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[3] Alkyl Glycoside Detergents: Synthesis and Applications to the Study of Membrane Proteins

By T. VANAKEN, S. FOXALL-VANAKEN, S. CASTLEMAN, and S. FERGUSON-MILLER

In 1975, Baron and Thompson[1] introduced the nonionic detergent octyl glucoside, designed to be easily removed by dialysis to facilitate reconstitution of membrane proteins into phospholipid vesicles. Our interest was in studying and reconstituting cytochrome oxidase, an enzyme that is highly sensitive to the detergent environment and not satisfactorily activated and dispersed by any commercially available detergents. Octyl glucoside was not effective in activating and dispersing with this protein, but since pure, homogeneous analogs could be synthesized using different sugars and alkyl tails, we decided to examine some of these detergents in the hope of finding a version that retained the advantageous qualities of octyl glucoside while supporting optimal activity of cytochrome oxidase. To accomplish this we devised a synthesis[2] for alkyl glycosides that facilitates large scale production by eliminating some of the more complicated procedures and employing a one-step purification on Dowex 1 (hydroxide form). A variety of detergents prepared by this method were investigated for their ability to disperse and activate cytochrome oxidase. Laurylmaltoside (dodecyl-β-D-maltopyranoside) was found to be the most ef-

[1] C. Baron and T. E. Thompson, *Biochim. Biophys. Acta* **382,** 276 (1975).
[2] P. Rosevear, T. VanAken, J. Baxter, and S. Ferguson-Miller, *Biochemistry* **19,** 4108 (1980).

fective.[2-4] Other investigators have found it to be the detergent of choice for studying rhodopsin[5] and photosynthetic reaction centers,[6] suggesting that this detergent may have generally useful properties for studies on a variety of intrinsic membrane proteins. Other analogs may prove to be equally valuable as agents for solubilizing, reconstituting, and crystallizing proteins with different specific requirements.

Synthetic Procedure

The method outlined is applicable to the preparation of all the detergents shown in the table, but will be discussed in terms of laurylmaltoside synthesis. It is scaled up 10-fold over the previously published procedure,[2] and uses higher levels of dodecanol to increase the yield. In cases where the acetobromo derivative of the desired sugar is not commercially available, the procedure for making it has been detailed in the original publication.[2]

Materials

The following materials were obtained from the sources indicated: acetobromomaltose, and Dowex 1 (Cl), 2% cross-linked, 200–400 mesh (Sigma Chemical Co.); dodecanol, dichloromethane, silver carbonate (Aldrich Chemical Co. Milwaukee, WI); silica gel G plates, 250 μm thick (Analtech). All other solvents and reagents were the highest grade available.

Methods

Thin-Layer Chromatographic Systems for Analyzing the Reaction Progress and Products. Solvent system I, ethyl acetate/hexane (1:1 v/v); solvent system II, ethyl acetate/methanol (4:1 v/v). The TLC plates are developed by spraying with 2 *N* sulfuric acid and heating in a 90° oven.

Alkyl Glycoside Formation from Acetobromo Sugar and Alcohol (Large Scale). In a foil-covered, 4-liter round bottom flask, 280 mmol (200 g) of acetobromomaltose is stirred with 2800 ml dichloromethane. To this is added 660 ml dodecanol (previously stirred with Drierite for 24 hr at 60° and stored over Drierite), 60 g silver carbonate (dried for 24 hr), 5 g

[3] S. Ferguson-Miller, T. VanAken, and P. Rosevear, *in* "Electron Transfer and Oxygen Utilization" (H. Chien, ed.), p. 297. Elsevier Biomedical, Amsterdam. 1982.
[4] D. A. Thompson and S. Ferguson-Miller, *Biochemistry* **22,** 3178 (1983).
[5] P. Knudsen and W. L. Hubbell, *Membr. Biochem.* **1,** 297 (1978).
[6] M. W. Kendall-Tobias and M. Seibert, *Arch. Biochem. Biophys.* **216,** 255 (1982).

SUMMARY OF PROPERTIES OF ALKYL GLYCOSIDE DETERGENTS

Alkyl glycoside	Critical micelle concentration[a] (m*M*)	Critical micelle temperature[b] (Krafft point)	Solubility at 25°	Micelle size[c] (Da), Stokes radius (Å)	Effect on cytochrome oxidase
Octyl-β-D-glucoside	23.4	0°	(++)	8,000 (15 Å)	Aggregates
Octyl-α-D-glucoside	—	40°	(−)	—	—
Octyl-β-D-lactoside	—	43°	(−)	—	—
Lauryl-β-D-lactoside	—	73°	(−)	—	—
Lauryl-β-D-cellobioside	—	48°	(−)	—	—
Octyl-β-D-maltoside	23.4	0°	(++)	10,000 (15.5 Å) 23,000 (21 Å)	Aggregates
Lauryl-β-D-maltoside	0.165	0°	(++)	50,000 (29 Å) 76,000[d]	Activates and disperses
Lauryl-α-D-maltoside	0.156	0°	(++)	46,000 (27.5 Å)	Activates and disperses
Oleoyl-β-D-maltoside	—	—	gel (0–90°)	—	—
Oleoyl-β-D-maltotrioside	<0.005	0°	(++)	125,000 (42 Å)	Stabilizes dilute enzyme

[a] Critical micelle concentrations were measured using Cibacron blue F3GA and 2-*p*-toluidinylnaphthalene 6-sulfonate (TNS) as spectral and fluorescent indicators of micelle formation, following a titration procedure similar to that described by Mast and Haynes.[30] An increase in absorbance at 324 nm (compared to 362 nm) was followed with Cibacron blue, and an increase in fluorescence intensity (excitation: 360 nm; emission, 460 nm) was followed with TNS.

[b] Critical micelle temperatures were measured as described in the legend to Fig. 1.

[c] The micelle size was measured by gel filtration on LKB-Ultrogel 34 or 54 as described in Rosevear *et al.*[2]

[d] This value of the micelle size was determined by sedimentation equilibrium analysis as described in Suarez *et al.*[11]

iodine, and 200 g Drierite, in that order. The mixture is stirred for 12 hr with a propeller-type stirrer inserted through the neck of the vessel. (Magnetic stirrers have been found unsuccessful with this mass of material.) The reaction mixture is checked by TLC in solvent system I and should show two main spots: laurylmaltoside peracetate (R_f 0.70) and maltose peracetate (R_f 0.38). The reaction mixture is filtered through a pad of celite on a 2-liter scintered glass funnel (small pore) and washed with 1000 ml dichloromethane. The filtrate is concentrated to a syrup by rotary evaporation using a 50° water bath.

Hydrolysis of Orthoester, Deacetylation, and Removal of Sugar By-products. Orthoester side product[7] is hydrolyzed by dissolving the syrup in 3 liters of 0.01 *N* H_2SO_4 in 90% aqueous acetone, allowing it to stand for 30 min, and then neutralizing with pyridine until a slight cloudiness appears (pH 6 with pH paper). The mixture is again concentrated to a syrup. Deacetylation is performed by dissolving in 2000 ml methanol:triethylamine:water (2:1:1) and letting stand overnight. Two main spots are then apparent in solvent system II: lauryl-β-D-maltoside (R_f 0.43) and maltose (R_f 0.13). Dodecanol runs at the solvent front. The deacetylated mixture is concentrated until the methanol and triethylamine are removed but the water remains (mixture begins to foam), and then allowed to separate into two phases in a separatory funnel. The lower phase (brown, aqueous) contains the unreacted sugar and by-products, and can be discarded. The upper phase contains dodecanol and detergent, and can be washed several times with water in the separatory funnel until most of the brown color is removed. If it is then allowed to stand for several days, a crude precipitate of laurylmaltoside forms, from which the excess dodecanol can be decanted (but the dodecanol should be checked by TLC to determine whether a significant amount of laurylmaltoside remains).

Purification by Dowex 1 (OH) Chromatography. Aliquots of the laurylmaltoside precipitate or the dodecanol–detergent mixture are dissolved in a minimum amount of anhydrous methanol and applied in 50 to 100 ml portions to a 3.0 × 150 cm column of Dowex 1 (OH) previously converted from the chloride form by extensive washing with 2 *N* NaOH (20 liters, or until the silver nitrate test for chloride is negative), followed by distilled H_2O until no longer alkaline, and 3 liters of anhydrous methanol. (This same procedure is used to regenerate the column when it has turned from brown to black, indicating accumulation of sugar products.[2]) The column is eluted with methanol at a rate of 10 ml/hr. After collecting about 1200 ml in bulk, fractions of 10 to 20 ml are collected, and the alcohol and

[7] N. K. Kochetkov, A. J. Khorlin, and A. F. Bochkov, *Tetrahedron* **23**, 693 (1967).

detergent peaks are located by adding 1 ml 5% aqueous phenol to 100-μl aliquots of each tube (cloudiness indicates dodecanol is present) followed by 5 ml concentrated sulfuric acid (orange-brown color indicates detergent). The alcohol runs ahead of the detergent and is usually found in the initial 1200 ml. Any lauryl-α-D-maltoside moves ahead of the β-form, and can be detected in solvent system II, the α being the slower moving spot (R_f 0.38). The α- and β-laurylmaltoside fractions are pooled separately and any overlapping region can be concentrated and added to the next sample to be chromatographed. When a large (10-fold) excess of dodecanol is used as described in this preparation, no α-form is observed, possibly because it is more soluble in dodecanol and moves ahead with that peak. The methanol is removed by rotary evaporation and the detergent is taken up in water, lyophilized, and stored in a desiccator. Any stock solutions of the detergents in water are kept frozen to avoid any possibility of hydrolysis. A yield of 100 g of detergent can be obtained from the reaction described (approximately 70% yield, from acetobromomaltose).

Properties of Alkyl Glycoside Detergents

The characteristics of the detergents studied so far are summarized in the table. It was found[2,3] that only the α 1,4-linked disaccharides made suitable head groups, since the β-linked sugars (cellobiose, lactose) formed insoluble glycosides, possibly because of dimer formation between sugar head groups.

Octylmaltoside was synthesized to determine if the head group would affect any of the micelle properties, hopefully creating a more stable, better dispersing micelle than octyl glucoside. However, no difference was observed in its physical properties or in its effects on cytochrome oxidase compared to octyl glucoside.

In contrast to the striking differences between the α and β forms of octyl glucoside[8,9] (see the table), α- and β-laurylmaltoside behaved in an almost identical manner, though the tendency of the α-laurylmaltoside to be contaminated with dodecanol (because it runs closer to the alcohol peak on the column) originally led us to believe that it had different properties, including the ability to denature cytochrome oxidase. This deleterious effect was not observed when the α-form was repurified. The β-form does have the distinguishing feature that when it is contaminated

[8] H. Schindler and J. P. Rosenbusch, *Proc. Natl. Acad. Sci. U.S.A.* **78,** 2302 (1981).
[9] D. L. Dorset and J. P. Rosenbusch, *Chem. Phys. Lipids* **29,** 299 (1981).

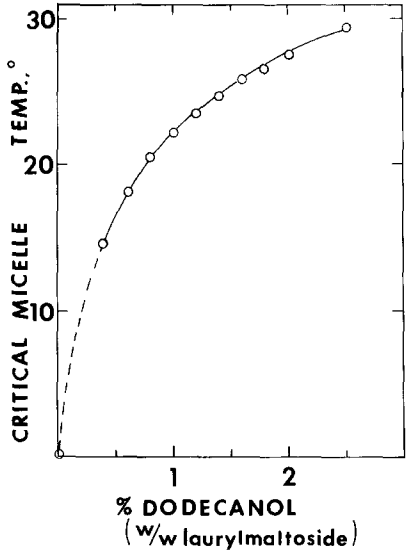

FIG. 1. Effect of dodecanol on the critical micelle temperature of laurylmaltoside. To a solution of pure laurylmaltoside in water (10 g/100 ml) was added various amounts of dodecanol to give effective contamination levels of 0.5 to 2.5% with respect to the weight of laurylmaltoside (1% dodecanol = 0.1 g dodecanol added per 10 g laurylmaltoside). The critical micelle temperature was measured in a capillary tube in a controlled temperature bath, as the temperature at which the detergent/dodecanol solution redissolved after precipitating out at lower temperatures.

with even small amounts of dodecanol it will form a white precipitate at 4° in aqueous solution. Figure 1 illustrates the effect of dodecanol contamination on the solubility of β-laurylmaltoside. α-Laurylmaltoside remains soluble in the presence of substantial dodecanol contamination.

Oleoylmaltotrioside is highly soluble and produces large discrete micelles, but does not disperse or activate cytochrome oxidase to the same extent as laurylmaltoside. It is, however, more effective as a stabilizing agent for lipid-depleted enzyme preparations in dilute solution, as evidenced by a linear rate of oxygen consumption by cytochrome oxidase in an assay system containing 50 m*M* potassium phosphate, 0.06% oleoylmaltotrioside, pH 6.5, conditions under which laurylmaltoside supports higher initial rates but the activity drops off rapidly.[10]

[10] D. A. Thompson, Ph.D. thesis, Michigan State University, 1984.

Applications of Alkyl Glycoside Detergents

Studies on Cytochrome Oxidase

We have found that laurylmaltoside is able to both disperse and activate purified cytochrome oxidase better than any other detergent tested. Although considerable variability in activity is found among different beef heart oxidase preparations, a range of molecular activities from 200 to 700 electrons per second per heme aa_3 has been observed in laurylmaltoside. Cytochrome oxidase prepared in cholate and dissolved in laurylmaltoside behaves consistently on gel filtration columns at medium ionic strength (100 ml KCl, 10 m*M* Tris–Cl, pH 7.8) revealing a major homogeneous species with an apparent molecular weight of 320,000 to 370,000. All other detergents tested (Tween 20, Tween 80, Triton X-100, deoxycholate, octyl glucoside, oleoylmaltotrioside, octylmaltoside) gave a predominance of a much larger molecular weight species (>1,000,000) under these conditions. More rigorous analysis of beef heart cytochrome oxidase by sedimentation equilibrium[11] shows it to be a monomer (2 heme *a*, 2 copper atoms) in laurylmaltoside with a protein moiety of 194,000 Da and associated detergent equal to 108,000 Da (~200 molecules of laurylmaltoside). Although laurylmaltoside does not dialyze out rapildy (half-time of dialysis ~8 hr),[12] when the enzyme is dialyzed for 20 hr with cholate-solubilized phospholipids, it is readily incorporated into vesicles that exhibit high levels of respiratory controls,[4] indicating that little detergent remains.

Purification of cytochrome oxidase from whole mitochondria by a one-step cytochrome *c* affinity chromatography procedure is greatly facilitated with respect to yield, purity, and activity by use of laurylmaltoside as the solubilizing agent, apparently because of its superior dispersing ability compared to Triton X-100, the only other detergent shown to be effective for this purpose.[4,13,14]

Purification and Stabilization of Other Membrane Proteins

The unusual effectiveness of laurylmaltoside compared to several ionic detergents as a protein stabilizing agent was first observed by Knud-

[11] M. D. Suarez, A. Revzin, R. Narlock, E. S. Kempner, D. A. Thompson, and S. Ferguson-Miller, *J. Biol. Chem.* **259,** 13791 (1984).
[12] W. J. DeGrip and P. H. M. Bovee-Geurts, *Chem. Phys. Lipids* **23,** 321 (1979).
[13] H. Weiss and B. Juchs, *Eur. J. Biochem.* **88,** 17 (1978).
[14] K. Bill, C. Broger, and A. Azzi, *Biochim. Biophys. Acta* **679,** 28 (1982).

sen and Hubbell[5] studying rhodopsin. They concluded that large micelle size and high packing density of the nonionic head groups were responsible for preventing access of water and protein denaturation. Evidence from ^{13}C NMR studies on laurylmaltoside micelles in aqueous solution (T. VanAken and S. Ferguson-Miller, unpublished) support the idea that the maltose head groups are indeed relatively immobilized, compared to glucose in an octyl glucoside micelle.

More recently, laurylmaltoside has been reported to increase the stability of photosynthetic reaction centers that were purified in lauryldimethylamine oxide (LDAO) from *Rhodopseudomonas sphaeroides*.[6] Similarly, a final sucrose-gradient purification step in laurylmaltoside of photosystem II from thermophilic blue–green algae results in a significant increase in purity, activity, and stability of the complex.[15]

Octyl glucoside has been used successfully in numerous purification procedures[16–18] and has led to the development of some novel purification schemes.[19–21]

Preparation of Lipid Vesicles and Enzyme Reconstitution

The high critical micelle concentration of octylglucoside has proved useful not only in facilitating reconstitution of enzymes into artificial vesicles,[1,22,23] but also in the preparation of specific types of phospholipid vesicles.[23,24] In addition, alkyl glucosides of different chain lengths have been used to produce vesicles of various sizes in relatively homogeneous populations.[25]

Crystallization of Membrane Proteins

An important recent breakthrough in the study of membrane proteins is the demonstration that three-dimensional crystals can be produced that

[15] J. Bowes, A. C. Stewart, and D. S. Bendall, *Biochim. Biophys. Acta* **725,** 210 (1983).
[16] G. W. Stubbs, H. Smith, Jr., and B. J. Litman, *Biochim. Biophys. Acta* **425,** 46 (1976).
[17] J. T. Lin, S. Reidel, and R. Kinne, *Biochim. Biophys. Acta* **577,** 179 (1979).
[18] B. Wittenberger, D. Raben, M. A. Lieberman, and L. Glaser, *Proc. Natl. Acad. Sci. U.S.A.* **75,** 5457 (1978).
[19] P. Felgner, J. Messer, and J. E. Wilson, *J. Biol. Chem.* **254,** 4946 (1979).
[20] M. Stadt, P. A. Banks, and R. D. Kobes, *Arch. Biochem. Biophys.* **214,** 223 (1982).
[21] J. E. Wilson, J. L. Messer, and P. L. Felgner, this series, Vol. 97, p. 469.
[22] E. Racker, B. Violand, S. O'Neal, M. Alfonzo, and J. Telford, *Arch. Biochem. Biophys.* **198,** 470 (1979).
[23] L. T. Mimms, G. Zampighi, Y. Nozaki, C. Tanford, and J. A. Reynolds, *Biochemistry* **20,** 833 (1981).
[24] O. Zumbuehl and H. G. Weder, *Biochim. Biophys. Acta* **640,** 252 (1981).
[25] R. A. Schwendener, A. Asanger, and H. G. Weder, *Biochem. Biophys. Res. Commun.* **100,** 1055 (1981).

are suitable for high resolution X-ray analysis. In the first successful crystallizations reported[26,27] octyl glucoside was used as the solubilizing agent. More recently other small detergents also have been found to be effective[28,29] and it appears that different proteins will have different specific requirements for a detergent that can be incorporated into the crystal lattice and yet also maintain a stable homogeneous form of the enzyme.[29] A variety of pure alkyl glycosides may prove useful for this purpose.

Acknowledgment

This work was supported by National Institutes of Health Grant GM 26916 to SFM.

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[27] R. M. Garavito and J. P. Rosenbusch, *J. Cell Biol.* **86,** 327 (1980).
[28] H. Michel, *J. Mol. Biol.* **158,** 567 (1982).
[29] H. Michel, *Trends Biochem. Sci.* **8,** 56 (1983).
[30] R. C. Mast and L. V. Haynes, *J. Colloid Interface Sci.* **53,** 35 (1975).

[4] Lipid Enrichment and Fusion of Mitochondrial Inner Membranes

By CHARLES R. HACKENBROCK and BRAD CHAZOTTE

Introduction

Membrane engineering, reconstitution, and modification are important approaches in facilitating the analysis of the activities and interactions of catalytic membrane components. It is the purpose of this chapter to present the details of methods developed in our laboratory over the past few years which permit a significant incorporation or bulk enrichment of the mitochondrial inner membrane with a variety of membrane lipids.[1,2] Related techniques which permit the conversion of the typically small mitochondrial inner membranes to ultralarge inner membranes through a fusion process are also presented. Significantly, one method provides osmotically active, ultralarge (>200 μm) individual, spherical inner mem-

[1] H. Schneider, J. J. Lemasters, M. Höchli, and C. R. Hackenbrock, *Proc. Natl. Acad. Sci. U.S.A.* **77,** 442 (1980).
[2] H. Schneider, J. J. Lemasters, M. Höchli, and C. R. Hackenbrock, *J. Biol. Chem.* **255,** 3748 (1980).