Oligomeric States of the Detergent-solubilized Human Serum Paraoxonase (PON1)*

Received for publication, January 4, 2002, and in revised form, June 5, 2002
Published, JBC Papers in Press, June 21, 2002, DOI 10.1074/jbc.M200108200

Denis Josse‡‡, Christine Ebel‡, David Stroebel‡, Alice Fontaine‡, Frédéric Borges‡, Aude Echalier‡, Delphine Baud‡, Frédérique Renault‡, Marc le Maire‡, Eric Chabrèièrs** and Patrick Masson‡

From the ‡Unité d’Enzymologie, Centre de Recherches du Service de Santé des Armées, 24 avenue des Maquis du Grésivaudan, BP 87, 38702 La Tronche Cedex, France, *Laboratoire de Biophysique Moléculaire, Institut de Biologie Structurale J. P. Ebel, UMR 5075 Commissariat à l’Énergie Atomique-CNRS-UJF, 41 rue Jules Horowitz, F-38027 Grenoble Cedex 01, France, †DBJC/SBPM, URA CNRS 2096 et LRA17V (Commissariat à l’Énergie Atomique/University of Paris XI) Commissariat à l’Énergie Atomique de Saclay, 91191 Gif-sur-Yvette cedex, France, and **LCM3B, Université Henri Poincaré, Nancy 1, B.P. 239, 54506 Vandoeuvre-les-Nancy, France

Human plasma paraoxonase (HuPON1) is a high density lipoprotein (HDL)-bound enzyme exhibiting antiatherogenic properties. The molecular basis for the binding specificity of HuPON1 to HDL has not been established. Isolation of HuPON1 from HDL requires the use of detergents. We have determined the activity, dispersity, and oligomeric states of HuPON1 in solutions containing mild detergents using nondenaturing electrophoresis, size exclusion chromatography, and cross-linking. HuPON1 was active whatever its oligomeric state. In nonmicellar solutions, HuPON1 was polydisperse. In contrast, HuPON1 exhibited apparent homogeneity in micellar solutions, except with CHAPS. The enzyme apparent hydrodynamic radius varied with the type of detergent and protein concentration. In C12E8 micellar solutions, from sedimentation velocity, equilibrium analytical ultracentrifugation, and radioactivity detergent binding, HuPON1 was described as monomers and dimers in equilibrium. A decrease of the detergent concentration shifted this equilibrium toward the formation of dimers. About 100 detergent molecules were associated per monomer and dimer. The assembly of amphiphilic molecules, phospholipids in vitro, in sufficiently large aggregates could be a prerequisite for anchoring of HuPON1 and then allowing stabilization of the enzyme activity. Changes of HDL size and shape could strongly affect the binding affinity and stability of HuPON1 and result in reduced antioxidative capacity of the lipoprotein.

Human serum paraoxonase (HuPON1)† is a high density lipoprotein (HDL)-bound protein exhibiting antioxidative properties (1, 2). It has been shown to hydrolyze phospholipid oxidation products (3), the platelet-activating factor (4), and the l-homocysteine thiolactone (5), thereby contributing to the prevention of atherogenesis and inflammation in blood vessel walls. In addition, HuPON1 has large substrate specificity toward different unnatural compounds: arylesters, organophosphates (OP), including the nerve agents sarin and soman (6), and lactones (7). The physiological importance of PON1 was demonstrated through studies on PON1 knockout mice, which were more sensitive to OP poisoning and more susceptible to atherogenesis relative to wild-type mice (8). In serum, PON1 is exclusively associated with HDL-type complexes, which stabilize the enzyme activity (9, 10) and, presumably, provide the enzyme an optimal environment for interacting with its physiological substrates. The binding of HuPON1 to HDL has been shown to occur at the cell surface, where the enzyme is anchored prior to secretion (10). The molecular basis for the binding selectivity of HuPON1 to HDL-type complexes has not been established. The three-dimensional structure of HuPON1 has not yet been determined. However, as demonstrated by Sorensen et al. (9), the enzyme hydrophobic N terminus, which corresponds to a retained signal peptide, is essential for binding to HDL and phospholipid micelles. Besides interacting with HDL-type complexes, HuPON1 has the capability to associate with other amphiphilic complexes such as phospholipid micelles (9, 10) and, although not demonstrated, detergent molecules. For instance, the nonionic detergent Triton X-100 was used to isolate HuPON1 from other HDL-bound proteins and, similarly to HDL in serum, allowed stabilization of the enzyme activity in vitro (11).

The objectives of this study were to structurally and functionally characterize HuPON1 in detergent solutions and determine whether the detergent type and concentration modulate the activity and interactions of HuPON1. Using detergent molecules as models of amphiphiles, we intended to establish the molecular basis for the interaction selectivity between amphiphilic molecules and HuPON1. Our long term goal is to design HuPON1 mutants with an increased OP hydrolyase activity.

β-D-O-maltopyranoside; HDL, high density lipoprotein; LDAO, n-dodecyl N,N-dimethylamine-N-oxide; CYMAL-6, cyclohexyl-hexyl-β-D-maltoside; FOS-CHOLINE-12, N-dodecylphosphocholine; apoA-I, apolipoprotein A-I; apoA-II, apolipoprotein A-II; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; DTSSP, 3,3’-di-thiobis(sulfosuccinimidylpropionate); DSP, dithiobis(succinimidylpropionate); α-PDM, N,N’-o-phenylenediaminomaleimide; p-PDM, N,N’-p-phenylenediaminomaleimide.

This paper is available on line at http://www.jbc.org
tivity for use as a therapeutic in OP poisoning. A rational design of such mutants ultimately will benefit from the knowl-
edge of the catalytic mechanism for the hydrolysis of OP, based on the HuPON1 three-dimensional structure. Previously, site-
directed mutagenesis has been used to identify residues essential for the HuPON1 OP hydrolase activity (12). However, in the absence of a structural model, it is difficult to confirm whether these residues are located in the enzyme active site and what their functional role is. One of our aims was to find a rationale for crystallization properties of HuPON1, a prerequi-
site for the determination of its three-dimensional structure through x-ray diffraction. Crystallization of HuPON1 first re-
quires the preparation of a pure and homogeneous sample. The purified plasma HuPON1 is a 354-amino acid protein that exhibits 1–3 bands at 37–44 kDa following SDS-PAGE (13). These bands have been shown to correspond to protein glyco-
forms (13). Therefore, homogeneity of HuPON1 pure samples a priori depends on two main factors: the protein glycosylation and oligomeric states. Earlier attempts have been made to determine the homogeneity and apparent molecular mass of the native PON1 isolated from human, rabbit, sheep, and bo-
vine sera (see Ref. 14 for a review). The apparent molecular mass of the native PON1 was found to be variable and ranged within 70–500 kDa. This polydisperse behavior suggests that PON1 exhibits multiple oligomeric states or that the apparent molecular mass is greatly affected by the amount of bound lipids or detergents that were used to dissociate the enzyme from HDL. Since then, the issue of homogeneity and oligomeric states of the native HuPON1 and the influence of detergent in the stoichiometry of the enzyme had not been addressed. In addition, although it was shown that the rabbit PON1 (RaPON1) and HuPON1 monomers retained some activity af-
after SDS-PAGE (15), it had not been clearly established whether the HuPON1 native monomer was active.

In the present work, nondenaturing polyacrylamide gradient gel electrophoresis (PAGGE), size exclusion chromatography, and sedimentation velocity in the presence of mild nonionic or zwitterionic detergents were used to measure the apparent hydrodynamic radius (Rh) and sedimentation coefficient (s) of HuPON1 and establish whether the HuPON1 dispersity was affected by the detergent type and concentration. The specific arylesterase activity of HuPON1 was determined on fractions eluted from size exclusion chromatography, or semiweight-
tively estimated on gel following nondenaturing electrophore-
sis. Chemical cross-linking and analytical ultracentrifugation equilibrium experiments were performed at different detergent concentrations in order to characterize the HuPON1 oligomeric states and determine the effects of the detergent concentration on the enzyme oligomerization. Gel filtration chromatography was used to determine the amount of radioactive detergent molecules bound to HuPON1 in micellar solutions.

The results of this study allowed us to determine the quan-
tary structure and activity of HuPON1 in solutions as a function of detergent type and concentration and establish how the transitions between HuPON1 oligomers are modulated by the detergent concentration. In addition, a model of interaction between HuPON1 and detergent molecules was designed. Fi-
ally, given the structural and functional similarities between the detergent- and HDL-bound HuPON1, the biological signif-
ificance of these findings was addressed.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—The detergents Triton X-100, n-dodecyl-β-maltopyra-
oside (C12-maltoside), and CHAPS were from Sigma; n-dodecyl-N,N-
dimethylethynle-N-oxide (LDAO) and octaethylene glycol monododecyl ether (C12-E8) were from Fluks; ethoxyethoxy- hexyl-β-maltoside (CY-
MAL-6) and N-dodecylphosphocholine (FOS-CHOLINE-12) were from Anatrace. Radioactive C12-E8 at 54.2 μCi/mmol was from CEACelsay, Service des Molecules Marqueses. High and low molecular weight standard proteins were purchased from Amersham Biosciences or Sigma. Human apolipoprotein A-I (apoA-I) and apolipoprotein A-II (apoA-II) were provided by Calbiochem. Phenyacetate, β-naphtyl acetate, and Fast Blue RR were from Sigma.

**PON1 Purification, Activity, and Concentration**—Human and rabbit PON1 were purified according to the method of Gan et al. (11). Briefly, an affinity chromatography on blue Cibacron allowed the isolation of hydrophobic particles from plasma, mainly lipoproteins. Then PON1 was separated from other HDL-bound proteins, mainly apoA-I, using Triton X-100 and DEAE anion exchange chromatography. Enzyme as-
says were performed, at 25 °C, in 50 mM Tris/HCl buffer, pH 8.0, containing 1 mM CaCl2 (buffer A) as described by Gan et al. (11). One unit of enzyme activity corresponded to the hydrolysis of 1 μmol of phenylacetate/min. The protein concentration of PON1 samples was determined using the BCA or micro-BCA kit (Pierce).

**SDS-PAGE and Western Blot Analysis**—PAGE under denaturing conditions (0.1% SDS) was carried out using 4–20% polyacrylamide gradient separating gels according to the method of Laemmli (16). Prior to loading on a 4% polyacrylamide stacking gel, samples were dena-
tured in a loading buffer containing SDS 1% (w/v) and glycerol 10% (v/v). Prestained molecular weight markers (Kaleidoscope; Bio-Rad) were loaded on each gel. SDS-PAGE was performed at constant voltage (100 V) for 8 h with tap water cooling. Electrottransfer (0.5 A, 1 h) onto nitrocellulose membranes (Bio-Rad) was carried out at 100V for 4 h. The membranes were then washed in Tris-buffered saline buffer containing 5% milk and incubated at 25 °C for 2 h with an anti-mouse IgG, horseradish peroxidase-linked antibody from sheep (Amersham Bio-
siences). After a final step washing in Tris-buffered saline buffer, the peroxidase activity was revealed with a chemiluminescent detection kit (ECL kit, Amersham Biosciences).

**Nondenaturing PAGE**—Nondenaturing electrophoresis was performed in 1.5-mm-thick 4–20% polyacrylamide gradient gels, in the alkaline discontinuous buffer system of Ornstein (18) and Davis (19). Samples (~1–10 μg) in buffer A containing 1.5 mM Triton X-100, 0.54 mM C12-maltoside, or 0.18 mM C12-E8 were loaded on 4% polyacrylamide stacking gel. The separating gel was a linear gradient polyacrylamide gel (5–20%). Each of various detergents, nonionic (0.024 or 0.48 mM Triton X-100, 0.34 mM C12-maltoside, 0.18 mM C12-E8, 2.1 mM FOs-CHOLINE-12, 1.19 mM Cymal-6) or zwitterionic (8.1 mM CHAPS, 2.26 mM LDAO), and 1 mM CaCl2 were mixed to the acrylamide/bisac-
rylamide (37.5:1) solution before adding the polymerization agents ammonium persulfate and TEMED. Electrophoresis was carried out at constant voltage (100 V), for 24 h, at 4–6 °C. A calibration curve relating the logarithm of Rh and electrophoretic mobility (Rf), defined as the equilibrium migration distance relative to the size of the gel or to the migration distance of a protein standard, was generated using protein standards of known Rh (21): thyroglobulin (6.6 nm), ferritin (8.3 nm), catalase (5.2 nm), lactate dehydrogenase (4.1 nm), albumin (3.5 nm), and ovalbumin (2.8 nm).

**Staining of Gels for Protein Detection and Arylesterase Activity**—Gels were stained for arylesterase activity according to Furlong et al. (15). Gels were placed in a 100 ml solution of 50 mM Tris/HCl buffer, pH 8.0, containing 1 mM CaCl2 and 50 μg of β-naphtyl acetate dissolved in 1 ml ethanol. Reaction between β-naphtol, generated upon enzymatic hydrolysis of β-naphtyl acetate, and Fast Blue RR (50 mg) yielded red bands. Gel fixing and staining for proteins were performed in 60 mg/liter Coomassie Brilliant Blue, 5% methanol, and 7.5% acetic acid, with destaining in 10% acetic acid. Detection limit of the activity staining was ~0.1 μg of active PON1 per band; it was ~1 μg for the protein staining using Coomassie Blue.

**Detergent Exchange**—Prior to size exclusion chromatography and analytical ultracentrifugation, Triton X-100 in the purified enzyme was exchanged for C12-E8 or C12-maltoside using anion exchange chromatography on a Q-Sepharose fast flow gel (Amersham Biosciences). Five milliliters of gel were placed on an 8-mm-diameter column (Amersham Biosciences). The gel was first equilibrated with 25 ml of buffer A containing an appropriate concentration of C12-E8 (buffer B). Purified HuPON1, stabilized in 1.5% Triton X-100, was first diluted by a 2-fold factor in buffer B and then injected on the gel at a 2 ml/min flow rate. The gel was then washed with 25 ml of buffer B. Elution of HuPON1 was performed with buffer B containing an appropriate concentra-
tion of NaCl.

**Size Exclusion Chromatography**—Size exclusion chromatography was performed at 25 °C using a 30-ml Superdex 75 HR 10/30 column
(Amersham Biosciences). The gel was equilibrated in buffer A containing 0.36 mM C$_2$E$_9$ or 0.68 mM C$_{12}$-maltoside. Samples of HuPON1 (100 μl, −0.5 mg/ml) were loaded on the gel after Triton X-100 exchange with C$_2$E$_9$ or C$_{12}$-maltoside, as already described. The flow rate was 0.5 ml/min, and 0.5-ml fractions were collected. The absorbance at 280 nm and the total detergent concentration of the solvent were accurately measured. The CMC was determined by the conductometric method or the adsorption method. The CMC of the solvents was, respectively, 0.7265 ml/g, 0.731 ml/g, and 0.7400 ml/g. We related the activity of the detergent to that of the monomer detergent, $a_{	ext{monomer detergent}}$, which is linked to the presence of the micelle species, $a_{	ext{micelle}}$, since, for a detergent undergoing auto-association to micelles with an association number $n$ (28), the following equation is true: $\mu_{\text{monomer detergent}} - \mu_{\text{monomer detergent}}$ is the gas constant (8.315 J/mol·K) and $T$ is the absolute temperature. Thus, equation (1) can be written in the following form.

$$\ln \frac{K_d}{\ln a_{\text{micelle}}} = -N_{\text{int}}$$

(Eq. 1)

The effects of hydration, emphasized by Tanford (27), were ignored here in view of the low detergent concentration relative to that of water. We related the activity of the detergent to that of the "free" monomer detergent, $a_{\text{monomer detergent}}$, which is linked to the presence of the micelle species, $a_{\text{micelle}}$, since, for a detergent undergoing auto-association to micelles with an association number $n$ (28), the following equation is true: $\mu_{\text{monomer detergent}} - \mu_{\text{monomer detergent}}$ is the gas constant (8.315 J/mol·K) and $T$ is the absolute temperature. Thus, equation (1) can be written in the following form.

$$\ln \frac{K_d}{\ln a_{\text{micelle}}} = N_{\text{int}}$$

(Eq. 2)

We considered for $a_{\text{micelle}}$, the micelle concentration calculated from the total detergent concentration of the solvent, the CMC, and $n$ as follows.

$$[\text{micelle}] = (\text{[total detergent]} - \text{CMC})/n$$

(Eq. 3)

The total detergent concentration of the solvent was accurately known for HuPON1 samples after the chromatography on Q-Sepharose as described above. We neglected the variation of the bulk detergent related to HuPON1 dissociation upon dilution. For C$_{12}$E$_9$, we used $n = 89$, and CMC = 0.09 mM (29).

**Evaluation of the Amount of Detergent Released upon Protein Association**—Wymann (26) demonstrated the influence of ligand binding on protein equilibrium processes through the theory of linked functions. The dissociation of dimer is characterized by the apparent dissociation constant, $K_d$. $K_d$ is an apparent value, since it is determined in view of the concentrations of monomers and dimers only. If the dissociation process is accompanied by a change in the number of bound molecules of detergent ($\Delta N_{\text{int}}$), $K_d$ evolves with the activity $a_{\text{monomer detergent}}$ as follows.

$$\ln \frac{K_d}{\ln a_{\text{micelle}}} = -N_{\text{int}}$$

(Eq. 4)

We evaluated $N_{\text{int}}$ by using $a_{\text{micelle}}$ and $a_{\text{monomer detergent}}$ obtained from the sedimentation coefficient $s$ and the partial specific volume $\psi$ of HuPON1. The protein con-
The amount of bound detergent, $\Delta_{\text{det}}$, was calculated using the equation,

$$\Delta_{\text{det}} = \frac{(\text{cpm}_{\text{peak}} - \text{cpm}_{\text{bl}})/\text{cpm}_{\text{bl}} - C_{\text{det}}/C_{\text{HuPON1}}}{(C_{\text{det}}/C_{\text{HuPON1}})}$$

where $C_{\text{det}}$ and $C_{\text{HuPON1}}$ correspond to the concentration of the detergent and HuPON1 in the solvent, respectively.

**RESULTS**

Influence of Detergent Type and Concentration on the Dispersion and Apparent Rh of HuPON1—The size dispersity and Rh of HuPON1 were determined by nondenaturing PAGGE and size exclusion chromatography. Upon nondenaturing electrophoresis in a gel made of a continuous concentration gradient of polyacrylamide, the proteins are forced to migrate through progressively smaller pores, the sizes of which depend on the polyacrylamide concentration (30). The proteins stop migrating when they reach pores narrower than their diameter. Similarly to size exclusion chromatography, this allows resolution of proteins according to their size. However, by contrast to size exclusion chromatography, which leads to dilution of resolved proteins in the eluate, nondenaturing PAGGE results in concentration of resolved proteins in thin bands.

As illustrated in Fig. 1, the dispersity of HuPON1 from the electrophoresis experiments mainly depended on the presence or absence of detergent in the polyacrylamide gel. With the exception of CHAPS, a unique red band corresponding to active HuPON1 was observed when the detergent concentration incorporated in the gel was slightly higher than the CMC (Fig. 1A). Electrophoresis in gels that did not include detergent molecules (Fig. 1B) or that incorporated a Triton X-100 concentration 10 times lower than the CMC (not shown), or 8.1 mM CHAPS (not shown) led to streaking of HuPON1 and resolution of multiple oligomeric species. As shown in Table 1, the main HuPON1 species had Rh of 3.8, 4.2, and 4.6 nm. This indicated enzyme heterogeneity in media containing nonmicellar concentrations of detergent. Identical electrophoretic profiles were obtained for HuPON1 initially solubilized in buffer A containing 0.18 mM C12E8, 0.34 mM C12-maltoside, or 1.5 mM Triton X-100 (Fig. 1B, lanes 6–8). This suggested that the HuPON1 oligomers resolved in the gel, if associated with detergent molecules, had the same general structure in the presence of C12E8, C12-maltoside and Triton X-100 molecules or that the HuPON1 self-associated species were no longer bound to nonionic detergent molecules as a result of dissociation during electrophoresis. In addition, the electrophoretic pattern of HuPON1 did not change when the amount of protein loaded on the gel was reduced from 10 to 1 μg (Fig. 1B, lanes 6 and 5), suggesting that the dispersity of HuPON1 in nonmicellar media was not dependent of the protein concentration.

Rh of HuPON1 could be determined using the linear relationship between the logarithm of Rh and Rf of protein standards when compared with the HDL-binding proteins apoA-I, apoA-II, and PON1. With a detergent concentration higher than the CMC, the Rh of HuPON1 varied between 3.8 and 4.8 ± 0.1 nm, depending on the type of detergent incorporated in the gel (Table 1). This is indicative of interaction between HuPON1 and detergent molecules incorporated in the gels. Additionally, as shown in Table 1, there was a positive correlation between Rh of the HuPON1 and the size of the detergent micelles, suggesting either that HuPON1 could be associated with micelles in gels or that HuPON1 could autoassociate in a detergent type-dependent way (see “Discussion”).

At detergent concentrations below the CMC, the Rh values can result from several scenarios. The lowest value determined for the HuPON1 Rh after PAGGE without detergent in gel was found to be 3.8 nm, corresponding to a globular protein of ~90 kDa. The 4.2- and 4.6-nm Rh species, of 130- and 170-kDa apparent molecular mass, respectively, could correspond to HuPON1 globular trimers and tetramers. Alternatively, this could be the result of species of lower stoichiometry with associated detergent or elongated shapes. Comparison of band intensity indicated that the 3.8-nm and, to a lesser extent, the 4.6-nm species were the predominant forms when compared with the 4.2-nm ones.

In gels incorporating micellar concentrations of detergents (see Fig. 1A for Triton X-100), RaPON1 was slightly larger than HuPON1. This could result from the 4-amino acid differences
between the polypeptide chain size of HuPON1 and RaPON1. It could also be due to a different shape of detergent-enzyme complexes or to distinct protein-protein or protein-detergent interactions for RaPON1 and HuPON1.

HuPON1 forms and temperature of analysis were distinct (see Experimental Procedures). While measuring the amount of bound detergent (see below; Fig. 7), we clearly determined that the volume of elution was dependent on the loading concentration with, in the presence of 0.36 mM C12E8, apparent Rh of 4.6 and 4.4 ± 0.1 nm for loading concentrations of 1.2 and 0.3 mg/ml, respectively, indicating an autoassociation process for HuPON1.

Cross-linking of HuPON1—The homobifunctional Lys-selective DSP and DTSSP and Cys-selective o-PDM and p-PDM were used as cross-linking reagents of HuPON1. In the control without cross-linking agent, the ~40-kDa SDS-denatured HuPON1 monomeric glycoforms were the only species detected (Fig. 3B, lane 4). By contrast, following cross-linking with DTSSP, DSP, o-PDM, or p-PDM, multiple SDS-resistant oligomeric proteins were detected by Western blot analysis. Cross-linking of HuPON1 with o-PDM and p-PDM suggested that the free Cys residues of HuPON1 monomers were accessible, correctly oriented within an adequate distance for reacting with neighboring subunits of a complex. As shown in Fig. 3, the proportion of HuPON1 SDS-resistant oligomers depended on type and concentration of cross-linking reagents and concentration of detergent relative to the CMC. The proportion of HuPON1 oligomers suggested heterogeneity of reactions between HuPON1 and the reagents used, or it possibly came from lys residues in a preferentially hydrophilic or hydrophobic environment, respectively. After dithiothreitol treatment, the DTSSP- and DSP-cross-linked HuPON1 were converted into monomers, demonstrating reversibility of Lys-selective cross-linking reactions (not shown).

When the detergent concentration was lower than the CMC, the HuPON1-cross-linked oligomers had ~80–180-kDa apparent molecular mass. The broadness of bands corresponding to HuPON1 oligomers suggested heterogeneity of reactions between HuPON1 and the reagents used, or it possibly came from enzyme aggregation (Fig. 3B). The proportion of HuPON1-cross-linked oligomers was much lower in micellar media as opposed to that in nonmicellar media. Reaction of HuPON1 with DTSSP and DSP led to the formation of mainly 80-kDa oligomers and, at a lower extent, to 120-kDa species (Fig. 3A, lanes 3 and 4). Given that the HuPON1 monomer has a molecular mass of ~40 kDa, the 80- and 120-kDa cross-linked oligomers could correspond to HuPON1 dimers and trimers, respectively. Reaction of HuPON1 with o-PDM and p-PDM led to

---

**TABLE I**

Apparent hydrodynamic radii of HuPON1 as determined using nondenaturing PAGGE in gels incorporating or not incorporating detergent molecules

| Detergents          | CMC * | Concentration in gel | HuPON1 Rh † | Micelle Rh ‡ |
|---------------------|-------|----------------------|-------------|-------------|
|                     |       |                      | ms          | nm          | nm          |
| Triton X-100        | 0.23  | 0.17                 | 4.8         | 3.42        |
| C12E8               | 0.18  | 0.34                 | 4.4         | 3.22        |
| C12-maltoside       | 0.09  | 1.91                 | 4.2         | 2.99        |
| CÝMAL-6             | 0.56  | 1.19                 | 4.0         | —           |
| LDAO                | 1.5   | 2.26                 | 4.0         | 2.30        |
| FOS-CHOLINE-12      | 1.5   | 2.1                  | 3.9         | —           |
| CHAPS               | 8     | 8.1                  | 3.8;4.2;4.6 | —           |
| Triton X-100        | 0.23  | 0.024                | 4.2;4.2;4.6 | —           |
| or without detergent| 0     | 4.6;4.2;4.6          |             |             |

* The CMC values of detergents were taken from Ref. 29 for C12E8 and Anatrace company technical data for the other detergents.
† HuPON1 Rh was determined from the linear relationships between the logarithm of Rh and Rf of protein standards. From two experiments performed with HuPON1 in each detergent solution, we determined that variations in calculated apparent Rh values were less than 0.1 nm.
‡ The Rh values of detergent micelles were from Ref. 20.
—, data not found in the literature.
the formation of two cross-linked species. They had apparent molecular masses of 100 and 120 kDa, respectively, higher than expected for SDS-denatured HuPON1 covalent dimers. However, since we did not get evidence for HuPON1 trimers by sedimentation equilibrium (see below), the bands of 100 and 120 kDa are higher than expected for SDS-denatured HuPON1 covalent dimers. Cross-linking experiments were performed in solutions containing (A) or not containing (B) C12E8 micelles, as described under "Experimental Procedures." The mass (in kDa) of cross-linked HuPON1 species are indicated on the left side of A and on the right side of B. A, lane 1, 0.1 mM p-PDM; lane 2, 0.5 mM p-PDM; lane 3, 1 mM DTSSP; lane 4, 0.1 mM DTSSP. B, lane 1, 0.1 mM DSP; lane 2, 1 mM DTSSP; lane 3, 0.1 mM DTSSP; lane 4, control without cross-linking reagent; lane 5, 0.1 mM o-PDM; lane 6, 0.5 mM o-PDM.

FIG. 3. Western blot analysis of HuPON1 cross-linked species. C12E8-solubilized HuPON1 (0.5 µg/lane) was cross-linked with homobifunctional Lys-specific (DTSSP and DSP) or Cys-specific (o-PDM and p-PDM) reagents with linker lengths of 12 (DTSSP, DSP), 10 (p-PDM), and 6 Å (o-PDM). Cross-linking experiments were performed in solutions containing (A) or not containing (B) C12E8 micelles, as described under “Experimental Procedures.” The mass (in kDa) of cross-linked HuPON1 species are indicated on the left side of A and on the right side of B. A, lane 1, 0.1 mM p-PDM; lane 2, 0.5 mM p-PDM; lane 3, 1 mM DTSSP; lane 4, 0.1 mM DTSSP. B, lane 1, 0.1 mM DSP; lane 2, 1 mM DTSSP; lane 3, 0.1 mM DTSSP; lane 4, control without cross-linking reagent; lane 5, 0.1 mM o-PDM; lane 6, 0.5 mM o-PDM.

Effects of the Protein and Detergent Concentration on the Sedimentation Coefficient of C12E8-solubilized HuPON1—Sedimentation velocity experiments were performed at various HuPON1 and C12E8 concentrations. They were analyzed in terms of distribution of sedimentation coefficients, which allowed a qualitative evaluation of the enzyme homogeneity and autoassociation capability. Fig. 4A presents the distribution obtained for HuPON1 at 18 and 6 µM in 50 mM Tris/HCl, pH 8.0, containing CaCl2, 1 mM and C12E8 0.36 mM. In this experiment, Triton X-100 was exchanged for C12E8 by membrane ultrafiltration. We observed a complex pattern, with two main peaks, the position of the main one being shifted from $s_{20,w} = 4.9$ S to $s_{20,w} = 4.6$ S when the protein concentration was decreased, which suggested an autoassociation equilibrium process. The light species at 3.5 S was not related to remaining micelles of Triton X-100; considering a molar mass of 90 kDa and a partial specific volume of 0.908 cm3/g (28), we calculated a $s_{20,w}$ value of 1.9 S. Complexity of the HuPON1 sedimentation profile could result from only partial Triton X-100 substitution for C12E8 when using membrane ultrafiltration for the detergent exchange. Consequently, part of HuPON1 could still be associated with Triton X-100, another part with C12E8 or with a mixture of both detergents. Accordingly, when the detergent was exchanged by using Q-Sepharose chromatography, the sedimentation profile of HuPON1 suggested apparent homogeneity of the sample, possibly as a result of complete Triton X-100 substitution for C12E8. We thus determined the effect of the detergent concentration on the sedimentation pattern of HuPON1, by using Q-Sepharose chromatography for the detergent exchange. From Fig. 4B, it is clear that increasing the C12E8 concentration from 0.81 to 7.29 mM, while maintaining the HuPON1 concentration in the 5–8 µM range, decreased the sedimentation coefficients, $s_{20,w}$, from 3.5 to 3 S. This suggested a dissociation of a multimer when the detergent concentration increased. An equilibrium between monomers and dimers was suggested from the 2–5 S range of experimental $s_{20,w}$, since for two noninteracting globular proteins of molar masses 40 and 80 kDa, corresponding to the HuPON1 monomer and dimer, we calculated theoretical values for $s_{20,w}$ of 3.4 and 5.4 S, respectively, and the presence of the detergent or the form asymmetry would decrease these values. However, the analysis of the distribution of sedimentation coefficients in the case of interconverting system is complex, even in the absence of interactions with detergents, and we used sedimentation equilibrium to characterize the stoichiometry of HuPON1 in solution.

Molecular Mass of C12E8-solubilized HuPON1 by Sedimentation Equilibrium—For sedimentation equilibrium experiments, we used samples obtained after detergent exchange through a chromatographic step prior to eventual precise dilution in order to control the detergent concentration. Table II indicates the range of protein and C12E8 concentrations investigated. The detergent C12E8 was chosen because, as opposed to...
Oligomeric States of Human PON1

FIG. 4. Sedimentation velocity experiment of PON in the presence of C_{12}E_8. Sedimentation velocity experiments performed at 6–8 °C and 40,000 rpm were analyzed in terms of a continuous distribution of sedimentation coefficients s_{20,w} (see “Experimental Procedures”). The vertical scale was adapted to assign for the various samples the same arbitrary concentration for main sedimenting species. A, the distributions obtained for HuPON1 at 18 μM (continuous line) and 6 μM (dashed line) in 50 mM Tris/HCl, 1 mM CaCl_2, 0.36 mM C_{12}E_8 (the solvent was exchanged by membrane ultrafiltration). B, the distributions for HuPON1 at ~5–8 μM in 50 mM Tris/HCl, 1 mM CaCl_2, and 0.81 mM C_{12}E_8, 280 mM NaCl (dashed line), 1.54 mM C_{12}E_8, 280 mM NaCl (dotted line), 7.29 mM C_{12}E_8, and 140 mM NaCl (continuous line).

Table II
Equilibrium sedimentation of HuPON1 in the presence of C_{12}E_8

| C_{12}E_8 | HuPON1 | Micelle of C_{12}E_8 | IV | K_d |
|-----------|--------|----------------------|----|-----|
| mM       | μM    | μM                   | μM| μM  |
| 7.99      | 7.6   | 80.9                 | 7.4|     |
| 4.86      | 5.1   | 53.6                 | 4.4|     |
| 2.43      | 2.5   | 26.3                 | 2.0|     |
| 1.54      | 8.3   | 16.3                 | 3.2|     |
| 1.02      | 5.6   | 10.4                 | 1.3|     |
| 0.81      | 5.5   | 8.1                  | 1.1|     |
| 0.54      | 3.7   | 5.1                  | 0.3|     |
| 0.51      | 2.8   | 4.7                  | 0.4|     |
| 0.27      | 1.8   | 2.0                  | 0.1|     |

Triton X-100, it does not absorb light at 277 nm, and the reported values of its partial specific volume are close to 1 (0.973 cm^3/g, according to Ref. 29), and thus its density (approximately the inverse of the partial specific volume) is close to that of the solvent. Thus, we can consider that free or bound C_{12}E_8 are not detected in the sedimentation equilibrium profiles, which essentially address the protein stoichiometry. This strategy was used previously to address by equilibrium sedimentation the oligomerization of transmembrane fragments (32) and membrane proteins (33, 34).

When the equilibrium sedimentation profiles were fit according to a one-species model, the molecular mass measured, 60 kDa, was intermediate between the molecular mass of a monomer (~40 kDa) and of a dimer (~80 kDa). This agreed with the sedimentation velocity experiments and suggested that PON1 monomers and dimers were in equilibrium in C_{12}E_8 micellar solutions. Thus, the data corresponding to the same solvent composition at four rotor speeds were then modeled together assuming a monomer-multimer equilibrium. We first fitted the monomer molar mass, the association number, and the association constant. The derived molar masses for the monomer being always close to the theoretical value for the HuPON1 polypeptide chain and the association number being close to the value of 2, we then fixed this monomer molar mass value to the theoretical one and fitted only the association constant. Considering an equilibrium between monomers and dimers gave better results than monomer-trimer and monomer-tetramer equilibrium. We were unable to fit our data with a model consisting of three species in equilibrium (monomers, trimers, and tetramers). Using the monomer-dimer model, the quality of the fit was reasonably good, even if the residuals were not completely random, as can be seen for one example on Fig. 5. The reason could be that the detergent bound in different amounts by HuPON1 in its different oligomeric forms would not be completely masked. So, even if multimers of higher stoichiometry in small amounts are not definitively excluded from our data, we restricted our analysis to a monomer-dimer equilibrium.

Effect of the C_{12}E_8 Concentration on the Equilibrium Constant (K_d) Corresponding to the HuPON1 Monomer-Dimer Equilibrium—The values of the dissociation constants, K_d, corresponding to the monomer-dimer equilibrium, measured at different concentrations of C_{12}E_8, are reported in Table II. It was clear that an increase of the detergent concentration was accompanied by an increase of K_d. This was in qualitative agreement with the sedimentation velocity experiments described above. The fact that K_d varied with the C_{12}E_8 concentration means that the detergent had distinct interactions for the monomer and the dimer. Consequently, the detergent is a partner of the equilibrium. Increasing the C_{12}E_8 concentration favored the species that interact the most efficiently with the detergent; as shown in Table II, in the case of HuPON1, it was clearly the monomeric forms. To quantify the difference in the bound detergent between the HuPON1 monomeric and dimeric forms, we plotted the logarithm of K_d as a function of logarithm of the detergent concentration in the solvent. As seen in Fig. 6, the data can be fit with a straight line. The inferred slope of 1.1, expressed in micelle units, is the number of detergent mole-
cules (i.e., 98) that were released upon the HuPON1 dimerization.

**Evaluation of the Amount of Bound Detergent**—Fig. 7 shows the elution profiles of 200 and 100 μl of HuPON1 injected at 0.3 and 1.2 mg/ml, respectively, in the presence of 0.36 mM radioactive 14C12E8 and 0.28 M NaCl. The lines correspond to the fitted curves and were obtained considering a monomer-dimer equilibrium with a dissociation constant of 1.1 μM. B, the differences, normalized by the statistical error of experimental absorbance δA, between the experimental A and calculated A₀ values of the absorbance. The interval between the ticks has a value of 5.

A

**Fig. 5. Global fitting of the sedimentation equilibrium.** A, the experimental sedimentation equilibrium profiles of absorbance at 277 nm as a function of the radial position in the ultracentrifuge obtained at 6 °C, at 10,000 (∗), 12,000 (+), 15,000 (□), and 20,000 (○) rpm for HuPON1 at 5.5 μM in the presence of 0.81 mM C₁₂E₈ and 0.28 M NaCl. The lines correspond to the fitted curves and were obtained considering a monomer-dimer equilibrium with a dissociation constant of 1.1 μM. B, the differences, normalized by the statistical error of experimental absorbance δA, between the experimental A and calculated A₀ values of the absorbance. The interval between the ticks has a value of 5.

B

The maximum concentrations of elution of HuPON1 were 61 and 31 μg/ml, respectively. A significantly lower ratio of 0.45 μg/g was measured for the fractions eluted from the Q-Sepharose. However, the measurement of low levels of radioactive bound detergent is generally reported for other proteins after ion exchange chromatography (29), and we will not consider this result here. From the gel filtrations, the value of $\text{det}$ is in the range usually found for membrane proteins (29). It corresponds to about 80 mol of C₁₂E₈/mol of polypeptide. A value of $K_d$ = 0.24 μM is interpolated from our results in 0.36 mM C₁₂E₈. It provides, for a
the number of detergent molecules bound to the monomer, molecules of detergent/monomer upon HuPON1 dimerization, considered fractions), a value for the proportion \( f \) of detergent micelle \((\text{micelle})\). The linkage relationship between HuPON1 dissociation constant \( K_d \) and the detergent activity provides the number of detergent molecules released upon dissociation, \( \Delta N_{\text{det}} \), obtained in units of detergent micelle \((\text{micelle})\), where \( n \) is the aggregation number of the micelle: \( \ln K_d / \ln a_{\text{micelle}} = \Delta N_{\text{det}} / n \). The linear fit of the plotted data provides for \( \Delta N_{\text{det}} / n \) a value of 1.1.

HuPON1 concentration of 40 \( \mu \text{g/ml} \) (i.e. close to that of the considered fractions), a value for the proportion \( f_{\text{mono}} \) of the polypeptide chain in the monomer form of 0.5. Considering, from equilibrium sedimentation, the release of \( \Delta N_{\text{det}} / 2 = 50 \) molecules of detergent/monomer upon HuPON1 dimerization, the number of detergent molecules bound to the monomer, \( \Delta_{\text{det(monomer)}} \), can be estimated from the following:

\[
\Delta_{\text{det}} \text{ (mol/mol)} = f_{\text{mono}} \cdot \Delta_{\text{det(monomer)}} + (1 - f_{\text{mono}}) \cdot (\Delta_{\text{det(monomer)}} \cdot \Delta N_{\text{det}} / 2) \quad (\text{Eq. 5})
\]

This gives a value of 105 and 110 mol of \( C_{12}E_8 \) that would be bound per mole of monomer and dimer, respectively. This measurement indicates that the monomer of HuPON1 is associated with a number of \( C_{12}E_8 \) molecules corresponding to nearly a micelle. Note, however, that, as first pointed out by Tanford (35), this result does not demonstrate that the \( C_{12}E_8 \) molecules are actually arranged as in a protein-free micelle (see “Discussion”).

**DISCUSSION**

In this work, we have characterized different oligomeric states of detergent-solubilized HuPON1 purified from plasma. They all exhibited arylesterase activity. We demonstrated that the detergent type and concentration could strongly affect both the HuPON1 dispersity and oligomeric state. In micellar solutions of \( C_{12}E_8 \), a monomer to dimer equilibrium, regulated by the detergent concentration, with the number of bound detergent molecules nearly 100 per monomer and per dimer was found to be the most reliable model. This number corresponds roughly to one micelle for the detergent in solution. In nonmicellar media, HuPON1 was shown to self-associate in multiple oligomeric forms and aggregates.

**Detergent-dependent Interactions**

Similarly to membrane proteins, we found that protein-protein and protein-detergent interactions specific to HuPON1 were strongly affected by the detergent concentration relative to the CMC and the detergent type.

**Nonmicellar Solutions**—In nonmicellar solutions, the sizes of the main oligomeric species observed by nondenaturing PAGGE could correspond to globular dimers, trimers, and tetramers of HuPON1. Accordingly, HuPON1 covalent dimers, trimers, and tetramers were formed upon cross-linking in nonmicellar solutions. Oligomerization in nonmicellar solutions is a usual trait for membrane proteins and has been demonstrated to occur for other HDL-bound proteins: apoA-I (36), cholesteryl ester transfer protein (37), and lecithin:cholesterol acyltransferase (38). It has been shown to come from the tendency of hydrophobic domains of distinct subunits to autoassociate as a result of unmasking by bound lipids or detergents.

The existence of exposed hydrophobic regions for HuPON1 cannot be firmly established without knowledge of the enzyme three-dimensional structure. However, when combining the Kyte-Doolittle hydropathy profile (39) with predictions for locations of transmembrane regions using TMPred (available on the World Wide Web at www.isrec.isb-sib.ch/ftp-server/tmpred/ www/TMPRED_form.html), we found that HuPON1 could have one transmembrane \( \alpha \)-helical region corresponding to the first 19 amino acids from its N terminus. Therefore, this putative transmembrane \( \alpha \)-helix, which actually corresponds to the retained N-terminal signal peptide of HuPON1, could be involved in the protein autoassociation process in nonmicellar solutions. Whether or not the HuPON1 oligomers formed in these conditions interact with detergent molecules has not been established. This could actually depend on whether the protein-protein interactions in the HuPON1 oligomers lead to total or only partial shielding of the exposed hydrophobic domains.

**Micellar Solutions**—In micellar solutions, sedimentation velocity and equilibrium indicated that the oligomeric state of \( C_{12}E_8 \)-solubilized HuPON1 could change upon varying the detergent concentration. The most reliable model that fit the sedimentation equilibrium data was that of a monomer-dimer reversible equilibrium. The tendency of HuPON1 to autoassociate was qualitatively confirmed in the cross-linking experiments demonstrating the existence of large species of \( 80–120 \)-kDa apparent molecular mass. The number of \( C_{12}E_8 \) molecules bound to the HuPON1 monomer and dimer was shown to be close to the aggregation number for this detergent (i.e. 100). Additionally, from the relationship between \( K_p \), the dissociation constant corresponding to the monomer-dimer equilibrium, and the detergent concentration, we determined that the dimerization process led to the liberation of \(-100\) molecules of \( C_{12}E_8 \). The results of cross-linking studies indicated that HuPON1 had fewer amino acids accessible and that its paired Cys residues were more specifically distant and oriented in micellar solutions when compared with nonmicellar solutions. These results suggest that the HuPON1 dimers formed in nonmicellar and micellar solutions are structurally distinct. In micellar solutions, detergent molecules associated with HuPON1 dimers could prevent direct protein-protein interactions between protected hydrophobic domains.

Bovine cytochrome \( c \) oxidase, which is a dimer within most crystals and assumed to be a dimeric complex in the membrane, was characterized in a variety of detergent solutions as a mixture of monomers and dimers and/or aggregates (33). This system presented a lot of similarities with the HuPON1 one. In
particular, the relative amount of monomers and dimers was found to depend on the type and concentration of the detergent, whereas the monomeric form was functional for all of the activities evaluated. In a recent paper, it was found that the addition of bile salts stabilized the dimer form and caused, in appropriate conditions, complete reassociation of the monomer (40). It would be interesting to see whether, as it is the case for cytochrome c oxidase, a low amount of cholate stabilizes the dimeric form of HuPON1.

Anchoring Versus Shielding of a Large Hydrophobic Area by Detergent Molecules

At least two different models of detergent binding by HuPON1 can be considered. One could simply result from anchoring of the hydrophobic N-terminal signal peptide of HuPON1 to detergent aggregates. Assuming that the C12Es molecules bound to HuPON1 are organized as micelles, a reasonable model for the association process of HuPON1 in micellar media would thus consist of two HuPON1 monomeric forms, each associated with one detergent micelle leading to the formation of a HuPON1 dimeric form associated with one detergent micelle. In this model, the dimerization process would be associated with the release of one detergent micelle. At the present stage of knowledge, reviewed by le Maire et al. (41), detergent micelles may provide a suitable membrane-like environment around small membrane peptides. This anchoring could require a minimal size and specific shape of detergent micelles. Accordingly, the autoassociation of HuPON1 in nonmicellar media or in media containing 6-kDa CHAPS micelles could result from an incapacity of HuPON1 to bind free detergent molecules or very small micelles. However, the more general model for detergent binding consists of a monolayer on the hydrophobic surface of the protein. In this model, for large proteins, the number of bound detergent molecules was related to the exposed hydrophobic surface. Can our results on HuPON1 give insight concerning the surface that is exposed upon dimer dissociation when the detergent concentration is increased? We calculated that about 100 molecules of released detergent with a cross-sectional area of 0.5 nm² would correspond to an exposed surface of 50 nm² (41). Bacteriorhodopsin is formed of seven trans-membrane helices and described to bind nearly the same amount (119 molecules) of C12Es (29). These arguments suggest that the hydrophobic exposed surface is quite large and would correspond to contact surfaces in addition to that of an entirely extended N terminus for the monomer. Thus, this would not be consistent with the structural data predicted from the HuPON1 amino acid sequence.

Effect of the Detergent Type

Assuming that HuPON1 binds to detergent molecules organized as micelles, the positive correlation of apparent Rh of the HuPON1-detergent complex with that of the detergent micelle could result from a higher affinity of the HuPON1 dimer, when compared with that of the monomer, for the largest micelles. Alternatively, the size of the interacting species could change with the detergent type, as has already been observed for the monomer of the integral membrane protein bacteriorhodopsin (42). In that case, the dimension of detergent-solubilized HuPON1 would be determined by the size of the free micelle.

Structural and Functional Similarities between HDL- and Detergent-bound Forms of HuPON1

Structural Features—In serum, the apolipoprotein apoA-I is involved in the assembly of phospholipid micelles, thus generating nascent HDL. Whereas large reconstituted apoA-I-HDL of 8.5-nm Rh were very efficient to release HuPON1 from transfected Chinese hamster ovary cells (10), in human plasma, PON1 has been shown by electron microscopy to be associated with an HDL subspecies of about 4.8 ± 0.6-nm Rh (17). The latter Rh values are of the same order as those of PON1-detergent complexes, 3.8–4.8 nm, as determined using nondenaturing PAGE and size exclusion chromatography, suggesting that the detergent aggregate can at least partially mimic the hydrophobic environment of the protein bound to HDL although the HuPON1-detergent complexes characterized in this work lack protein components, such as apoA-I, apoJ.
compared with relatively large detergent micelles (i.e. compatible amphiphilic compounds that will preserve the enzyme activity). In serum, HuPON1 was exclusively found associated with HDL-type complexes, which differ in size and composition from the much larger low density and very low density lipoproteins of 20-nm and 30–80-nm diameter, respectively (45). As hypothesized by Oda et al. (46), since the HuPON1-HDL lipoparticles characterized in plasma were mainly found associated with apoA-I (9, 17) and HDL-like complexes containing apoA-I were found to be necessary for optimal release and stabilization of HuPON1 (10), the association specificity of HuPON1 with HDL might rely on transient interaction of HuPON1 with apoA-I. However, interaction with apoA-I was not an absolute requirement for binding of HuPON1 to HDL-like complexes in apoA-I-deficient sera (9, 47, 48), to phospholipid micelles (9, 10), and, as suggested by the present work, to detergent micelles.

Alternatively, results of a recent study from Deakin et al. (10) and this work suggest that the size of detergent or phospholipid complexes might be the predominant factor that affects the binding affinity and stabilization of HuPON1 in serum and in vitro. Thus, subpopulations of apoA-I-containing lipoparticles of specific size and shape could provide optimal hydrophobic environment for anchoring HuPON1 and stabilizing the enzyme activity. This interpretation was suggested for cholesteryl ester transfer protein, which also specifically binds to apoA-I-containing HDL (49).

From the already mentioned predictive program of transmembrane regions, TMpred, the HuPON1 homologue, HuPON3, is predicted to have one transmembrane α-helix at its N-terminal extremities. This suggests that, as predicted for HuPON1, HuPON3 could interact specifically with HDL through anchoring of an N-terminally located hydrophobic α-helix. Accordingly, the HuPON1 homologous HuPON2, which was expressed exclusively intracellularly (50), is not predicted to exhibit an N-terminal transmembrane domain.

Apo A-I consists of several amphipathic α-helices that interact with the acyl chains of the lipid bilayers on the HDL side (51). The HDL-binding mode of HuPON1 and HuPON3 would thus be distinct from that of apoA-I and other apolipoproteins involved in recruiting phospholipids and generating nascent lipoparticles.

Functional Features—In apoA-I-deficient sera (9, 47, 48) or cell culture media (9, 10, 46), PON1 was found to be associated with relatively small and dense HDL-type complexes. The enzyme was active, but it displayed a specific activity toward phenylacetate significantly lower than that in apoA-I-containing media probably as a result of enzyme partial inactivation. Binding of HuPON1 to the larger apoA-I-containing HDL was thus assumed to be required for optimal specific activity and stability of the enzyme (9, 10, 46). This is consistent with preliminary results indicating that, in vitro, HuPON1 storage stability was much less important in nonmicellar solutions than that in micellar solutions and had very low stability in solutions containing small detergent micelles (i.e. CHAPS) compared with relatively large detergent micelles (i.e. Triton X-100) (current work in progress). As shown using nondenaturing PAGE, enzyme inactivation in nonmicellar media could result from protein aggregation. This could also be related to aggregation and inactivation of recombinant HuPON1 when secreted in culture media by Hi-5 insect cells or when expressed in Escherichia coli (52). A practical consequence of this is that formulation of HuPON1 for a use as therapeutics in OP poisoning should include adequate concentration of biocompatible amphiphilic compounds that will preserve the enzyme stability on storage and activity in vivo. In addition, the low specific activity of HuPON1 in serum associated with certain pathologies, such as that reported for diabetes (53–55), might in fact be due to changes in HDL size and/or shape, which affect the binding affinity and, as a result, stability of the HuPON1 enzyme activity.

Choice of the Detergent Type and Concentration to Obtain a Homogeneous and Active HuPON1 Sample

Activity of HuPON1 Samples—In this work, we showed that the arylesterase activity of HuPON1 toward β-naphthyl acetate was preserved after electrophoresis in gels containing or not containing nondenaturing concentrations of the nonionic C12E8, C12-maltoside, Triton X-100, FOS-CHOLINE-12, or CYMAL-6 detergent or the zwitterionic LDAO or CHAPS detergent. In addition, size exclusion chromatography strongly suggested that the HuPON1 monomers and dimers, in equilibrium in C12E8 micellar solutions, exhibited the same arylesterase-specific activity. Thus, in the time scale of the experiments performed in this work, the activity of HuPON1 was preserved in a variety of detergent-solubilized forms, independently of the detergent concentration. However, as already mentioned, the stability of the enzyme activity could be strongly affected by the detergent type and concentration relative to the CMC.

Dispersity of HuPON1 Samples: Importance of the Protein and the Detergent Concentration—Nondenaturing PAGE and cross-linking clearly showed a highly polydisperse HuPON1 when the detergent concentration was lower than the CMC. In micellar solutions, the apparent homogeneity of C12E8-solubilized HuPON1 observed by nondenaturing PAGE and size exclusion chromatography is probably related to the fast interconversion between the monomeric and dimeric forms. Given the apparent homogeneity of HuPON1 in gels containing Triton X-100, C12-maltoside, FOS-CHOLINE-12, CYMAL-6, or LDAO, it is reasonable to assume that HuPON1 exhibits a similar monomer-dimer equilibrium model in micellar solutions of other nondenaturing detergents. In the presence of CHAPS above the CMC, HuPON1 was highly heterogeneous. This probably extends to media containing micelles of detergents not tested in the present work.

According to our data, HuPON1 dimers would be the main oligomeric species in solutions containing a low concentration of C12E8 micelles in the solvent. For instance, from the linear relationship between $K_d$ and the micelle concentration (Fig. 6), we determined that extremely low concentrations of C12E8 micelle of $\sim 0.1$ or $\sim 0.01 \mu M$ would be required to obtain 95% of HuPON1 dimers in solutions containing 20 or 2 $\mu M$ HuPON1, respectively. Conversely, the proportion of HuPON1 monomers increases with the C12E8 concentration. We calculated that a very high C12E8 micelle concentration of $\sim 1$ mM would be required to obtain 95% of HuPON1 monomers in a medium containing 20 $\mu M$ of HuPON1. However, there might be a critical C12E8 concentration above which HuPON1 undergoes denaturation. Monomers and dimers coexist in most of the C12E8 solutions, above the CMC. Thus, results of this study clearly showed that choice of the type and concentration of detergent relative to the CMC is crucial to obtain homogeneous HuPON1 samples suitable for crystallization trials.

CONCLUSIONS

Altogether, the results of this study indicate that the nonionic detergent-solubilized forms of HuPON1 in vitro are reasonable models of its in vivo lipid-bound forms. In this regard, nonionic detergent aggregates probably mimic the HDL environment for proteins such as PON1, which exhibits hydrophobic anchors, in a better way than the phospholipid bilayers for integral membrane proteins. However, to what extent the de-
tergent can mimic the hydrophobic environment of the protein is a question that remains open. Consequently, the effective stoichiometry of HuPON1 in vivo cannot be definitively established from the present study. The results of the present work strongly suggest that assembly of amphiphilic molecules in aggregates of specific size and shape could be a prerequisite for anchoring of HuPON1. Consequently, the lower antioxidative capacity of HDL associated with certain diseases could be related to subtle changes of HDL size and/or shape, which directly affect the binding affinity of enzymes such as HuPON1, lecithin:cholesterol acyltransferase, cholesteryl ester transfer protein, and HuPON3, involved in the lipoprotein metabolism.

Acknowledgments—We thank Richard W. James (University Hospital, Geneva, Switzerland) for the gift of anti-human PON1 monoclonal antibodies. Thanks are also due to Jean-Luc Popot and Daniel Picot for useful advice and for the use of facilities at Institut de Biologie Physico-Chimique (IBPC) (Paris). Clarence Broomfield (United States Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, MD) and Brian Bahnson (University of Delaware, Newark, DE) are acknowledged for helpful discussion and critical reading of the manuscript.

REFERENCES

1. Navab, M., Hama, S. Y., Anantharamaiah, G. M., Hassan, K., Hough, G. P., Watson, A. D., Reddy, S. T., Sevanian, A., Fonarow, G., and Fogelman, A. M. (2000) J. Lipid Res. 41, 1495–1508
2. Mackness, M. I., Durrington, P. N., and Mackness, B. (2000) Curr. Opin. Lipidol. 11, 383–388
3. Ahmed, Z., Ravandi, A., Maguire, G. F., Emili, A., Draganov, D., La Du, B. N., Kuksis, A., and Connelly, P. W. (2001) J. Biol. Chem. 276, 24473–24481
4. Rodriguez, L., Mackness, B., Durrington, P., Hernandez, A., and Mackness, M. I. (2001) Biochem. J. 354, 1–7
5. Fukusho, H. (2000) J. Biol. Chem. 275, 3957–3962
6. Davies, H. G., Richter, R. J., Keifer, M., Broomfield, C. A., Sowalla, J., and Furlong, C. E. (1999) Nat. Genet. 14, 334–336
7. Billecke, S., Draganov, D., Connell, R., Stetson, P., Watson, C., Hsu, C., and La Du, B. N. (2000) Drug Metab. Dispos. 28, 1325–1342
8. Shih, D. M., Xu, Y. R., Navab, M., Li, W. F., Hama, S., Sowalla, J., and Furlong, C. E. (1999) Arterioscler. Thromb. Vasc. Biol. 19, 2214–2225
9. Dekin, S., Leviev, I., Gumaraschi, M., Cabarlesi, L., Franceschini, G., and James, R. W. (2000) J. Biol. Chem. 275, 4031–4038
10. Gan, K. N., Smolen, A., Eckerson, H. W., and La Du, B. N. (1991) Drug Metab. Dispos. 19, 100–106
11. Josse, D., Xie, W., Renaud, F., Rochu, D., Schopfer, L. M., Masson, P., and Lockridge, O. (1999) Biochemistry 38, 2816–2825
12. Kuo, C. L., and La Du, B. N. (1995) Drug Metab. Dispos. 23, 935–944
13. Geldmacher-von Mallinckrodt, M., and Diepgen, T. L. (1987) Toxicol. Environ. Chem. 18, 79–136
14. Furlong, C. E., Richter, R. J., Chapline, C., and Crabh, J. W. (1991) Biochemistry 30, 10133–10140
15. Lennm€uller, U. K. (1970) Nature 227, 680–685
16. Blatter, G., James, R. W., and Thomas, M. R. (1991) Eur. J. Biochem. 211, 871–879
17. Ornstein, L. (1964) Ann. N. Y. Acad. Sci. 121, 321–349
18. Davis, B. J. (1964) Ann. N. Y. Acad. Sci. 121, 404–427
19. le Maire, M., Aggerbeck, L. P., Montellier, C., Andersen, J. P., and Møller, J. V. (1986) Anal. Biochem. 154, 525–535
20. Harlan, J. E., Picot, D., Lell, P. J., and Garavito, R. M. (1995) Anal. Biochem. 224, 557–563
21. Kwaw, I., Sun, J., and Kaback, H. R. (2000) Biochemistry 39, 3134–3140
22. Nagy, J. K., Lau, F. W., Bowie, J. U., and Sanders, C. R. (2000) Biochemistry 39, 4154–4164
23. Schuck, P. (2000) Biophys J. 78, 1606–1619
24. Schuck, P., and Demeler, B. (1999) Biophys J. 76, 2288–2296
25. Wyman, J. (1964) Adv. Protein Chem. 19, 224–284
26. Tanford, C. (1969) J. Mol. Biol. 39, 539–544
27. Tanford, C. (1974) J. Phys. Chem. 78, 2449–2479
28. Møller, J. V., and le Maire, M. (1993) J. Biol. Chem. 268, 18659–18672
29. Møller, J. V., and le Maire, M. (2000) Biochim. Biophys. Acta 1508, 86–111
30. de la Fuente, M., Neumann, J.-M., and Rigaud, J.-L., (1991) J. Biol. Chem. 266, 10066–10069
31. Kelsinger, C. G., Gruenewald, C. H., Jordan-Starcher, T. C., and Hulten, J. A. (1994) J. Biol. Chem. 269, 832–839
32. Reddy, S. T., Wadleigh, D. J., Grijabal, V., Ng, C., Hama, S., Gangopadhyay, A., Shiib, D. M., Lusis, A. J., Navab, M., and Fogelman, A. M. (2001) Arterioscler. Thromb. Vasc. Biol. 21, 542–547
33. Rudel, L. L., Marzetti, C. A., and Johnson, F. L. (1986) Methods Enzymol. 129, 45–56
34. Kuo, C. L., and La Du, B. N. (1995) Drug Metab. Dispos. 23, 935–944
35. Kuo, C. L., and La Du, B. N. (2000) Drug Metab. Dispos. 28, 1325–1342
36. Laemmli, U. K. (1970) Nature 227, 680–685
37. Blatter, G., James, R. W., and Thomas, M. R. (1991) Eur. J. Biochem. 211, 871–879
38. Ornstein, L. (1964) Ann. N. Y. Acad. Sci. 121, 321–349