Detergent Binding as a Measure of Hydrophobic Surface Area of Integral Membrane Proteins*

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The mode by which four nonionic detergents interact with integral membrane proteins was studied by relating detergent binding to characteristic detergent monolayer and micellar dimensions and to the surface area of the hydrophobic sector of the proteins. The detergents used (C₁₂E₅, Triton X-100, dodecyl-β-D-maltoside, and dodecyl(dimethylamino)oxide) are efficient membrane solubilizers, which, according to our hydrodynamic analysis, form small approximately spherical micelles. Their binding by protomeric forms of bacteriorhodopsin, photosynthetic reaction center, sarcoplasmic reticulum Ca²⁺-ATPase, and cytchrome oxidase was measured after equilibration and delipidation, both by the use of successive chromatographies on silica gel and the use of agarose gel columns in combination with DEAE-cellulose chromatography. It was found that, despite detergent binding by silica gel, both chromatographic procedures gave equivalent and consistent results. The data obtained show systematic trends; thus dodecyl(dimethylamino)oxide exhibited the highest binding levels, followed by dodecylmaltoside, whereas the polyethylene glycol detergents C₁₂E₅ and Triton X-100 had the lowest degree of binding. This ranking order is inversely related to the cross-sectional areas of these detergents in monolayers at an air-water interface and in micelles. Binding was reduced by aggregation of protomers, whereas the presence of strongly bound residual lipid slightly increased it. Among the membrane proteins with a high molecular mass (reaction center, Ca²⁺-ATPase, and cytochrome oxidase), relative binding could be related to the size of their hydrophobic sectors. On the other hand, bacteriorhodopsin bound somewhat more detergent per transmembrane helix, probably because of less steric hindrance, caused by the absence of a sizable hydrophilic domain in this protein. Theoretical calculations indicated monolayer binding of detergent to the hydrophobic surface of membrane proteins to be a better model for interaction with membrane proteins than binding of the detergents in micellar form.

Detergents are means of disintegrating the structure of biological membranes and thus play important roles in the purification and characterization of membrane proteins. In this way, proteins may be solubilized in a native-like state with retention of functional properties, provided that solubilization occurs in a gentle manner. This can often be ensured by the use of nonionic detergents, especially those of an intermediate size such as C₁₂E₅, dodecylmaltoside, and Triton X-100 (Møller et al., 1986; Lund et al., 1989; Sami et al., 1992; le Maire et al., 1992). The mode of interaction and amount of bound detergent are important factors which contribute significantly to the shape and molecular mass of the detergent-solubilized membrane protein complex. In addition, the extent of detergent binding may provide important information on the properties of the membrane-embedded sector of these proteins.

From previous investigations, a few generalizations concerning protein-detergent interactions can be made. Detergent binding to integral membrane proteins involves both detergent-detergent interaction as well as protein-detergent interaction (Robinson and Tanford, 1975; Makino et al., 1975). On the other hand, water-soluble proteins, in general, interact only weakly, if at all, with non-denaturing detergents or, like serum albumin, only do so at a few discrete binding sites (Tanford and Reynolds, 1976, Clarke, 1975). Detergent solubilization of membrane proteins has been depicted to occur by insertion of the delipidated or partially delipidated protein into detergent micelles (Robinson and Tanford, 1975; Makino et al., 1975). However, we have questioned the applicability of this concept, at least in the case of large proteins, for which micelle sizes would not be large enough to accommodate the lipid-embedded region of many membrane proteins (le Maire et al., 1983; Møller et al., 1986). As alternatives, we have pointed out that detergent molecules may form a torus or ring around the hydrophobic sector of membrane proteins or be present as a monolayer, similar to what is observed at an air-water interface (le Maire et al., 1983).

Important parameters to be evaluated in connection with these hypotheses are micelle sizes and the packing of detergent molecules around the hydrophobic surface of the solubilized membrane protein. Our previous measurements of the binding of detergents were mainly performed with the aid of hydroxypropyl or DEAE-cellulose columns (le Maire et al., 1978; Rivas et al., 1980; le Maire et al., 1983). In agreement with previous data (reviewed in le Maire et al. (1983)), they

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1 The abbreviations used are: C₁₂E₅, octa(ethylene glycol)dodecyl monooether; DDAG, N,N-dimethyldecylamine-N-oxide; DM, n-dodecyl β-D-maltoside; CMS, critical micellar concentration; Tes, N-tris(hydroxyethyl) methyl-2-aminomethanesulfonic acid; HPLC, high performance liquid chromatography; Tricine, N-(2-hydroxy-1,1-bis(hydroxyethyl)methyl)glycine; CSA, cross-sectional area.

suggested a very limited size of the hydrophobic surface area of sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase and other membrane proteins (le Maire et al., 1983). However, subsequent characterization of detergent-solubilized Ca\textsuperscript{2+}-ATPase by high performance liquid chromatography (HPLC) suggested larger values for binding of C\textsubscript{12}E\textsubscript{8} to Ca\textsuperscript{2+}-ATPase than those measured by the previous desorption-chromatography methods (Andersen et al., 1986). These discrepant findings have necessitated a reinvestigation of the methods used for detergent binding, which is one of the subjects of the present investigation. In addition, we have extended the scope of our investigation by including other proteins and other types of nonionic detergents (dodecylmaltoside and DDAO) besides the polyoxyethylene glycol detergents, Triton X-100 and C\textsubscript{12}E\textsubscript{8}, that were mainly used previously. As a result, we have been able to determine the reasons for chromatographic discrepancies in the estimation of detergent binding and to provide a new set of membrane protein binding data. The revised data are generally higher than those previously obtained. They have made possible not only a comparison of detergent binding of various representative integral membrane proteins (sarco- plasmic reticulum Ca\textsuperscript{2+}-ATPase, bacteriorhodopsin, photosynthetic reaction center, and cytochrome oxidase), but also a comparison of the propensity of different types of detergent to combine with these membrane proteins. In combination with an investigation on the size properties of micelles of the pure detergents, our data lend support to the monolayer model as the fundamental basis for detergent interaction with membrane proteins.

**EXPERIMENTAL PROCEDURES**

**Materials**—The following detergents were used. Octaethyleneglycolmonooleinate (C\textsubscript{12}E\textsubscript{8}), from Nikko Chemicals, Tokyo and 1\textsuperscript{-[14C]}C\textsubscript{12}E\textsubscript{8} from the Commissariat à l’Énergie Atomique, Centre d’Etudes de Saclay; dodecylmaltoside from Biochemica Boehringer, Mannheim, Germany; 1\textsuperscript{-[14C]}dodecylmaltoside (specific activity, 2 GBq/mmol) was synthesized by the Centre d’Etudes de Saclay, France, as described previously (Kragh-Hansen et al., 1993). 1\textsuperscript{-[14C]}-Labeled and unlabeled DDAO were also obtained from Saclay; unlabeled Triton X-100 and [phenyl-\textsuperscript{3}H]Triton X-100 were obtained from Serva (Heidelberg, Germany) and Du Pont (Dreieichenhain, Germany), respectively.

Sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase (containing 65 mol of phospholipid/mol of polypeptide) was purified by elution with a low concentration of deoxycholate (Malmberg et al., 1973) 3-20 times the sarcoplasmic reticulum vesicles, prepared from rabbit skeletal muscles according to the procedure of de Meis and Hasselbach (1971). Bacteriorhodopsin (containing 11 mol of archaeobacterial phospholipid/mol of polypeptide) was prepared as purple membranes from Halobacterium halobium according to the procedure described by Rigaud et al. (1980). Bacterial photosynthetic reaction center (wild type, Y, lipid-free) was prepared from Rhodobacter sphaeroides after solubilization and purification in DDAO (Rivas et al., 1980). Cytochrome oxidase (containing 12-18 mol of phospholipid/protomer of 13 polypeptides) was prepared from bovine heart mitochondria by ammonium sulfate fractionation (Suarez et al., 1984).

**Chromatographic Procedures—**Detergent binding of the detergent-solubilized membrane proteins was studied by equilibrium chromatography based on previously published procedures (le Maire et al., 1983; Rivas et al., 1982; Andersen et al., 1986). The following chromatographic columns were used: HPLC silica gel columns 3000 SW or 3000 TL (Toyo Soda, Tokyo), 0.75 x 60 or 0.75 x 30 cm, with a 0.75 x 5-cm precolumn, in combination with an HPLC isocratic system (Pharmacia, Uppsala, Sweden). In addition gel chromatography was performed on different agarose matrices (0.5 m and 1.5 m, Bio-Gel, Bio-Rad and Superose 6, Pharmacia). Prior to use the columns were equilibrated with detergent containing 10-20 mM Tes buffer (pH 7.0 or 7.5), detergent at a concentration 3-20 mg/ml, 50-100 mM NaCl, and other additions as mentioned in figure and table legends. All measurements were performed at room temperature. No effect of detergent concentration on binding to mem-
the perimeter, we first used the α-carbon representation and divided the contour into 8–10 short straight segments. The summed length of these gave 14.2 nm for level (i), 14.6 nm for level (ii), 15.3 nm for level (iii), and 16.0 nm for level (iv), i.e. a mean value of 15.6 nm. The contours were sector-shaped on the cytoplasmic side but became oval shaped on the external side. The alpha-carbon atoms of the amino acid side chains attached to the helices, increased these perimeters by about 3 nm. On the basis of these data, we used 16.5 nm as a representative value for the circumference of the reaction center, taking partially hydrated bacteriorhodopsin as a 3-nm-diameter sphere. For these measurements, we used dextran as a gradient stabilizer as described previously (Le Maire et al., 1980). If we assume that the dimensions of the reconstituted image should be increased equally in all directions (Bisson, 1990), we estimate a mean value for the transmembranous perimeter of bacteriorhodopsin of around 10 nm.

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Measurements of micelle molecular mass were obtained, e.g. (Rivas et al., 1982; Le Maire et al., 1983). Results consistent with agarose chromatography were obtained, e.g. for DDAO binding by reaction center (Rivas et al., 1980) or of Tween 80 by Ca2+-ATPase oligomer (Le Maire et al., 1978). However, for C14Es, significantly higher binding levels (0.5–0.7 g/g protein) were obtained by silica gel HPLC (Andersen et al., 1986) than by DEAE-cellulose or hydroxyapatite chromatography (0.25 g/g protein). It should be noted that, despite the absence of anomalies during elution of protein (constant binding ratios across the protein peak, and no troughs, cf. Fig. 3A), the use of desorption chromatography presupposes that detergent binding by the adsorbed protein corresponds to that of the detergent-solubilized complex in solution. In addition, this method does not inform us about the aggregational state of the protein eluted from these columns.

In the latter respect, silica gel HPLC is a superior technique for separation of solubilized lipid from protein, as well as of the membrane proteins in their monomeric and oligomeric forms, cf. Figs. 1A and 2 as illustrative examples with Ca2+-ATPase and cytochrome oxidase. Furthermore, the resolving power of these columns is sufficiently sharp that enough material is present in the protein peak fraction to enable a binding determination to be repeated once or twice by rechromatography on the same silica gel column. In general, this is sufficient to efficiently remove lipid and to obtain

RESULTS

Methodological Aspects of Detergent Binding Determination—There are a number of problems associated with the correct determination of detergent binding of membrane proteins in a well defined functional state. Some of these arise from nonideal properties of the available chromatographic matrices. In using gel chromatography on agarose, detergent binding of membrane protein protomer is usually overshadowed by a massive peak of solubilized lipid and detergent, see Fig. 1B for an illustrative example with Ca2+-ATPase and DM. On the other hand, we found previously that adsorption chromatography on DEAE-cellulose or hydroxyapatite columns can be used as an efficient way of removing solubilized lipid and excess detergent. By this method, it is possible to establish equilibrium conditions before desorbing the protein from the matrix (Rivas et al., 1982; Le Maire et al., 1983). Results consistent with agarose chromatography were obtained, e.g. for DDAO binding by reaction center (Rivas et al., 1980) or of Tween 80 by Ca2+-ATPase oligomer (Le Maire et al., 1978). However, for C14Es, significantly higher binding levels (0.5–0.7 g/g protein) were obtained by silica gel HPLC (Andersen et al., 1986) than by DEAE-cellulose or hydroxyapatite desorption chromatography (0.25 g/g protein).
**Detergent Binding by Membrane Proteins**

**TABLE I**

<table>
<thead>
<tr>
<th>Detergent</th>
<th>Molecular mass</th>
<th>Aggregational number (n)</th>
<th>CSA$^a$</th>
<th>cmc, 20 °C$^c$</th>
<th>$\bar{v}_{max}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>C$_{12}$E$_8$</td>
<td>538</td>
<td>89</td>
<td>98$^a$, 120$^a$</td>
<td>0.49–0.66</td>
<td>0.09</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>623</td>
<td>75</td>
<td>146 (96–165)$^b$</td>
<td>0.49–0.64</td>
<td>0.25</td>
</tr>
<tr>
<td>DM</td>
<td>598</td>
<td>110</td>
<td>126 (111–140)$^d$</td>
<td>(0.38)–0.50$^b$</td>
<td>0.18</td>
</tr>
<tr>
<td>DDAO</td>
<td>229</td>
<td></td>
<td>71 (69–73)$^e$</td>
<td>(0.33)–0.40$^a$</td>
<td>2.2</td>
</tr>
</tbody>
</table>

$^a$ Determined by sedimentation equilibrium with radioactively labeled detergent in this and a previous publication (le Maire et al., 1989). For Triton X-100 also by analytical ultracentrifugation in the Beckman model E ultracentrifuge.

$^b$ Cross-sectional areas of detergent at the cmc, calculated from surface tension data by the use of the Gibbs adsorption isotherm. Note that the values obtained in different laboratories are somewhat variable so that a range of values is listed.

$^c$ Determined by dye uptake (Møller et al., 1986) or by gel chromatography (le Maire et al., 1983). Note that DDAO becomes progressively cationic below pH 7; cmc is affected both by pH and changes in ionic strength (Ikeda et al., 1985; Lange, 1967). Note that DDAO becomes progressively cationic below pH 7; cmc is affected both by pH and changes in ionic strength (Ikeda et al., 1985; Lange, 1967).

$^d$ Detergent molecular mass present (mm) cm$^2$/molecule cm/g

$^e$ Determined by sedimentation equilibrium with radioactively labeled detergent in this and a previous publication (le Maire et al., 1989). For Triton X-100 also by analytical ultracentrifugation in the Beckman model E ultracentrifuge.

$^f$ Cross-sectional areas of detergent at the cmc, calculated from surface tension data by the use of the Gibbs adsorption isotherm. Note that the values obtained in different laboratories are somewhat variable so that a range of values is listed.

$^g$ Determined by dye uptake (Møller et al., 1986) or by gel chromatography (le Maire et al., 1983). Note that DDAO becomes progressively cationic below pH 7; cmc is affected both by pH and changes in ionic strength (Ikeda et al., 1985; Lange, 1967).

**FIG. 1.** Chromatographic resolution of protein and detergent-solubilized lipid by silica gel (A) or Superose chromatography (B). Membranous Ca$^{2+}$-ATPase (6 mg of protein) was solubilized by DM (40 mg, with added $^{14}$C-labeled detergent) in a total volume of 0.73 ml at pH 7.0, followed by airfusing for 30 min at 100,000 × g. The supernatant (0.5 ml) was applied to either a silica gel 3000 SW column (0.75 × 60 cm), with a precolumn (A), or a Superose 6 column (1 × 30 cm) (B). The columns were equilibrated and eluted with 20 mM Tes (pH 7.0), 100 mM NaCl, 0.1 mM Ca$^{2+}$, and 0.5 mg of DM/ml, including radioactive label at the same specific activity as used for solubilization (200,300 cpn/mg). The volume of the fractions collected was 0.75 ml. The symbols denote the following: O, radioactive DM; •, protein concentration (Lowry et al., 1951); □, phospholipid (PL). After silica gel chromatography (A), the protein peak, with remaining associated phospholipid (0.08 g/g, corresponding to 11–12 mol of phospholipid/mol of ATPase) is clearly separated from the peak of mixed phospholipid and DM micelles, whereas these peaks are merged after Superose chromatography. Binding of DM from A was calculated to be 0.92 g/g of Ca$^{2+}$-ATPase monomer. This value was reduced to 0.80 g/g in two rechromatographies which resulted in complete delipidation.

a stable detergent base line. However, silica gel columns, due to their sensitivity to the nature of the eluant, exhibit a number of complicating features which often lead to rapid column degeneration with delayed or no elution of membrane protein, especially when used in conjunction with detergents. (For this reason we abandoned the use of Triton X-100 for silica gel chromatography, since the use of this detergent led to rapid deterioration of these expensive columns). As shown below, silica gel columns bind significant amounts of detergent, and it was therefore imperative to establish the reliability of these materials for binding determinations, relative to the more inert agarose. To obtain correct values for detergent
binding by membrane protein, we used combined DEAE-cellulose and agarose chromatography. The rationale of this approach was that the DEAE-cellulose step removes solubilized lipid and excess detergent from the detergent-solubilized membrane preparation, before measurement of binding properties is undertaken in the next step by classical gel chromatography. Fig. 3 shows an experiment in which Ca-ATPase, after solubilization with Triton X-100, was delipidated and equilibrated on a DEAE-cellulose column, before desorption by addition of 0.4 M NaCl (Fig. 3A). The eluted protein peak was then chromatographed on a type 0.5-m agarose (Fig. 3B). During this step, a biphasic peak appears which is attributable to elution of Ca-ATPase in monomeric (M) and dimeric (D) form (Andersen et al., 1986). The protein peaks arising from DEAE-cellulose and agarose chromatography are associated with a rise in detergent concentration above the baseline, but note that in the agarose experiment this is followed by a trough, indicating additional binding of detergent by protein during this step. In agreement with this view, the position of the trough corresponds to the elution position after application of pure detergent micelles. The following binding levels were calculated from the increase in detergent concentration. After DEAE-cellulose chromatography, there was bound 0.25 g of Triton X-100/g of protein, after agarose gel chromatography there was bound 0.55 g of Triton X-100/g of monomeric Ca-ATPase, and 0.27 g of Triton X-100/g of dimeric Ca-ATPase. The trough in Fig. 3B is thus accounted for by increased binding of detergent to Ca-ATPase during agarose chromatography. Therefore, we must conclude that although the protein was truly equilibrated when adsorbed on the DEAE-cellulose column (no trough being present during elution), in this state it binds less detergent than when present in solution. This leads to a decrease in the concentration of micellar detergent in the protein-containing eluate. This decrease is detected as a trough during the subsequent agarose chromatographic step.

Table II summarizes binding data from similar experiments with C2E6, DM, and Triton X-100 that were calculated from the elution patterns of DEAE-cellulose columns, combined DEAE-cellulose/agarose, combined DEAE-cellulose/silica gel or by repeated chromatography on silica gel columns. The binding levels observed by DEAE-cellulose chromatography alone were, typically, around 60% of those found during subsequent agarose chromatography, but could be as low as 40% (C2E6 binding by bacteriorhodopsin and Ca-ATPase) and as high as 90% (Triton X-100 binding by cytochrome oxidase). By contrast, there was good correspondence between data obtained by DEAE-cellulose/agarose chromatography.

Fig. 2. HPLC resolution of detergent-solubilized cytochrome oxidase in protomeric and aggregated forms and their DM binding level. Membranous cytochrome oxidase (3 mg of protein) was solubilized with 15 mg of DM in a total volume of 0.22 ml at pH 7.0 and airfuged for 20 min at 100,000 × g 1 h after the addition of DM. Then 180 μl of supernatant was applied to a 0.75 × 60-cm TSK G3000 SW column, with a precolumn which was equilibrated and eluted at 0.5 ml/min with 0.5 mg [14C]DM/ml dissolved in 20 mM Tes/Tris (pH 7.0), 100 mM NaCl, 1 mM EDTA, and 1 mM azide. The volume of the fractions collected was 0.75 ml. The symbols denote the following: C, radioactive DM; 0, protein (Lowry); ---, light absorption at 422 nm; Cl, phospholipid. Three peaks of cytochrome oxidase are seen; the first one (from left to right) represents aggregated cytochrome oxidase which elutes in the void volume; the second peak whose existence is indicated by the UVicord trace at 280 nm (---) probably corresponds to dimeric cytochrome oxidase (ε280 = 13 S, cf. Robinson and Talbert (1986)); and the third peak, labeled M, corresponds to protomeric cytochrome oxidase (with ε280 = 9 S and 13 polypeptide bands by SDS-gel electrophoresis). Detergent micelles, together with part of the phospholipid in the cytochrome oxidase preparation, elute as the large off-scale peak subsequent to the protein peaks. Binding of DM by cytochrome oxidase, calculated from the increment in the baseline, gradually increased from 0.42 g of DM/g of protein in the void volume to 0.70 g of DM/g of protein protomer.

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Fig. 3. Determination of Triton X-100 binding to Ca<sup>2+</sup>-ATPase by combined DEAE-cellulose (A) and agarose (B) chromatography. Membrane-bound Ca<sup>2+</sup>-ATPase (5 mg of protein) was solubilized with 25 mg of Triton X-100 and applied to a DEAE column (1 x 2 cm), equilibrated with 0.5 mg of [3H]Triton X-100/ml, 10 mM Tris/Tris (pH 7.5), 50 mM NaCl, 0.1 mM Ca<sup>2+</sup>, and 1 mM Mg<sup>2+</sup> (Buffer I). After flushing the column with 30 ml of this buffer to remove lipid and excess Triton X-100, the protein was eluted with Buffer II, similar to Buffer I, but containing 400 mM NaCl instead of 50 mM NaCl. From the protein-rich fractions (Fractions 2 and 3), an aliquot was applied to an 0.5-m agarose column (1.7 x 25 cm), equilibrated with Buffer I. Note that after elution from the DEAE column, the protein and detergent concentration profiles are similar, whereas after gel chromatography the peaks of dimeric (D) and monomeric (M) Ca<sup>2+</sup>-ATPase are different (due to less binding of detergent by dimeric Ca<sup>2+</sup>-ATPase) and are followed by a trough in the detergent curve, which corresponds to the elution position of Triton X-100 micelles. Binding levels are calculated from the curves, see text.

Table II

<table>
<thead>
<tr>
<th>Protein</th>
<th>Detergent</th>
<th>Detergent binding&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Detergent binding&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Si-3000 SW&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>DEAE-cellulose</td>
<td>Agarose&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Si-3000 SW&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;-ATPase</td>
<td>C&lt;sub&gt;3&lt;/sub&gt;E&lt;sub&gt;8&lt;/sub&gt;</td>
<td>0.25 ± 0.02</td>
<td>0.45 ± 0.06 (0.40)</td>
<td>0.55 ± 0.07</td>
</tr>
<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;-ATPase</td>
<td>DM</td>
<td>0.45 ± 0.02</td>
<td>0.70 ± 0.06 (0.58)</td>
<td>0.75 ± 0.08</td>
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<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;-ATPase</td>
<td>Triton X-100</td>
<td>0.27 ± 0.04</td>
<td>0.44 ± 0.06 (0.39)</td>
<td></td>
</tr>
<tr>
<td>Bacteriorhodopsin</td>
<td>C&lt;sub&gt;3&lt;/sub&gt;E&lt;sub&gt;8&lt;/sub&gt;</td>
<td>0.90 ± 0.05&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.2 ± 0.2&lt;sup&gt;e&lt;/sup&gt;</td>
<td>2.6 ± 0.3&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Bacteriorhodopsin</td>
<td>DM</td>
<td>2.50 ± 0.20&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4.2&lt;sup&gt;e&lt;/sup&gt;</td>
<td>4.0 ± 0.4&lt;sup&gt;e&lt;/sup&gt;</td>
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<tr>
<td>Bacteriorhodopsin</td>
<td>Triton X-100</td>
<td>1.50 ± 0.10&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reaction center</td>
<td>C&lt;sub&gt;3&lt;/sub&gt;E&lt;sub&gt;8&lt;/sub&gt;</td>
<td>0.48 ± 0.03</td>
<td>0.76 ± 0.07</td>
<td>0.87 ± 0.04</td>
</tr>
<tr>
<td>Reaction center</td>
<td>DM</td>
<td>0.66 ± 0.04</td>
<td>0.92</td>
<td></td>
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<tr>
<td>Reaction center</td>
<td>Triton X-100</td>
<td>0.36 ± 0.04</td>
<td>0.74 ± 0.11</td>
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<tr>
<td>Cytochrome oxidase</td>
<td>C&lt;sub&gt;3&lt;/sub&gt;E&lt;sub&gt;8&lt;/sub&gt;</td>
<td>0.40</td>
<td>0.45 ± 0.06 (0.50)&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.50&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cytochrome oxidase</td>
<td>DM</td>
<td>0.45 ± 0.03</td>
<td>0.60 (0.94)&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.59 ± 0.05&lt;sup&gt;f&lt;/sup&gt;</td>
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<tr>
<td>Cytochrome oxidase</td>
<td>Triton X-100</td>
<td>0.45</td>
<td>0.50 ± 0.07 (0.40)&lt;sup&gt;f&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Data are averages of two to eight experiments (±S.D., when four to eight experiments were performed).

<sup>b</sup>Binding in combined DEAE-cellulose and agarose experiments; the binding figure refers to binding by protomer ± S.D.; figures in parenthesis represent weighted average binding of detergent by protomer and irreversibly aggregated protein in all protein containing fractions eluting from the column, for direct comparison with DEAE-cellulose data (see text).

<sup>c</sup>Data obtained by chromatography on 3000 SW silica gel, either by successive chromatography or by combined DEAE-cellulose and silica gel chromatography (these procedures gave equivalent results).

<sup>d</sup>The lipid content was 10-11 mol of mesobacterial phospholipid/mol of bacteriorhodopsin, corresponding to the lipid content of purple membranes.

<sup>e</sup>The phospholipid content of bacteriorhodopsin was reduced to 2-6 mol of archael bacterial lipid/mol of bacteriorhodopsin after silica gel rechromatography.

<sup>f</sup>The lipid content of cytochrome oxidase remaining after gel chromatography was 6-8 mol of phospholipid/mole cytochrome oxidase.

and by HPLC on silica gel columns, both when the latter technique was used alone or in combination with DEAE-cellulose columns. These results attest to the usefulness of HPLC silica gel columns for rapid and accurate determination of detergent binding.

We generally observed a decrease in binding after protein aggregation, cf. Figs. 2 and 3. On the other hand, retention of moderate amounts of phospholipid strongly bound to the protein did not seem to have a pronounced effect on detergent binding. This was examined specifically for the interaction of Ca<sup>2+</sup>-ATPase and DM, where we could achieve gradual removal of lipid during rechromatography on silica gel. After the first chromatography, we calculated binding of DM to Ca<sup>2+</sup>-ATPase from three experiments to be 0.88 g of detergent/g of protein, at a phospholipid level of 7-10 mol/mol of ATPase; this value of detergent binding decreased to 0.80 g/
g of protein after complete delipidation by two successive chromatographies. This slight difference could be attributed to the continuous removal of lipid from ATPase before complete delipidation. In the case of bacteriorhodopsin, when detergent-solubilized protein was processed by combined DEAE-cellulose/agarose chromatography, no phospholipid was removed from the protein, whereas the lipid content decreased from 10–11 mol to 2–6 mol/mol of protein by repeated chromatography on silica gel. The binding data concerning bacteriorhodopsin listed in Table II, therefore, refer to different phospholipid levels, but as can be seen, this did not result in differences in detergent binding. On the other hand, attempts to remove phospholipid prior to binding estimation by extraction with deoxycholate, as suggested by Seigneuret et al. (1991), led to partial bleaching, and it is our impression that there was an increased binding of detergent under these conditions. Rather than to insist on complete delipidation of those proteins, for which this was difficult to obtain (bacteriorhodopsin and cytochrome oxidase), it was therefore our goal not to carry the process of delipidation further than was consistent with retention of native structural features, as evidenced by maintenance of spectral and functional properties. It was possible to obtain this objective for all membrane proteins after solubilization in DM, C12E8, Triton X-100, and for DDAO in the case of reaction center.

With the latter detergent, 50% enzyme activity was retained for protomeric cytochrome oxidase, whereas monomeric Ca2+-ATPase lost activity, but probably retained gross conformational features, as evidenced by elution from agarose in the normal position. Definite evidence of denaturation was obtained when Triton X-100-solubilized bacteriorhodopsin was replaced with DDAO, as evidenced by bleaching and irreversible aggregation of the protein. This was accompanied by detergent binding at an anomalously high level (5–7 g/g of protein). Despite the more precarious situation for DDAO, we have included binding data for this detergent, except for bacteriorhodopsin, in our summary of detergent binding data (Table III).

A factor leading to decreased binding levels by protein after DEAE-cellulose chromatography is the presence of aggregated forms, as observed by subsequent gel chromatography of cytochrome oxidase (Fig. 2) and Ca2+-ATPase (Figs. 3 and 4). We found by repeated chromatography or sedimentation equilibrium analysis that the aggregation state of the proteins, eluted well above the detergent cmc, is independent of protein concentration. Hence, the oligomeric forms probably represent irreversibly aggregated protein. In Table II, binding data in parentheses correct for the effect of these irreversible aggregation processes on binding after elution from DEAE-cellulose columns. This was done by calculating a weighted mean of the binding of detergent by both monomeric and aggregated forms after the agarose chromatography. It is seen that after implementation of this correction there is, for cytochrome oxidase, no significant difference between the DEAE-cellulose and agarose data, whereas detergent binding by Ca2+-ATPase is still less after elution from the DEAE-cellulose column. The same was the case for bacteriorhodopsin and reaction center (these latter proteins were only observed as monomeric species after gel chromatography). Accordingly, it seems that in addition to protein aggregation, the interaction of the protein with the DEAE-cellulose results in decreased binding of detergent. A possible reason is that the chromatographic matrix exerts steric hindrance to binding of detergent, despite that the interaction presumably occurs via the hydrophilic domain for the membrane protein. In this connection, it is of interest that cytochrome oxidase, the largest membrane protein examined, after binding to DEAE-cellulose exhibited the least interference with detergent binding, in accordance with the concept of steric hindrance.

Detergent Binding by the Protomeric Membrane Proteins and Micellar Sizes—The binding data obtained by the various correct methods, involving at least two successive chromatographies, have been averaged and summarized in Table III. They represent a consistent set of results which extend and revise our previous binding data (le Maire et al., 1983). By inspection of Table III, certain trends are recognizable. Irrespective of the detergent used, detergent binding capacity among the various membrane proteins (expressed on a gram/g basis) varies in almost the same order (Ca2+-ATPase < cytochrome oxidase < reaction center << bacteriorhodopsin). On the other hand, when detergent binding is expressed on a mole/mol basis binding differences are much less pronounced and are in a slightly different order (Ca2+-ATPase < cytochrome oxidase < reaction center << bacteriorhodopsin), suggesting that despite the wide range of protomer molecular masses (from 27 to 190 kDa), the size of their membrane-embedded sectors are much more similar. Except for bacteriorhodopsin, the ranking order for protein binding on a molar basis agrees with the estimated size of the transmembranous sectors (see "Experimental Procedures"). However, bacteriorhodopsin with its seven intramembranous helices binds relatively more detergent than the other membrane proteins.

Despite the similar size of the hydrophobic chains of the detergents used, there are also characteristic differences in the number of moles that are bound per mol of protein protomer (Triton X-100 ≪ C12E8 < DM < DDAO). These differences cannot be related in any simple way to differences in their micellar aggregational states (cf. Table I), as might be considered to be the case by binding of the detergents in a micellar mode (le Maire et al., 1983). This is most clearly seen by reference to DDAO, which has a minimal aggregation number in micellar form, but the largest binding capacity. On the other hand, binding levels are inversely related to the bulkiness of the hydrophilic heads of the detergent molecules.

### Table III

**Detergent binding capacity of protomeric membrane proteins**

<table>
<thead>
<tr>
<th>Detergent</th>
<th><strong>Bacteriorhodopsin</strong></th>
<th><strong>Ca2+-ATPase</strong></th>
<th><strong>Reaction center</strong></th>
<th><strong>Cytochrome oxidase</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gram/g</td>
<td>Mol/mol</td>
<td>Gram/g</td>
<td>Mol/mol</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>2.9</td>
<td>124</td>
<td>0.44</td>
<td>77</td>
</tr>
<tr>
<td>C12E8</td>
<td>2.4</td>
<td>119</td>
<td>0.50</td>
<td>103</td>
</tr>
<tr>
<td>DM</td>
<td>4.1</td>
<td>207</td>
<td>0.73</td>
<td>152</td>
</tr>
<tr>
<td>DDAO</td>
<td>0.5</td>
<td>240</td>
<td>0.76</td>
<td>289</td>
</tr>
</tbody>
</table>
and cross-sectional areas as estimated from surface tension measurements at the cmc at an air-water interface (cf. Table I). This raises the question if detergent binding, in a fairly simple way, reflects the number of detergent molecules required to cover the hydrophobic surface of the membrane-embedded sector of the membrane proteins with a monolayer of detergent. To obtain an idea of the compactness with which detergents in bulk solution can pack around the curved surface of the hydrophobic sector of the membrane proteins, we have performed an analysis of the hydrodynamic size (Stokes radius) of the detergent micelles (Table IV). From measurements of the intrinsic viscosity and molecular mass (Table I), we obtained values of \( R_e \) ranging from 2.30 nm for DDAO to 3.42 nm for Triton X-100. The values of \( R_e \) for DM and DDAO agree closely with gel chromatographic estimates of Stokes radius \( (R_0) \) obtained by comparison with the elution of water-soluble globular proteins (Le Maire et al., 1989, 1991), whereas the apparent \( R_0 \) for Triton X-100 and \( C_{12}E_8 \) is somewhat larger. As discussed previously, measurement of relatively high \( R_0 \) values by gel chromatography is not an unexpected finding for polyethylene glycol detergents, but can be attributed to the flexibility of the polyethylene glycol chains, which may result in too early elution of these detergents as a function of compactness or of compact protein (Le Maire et al., 1989). The frictional ratio \( (R_f/R_m) \), calculated from the intrinsic viscosity data, is only slightly above unity for DDAO, DM, and \( C_{12}E_8 \), indicating that the overall micellar shape can be approximated by a sphere with a moderate amount of bound water (last column of Table IV). Values for micelle-bound water, in agreement with those calculated for a perfect spherical shape in Table IV, have been obtained previously, for DM, by sedimentation equilibrium in a sucrose gradient (de Vitry et al., 1991) and for \( C_{12}E_8 \) from the decrease of \( D_2O \) self-diffusion in detergent solution (Nilsson, 1984). For DDAO, a neutron scattering study (Timmins et al., 1988) has led to the conclusion that this detergent forms spherical micelles, but with a slightly smaller radius (2.07 nm) than found here, resulting in a lower estimate of bound water (0.2 g/g of detergent). Even for Triton X-100, the estimated higher water-trapping capacity (1.25 g/g of detergent) is consistent with a spherical or only slightly elongated model for the micelle shape (this differs from previous conclusions (Robson and Dennis, 1977), mainly because, in our sedimentation equilibrium studies, we consistently found lower aggregation numbers than previously reported (Table I). From the volume of the sphere and micelle aggregation number of the various detergents, we have calculated the mean cross-sectional area of detergent molecules in the micelles (CSA column of Table IV) and cross-sectional areas as estimated from surface tension measurements at the cmc at an air-water interface (cf. Table I). This raises the question if detergent binding, in a fairly simple way, reflects the number of detergent molecules required to cover the hydrophobic surface of the membrane-embedded sector of the membrane proteins with a monolayer of detergent.

**TABLE IV**

<table>
<thead>
<tr>
<th>Detergent</th>
<th>( [\eta]_{in} )</th>
<th>( R^2 )</th>
<th>( R_0 )</th>
<th>CSA (( n_m ))</th>
<th>( \delta_{HP} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>CM-100</td>
<td>0.587</td>
<td>1.45</td>
<td>1.33</td>
<td>0.49</td>
<td>0.79</td>
</tr>
<tr>
<td>C12E8</td>
<td>0.54</td>
<td>1.22</td>
<td>1.22</td>
<td>0.49</td>
<td>0.79</td>
</tr>
<tr>
<td>TMX-100</td>
<td>0.54</td>
<td>1.22</td>
<td>1.22</td>
<td>0.49</td>
<td>0.79</td>
</tr>
</tbody>
</table>

* Calculated from intrinsic viscosity (column 2) and micelle aggregation number (Table I, present data), according to Equation 1.

* Stokes radius, estimated from elution position on agarose columns, calibrated with water-soluble globular proteins (Le Maire et al., 1989, 1991).”}

* Calculated from intrinsic viscosity and Equation 2.

* Cross-sectional area, calculated from the volume of each detergent molecule in the micelle (4/3\( R^2/m \)) and micelle radius (\( R_m \)), according to Equation 3.

* Bound and trapped water, calculated on the assumption of micelles being perfectly spherical with radius \( R_m = \frac{1}{2} \) (2.5 for a sphere, cf. Tanford (1961)).
IV), according to Equation 3. As might be expected, the cross-sectional areas generally increase with the bulkiness of the hydrophilic group. Cross-sectional areas are at the same level or somewhat lower than observed for monolayers at the air-water interface (Table I). This implies no fundamental difference in the properties of the detergent molecules when present in monolayer or micellar form. The micellar data on cross-sectional areas have been used to perform numerical estimations of detergent binding based on binding of detergent as monomers or in micellar form by the membrane proteins (see "Discussion").

Comparison with Other Determinations of Detergent Binding—In the past, detergent binding ratios have been determined as part of the biochemical characterization of detergent-solubilized membrane proteins. Nevertheless, for the proteins under consideration here (especially bacteriorhodopsin and reaction center) there is a paucity of detergent binding data which no doubt to a large extent reflects the difficulties that have prevailed in obtaining the detergents (except Triton X-100) in radiolabeled forms. Instead, in a number of cases, detergent binding has been estimated from measurements of the buoyancy of membrane protein-detergent complexes as a function of solvent density in D2O/H2O mixtures (Reynolds and Tanford, 1976). For bacteriorhodopsin, application of this technique led to an estimated binding of Triton X-100 of 4.7 (4.2-5.1) g/g of monomeric bacteriorhodopsin (Reynolds and Stoeckenius, 1977), a much higher level than measured here by gel chromatography (2.9 g/g, Table III). However, we consider that the high value obtained from the dependence of buoyancy on solvent density very likely is an overestimate, resulting from the limited accuracy of the method. This is corroborated by the fact that, by the usual application of the sedimentation equilibrium technique (Tanford et al., 1974), we obtained a correct value for protein molecular mass (26 kDa, to be compared with 26,750 Da calculated from the amino acid sequence), by the use of the present gel chromatographically determined binding ratios for Triton X-100 and C14E6 (Garrigos et al., 1993). In these experiments, the use of a binding of 4.7 g/g of Triton X-100 of bacteriorhodopsin monomer would have resulted in an underestimated value of the monomeric protein molecular mass of 19 kDa (this was also, to some extent, the case in the experiments of Reynolds and Stoeckenius (1977), from which protein molecular masses of 23,000-25,500 Da were reported).

In the case of the cytochrome oxidase monomer, application of the solvent density variation technique has led to estimations of binding ratios for Triton X-100 (Robinson and Talbert, 1986) and DM (Suarez et al., 1984) of 0.6 g/g which agree with the present data (Table III). The same binding level of Triton X-100 was also obtained for Triton X-100 in gel chromatographic experiments (Saraste et al., 1990), whereas Bolli et al. (1985) in chromatographic experiments reported a higher level for binding of DM (0.96 g/g), using tritiated dodecylmaltoside. For binding of Triton X-100 by Ca2+-ATPase, our binding ratio is intermediate in a range of values, obtained by gel chromatography or sucrose density centrifugation by various authors (Walter and Hasselbach, 1973; Clarke, 1975; Dean and Suarez, 1981). For reaction center (R. sphaeroides), Vadeboncoeur et al. (1979) reported a higher value of 1.2 g/g. This is a major discrepancy for which we have no explanation, since by use of the same technique (DEAE-cellulose chromatography alone) we obtained a value of 0.4 g/g, which increased to 0.74 g/g by subsequent gel chromatography.

We have been unable to find any previous documented values for binding of C14E6 to bovine cytochrome oxidase, reaction center, and bacteriorhodopsin. For C14E6, binding to the Ca2+-ATPase monomer, prepared by HPLC gel chromatography at 0 °C, Kijima et al. (1990) reported a binding ratio of 0.81 g/g of protein from measurements of the refractive index increment. This is greater than the detergent binding capacity of monomeric Ca2+-ATPase found here by HPLC chromatography at 20 °C (0.55 g/g of protein, Table II). It is also higher than the value of 0.58 g/g found previously by the Japanese group in similar experiments on C14E6 binding by Na+,K+-ATPase (Hayashi et al., 1989), but this could perhaps be ascribed to the presence of the relatively hydrophilic glycoprotein (β-subunit) in Na+,K+-ATPase. We have attempted to repeat the former binding determination at 0 °C, following the protocol given by Kijima et al. (1990) for preparation of Ca2+-ATPase monomer, but determining binding by the use of radiolabeled C14E6 instead of the refractive index increment. We observed a rise in detergent base line concentration corresponding to an apparent binding of 0.7-0.8 g of C14E6/g of protein. However, it was clear that this value was an overestimate, because it contained a contribution from the following major peak of C14E6.

Effect of Detergent Concentration on Membrane Protein Binding—In the present study detergent binding capacity was determined by successive chromatography at detergent concentrations well above the cmc to provide optimal conditions for delipidation and to keep the membrane proteins in a protomeric state. However, our previous evidence (le Maire et al., 1983) has indicated, in accordance with the generally accepted view (e.g. Tanford and Reynolds, 1976), that detergent binding by integral membrane proteins is maintained unaltered down to the cmc. Below the cmc we found that detergent binding by bacteriorhodopsin and reaction center drops precipitously, concomitant with the formation of large aggregates (data not shown). On the other hand, for SR Ca2+-ATPase it is possible to maintain the lipid-depleted protein in a soluble state below the cmc (Lund et al., 1989). Fig. 4 shows the details of an experiment in which we first solubilized Ca2+-ATPase with DM and removed the major part of the solubilized lipid by passage through a silica gel column, equilibrated and eluted with DM well above the cmc of the detergent. The peak fraction of monomeric Ca2+-ATPase was then passed through the column after it had been re-equilibrated at a lower detergent concentration either (i) at 0.092 mg/ml, corresponding to the cmc of DM, or (ii) slightly below the cmc of DM (0.070 mg/ml). It is seen from the figure that the monomeric state was maintained at the cmc, whereas partial conversion to oligomeric forms took place at 0.070 mg/ml. At the cmc the same binding level was obtained as observed previously for partially delipidated Ca2+-ATPase above the cmc (0.9 g/g of protein, see above). By contrast, some decrease in DM binding by monomeric Ca2+-ATPase (from 0.96 to 0.70 g/g of protein) took place below the cmc, at 0.070 mg of DM/ml. These binding levels were maintained by one further chromatography through the column, which was undertaken to ensure that measurements were performed at a stable baseline of free detergent. Oligomeric Ca2+-ATPase bound less detergent than monomeric Ca2+-ATPase (0.4-0.5 g/g). Note that, in these experiments, there is further conversion of monomer to oligomer during the second chromatography. Furthermore, residual protein-associated lipid is present in both monomeric and oligomeric forms. This is a consequence of the low detergent concentrations which were insufficient in removing the last traces of phospholipid from Ca2+-ATPase. In our normal procedure both the first and successive chromatographies were performed well above the

3 J. V. Mäller and M. Le Maire, unpublished observations.
cmc to ensure optimal conditions for removal of lipid.

Interaction of Detergent with Silica Gel Columns—As mentioned above, silica gel columns bind detergent to a significant extent. To understand the basis for this interaction, we performed a number of equilibration experiments with pure detergent. Fig. 5 shows that after flowing DM through a silica gel 3000 SW column at a constant concentration above the cmc, the detergent appears abruptly in the eluate, at the same concentration as in the infusate, but at a position later than that corresponding to the total volume of the column. Discontinuing the detergent supply, on the other hand, results in a biphasic outflow pattern. During the first phase, the concentration of detergent is unchanged and identical to that in the fluid which was added to the column in the previous period. This phase ends abruptly after elution of a volume of detergent which corresponds to the V₁ (27 ml). The second phase, which has a lower plateau value, therefore must represent bound detergent which is relatively slowly released from the silica gel. Interestingly, the elution of detergent during this phase is found to take place at a detergent concentration (170 μM) which corresponds to the cmc of the detergent (cf. Table I). This was found to be the case, irrespective of the concentration of micellar detergent used during the preceding equilibration of the column. This limiting value in the concentration of detergent during the second phase of the elution process, corresponding to the cmc, presumably, reflects bound detergent in the column in equilibrium with nonmicellar detergent. This view is supported by measurements of binding which reach a maximum at the cmc (see inset). Furthermore, after equilibration of detergent on the column below cmc, the outflow is monophasic, i.e. it only takes place at the same concentration as that used for equilibration (not shown). Under these conditions, column binding was dependent on detergent concentration (see inset).

From these results, which were similar to those obtained with C₁₂E₈, we conclude that the silica gel columns have binding sites that interact in a reversible manner with free (nonmicellar) detergent. This means that, after equilibration with detergent above the cmc, binding is at a maximum so that the column binding sites do not interfere with the detergent-solubilized membrane proteins. Probably, the interaction of silica gel with nonmicellar detergent only is an indication that it is the hydrophobic tail of the detergent which interacts with the silica gel coating. Therefore, there is no reason to expect interactions to occur between the solubilized membrane-protein complexes and silica gel during chromatography. That is, if it is assumed that, in these complexes, hydrophobic surfaces are sufficiently shielded from hydrophobic contact with the gel by a layer of detergent molecules with the hydrophilic head facing outwards. These conclusions provide an explanation for the empirical finding that HPLC columns can be used for reliable binding estimations (Table II).

**DISCUSSION**

The present data demonstrate the feasibility of using modified versions of gel equilibrium chromatography, with preliminary separation on DEAE-cellulose columns, or repeated chromatography on silica gel, to remove lipid and detergent mixed micelles. This is required to obtain reliable measurements of detergent binding by fully solubilized protomeric membrane proteins. The revised methods in general have led to higher values for binding than were previously estimated by us (le Maire et al., 1983), but they are comparable with most of the relatively few determinations reported by others. The question remains as to how these values compare with those required to form a contiguous layer of detergent around the hydrophobic sector of the membrane proteins to shield it from contact with the solvent. We have previously pointed out that coverage of hydrophobic membrane surfaces could...
The size of the micelle would thus be dependent on the length of the oblate discs, where the edges are under strain, causing a blurring of the hydrophobic/hydrophilic border region. This would make it possible for detergent molecules outside the center to extend further outwards in the radial direction, providing sufficient room for packing of the hydrophobic tail of proteins. The hydrophobic sector of the membrane protein is depicted by the hatched area and the outer boundary of detergent by the broken lines. Detergent molecules are depicted with a hydrophobic tail and a hydrophilic head. In A, the monolayer is shown in pure form, without regard to the exposure of hydrophobic chains at the ends of the cylinder; in B, a modified monolayer arrangement is shown, with the same dimensions, but providing hydrophilic coverage of the whole monolayer.

For a micellar mode of arrangement, detergent molecules will form an ellipsoidal or spherical, rather than a cylindrical, ring around the hydrophobic sector. In Fig. 6C we have assumed the micellar shape to be spherical, in keeping with our hydrodynamic data (Table IV) and with the presently held view (Small, 1986) that small well defined micelles approximate a spherical or slightly prolate (Small, 1986), rather than an oblate shape (Tanford et al., 1977; Robson and Dennis 1977). As discussed by Small (1986), a spherical shape requires that the hydrophobic core of the detergent micelles corresponds to the fully extended hydrocarbon chain. This is to provide sufficient room for packing of the hydrophobic tail of the detergent molecules at the periphery of the micelle. For a C12 detergent, this would give rise to a diameter of the hydrocarbon core of the micelle of 3.1 nm, sufficient to cover the hydrophobic sector of the membrane protein in the transmembranous direction. From these models it is possible to estimate the volume of bound detergent, VD, in a fairly straightforward manner (see the "Appendix"), from the following equations for a cylindrical (cyl) monolayer binding mode (Equation 4a) and a spherical (sph) micellar binding mode (Equation 4b), respectively.

\[
V_D(\text{cyl}) = H l_0 \hat{\rho} + \pi H l_0^2 \tag{4a}
\]
\[
V_D(\text{sph}) = \frac{\pi}{2} l_0^3 \hat{\rho} + \frac{4}{3} \pi l_0^3 \tag{4b}
\]

In these equations, \(\hat{\rho}\) represents the average perimeter and \(H\) the height of the hydrophobic sector of the membrane protein, \(l_0\) is the radius of the detergent molecules in the extended conformation. From Equations 4a and 4b, \(n_0\) the number of molecules detergent bound per membrane protein protomer is obtained by dividing \(V_D\) by the hydrated volume of each detergent molecule (calculated as \(4/3 \pi R^3/6\) or by the equivalent expression \(R \times \text{CSA}\)).

The results of such calculations are shown in Table V, using as values of \(l_0\) the radius of detergent micelles (\(R_m\) Table IV), \(H = 3.0\) nm, and different values of \(\hat{\rho}\) (10, 14, 16.5, and 21 nm) corresponding to those estimated to represent the perimeter of bacteriorhodopsin, Ca\(^{2+}\)-ATPase, reaction center, and cytochrome oxidase, respectively, see "Experimental Procedures." It is seen from Table V, that for Triton X-100, C12E5, and DM, a monolayer arrangement around a circumference, corresponding to that of bacteriorhodopsin (10 nm), agrees well with the actual experimentally determined binding data (deviations from \(-23\) to \(+3\)%); whereas binding according to the micellar model is somewhat overestimated (from \(+20\) to \(+61\)%). For circumferences corresponding to perimeters of Ca\(^{2+}\)-ATPase, reaction center, and cytochrome oxidase, the monolayer model agrees with, or somewhat overestimates, the experimentally determined binding by these three larger detergent molecules (deviations from \(-4\) to \(+54\)%); whereas application of the micellar model results in larger discrepancies (overestimations ranging from 66 to 147%). On the other hand, for DDAO, the detergent molecule with the shortest length, application of both models tends to slightly underestimate binding by Ca\(^{2+}\)-ATPase, reaction center, and cytochrome oxidase (deviations from \(-21\) to \(-3\)%). It may be noted that, for reaction center, there is direct structural evidence from neutron scattering experiments that detergent coverage closely follows the transmembranous perimeter over a transmembranous height of 2.5–3.0 nm (Roth et al., 1989, 1991).

Bacteriorhodopsin, compared with the other membrane proteins, is distinguished by a relatively higher capacity for detergent binding. This can also be seen directly from Table III by realizing that detergent binding by bacteriorhodopsin with its 7 transmembranous helices corresponds to that of reaction center with 11 transmembranous helices and other intramembranous components of low molecular mass (comprising bacteriochlorophyll, bacteriopheophytin, ubiquinone, and spheroidene (Rivas et al., 1980), representing a total mass of 7.8 kDa). Bacteriorhodopsin differs from the other membrane proteins by having a lower molecular mass and an

\*DDAO data are not available for bacteriorhodopsin (Table III). Note that the convergence of the binding levels for the two models in the case of DDAO is due to the relatively small size of the hydrophilic head of this detergent. This narrows the difference in detergent dioposition especially in the transmembranous (vertical) direction (cf. Fig. 6, which, in fact, illustrates the situation for a detergent with a small hydrophilic group like DDAO).
absence of prominent hydrophilic regions. Accordingly, the better agreement between the experimental and theoretical calculations in this case may indicate the absence of complications arising from steric hindrance by protruding hydrophilic regions. On the other hand, steric effects would be expected preferentially to reduce binding of detergents with bulky hydrophilic heads by Ca\(^{2+}\)-ATPase, reaction center, and cytochrome oxidase, and this is what is observed. Another factor is the effect of having puckered membrane surfaces, caused by the presence of amino acid side chains sticking out from the transmembranous helices. This conceivably may reduce the requirement for detergent binding by interdigitation of detergent and the hydrocarbon amino acid side chains. Finally, it should be pointed out that the calculations in Table V have been made on the assumption that detergent molecules are bound in an extended conformation. If this is not the case (i.e. if \(l_b < R_h\)) cross-sectional areas would increase, resulting in a lowering of detergent binding.

Comparing the binding capacity of the various detergents, it is seen from Table V that, for a micellar type interaction, the ranking order is different from that found by experiment. That is binding of DDAO, instead of being larger than for the other detergents, corresponds to that of C\(_{12}\)E\(_6\) and is definitely less than that of DM.

Whereas for the monolayer arrangement, the model calculations nearly reproduce the correct ranking order for binding of detergent (calculated: Triton X-100 \(<\) C\(_{12}\)E\(_6\) \(<\) DM \(\approx\) DDAO, Table V; observed: Triton X-100 \(<\) C\(_{12}\)E\(_6\) \(<\) DM \(\approx\) DDAO, Table III). The somewhat lower binding levels of DM than of DDAO by reaction center, Ca\(^{2+}\)-ATPase and cytochrome oxidase that we find in our experiments (Table III) may reflect steric hindrance by the bulky hydrophilic head of the DM molecule. (Unfortunately comparative binding data under conditions where steric hindrance is at a minimum (bacteriorhodopsin) could not be obtained (see "Results").

In conclusion, we have obtained data suggesting that detergent binding primarily reflects the surface area of the hydrophobic sector of membrane proteins. Conceptually, such a relationship is most easily visualized on the basis of a monolayer type of detergent binding. Model calculations, taking into consideration reductions arising from steric hindrance by bulky hydrophilic heads and other nonideal effects, seem consonant with this mode of interaction. Accordingly, we propose a monolayer rather than a micellar type of interaction as the basis for solubilization of membrane proteins by detergents.

**Acknowledgments**—We are grateful especially to Bitten Holm and Inger Andersen for meticulous care with the chromatographic experiments, to Dr. Françoise Reiss-Husson for preparations of R. sphaeroides reaction center, and to Dr. Ulrik Brandt for a preparation of cytochrome oxidase at a critical time. We also thank Drs. J.-L. Rigaud, M. Seigneur, and N. Assou for preparations of bacteriorhodopsin. Furthermore, we are indebted to Dr. Manuel Garrigos for help with some of the sedimentation equilibrium experiments, to Drs. Bernardette Arnoux and Jean-Luc Popot for help with the calculation of transmembrane perimeters of reaction center and bacteriorhodopsin, and to Lars Anderson for discussions on various aspects of detergent binding.

**APPENDIX**

In our previous model calculations of detergent binding (Le Maire et al., 1983), we assumed the hydrophobic sector of membrane proteins to have a cylindrical shape and calculated the volume of bound detergent as ellipsoidal or cylindrical bodies, formed by rotation around the cylindrical protein core. However, from the standpoint of present knowledge, the structure of hydrophobic membrane sectors generally cannot be considered to have an overall cylindrical shape. In most cases, the available evidence indicates an oblong organization of, e.g. a double row of transmembranous helices with a somewhat irregular outline. In addition, the transmembrane helices may be tilted, resulting in different cross-sections as a function of position within the lipid bilayer.

Despite this, it can be shown that for a specified monolayer or micellar model a simple relationship exists between detergent binding and perimeter, \(p\), regardless of the shape of the hydrophobic sector. We first consider the case of monolayer binding, assuming the circumference of the hydrophobic sector parallel to the plane of the membrane to be irregular, but projecting with the same shape perpendicular to the plane of the membrane (Fig. 7A). As an approximation, we divide the periphery into a convex polygon with \(n\) edges of lengths \(p_i\). Each of these edges can be considered to be covered with blocks of detergent sticking out a distance \(l_0\) parallel to the membrane plane over a transmembranous height \(H\). At the ends the detergent blocks are joined by cylindrical sectors, with angles \(\omega_1, \omega_2, \omega_3, \ldots, \omega_n\) to form a continuous detergent layer around the periphery (Fig. 7B). The total volume of bound detergent \((V_p)\) is given by the following equation.

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**Table V**

Calculations were based on Equations 4a and 4b, using detergent lengths \((l_b)\) and cross-sectional areas obtained from the hydrodynamic characterization of detergent micelles \((R_h, \text{and } \text{CSA})\) in Table IV. A value of 3 nm was used for the height \((H)\) of the detergent cylinder in the monolayer calculations (Equation 4a). The values of perimeters chosen were those estimated to be representative for bacteriorhodopsin (10 nm), Ca\(^{2+}\)-ATPase (14 nm), reaction center (16.5 nm), and cytochrome oxidase (21 nm), see "Experimental Procedures." Based on these assignments of perimeters, percentages in parenthesis indicate the ratio between calculated values and the experimentally determined molar detergent binding by the four membrane proteins (Table III).

| Detergent | \(p = 10\) nm | | \(p = 14\) nm | | \(p = 16.5\) nm | | \(p = 21\) nm |
|-----------|----------------|----------------|----------------|----------------|----------------|----------------|
| Triton X-100 | 95 (77%) | 158 (127%) | 114 (148%) | 190 (247%) | 125 (122%) | 211 (205%) | 146 (96%) | 252 (166%) |
| C\(_{12}\)E\(_6\) | 123 (103%) | 192 (161%) | 147 (143%) | 233 (226%) | 162 (126%) | 259 (200%) | 190 (119%) | 305 (192%) |
| DM | 171 (83%) | 248 (120%) | 206 (136%) | 303 (199%) | 228 (154%) | 338 (228%) | 267 (125%) | 400 (186%) |
| DDAO | 166 | 187 | 204 (85%) | 233 (97%) | 228 (79%) | 262 (91%) | 271 (82%) | 314 (95%) |
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