lipids 38, 205-222). We demonstrate here that the two polypeptides (referred to as 58-kDa and 88-kDa, respectively) are associated in a protein-protein complex, and that the triglyceride transfer activity is associated with this complex. Antibodies specific for either polypeptide immunoprecipitated both the 58-kDa and 88-kDa polypeptides as well as the lipid transfer activity. The 58-kDa subunit of the microsomal transfer protein complex was identified as protein disulfide-isomerase (PDI) (EC 5.3.4.1) by 1) a comparison of the amino-terminal sequence of PDI and the 58-kDa subunit of the transfer protein, 2) a comparison of the reverse phase high performance liquid chromatography peptide maps of CNBr digests of PDI and the lipid transfer protein, 3) immunoprecipitation competition experiments in which PDI was found to compete with the lipid transfer protein for immunoprecipitation by the anti-58-kDa polyclonal antibodies, 4) immunological cross-reactivity of the microsomal triglyceride transfer protein complex with polyclonal antibodies raised against PDI, and 5) the appearance of protein disulfide isomerase activity following the dissociation of purified microsomal transfer protein complex with guanidine HCl. In conclusion, the microsomal triglyceride transfer protein has a multi-subunit structure which is unique compared to other intracellular lipid transfer proteins which have been described to be single polypeptides. The unexpected finding that PDI is a component of the microsomal triglyceride transfer protein complex suggests a new previously undescribed role for protein disulfide isomerase.

A protein which catalyzes the transport of triglyceride (TG), \(^{1}\) cholesteryl ester, and, to a lesser extent, phosphatidylcholine (PC) between membranes has been isolated from bovine (1) and rat liver (2). It has also been detected in the intestinal mucosa of rats; however, it was not detected in appreciable quantities in the brain, heart, kidneys, or plasma. When rat liver homogenates were fractionated, most of the TG transfer activity was found in the microsomal fraction. The protein appears to reside within the lumen of the microsomes (2) and thus has been designated the microsomal lipid transfer protein (MTP). This neutral lipid transfer protein appears unique in that it prefers to transfer TG relative to cholesteryl ester.

The lipid transport properties as well as the tissue and cellular location of MTP has led to speculation that it may play a role in plasma lipoprotein biogenesis (2-4). Recent evidence suggests that plasma very low density lipoproteins are assembled by the sequential addition of lipid to pre-existing nascent particles in the lumen of the endoplasmic reticulum and Golgi apparatus (4-7). MTP may mediate the transport of newly synthesized triglyceride into nascent very low density lipoproteins in the liver or chylomicrons in the intestine.

MTP has been purified from bovine liver by a series of five column chromatography steps (8). It has an apparent molecular weight of about 220,000, as determined by the elution position of lipid transfer activity on a calibrated Sephadex G-200 gel permeation column. Transfer activity could be recovered from nondenaturating polyacrylamide electrophoresis gels at a position which coincided with that of the single stained protein band. When the protein was electrophoresed in the presence of SDS (in the presence or absence of \(^{2}\) mercaptoethanol), two bands of molecular masses 58,000 and 88,000 were observed. This data suggested that a 58-kDa-88-kDa protein complex may be the lipid transfer protein. Alternatively, the MTP preparations may contain two independent proteins which have similar properties and co-purify. The purpose of this work was to distinguish between these possibilities and to begin to characterize the subunits of MTP.

Polyclonal antibodies specific for the 58-kDa and 88-kDa polypeptides were generated in rabbits. Immunoprecipitation with either anti-58-kDa or anti-88-kDa removed both the 58-kDa and 88-kDa polypeptides from solution, as well as the triglyceride transfer activity. The 58-kDa subunit was iso-

\(^{1}\) The abbreviations used are: TG, triglyceride; SDS, sodium dodecyl sulfate; MTP, microsomal triglyceride transfer protein or microsomal lipid transfer protein; 58-kDa, the subunit of the microsomal lipid transfer protein which has a molecular weight of 58,000; 88-kDa, the subunit of the microsomal lipid transfer protein which has a molecular weight of 88,000; PDI, protein disulfide isomerase; ER, endoplasmic reticulum; HPLC, high pressure liquid chromatography; PAG, polyacrylamide gel electrophoresis; FC, phosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; TFA, trifluoroacetic acid; Ts, 3,5,3’-triiodo-1-thyronine.

9800
lated, and the sequence of the amino terminal 25 amino acids was determined. The sequence was found to have perfect homology with the bovine microsomal protein, protein disulfide isomerase (PDI). The identity of the 58-kDa polypeptide as PDI was further supported by biochemical and immunochemical characterization of MTP. We propose that the microsomal protein which catalyzes the transfer of triglyceride between membranes is a complex of two proteins. The smaller, 65-kDa component is protein disulfide isomerase.

**MATERIALS AND METHODS**

**Assay for Triglyceride Transfer Activity**—Triglyceride transfer activity was measured by following the transfer of [14C]triolein from donor small unilamellar vesicles (35 nmol of egg PC, 0.175 nmol of TG, 0.1% butyryl hydroxytoluene) to acceptor small unilamellar vesicles (175 nmol of egg PC, 8.8 nmol of cardiolipin, 0.875 nmol of TG, and 0.1% butyryl hydroxytoluene). Donor vesicles, acceptor vesicles, and transfer protein were incubated for varying times at 37 °C in 15 mM Tris, 7.4, 35 mM NaCl, 1 mM EDTA, and 0.02% NaN3 (hereafter referred to as 15:35 buffer). Following a transfer reaction, an aliquot of DEAE-cellulose was added, and the mixture was agitated for 4 min. The DEAE-cellulose with bound acceptor membranes (due to the negative charge from the cardiolipin) was pelleted by low speed centrifugation. Triglyceride transfer was quantitated by the loss of radiolabeled TG from the donor membranes in the supernatant. Donor vesicle recovery was quantitated by the recovery of [3H]HDLV phosphorylated (DPPC) incorporated into the donor vesicles. MTP-stimulated transfer of [3H]DPPC is negligible when TG transfer is less than 30% (8). A blank assay containing donor and acceptor membranes without transfer protein was used to correct for the spontaneous transfer of [14C]triolein from donor to acceptor vesicles (less than 2% for a 1-h incubation). Additional details for the preparation of vesicles and the transfer assay have been described previously (8).

Total triglyceride transfer was calculated by assuming first order kinetics, and the fraction of labeled TG present at time 0 and time t, respectively, and is the fraction of donor TG replaced per unit time. The percentage of donor lipid transferred equals k x 100.

**Purification of the Microsomal Lipid Transfer Protein**—A modification of a procedure described previously was used to isolate and purify MTP (8). This protein was used for immunoprecipitation experiments and the HPLC isolation of the 58-kDa polypeptide. The procedure described for the preparation of crude MTP was further purified by ammonium sulfate precipitation, cation exchange, and hydroxylapatite chromatography. The latter three or four steps resulted in about a 7-19-fold purification. Blue-stained protein bands upon SDS-PAGE were cut from the gel, and the proteins were eluted at 175-200 mM potassium phosphate. Recoveries ranged from 0.5 to 3.0 mg of MTP.

**Isolation of Protein Disulfide Isomerase**—PDI was isolated from the supernatant of the microsomal fraction of bovine liver by the same procedure described for the preparation of crude MTP. PDI was further purified by an ammonium sulfate precipitation, cation exchange chromatography, and anion exchange chromatography as described by Hillson et al. (14). In some instances, an additional Sephacryl S-300 column chromatography step was added to the purification. The latter three or four steps resulted in a 7-19-fold purification (range for three purifications) and a single Coomassie Blue-stained protein band upon SDS-PAGE. The purified protein had an apparent molecular mass of 58,000 which corresponds well to the reported 57,000 for bovine PDI (14).

**Preparation of Transferase**—First, the protein was eluted with 0.1% SDS, 0.04 M Tris, 0.1% TFA, H2O, then eluted with a 30-55% linear gradient of 0.1% TFA, acetonitrile. The 58-kDa polypeptide eluted at 52% acetonitrile.

**Protein Disulfide Isomerase Activity**—Protein disulfide isomerase (PDI) activity was determined by the ability of PDI to reactivate previously reduced and denatured ribonuclease. Ribonuclease activity was measured as the rate of RNA hydrolysis to acid-soluble oligonucleotides (12). Ribonuclease, 0.1 ml, was added to 0.1 ml of 1% RNA (Sigma, Type VI from Torula yeast) in 0.1 M sodium acetate, pH 5.0, and incubated at 37 °C. At times of 0 and 7-8 min, duplicate samples were removed, 0.1 ml of ice cold 5% perchloric acid (1.75% trichloroacetic acid was added, and the mixture was placed on ice for 5 min. Following a low speed centrifugation to pellet undissolved RNA, digested RNA in the supernatant was quantitated by diluting 0.1 ml of supernatant with 3.0 ml of water and measuring the optical density at 260 nm.

Ribonuclease was denatured in 8 M urea and reduced with 1 μl of β-mercaptoethanol/mg of protein as described previously (13). Inactivated ribonuclease was eluted from a Sephadex G 25 column (Pharmacia) with 0.1 M acetic acid, adjusted to 5 mg/ml with 0.1 M acetic acid, and stored at 4 °C. It was diluted with H2O, 1:9 (ribonuclease-urea) immediately before it was used in the assay.

To measure PDI activity, protein in 1 ml of 50 mM sodium phosphate, pH 7.5, was adjusted to 10−4 M dithiothreitol and incubated for 4 min at room temperature. Alternatively, in some cases, the dithiothreitol treatment was omitted. Reduced and denatured ribonuclease, 0.1 ml, was added, and the solution was kept at 37 °C. At varying times, an aliquot of the solution was removed, and the ribonuclease activity was determined. PDI activity was defined by the increase in ribonuclease activity with time of incubation.

Isolation of Protein Disulfide Isomerase—PDI was isolated from the supernatant of the microsomal fraction of bovine liver by the same procedure described for the preparation of crude MTP. PDI was further purified by an ammonium sulfate precipitation, cation exchange chromatography, and anion exchange chromatography as described by Hillson et al. (14). In some instances, an additional Sephacryl S-300 column chromatography step was added to the purification. The latter three or four steps resulted in a 7-19-fold purification (range for three purifications) and a single Coomassie Blue-stained protein band upon SDS-PAGE. The purified protein had an apparent molecular mass of 58,000 which corresponds well to the reported 57,000 for bovine PDI (14).

**Preparation of Transferase**—First, the protein was eluted with 0.1% SDS, 0.04 M Tris, 0.1% TFA, H2O, then eluted with a 30-55% linear gradient of 0.1% TFA, acetonitrile. The 58-kDa polypeptide eluted at 52% acetonitrile.

**Protein Disulfide Isomerase Activity**—Protein disulfide isomerase (PDI) activity was determined by the ability of PDI to reactivate previously reduced and denatured ribonuclease. Ribonuclease activity was measured as the rate of RNA hydrolysis to acid-soluble oligonucleotides (12). Ribonuclease, 0.1 ml, was added to 0.1 ml of 1% RNA (Sigma, Type VI from Torula yeast) in 0.1 M sodium acetate, pH 5.0, and incubated at 37 °C. At times of 0 and 7-8 min, duplicate samples were removed, 0.1 ml of ice cold 5% perchloric acid (1.75% trichloroacetic acid was added, and the mixture was placed on ice for 5 min. Following a low speed centrifugation to pellet undissolved RNA, digested RNA in the supernatant was quantitated by diluting 0.1 ml of supernatant with 3.0 ml of water and measuring the optical density at 260 nm.

Ribonuclease was denatured in 8 M urea and reduced with 1 μl of β-mercaptoethanol/mg of protein as described previously (13). Inactivated ribonuclease was eluted from a Sephadex G 25 column (Pharmacia) with 0.1 M acetic acid, adjusted to 5 mg/ml with 0.1 M acetic acid, and stored at 4 °C. It was diluted with H2O, 1:9 (ribonuclease-urea) immediately before it was used in the assay.

To measure PDI activity, protein in 1 ml of 50 mM sodium phosphate, pH 7.5, was adjusted to 10−4 M dithiothreitol and incubated for 4 min at room temperature. Alternatively, in some cases, the dithiothreitol treatment was omitted. Reduced and denatured ribonuclease, 0.1 ml, was added, and the solution was kept at 37 °C. At varying times, an aliquot of the solution was removed, and the ribonuclease activity was determined. PDI activity was defined by the increase in ribonuclease activity with time of incubation.

Isolation of Protein Disulfide Isomerase—PDI was isolated from the supernatant of the microsomal fraction of bovine liver by the same procedure described for the preparation of crude MTP. PDI was further purified by an ammonium sulfate precipitation, cation exchange chromatography, and anion exchange chromatography as described by Hillson et al. (14). In some instances, an additional Sephacryl S-300 column chromatography step was added to the purification. The latter three or four steps resulted in a 7-19-fold purification (range for three purifications) and a single Coomassie Blue-stained protein band upon SDS-PAGE. The purified protein had an apparent molecular mass of 58,000 which corresponds well to the reported 57,000 for bovine PDI (14).
was visualized with goat anti-rabbit IgG coupled to horseradish peroxidase (Bio-Rad Laboratories).

Protein disulfide isomerase was isolated as described above. Antibodies were prepared in a manner identical with that for the 58-kDa and 88-kDa polypeptides except 100 µg of protein per injection was used. A maximum titer of 2500 was achieved.

**Immunoprecipitation Reactions—** IgG was adsorbed to protein A-agarose (Bio-Rad Laboratories) at a ratio of 1.25:1.0 (ml:ml, serumprotein A-agarose suspension). Following a two-step wash of the protein A-agarose and bound IgG with 18:35 buffer, aliquots of the antibody-protein A-agarose suspension were added to MTP, and the mixture was agitated overnight at 4°C. Following low speed centrifugation, the depletion of protein or TG transfer activity from solution was determined. The immunoprecipitations were performed in 18:35 buffer or 18:35 buffer supplemented with bovine serum albumin when transfer activity was being measured. Omitting albumin from immunoprecipitation reactions facilitated subsequent electrophoretic analysis of the supernatant proteins. Protein concentrations were estimated by the method of Lowry *et al.* (15) with bovine serum albumin as a standard. Alternatively, optical density at 280 nm was used to estimate the protein concentration. Lowry and optical density measurements were made on control samples of MTP and PDI and used to normalize the determination by optical density to that by the Lowry technique.

125I-MTP was used to determine if both the 58-kDa and 88-kDa polypeptides were depleted from solution following an immunoprecipitation reaction. MTP was labeled with 125I by the method of Bolton and Hunter (16), utilizing commercially available Bolton-Hunter reagent (DuPont-New England Nuclear Research Products). Following the immunoprecipitation reaction, an aliquot of the supernatant was removed and fractionated by SDS-PAGE (9). The gel was dried, and an autoradiogram was developed. The bands corresponding to the 58-kDa and 88-kDa polypeptides were excised from the gel, and the 125I-protein was quantified by γ counting in a 1185 Series Automatic Gamma Counting System (Searle Analytic Inc.).

**Protein Sequencing—** The amino-terminal sequence of the HPLC-isolated 58-kDa protein was determined in the Protein Chemistry Core Laboratory (Dr. Terry Kirley, Director) in the Department of Pharmacology and Cell Biophysics at the University of Cincinnati. Gas phase sequence technology on an Applied Biosystems 470A Protein Sequencer was used to generate phenylthiohydantoin amino acids which were identified by HPLC analysis.

**Cyanogen Bromide Digestion and HPLC Peptide Maps—** One mg of MTP or PDI was adjusted to a concentration of 1 mg/ml and reduced overnight with 5% mercaptoethanol in the dark under a N2 atmosphere. The protein was dialyzed into 70% formic acid. Thirty mg of solid cyanogen bromide (Sigma) was added, and the mixture was incubated at room temperature overnight in the dark under a N2 atmosphere. The digest was dialyzed extensively into 0.01 N HCl (Spectra/Par 6, 1000 molecular weight cut off from Spectrum Medical Industries). An aliquot of the digest was loaded onto a Vydac standard bore C4 reverse phase HPLC column and eluted with a 20-60% linear gradient of increasing 0.1% TFA, acetonitrile in 0.1% TFA, water. Eluting protein was detected by optical density at 220 and 280 nm.

**RESULTS**

**The Microsomal Triglyceride Transfer Protein Is a Protein Complex—** A highly purified preparation of the microsomal triglyceride transfer protein contains two polypeptides of molecular mass 58,000 and 88,000. To obtain a tool to investigate the role of these two proteins in the lipid transport process, polyclonal antibodies against the isolated 58-kDa and 88-kDa polypeptides were generated. The specificity of each antiserum was examined by immunoblot analysis of bovine liver homogenate and purified MTP, following their fractionation by SDS-PAGE (see Fig. 1). Anti-58-kDa reacted with only a 58,000 molecule mass protein in the whole liver homogenate or purified MTP. Anti-88-kDa reacted with an 88,000 molecule mass protein in the whole liver homogenate and purified MTP. Anti-88-kDa in whole liver homogenate also reacted with a protein with a mobility intermediate between the 58-kDa and 88-kDa proteins. The relationship between this and the 88-kDa protein is not known. Because this cross-reactivity does not influence experiments performed with purified MTP, its origin was not pursued at this time.

To determine if the 58-kDa and 88-kDa polypeptides actually formed a 58-kDa-88-kDa protein complex, purified MTP was iodinated to a specific activity of 1000 cpm/ng of protein and then immunoprecipitated with antibodies specific for each polypeptide. Using Bolton-Hunter reagent for iodination, approximately 10 times more 125I was incorporated into the 88-kDa polypeptide than the 58-kDa polypeptide. Fig. 2, *left panel,* illustrates the immunoprecipitation performed with the anti-58-kDa antisera. Preimmune IgG immunoprecipitated neither the 58-kDa polypeptide nor the 88-kDa polypeptide. However, postimmune anti-58-kDa IgG was equally effective in immunoprecipitating the 58-kDa and 88-kDa polypeptides. Since anti-58-kDa did not recognize the 88-kDa polypeptide (Fig. 1), these data suggest that the immunoprecipitation of the 88-kDa polypeptide occurs because it forms a stable complex with the 58-kDa polypeptide.

A similar experiment was performed with the anti-88-kDa antisera (Fig. 2, *right panel*). The preimmune IgG did not immunoprecipitate either polypeptide, whereas the postimmune antisera immunoprecipitated both 125I-labeled polypeptides. These results confirm that the two polypeptides form a 58-kDa-88-kDa protein complex.

To determine if the triglyceride transfer activity was associated with the 58-kDa-88-kDa protein complex, both antisera were used to immunoprecipitate TG transfer activity. Neither preimmune antisera was able to deplete transfer activity from solution in an immunoprecipitation reaction. In contrast, both the anti-58-kDa and anti-88-kDa antisera were able to deplete the solution of transfer activity (Fig. 3). These results, in conjunction with the 125I-MTP immunoprecipitation results, demonstrate that the triglyceride transfer protein is a complex of two proteins of molecular weights 58,000 and 88,000.

**Identification of Protein Disulfide Isomerase in the Purified Microsomal Triglyceride Transfer Protein—** To isolate the 58-kDa polypeptide, pure MTP was applied to an HPLC reverse
kDa or rabbit anti-88-kDa polyclonal antibodies. 

\[ \text{[Equation]} \]

The protein was fractionated by SDS-PAGE, and the bands on the gel were cut from the gel, and the radioactivity was quantitated. The results are expressed as percent counts/min recovered in control immunoprecipitations (preimmune serum absorbed to an equal volume of protein A-agarose). In the left panel, anti-58-kDa IgG was used. Control samples contained 996 ± 135 (± S.D.) and 8499 ± 1159 cpm of 

\[ ^{125}I \]

in the 58-kDa and 88-kDa bands, respectively. In the right panel, anti-88-kDa IgG was used. Control samples contained 432 ± 31 (± S.D.) and 4014 ± 348 cpm of 

\[ ^{125}I \]

in the 58-kDa and 88-kDa bands, respectively.

Increasing concentrations of anti-58-kDa or anti-88-kDa IgG preabsorbed to protein A-agarose were incubated overnight with 80 and 50 ng of MTP, respectively, in 0.6 ml of 15:35 buffer supplemented with 1% bovine serum albumin. The protein A-agarose was pelleted by low speed centrifugation, and the aliquot of the supernatant was removed. The protein was fractionated by SDS-PAGE, the bands corresponding to the 58-kDa and 88-kDa polypeptides were cut from the gel, and the radioactivity was quantitated. The results are expressed as percent counts/min recovered in control immunoprecipitations (preimmune serum absorbed to an equal volume of protein A-agarose). In the left panel, anti-58-kDa IgG was used. Control samples contained 996 ± 135 (± S.D.) and 8499 ± 1159 cpm of 

\[ ^{125}I \]

in the 58-kDa and 88-kDa bands, respectively. In the right panel, anti-88-kDa IgG was used. Control samples contained 432 ± 31 (± S.D.) and 4014 ± 348 cpm of 

\[ ^{125}I \]

in the 58-kDa and 88-kDa bands, respectively.

Increasing concentrations of anti-58-kDa or anti-88-kDa IgG preabsorbed to protein A-agarose were incubated overnight with 80 and 50 ng of MTP, respectively, in 0.6 ml of 15:35 buffer supplemented with 1% bovine serum albumin. The protein A-agarose was pelleted by low speed centrifugation, and the aliquot of the supernatant was removed. The protein was fractionated by SDS-PAGE, the bands corresponding to the 58-kDa and 88-kDa polypeptides were cut from the gel, and the radioactivity was quantitated. The results are expressed as percent counts/min recovered in control immunoprecipitations (preimmune serum absorbed to an equal volume of protein A-agarose). In the left panel, anti-58-kDa IgG was used. Control samples contained 996 ± 135 (± S.D.) and 8499 ± 1159 cpm of 

\[ ^{125}I \]

in the 58-kDa and 88-kDa bands, respectively. In the right panel, anti-88-kDa IgG was used. Control samples contained 432 ± 31 (± S.D.) and 4014 ± 348 cpm of 

\[ ^{125}I \]

in the 58-kDa and 88-kDa bands, respectively.

Increasing concentrations of anti-58-kDa or anti-88-kDa IgG preabsorbed to protein A-agarose were incubated overnight with 80 and 50 ng of MTP, respectively, in 0.6 ml of 15:35 buffer supplemented with 1% bovine serum albumin. The protein A-agarose was pelleted by low speed centrifugation, the supernatant was removed, and the transfer activity was quantitated. Control immunoprecipitations were performed with preimmune serum absorbed to protein A-agarose. The results are expressed as the percent of the TG transfer activity recovered in control incubations. For the anti-58-kDa and anti-88-kDa control immunoprecipitations, the TG transfer activity (percent donor TG transferred per 1-h incubation) was 15.5 ± 0.6 (± S.D.) and 21.9 ± 2.5, respectively.

\[ \text{Peptide} \]

One amino-terminal sequence of the 58-kDa subunit of MTP is protein disulfide isomerase. Particularly evident amino acid sequence analysis of the HPLC 58-kDa polypeptide. Two amino acids were identical in some cycles. The most probable sequence for the protein (based upon recovery of amino acids at each cycle with consideration for our typical recovery of each amino acid from standard proteins) matched exactly the sequence of bovine protein disulfide isomerase (17). Portions of a second, minor sequence were apparent. This second sequence also was identical with that of PDI; however, it appeared to be two amino acids shorter than the native form of PDI. For example, what appeared in cycle 4 for the most probable sequence, appeared in cycle 2 of the minor sequence.

Protein disulfide isomerase was isolated from bovine liver by a modification of the procedure of Hillson et al. (14). PDI and the 58-kDa subunit of MTP co-migrated on SDS-PAGE (Fig. 4). To confirm the identity of the MTP 58-kDa subunit, MTP and conventionally purified PDI were digested with cyanogen bromide, and reverse phase HPLC peptide maps were generated (Fig. 5). Peptides from the PDI digest (middle panel) can be clearly identified in the MTP digest (see arrows, top panel), providing further evidence that the 58-kDa subunit of MTP is protein disulfide isomerase. Particularly evident are the cluster of peptides at around 40% solvent B and the two major peaks at 46 and 48% B. Few peptides which appear unique to MTP (and therefore thought to represent digested 88-kDa polypeptide) could be identified. The poor recovery of the 88-kDa peptides is consistent with our inability to recover the intact 88-kDa polypeptide from HPLC. The 88-kDa polypeptide apparently has properties which are not amenable to reverse phase HPLC purification.

Protein Disulfide Isomerase is the 58,000 Molecular Mass Component of the Microsomal Triglyceride Transfer Protein Complex—Having established that PDI is a component of our purified MTP, we wanted to confirm that PDI is a component of our purified MTP.

**TABLE I**

| Amino acid | Bovine PDI* | 58-kDa subunit* | Second sequence* | Isolated PDI* |
|------------|------------|----------------|-----------------|---------------|
| 1          | Ala        | Ala-73         | Ala-185         |               |
| 2          | Pro        | Pro-33         | Pro-65          |               |
| 3          | Asp        | Asp-31         | Asp-175         |               |
| 4          | Glu        | Glu-56         | Glu-86          |               |
| 5          | Glu        | Glu-70         | Glu-60          |               |
| 6          | Asp        | Asp-40         | Asp-145         |               |
| 7          | His        | His-10         | His-39          |               |
| 8          | Val        | Val-62         | Val-78          |               |
| 9          | Leu        | Leu-76         |                 |               |
| 10         | Val        | Val-53         |                 |               |
| 11         | Leu        | Leu-85         |                 |               |
| 12         | His        | His-10         | Gly-18          |               |
| 13         | Lys        | Lys-11         | Asn-10          |               |
| 14         | Gly        | Gly-39         | Phe-23          |               |
| 15         | Asn        | Asn-32         |                 |               |
| 16         | Phc        | Phc-38         | Glu-24          |               |
| 17         | Asp        | Asp-40         | Ala-33          |               |
| 18         | Glu        | Glu-47         | Leu-34          |               |
| 19         | Ala        | Ala-74         |                 |               |
| 20         | Leu        | Leu-59         |                 |               |
| 21         | Ala        | Ala-69         |                 |               |
| 22         | Ala        | Ala-68         | Lys-27          |               |
| 23         | His        | His-13         |                 |               |
| 24         | Lys        | Lys-45         |                 |               |
| 25         | Tyr        | Tyr-32         |                 |               |

* Bovine PDI sequence was from the work of Yamauchi et al. (17).

The protein sequence and the reported picomole recovery of each amino acid.

Protein disulfide isomerase isolate as described under "Materials and Methods."
of the lipid transfer protein. To accomplish this, immunoprecipitation competition experiments were performed. If PDI is the 58-kDa component of MTP, then the addition of PDI to an immunoprecipitation reaction with anti-58-kDa should prevent the precipitation of MTP.

The HPLC-isolated 58-kDa polypeptide which was identified as PDI by amino-terminal sequence analysis or PDI purified by conventional column chromatography were tested for their ability to compete with 125I-MTP or triglyceride transfer activity in an immunoprecipitation reaction with the anti-58-kDa antibody. Following the immunoprecipitation of 125I-MTP with 50 μl of anti-58-kDa, only 15% of the radiolabeled 58-kDa and 88-kDa polypeptides remained in solution (Fig. 6, 0 μg of competing PDI). When increasing concentrations of HPLC-isolated 58-kDa polypeptide were added to the immunoprecipitation reactions, increasing amounts of radiolabeled protein remained in the supernatant. This demonstrated that the HPLC-isolated 58-kDa protein competed with the 125I-58-kDa-88-kDa protein complex in the immunoprecipitation. Similar competition was observed with PDI isolated by a modification of the method of Hillson et al. (14).

Fig. 7 illustrates that the 58-kDa protein isolated by HPLC or PDI isolated by a modification of the method of Hillson et al. (14) compete for the immunoprecipitation of TG transfer activity by anti-58-kDa. Collectively, these results indicate that the 58-kDa polypeptide of the lipid transfer protein complex is protein disulfide isomerase.

**Polyclonal Antibodies Raised Against Protein Disulfide Isomerase Cross-react with the Microsomal Triglyceride Transfer Protein Complex**—Polyclonal antibodies against PDI were raised in rabbits and tested for their ability to cross-react with MTP. Fig. 8 is an immunoblot analysis of a bovine liver homogenate and MTP. Anti-PDI cross-reacts with the 58,000 molecular weight component of MTP.

Anti-PDI was tested for its ability to immunoprecipitate MTP. As is illustrated in Fig. 9, these antibodies which were raised against a protein which has no TG transfer activity (see Table II) readily deplete TG transfer activity from solution.

**Treatment of MTP with a Denaturant, Guanidine HCl, Results in the Loss of TG Transfer Activity and Enhanced Expression of Protein Disulfide Isomerase Activity**—Protein disulfide isomerase catalyzes the rearrangement of protein disulfide bonds. It can reactivate denatured proteins by correctly pairing their cysteine residues into disulfide bonds, thus allowing the protein to fold to its enzymatically active state (for review, see Ref. 18). We tested the ability of MTP to express PDI activity by measuring its ability to reactivate previously reduced and denatured ribonuclease.

Table II is a comparison of the TG transfer activity and protein disulfide isomerase activity expressed by MTP and PDI. Native PDI expresses no TG transfer activity, even at protein concentrations over 1000 times higher (up to 350 μg tested) than that normally used to measure MTP transfer activity. MTP has some disulfide isomerase activity, but in this example, only about 5% of an equal mass of PDI. PDI and MTP were isolated independently from three differ-
Microsomal Triglyceride Transfer Protein Subunit Structure

FIG. 6. HPLC-isolated 58-kDa polypeptide competes with 125I-MTP for immunoprecipitation by rabbit anti-58-kDa. Radiolabeled MTP (30 ng) was immunoprecipitated with 50 μl of an anti-58-kDa-protein A-agarose suspension. Three additional immunoprecipitation reactions were performed in the presence of increasing concentrations of HPLC-isolated 58-kDa polypeptide. Following the immunoprecipitation, an aliquot of protein in the supernatant was fractionated by SDS-PAGE, the bands corresponding to the 58-kDa and 88-kDa polypeptides were cut from the gel, and the radioactivity was quantitated. The results are expressed as percent cpm recovered in control incubations performed in the absence of the anti-58-kDa protein A-agarose suspension.

FIG. 7. HPLC-isolated 58-kDa polypeptide and authentic PDI compete with TG transfer activity for immunoprecipitation by rabbit anti-58-kDa. MTP (150 ng) was immunoprecipitated with 150 μl of an anti-58-kDa-protein A-agarose suspension in 500 μl of 15:35 buffer supplemented with 0.1% bovine serum albumin. Additional immunoprecipitations were performed in the presence of increasing concentrations of HPLC-isolated 58-kDa polypeptide (C) or authentic PDI (⋄) isolated by the method of Hillson et al. (14). The protein A-agarose was pelleted by low speed centrifugation, and the TG transfer activity in the supernatant was determined. The results are expressed as the percent of the TG transfer activity in control incubations performed in the absence of the anti-58-kDa protein A-agarose suspension. Control transfer activities were 15.4 and 16.2% TG transfer per 1-h incubation for the HPLC-isolated 58-kDa polypeptide and authentic PDI competition experiments, respectively.

FIG. 8. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblot analysis of MTP with anti-PDI. Polyclonal antibodies against conventionally purified PDI were raised in rabbits. Bovine liver homogenate (35 μg) and MTP (10 μg) were fractionated by SDS-PAGE, transferred to nitrocellulose, and probed with anti-PDI. Horseradish peroxidase-conjugated secondary antibody was used to detect immunoreactive proteins. The mobility of the 58-kDa and 88-kDa polypeptides is indicated in the right margin.

FIG. 9. Immunoprecipitation of TG transfer activity by rabbit anti-PDI. Increasing concentrations of anti-PDI IgG preabsorbed to protein A-agarose were incubated overnight with 25 ng of MTP in 0.8 ml of 15:35 buffer supplemented with 1% bovine serum albumin. The protein A-agarose was pelleted by low speed centrifugation, the supernatant was removed, and the transfer activity was quantitated. Control immunoprecipitations were performed with preimmune IgG absorbed to protein A-agarose. The results are expressed as the percent of the TG transfer activity recovered in control incubations. The TG transfer activity (percent donor TG transferred per 1-h incubation) in the control incubations was 21.7 ± 1.1 (± S. D.).

DISCUSSION

The data reported here indicate that the microsomal triglyceride transfer protein is a complex of two proteins of molecular mass 58,000 and 88,000. Polyclonal antibodies specific for either the 58-kDa or 88-kDa polypeptide immunoprecipitated both the 58-kDa and 88-kDa polypeptides. In addition, both antisera immunoprecipitated triglyceride transfer activity. The 58-kDa subunit of MTP was identified as protein of MTP results in a complete loss of TG transfer activity; however, a 6-7-fold increase in disulfide isomerase activity was observed. PDI, which was treated in a similar manner as a control, lost some disulfide isomerase activity. In summary, the native lipid transfer protein complex expresses little disulfide isomerase activity. Once the complex is disrupted by guanidine HCl, the lipid transfer activity is lost and the disulfide isomerase activity expressed increases to a level comparable to native PDI. (The specific activity of MTP is expected to be lower than PDI due to the mass of the 88-kDa polypeptide).
Microsomal Triglyceride Transfer Protein Subunit Structure

Comparison of the catalytic activities expressed by the microsomal triglyceride transfer protein complex and protein disulfide isomerase

| Control  | MTP | PDI | Guanidine HCl treatment |
|----------|-----|-----|-------------------------|
|          |     |     |                         |
| MTP      | +   | 0.7 ± 0.2 | 4.6 ± 0.8   |
| PDI      | -   | 13.7 ± 1.7 | 8.2 ± 1.9   |

*Disulfide isomerase activity was measured as the increase in activity of reduced and denatured ribonuclease following a 20-min incubation at 37°C. The spontaneous reactivation of ribonuclease was subtracted from the total reactivation to determine the protein-stimulated reactivation. Ribonuclease activity was a measure of the RNA digested in a 7-min assay at 37°C. Digested RNA was quantitated by optical density at 260 nm following a perchloric acid, uranyl acetate precipitation of undigested RNA. The disulfide isomerase activity is expressed as the change in the ribonuclease activity (ΔA260) per mg of MTP or PDI. The activity was measured four times, and the results are expressed as the average ± S.D. See “Materials and Methods” for additional details of the assay.

|          | PDI | MTP |
|----------|-----|-----|
| Control  | 13.7 ± 1.7 | 4.6 ± 0.8 |
| Guanidine HCl treatment |
| MTP      | -   | 4.6 ± 0.8 |
| PDI      | -   | 8.2 ± 1.9 |

In MTP appears to drastically decrease the expression of disulfide isomerase activity.

in addition to its role in protein assembly and prolyl 4-hydroxylase, bovine liver PDI has been reported to be a thyroid hormone binding protein. T3 binds to PDI with a dissociation constant of 57 nM (17). Recently, a protein which appears highly similar if not identical with PDI was identified at the glycosylation site binding protein, a component of oligosaccharyl transferase (34). These results, in conjunction with those we have presented here, suggest that PDI is a multifunctional protein that participates in a variety of cellular processes.

The association between the 88-kDa subunit and PDI may play a role in the intracellular targeting of the lipid transfer activity. Proteins which contain the carboxyl-terminal sequence, Lys-Asp-Glu-Leu, or are associated with a protein which contains this sequence, appear to be selectively retained in the ER (35). For example, PDI, BiP (the immunoglobin heavy chain binding protein), and grp94 all contain this sequence and are all retained in the ER. In addition, improperly folded proteins or β-glucuronidase which do not contain the retention sequence are thought to be retained in the ER because they form a complex with proteins which are retained, BiP (36) and eogyn (37), respectively. Perhaps PDI, which contains the four-amino acid carboxyl-terminal retention sequence, is playing a similar role in the retention of the 88-kDa polypeptide within the ER.

The role of the subunits in the lipid transfer protein complex is not known. One of the two subunits may be the TG transfer protein and the second subunit may have another, yet to be defined role. Alternatively, both subunits may be required to express lipid transfer activity. If one subunit is the transfer protein, one would predict it as 88-kDa, PDI, in a purified form does not express TG transfer activity (see Table II). To date, we have been unable to isolate catalytically active 88-kDa. Treatment of MTP with detergents, denaturants, or chaotropic agents at concentrations necessary to disrupt the MTP complex appear to inactive MTP. The characterization of 88-kDa has been further hampered by its tendency to degrade following its dissociation from PDI and our difficulty isolating it by HPLC.

Our working model of the microsomal triglyceride transfer protein complex is that the uncharacterized 88-kDa component is the catalytic subunit or that this subunit when complexed with PDI confers transfer activity to the protein complex. Based upon our inability to isolate catalytically active 88-kDa, we hypothesize that both subunits are required for an active lipid transfer protein. The role of PDI in the TG transfer protein complex is intriguing. The interfacial interaction between the two subunits may be necessary to create the active site or to stabilize the transfer protein in an active conformation. The role of PDI in the transfer protein complex may have similarities to its role in prolyl 4-hydroxylase. The α subunit of this tetrameric protein has no catalytic activity unless it is complexed with PDI (38). One could speculate that the ability of PDI to catalyze the rearrangement of disulfide bonds within a protein may play a role in the lipid transfer process. Perhaps this modulates conformational changes during the transfer reactions. A role for cysteine residues in neutral lipid transport may be suggested by the sensitivity of the plasma neutral lipid transfer protein to sulfhydryl reagents (38).

Acknowledgments—We would like to thank Gwen Kraft and Terri
REFERENCES

1. Wetterau, J. W., and Zilversmit, D. B. (1984) J. Biol. Chem. 259, 10863-10866
2. Wetterau, J. R., and Zilversmit, D. B. (1990) Biochim. Biophys. Acta 1015, 610-617
3. Chao, F.-F., Stiers, D. L., and Ontko, J. A. (1986) J. Lipid Res. 27, 1174-1181
4. Boström, K., Borén, J., Wettersten, M., Sjöberg, A., Bondjers, G., Wiklund, O., Carlsson, P., and Olofsson, S.-O. (1988) J. Biol. Chem. 263, 4474-4482
5. Howell, K. E., and Palade, G. E. (1982) J. Cell Biol. 92, 833-845
6. Janero, D. R., and Lane, M. D. (1983) J. Biol. Chem. 258, 14496-14504
7. Higgins, J. A., and Hutson, J. L. (1984) J. Lipid Res. 25, 1295-1305
8. Wetterau, J. R., and Zilversmit, D. B. (1985) Chem. Phys. Lipids 38, 205-222
9. Iwasaki, H. (1970) Nature 227, 680-685
10. Neville, D. M., Jr. (1971) J. Biol. Chem. 246, 6328-6334
11. Hunkapiller, M. W., and Lujan, E. (1986) in Methods of Protein Microcharacterization (Shively, J. E., ed) pp. 89-101, Humana Press, Clifton, NJ
12. Kalnitsky, G., Hummel, J. P., and Dierks, C. (1959) J. Biol. Chem. 234, 1512-1516
13. Anfinsen, C. B., and Haber, E. (1961) J. Biol. Chem. 236, 1361-1368
14. Hillson, D. A., Lambert, N., and Freedman, R. B. (1984) Methods Enzymol. 107, 281-294
15. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275
16. Bolton, A. E., and Hunter, W. M. (1973) Biochem. J. 133, 529-539
17. Yamauchi, K., Yamamoto, T., Hayashi, H., Koya, S., Takikawa, H., Toyoshima, K., and Horiuchi, R. (1987) Biochem. Biophys. Res. Commun. 146, 1485-1492
18. Freedman, R. B. (1984) Trends Biochem. Sci. 9, 438-441
19. Wirtz, K. W. A. (1982) in Lipid-Protein Interactions (Griffith, O. H., and Jost, P. C., eds) Vol. 1, pp. 151-231, John Wiley & Sons, Inc., New York
20. Wetterau, J. R., and Zilversmit, D. B. (1984) Methods Biochem. Anal. 30, 199-226
21. Kessen, C. B., Swenson, T. L., and Tall, A. R. (1981) J. Biol. Chem. 262, 2275-2282
22. Jarnagin, A. S., Kohr, W., and Fielding, C. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 1854-1857
23. Drayna, D., Jarnagin, A. S., McLean, J., Hensel, W., Kohr, W., Fielding, C., and Lawn, R. (1987) Nature 327, 629-634
24. Bulleid, N. J., and Freedman, R. B. (1988) Nature 335, 649-651
25. Goldberger, R. F., Epstein, C. J., and Anfinsen, C. B. (1963) J. Biol. Chem. 238, 628-635
26. Goldberger, R. F., Epstein, C. J., and Anfinsen, C. B. (1964) J. Biol. Chem. 239, 1406-1410
27. Steinher, R. F., De Lorenzo, P., and Anfinsen, C. B. (1965) J. Biol. Chem. 240, 4648-4651
28. Roth, R. A., and Koshland, M. E. (1981) Biochemistry 20, 6594-6599
29. Edman, J. C., Ellis, L., Blacher, R. W., Roth, R. A., and Rutter, W. J. (1985) Nature 317, 267-270
30. Brockway, B. E., Forster, S. J., and Freedman, R. B. (1990) Biochem. J. 191, 873-876
31. Koivu, J., Myllylä, R., Helaakoski, T., Pihlajaniemi, T., Tasanen, K., and Kivirikko, K. I. (1987) J. Biol. Chem. 262, 6447-6449
32. Pihlajaniemi, T., Helaakoski, T., Tasanen, K., Myllylä, R., Huhthala, M., Koivu, J., and Kivirikko, K. I. (1987) EMBO J. 6, 643-649
33. Kivirikko, K. I., Myllylä, R., and Pihlajaniemi, T. (1989) FASEB J. 3, 1609-1617
34. Geetha-Habib, M., Nova, K., Kaplan, H. A., and Lenntz, W. J. (1988) Cell 54, 1053-1060
35. Munro, S., and Pelham, H. R. B. (1987) Cell 48, 899-907
36. Kassenbrock, C. K., Garcia, P. D., Walter, P., and Kelly, R. B. (1988) Nature 333, 90-93
37. Pelham, H. R. B. (1988) EMBO J. 7, 913-918
38. Morton, R. E., and Zilversmit, D. B. (1982) J. Lipid Res. 23, 1058-1067
Protein disulfide isomerase is a component of the microsomal triglyceride transfer protein complex.

J R Wetterau, K A Combs, S N Spinner and B J Joiner

*J. Biol. Chem.* 1990, 265:9801-9807.

Access the most updated version of this article at [http://www.jbc.org/content/265/17/9801](http://www.jbc.org/content/265/17/9801)

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at [http://www.jbc.org/content/265/17/9801.full.html#ref-list-1](http://www.jbc.org/content/265/17/9801.full.html#ref-list-1)