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A colorimetric determination for glycosidic and bile salt-based detergents: applications in membrane protein research

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Abstract

Detergents are crucial to the isolation of integral membrane proteins. During membrane protein purification, it is useful to accurately quantify detergent, especially if concentration steps have been used. Previously, this has been difficult and time-consuming. We present a simple, rapid, and sensitive procedure for the quantification of glycosidic and bile salt-based detergents such as dodecylmaltoside, octylglucoside, and CHAPS. The method directly quantifies sugar or cholate moieties via colorimetric reactions with phenol and sulfuric acid. A number of detergents have been screened, and the assay has been validated in the presence of commonly used reagents. In addition to determining the overall detergent concentration in solution, the procedure allows accurate quantification of specific binding of glycosidic or bile salt-based detergents to purified membrane proteins. Both the colorimetric method and the radiometric ^{14}C method were used to determine detergent binding to two integral membrane proteins: the cytochrome cbb_3 oxidase from *Pseudomonas stutzeri* and the turkey β-adrenergic receptor. Both methods gave similar results. After separating monomeric glycosidic detergent from micellar solutions by ultrafiltration, we used the colorimetric method to determine the concentration of monomeric detergent present. We observed that values obtained are in close agreement with previously determined critical micelle concentrations.

Keywords: Membrane proteins; Detergents; Specific detergent binding; Crystallization; Critical micelle concentration

Recent developments in genome sequencing have shown that 20–30% of genes encode integral membrane proteins [1] and that 50% of these may be potential drug targets [2], emphasizing the importance of this class of proteins. Research on integral membrane proteins is particularly demanding due to the requirement for functional mimetics of the biological membrane in their investigation. Detergents are solubilizing agents used in the isolation and purification of integral membrane proteins [3]. The nature of the detergent used to purify an integral membrane protein strongly affects the functional and structural properties of the solubilized protein. Therefore, the choice of appropriate detergents in the investigation of membrane proteins is critical.

Glycosidic nonionic detergents, such as dodecylmaltoside and octylglucoside, have been used to solubilize a wide variety of membrane proteins in a stable functional form, allowing their purification and crystallization. Indeed, most of the membrane protein structures

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so far deposited in the Protein Data Bank (PDB)[1] have been obtained from crystals grown in the presence of such glycosidic detergents, and dodecylmaltoside and octylglucoside have proven to be the two most successful detergents for α-helical membrane proteins [4].

To correctly use detergents in membrane protein investigations, it is important to consider the interaction of the membrane protein and the detergent moiety. This interaction is directly correlated to the chemical and physical properties of the detergent itself such as the critical micelle concentration, the length of the alkyl chain, and the nature of the polar head group [3] The overall effect of a detergent on an integral membrane protein can be evaluated by monitoring, where possible, the functional stability of the protein in the detergent of choice. It is more demanding to determine the amount of detergent bound to the purified membrane protein, and the determination of this parameter may be important in the selection of detergents for three-dimensional crystallization experiments and for the determination of membrane protein oligomeric states. Quantification of detergent binding to membrane proteins has been carried out mostly with the use of radioactively labeled detergents. The use of this method has been limited by the poor availability of such detergents and by the laborious nature of the procedure [5,6]. More recently, a Fourier transform infrared method for determination of both detergent:protein and lipid:protein ratios has been described [7]. This method requires instrumentation and expertise that are not widely available. To overcome these limitations, we have applied a colorimetric method that has previously been used for the determination of free sugar concentrations [8] to simply, rapidly, and reproducibly determine glycosidic detergent concentrations. The assay uses the condensation reactions of furfural derivatives, generated by the dehydration of sugars in concentrated sulfuric acid, with aromatic molecules such as phenol. The *p*-semiquinonoid chromogen formed is quantified by absorbance in the visible region (490 nm). We have also observed that detergents belonging to the bile salt class (e.g., CHAPS and cholate) can be quantified by the absorbance increase at 389 nm arising from sulfonation of the cholate ring [9]. We describe the basic procedure of this method and its application in comparison with the radiometric ^{14}C method to the determination of detergent binding to two integral membrane proteins solubilized and purified in dodecylmaltoside.

In the preparation of membrane proteins for three-dimensional (3D) crystallization, it is generally thought to be prudent to ensure that the final concentration of free micellar detergent is limited to two to three times the critical micellar concentration (CMC) [10]. This figure may be difficult to achieve when the final step involves the concentration of the protein and may, in any case, be unknown without a method of quantification. To select appropriate molecular weight cut-off (MWCO) centrifugal sample concentrators for membrane proteins so as to avoid the concentration of micellar detergent, the colorimetric assay was carried out on filtrates and concentrates from these devices. In the course of investigating the suitability of these devices, we observed that when the monomeric detergent fraction was separated from a micellar detergent solution by centrifugal ultrafiltration, values for detergent concentrations obtained from the flow-through fraction were in agreement with published CMC values. Aqueous detergent solutions are monomeric up to the CMC. Further increases in concentration result in the formation of micelles that coexist with monomers at a concentration approximately equal to the CMC, and this value does not vary greatly with moderate increases in concentration of micellar detergent [11]. CMC values for detergents have previously been determined by a variety of methods that usually require the generation of a series of detergent concentrations and a number of determinations of the change in physico-chemical characteristics (e.g., fluorimetry, dynamic light scattering, surface tension) [12] to infer the concentration at which micelles are formed. The formation of micelles and the accompanying physical changes being followed by these methods actually occur over a range of concentrations, and CMC values obtained by these methods correspond to an inferred midpoint of a range of values [13]. CMC values for glycosidic detergents are freely available [14], but these may vary with ionic strength and additives [15] as well as with batch purity [16], factors that indicate the necessity of routine CMC determinations. The quantization of monomeric glycosidic detergent concentration present in a micellar solution by a combination of the colorimetric method and ultrafiltration may represent a simplified method for routine CMC estimation without the need for extensive titration of detergent to determine the concentration at which micelles are formed. The method requires the use of only a basic photometer and a centrifuge.

The simplicity of the colorimetric assay that we present here and the variety of problems that can be addressed only with accurate detergent quantification are strong reasons for its routine implementation wherever membrane proteins are studied.

[1] *Abbreviations used:* PDB, Protein Data Bank; 3D, three-dimensional; CMC, critical micellar concentration; MWCO, molecular weight cut-off; CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfate; Fos-Choline-12, *N*-dodecylphosphocholine; LDAO, *n*-dodecyl-*N,N*-dimethylamine-*N*-oxide; SDS, sodium dodecyl sulfate; HECAMEG, methyl-6-*O*-(*N*-heptylcarbamoyl)-β-D-glucopyranoside; $C_{12}E_8$, octaethylene glycol monotridecyl ether; BCA, bicinchoninic acid; HPLC, high-performance liquid chromatography; FPLC, fast protein liquid chromatography; LOQ, limit of quantization; DTT, dithiothreitol; BSA, bovine serum albumin; IgG, immunoglobulin G; ATP/ADP, adenosine triphosphate/adenosine diphosphate.

Materials and methods

Materials

All detergents employed were of the highest purity grade available and were used without further purification. Dodecyl(-β-D-)maltoside, undecyl(-β-D-)maltoside, decyl(-β-D-)maltoside, CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfate), sodium cholate, octyl-β-D-glucoside, sucrose monododecanoate, Triton X-100, Fos-Choline-12 (*N*-dodecylphosphocholine), LDAO (*n*-dodecyl-*N,N*-dimethylamine-*N*-oxide), and sodium dodecyl sulfate (SDS) all were obtained from Anatrace. HECAMEG (methyl-6-*O*-(*N*-heptylcarbamoyl)-β-D-glucopyranoside) and $C_{12}E_8$ (octaethylene glycol monotridecyl ether) were obtained from Calbiochem. [^{14}C]Dodecyl(-β-D-)maltoside (55 mCi/mmol) was a gift from Dr. M. le Maire (CNRS, Gif-Sur Yvette, France).

Preparation of membrane proteins

The (cbb_3)-type cytochrome *c* oxidase was purified from the membrane fraction of aerobically grown *Pseudomonas stutzeri*. The three-subunit protein complex (ccoNOP) so purified has a molecular weight of 114 kDa [17]. Protein quantification was achieved by the bicinchoninic acid method (BCA, Pierce) and by quantitation of the heme content of the protein assuming a 2:3 stoichiometry of hemes *b:c* in the pyridine hemochrome inverse matrix spectral deconvolution [18].

A truncated construct of the turkey β-adrenergic receptor [19] encoding amino acids 20–424 with the mutation Cys^{116}Leu and a C-terminal hexahistidine tag was expressed in insect cells with baculovirus and purified as described previously [20]. The construct had a predicted molecular weight of 46 kDa and was nonglycosylated. Protein concentration was determined by a modified amido black method [21] that was calibrated by quantitative amino analysis.

Basic colorimetric assay procedures

The colorimetric assay procedure for glycosidic detergents was evaluated with 50-μl samples of aqueous solutions of detergent. Samples containing glycosidic detergents were pipetted into 2-ml Safe-Lock polypropylene tubes (Eppendorf). Then, under a fume hood, 250 μl of 5% phenol was added, followed by 600 μl concentrated sulfuric acid. Both reagents were dispensed with a Combitip (Eppendorf), and appropriate protective clothing was worn. On the addition of the concentrated sulfuric acid, a strong exothermic reaction occurred. The tubes were then closed (i.e., locked), and the samples were mixed by vortexing. As the temperature increased to 110–120 °C, the tubes were first allowed to cool to room temperature before transferring their contents to plastic semi-micro cuvettes (1 cm optical path length) and determining absorbances at 490 nm. Unknown detergent concentrations in samples of proteins purified in dodecylmaltoside were inferred using standard curves prepared with 50-μl samples containing 4–20 μg detergent.

A similar procedure was followed for bile salt-based detergents, where 50-μl samples containing bile salt detergent were pipetted into 2-ml polypropylene tubes before adding 800 μl of concentrated sulfuric acid. Tubes were vortexed and allowed to cool to room temperature, and absorbances were determined at 389 nm. Unknown bile salt detergent concentrations could be inferred from a standard curve prepared with 50-μl samples containing 4–20 μg detergent.

We investigated the susceptibility of the colorimetric reaction of glycosidic detergents to reagents commonly used in protein research. Possible interference from these reagents with color development at 490 nm was screened by assaying 50 μl of 0.16 mM dodecylmaltoside (4 μg) with and without the investigated compound. Increases in absorbance at 490 nm were judged to be insignificant if they were less than 3 standard deviations above the mean of multiple blank determinations.

Estimation of specific binding of detergent to membrane proteins

Colorimetric determination of specific binding of dodecylmaltoside to purified membrane proteins was performed on samples that had been eluted from size exclusion columns without any subsequent protein concentration step. A TSK Gel G2000 SW XL column on a Gilson high-performance liquid chromatography (HPLC) system was used for the (cbb_3)-type oxidase from *P. stutzeri*, and a Superdex 200 HR10/30 column on a fast protein liquid chromatography (FPLC) system (Pharmacia) was used for the turkey β-adrenergic receptor. Elution profiles were monitored at 280 nm, and 0.5-ml fractions were assayed for glycoside and protein content. Specific detergent binding was calculated by dividing the detergent concentration of the peak by the protein concentration after first subtracting the baseline concentration of detergent in the equilibration buffer. The protein samples were also subjected to size exclusion chromatography with [^{14}C]dodecylmaltoside [5,6] to validate the colorimetric assay. Purified protein samples (cytochrome cbb_3: 13.5 mg/ml, 0.02% dodecylmaltoside; β-adrenergic receptor: 5.3 mg/ml, 0.015% dodecylmaltoside) were incubated with traces (300 cpm/μl) of [^{14}C]detergent overnight. Samples were run at a flow rate of 0.05 ml/min on gel filtration columns equilibrated with buffers containing 0.02 and 0.015% dodecylmaltoside, respectively (~30 cpm/μl of [^{14}C]dodecylmaltoside). Elution time for the protein

peak was 4 h, allowing further opportunity for equilibration. The specific binding was calculated according to the following equation:

$$\frac{\text{g detergent}}{\text{g protein}} = \frac{\text{cpm(peak)} - \text{cpm(baseline)}}{\text{cpm(baseline)}} \frac{[\text{detergent}]}{[\text{protein}]},$$

where cpm(peak) is the cpm in the protein peak fractions, cpm(baseline) is the average value of cpm in the baseline fractions, [detergent] is the concentration of detergent in the equilibration buffer (mg/ml), and [protein] is the protein concentration (mg/ml).

Centrifugal ultrafiltration experiments

For selection of appropriate sample concentrators, micellar solutions of glycosidic detergents were prepared at concentrations representing typical working concentrations that may be employed in the latter part of a purification procedure. Detergents and concentrations used were dodecylmaltoside (0.02%), undecylmaltoside (0.075%), and decylmaltoside (0.2%). Samples (2 ml) were placed on Centricon concentrators (Amicon) with MWCOs of 10–100 kDa and concentrated as far as possible by centrifugation at the recommended speed in a Sorvall SS-34 fixed-angle rotor at 20 °C. This resulted in an 80-fold concentration factor if micellar detergent was retained and the residual concentrate volume was 25 µl. Samples of concentrates were assayed colorimetrically for glycosidic detergent content.

For colorimetric assays on filtrates from Centricon concentrators, 10-kDa MWCO concentrators were used for alkylmaltosides, whereas 5-kDa MWCO concentrators were used for octylglucoside (1%) and nonylglucoside (0.3%). Alkylmaltoside concentrations applied were as described above. Samples of filtrates were taken at intervals during the concentration procedure.

Results and discussion

Glycosidic detergents

The visible absorption spectrum of 50 µl 0.4 mM (10 µg) dodecylmaltoside reacted with sulfuric acid and phenol is shown in Fig. 1. The visible band at 490 nm arises from *p*-semiquinonoid chromogen formation via the addition of the furfural aldehydic group to the hydroxyl moiety of phenol, and the shoulder in the region at 400–440 nm arises from *p*-sulfonation of the phenol [22,23]. The yield of this side product in this single-step reaction setup is dependent on the specific reactivity of the furfural derivative with phenol. The UV absorption bands in the region at 320–330 nm are due to the electronic transition of the furfural derivatives and, therefore, are dependent on the glycoside structure and composition [23,24]. Fig. 2 shows typical calibration curves for dodecylmaltoside (4–20 µg) and octylglucoside (4–20 µg) at 490 nm (determined in triplicate, $R > 0.99$). Absorbance is plotted against millimolar concentration in the 50-µl samples to illustrate the ratio of the increment of absorbance per concentration of maltoside over glucoside, which is approximately 2, as expected from the release of two glucose groups from each maltoside group. Precision of the assay for dodecylmaltoside and octylglucoside was determined by replicate assays on samples containing 10 µg detergent. The coefficients of variation determined for 10 samples were

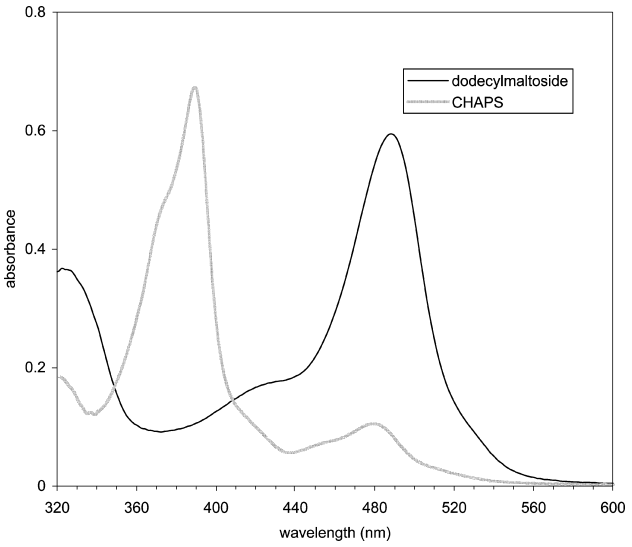

Fig. 1. Spectra obtained from dodecylmaltoside reacted with phenol and sulfuric acid and CHAPS reacted with sulfuric acid.

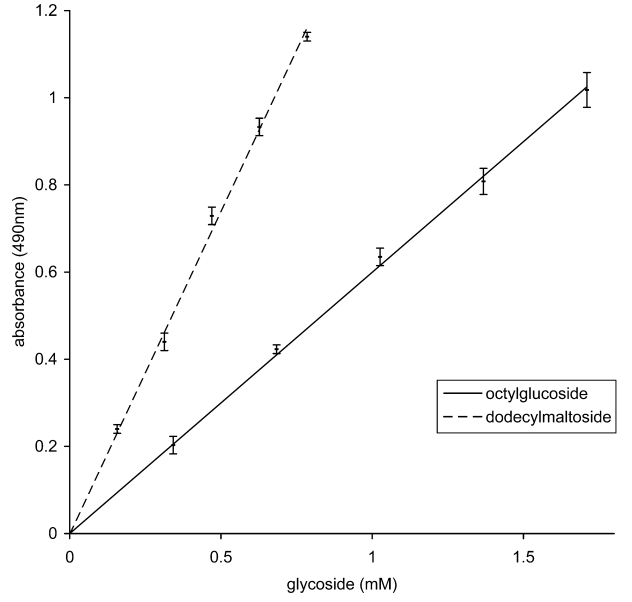

Fig. 2. Calibration curves obtained for octylglucoside and dodecylmaltoside. Each error bar indicates 1 standard deviation.

Table 1
LOQs for glycosidic detergents

Detergent	Concentration (mM)	Quantity (μg)	CMC (mM)
Dodecylmaltoside	0.096	2.45	0.17
Decylmaltoside	0.095	2.29	1.80
Sucrose monododecanoate	0.088	2.30	0.30
Octylglucoside	0.188	2.75	19.00
HECAMEG	0.240	4.00	19.50

2.0% for dodecylmaltoside and 2.5% for octylglucoside. The absorbance generated at 490 nm was quite stable, declining by only approximately 10% over 4 h.

All glycosidic detergents investigated gave detectable colorimetric reactions. For the purpose of detergent estimation, the limit of quantization (LOQ) at 490 nm has been calculated for some commonly used detergents. An absorbance increase at this wavelength was common to all glycosidic detergents investigated and was least prone to interference effects. The LOQ is the smallest concentration at which it is possible to accurately quantify and corresponds to a signal that is more than 10 standard deviations from the average of the blanks [25]. In Table 1, these values are compared with the CMC values of each detergent that were obtained from the respective manufacturers' literatures [14].

Interfering effects and blank detergents (assay for glycosidic detergents)

The following buffers were tested: Hepes–KOH (100 mM), Tris–HCl (100 mM), and sodium phosphate (50 mM). These had a negligible effect on the absorbance at 490 nm in both the presence and absence of detergent. Dithiothreitol (DTT) and glycerol react with phenol/sulfuric acid, producing colored compounds whose spectra in the visible region interfere with the reading at 490 nm. Glycerol (20%) and DTT (10 mM) contribute 0.14 and 0.18 (Abs cm^{-1}), respectively, to the absorbance at 490 nm. However, the contributions of these compounds can be subtracted by an appropriate blank control. Proteins contain aromatic amino acids that might potentially react either with the furfural derivatives or with the phenol. In addition, further interference to color development in the assay could arise from glycosylation of proteins or prosthetic heme groups. To evaluate these contributions, a number of proteins were tested. Bovine serum albumin (BSA, 80 μg) was chosen to evaluate interference from the polypeptide chain, mouse immunoglobulin G (IgG, 80 μg) was chosen to address the contribution of glycosylation, and horse cytochrome *c* (150 μg) was selected to test for interference arising from its prosthetic group (heme *c*). Polypeptides do not significantly interfere with the assay. Heme-containing proteins provide a contribution to the absorbance at 490 nm that is due to the Soret absorbing band at around 400 nm. This was estimated as 0.00026 absorbance units cm^{-1} per micromolar of heme group in the sample. As expected, glycosylated proteins gave rise to a significant colored reaction when assayed with this method, and 80 μg IgG returned an absorbance equivalent to 4 μg dodecylmaltoside. The range of specific detergent-binding values for membrane proteins purified in dodecylmaltoside can vary from 0.6 g detergent/g protein for a medium-sized membrane protein complex [17] up to 4.0 g detergent/g protein for a small hydrophobic membrane protein [26]. Because the colorimetric assay for glycosidic detergents is so sensitive and the limit of quantization for dodecylmaltoside (0.096 mM) corresponds to 2.5 μg, detergent quantization is actually performed on samples containing only 2.5–10.0 μg membrane protein. This means that in most cases a significant dilution of the protein sample is necessary, and this may reduce the level of interferences that we have listed while maintaining a strong signal for the glycosidic detergent. In the case of a protein with few glycosylation sites, the contribution from the glycosyl groups will be very small relative to the bound detergent. Particularly for more extensively glycosylated proteins, an accurate quantification of glycosidic detergent binding can be achieved only by first subtracting the glycosidic contribution arising from the protein. To do this, one could prepare glycosylated proteins in detergents that do not have cholate or sugar moieties and, therefore, do not give a color reaction with sulfuric acid or phenol/sulfuric acid. Table 2 reports the results of assays with phenol/sulfuric acid on such unreactive detergents together with the highest concentration assayed and their CMC values [14].

Table 2
Highest concentrations tested of unreactive detergents

Unreactive detergent	Concentration tested (mM)	CMC (mM)
LDAO	40.0 (2%)	1.40
SDS	8.7 (0.25%)	2.60
Triton X-100	15.0 (1%)	0.23
$C_{12}E_8$	18.6 (1%)	0.11
FOS-Choline-12	32.0 (1%)	1.50

Note. Absorbance values at 490 nm were within 3 standard deviations of the mean of the blank.

Assay of bile salt detergents

In screening for interfering chemical substances, we found that color development was also observed when the phenol/sulfuric acid treatment was carried out with bile salt-based detergents such as CHAPS and cholate. In this case, color development was not dependent on the presence of phenol because it directly involved the reaction of the sulfuric acid with the hydroxyl group of the cholate ring [9]. The resulting visible absorption spectra arising from the reaction of 50 μl of 0.4 mM

Table 3
LOQs for bile salt-based detergents compared with CMC values

Detergent	LOQ (mM)	CMC (mM)
CHAPS	0.249	0.658
Cholate	0.237	0.464

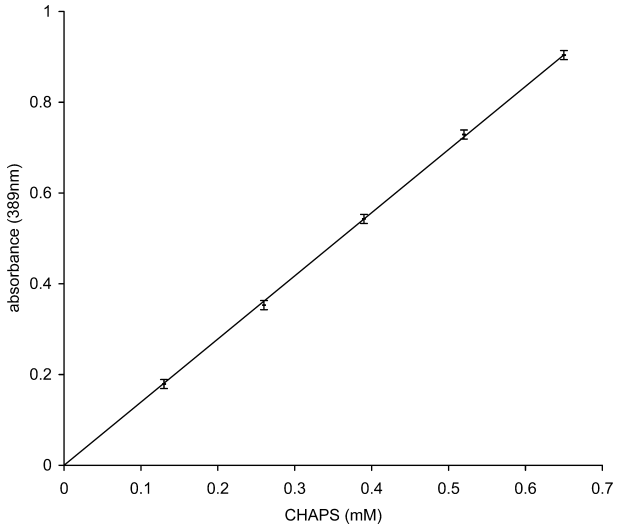

Fig. 3. Calibration curve obtained for CHAPS. Each error bar indicates 1 standard deviation.

(12.5 μg) CHAPS is shown in Fig. 1 and features an intense transition bell centered at 389 nm and two much weaker signals at 450 and 479 nm. To provide a general method for the quantization of detergents belonging to the bile salt group, it was decided to develop the assay based on the signal at 389 nm using a sample volume of 50 μl assayed with the addition of 800 μl concentrated sulfuric acid. The LOQ values at 389 nm have been measured for the bile salt-based detergents reported in this investigation and are compared with their CMCs in Table 3. Fig. 3 shows a typical calibration curve for CHAPS (4–20 μg, determined in triplicate, $R > 0.99$). Precision was determined by replicate assays on samples containing 10 μg CHAPS, and the coefficient of variation determined for 10 samples was 2.7%. The absorbance generated at 389 nm was quite stable, increasing by only approximately 5% over 4 h.

Specific binding of detergent (dodecylmaltoside) to membrane proteins

The colorimetric assay has been tested and compared with the previously described [5,6] radiometric assay for dodecylmaltoside on the *P. stutzeri* (cbb_3)-type cytochrome *c* oxidase and on a truncated and nonglycosylated turkey β-adrenergic receptor construct. The aim of this experiment was to accurately quantify the proportion of detergent bound in each protein–detergent complex. The procedure requires detergent quantification for protein peak fractions as well as for the background contribution from the running buffer that must be subtracted. An accurate protein determination calibrated to an amino acid analysis is also essential. The specific detergent binding is given as the ratio of grams of detergent per gram of protein for both colorimetric and radiometric detergent assay methods for the membrane proteins investigated in Table 4. The results are mean values of three independent experiments except for the radiometric determination applied to the turkey β-adrenergic receptor, which was performed only once. Both methods gave quite similar values of specific detergent binding, giving us the confidence to routinely implement the use of the colorimetric assay in our laboratories [17,20,26]. In addition to being helpful in the interpretation of gel filtration chromatography experiments [20], the determination of specifically bound detergent is essential to perform analytical centrifugation of membrane proteins [6], and this parameter has been determined with the colorimetric method for such studies [17,26].

Table 4
Specific detergent binding to membrane proteins

Protein	g detergent/g protein (^{14}C)	g detergent/g protein (phenol/sulfuric acid)
cbb_3 type cytochrome *c* oxidase	0.57 ± 0.017	0.61 ± 0.046
β-Adrenergic receptor	2.16	2.27 ± 0.16

Centrifugal ultrafiltration experiments

To address the problem of which sample concentrator to choose for use with alkylmaltoside detergents, we used a variety of MWCO Centricon sample concentrators to concentrate aqueous micellar solutions of decylmaltoside, undecylmaltoside, and dodecylmaltoside to completeness, normally a concentration factor of 80. The results of colorimetric assays carried out on the concentrates are summarized in Table 5. Estimates for the molecular weight of the dodecylmaltoside micelle vary from 34 to 70 kDa [6,14]. It can be concluded from the data presented in this table that for the alkylmaltosides tested, the use of a 100-kDa MWCO is appropriate if one wishes to avoid concomitant concentration of free micellar detergent along with the desired concentration of the protein–detergent complex. In the case of dodec-

Table 5
Final concentrations of detergent obtained with 30- to 100-kDa MWCO Centricon sample concentrators

Detergent	Initial concentration (%)	30 kDa (%)	50 kDa (%)	100 kDa (%)
Dodecylmaltoside	0.020	0.81	0.75	0.024
Undecylmaltoside	0.075	3.18	2.80	0.097
Decylmaltoside	0.200	5.86	0.64	0.210

ylmaltoside, the difference in molecular weight between the protein–detergent complex of a relatively small integral membrane protein, such as the 46-kDa turkey β-adrenergic receptor, and free detergent micelles is sufficient to allow its concentration with 100-kDa MWCO concentrators [20]. In the preparation of the turkey β-adrenergic receptor for crystallization in various alkylmaltoside detergents, specific detergent binding is determined at dialysis equilibrium and is routinely monitored after final concentration to make sure that micellar detergent has not been excessively concentrated.

Excessive concentration of free detergent micelles on smaller MWCO concentrators can be avoided if high-concentration factors are not employed and there are examples of successful membrane protein crystallization subsequent to the use of inappropriate concentrators [27]. However, it has also been reported recently that in the case of the bovine adenosine triphosphate/adenosine diphosphate (ATP/ADP) carrier, removal of excess detergent after concentration with a 30-kDa membrane was essential for successful crystallization [28].

Determinations of monomeric detergent concentrations in filtrates from Centricon concentrators

It was observed during preliminary determinations of detergent concentration in the filtrate from Centricons that retained detergent micelles that concentrations obtained were in close agreement with published CMC values. It can be seen from the results in Table 5 that there was nearly complete retention of alkylmaltoside micelles on 30-kDa MWCO Centricons. This can be assumed because multiplication of micellar detergent concentration (obtained by subtraction of CMC from initial detergent concentration) by the concentration factor (80) gives a value in close agreement with the concentration of the retained micelles. However, to enhance a complete separation of detergent micelles from monomeric detergent, 10-kDa MWCO concentrators were used for alkylmaltoside detergents and 5-kDa MWCO concentrators were used for octylglucoside and nonylglucoside. The results of assays carried out in triplicate on filtrates obtained during the first half of the concentration procedure are given in Table 6. It was observed that concentrations obtained from samples of filtrate taken at the very end of the concentration procedure, when the micellar solution retained above the filter was rather viscous, were slightly elevated (results not shown); this could represent either a change in the CMC or nonideal behavior of the ultrafiltration membrane. However, the concentrations of monomeric detergent in the filtrate obtained during the earlier stages from these micellar solutions were mostly in good agreement with the manufacturer's CMC values [14]. For octylglucoside and dodecylmaltoside, the values are not as close as they are with the other detergents tested. However, in the case of octylglucoside, it should be noted that CMC values of 20–25 mM have also been determined [6], whereas in the case of dodecylmaltoside, the concentration determined is close to the LOQ for this detergent (0.096 mM). The close agreement of the two sets of values for the five detergents tested suggests that the combination of ultrafiltration and the colorimetric assay represents a novel method for estimating the CMC. This differs from other procedures in that only one detergent concentration slightly above the CMC (which may be judged by change in surface tension or foam generation) is required.

Table 6
Concentrations of monomeric detergent present in micellar detergent solutions compared with the maufacturer's CMC values

Detergent	Initial concentration (%)	Concentration in filtrate (mM)	CMC value (mM)
Dodecylmaltoside	0.020 (0.39 mM)	0.125	0.17
Undecylmaltoside	0.075 (1.50 mM)	0.560	0.59
Decylmaltoside	0.200 (4.10 mM)	1.900	1.80
Nonylglucoside	1.000 (34.20 mM)	5.700	6.50
Octylglucoside	0.300 (9.80 mM)	25.300	19.00

Conclusions

In this study, we have described and evaluated a method for the accurate quantization of glycosidic and bile salt detergents. The method is rapid and robust and does not require specialized reagents or equipment; therefore, it could be implemented in most laboratories where membrane proteins are studied. In our laboratories, the method is performed routinely during membrane protein purification procedures. We have found that in addition to determining specific detergent binding to membrane proteins—an essential parameter for determination of oligomeric states—it can be used to monitor the concentration of unbound micellar detergent during protein concentration steps, the progress of detergent dialysis, and the efficiency of glycosidic detergent removal when a glycosidic detergent is exchanged for a nonreactive detergent. In addition, by quantifying the monomeric fraction in micellar solutions, it may be used to give a rapid CMC estimation.

All of these parameters have been laborious to obtain with the available methods, thereby limiting the rationale of the experimental design in membrane protein investigation. Therefore, the method that we have introduced represents a useful addition to the methods available to those engaged in structural work on membrane proteins.

References

[1] A. Krogh, B. Larsson, G. von Heijne, E.L. Sonnhammer, Predicting transmembrane protein topology with a hidden Mar-

kov model: application to complete genomes, J. Mol. Biol. 305 (2001) 567–580.
[2] R.B. Russell, D.S. Eggleston, New roles for structure in biology and drug discovery, Nat. Struct. Biol. (2000) 928–930.
[3] M. le Maire, P. Champeil, J.V. Møller, Interaction of membrane proteins and lipids with solubilizing detergents, Biochim. Biophys. Acta 1508 (2000) 86–111.
[4] S. Iwata, B. Byrne, Introduction, in: S. Iwata (Ed.), Methods and Results in Crystallization of Membrane Proteins, IUL, La Jolla, CA, 2003.
[5] M. le Maire, S. Kwee, J.P. Andersen, J.V. Møller, Mode of interaction of polyoxyethyleneglycol detergents with membrane proteins, Eur. J. Biochem. 129 (1983) 525–532.
[6] J.V. Møller, M. le Maire, Detergent binding as a measure of hydrophobic surface area of integral membrane proteins, J. Biol. Chem. 268 (1993) 18659–18672.
[7] C.J. daCosta, J.E. Baenziger, A rapid method for assessing lipid:protein and detergent:protein ratios in membrane–protein crystallization, Acta Cryst. D 59 (2003) 77–83.
[8] M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, F. Smith, A colorimetric method for the determination of sugars, Nature 168 (1951) 167.
[9] E. Heftmann, S.T. Ko, R.D. Bennett, Response of steroids to sulfuric acid in thin-layer chromatography, J. Chromatogr. 21 (1966) 490–494.
[10] S. Iwata, Crystallization informatics of membrane proteins, in: S. Iwata (Ed.), Methods and Results in Crystallization of Membrane Proteins, IUL, La Jolla, CA, 2003.
[11] C. Tanford, The Hydrophobic Effect: Formation of Micelles and Biological Membranes, John Wiley, New York, 1980.
[12] M. Zulauf, U. Furstenberger, M. Grabo, P. Jaggi, M. Regenass, J.P. Rosenbusch, Critical micellar concentrations of detergents, Methods Enzymol. 172 (1989) 528–538.
[13] R.M. Garavito, S. Ferguson-Miller, Detergents as tools in membrane biochemistry, J. Biol. Chem. 276 (2001) 32403–32406.
[14] (a) Anatrace Inc., Available from: <www.anatrace.com>;
(b) EMD Biosciences, Available from: <www.emdbiosciences.com>.
[15] A. Walter, G. Kuehl, K. Barnes, G. VanderWaerdt, The vesicle-to-micelle transition of phosphatidylcholine vesicles induced by nonionic detergents: effects of sodium chloride, sucrose, and urea, Biochim. Biophys. Acta 1508 (2000) 20–33.
[16] R.M. Garavito, D. Picot, The art of crystallising membrane proteins, Methods: A Companion to Methods in Enzymology 1 (1990) 57–69.
[17] A. Urbani, S. Gemeinhardt, A. Warne, M. Saraste, Properties of the detergent solubilised cytochrome *c* oxidase (cytochrome cbb_3) purified from *Pseudomonas stutzeri*, FEBS Lett. 508 (2001) 29–35.
[18] E.A. Berry, B.L. Trumpower, Simultaneous determination of hemes *a*, *b*, and *c* from pyridine hemochrome spectra, Anal. Biochem. 161 (1987) 1–15.
[19] Y. Yarden, H. Rodriguez, S.K. Wong, D.R. Brandt, D.C. May, J. Burnier, R.N. Harkins, E.Y. Chen, J. Ramachandran, A. Ullrich, The avian beta-adrenergic receptor: primary structure and membrane topology, Proc. Natl. Acad. Sci. USA 83 (1986) 6795–6799.
[20] T. Warne, J. Chirnside, G.F. Schertler, Expression and purification of truncated, non-glycosylated turkey beta-adrenergic receptors for crystallization, Biochim. Biophys. Acta 1610 (2003) 133–140.
[21] R.S. Kaplan, P.L. Pedersen, Sensitive protein assay in presence of high levels of lipid, Methods Enzymol. 172 (1989) 393–399.
[22] H.M. Mallya, T.N. Pattabiraman, Effect of acid concentration on chromogen formation from hexoses in sulfuric acid-based reactions, Anal. Biochem. 251 (1997) 230–299.
[23] P. Rao, T.N. Pattabiraman, Reevaluation of the phenol–sulfuric acid reaction for the estimation of hexoses and pentoses, Anal. Biochem. 181 (1989) 18–22.
[24] M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, F. Smith, Colorimetric method for determination of sugars and related substances, Anal. Chem. 28 (1956) 350–356.
[25] H.T. Karnes, G. Shiu, V.P. Shah, Validation of bioanalytical methods, Pharm. Res. 8 (1991) 421–426.
[26] P.J. Butler, I. Ubarretxena-Belandia, T. Warne, C.G. Tate, The *Escherichia coli* multidrug transporter EmrE is a dimer in the detergent-solubilised state, J. Mol. Biol. 340 (2004) 797–808.
[27] G. Von Jagow, H. Schägger, C. Hunte (Eds.), Membrane Protein Purification and Crystallization: A Practical Guide, second ed., Academic Press, San Diego, 2002.
[28] C. Dahout-Gonzalez, G. Brandolin, E. Pebay-Peyroula, Crystallization of the bovine ADP/ATP carrier is critically dependent upon the detergent-to-protein ratio, Acta Cryst. D 59 (2003) 2353–2355.