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Influence of Detergent Polar and Apolar Structure upon the Temperature Dependence of Beef Heart Cytochrome *c* Oxidase Activity†

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ABSTRACT: The temperature dependence of lipid-depleted beef heart cytochrome *c* oxidase activity was studied in a series of chemically homogeneous detergents. The detergents that were tested included C_{10} to C_{18} maltosides, C_8 to C_{12} glucosides, C_8 to C_{16} Zwittergents, and C_{12} poly(oxyethylene) ethers. The observed rates of electron transport were dependent upon the structure of the polar head group and the length of the hydrocarbon tail. Of the detergents tested, the alkyl maltosides were the best in terms of both high rates of electron transport and superior enzyme stability. With the maltosides, changing the length of the alkyl tail affected the activity of cytochrome *c* oxidase in a manner quite similar to that reported with synthetic phosphatidylcholines and phosphatidylethanolamines [Vik, S. B., & Capaldi, R. A. (1977) *Biochemistry 16*, 5755–5759], suggesting that the alkyl maltosides can mimic some of the features of the membrane environment. In each of the detergents, the activation enthalpy (determined from the slope of an Arrhenius plot) was nearly identical, suggesting that the same electron-transfer step within cytochrome *c* oxidase is rate limiting. This result has been interpreted as evidence for the existence of two or more conformers of cytochrome *c* oxidase, one of which is significantly more active than the other(s). The enzyme turnover number, which changes by 2 orders of magnitude depending upon the structure of the bound detergent, may reflect the ability of each detergent to alter the equilibrium between the active and nearly inactive conformers.

The electron-transport activity of cytochrome *c* oxidase is known to be influenced by either the phospholipid or detergent environment that surrounds its two hydrophobic, intramembrane domains. Reconstituting the purified enzyme into a variety of phosphatidylcholines and phosphatidylethanolamines has shown that the fatty acid composition of the boundary layer phospholipids affects the enzymatic turnover number more so than does the nature of the polar head group (Vik & Capaldi, 1977). Limited activity measurements using the detergent-solubilized enzyme, i.e., enzyme solubilized in either lysophosphatidylcholines or in Tween detergents, have shown a similar but less pronounced dependence of the enzymatic activity upon the structure of the detergent's hydrocarbon tail and polar head group (Vik & Capaldi, 1977; Robinson & Capaldi, 1977). Unfortunately, extension of this type of study to other classes of detergents in order to more fully understand the structural requirements at the protein–amphiphile interface has been difficult since most commercially available detergents, including the Tween detergents used in the studies mentioned above, are heterogeneous mixtures and contain significant amounts of inherent impurities, especially peroxides and free radicals. Therefore, it is almost impossible to determine whether the enzymatic rate changes that are observed in each detergent are caused by important structural differences in the detergents or are the result of inactivation of cytochrome *c* oxidase by the different amounts or types of impurities that are present in the detergents.

In the past several years, a variety of homogeneous, structurally defined detergents have become available that potentially could be used to assess the structure–function relationships at the hydrophobic surface of cytochrome *c* oxidase. Some of the most promising classes of highly purified detergents are the alkyl glycosides, Zwittergents,[1] and homogeneous

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[1] Abbreviations: Tris-HCl, tris(hydroxymethyl)aminomethane base titrated to the appropriate pH with HCl; EDTA, ethylenediaminetetraacetic acid; cmc, critical micelle concentration; $C_{12}E_8$, octylethylene glycol dodecyl ether; $C_{12}E_9$, nonaethylene glycol dodecyl ether; octyl glucoside, octyl β-D-glucopyranoside; decyl glucoside, decyl β-D-glucopyranoside; lauryl glucoside, dodecyl β-D-glucopyranoside; decyl maltoside, decyl β-D-maltopyranoside; lauryl maltoside, dodecyl β-D-maltopyranoside; myristyl maltoside, tetradecyl β-D-maltopyranoside; cetyl maltoside, hexadecyl β-D-maltopyranoside; stearyl maltoside, octadecyl β-D-maltopyranoside; Zwittergent 3-10, 3-(*N*-decyl-*N*,*N*-dimethylammonio)-1-propanesulfonate; Zwittergent 3-12, 3-(*N*-dodecyl-*N*,*N*-dimethylammonio)-1-propanesulfonate; Zwittergent 3-14, 3-(*N*-tetradecyl-*N*,*N*-dimethylammonio)-1-propanesulfonate; Zwittergent 3-16, 3-(*N*-hexadecyl-*N*,*N*-dimethylammonio)-1-propanesulfonate; CL, cardiolipin.

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poly(oxyethylene) alkyl ethers. Rosevear et al. (1980) have shown that one of the alkyl glycosides, lauryl maltoside, is especially effective for solubilizing cytochrome *c* oxidase and achieving high levels of electron-transport activity. Other alkyl glycosides that they synthesized, lauryl lactoside and lauryl cellobioside, were not useful because of their high critical micelle temperatures; octyl glucoside resulted in low enzymatic activity and irreversible inactivation of cytochrome *c* oxidase. The procedures that have been used to synthesize octyl glucoside and lauryl maltoside, however, provide the possibility of synthesizing a variety of other homogeneous glucosides and maltosides containing different alkyl chains. Because these detergents would be chemically well defined, would have a high degree of purity, and would not spontaneously produce peroxides like many other neutral detergents, e.g., the poly(oxyethylene) alkyl ethers, these detergents would be good candidates for studies of the amphiphile specificity of cytochrome *c* oxidase.

In this paper, we report the synthesis of five alkyl maltosides that contain saturated, even-numbered hydrocarbon chains (C_{10} to C_{18}) and two alkyl glucosides C_{10} and C_{12}. The activity of fully lipid-depleted cytochrome *c* oxidase and its temperature dependence in each of these detergents together with identical studies in several other highly purified commercially available detergents, i.e., the Zwittergents, poly(oxyethylene) alkyl ethers, and octyl glucoside, are the subjects of this paper.

Experimental Procedures

Materials. Triton X-100 was purchased from Research Products International; octyl and decyl glucosides, lauryl maltoside, octaethylene glycol dodecyl ether ($C_{12}E_8$), nonaethylene glycol dodecyl ether ($C_{12}E_9$), and the Zwittergents 3-08, 3-10, 3-12, 3-14, and 3-16 were from Calbiochem-Behring; cytochrome *c* (type III) was from Sigma Chemical Co. All other chemicals were reagent grade.

Beef heart cytochrome *c* oxidase was isolated by the method of Fowler et al. (1962) as modified by Capaldi & Hayashi (1972) from Keilin-Hartree heart muscle particles that had been prepared according to Yonetani (1960). After the final ammonium sulfate precipitation, the cytochrome *c* oxidase precipitate was dissolved in 0.02 M Tris-HCl buffer at pH 8 containing 0.09 M NaCl, 1 mM EDTA, and 15 mM Triton X-100 and stored until used in liquid N_2. The isolated complex contained 9.2–10.0 nmol of heme A/mg of protein, on the basis of $\Delta\epsilon_{605nm-630nm}$ (reduced) = 16.5 mM^{-1} cm^{-1} (Griffiths & Wharton, 1961) and protein determination according to Gornall et al. (1949), and had a molecular activity of 300 and 320 s^{-1} when assayed in 0.025 M phosphate buffer at pH 7.0 containing 0.5% Tween 80 or 10 mM lauryl maltoside, respectively.

Methods. Cytochrome *c* oxidase and Triton X-100 concentrations were measured spectrophotometrically with $\epsilon_{422} = 1.54 \times 10^5$ M^{-1} cm^{-1} (van Gelder, 1978) and $\epsilon_{277} = 1.50 \times 10^3$ (Robinson & Tanford, 1975), respectively. Triton X-100 concentrations in solutions that also contained cytochrome *c* oxidase were calculated after correcting for the protein absorbance at 277 nm by using the measured A_{422} and $A_{277}/A_{422} = 2.40$ for cytochrome *c* oxidase in the absence of Triton X-100. The concentrations of glucosides and maltosides were determined by the anthrone assay for glucose (Seifter et al., 1950).

Delipidation of Cytochrome c Oxidase. Glycerol gradient delipidation of cytochrome *c* oxidase by Triton X-100 was performed as previously described (Robinson et al., 1980) with the exception that 0.2 M Tris-HCl buffer at pH 8.0 containing 0.1 mM EDTA rather than the 0.02 M Tris-HCl buffer was used during the 5% Triton X-100 incubation and centrifugation. With this modified buffer system, a single glycerol gradient centrifugation was sufficient to remove all but the three tightly bound cardiolipins. The phospholipid content and subunit composition of the delipidated cytochrome *c* oxidase preparations were performed as previously described (Robinson, 1982).

Spectrophotometric Enzymatic Activity Measurements. Determination of the cytochrome *c* oxidase activity was performed as described previously (Robinson, 1982) with the exception that the data collection and calculation of molecular activities were done automatically by an Apple II Plus microcomputer that was connected via an Adalab A/D converter (Interactive Microware) to the analog output of the Zeiss PM6K spectrophotometer. This assay procedure involves a 20-fold dilution of the cytochrome *c* oxidase from the 0.8 mM Triton X-100 solution at pH 8 into a solution at pH 8 containing 0.02 M tris, 0.09 M NaCl, and 10 mM of the detergent of choice. After at least a 10-min incubation at 0 °C, 5 μL of this solution was used for the spectrophotometric assay, which was performed in 0.7 mL of 0.025 M phosphate buffer at pH 7.0 that contained 10 mM of the detergent as micelles; i.e., total concentration = cmc + 10 mM. During the 30-s assay, the cuvette holder was maintained at the appropriate temperature with a Zeiss piezoelectric temperature controller that directly reads the temperature of the cuvette holder; the solution temperature in the cuvette was directly measured with a thermistor probe connected to a Markson Model 94 temperature meter, and enzymatic assays were not begun until temperature equilibration had occurred. To minimize this equilibrium time, the assay buffer was maintained at the assay temperature with a temperature-controlled water bath. Dry air was blown through the cuvette chamber to minimize condensation on the cuvette during assays performed below 15 °C.

Synthesis of Alkyl Maltosides. Decyl, lauryl, myristyl, cetyl, and stearyl maltosides were synthesized from heptaacetobromomaltose and the corresponding alcohol as described by Rosevear et al. (1980) with the following modifications: (1) freshly prepared Ag_2O was used as the catalyst (de Grip et al., 1979); (2) 0.1 M NaCl was added to the deacetylation mixture to precipitate any dissolved Ag(I) that was still present; (3) after deacetylation, excess triethylamine was converted into the chloride salt and removed by phase separation with chloroform–methanol–water (10:3:5); (4) final purification was performed on a 0.9 × 60 cm silicic acid, HPLC column filled with Sigma Sil-LC (<325 mesh), a CC-4 silicic acid precolumn, and a chloroform–methanol gradient (100:0 to 70:30 v/v). The synthesized detergents were judged homogeneous by the thin-layer chromatography systems described by Rosevear et al. (1980).

Electrophoretic Exchange and Removal of Detergents. Excess Triton X-100 that was present after the glycerol gradient delipidation of cytochrome *c* oxidase was removed or exchanged for another neutral detergent, e.g., lauryl maltoside, in an ISCO electrophoretic concentrator. A 2–3-mL sample containing 1–2-mg of cytochrome *c* oxidase/mL, 20% glycerol, and 15 mM Triton X-100 was placed in the cathode chamber of the concentrator, and pH 8.9 buffer containing 25 mM Tris base (titrated to pH 8.9 with HCl) and either 0.8 mM Triton X-100 or 10 mM lauryl maltoside was used to fill the remainder of the electrophoresis cell. The 25 mM Tris pH 8.9 buffer without detergent was used to fill the large buffer chamber. After electrophoresis at 5 °C for 2.75 h, the cytochrome *c* oxidase was concentrated against the dialysis mem-

brane covering the anode chamber. The 2-3 mL at the bottom of the anode chamber that contained the cytochrome *c* oxidase in either 0.8 mM Triton X-100 or 10 mM lauryl maltoside were carefully removed with a transfer pipet after reversing the current for 60 s to move the protein slightly away from the dialysis membrane. The protein solution that was now in either 0.8 mM Triton X-100 or 10 mM lauryl maltoside was stored frozen in $N_2(l)$ after adjustment of the pH to 8.0. Recovery of protein during the electrophoretic exchange of detergents was 70–95%.

RESULTS

Two factors must be controlled before the dependence of cytochrome *c* oxidase activity upon the structure of boundary layer amphiphiles can be adequately determined. First, the influence of the endogenous bound phospholipid must be minimized, and second, the aggregation state of the solubilized complex in each of the detergents to be tested must be as similar as possible.

To eliminate the effects of endogenous phospholipid, cytochrome *c* oxidase was delipidated of all phospholipids except for the very tightly bound cardiolipin by glycerol gradient centrifugation at pH 8.0 in 15 mM Triton X-100 as described under Methods. Enzyme delipidated in this manner contained only 1–1.5 molecules of CL per complex, thereby, eliminating the contribution of endogenous phospholipid upon the function and the structural stability of the complex. An added advantage of the Triton X-100 delipidation procedure was the resulting molecular homogeneity of the cytochrome *c* oxidase. As we have previously shown by sedimentation equilibrium in H_2O/D_2O, this type of delipidated complex is a heme aa_3 monomer having a protein molecular weight of 200 000 (Robinson & Talbert, 1985). The only disadvantage of using this delipidated preparation of cytochrome *c* oxidase for systematically studying the influence of detergents on the enzymatic activity is the high concentration of Triton X-100 that is present in the delipidated sample, e.g., 10 mg of Triton X-100/mL.

Electrophoretic Removal of Excess Triton X-100. To minimize the influence of Triton X-100 on subsequent experiments, the complex was electrophoretically transferred from the 15 mM Triton X-100 solution into a solution containing the minimum amount of Triton X-100 that is necessary for maintaining complete enzyme solubility, i.e., 0.8 mM Triton X-100. This was accomplished with an ISCO electrophoretic concentrator as described under Methods. Subsequent activity measurements on this delipidated enzyme could be made in another detergent without significant contamination by Triton X-100 since the spectrophotometric electron-transport assay involved a 3000-fold dilution into the second detergent. By dilution and performance of the assay in 10 mM of the second detergent, 15 000 times as much of the second detergent was present in the assay cuvette as Triton X-100; therefore, on the average only 1% of the enzyme molecules had a Triton X-100 molecule bound at their surface while 99% of the enzyme was completely free of Triton X-100. The validity of using this low Triton X-100 sample for investigating the effects of other detergents was verified by electrophoresing the delipidated cytochrome *c* oxidase from the 15 mM Triton X-100 into 10 mM lauryl maltoside. In this case, 98–99% of the Triton X-100 was removed during the electrophoresis. The enzymatic activity of this enzyme sample after the additional 3000-fold dilution into lauryl maltoside was found to be identical with the activity measured with the 0.8 mM Triton X-100 sample that had been diluted into lauryl maltoside.

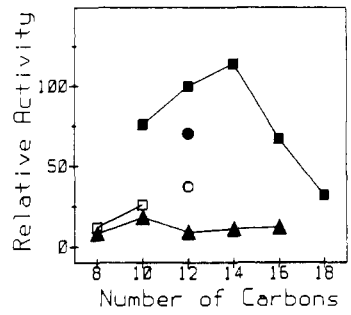

FIGURE 1: Effect of detergent structure upon cytochrome *c* oxidase electron-transport activity at pH 7.0 and 25 °C. All activities are expressed as the percent of the activity observed in 10 mM lauryl maltoside, which was 210 s^{-1}. The number of carbons labeling the abscissa refers to the number of carbons in the alkyl hydrocarbon tail of each detergent. The activities in each detergent are represented by the following symbols: filled squares, alkyl maltosides; open squares, alkyl glucosides; filled circle, $C_{12}E_9$; open circle, $C_{12}E_8$; filled triangles, Zwittergent detergents.

Effect of Detergent Structure upon Activity at 25 °C. The electron-transport activity of cytochrome *c* oxidase in a variety of homogeneous detergents is compared in Figure 1. Two major conclusions can be reached. First, it is apparent that the nature of the polar head group is very important in determining the rate of electron transport through cytochrome *c* oxidase. As can be seen in Figure 1, with any given length of hydrocarbon tail, the activity was the greatest in the presence of the maltoside detergents, e.g., the activity in the maltosides was 5–10 times greater than the activity in the corresponding Zwittergents. Small changes in the polar head group also greatly affected the activity of the complex; e.g., decreasing the number of poly(oxyethylene) groups from nine to eight decreased the activity approximately 2-fold if both detergents had 12 carbon tails; removing a glucosyl moiety from the polar head group of decyl maltoside decreased the activity by a factor of 3. It is possible that the difference in activity of cytochrome *c* oxidase in $C_{12}E_8$ and $C_{12}E_9$ is not entirely due to the slight alteration in the polar head group, but rather reflects the presence of chemical impurities in one of the poly(oxyethylene) lauryl ethers since the source of these detergents did affect the results that were obtained (50% less enzymatic activity was observed if $C_{12}E_9$ was purchased from Sigma Chemical Co. rather than Calbiochem-Behring; 10% higher activity was found if the $C_{12}E_8$ was obtained from K. K. Koh-yo Trading Co in Japan rather than Calbiochem-Behring).

The second major conclusion that can be reached from the data in Figure 1 is that changing the length of the hydrocarbon tail of a detergent also affects the turnover number of cytochrome *c* oxidase. For example, with the maltosides, altering the number of carbon atoms in the alkyl tail caused the activity at 25 °C to change by about a factor of 2.[2] The length of the alkyl chain also had a slight effect on the activity in the Zwittergent detergents, but the effect was not as great as that observed with the maltosides. As described later, cytochrome *c* oxidase was much less stable in the Zwittergents than in the maltosides, and detergent-induced denaturation may complicate the interpretation of the Zwittergent data. The two results obtained with the glucoside detergents also suggest that cytochrome *c* oxidase activity may be dependent upon the length of the alkyl chain; however, the experiments in the

[2] C_{18} maltoside decreased the activity of cytochrome *c* oxidase 4-fold as compared with the activity in myristyl maltoside, but this value may be misleading since the comparison was at 42 °C rather than at 25 °C due to the insolubility of stearyl maltoside below 42 °C.

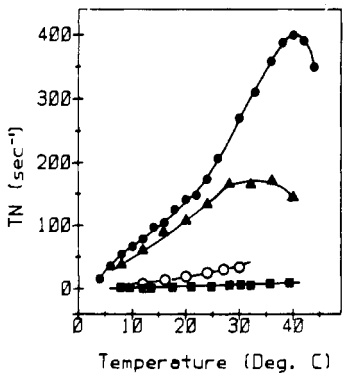

FIGURE 2: Temperature dependence of the turnover number (TN) of cytochrome *c* oxidase in pH 7.0 buffer containing lauryl maltoside (filled circles), $C_{12}E_9$ (filled triangles), decyl glucoside (open circles), and Triton X-100 (filled squares).

glucosides were hampered by detergent-solubility problems.[3] Due to the complications in both the glucosides and Zwittergents and the lack of another detergent series, it is difficult to determine if the dependence of cytochrome *c* oxidase activity upon alkyl hydrocarbon length that is seen in the maltosides is a general result or something that is specific for this one detergent class.

Effect of Detergent Structure upon the Temperature Dependence of Cytochrome c Oxidase Activity. The electron-transport activity of cytochrome *c* oxidase was measured as a function of temperature in several detergents, e.g., Triton X-100, as well as several of the glucoside, maltoside, Zwittergent, and poly(oxyethylene) detergents, in order to determine whether the detergent environment at the apolar surface of the protein influenced the temperature dependene of either the catalytic rate or the protein structure. The activity of cytochrome *c* oxidase as a function of temperature in the presence of four detergents that had shown very different steady-state rates of electron transport at 25 °C is shown in Figure 2. At each temperature the turnover number of cytochrome *c* oxidase was the highest when lauryl maltoside was bound at the apolar surface, was significantly decreased with decyl glucoside at the surface, and was nearly zero when Triton X-100 was bound to the protein. In the poly(oxyethylene) alkyl detergent $C_{12}E_9$, the behavior was more complicated since the activity was only slightly lower in this detergent than in lauryl maltoside at temperatures lower than 25 °C but was significantly lower at temperatures above 25 °C. Apparently, temperature-induced denaturation of cytochrome *c* oxidase begins to occur at a much lower temperature with $C_{12}E_9$ bound to the apolar surface than with lauryl maltoside bound to this surface.

The very different rates of electron transport in the various detergent environments reflect differences in the turnover number of cytochrome *c* oxidase, not altered cytochrome *c* binding since saturating concentrations of cytochrome *c* were used in each assay, i.e., [cyt *c*] > 30 μM. Saturation by cytochrome *c* was confirmed by decreasing the cytochrome *c* concentration to 5 μM, which caused less than a 10% reduction in the rate. The different enzymatic turnover numbers in each detergent, therefore, must reflect changes in the rate-limiting step of cytochrome *c* oxidase, i.e., the transfer of electrons from the heme *a*, Cu_A center to the heme a_3, Cu_B center (Chance, 1981; Brunori & Wilson, 1982).

[3] The longer chain glucosides were found to be very insoluble; decyl and lauryl glucosides had maximum solubilities at 25 °C of only 3 and 0.2 mM, respectively. These values are each very close to the theoretical cmc values, which means that micelles are not present at 25 °C. Activity measurements in decyl glucoside were performed at the saturating concentration of 3 mM, but measurements in the other long-chain glucosides were impossible. Because of the low concentration of decyl glucoside in this experiment, the result may not be experimentally comparable to the other data.

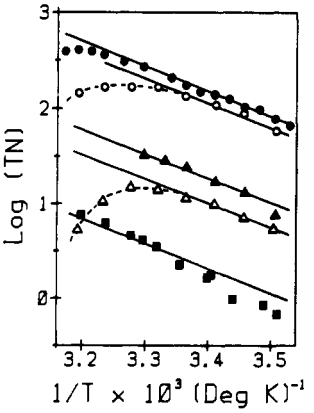

FIGURE 3: Arrhenius plot of the turnover number (TN) of cytochrome *c* oxidase in pH 7.0 buffer containing lauryl maltoside (filled circles), $C_{12}E_9$ (open circles), decyl glucoside (filled triangles), Zwittergent 3-12 (open triangles), and Triton X-100 (filled squares). The solid line through the lauryl maltoside data is the best linear fit to the data measured below 33 °C; the other four lines are lines drawn parallel to this line through the lauryl maltoside data for comparison purposes. It should be noted that the turnover numbers for cytochrome *c* oxidase that were measured in Triton X-100 at low temperatures were very low, i.e., less than 1 s^{-1}, and are within experimental error of the line drawn parallel to the lauryl maltoside data.

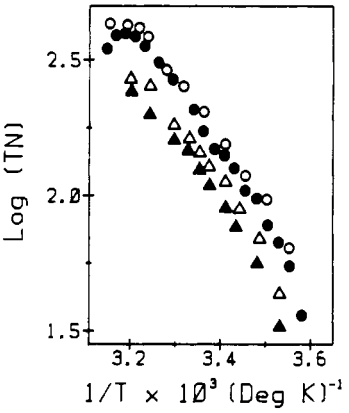

FIGURE 4: Arrhenius plot of the turnover number (TN) of cytochrome *c* oxidase in a series of alkyl maltosides. The detergents used were decyl maltoside (open triangles), lauryl maltoside (filled circles), myristyl maltoside (open circles), and palmityl maltoside (filled triangles).

The manner by which the detergent environment affects the rate-limiting step of cytochrome *c* oxidase is clarified if the data are compared with an Arrhenius plot that permits the comparison of activities that differ by several orders of magnitude. The data collected in all of the detergents having a 12-carbon alkyl tail together with the data in decyl glucoside and Triton X-100 are summarized in Figure 3. A similar comparison of the rate of electron transfer of cytochrome *c* oxidase in each of the maltosides is shown in Figure 4. Surprisingly, the temperature dependence of the rate-limiting step of cytochrome *c* oxidase, i.e., the activation enthalpy, is approximately the same in each detergent as is evident by the nearly identical slopes for each set of data prior to the enzyme denaturation at higher temperatures (the slope corresponds to a ΔH_{act} = 11 kcal/mol). Even in Triton X-100, a detergent in which cytochrome *c* oxidase has less than 1% of the electron-transport activity that it has in lauryl maltoside, the

response to increasing temperature is nearly identical. Apparently, the enthalpy changes involved in the rate-limiting step of electron transfer are independent of the nature of the bound detergent even though the maximum rate may differ by several orders of magnitude. Therefore, the rate-limiting step in each detergent is probably identical, and either the detergent environment must not affect the rate limiting step, or it must only affect the entropy of the rate-limiting step via a change in the protein conformation or ordering of the solutes, i.e., detergent or water molecules. One of the most reasonable explanations of these large changes in turnover number would be that of the number of active cytochrome *c* oxidase molecules in each detergent solution is a function of the bound detergents, i.e., the detergent environment alters the protein conformation.

In each of the detergents, the activation enthalpy did exhibit a slight temperature dependence throughout the temperature range that was tested, but in each case the small amount of curvature in the Arrhenius plots was nearly identical. The only exceptions were with Zwittergent 3-12 and $C_{12}E_8$ in which the temperature-induced inactivation of cytochrome *c* oxidase began to occur as low as 25 °C. Because the small temperature-dependent changes in the activation enthalpy were nearly identical in each detergent and since detergents would not be expected to exhibit phase transitions in this temperature range, these changes must reflect temperature-induced changes in the protein structure that can alter the rate of electron transport not temperature-induced changes in the hydrocarbon environment that indirectly affect the enzymatic rate.

In each detergent, large decreases in activity were observed at temperatures high enough to cause thermal inactivation of the enzyme; however, the temperature at which this inactivation occurs was dependent upon the detergent environment. In lauryl maltoside inactivation did not begin to occur until ca. 40 °C while in Zwittergent 3-12 and $C_{12}E_8$ the inactivation was evident in the assays performed as low as 25 °C. Since little deviation in the first-order rate constant was observed during the 30-s activity assay, the inactivation probably occurred during the incubation with detergent at 0 °C prior to the assay. This was confirmed by following the rate of inactivation of cytochrome *c* oxidase during incubation with Zwittergent 3-12 at 0, 25, and 37 °C as compared with the rate of its inactivation in lauryl maltoside or Triton X-100. Cytochrome *c* oxidase was irreversibly inactivated 20–50 times faster in Zwittergent 3-12 than in the other two detergents. A similar irreversible inactivation of cytochrome *c* oxidase by octyl glucoside was also observed. Additional evidence for the instability of cytochrome *c* oxidase in these detergents was also obtained spectrally. Incubation of cytochrome *c* oxidase in Zwittergent 3-12 resulted in spectral shifts in the oxidized Soret band (A_{max} = 416 nm in Zwittergent 3-12; A_{max} = 421 nm in lauryl maltoside) as well as an inability to be completely reduced by dithionite. Obviously, the detergent environment surrounding cytochrome *c* oxidase is not identical in each of the protein–detergent complexes, and the molecular structure of the bound detergent is important not only in determining the maximum rate of electron transport between cytochrome *c* and oxygen but also in influencing the conformational stability of the protein.

Discussion

The enzymatic turnover number and the temperature dependence of the electron-transport rate of cytochrome *c* oxidase were measured in a series of homogeneous, chemically pure detergents in order to determine which detergents, or class of detergents, most closely mimic the native inner mitochondrial membrane environment of the enzyme. The conclusions that can be reached from these studies can be divided into three main areas: (1) the structural requirements of detergents bound to the surface of cytochrome *c* oxidase that are essential for maximum enzymatic activity; (2) the temperature dependence of the electron-transfer reactions within cytochrome *c* oxidase; (3) the mechanisms by which the rate of electron transfer can be altered by 2 orders of magnitude depending upon the structure of the detergent bound to the apolar protein surface.

(1) Structural Requirements of Bound Detergents Essential for Maximum Activity. (a) Effect of Altering the Detergent's Polar Head Group upon Activity. Altering the polar head group of the detergent bound to cytochrome *c* oxidase was found to have more influence over the turnover number of the enzyme than changes in the length of the alkyl chain. For example, the enzymatic activity in detergents having a 12-carbon hydrocarbon tail was found to vary as much as 10-fold depending upon the polar head group that was used but only a maximum of a factor of 2 as the chain was varied from 10 to 16 carbons (refer to Figure 1). Of all the detergents that were tested, the maltosides were clearly superior. Not only were the highest enzymatic turnover numbers achieved in the maltosides, but the enzymatic inactivation that occurred in the glucosides, Zwittergents, and poly(oxyethylene) alkyl ethers was not found with the maltosides. The only other detergent in which cytochrome *c* oxidase had a similar long-term stability was Triton X-100; however, the enzymatic activity in Triton X-100 was less than 1% of what it was in lauryl or myristyl maltoside.

The importance of the polar head group for maintaining maximum activity was also evident from the rather large alteration in the rate of electron transport that resulted from relatively small changes in the polar head group structure; e.g., addition of an oxyethylene group to $C_{12}E_8$ increased the enzymatic activity 2-fold; removal of a glucosyl moiety from decyl maltoside decreased the activity 3-fold. In the latter case, the effect may have been caused by the altered detergent properties since the removal of this one glucosyl moiety also prevented the formation of micelles at 25 °C. The lack of decyl glucoside micelles probably prevented binding of the detergent to cytochrome *c* oxidase and, therefore, interfered with the activity measurements that were performed in the saturated detergent solution. A similar, but more serious solubility problem was encountered after removal of a glycosyl moiety from lauryl maltoside that prevented activity measurements from even being performed in this detergent.

The conclusion that the maltosides are the best choice for solubilizing and maintaining high levels of electron-transport activity supports the earlier work of Rosevear et al. (1980), who originated the use of lauryl maltoside with cytochrome *c* oxidase. On the basis of the solubility properties of several alkyl glycosides and the cytochrome *c* oxidase kinetics in octyl glucoside and lauryl maltoside, they had concluded that lauryl maltoside was a better detergent to use with cytochrome *c* oxidase than octyl glucoside, octyl or lauryl lactoside, lauryl cellobioside, Tween 20, or Triton X-100. We can now extend their conclusions to add decyl, myristyl, and palmityl maltosides as other excellent detergents for use with cytochrome *c* oxidase and add several glucosides and Zwittergents as poor choices ($C_{12}E_8$ and $C_{12}E_9$ may also be useful if they are used below 20 °C).

(b) Effect of Detergent's Alkyl Chain Length upon Activity. Within at least one class of detergents, the maltosides, the length of the alkyl chain also affects the turnover number of cytochrome *c* oxidase. Whether this phenomenon is general

is difficult to decide since the only other class of detergents that was available as an entire series having different lengths of alkyl chains was the Zwittergent series, which also acted as cytochrome *c* oxidase denaturants.[4] Interestingly, the effect of alkyl chain length upon the activity of cytochrome *c* oxidase in the maltosides is quite similar to the results Vik & Capaldi (1977) obtained with the phosphatidylcholines and phosphatidylethanolamines. With either the maltosides or the phospholipids, higher rates of electron transport occurred with the amphiphiles containing myristyl (myristoyl) hydrocarbon chains than with amphiphiles containing lauryl (lauroyl), palmityl (palmitoyl), or stearyl (stearoyl) hydrocarbon chains. Therefore, not only are the maltosides the most useful detergents for producing the highest turnover number for cytochrome *c* oxidase, but structural alterations in the maltosides affect the activity in a manner similar to phospholipids. At this time, the maltosides appear to be the best choice for detergents that are able to mimic the native lipid environment of the mitochondrial inner membrane.

(*2*) *Temperature Dependence of Cytochrome c Oxidase Activity.* The temperature dependence of cytochrome *c* oxidase catalyzed electron transport in each detergent that we tested agrees quite well with the previous studies that have been performed in phospholipid, detergent, or membrane environments, e.g., Smith & Newton (1968), Raison et al. (1971), Erecinska & Chance (1972), Denes & Stanacev (1979), Yoshida et al. (1979), Vanderkooi & Chazotte (1982), and De Cuyper & Joniau (1980). The largest apparent discrepancy between the present data and those that had been obtained earlier is the absence of a clear break in any of the Arrhenius plots of our data between 20 and 25 °C (refer to Figures 3 and 4) although some previous data also lacked a clear break (Denes & Stanacev, 1979; Yohsida et al., 1979; Vanderkooi & Chazotte, 1982). In each of our detergent systems, some curvature in the Arrhenius plot was evident, and two lines could have been drawn through the data that intersected between 20 and 25 °C. We chose not to interpret the data in this manner since there were not two clear linear portions with any of the data. Nevertheless, our data are very similar to those previously reported in other systems and fit reasonably well the two lines that have been previously drawn thorough other data. Therefore, measurements that we have made in the complete absence of any boundary layer lipid behave the same with respect to their temperature dependence as cytochrome *c* oxidase in the inner mitochondrial membrane or phospholipid vesicles. Most likely, the curvature in the Arrhenius plot with increasing temperature is the result of protein conformational alterations rather than phase transitions in the phospholipid or detergent environment since alkyl detergents with up to 16 carbons are generally thought to form micelles with a fluid hydrocarbon interior at room temperature (Tanford, 1980). A similar conclusion that temperature-induced conformational changes in the protein explain the temperature dependence of cytochrome *c* oxidase activity has been reached previously by both Denes & Stanacev (1979) and Yoshida et al. (1979).

(*3*) *Mechanisms by Which Bound Detergent Could Influence Electron-Transfer Activity.* The most surprising aspect of the temperature dependence of cytochrome *c* oxidase activity was the nearly identical response of the activity to increasing temperature in each of the detergent systems tested, i.e., the very similar activation enthalpies. Even though the steady-state rate increased more than 2 orders of magnitude as the bound detergent was changed from Triton X-100 to lauryl maltoside, the Arrhenius plot had nearly an identical slope in these two detergents. Replacement of Triton X-100 by other detergents, e.g., Zwittergent 3-12, decyl glucoside, or $C_{12}E_8$, led to intermediate activities, which once again had nearly identical slopes in the Arrhenius plot. As mentioned under Results, this finding is most consistent with the bound detergent affecting the percentage of enzyme in a fully active conformation. The idea of the protein conformation affecting the electron-transfer reactions between the metal centers in cytochrome *c* oxidase is not a new concept but was proposed by Antonini et al. (1977) to explain anomalies in the transient kinetics, i.e., the "resting" and "pulsed" conformations. Conformational flexibility of cytochrome *c* oxidase has also been detected by a variety of physical and optical methods (Kornblatt et al., 1975; Kawato et al., 1980; Brudvig et al., 1981).

The detergent environment could potentially affect the maximum rate of electron-transport activity as detected by the spectrophotometric assay procedure by mechanisms other than alterations in the internal electron-transfer reactions of cytochrome *c* oxidase. For example, if either the association of reduced cytochrome *c* or the release of oxidized cytochrome *c* were impaired, decreased enzymatic rates would be observed. Alternatively, self-association of monomeric cytochrome *c* oxidase may be favored in some detergents that could possibly affect the rate of electron transfer. We do not believe that any of these possibilities explain the dependence of cytochrome *c* oxidase activity upon its detergent environment because (1) the observed rates were not significantly decreased by a 10-fold decrease in the cytochrome *c* concentration, which suggests that impaired cytochrome *c* binding is not the reason for the lower activities in some detergents, (2) the observed rate of cytochrome *c* oxidation was first order in the presence of each detergent, which indicates that release of oxidized cytochrome *c* must not be the rate-limiting step as suggested by Sinjorgo et al. (1984),[5] and (3) cytochrome *c* oxidase was almost certainly monomeric during the enzyme assay since we have shown that the Triton X-100 enzyme preparation that was used for these studies was monomeric prior to its dilution into each detergent (Robinson & Talbert, 1985) while Suarez et al. (1984) have shown that the lauryl maltoside solubilized enzyme is also monomeric, ruling out self-association as an explanation of altered electron transport. We have, therefore, concluded that changes in the electron-transfer reactions within cytochrome *c* oxidase rather than these other explanations must be the mechanism by which the detergent environment must influence the overall rate of cytochrome *c* oxidation.

A possible mechanism by which the detergent environment could alter the internal electron-transfer reactions within cytochrome *c* oxidase would be via conformational flexibility of the protein, i.e., the structure of the bound detergent would restrict the percentage of cytochrome *c* oxidase that could switch from the resting to the pulsed conformation during the enzyme assay. Those detergents in which the enzyme has very low activity, e.g., Triton X-100, may stabilize the resting conformation and, thereby, cause very low enzymatic activity while those detergents that support high activity, e.g., lauryl or myristyl maltoside, favor the pulsed conformation. This

[4] The short-chain glucosides are also commercially available, but their high monomer solubility makes it difficult to use them as protein-solubilizing detergents.

[5] If the release of oxidized cytochrome *c* were rate limiting, the overall reaction would be zero order rather than first order with respect to reduced cytochrome *c* since the reaction would proceed in a fashion much like an active site titration of a hydrolytic enzyme, e.g., Bender et al. (1966).

would be consistent with the scheme described by Brunori & Wilson (1982) in which the conversion from the resting to pulsed conformation increases the first-order rate constant for the reduction of heme a_3 from 0.5 s^{-1}, a value similar to the turnover number we measure in Triton X-100, to 7000 s^{-1}. Because similar alterations in the turnover number of cytochrome *c* oxidase have been observed as the phospholipid environment is altered or as local anesthetics are added to membrane preparations, these systems may also affect the equilibrium between the resting and pulsed conformations of cytochrome *c* oxidase, but further experimental data are required before this proposal can be any more than a working hypothesis.

Registry No. $C_{12}E_8$, 3055-98-9; $C_{12}E_9$, 3055-99-0; zwittergent 3-08, 15178-76-4; zwittergent 3-10, 15163-36-7; zwittergent 3-12, 14933-08-5; zwittergent 3-14, 14933-09-6; zwittergent 3-16, 2281-11-0; octyl glucoside, 29836-26-8; decyl glucoside, 58846-77-8; lauryl glucoside, 59122-55-3; decyl maltoside, 82494-09-5; lauryl maltoside, 69227-93-6; myristyl maltoside, 18449-82-6; cetyl maltoside, 98064-96-1; stearyl maltoside, 93911-17-2; cytochrome *c* oxidase, 9001-16-5.

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