Huxley, H. E. (1969) *Science 164*, 1356–1366.
Kretzschmar, K. M., Mendelson, R. A., & Morales, M. F. (1978) *Biochemistry 17*, 2314–2318.
Lowey, S., Slayter, H. S., Weeds, A. G., & Baker, H. (1969) *J. Mol. Biol. 42*, 1–29.
Lymn, R. W., & Cohen, G. H. (1975) *Nature (London) 258*, 771–772.
Margossian, S., & Stafford, W. (1979) *Biophys. J. 25*, 20a (Abstr.).
Margossian, S., Lowey, S., & Barshop, B. (1975) *Nature (London) 258*, 163–166.
Mendelson, R. A., & Cheung, P. (1976) *Science 194*, 190–192.
Mendelson, R. A., & Morales, M. F. (1977) *Biochim. Biophys. Acta 459*, 578–595 (Appendix).
Mendelson, R. A., Morales, M. F., & Botts, J. (1973) *Biochemistry 12*, 2250–2255.
Mendelson, R. A., Putnam, S., & Morales, M. F. (1975) *J. Supramol. Struct. 3*, 162–168.
Miller, A. M., & Tregear, R. T. (1972) *J. Mol. Biol. 70*, 85.
Moore, P. B., Huxley, H. E., & DeRosier, D. J. (1970) *J. Mol. Biol. 50*, 279–295.
Murthy, N. S., & Knox, J. R. (1977) *J. Appl. Crystallogr. 10*, 137–140.
Offer, G., & Elliott, A. (1978) *Nature (London) 271*, 325–329.
Porod, G. (1951) *Kolloid Z. 124*, 83–87.
Reedy, M. K., Holmes, K. C., & Tregear, R. T. (1965) *Nature (London) 207*, 1276–1280.
Richards, F. M. (1977) *Annu. Rev. Biophys. Bioeng. 6*, 151–176.
Sakura, J. D., & Reithel, F. J. (1972) *Methods Enzymol. 26*, 107–119.
Squire, J. M. (1975) *Annu. Rev. Biophys. Bioeng. 4*, 137–163.
Stuhrman, H. B. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2316–2320.
Takahashi, T. (1978) *J. Biochem. (Tokyo) 83*, 905–908.
Tregear, R. T., & Mendelson, R. A. (1975) *Biophys. J. 15*, 455–467.
Yang, J. T., & Wu, C. C. (1977) *Biochemistry 16*, 5785–5789.

Alkyl Glycoside Detergents: A Simpler Synthesis and Their Effects on Kinetic and Physical Properties of Cytochrome *c* Oxidase†

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ABSTRACT: Octyl glucoside is an effective, nonionic, solubilizing agent for membrane proteins with the advantage of ease of removal by dialysis. In order to study the detergent-sensitive activity of cytochrome *c* oxidase, we chose this detergent because of its simple structure and the possibility of synthesizing analogues to test the structural dependence of the detergent specificity. A procedure was therefore developed that facilitates large-scale preparation of octyl glucoside and related alkyl glycosides, improving on previous methods by eliminating crystallization steps and employing a one-step purification of the final product on Dowex 1. This new purification procedure is particularly important for achieving the level of purity required to obtain the disaccharide, longer alkyl chain detergents in soluble form. Of the alkyl glycosides prepared (octyl β-D-glucopyranoside, octyl β-D-lactopyranoside, dodecyl β-D-lactopyranoside, dodecyl β-D-cellobiopyranoside, and dodecyl β-D-maltopyranoside), lauryl (dodecyl) maltoside was found to be the most successful as an activator of purified beef and *Neurospora* cytochrome oxidases, giving two- to tenfold higher activities than octyl glucoside and other commercially available detergents, Tween-20 and Triton X-100. Kinetic studies using two different steady-state assay systems indicate that the activity changes are not the result of altered binding of the substrate but rather reflect a detergent effect on the state of association of the enzyme (as a monomer, dimer, or polymer) as well as on its intrinsic activity. By gel filtration procedures, lauryl maltoside and octyl glucoside were found to exist as monodisperse populations of micelles of 50 000 and 8000 daltons, respectively. The small uniform micelles and chemically well-defined structures of lauryl maltoside and octyl glucoside make them superior to other nonionic detergents for the study of membrane proteins in general and cytochrome oxidase in particular, since its activity in lauryl maltoside most closely approaches that of the physiological state.

Octyl glucoside has recently been found to be an effective solubilizing agent for a number of membrane proteins (Baron & Thompson, 1975; Stubbs & Litman, 1978; Wittenberger et al., 1978; Felgner et al., 1979; Petri & Wagner, 1979). It is a small molecule of simple, defined structure which has a high critical micelle concentration that permits easy removal by dialysis. These properties have facilitated the purification and reconstitution of hexokinase-binding protein from mitochondrial outer membranes (Felgner et al., 1979), an isolation previously unattainable with numerous other detergents.

Mitochondrial cytochrome *c* oxidase has been isolated from many sources, but the electron transfer activity of the purified, multisubunit enzyme is found to be very sensitive to its detergent environment (Chuang & Crane, 1973; Yu et al., 1975; Robinson & Capaldi, 1977; Yu et al., 1979). It appears that the structure of the hydrophobic portion of the detergent is an important factor in the activity changes, but the kinetic characteristics and the molecular basis of the detergent effects have not been determined. With the ultimate aim of understanding the influence of membrane structure and other electron transfer proteins on cytochrome oxidase activity, the alkyl glycoside detergents were chosen to investigate the

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molecular requirements for maximizing cytochrome oxidase function.

Octyl glucoside is available commercially, but at high cost, and the published synthetic methods involve lengthy crystallizations, silicic acid chromatography, and steam distillation (Noller & Rockwell, 1938; Baron & Thompson, 1975; Keana & Roman, 1978; Knudsen & Hubbell, 1978; De Grip & Bovee-Geurts, 1979). It was therefore of interest to develop a synthesis that was simpler to perform and that would allow preparation of both radioactive and unlabeled octyl glucoside in sufficient quantities for enzyme purification purposes, as well as structural analogues for defining the effects of detergent on protein activity. The synthesis of the alkyl glycoside detergents reported here involves a modification of the classical Koenigs–Knorr reaction using silver carbonate–iodine (Chiang et al., 1979) as the catalyst and Dowex 1 (hydroxide) column chromatography for purification. Lauryl (dodecyl) glycosidic derivatives of disaccharides were synthesized to determine if a longer alkyl chain could be balanced by a larger hydrophilic head group and maintain the desirable features of octyl glucoside while increasing the ability to extract and activate cytochrome *c* oxidase. The disaccharide detergent, lauryl maltoside, was found to be superior to other alkyl glycosides and to commercially available nonionic detergents, with respect to its physical properties (small, uniform micelles) and its ability to enhance the activity of cytochrome *c* oxidase. The physical and kinetic characteristics of cytochrome oxidase in the alkyl glycoside detergents are described.

Experimental Procedures

Synthesis of Glycoside Detergents. (*1*) *Methods.* Thin-layer chromatography was performed on glass plates coated with Silica Gel G, 250–500 μm thick (Analtech), by using solvent systems I [ethyl acetate/hexane (1:1 v/v)] and II [ethyl acetate/methanol (4:1 v/v)]. Plates were activated by heating for 30 min at 110 °C before use in solvent system I. Unactivated plates were used with solvent system II. Compounds were visualized by spraying the plates with 2 N sulfuric acid and charring them on a hot plate.

Carbon-13 nuclear magnetic resonance spectra were obtained with a Bruker WP-60, 16.08-MHz Fourier transform spectrometer equipped with quadrature detection. Spectra were obtained with 4000 spectral points at 36 °C. Chemical shifts are given relative to external tetramethylsilane and are accurate to within 0.1 ppm.

Evaporations were performed under reduced pressure at 40 °C unless otherwise stated.

(*2*) *Chemicals.* [U-^{14}C]Glucose was obtained from New England Nuclear. Anhydrous silver carbonate was prepared by the procedure of Wolfram & Lineback (1963). The silver carbonate prepared in this way remained light yellow and active for at least 2 months when stored in the dark in a desiccator over $Mg(ClO_4)_2$. Darkening of the compound to black indicates that the silver carbonate is no longer suitable for use as a catalyst.

Glucose pentaacetate can be purchased or prepared by a modified procedure of Bates et al. (1942). Glucose (100 g, 0.55 mol), containing 2×10^7 cpm [U-^{14}C]glucose if desired, is added to 50 g of anhydrous sodium acetate in 700 mL of acetic anhydride previously equilibrated in a bath at 100 °C. Complete solution of the reaction mixture is obtained after 30 min. Thin-layer chromatography in solvent system I after 1.5 h reveals only one spot with an R_f of 0.56 which migrated with authentic glucose pentaacetate. The reaction is poured hot onto 4 L of ice and left standing for 2 h. A gummy precipitate is formed and the supernatant is decanted from it. The precipitate is dissolved in 500 mL of dichloromethane and extracted 1 time with 500 mL of ice water and 4 times with 500 mL of cold, saturated sodium bicarbonate. The pH of the last extraction with sodium bicarbonate should be >7. The dichloromethane layer is then extracted 2 times with 500 mL of water, decolorized with 5 g of activated charcoal, filtered, concentrated to a thick syrup by rotatory evaporation, dried overnight on an oil vacuum pump, and found to be homogeneous by TLC in solvent system I. The yield of a white powder of glucose pentaacetate is 90% by radioactivity.

Acetobromoglucose (2,3,4,6-tetraacetylglucopyranosyl bromide) can be purchased or prepared by dissolving 20 g of glucose pentaacetate (52 mmol) in 40 mL of glacial acetic acid. To this is added 40 mL of 30% hydrogen bromide in glacial acetic acid (Eastman). After 30 min, TLC in solvent system I shows 90% one spot with an R_f of 0.70. Traces of glucose pentaacetate are still present. The reaction mixture is immediately diluted with 200 mL of dichloromethane and poured onto 300 mL of ice in a separatory funnel. Prolonged reaction times result in the formation of degradation products of acetobromoglucose. The dichloromethane layer is washed 1 time with 200 mL of H_2O and 3 times with 200 mL of cold, saturated sodium bicarbonate with the pH of the sodium bicarbonate in the last extraction being >7. The dichloromethane layer is then washed 3 times with 200 mL of water, dried over anhydrous $MgSO_4$ for 30 min, and filtered through a pad of Celite. This product is unstable and should be used immediately for best results in the synthesis of the glycoside detergents.

Maltose octaacetate is prepared similarly to glucose pentaacetate. Maltose (20 g, 56 mmol) is added over a 20-min period to 8 g of anhydrous sodium acetate dissolved in 125 mL of acetic anhydride previously equilibrated at 100 °C. After 6 h the reaction is judged to be complete by TLC in solvent system I. Maltose octaacetate has an R_f of 0.38. Workup of the reaction mixture is identical to that given for glucose pentaacetate.

Acetobromomaltose can be purchased or prepared by dissolving 20 g of maltose octaacetate in 50 mL of glacial acetic acid, adding 50 mL of hydrogen bromide (30% in glacial acetic acid), and stirring at room temperature for 45 min. Thin-layer chromatography in solvent system I shows mainly one spot with an R_f of 0.48. The acetobromomaltose is worked up similarly to the acetobromoglucose.

Glycoside Synthesis. (*1*) *Octyl β-D-glucopyranoside* is synthesized from 2,3,4,6-tetraacetylglucopyranosyl bromide and 1-octanol in the presence of silver carbonate as a catalyst. The 2,3,4,6-tetraacetylglucopyranosyl bromide (~52 mmol) is diluted to 450 mL with dichloromethane and placed in a foil-covered reaction flask. With efficient stirring, the following additions are made: 8.7 mL (55 mmol) of 1-octanol that has been stored over 3-Å molecular sieves, 10.0 g of silver carbonate that had been dried for at least 24 h, 0.7 g of iodine, and 20 g of 4-Å molecular sieves. The octyl β-D-glucopyranoside peracetate formed has an R_f in solvent system I of 0.76. Although the condensation reaction is usually complete after 5 h, reactions routinely are allowed to proceed overnight. The suspension is then filtered through a pad of Celite and washed with 100 mL of dichloromethane. The filtrate is concentrated to a syrup and dissolved in 200 mL of methanol/triethylamine/water (2:1:1) and allowed to stand for 10 h at room temperature. Deacetylation is judged to be complete by TLC in solvent system II. Two main spots for the reaction mixture correspond to octyl β-D-glucopyranoside and glucose with R_f values of 0.63 and 0.28, respectively.

Unreacted octanol is found as a fast-moving diffuse spot near the solvent front. The deacetylated reaction mixture is concentrated to a syrup. Most of the unreacted octanol is removed by repeated evaporations of water from the syrup at 60 °C on an oil pump vacuum for ~2 h. The syrup is dissolved in methanol and placed on a 3.1 × 100 cm column of Dowex 1 (2% cross-linked; hydroxide form; 200–400 mesh) previously equilibrated with methanol and eluted with the same solvent. Fractions (10 mL) are collected and the octyl glucoside is assayed by radioactivity when [U-^{14}C]glucose is used or by the phenol–sulfuric acid test for glycosides and sugars (Dubois et al., 1956; Hodge & Hofreiter, 1962). The remaining unreacted octanol elutes immediately before the octyl glucoside. Octanol can be detected down to 0.3% (v/v) by the presence of a precipitate when an aliquot (100 μL) from the fraction is added to 1 mL of 5% aqueous phenol, the first step in the assay for sugar by the phenol–sulfuric acid test. The Dowex 1 column can be regenerated by washing with 3 L of 1 M NaOH, 3 L of water, and finally 2 L of methanol. The purified octyl β-D-glucoside, obtained as a white powder after lyophilization in ~60% yield from starting glucose, is homogeneous in solvent system II and comigrates with an authentic sample. Carbon-13 NMR revealed the presence of only one peak in the anomeric region, at 104.2 ppm, characteristic of a β-glycosidic linkage of glucose.

(*2*) *Lauryl β-D-maltopyranoside* is synthesized from acetobromomaltose and lauryl alcohol in the presence of silver carbonate as the catalyst. To a well-stirred dichloromethane solution of acetobromomaltose (28 mmol in 280 mL) in a foil-covered reaction vessel are added 6.6 mL (29 mmol) of 1-dodecanol, which has been stirred at 60 °C over Drierite for 24 h and stored over Drierite, 6 g of silver carbonate, which had been dried for at least 24 h, 0.4 g of iodine, and 20 g of 4-Å molecular sieves. After 12 h, TLC in solvent system I shows two main spots corresponding to lauryl β-D-maltoside peracetate and maltose peracetate with R_f values of 0.70 and 0.38, respectively. Unreacted dodecanol appears as a fast-moving diffuse spot near the solvent front. The reaction mixture is filtered through a pad of Celite, washed with 100 mL of dichloromethane, and taken to dryness by rotary evaporation. The resulting syrup is dissolved in 350 mL of 0.01 N H_2SO_4 in 90% (aqueous) acetone and allowed to stand for 25–30 min. This treatment hydrolyzes any ortho ester formed as a side product during the condensation reaction. The solution is then neutralized by the addition of pyridine and concentrated to a syrup. Deacetylation is carried out by dissolving the syrup in 200 mL of methanol/triethylamine/water (2:1:1) and letting it stand overnight at room temperature. Thin-layer chromatography in solvent system II reveals two spots corresponding to lauryl β-D-maltoside and maltose with R_f values of 0.43 and 0.13, respectively. Chromatography was carried out on a 3.1 × 100 cm column of Dowex 1 (hydroxide) equilibrated in methanol. The purified lauryl β-D-maltoside from the column is detected by using the phenol–sulfuric acid test for glycosides. Unreacted lauryl alcohol elutes well before the lauryl maltoside and is detected down to 0.1% (v/v) by the presence of a precipitate when 1 mL of 5% aqueous phenol is added to a 100-μL aliquot of the fraction. Concentration of the lauryl glycoside is best achieved by rotatory evaporation of the pooled fractions from the column, addition of water, and lyophilization. The lauryl β-D-maltopyranoside, 25% yield from starting maltose, was found to be homogeneous by carbon-13 NMR and by TLC in solvent system II with an R_f of 0.43.

Octyl and lauryl β-D-glycosides of lactose and cellobiose were prepared as described for lauryl β-D-maltopyranoside.

Enzyme Studies. Beef heart mitochondrial particles, depleted of cytochrome *c*, were prepared as described by Ferguson-Miller et al. (1976). Rat liver mitochondria were prepared from Sprague–Dawley rats according to Johnson & Lardy (1967), and inner mitochondrial membranes (mitoplasts) were obtained by a modification of the method of Sottocasa et al. (1967) as described by Felgner et al. (1979) and depleted of cytochrome *c* by washing in 0.45 M sucrose and 0.15 M KCl. Neurospora mitochondrial cytochrome *c* oxidase, purified in Triton X-100, was the gift of Dr. H. Weiss (EMBL, Heidelberg). Beef heart cytochrome *c* oxidase, purified in cholate and depleted of phospholipids according to the procedure of Hartzell & Beinert (1974) was the gift of Dr. G. Babcock (Michigan State University). Beef heart oxidase was also prepared by a modification of the method of Kuboyama et al. (1972). Cytochrome *c* (Sigma Type VI) was purified before use according to the procedure of Brautigan et al. (1979).

Mitochondrial membranes were solubilized at varying concentrations of salt, detergent, and protein (measured by the biuret procedure) in 0.66 M sucrose, 0.05 M Tris-HCl, and 1 mM histidine, pH 8.0. The concentrations of the cytochromes in the 45000*g* × 1 h supernatant were determined by measuring the dithionite-reduced minus the ferricyanide-oxidized difference spectra from 500 to 650 nm with an Aminco DW 2A dual-beam, dual-wavelength spectrophotometer and millimolar extinction coefficients for cytochrome aa_3 at 605 minus 630 nm of 24, cytochrome *b* at 560 minus 575 nm of 23.4, and cytochrome c_1 at 553 minus 542 nm of 18.7. No corrections were made for the contributions of the other cytochromes at the specified wavelengths. Oxidase activities were measured polarographically in 25 mM Tris–acetate, pH 7.9, 2.5 mM ascorbate, 0.6 mM *N,N,N′,N′*-tetramethylphenylenediamine (TMPD), and 20 μM cytochrome *c*.

The purified cytochrome oxidases were equilibrated into different detergents by gel filtration through a 1 × 26 cm column of LKB Ultrogel 54 in 100 mM KCl, 10 mM Tris-HCl, pH 7.8, 1 mM EDTA, and 30 mM octyl glucoside, 0.1% lauryl maltoside, or 0.1% Tween-20 or as indicated in the figure legend. The flow rate was 5 mL/h. The oxidase (20–100 μM) was applied as a 200-μL sample in two- to fivefold the concentration of detergent present on the column.

The kinetics of the reaction between cytochrome *c* and cytochrome oxidase were studied polarographically and spectrally as described by Ferguson-Miller et al. (1978) in 25 mM Tris–OAc, pH 7.9 or 50 mM potassium phosphate, pH 6.5, at the detergent concentrations indicated in the figure legends. Turnover numbers (TN), or molecular activity, were calculated by dividing the velocity, in moles of cytochrome *c* per second, by the moles of cytochrome oxidase present in the reaction vessel.

The size of the detergent micelles was estimated by gel filtration through 1 × 26 cm columns of LKB Ultrogel 34 (20000–350000 molecular weight range) and 54 (5000–70000 molecular weight range) equilibrated in 100 mM KCl, 10 mM Tris-HCl, pH 7.8, and 0.25 mM lauryl maltoside or 30 mM octyl glucoside. The Ultrogel 34 columns were calibrated with Blue Dextran (2000000 daltons), ferritin (450000 daltons), catalase (240000 daltons), aldolase (158000 daltons), bovine serum albumin (68000 daltons), ovalbumin (45000 daltons), chymotrypsinogen A (25000 daltons), cytochrome *c* (12500 daltons), and ferricyanide (totally included). Ultrogel 54 columns were calibrated with Blue Dextran, bovine serum

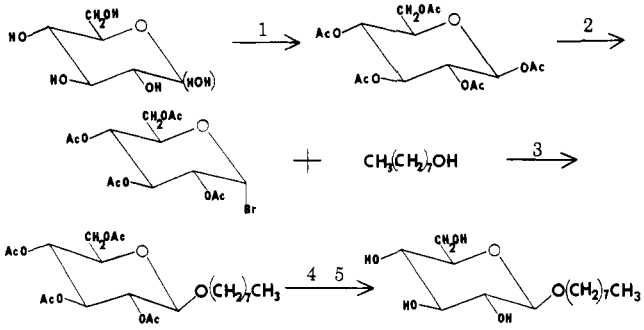

FIGURE 1: The reaction scheme for the synthesis of the alkyl glycoside detergent octyl β-D-glucopyranoside. Glucose is acetylated (1) in sodium acetate and acetic anhydride at 100 °C, yielding glucose pentaacetate which is then brominated (2) with hydrobromic acid in glacial acetic acid at room temperature. The acetobromoglucose in dichloromethane is condensed (3) with 1-octanol in a container shielded from light, with silver carbonate and iodine present as the catalyst. The resulting octyl β-D-glucopyranoside peracetate is deacetylated (4) in methanol–triethylamine–water, and, finally, the desired product, octyl β-D-glucopyranoside, is purified (5) by column chromatography on Dowex 1 (hydroxide) developed with methanol.

albumin, ovalbumin, chymotrypsinogen A, cytochrome *c*, and ferricyanide. Fractions of 1 mL were collected at a flow rate of 5 mL/h. The position of elution of the detergent was monitored by the phenol–sulfuric acid test for glycosides (Dubois et al., 1956) which produces a colored product absorbing at 490 nm or by radioactivity by using ^{14}C-labeled octyl glucoside.

The molecular size of cytochrome oxidase in octyl glucoside and lauryl maltoside was determined by equilibrating the purified oxidase through an Ultrogel 54 column in 30 mM octyl glucoside or 10 mM lauryl maltoside under the same conditions as described above and then putting the equilibrated sample through a second column of Ultrogel 34, also in 30 mM octyl glucoside or 2 mM lauryl maltoside, calibrated as indicated for the micelle size determination. The position of elution was monitored by absorbance at 280 and 418 nm.

Results

The reaction scheme for the synthesis of alkyl glycoside detergents is summarized in Figure 1. The important features of this procedure are the purification of the acetobromo compounds by extraction with dichloromethane to prevent decomposition, the use of silver carbonate plus iodine as a catalyst, favoring rapid production of the β anomer with little side-product formation, and chromatography on Dowex 1 (hydroxide), which converts the unreacted sugar to strongly binding acid forms and separates the alcohol ahead of the glycoside in a one-step purification that eliminates the need for crystallization. This method gives a white powder on lyophilization that is homogeneous by TLC and carbon-13 NMR.

In the synthesis of lauryl maltoside, the low volatility of the 1-dodecanol prevents prior removal of the unreacted alcohol by evaporation, as in the case of octanol, but Dowex 1 (hydroxide) chromatography in 100% methanol successfully separates the alcohol and the detergent. Trace impurities in the lauryl maltoside are found to markedly affect its solubility in water, and occasionally rechromatography in the same system was necessary to achieve the level of purity required to give a product that is freely soluble in water down to 0 °C.

The lauryl glycoside of lactose was very insoluble in water (<100 mg/L at 25 °C) even in a highly purified form and after treatment with Chelex to remove any possible divalent ion contaminants. The solubility of lauryl lactoside increased sharply at 73 ± 1 °C, independent of concentration, indicating that this is the Krafft point of the detergent (the temperature at which micelle formation occurs). Similarly, the octyl lactoside was highly insoluble, whereas the lauryl cellobioside gave an intermediate solubility, with a Kraft point of 43 °C.

The natural abundance carbon-13 NMR spectra of octyl β-D-glucoside, dodecyl β-D-maltoside, and dodecyl β-D-lactoside are shown in Figure 2. In the spectrum of octyl glucoside, obtained in deuterium oxide, the resonances of the carbohydrate moiety are found downfield of tetramethylsilane at 104.2, 77.6, 77.4, 74.7, 71.2, and 62.5 ppm, in good agreement with those observed for a β-glycoside of glucose (Walker et al., 1976). The lack of resonances between 100 and 90 ppm indicates the detergent is free from contaminating glucose.

The spectrum of lauryl maltoside was performed in deuterated dimethyl sulfoxide in order to obtain very high concentrations of detergent so any trace contaminants could be detected. The resonance at 104.4 ppm (Figure 2B, peak 2) represents the C1′ of maltose β-linked to dodecanol. The second anomeric resonance at 101.9 ppm (Figure 2B, peak 3) represents the C1 of glucose α-linked to the C4′ of glucose in the maltose moiety. The equal intensity of these two resonances, as well as the lack of any resonances between 100 and 90 ppm where C1 of the reducing sugar would be located, is strong evidence that no contaminating maltose is present.

The carbon-13 NMR of lauryl lactoside, also determined in dimethyl sulfoxide, is shown in Figure 2C and demonstrates the absence of contaminating lactose by the same criteria discussed for the lauryl maltoside: equal heights of resonances 4 and 5 in Figure 2C and lack of resonances between 90 and 100 ppm.

Enzyme Studies. The effectiveness of octyl glucoside as a solubilizing agent for mitochondrial membranes is shown in Figure 3. Higher levels of detergent were required to release the cytochromes from the mitochondrial membranes of beef heart (Figure 3A) than from rat liver (Figure 3B). The optimum detergent concentration for extracting cytochrome *c* oxidase from beef heart mitochondria was 45 mM, at 10 mg of protein/mL and 0.5 M KCl. At higher salt (1 M KCl) only 30 mM octyl glucoside was required to completely solubilize the oxidase, but the differential extraction of cytochromes *b* and c_1, observed in 0.5 M KCl (Figure 3A), was eliminated.

The optimal octyl glucoside concentration for complete solubilization of rat liver mitochondrial membranes was 20 mM, at 20 mg of protein/mL and 0.6 M KCl (Figure 3B). No evidence of differential extraction of cytochromes *b*, c_1, or aa_3 was found at any level of salt or detergent in the rat liver tissue. However, preliminary experiments indicate that an effective purification of cytochrome oxidase can be achieved by selective reconstitution of oxidase into a membranous phase by dialyzing out the octyl glucoside, a procedure similar to that described by Felgner et al. (1979) for the purification of hexokinase-binding protein from rat liver mitochondrial outer membranes.

Lauryl maltoside solubilized rat liver mitochondrial membranes at 10–20 mM, in 1 M KCl and 20 mg of protein/mL. Differential extraction of cytochromes *b* and c_1 was observed between 3 and 5 mM lauryl maltoside (Figure 4) and is accentuated in 0.5 M KCl. By use of these latter conditions, a very efficient, high-yield purification of rat liver cytochrome oxidase has been developed (D. A. Thompson and S. Ferguson-Miller, unpublished experiments; VanAken et al., 1980).

Octyl glucoside and lauryl maltoside were compared to other commercially available detergents as to their effects on the kinetics and activity of cytochrome oxidase with cytochrome

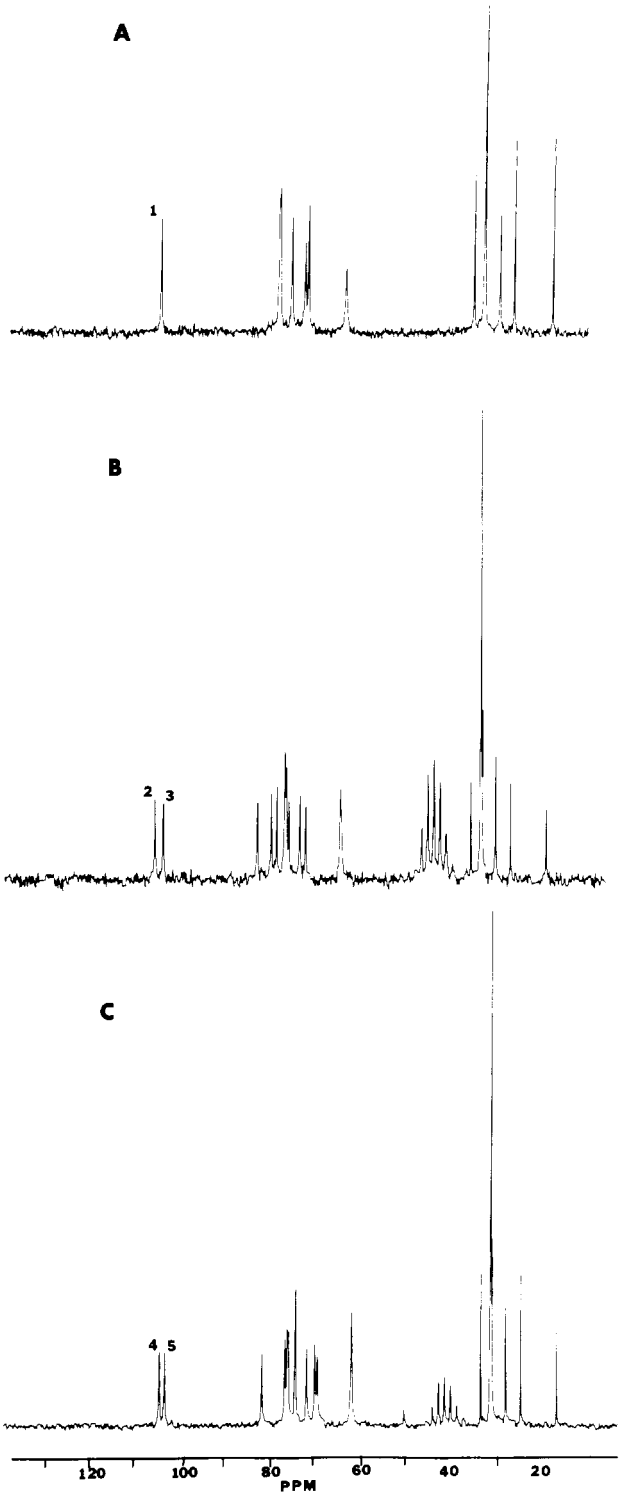

FIGURE 2: The 15.08-MHz, proton-decoupled ^{13}C NMR spectra of (A) octyl β-D-glucoside, (1) the C1 of glucose β-linked to 1-octanol; (B) lauryl β-D-maltoside, (2) the C1′ of maltose β-linked to 1-dodecanol and (3) the C1 of glucose α-linked to the C4′ of glucose in the maltose moiety; (C) lauryl β-D-lactoside, (4) the C1 of galactose β-linked to C4′ of glucose in the lactose moiety and (5) C1′ of glucose β-linked to dodecanol.

c. The purified beef and Neurospora oxidases were equilibrated with different detergents by gel filtration (see Experimental Procedures). The Neurospora enzyme was very unstable in octyl glucoside at 5–30 mM under the conditions used for gel filtration, apparently dissociating into subunits. Therefore, no data were obtained concerning its activity in this detergent. However, a comparison between lauryl maltoside,

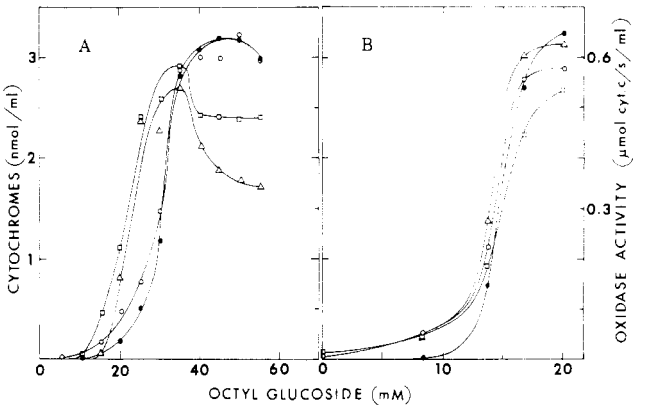

FIGURE 3: Extraction of cytochromes from mitochondrial membranes by octyl glucoside. (A) Beef heart mitochondrial particles were incubated on ice for 10 min, at 10 mg of protein/mL, in 0.5 M KCl, 0.66 M sucrose, 0.01 M Tris-HCl, 1 mM histidine, pH 8.0, and the indicated concentrations of octyl glucoside. The concentrations of cytochromes *b* (△), c_1 (□), and aa_3 (○) were measured in the 45000*g* × 1 h supernatant, as described under Experimental Procedures. The concentration of cytochrome aa_3 in the particles before extraction was 0.4 nmol/mg of protein. Cytochrome *c* oxidase activity (●) was measured polarographically as described under Experimental Procedures, giving turnover numbers of ~200 s^{-1}. (B) Rat liver inner mitochondrial membranes were incubated on ice for 10 min, at 20 mg of protein/mL and 0.6 M KCl, under the conditions described in (A). The concentration of cytochrome aa_3 in the membranes before extraction was 0.15 nmol/mg of protein.

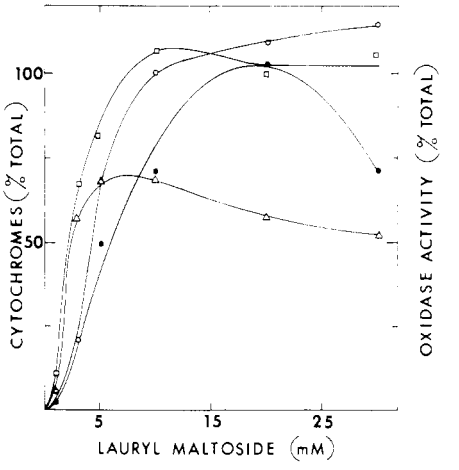

FIGURE 4: Extraction of cytochromes from mitochondrial membranes by lauryl maltoside. Rat liver inner mitochondrial membranes were incubated on ice for 10 min, at 20 mg of protein/mL, in 1 M KCl, 0.33 M sucrose, 0.01 M Tris-HCl, pH 8.0, and the indicated concentrations of lauryl maltoside. Other procedures were as described in Figure 3. The percent of the total cytochrome extracted was calculated from the original concentrations in the membranes (in nmol/mg of protein): cytochrome aa_3 (○) = 0.13; cytochrome *b* (△) = 0.10; cytochrome c_1 (□) = 0.10. The activity of oxidase (●) is given as the percent of the original membranes (34 nmol of cytochrome *c* oxidized per s per mg of protein; TN = 260 s^{-1}).

Tween-20, and Triton X-100 (Figure 5) indicates that equilibration and assay in lauryl maltoside produces the most active form of the Neurospora enzyme. The major change in activity is seen to be an increase in the turnover number of both phases of the biphasic Eadie–Hofstee plot and a slight increase in the apparent K_m value of the initial phase.

The beef oxidase was stable in all the detergents tested. Equilibration in lauryl maltoside again produced a significantly more active form of the enzyme than did octyl glucoside or Tween-20 (Figure 6A). Equilibration at higher concentrations of all three detergents (50 mM octyl glucoside, 0.5–1% lauryl maltoside, or Tween-20) increased the activity in lauryl

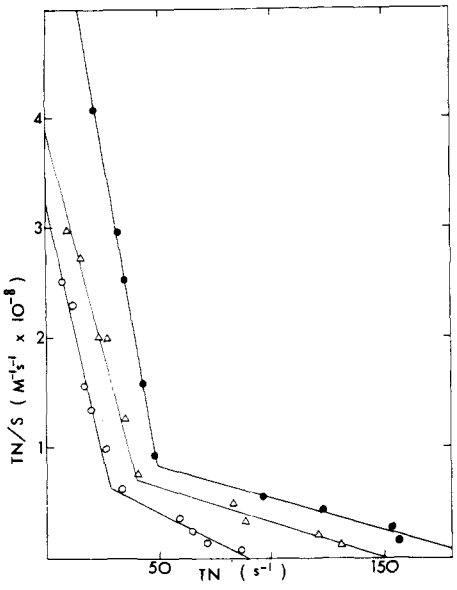

FIGURE 5: An Eadie–Hofstee plot of the kinetics of oxidation of cytochrome *c* by purified Neurospora cytochrome oxidase equilibrated in 0.05% Tween-20 (○), 0.05% Triton X-100 (△), and 0.05% lauryl maltoside (●). Rates of oxygen consumption were measured polarographically in 25 mM Tris–OAc, pH 7.9, as described under Experimental Procedures. Velocities were expressed as rates of cytochrome *c* oxidation by multiplying by 4 and converted to turnover numbers by dividing by the concentration of cytochrome oxidase (4×10^{-8} M). The range of horse heart cytochrome *c* concentrations used was 0.03–20 μM.

maltoside by a factor of 2 but decreased the activities in the other detergents.

The activity of the beef oxidase was measured by the spectral assay (Figure 6B) as well as polarographically (Figure 6A) since the two assay procedures appear to involve different rate-limiting steps (Ferguson-Miller et al., 1978, 1979) and therefore can give complementary information concerning the nature of the activating effect of the detergent. Even greater activity differences were observed by the spectral assay procedure between the octyl glucoside and lauryl maltoside equilibrated enzymes, the latter being ~5 times as active as the former. The detergent concentrations used in the assays, as detailed in the figure legends, were those that gave the highest activity for the particular detergent. The conditions used, high pH and low ionic strength, were those that give the highest turnover rates in the polarographic assay, by favoring tight binding of cytochrome *c* to the oxidase and permitting rapid reduction of the cytochrome *c*–cytochrome oxidase complex by TMPD (Ferguson-Miller et al., 1976, 1978, 1979; Smith et al., 1979). Because turnover in the spectral assay, in the absence of TMPD, requires dissociation of cytochrome *c* from the oxidase, lower rates of oxidation of cytochrome *c* are measured spectrophotometrically. In potassium phosphate (50 mM; pH 6.5), conditions that favor rapid dissociation, turnover numbers of 475 s^{-1} were observed for the oxidase equilibrated in 10 mM lauryl maltoside.

The critical micelle concentrations for octyl glucoside (25 mM) (Shinoda et al., 1961) and lauryl maltoside (0.16–0.19 mM) (Knudsen & Hubbell, 1978; De Grip & Bovee-Geurts, 1979) have been previously reported. However, the micelle size and degree of dispersity have not been reported for either detergent. In filtration studies with LKB Ultrogel 54 (molecular weight range 5000–70000), lauryl maltoside gave a very sharp band, indicating a monodisperse population of micelles at ~50000. Octyl glucoside gave a well-defined peak corresponding to a molecular weight of ~8000.

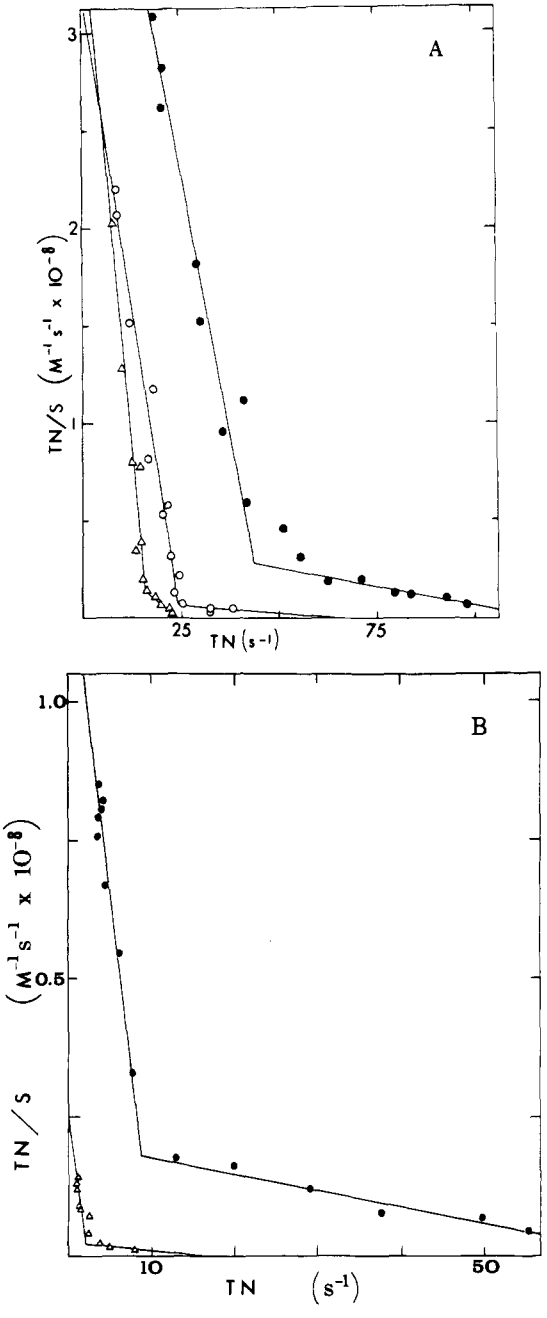

FIGURE 6: An Eadie–Hofstee plot of the kinetics of oxidation of cytochrome *c* by purified beef heart cytochrome oxidase equilibrated in 0.1% lauryl maltoside (●), 0.1% Tween-20 (○), and 30 mM octyl glucoside (△). (A) Rates of oxygen consumption were measured polarographically as described in Figure 5, at a cytochrome oxidase concentration of 5×10^{-8} M in the presence of 0.25 mM (0.0125%) lauryl maltoside (●), 20 mM octyl glycoside (△), and 0.25 mM (0.03%) Tween-20 (○). (B) Rates of cytochrome *c* oxidation were measured spectrally as described under Experimental Procedures, at a cytochrome oxidase concentration of 0.5×10^{-9} M in the presence of 0.25 mM lauryl maltoside (●) and 20 mM octyl glucoside (△) and cytochrome *c* concentrations of 0.05–23 μM.

The molecular form of beef heart cytochrome oxidase after equilibration in octyl glucoside was measured by gel filtration as described under Experimental Procedures. Three major peaks were observed (Figure 7A), one in the excluded volume, one at ~280000, and a broad shoulder at a lower molecular weight. The largest molecular weight species appeared to be denatured aggregated oxidase, since it had little absorbance at 418 nm (280 nm/418 nm = 35). The 280000-dalton peak had the spectral properties of native cytochrome oxidase (280 nm/418 nm = 4.5) but had very low activity (TN = 35 s^{-1}).

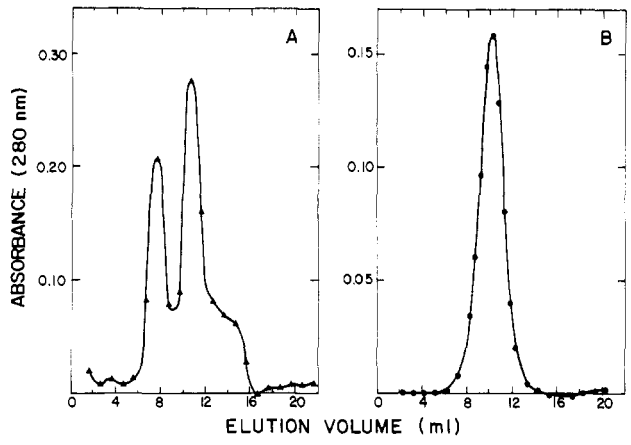

FIGURE 7: (A) Gel filtration of beef heart cytochrome oxidase through Ultrogel 34, after previous equilibration in 30 mM octyl glucoside. The column (1 × 26 cm) was run in 100 mM KCl, 30 mM octyl glucoside, and 10 mM Tris-HCl, pH 7.8. Cytochrome oxidase (200 μL, 22 μM) was applied, and 0.5-mL fractions were collected. (B) Gel filtration of beef heart cytochrome oxidase through Ultrogel 34, after previous equilibration in 0.5% (10 mM) lauryl maltoside. The same column was used as in (A), in 100 mM KCl, 0.1% (2 mM) lauryl maltoside, and 10 mM Tris-HCl, pH 7.8. Cytochrome oxidase (200 μL, 7.2 μM) was applied, and 0.5-mL fractions were collected. The column was calibrated as described under Experimental Procedures.

However, addition of lauryl maltoside to the assay medium caused immediate partial reactivation (TN = 114 s^{-1}).

After equilibration in 10 mM (0.5%) lauryl maltoside (Figure 7B), beef heart oxidase appeared as a single peak on Ultrogel 34 with a molecular of weight ~300000. Molecular activities of 325–475 s^{-1} were observed for this species.

Discussion

The studies reported in this paper on alkyl glycoside detergents indicate that octyl glucoside and lauryl maltoside are of great interest biochemically because of their purity, solubility, simplicity of structure, small uniform micelles, and dramatically different abilities to activate cytochrome *c* oxidase. These properties would be advantageous in the investigation of many membrane proteins. However, an improved synthesis was essential for exploiting their potential, since lauryl maltoside was not available commercially and octyl glucoside was available only at high cost.

The synthesis described above is simpler to perform and gives higher yields than those previously reported. The main reasons for this are the elimination of crystallization steps, the employment of silver carbonate plus iodine as a catalyst that gives the β anomer as the main product, and the use of a one-step purification on Dowex 1. Chromatography on Dowex 1 (hydroxide) is necessary to achieve the level of purity needed for the disaccharide detergents to be optimally soluble. In contrast to octyl glucoside, slight impurities in the disaccharide detergents were found to greatly decrease their solubility.

The α and β anomers of the alkyl glycosides are different in their detergent properties and solubility (Brown et al., 1970), and, therefore, the production of one anomer is important to obtain good yields and facilitate purification. Other procedures have been found to give a mixture of anomers (Brown et al., 1970; Weigel et al., 1979). The use of a trace of iodine with silver carbonate as catalyst appears to be important for preventing side reactions (Chiang et al., 1979) and favoring the β anomer.

Unexpectedly, the three lauryl disaccharide detergents, lauryl maltoside, lauryl lactoside, and lauryl cellobioside, had strikingly different properties, implicating the conformation of the sugar moiety as the critical factor in influencing the ability of the detergent to form micelles. Specifically, the β1–4 linkage in the disaccharides lactose and cellobiose appears to give a head-group conformation that lowers the solubility of the detergent, as discussed by Ferguson-Miller et al. (1980).

Characterization of the physical properties of solubilized membrane proteins is facilitated by the use of detergents with micelles of a uniform, small size (Tanford, 1972; Tanford & Reynolds, 1976). Lauryl maltoside and octyl glucoside should be valuable for such studies since they form monodisperse populations of micelles of approximately 50000 and 8000 molecular weight, respectively. These are unusually small micelles compared to most nonionic detergents (Helenius & Simons, 1975) and will be advantageous for gel filtration purification as well as for physical studies. The very small size of the octyl glucoside micelle may also account for its rapid removal by dialysis.

The observations reported here on the extraction of cytochrome oxidase and its kinetic behavior in alkyl glycoside detergents indicate that both octyl glucoside and lauryl maltoside are useful for purifying cytochrome oxidase, giving essentially 100% extraction of active oxidase, while lauryl maltoside may be the most valuable detergent for kinetic and physical studies on this enzyme. Octyl glucoside did not support activities comparable to those observed with other detergents in kinetic studies with the purified oxidases from beef or Neurospora. In contrast, purified oxidase equilibrated in lauryl maltoside gave turnover numbers two- to tenfold greater than in Tween-20 or octyl glucoside. Previous investigators have found that Tween-20 (or Emasol 1130) supports the highest oxidase activities of the commercially available detergents (Yu et al., 1975; Robinson & Capaldi, 1977). The fluidity of the hydrophobic phase provided by the detergents has been suggested as an important factor in influencing oxidase activity (Robinson & Capaldi, 1977). It is likely, however, that other factors contribute in this case, since lauryl maltoside has the same alkyl chain as Tween-20 [poly(oxyethylene glycol) sorbitan monolaurate] and yet gives significantly higher activity.

Most previous studies have based their activity comparisons on maximal activities of the oxidase or on pre-steady-state rates of electron transfer as a function of the detergent or phospholipid environment. Such activity measurements do not provide information concerning possible changes in the affinity of cytochrome *c* for cytochrome oxidase. Evidence relating to this question has been limited and conflicting (Brierley & Merola, 1962; Tzagoloff & MacLennan, 1965; Chuang & Crane, 1973). The steady-state kinetic studies presented in this paper show that the change in affinity for cytochrome *c* in response to the detergent environment is small in the initial high-affinity phase of the reaction, as revealed by little alteration in the apparent K_m in either the polarographic or spectral assay systems (Ferguson-Miller et al., 1976, 1978, 1979; Errede & Kamen, 1978). This result is in agreement with the original studies by Brierley & Merola (1962) and is also consistent with structural studies on oriented cytochrome oxidase membranes (Fuller et al., 1979) which indicate that the majority of the oxidase protein protrudes outside the bilayer.

It seemed likely that the lower activities observed in some of the detergents might simply reflect less effective dispersion of the enzyme with a resultant limited accessibility to the substrate, rather than inhibition of the intrinsic electron-transfer ability of the enzyme, as shown to occur with lipid-depleted oxidase (Yu et al., 1975). None of the oxidase

preparations studied were totally depleted of phospholipid, and therefore the detergents were presumably substituting only for the lipid bilayer or "boundary" lipids [Jost et al. (1973)] rather than tightly bound cardiolipin. [The lowest phospholipid content was about 0.05 mg/mg of protein in the enzyme prepared according to Hartzell & Beinert (1974).] Alternatively, it was possible that some detergents preferentially maintained the oxidase in a more active dimer form (280000 daltons; 4 hemes, 4 Cu) as suggested by Robinson & Capaldi (1977).

The results of the kinetic and gel filtration studies reported in this paper and elsewhere (Ferguson-Miller et al., 1980) demonstrate that a number of factors are involved in the effects of the alkyl glucoside detergents on cytochrome oxidase. After equilibration in octyl glucoside, the beef heart oxidase was found to exist as a mixture of polymer, dimer, and lower molecular weight forms. The polymer had lost the spectral characteristics of native oxidase, indicating true denaturation had occurred and accounting for part of the inactivating effect of octyl glucoside. The dimer, however, had normal spectral properties but very low activity. It could be reactivated by the addition of lauryl maltoside to the assay medium, indicating that the intrinsic electron-transfer ability of the dimer was inhibited by the hydrophobic environment provided by octyl glucoside. The lower molecular weight forms could not be activated by lauryl maltoside.

In contrast, the enzyme equilibrated in lauryl maltoside was found to exist as a monodisperse population with a molecular weight of 300000. The high activity of this form (TN = 475 s^{-1}) supports the concept that the dimer is the most active molecular species of cytochrome oxidase. The ability of lauryl maltoside to maintain the oxidase 100% in this form appears to account for part of its superior activating ability. The fluidity of the bilayer it produces may be another important contributing factor.

Acknowledgments

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References

Baron, C., & Thompson, T. E. (1975) *Biochim. Biophys. Acta 382*, 276.

Bates, F. J. (1942) *Polarimetry, Saccharimetry and the Sugars*, p 488, U.S. Government Printing Office, Washington, DC.

Brautigan, D. L., Ferguson-Miller, S., & Margoliash, E. (1979) *Methods Enzymol. 53D*, 128.

Brierley, G. P., & Merola, A. J. (1962) *Biochim. Biophys. Acta 64*, 205.

Brown, G. M., Dubreuil, F. M., Ichhaporia, F. M., & Desnoyers, J. E. (1970) *Can. J. Chem. 48*, 2525.

Chiang, C. K., McAndrew, M., & Barker, R. (1979) *Carbohydr. Res. 70*, 93.

Chuang, T. F., & Crane, F. L. (1973) *Biochim. Biophys. Acta 4*, 563.

De Grip, W. J., & Bovee-Geurts, P. H. M. (1979) *Chem. Phys. Lipids 23*, 321.

Dubois, M., Gilles, K., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956) *Anal. Chem. 28*, 350.

Errede, B., & Kamen, M. D. (1978) *Biochemistry 17*, 1015–1027.

Felgner, P. L., Messer, J. L., & Wilson, J. E. (1979) *J. Biol. Chem. 254*, 4946.

Ferguson-Miller, S., Brautigan, D. L., & Margoliash, E. (1976) *J. Biol. Chem. 251*, 1104–1115.

Ferguson-Miller, S., Brautigan, D. L., & Margoliash, E. (1978) *J. Biol. Chem. 253*, 149–159.

Ferguson-Miller, S., Weiss, H., Speck, S. H., Brautigan, D. L., Osheroff, N., & Margoliash, E. (1979) *Dev. Biochem. 5*, 281–292.

Ferguson-Miller, S., VanAken, T., & Rosevear, P. (1980) in *Symposium on Interaction Between Iron and Proteins in Oxygen and Electron Transport*, Elsevier, Amsterdam (in press).

Fuller, S. D., Capaldi, R. A., & Henderson, R. (1979) *J. Mol. Biol. 134*, 305.

Hartzell, C. R., & Beinert, H. (1974) *Biochim. Biophys. Acta. 368*, 318–338.

Helenius, A., & Simons, K. (1975) *Biochim. Biophys. Acta 415*, 29–79.

Hodge, J. E., & Hofreiter, B. T. (1962) *Methods Carbohydr. Chem. 1*, 388.

Johnson, D., & Lardy, H. A. (1967) *Methods Enzymol. 10*, 232.

Jost, P. C., Griffith, O. H., Capaldi, R. A., & Vanderkooi, G. (1973) *Biochim. Biophys. Acta 311*, 141.

Keana, J. F., & Roman, R. B. (1978) *Membr. Biochem. 1*, 323.

Knudsen, P., & Hubbell, W. L. (1975) *Membr. Biochem. 1*, 297.

Kuboyama, M., Yong, F. C., & King, T. E. (1972) *J. Biol. Chem. 247*, 6375.

Noller, C. R., & Rockwell, W. C. (1938) *J. Am. Chem. Soc. 60*, 2076.

Petri, W. A., & Wagner, R. R. (1979) *J. Biol. Chem. 254*, 4313–4316.

Robinson, N. C., & Capaldi, R. A. (1977) *Biochemistry 16*, 375.

Shinoda, K., Yamaguchi, T., & Hori, R. (1961) *Bull. Chem. Soc. Jpn. 34*, 237.

Smith, L., Davies, H. C., & Nava, M. E. (1979) *Biochemistry 18*, 3140.

Sottocasa, G. L., Kuylenstierna, B., Ernster, L., & Bergstrand, A. (1967) *Methods Enzymol. 10*, 448–463.

Stubbs, G. W., & Litman, B. J. (1978) *Biochemistry 17*, 215.

Tanford, C. (1972) *J. Mol. Biol. 67*, 59–74.

Tanford, C., & Reynolds, J. A. (1976) *Biochim. Biophys. Acta 457*, 133.

Tzagoloff, A., & MacLennan, D. H. (1965) *Biochim. Biophys. Acta 99*, 476.

VanAken, T., Thompson, D. A., & Ferguson-Miller, S. (1980) ASBC/BS Abstract No. 2397.

Walker, T. E., London, R. E., Whaley, T. W., Barker, R., & Matwiyoff, N. A. (1976) *J. Am. Chem. Soc. 98*, 5807.

Weigel, P. H., Nasi, M., Roseman, S., & Lee, Y. C. (1979) *Carbohydr. Res. 70*, 103.

Wittenberger, B., Raben, D., Lieberman, M. A., & Glazer, L. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5457.

Wolfram, M. L., & Lineback, D. R. (1963) *Methods Carbohydr. Chem. 2*, 342.

Yu, C. A., Yu, L., & King, T. E. (1975) *J. Biol. Chem. 250*, 1383.

Yu, C. A., Yu, L., King, T. C. (1979) *Dev. Biochem. 5*, 219.