# Interaction of Detergents with Cytochrome *c* Oxidase†

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ABSTRACT: The binding of ionic and nonionic, nondenaturing detergents to cytochrome *c* oxidase has been examined. All bind and displace part but not all of the phospholipid that is associated with the enzyme after isolation. From 6 to 10 phospholipid molecules, depending on the detergent used, do not exchange and these are mostly diphosphatidylglycerol molecules as first shown by Awasthi et al. ((1971) *Biochim. Biophys. Acta 226,* 42). The binding of Triton X-100 and deoxycholate to the cytochrome *c* oxidase complex has been studied in detail. Both bind to the enzyme above their critical micelle concentrations: Triton X-100 in the amount of 180 ± 10 molecules per complex and deoxycholate in the amount of 80 ± 4 molecules per complex. In nonionic detergents, cytochrome *c* oxidase exists as a dimer (4 heme complex). The enzyme is dissociated into the monomer or heme $aa_3$ complex by delipidation in bile salts. Activity measurements in different detergents suggest that cytochrome *c* oxidase requires a flexible, hydrophobic environment for maximal activity and that the dimer or 4 heme complex may be the active species.

Cytochrome *c* oxidase, a protein complex associated with the inner mitochondrial membrane, is the terminal member of the electron transport chain and an integral part of coupling site III. The beef heart enzyme is composed of two heme a moieties (hemes a and $a_3$), two copper atoms (Kuboyama et al., 1972; Yonetani, 1961), and one or more copies of seven different polypeptides (Briggs et al., 1975; Downer et al., 1976). A complex of these components spans the mitochondrial inner membrane (Hackenbrock and Hammon, 1975; Schneider et al., 1972), allowing the vectorial transport of electrons across the membrane and coupling to ATP synthesis.

When the enzyme is isolated from the membrane, phospholipids or nondenaturing detergents are required for electron transfer activity (Brierley and Merola, 1962; Awasthi et al., 1971; Crane and Sun, 1973), and phospholipid vesicles are specifically required for the reconstitution of coupling functions (Racker and Kandrach, 1973).

The importance of different lipid head groups to cytochrome *c* oxidase activity has been examined in several laboratories but with variable results (Awasthi et al., 1971; Brierley and Merola, 1962; Yu et al., 1975). There are several possible reasons for the discrepant reports. Firstly, different laboratories have used different lipid depletion methods including organic solvent extraction (Brierley and Merola, 1962), phospholipase digestion (Awasthi et al., 1971), or ammonium sulfate precipitation in the presence of detergents (Yu et al., 1975), and these approaches may affect the protein structure differently. Secondly, all methods of lipid depletion used so far have yielded an insoluble preparation that must be redispersed for optimal activity (Smith and Camerino, 1963; Vanneste et al., 1974), and different approaches to lipid addition could have dispersed the enzyme to different extents. Thirdly, in the various studies discussed, attention has not been given to fatty acid composition of added lipids and, as the results to be presented here show, the character of the hydrocarbon portion of the amphiphile is probably more important to cytochrome *c* oxidase activity than the nature of the head group. Finally, where detergents have been used to displace lipids, the nature of the detergent protein complexes obtained has not been characterized, and residual lipid could be responsible for maintaining any activity that is seen.

From the foregoing it is clear that, in studying the lipid requirements for cytochrome *c* oxidase activity, it is important initially to use mild conditions of delipidation which retain the enzyme in solution, and then to add back synthetically obtained lipids of defined head group and fatty acid composition. As a first step in this direction we have looked at detergent exchange as an approach to delipidating the enzyme, i.e., replacing as much of the bound phospholipid as possible with each of a series of "nondenaturing" detergents while maintaining a soluble complex. Each of the detergent–phospholipid–protein complexes is soluble and of defined composition, and therefore conclusions can be made about the hydrophobic environment required for maximum cytochrome *c* oxidase electron transfer activity. Also important differences are found in the mode of action of the different "nondenaturing" detergents.

## Experimental Section

*Materials.* Cytochrome *c* oxidase was isolated from beef heart mitochondria as described previously (Capaldi and Hayashi, 1972; Downer et al., 1976). Sodium [$^{14}$C]deoxycholate (dCho)[1] (52 mCi/mmol) was obtained from Amersham/Searle and diluted with unlabeled dCho (obtained from Fisher Scientific Co.) that had been recrystallized from acetone–water as described by Makino et al. (1973). Triton X-100 (TX) was obtained from Packard Instrument Co. Tween 80, Tween 60, Tween 40, Tween 20, and sodium cholate were purchased from Sigma Chemical Co. (The Tween series of

† From the Institute of Molecular Biology and Department of Biology, University of Oregon, Eugene, Oregon 97403. *Received August 4, 1976.* This investigation was supported in part by a grant from the Oregon Heart Association, by a National Science Foundation Grant PCM 74-00957 A02, and an Institutional Grant No. GM-00715 from the National Institutes of Health. This is paper number 5 in a series on Components of the Mitochondrial Inner Membrane.

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[1] Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; TLC, thin-layer chromatography; ETP, beef heart submitochondrial particles (electron transfer particles); NaDodSO$_4$, sodium dodecyl sulfate; dCho, deoxycholate; TX, Triton X-100; DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI, phosphatidylinositol; TX–CO, Triton X-100–cytochrome *c* oxidase complex; dCho–CO, deoxycholate–cytochrome *c* oxidase complex; cmc, critical micelle concentration.

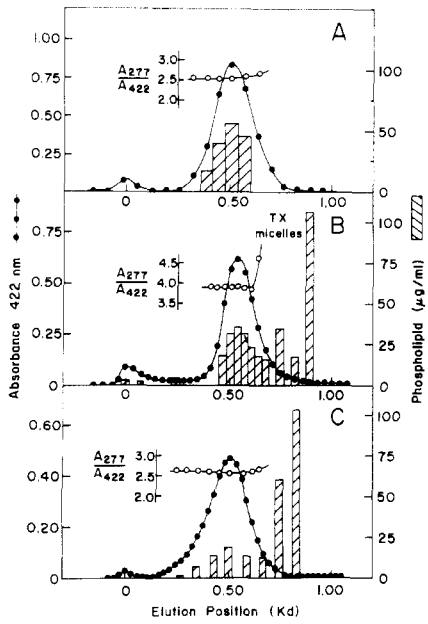


FIGURE 1: Phospholipid depletion of cytochrome *c* oxidase on Sepharose 4B with nondenaturing detergents, pH 8.10, 0.02 M Tris-HCl–0.09 M NaCl, 4 °C. Open circles illustrate $A_{277}/A_{422}$. (A) Tween 80 (0.5%) column: 1.1 × 45 cm, excluded volume = 14.5 ml, total volume = 43.0 ml, flow rate 5 ml/h. A 0.67-ml sample was applied containing 15 mg/ml protein, 0.5% Tween 80, and 5% glycerol. (B) Triton X-100 (1 mM) column: 0.90 × 58 cm, excluded volume = 14.4 ml, total volume = 37.7 ml, flow rate 2.6 ml/h, 0.80 ml/fraction. A 0.70-ml sample was applied; 13 mg/ml protein, 2.2 mg of TX/mg of protein (45 mM TX), 5% glycerol. (C) dCho (20 mM) column: 1.1 × 55 cm, excluded volume 21.3 ml, total volume = 53.1 ml, flow rate 2.6 mg/h, 0.80 ml/fraction. A 0.45-ml sample was applied; 12.5 mg/ml protein, 4.5 mg of dCho/mg of protein (135 mM dCho), 5% glycerol.

detergents was used without further purification; sodium cholate was recrystallized from acetone–water.)

*Methods.* Protein concentrations were measured by the method of Lowry et al. (1951) using bovine serum albumin as a standard, or by amino acid analysis using a Technicon automatic amino acid analyzer according to the method of Spackman et al. (1958). Heme a concentrations were estimated by pyridine hemochromagen difference spectra ($\epsilon_{587-620}$ = 21.7 $mM^{-1}$ $cm^{-1}$).

Cytochrome *c* oxidase activity was measured spectrophotometrically by following the oxidation of ferrocytochrome *c* at 550 nm in 0.05 M phosphate buffer, pH 7.0, at 25 °C in the appropriate detergent. Activities are expressed as molecular activity (MA) at 25 μM cytochrome *c* in units of μmol of cytochrome *c* oxidized per μmol of heme $a_3$ per s as described by Vanneste et al. (1974).

Total phospholipid content was measured using the method of Chen et al. (1956) to determine inorganic phosphate, assuming an average phospholipid molecular weight of 775. Quantitation of individual phospholipids was accomplished by extracting the phospholipid by a modification of the method of Bligh and Dyer (1959) as described by Awasthi et al. (1971) and separating the various phospholipids using two-dimensional thin-layer chromatography on Supelco Silica Gel Readi Coat Plates (05-8032). The solvent in the first dimension contained chloroform (65 ml), methanol (35 ml), water (4 ml), and 58% $NH_4OH$ (0.25 ml). The solvent for the second dimension was chloroform (50 ml), methanol (10 ml), water (5 ml), acetone (20 ml), and acetic acid (10 ml). Phospholipids were charred with $H_2SO_4$, scraped off, and analyzed for inorganic phosphate by the method of Chen et al. (1956).

All gel filtration experiments were done at 4 °C using columns that had been calibrated using Blue Dextran 2000 (Pharmacia Fine Chemicals) to determine the excluded volume ($V_0$) and β-mercaptoethanol to determine the total volume. The buffer used contained 0.02 M Tris-HCl, 0.2 mM EDTA, and 0.09 M NaCl at pH 8.10.

The molecular weight of cytochrome *c* oxidase detergent complexes was measured by sedimentation equilibrium using a Model E Beckman-Spinco analytical ultracentrifuge equipped with a photoelectric scanner. The protein molecular weight was determined by the method of Tanford et al. (1974) using a partial specific volume of 0.743 for cytochrome *c* oxidase calculated from the amino acid composition of Kuboyama et al. (1972) [verified for our preparation of cytochrome *c* oxidase by amino acid analysis] and using the partial specific volumes of the amino acid residues given by Cohn and Edsall (1943). The values used for the partial specific volume of bound dCho, TX, and phospholipid were those given by Tanford et al. (1974) ($\bar{v}_{dCho}$ = 0.778; $\bar{v}_{TX}$ = 0.908).

The binding of dCho and TX was determined by the gel filtration method of Makino et al. (1973) (see also Robinson and Tanford, 1975). Concentrations of [$^{14}$C]dCho were determined using a Packard TriCarb Model 3375 liquid scintillation counter by counting 100-μl samples in 5 ml of TX–toluene–Omnifluor scintillation fluid. Corrections for quenching by heme a were found to be unnecessary. Observed counts/minute were converted to concentrations of dCho by comparison with a standard solution of dCho made to the same specific activity. Concentrations of TX were determined using $\epsilon_{277}$ = 1465 (Robinson and Tanford, 1975).

## Results

*Phospholipid Composition of Purified Cytochrome c Oxidase.* Cytochrome *c* oxidase used in this study was isolated by the method of Capaldi and Hayashi (1972). Preparations contained between 120 and 150 μg of phospholipid per mg of protein. Three different lipids, DPG, PE, and PC, accounted for almost all of the phospholipid present (Table I), although small amounts of PI and lysolipid forms were seen in some samples. Among the lipids, only DPG was concentrated during the preparation of the complex as compared with its concentration in the mitochondrial inner membrane (i.e., ETP). DPG was also concentrated in enzyme preparations obtained as described by Sun et al. (1968) and Kuboyama et al. (1972) (Awasthi et al., 1971; Yu et al., 1975).

*Exchange of Bound Phospholipid with Detergents.* Exchange of detergent for phospholipid was accomplished by incubating cytochrome *c* oxidase in the presence of an excess of the various detergents and then separating the protein–complex from displaced phospholipid by gel filtration on a Sepharose 4B column equilibrated with a micellar concentration of detergent. The cytochrome *c* oxidase eluting from such a column is a protein–detergent complex (and residual phospholipid) as it exists in equilibrium with micellar detergent.

The observed elution patterns of cytochome *c* oxidase in Tween 80, TX, and dCho are illustrated in Figure 1. In each case the complex eluted as a single peak with a $K_d \simeq 0.5$ (a small amount of aggregated material usually eluted in the excluded volume). Each detergent–protein complex was homogeneous with respect to the relative amounts of protein, phospholipid, and detergent present, as judged by the fact that the protein/heme ratio (indicated by $A_{277}/A_{422}$), the phospholipid/protein ratio and, where measured, the detergent/protein ratio were the same in all peak samples. (With TX, the

TABLE I: Phospholipid Composition of Cytochrome *c* Oxidase.

| Phospholipid Class | Lipid in the Various Preparations in μg/mg Protein[a] | | | | |
|---|---|---|---|---|---|
| | Purified Cytochrome *c* Oxidase | TX-CO | Tween 80-CO | dCho-CO | Enzyme Treated with $CHCl_3$-$CH_3OH$ (2:1) |
| Total | 120 | 57 | 53 | 32 | 25 |
| DPG | 48 | 32 | 32 | 19 | 20 |
| PE | 43 | 8 | 12 | 5 | 1.5 |
| PC | 28 | 6 | 9 | 3 | 1.5 |
| Others | | 8 | | 4 | 1.5 |

[a] Amounts determined from TLC plates were corrected for losses during the procedure (10–15%).

TABLE II: Activity of Cytochrome *c* Oxidase in Various Detergents.

| Preparation | Act.[a] Measured in the Different Detergents[b] | | | | | |
|---|---|---|---|---|---|---|
| | dCho | TX | Tween 80 | Tween 60 | Tween 40 | Tween 20 |
| Purified cytochrome *c* oxidase (CO) | 4 | 2.5 | 110 | 67 | 49 | 104 |
| TX-CO | | 2 | 90 | | | |
| Tween 80-CO | | 3 | 95 | 70 | 52 | 101 |
| Cholate-CO (56 μg of phospholipid/mg of protein) | | | 66 | | | |
| dCho-CO (56 μg of phospholipid/mg of protein) | | | 62 | | | |
| dCho-CO (38 μg of phospholipid/mg of protein) | 5 | | 55 | | | |

[a] In micromoles of cytochrome *c* oxidized per second per micromole of heme $a_3$. [b] Assays were all performed in 0.5% detergent solution.

$A_{277}/A_{422}$ ratio increased sharply at the end of the protein peak due to the large $A_{277}$ of the overlapping TX micelle peak.)

*Phospholipid Composition of Detergent-Treated Enzyme.* Gel filtration in dCho, cholate, TX, and Tween 80 removed phospholipid from the enzyme, but none of these detergents displaced all of the lipid from the surface of the cytochrome *c* oxidase complex. Tween 80 and TX generally removed all but 45–58 μg of phospholipid from protein. (Specific examples of the phospholipid remaining are shown in Table I.) Repeated detergent treatment and rechromatography did not further deplete the enzyme of phospholipid and the amount of lipid removed by TX was not altered by high salt (1 M KCl).

The bile salts, cholate and dCho, removed more phospholipid from the surface of the enzymic complex (32–50 μg of phospholipid remaining per mg of protein). The lower limit was not exceeded by repeated detergent treatment and rechromatography and the value was not changed by including 25% ammonium sulfate in the detergent solutions.

The phospholipid composition of the tightly bound lipid after different detergent treatments was examined. All detergents removed considerably more PC and PE than DPG. Under conditions where approximately 50 μg of phospholipid/mg of protein remained (i.e., in TX, Tween 80, and under conditions of less than optimal removal of lipid by bile salts), about 80% of the PE and PC but only 30% of the DPG were replaced by detergent (Table I). Under optimal conditions of lipid displacement by dCho, almost all of the PE and PC were removed along with 60% of the DPG. This residual DPG was not removed with neutral chloroform–methanol in the conventional Bligh and Dyer (1959) extraction procedure. It was extracted by chloroform–methanol–ammonia as reported by Awasthi et al. (1971) and was also removed completely by chloroform–methanol alone if the complex was first dissociated with sodium dodecyl sulfate (results not listed). The detergent-unexchangeable phospholipids, therefore, are not covalently bound to the protein.

*Molecular Activity of Phospholipid Depleted Samples.* The activity of phospholipid depleted samples was measured in the presence of different detergents (Table II). All samples were essentially inactive when assayed in buffers containing TX or dCho. Enzyme depleted of lipid in TX or Tween 80 was active when assayed in the presence of detergents of the Tween series. Around 85% of the original activity of these samples was regenerated when the enzyme was diluted into Tween 80 (an oleate ester). Equally high activities were obtained in Tween 20 (a laurate ester), while somewhat lower values were measured in Tween 40 (a palmitate ester) and Tween 60 (a stearate ester).

Surprisingly, cytochrome *c* oxidase depleted of lipid by cholate or dCho was considerably less active than the original samples even in the presence of detergents of the Tween series. Samples eluted from a column in dCho showed 30–50% of the activity of untreated enzyme when assayed in Tween 80. The reduced activity of dCho-treated samples was also found in samples containing 50 μg of phospholipid/mg of protein; therefore, the reduced activity of dCho-treated samples was not a consequence of the removal of additional DPG by dCho.

*Polypeptide Composition of Lipid-Depleted Enzyme.* Samples of cytochrome *c* oxidase obtained by gel filtration in different detergents showed very similar polypeptide profiles as examined by polyacrylamide gel electrophoresis in either $NaDodSO_4$ or $NaDodSO_4$ and 8 M urea (Swank and Munkres, 1971; Downer et al., 1976). All samples contained the seven different polypeptides known to be associated with the active complex (Downer et al., 1976) and these were present in the same proportions as found in the untreated enzyme. The only difference between detergent-treated samples and the starting material was the smaller amount of trace

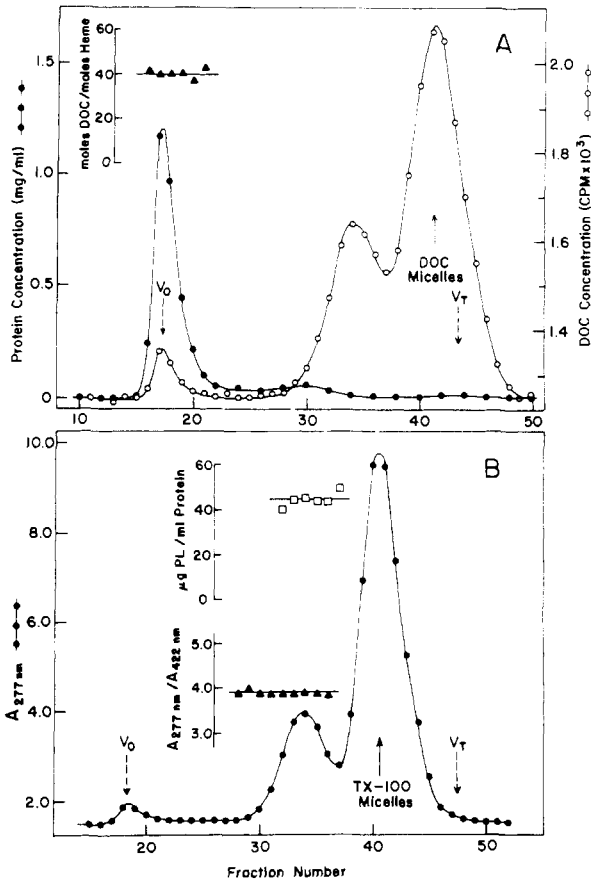


FIGURE 2: Measurement of detergent binding to cytochrome *c* oxidase by gel filtration; pH 8.10, 0.02 M Tris-Cl, 0.09 M NaCl, 4 °C. (A) Sephadex G-100 equilibrated with 5.36 mM [$^{14}$C]dCho. A 0.40-ml sample was applied (5 mg/ml protein in 80 mM [$^{14}$C]dCho–5% glycerol) to a 1.0 × 45 cm column; flow rate 3.6 ml/h, 0.80 ml/fraction. Protein concentrations (●) were determined by Lowry method; dCho concentration (○) was determined by counting 100-μl aliquots (specific activity = 2.335 × $10^3$ cpm/μmol dCho). Binding of dCho to cytochrome *c* oxidase (▲) is expressed as moles of dCho bound per mole of heme a. (B) Sepharose 4B equilibrated with 1.03 mM TX. Column dimensions, flow rate, and sample application are the same as in Figure 1B. The binding of TX to cytochrome *c* oxidase was calculated from the increased absorbance at 277 nm. Cytochrome *c* oxidase has an $A_{277}/A_{422}$ = 2.56 if no TX is bound, but $A_{277}/A_{422}$ = 3.90 (▲) after equilibration with 1 mM TX.

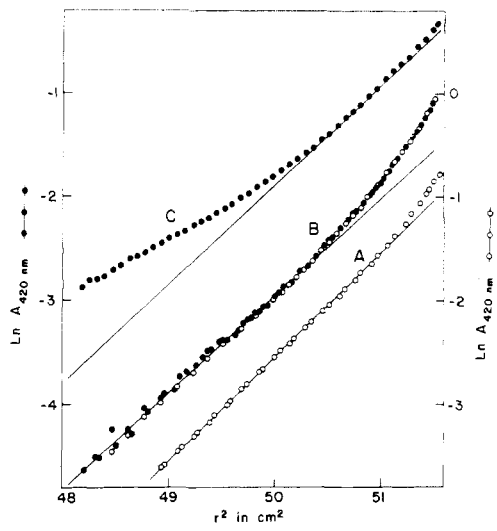


FIGURE 3: Sedimentation equilibrium of cytochrome *c* oxidase–detergent complexes. (A) CO–TX complex; the sample was obtained from complex eluting with a $K_d$ = 0.50 from a Sepharose 4B column (Figure 1B); data were collected after 23 h at 6400 rpm, 22.4 °C. (B) CO–dCho complex; the sample was obtained from complex eluting at the excluded volume from a Sephadex G-100 column (Figure 2A); data were collected after 24 h at 8000 rpm, 10.0 °C. (Identical data were obtained after 42 h.) ○ represents data obtained using an initial protein concentration of 0.470 mg/ml; ● represents data obtained using an initial protein concentration of 0.170 mg/ml. (C) CO–dCho complex; the sample was obtained from complex eluting with a $K_d$ = 0.50 from a Sepharose 4B column (Figure 1C); data were collected after 42 h at 8000 rpm, 10 °C. The initial protein concentration was 0.390 mg/ml.

contaminants in the former. Removal of essential polypeptides by dCho or cholate cannot account for the loss of activity when the enzyme is depleted of lipid by gel filtration in bile salts.

*Binding of Detergent.* The amount of detergent bound to phospholipid-depleted complexes was measured for the TX–cytochrome *c* oxidase (TX–CO) and dCho–CO complexes. Measurements of the binding of Tween 80 were not possible due to the lack of a quantitative assay for Tween 80. The binding of TX and dCho could be measured by the gel filtration method (Figure 2) because the unbound detergent was clearly separated from the detergent–protein–phospholipid complexes. The binding was measured by the increase in the amount of detergent that eluted with the protein (above that in the elution buffer). Experiments at 5 and 10 mM dCho, concentrations greater than the critical micelle concentration (cmc) for dCho (cmc = 2.3 mM), give identical results with 40 ± 2 mol of DOC bound per mol of heme (Figure 2A). With TX, the binding was measured by the increased absorbance of the complex at 277 nm. In the absence of TX, cytochrome *c* oxidase had an $A_{277nm}/A_{422nm}$ = 2.56 (i.e., in either dCho or Tween 80 solutions which do not absorb at 277 nm). In the presence of TX, the $A_{277nm}/A_{422nm}$ ratio increased to 3.90, indicating bound TX (Figure 2B). This increase in absorbance corresponds to 90 ± 5 mol of TX bound per mol of heme. The same value of TX binding was obtained when the equilibrium was approached from either direction, i.e., separating excess TX from the complex by gel filtration as in Figure 2B, or letting the complex bind TX as it moved down the column.

*Molecular Weight of the Phospholipid Depleted Cytochrome c Oxidase.* Because the TX–CO and dCho–CO complexes had been characterized in terms of the amount of phospholipid and detergent bound to each, the molecular weight of the protein associated with each complex could be measured by sedimentation equilibrium. The results obtained by the sedimentation equilibrium technique are shown in Figure 3. In TX (line A), the ln *c* vs. $r^2$ plot is linear throughout most of the cell with a small amount of curvature near the bottom of the cell. Correcting for the bound TX and phospholipid, a molecular weight of 345 000 was obtained. Since the heme content was found to be 11–12 nmol/mg for the isolated complex (i.e., 84–90 × $10^3$ g/mol of heme),[2] the TX–CO complex clearly contains four hemes or is a dimer of the heme $aa_3$ complex. In dCho, a more complicated result is obtained. Sedimentation equilibrium of the dCho–CO complex obtained from the excluded volume of a G-100 column, i.e., Figure 2A, gave a nonlinear plot of ln *c* vs. $r^2$. Analysis at two different initial concentrations of protein (see Figure 3, line

[2] The amount of protein per heme was here measured by amino acid analysis. We find that values for the protein concentration of cytochrome *c* oxidase samples are 15–20% lower as obtained by amino acid analysis than when measured by a Lowry determination using bovine serum albumin as a standard.

B) gives the same curved line, indicating that two or more independent species were present rather than a monomer–dimer type of equilibrium. Estimations of the protein molecular weight are 200 000 (or below) at the meniscus (corresponding to a monomer of the heme $aa_3$ complex) and 360 000 at the bottom of the cell (corresponding to a dimer of the heme $aa_3$ complex). Clearly, the dCho has disrupted a significant amount of the four heme complex of cytochrome *c* oxidase. If the sedimentation equilibrium was performed upon a sample of cytochrome *c* oxidase that had eluted from a Sepharose 4B column (a fraction eluting with a $K_d = 0.55$ in Figure 1C), the sedimentation behavior was not the same (line C in Figure 3). This material has a lower average molecular weight than the unfractionated material obtained from G-100. The molecular weight near the bottom of the cell was now 200 000 (i.e., a monomer). It appears that the Sepharose 4B column has separated the smaller 200 000 molecular weight fragments of the cytochrome *c* oxidase, the heme $aa_3$ monomer, from the larger four heme complex. Inspection of the elution pattern of cytochrome *c* oxidase in dCho on a Sepharose 4B column (Figure 1C) does show a more asymmetric elution profile than was seen in either Tween 80 or TX (Figures 1A and 1B).

## Discussion

Cytochrome *c* oxidase is an intrinsic membrane protein spanning the mitochondrial inner membrane and as such it must be at least partially surrounded by a hydrophobic environment in vivo. In this study we have used a series of nondenaturing detergents to probe the hydrophobic areas of the protein complex in order to determine the essential protein amphiphile interactions that are required for maximal electron transport activity. Our conclusions are necessarily restricted to the needs for electron transport since the coupling of electron transport to ATP synthesis or ion transport is lost in detergent solution (Racker, 1975).

The first step in this study was to substitute detergents for the endogenous lipids around the enzyme complex. The exchange of bound phospholipid molecules for detergent molecules was achieved by gel filtration at micellar concentrations of detergent. This method of delipidation used mild conditions, avoided organic solvents, and always maintained the complex in solution. Although detergent exchange has been effective in fully delipidating several membrane proteins including cytochrome $b_5$ (Spatz and Strittmatter, 1973), sarcoplasmic reticulum ATPase (Walter and Hasselbach, 1973), rhodopsin (Osborn et al., 1974), and Semiliki Forest virus (Simons et al., 1973), this approach did not remove all of the phospholipid from cytochrome *c* oxidase. Equilibration of the complex with dCho, cholate, TX or Tween 80, individually or sequentially left 35–55 μg of phospholipid/mg of protein (6–10 molecules of phospholipid/cytochrome *c* oxidase heme $aa_3$ complex) still associated with the protein, at least two-thirds of which was DPG. Repeated gel filtration of cytochrome *c* oxidase with large excesses of each detergent did not further deplete the complex of lipid. These phospholipids that could not exchange for detergent must be tightly associated with the protein and not merely poorly extracted by detergent micelles because they were not extracted by several different types of detergent and were not removed by extraction with organic solvents. Therefore, in addition to the two classes of lipid known to be involved with cytochrome *c* oxidase, i.e., bilayer lipid which under physiological conditions has fluid hydrocarbon chains, and boundary lipid which has restricted hydrocarbon chain motion (Jost et al., 1973), we must now add a third class, the tightly bound and detergent nonexchangeable phospholipid.

The role of the tightly bound phospholipid in cytochrome *c* oxidase activity and the location of this phospholipid in the complex cannot be readily determined because conditions needed to remove it from the enzyme are denaturing to the protein moiety. It is clear, however, that any specific interaction between lipid head groups and protein is limited at most to the interactions between the 6DPG, 2PE, and 2PC molecules that are tightly bound to the heme $aa_3$ complex since detergents containing polyoxyethylene sorbital head groups can replace all other lipid molecules in supporting high electron transfer activity.

Conclusions about the hydrophobic environment needed for maximal electron transport can also be drawn from these detergent exchange studies. Each detergent reactivated the lipid depleted enzyme to a different extent. This must be related to the ability of each detergent to provide the correct hydrophobic environment around the enzyme rather than to their effectiveness in solubilizing the protein complex because in each case the enzyme–detergent complex being examined was water soluble. Cytochrome *c* oxidase depleted of phospholipid by exchange with either TX or Tween 80 had very low activity when assayed in TX but high activity when assayed in Tween 80. Both detergents contain polyoxyethylene head groups but Tween 80 is an ester of an unsaturated fatty acid (oleate 18:1), which forms micelles with much greater hydrocarbon fluidity than the more rigid *p* octylphenyl hydrocarbon tail of TX (Tanford et al., 1974; Helenius and Simons, 1975). High activity was also restored to lipid depleted enzyme (obtained by TX exchange or exchange with Tween 80) by Tween 20, an ester of laurate (12:0), which also forms micelles with a fluid interior. In contrast, much less activity was regenerated by Tween 60 (stearate 18:0) or Tween 40 (palmitate 16:0), which contain long saturated hydrocarbon tails and produce micelles that are much less fluid than with either Tween 80 or Tween 20. Cytochrome *c* oxidase is essentially inactive when assayed in dCho or cholate, two bulky rigid, ionic detergents. These results are consistent with the interpretation that cytochrome *c* oxidase requires an environment of fluid or flexible hydrocarbon chains for maximal activity. It is not known which phospholipids provide this environment in vivo but DPG, which is enriched in cytochrome *c* oxidase preparations, is particularly rich in unsaturated fatty acids (Crane and Sun, 1973) and might fulfill this need.

In addition to the hydrocarbon fluidity requirements of cytochrome *c* oxidase, differences in the regenerability of the detergent-treated complex suggest important protein structural requirements for maximal electron transport activity. Enzyme depleted of lipid by Tween 80 or TX was highly active when assayed in Tween 80 (or Tween 20), but enzyme depleted of lipid by cholate or dCho was much less active even when assayed in activating detergents. It is unlikely that the differences in regenerability of activity are a consequence of the small variations in delipidation because samples of enzyme treated with bile salts, and yet retaining 50–60 μg of phospholipid/mg of protein, also lost activity when assayed in Tween 80. Instead, the different activities probably resulted from changes in the molecular organization of the protein complex. The association of different polypeptides into the cytochrome *c* oxidase complex (2 heme, 2 copper unit) was not altered by exchange of lipid for any of the detergents tested as shown by the $NaDodSO_4$–polyacrylamide gels of lipid depleted samples. However, as judged by sedimentation equilibrium, there were differences in the aggregation state of these units after the various detergent treatments. In TX, conditions which

left the enzyme in an active form (e.g., when assayed in Tween 80), cytochrome *c* oxidase was monodisperse with a protein molecular weight of 345 000 and thus, in this detergent, it exists as a 4 heme complex. In Tween 80 there is also evidence that cytochrome *c* oxidase exists as a 4 heme complex or higher, aggregation state (Kuboyama et al., 1972). The dCho–enzyme complex, however, was polydisperse, with a protein molecular weight range from below 200 000 to 360 000, indicating a disruption of the 4 heme complex. Since only the TX–enzyme complex, not the dCho–enzyme complex, could be regenerated by Tween 80, the cytochrome *c* oxidase dimer (4 heme complex) may be required for activity of the complex. This interesting possibility is consistent with the observed stoichiometries of electron transfer components. There are 4 heme a's (2 cytochrome *c* oxidase heme $aa_3$ monomers) for every 2 cytochrome *c*'s, 1 cytochrome $c_1$, and 2 cytochrome *b*'s (Slater, 1972; Vanneste, 1966). Studies of the antibody inhibition of cytochrome *c* oxidase activity also lead to the conclusion that the active species of the enzyme contains multiple units (Hackenbrock and Hammon, 1975).

Recent studies on detergent–protein interactions indicate that both the ionic bile salts and nonionic polyoxyethylene detergents bind to intrinsic membrane proteins and are equally effective in maintaining the native conformation of these proteins in aqueous solution (Robinson and Tanford, 1975; Spatz and Strittmatter, 1971, 1973; Helenius and Simons, 1975). It has been suggested that detergents, in general, form micelles around the exposed hydrophobic surface of membrane proteins (Robinson and Tanford, 1975). Cytochrome *c* oxidase bound 80 molecules of dCho and 180 molecules of TX per complex, amounts of detergent per gram of protein that are in the range found to bind to other membrane proteins (Helenius and Simons, 1975; Robinson and Tanford, 1975; Clarke, 1975). Although the bile salts and nonionic detergents were equally effective in disrupting the phospholipid–protein interactions in the cytochrome *c* oxidase complex, only the latter can maintain the organization of the complex that is required for optimal activity. The bile salts, dCho and cholate, disrupted intramolecular interactions in the cytochrome *c* oxidase complex that were not affected by TX. Presumably, based upon our activity measurements, the Tween series of detergents behave like TX in not disrupting these intramolecular interactions. Bile salts and nonionic detergents have also been shown to behave differently with respect to their solubilization of the spike proteins of the Semiliki Forest virus (Helenius and Simons, 1975); TX solubilized the spike proteins with their subunit structure intact, while dCho dissociated the spike complex into its three component polypeptides.

We conclude, therefore, that "nondenaturing" detergents are not all equivalent and fall into two broad classes: (1) nonionic detergents which are mild and less effective in disrupting protein–protein interactions and are, therefore, useful in maintaining active membrane complexes; (2) ionic bile salts (cholate and dCho) which are stronger detergents, more effective in disrupting protein–protein interactions, and can, therefore, destroy activity that is dependent on multisubunit associations. (They are considered to be nondenaturing since they do not cause unfolding and denaturation of individual polypeptide chains.) Although both types of nondenaturing detergents have been equally effective in studying single, polypeptide, intrinsic membrane proteins, the differences we have found with the multipolypeptide complex of cytochrome *c* oxidase suggest that these detergent differences must be considered when studying other intrinsic membrane protein complexes.

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# Properties of the Galactose Binding Protein of *Salmonella typhimurium* and *Escherichia coli*†

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ABSTRACT: The galactose binding protein implicated in transport and in chemotaxis has been purified to homogeneity from the shock fluids of *Salmonella typhimurium* and *Escherichia coli.* Both proteins are monomers of molecular weight 33 000 and exhibit cross-reactivity with antibody. The *Salmonella* galactose receptor showed binding of 1 mol of [$^{14}$C]galactose or 1 mol of [$^{14}$C]glucose at saturation. The dissociation constants were 0.38 and 0.17 $\mu$M, respectively. In light of the previously published report that the *E. coli* protein contains two binding sites with two different affinities, the binding characteristics of this protein were reexamined. Using highly purified radiolabeled substrate and homogeneous protein, a single binding site and single binding affinity were seen for galactose ($K_D$ = 0.48 $\mu$M) or for glucose ($K_D$ = 0.21 $\mu$M). The competition between glucose and galactose for the same site is intriguing in view of the competition between ribose and galactose at the receptor level.

Binding proteins for many different substances such as inorganic ions (Pardee, 1966, 1968; Medveczky and Rosenberg, 1970), sugars (Anraku, 1968a,b; Schleif, 1969; Boos, 1974), and amino acids (Penrose et al., 1968; Ames and Lever, 1972) have been isolated from bacteria by osmotic shock and have been implicated in the mechanisms for transport and chemotaxis. The galactose binding protein (GBP)[1] was first isolated from *E. coli* by Anraku (1968a,b) and was identified as one of the components of the $\beta$-galactoside permease system (Anraku, 1968a,b; Boos, 1969). GBP was also identified by genetic studies as the galactose chemoreceptor by Hazelbauer and Adler (1971). The purified protein has been studied and was found to bind 2 mol of galactose per mol of protein at two independent but nonequal sites (Boos and Gordon, 1971). The possible functions of such a dual affinity system were discussed in subsequent papers (Boos et al., 1972; Silhavy et al., 1974).

In the case of *Salmonella typhimurium,* a ribose receptor was isolated by Aksamit and Koshland (1974) which had a molecular weight similar to that of other binding proteins, but which binds only 1 mol of ribose per mol of protein. In the course of studying the properties of this protein in its interactions with galactose, Strange and Koshland (1975) demonstrated that there was competition between the galactose receptor and the ribose receptor in the signalling systems of *S. typhimurium* and *E. coli.* The galactose receptor of *Salmonella* was then isolated and preliminary results indicated that only one molecule of galactose was bound per molecule of protein. In view of the similarities of *E. coli* and *Salmonella,* the importance of this receptor interaction phenomena, and the apparent discrepancy between the two systems, the galactose binding proteins from both *Salmonella* and *E. coli* were purified and their properties investigated. The results are reported in this paper.

## Materials and Methods

*General.* All chemicals were of the highest purity available. Radiolabeled sugars were obtained from Amersham/Searle and were routinely purified by preparative thin-layer chromatography on silica gel in 1-butanol–ethanol–water, 7:4:2. The concentration of radiolabeled sugar was assayed using the method of Park and Johnson (1949). *Salmonella typhimurium* ST1 is a derivative of LT2 and was obtained as described previously (Aksamit and Koshland, 1974). *E. coli* W3092, a strain constitutive for the galactose binding protein, was obtained from Clement Furlong.

Liquid scintillation counting was carried out in aquasol–toluene, 2:1 (10 ml; New England Nuclear) on a Packard TriCarb 3320 liquid scintillation spectrometer. Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard. All chromatography, diaflow concentrations, and dialyses were carried out at 4 °C and pH's and conductivities were determined at 25 °C.

*Purification of Galactose Binding Protein.* For purification of the galactose binding protein from *Salmonella typhimurium,* 135 l. of ST1 were grown at 30 °C in Vogel–Bonner citrate based medium (Vogel and Bonner, 1956) to a cell density of about 8 × $10^8$ cells/ml. Bacteria were harvested using a steam-driven Sharples continuous centrifuge after the growth medium had been preconditioned by the additions of molar Tris-HCl buffer, pH 7.3, and molar NaCl to final concentrations of 33 mM. The cells were then osmotically shocked ac-

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‡ R.S.Z. is a recipient of a United States Public Health Service Fellowship.

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[1] Abbreviations used: GBP, galactose binding protein; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane; Temed, *N,N,N',N'*-tetramethylethylenediamine.