

Detergent-induced Solubilization of Cytochrome *c* Oxidase As Detected in a Novel Reconstituted System*

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Thomas D. Madden‡ and Pieter R. Cullis§

From the Department of Biochemistry, University of British Columbia, Vancouver, British Columbia, Canada V6T 1W5

A preparation of reconstituted cytochrome oxidase vesicles in which the enzyme is oriented facing inwards (such that it cannot interact with external cytochrome *c*) is described. No oxidase activity is expressed by these vesicles unless they are disrupted, allowing influx of cytochrome *c* or exposure of the oxidase-binding site to the external medium. We have exploited this property to follow detergent-induced solubilization of the membrane, a technique which allows membrane disruption and enzyme activity to be monitored simultaneously. This protocol can be employed to investigate the properties and mechanism of action of detergents as is illustrated for several ionic and nonionic detergents.

In order to study individual protein components of biological membranes, it is common practice to first extract them from the membrane employing various detergents, which allows subsequent purification and characterization. However, the detergent employed has to fulfill two roles which are not necessarily compatible. First, it must be able to solubilize the protein, preferably in a monodisperse form, and secondly, it must not induce denaturation. Different integral proteins exhibit different solubilization and denaturation behavior and thus, no single detergent can be universally employed. The optimum species and concentration of solubilizing agent must usually be determined empirically for any given protein. Particular difficulties are experienced for multisubunit membrane-bound enzymes, where the interactions between various subunits are often hydrophobic and thus susceptible to detergent-induced disruption. One of the best characterized proteins in this regard is cytochrome oxidase, the activity of which (in solubilized form) is highly sensitive to the detergent employed (1, 2). Optimum activity has been observed using lauryl maltoside (2), which maintains the oxidase in a dimeric state, whereas for octyl glucoside, oxidase polymers, dimers, and monomers, as well as lower molecular weight components, are observed which result in much reduced activity. It may be noted that standard techniques of oxidase isolation (3, 4) produce heterogeneity in the subunit composition of the isolated enzyme, suggesting that these procedures also induce some disruption of the oxidase complex during solubilization.

Currently available techniques for following detergent solubilization of membranes involve monitoring the release of

membrane components in a soluble form (for example, after centrifugation of any insoluble material) or employ physical techniques such as light scattering (5) or NMR (6) to follow gross disruption of membrane structure. In this work, we report on a novel technique by which detergent solubilization of oxidase in reconstituted cytochrome oxidase vesicles can be assayed. Briefly, vesicles were prepared containing cytochrome oxidase oriented facing inwards (such that it cannot interact with external cytochrome *c*), and the release of latent oxidase activity induced by addition of detergent followed. The protocol permits the simultaneous observation of membrane disruption and enzyme activity and is suited for studies on mechanisms of detergent action and the selection of appropriate detergents for enzyme solubilization. The properties of a number of ionic and nonionic detergents are examined.

EXPERIMENTAL PROCEDURES

Materials—Cytochrome *c* (horse heart, type VI), sodium cholate, Tween 80, oleoyllysophosphatidylcholine, carbonyl cyanide (trifluoromethoxy)phenylhydrazone, and valinomycin were obtained from Sigma. Triton X-100 was purchased from BDH Chemicals, whereas octyl β -D-glucopyranoside was obtained from Calbiochem Behring.

DOPC¹ was synthesized as described previously (7) and was at least 99% pure as determined by thin layer chromatography.

Preparation of Cytochrome Oxidase Vesicles—Cytochrome *c* oxidase was prepared by the method of Kuboyama *et al.* (8). The oxidase was finally resuspended in 1% Tween 80 and stored in liquid nitrogen at a protein concentration of approximately 50 mg/ml.

For reconstitution experiments, 75 mg of DOPC and 37.5 mg of cholate were hydrated in 5 ml of 75 mM potassium phosphate, pH 8.3. Large unilamellar vesicles were then prepared according to the LUVET procedure.² Essentially this involves extrusion of the multilamellar vesicles through two (stacked) polycarbonate filters (100-nm pore size) 10 times under 250 p.s.i. of nitrogen pressure. Cytochrome *c* oxidase (500 μ g) was then added, and the mixture was dialyzed (Spectrapor 2, 25-mm diameter dialysis tubing) against 400 volumes of 75 mM potassium phosphate, pH 8.3, for 6 h and then against the same volume of buffer for an additional 12 h. The mixture was then applied to a DEAE-Sephacel column (1.5 \times 30 cm) pre-equilibrated with 25 mM potassium phosphate, pH 8.3. The reconstituted vesicles were eluted with the same buffer at a flow rate of 5 ml h⁻¹.

Assay for Cytochrome Oxidase Activity—Enzyme activities were measured spectrophotometrically by following the oxidation of ferrocytochrome *c* (25 μ M) at 550 nm in 50 mM potassium phosphate, pH 7.4. Detergents were added from a stock solution to the desired concentration. Unless otherwise stated, the assays were performed at 25 °C.

Light Scattering Measurements—Detergent solubilization of vesicles was also followed by monitoring absorbance changes at 340 nm for vesicles suspended in 50 mM potassium phosphate, pH 7.4, using a Pye Unicam SP8-500 spectrophotometer.

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§ Medical Research Council of Canada Scientist.

¹ The abbreviations used are: DOPC, dioleoylphosphatidylcholine; OLPC, oleoyllysophosphatidylcholine; OGP, octyl β -D-glucopyranoside; CMC, critical micellar concentration; LUVET, large unilamellar vesicles by extrusion techniques.

² M. J. Hope, M. B. Bally, and P. R. Cullis, submitted to *Biochim. Biophys. Acta*.

RESULTS

We have shown previously that when cytochrome oxidase is reconstituted with DOPC at very high lipid to protein ratios (molar ratio 50,000:1), most of the protein-containing vesicles contain only a single oxidase dimer (10). We also observed that vesicles in which the oxidase is oriented facing outwards (such that it can interact with external cytochrome *c*) can be separated by DEAE chromatography from protein-free vesicles and vesicles containing inwardly oriented oxidase. A typical elution profile is shown in Fig. 1, where peak 1 consists of protein-free vesicles and vesicles containing inwardly oriented oxidase and peak 2 reflects largely (>90%) vesicles containing outwardly oriented enzyme. The orientation of the oxidase was determined using the spectroscopic procedure of Nicholls *et al.* (11).

Vesicles containing inwardly oriented enzyme show no activity with external ferrocytochrome *c* unless the membranes are disrupted, thus exposing the oxidase-binding site to the external medium. This property has been exploited to follow the disruption of the reconstituted vesicles by OGP as shown in Fig. 2A. At concentrations of OGP up to 15 mM, virtually no activity is observed. However, in a narrow concentration range between 16 and 18 mM, detergent, full release of oxidase activity is apparent. Higher concentrations of OGP have an inhibitory effect, as previously reported (2), presumably due to the gradual removal of phospholipid from around the protein and/or protein aggregation. The release of oxidase activity was correlated with solubilization of the vesicles by following changes in light scattering at 340 nm upon addition of OGP. Fig. 2B shows two traces, one for the reconstituted vesicles and one for large unilamellar vesicles of DOPC prepared by the LUVET procedure² but in the absence of detergent. For both samples, an abrupt decrease in light scattering between 17 and 18 mM OGP is observed, indicating solubilization of the vesicles. The fact that both samples behave similarly suggests that residual cholate in the reconstituted vesicles (approximately 1 mol of cholate/50 mol of phospholipid) does not affect their susceptibility to this detergent. The effect of OGP on pure phosphatidylcholine vesicles has also been studied by Jackson *et al.* (12) using a fluorescence polarization technique. In agreement with the above data, they observed solubilization of the phospholipid at between 16 and 20 mM OGP.

In Fig. 3, the influence of a zwitterionic detergent (oleoyl-

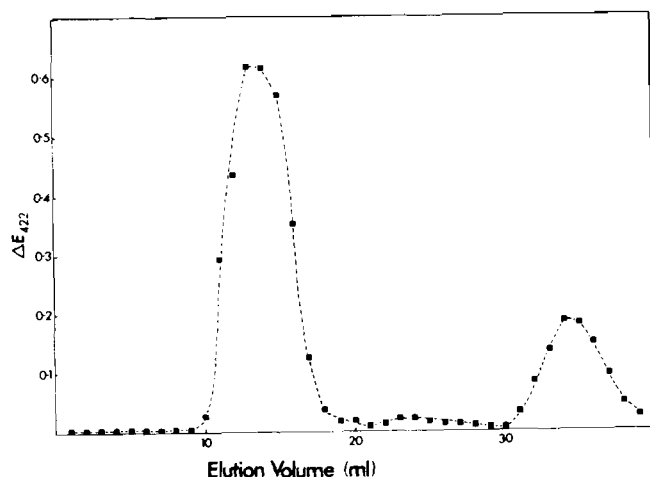


FIG. 1. Elution profile of reconstituted cytochrome oxidase vesicles on DEAE-Sephacel. Vesicles were prepared as described under "Experimental Procedures" and eluted in 15 mM potassium phosphate, pH 8.3, at a flow rate of 5 ml h⁻¹.

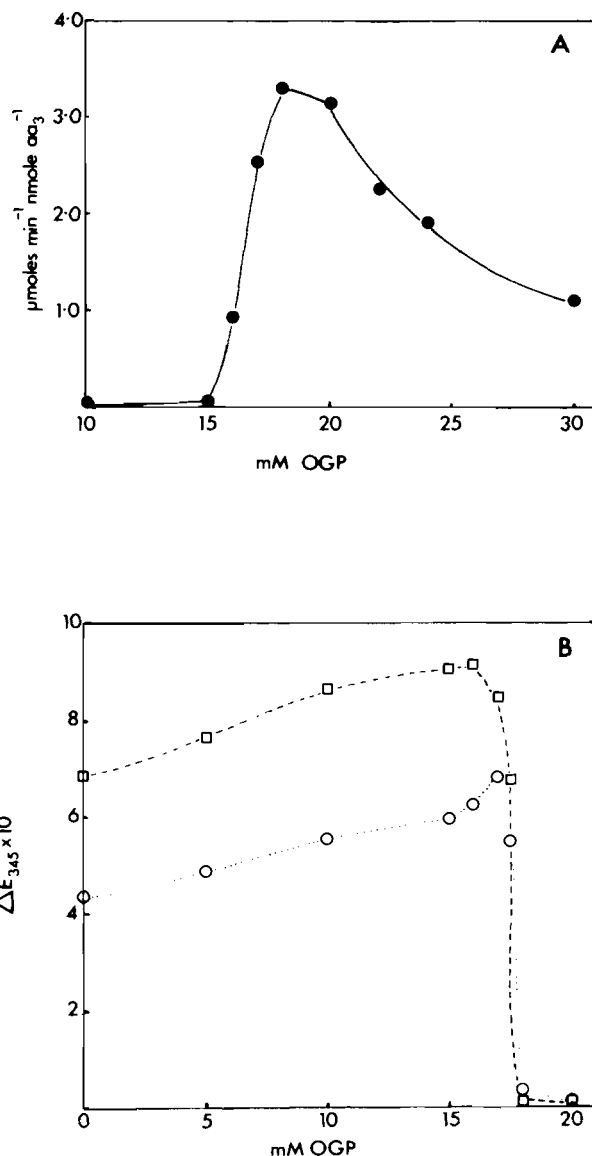


FIG. 2. Release of latent oxidase activity by OGP. Enzyme activity was assayed in reconstituted vesicles in the presence of varying concentrations of OGP (A). The assays were performed as described under "Experimental Procedures" at 25 °C, and OGP was added from a 200 mM stock solution. The concentrations of phospholipid and heme aa₃ were 230 μM and 50 nM, respectively. The corresponding effects of OGP on light scattering of the vesicles suspension is shown in B. Absorbance at 340 nm of reconstituted vesicles (○) and of large unilamellar vesicles composed of DOPC alone (□) is shown.

lysophosphatidylcholine) and a nonionic detergent (Triton X-100) on the reconstituted oxidase vesicles is shown. In both cases, full release of oxidase activity occurs over a fairly narrow detergent concentration range as observed for OGP. The CMC for OLPC determined using a dye inclusion technique (13) was 40–45 μM, while the CMC for Triton X-100 is reported to be 300 μM (14). These values are somewhat lower than the concentrations of each detergent required to disrupt the vesicles. However, a significant proportion of the added amphiphile is likely to be associated with the membrane, thereby lowering the monomer concentration. It seems reasonable to suggest that the detergent concentration leading to sudden release of oxidase activity corresponds to the detergent CMC and possible fusion between preformed detergent micelles and membrane or to the concentration at which

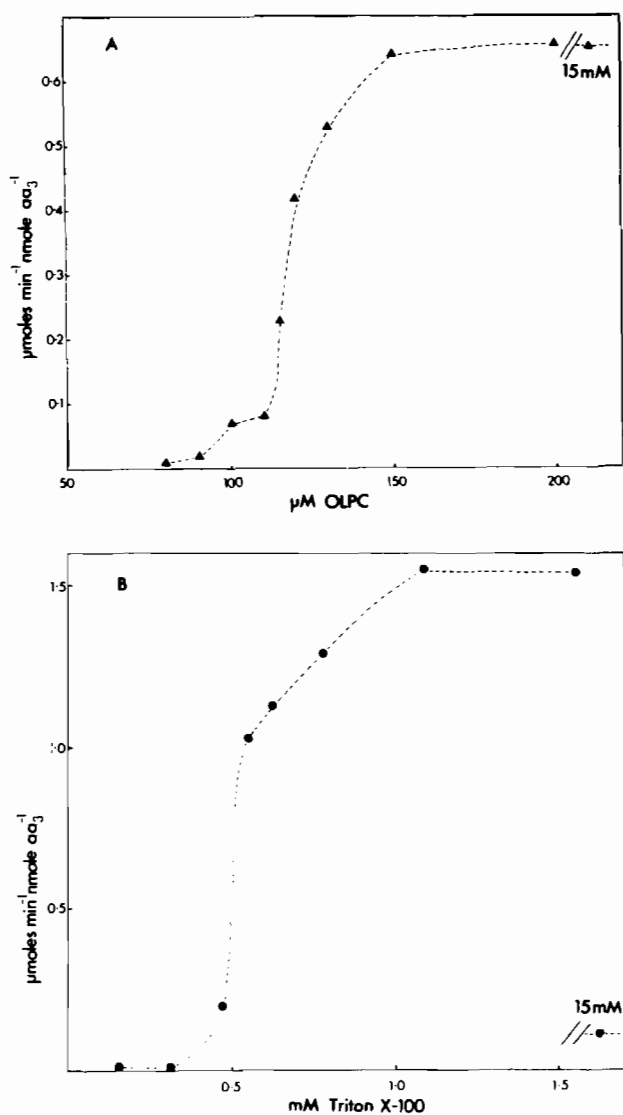


FIG. 3. Release of latent oxidase activity by OLPC and Triton X-100. Enzyme activities were determined at 25 °C. The assay cuvette contained 340 μM phospholipid and 70 nM heme aa₃. Cytochrome oxidase activities in the presence of OLPC (▲) and Triton X-100 (●) are shown.

mixed micelles of detergent, protein, and phospholipid are formed.

Also shown in Fig. 3 are the oxidase activities observed in 15 mM OLPC and 15 mM Triton X-100. It is interesting that no inhibitory effect of high concentrations of OLPC is observed, whereas, in agreement with previous reports (1), a strong inhibition by Triton X-100 is apparent. This inhibition by Triton X-100 presumably reflects stripping of phospholipid from the oxidase, aggregation of oxidase molecules, or subunit dissociation.

The detergents OGP, OLPC, and Triton X-100 all cause an immediate release of oxidase activity when introduced at levels sufficient to induce solubilization. This is not true, however, for Tween 80. As shown in Fig. 4, left, a concentration-dependent lag time is observed between addition of vesicles to the detergent solution and release of enzyme activity. Tween 80 is a considerably larger molecule than the other detergents tested, and this lag period may reflect a slower partitioning of Tween 80 into the vesicle membrane. In addition, it has the lowest CMC, 12 μM (15), of the detergents

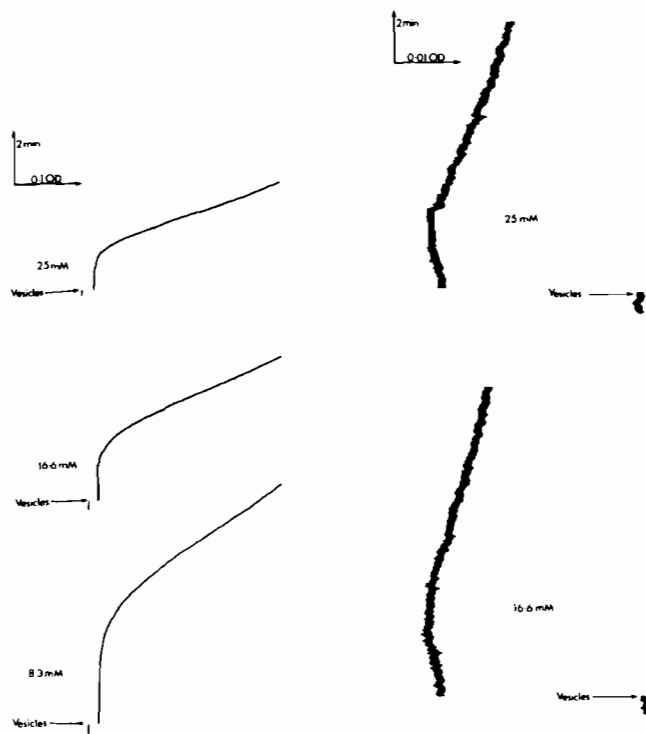


FIG. 4. Time course for release of oxidase activity by Tween 80. The time course for oxidation of ferrocytochrome *c* followed at 550 nm when vesicles are added to the assay medium containing 8.3, 16.6, or 25 mM Tween 80 is shown (left). The corresponding time course for changes in light scattering at 340 nm in the presence of 16.6 and 25 mM Tween 80 is shown (right). The concentrations of phospholipid and heme aa₃ in the assay medium were 230 μM and 50 nM, respectively.

studied and therefore the lowest monomer concentration. The changes in light scattering at 340 nm for vesicles suspended in Tween 80 are shown in Fig. 4, right. It is apparent that complete solubilization of the membrane occurs over a time period considerably longer than the lag time prior to release of oxidase activity. However, the release of enzyme activity occurs at the time at which changes in light scattering are first observed and probably correspond to lysis of the vesicles.

The influence of temperature on the time required for release of oxidase activity by Tween 80 is shown in Fig. 5. As would be expected, if the delay corresponded to the time required for partitioning and accumulation of detergent in the membrane, a strong temperature dependence is observed.

DISCUSSION

The release of latent cytochrome oxidase activity for "inwardly oriented" oxidase vesicles by OGP occurs over a narrow concentration range (16–18 mM). Over a similar range, this detergent solubilizes oxidase in rat liver inner mitochondrial membranes (2). The initial release of enzyme activity at 16–17 mM OGP likely reflects membrane lysis, as indicated by the light scattering studies which suggest that complete solubilization of the vesicles occurs only at 17–18 mM detergent. This result is similar to the behavior observed by Helenius *et al.* (16) for the solubilization of Semliki Forest virus. They observed that between 14 and 18 mM OGP, the membranes were lysed (such that viral RNA was degraded by externally added RNase) but the virus components remained associated. At 19 mM OGP, however, complete solubilization occurred. The CMC of this detergent is between 20 and 22

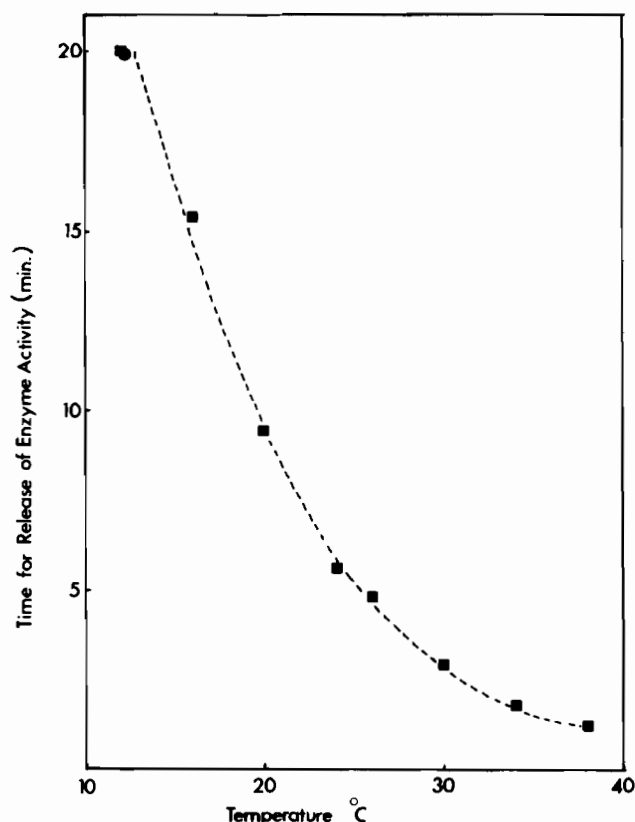


FIG. 5. Influence of temperature on the lag period for release of oxidase activity by Tween 80. The time course for ferrocytochrome *c* oxidation was followed between 12 and 38 °C in an assay medium containing 340 μ M phospholipid, 70 nM heme a_3 , and 16.6 mM Tween 80.

mM (Helenius *et al.* (16)),³ suggesting that membrane solubilization does not require the presence of OGP micelles. Benzonana (13) has shown that even fairly low concentrations of oleate caused a marked depression of the CMC for deoxycholate. It would therefore appear likely that mixed micelles of OGP, phospholipid, and protein can be formed below the CMC of the pure detergent.

The abrupt release of oxidase activity by OLPC and Triton X-100 would also be consistent with the onset of micelle formation. It is difficult, however, to assess the free concentrations of these amphiphiles in the assay medium because a considerable fraction is likely to be associated with the phospholipid. This problem is less prevalent for OGP as the detergent concentration is far higher than that of the lipid. Nevertheless, after allowing for the binding of OLPC and Triton X-100 to the vesicles, the concentrations required for expression of oxidase activity is fairly close to their respective CMC values.

The long time period over which reconstituted vesicles are solubilized by Tween 80 is intriguing. It suggests that, at least, initially, fusion of detergent micelles with vesicles does not occur. It may be suggested that monomers of Tween 80 partition into the membranes and at a critical membrane concentration cause lysis and eventual solubilization or per-

mit fusion with pure detergent micelles.

The studies detailed here indicate the utility of inwardly oriented oxidase vesicles in investigating membrane solubilization by detergents. The technique is sensitive and rapid and allows membrane disruption and enzyme activity to be monitored simultaneously. Detergents such as Triton X-100 which do not normally support cytochrome oxidase activity can be studied at concentrations which cause vesicle disruption but which do not deplete the protein of phospholipids. The present study also demonstrates the diverse properties of detergents with regard to their efficacy, their time course of action, and the influence of high detergent concentrations on enzymatic activity.

This protocol could be well suited to determining the optimum detergent concentrations required for enzyme extraction. In addition, the potential utility of alternative detergents can be rapidly screened in a straightforward manner. It should be noted that this technique should be applicable to membrane proteins other than cytochrome *c* oxidase. This is because the resolution by DEAE chromatography of reconstituted vesicles where the oxidase is oriented facing outwards from protein-free vesicles and vesicles containing inwardly oriented enzyme appears to rely on the fact that the polar region of the oxidase complex containing the cytochrome *c*-binding site is considerably larger than the polar region on the other side of the membrane (17). Such an asymmetry is also exhibited by a number of other membrane proteins including Ca^{2+} -ATPase from sarcoplasmic reticulum and ATP synthetase. It is likely that this property could be exploited to generate asymmetric reconstituted vesicles containing these enzymes using the procedure described for cytochrome oxidase.

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³ T. D. Madden and P. R. Cullis, personal observation.