Specificity and Binding Affinity of Phospholipids to the High-Affinity Cardiolipin Sites of Beef Heart Cytochrome *c* Oxidase†

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ABSTRACT: Beef heart lipid-depleted cytochrome *c* oxidase, containing only 50% of the two to three essential high-affinity cardiolipin molecules per heme aa_3 complex, was used to study the phospholipid requirements of this enzyme. The lipid-depleted complex had two-thirds of the electron transport activity as enzyme containing a full complement of essential cardiolipin (diphosphatidylglycerol or DPG) when it was assayed in Tween 80. However, incubation of the lipid-depleted enzyme with DPG in the presence of 1% Triton X-100 followed by a 140-fold dilution of the reconstituted complex into Tween 80 restored 100% of the initial activity. Similar incubations of the lipid-depleted enzyme with phosphatidylethanolamine, phosphatidylglycerol, phosphatidylserine, or phosphatidic acid did not stimulate the enzymatic activity in Tween 80 more than 5–10%. In the presence of 1% Triton X-100, bovine DPG (90% C18:2) reassociated with the vacant high-affinity sites with an apparent dissociation constant of 5 μM based upon the regeneration of electron transport activity in Tween 80. Bacterial DPG (0% C18:2) measured in a similar manner had a dissociation constant of 2 μM; therefore, the binding of exogenous DPG to the high-affinity sites on the enzyme does not have an absolute specificity for the linoleic acid tails present in bovine DPG. Reisolation of the complex by discontinuous glycerol gradient centrifugation after incubation with exogenous DPG in 1% Triton X-100 indicated that DPG reassociated only with the high-affinity cardiolipin sites; phosphatidylcholine did not bind to the complex under these conditions. On the basis of these results, we conclude that the unique structure of DPG, not the negatively charged head group or the composition of the hydrocarbon tails, is the structural feature necessary for the binding of exogenous phospholipids to the essential high-affinity sites of cytochrome *c* oxidase and the maintenance of full electron transport activity in Tween 80.

Cytochrome *c* oxidase, a multisubunit intrinsic membrane protein complex, spans the inner mitochondrial membrane and, therefore, contacts a layer of boundary phospholipids (PL).[1] Not all of these boundary layer PL's are removed from the protein during its solubilization by nondenaturing detergents and subsequent purification. Depending upon the method of purification, between 20 and 60% of the approximately 50 boundary layer PL's remain associated with the complex (Yu et al., 1979). The remainder of the boundary layer sites are usually occupied by bound detergent.

The most profitable approach for studying the specificity and functional importance of the boundary layer PL has been first to remove as much of the endogenous PL as possible without irreversibly altering either the conformation of the protein or its potential for full electron transport activity. Three approaches have been used for the preparation of lipid-depleted cytochrome *c* oxidase: (1) extraction of PL with mild organic solvents, e.g., aqueous acetone (Brierley & Merola, 1962); (2) repeated ammonium sulfate precipitation from detergent solution, usually cholate (Awasthi et al., 1971; Yu et al., 1975, 1979); (3) detergent–PL exchange followed by separation of the delipidated complex from the micelle-extracted PL by either gel filtration (Robinson & Capaldi, 1977) or glycerol gradient centrifugation (Robinson et al., 1980). Of these general methods, we have found detergent–PL exchange followed by glycerol gradient centrifugation to be the most successful since precipitation of the delipidated complex is avoided, a step that often causes irreversible loss of activity. On the basis of our studies, we have divided the boundary layer PL's into three broad categories: (1) 40–45 loosely associated PL molecules that are nonessential for activity since they can be replaced by detergents such as Tween 80 or Tween 20 without affecting the activity of the enzyme; (2) 6–8 molecules of more tightly bound PL that are nonessential for full activity of the complex and that can be extracted by high concentrations of detergent; (3) 2–3 molecules of very tightly bound DPG that are essential for full activity of cytochrome *c* oxidase.

As an attempt to better define the specificity and functional importance of the very tightly bound two to three DPG molecules in cytochrome *c* oxidase, we have developed a procedure for extracting 50–60% of these DPG molecules and for specifically reassociating exogenous PL's with only the high-affinity sites (Robinson et al., 1980). This method involves (1) preparation of lipid-depleted enzyme containing only 60% of the two to three essential DPG molecules per heme aa_3 complex and having only 60–65% of the activity in Tween 80 or Tween 20 as enzyme containing all of the essential DPG, (2) incubation of the lipid-depleted enzyme with exogenous PL in the presence of 1% TX, and (3) evaluation of the electron transport activity of the resulting complex after dilution into Tween 80. Using this procedure, we have been able to restore full enzymatic activity to lipid-depleted cytochrome *c* oxidase by specifically titrating only the vacant high-affinity binding sites. Continuing with this approach, we have investigated the PL specificity, binding affinity, and functional importance of the high-affinity DPG sites of cytochrome *c* oxidase. The results of such a study are the subject of this communication.

Experimental Procedures

Materials. Cytochrome *c* oxidase was isolated by the method of Fowler et al. (1962) as modified by Capaldi & Hayashi (1972) from beef heart mitochondria prepared according to Crane et al. (1956). Typical preparations had a molecular

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[1] Abbreviations: PL, phospholipids; TX, Triton X-100; DPG, diphosphatidylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PS, phosphatidylserine; PA, phosphatidic acid.

0006-2960/82/0421-0184$01.25/0

activity of 150–165 μmol of cytochrome *c* oxidized per s per μmol of heme aa_3 when assayed in 0.5% Tween 80 and contained 8.6–9.4 nmol of heme *a*/mg of protein. Lipid-depleted cytochrome *c* oxidase containing 3.6 g-atoms of P/mol of heme aa_3 complex, 90–95% of which was due to tightly bound DPG, was prepared by the TX–glycerol gradient procedure of Robinson et al. (1980). Type IV cytochrome *c*, Triton X-100 (TX), Tween 80, bovine heart diphosphatidylglycerol (DPG), type III-E phosphatidylcholine (PC), type III phosphatidylethanolamine (PE), phosphatidylserine (PS), grade I phosphatidylglycerol (PG), and grade I phosphatidic acid (PA) were purchased from Sigma Chemical Co. Bacterial diphosphatidylglycerol was purchased from Supelco, Inc. All other chemicals were A.C.S.-certified reagent grade and purchased from Fisher Chemicals.

Methods. All experiments, unless otherwise noted, were performed in 0.02 M tris(hydroxymethyl)aminomethane hydrochloride buffer at pH 8.1 containing 0.09 M NaCl and 0.1 mM ethylenediaminetetraacetic acid. Cytochrome *c* oxidase activities were measured spectrophotometrically by following the oxidation of reduced cytochrome *c* at 550 nm and 25.0 °C with a PM 6K Zeiss spectrophotometer equipped with a temperature-controlled cuvette holder. Typically, a 5-μL aliquot of an enzyme solution (2.3×10^{-7} M heme aa_3) in pH 8.1 buffer containing 1% TX and the appropriate concentration of PL (0–300 μM PL) was assayed following dilution into a temperature-equilibrated cuvette containing 0.7 mL of 30 μM reduced cytochrome *c* in 0.5% Tween 80–0.01 M sodium phosphate buffer, pH 7.0.[2] All PL–cytochrome *c* oxidase incubation solutions (100 μL) were prepared by mixing appropriate volumes of 1×10^{-6} M enzyme dissolved in 1% TX, pH 8.1 buffer (the concentration was based upon the absorbance at 422 nm with an $\epsilon_{422} = 1.54 \times 10^5$ M^{-1} cm^{-1}; van Gelder, 1978), 5×10^{-4} M PL (prepared by dissolving a known amount of N_2-dried PL in the 1% TX, pH 8.1 buffer), and 1% TX, pH 8.1 buffer. The solutions were incubated for 30 min at room temperature and then kept at 0 °C until they were assayed (0–2 h). Molecular activities (micromoles of cytochrome *c* oxidized per micromole of cytochrome *c* oxidase per second) were calculated from the first-order rate constants for the reactions (calculated by a linear regression analysis of the slopes of the first-order plots) and the cytochrome *c* and the heme aa_3 concentrations as described by Vanneste et al. (1974).

For the analysis of bound PL, cytochrome *c* oxidase was extracted by the method of Bligh & Dyer (1959), and TX was removed from these extracts by the thin-layer chromatography method using acetone as described by Robinson et al. (1980). The TX-free extracts and stock PL solutions were analyzed for phosphorus content by using the procedure of Chen et al. (1956) after digestion of the PL's in 0.5 mL of perchloric acid according to Marinetti (1962).

Results

Phospholipid Specificity of Delipidated Cytochrome c Oxidase. The head group specificity of the essential high-affinity PL sites on lipid-depleted cytochrome *c* oxidase was determined by monitoring the activity of the lipid-depleted complex in Tween 80 after its incubation with a variety of PL's dissolved in 1% TX. The concentration of each phospholipid in 1% TX was varied from 0 to 300 μM in order to assess the relative affinities of each PL for the lipid-depleted cytochrome *c* oxidase complex. The specific stimulation of the delipidated complex by DPG is clearly evident from the results summarized in Figure 1. Incubation of the complex with as little as 80–100 μM DPG in 1% TX resulted in full regeneration of activity while the other five PL's (PC, PE, PS, PG, and PA) were each ineffective in stimulating activity (ca. 10% stimulation of activity with 300 μM PL).[3] The negative charge on the DPG was not the most significant parameter for the reconstitution of full enzymatic activity since several other negatively charged PL's, i.e., PG, PS, and PA, were no more effective in stimulating the activity than the zwitterionic PL's, i.e., PE and PC. Presumably, either DPG is the only PL capable of binding to the lipid-depleted cytochrome *c* oxidase or the other PL's, if they do bind, are incapable of meeting the structural and/or functional requirements met by DPG.

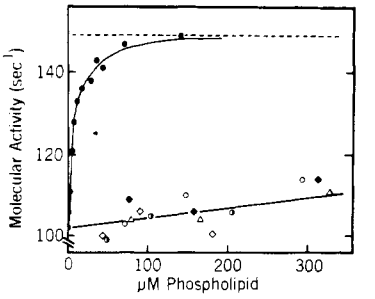

FIGURE 1: Effect of exogenous PL's upon the activity of lipid-depleted cytochrome *c* oxidase. All PL–enzyme incubations and determinations of molecular activities were performed as described under Methods. The dashed line represents the molecular activity of cytochrome *c* oxidase prior to delipidation. Cytochrome *c* oxidase (2.3×10^{-7} M heme aa_3) was incubated in 1% TX, pH 8.1, with (●) bovine DPG, (○) PC, (♦) PE, (◑) PS, (△) PG, or (◇) PA prior to assaying the PL–TX–cytochrome *c* oxidase complex in 0.5% Tween 80.

One of the structural differences between DPG and the other PL's is its high percentage of linoleic acid (bovine DPG contains 90% C18:2). So that the importance of the high proportion of linoleic acid for the stimulation of activity could be assessed, the effectiveness of bacterial DPG was measured and compared with that of bovine DPG (bacterial DPG contains 32% C16:0, 38% C18:1, 0% C18:2, and 30% cyclopropane fatty acids). For measurement of the relative affinities of each type of DPG, the concentration of each PL was varied from 0 to 30 μM during its incubation with cytochrome *c* oxidase in 1% TX. Either type of DPG was capable of stimulating the activity of lipid-depleted cytochrome *c* oxidase; in fact, bacterial DPG appeared to have a slightly higher affinity for the complex than bovine DPG since the same stimulatory effect was observed at somewhat lower DPG concentrations. Both types of DPG produced a hyperbolic type of stimulation of activity as a function of PL concentration with a maximum rate of electron transport approaching the initial activity of the nondelipidated complex. Apparently, the most important

[2] This concentration of cytochrome *c* is sufficient to saturate both the high- and low-affinity cytochrome *c* binding sites on cytochrome *c* oxidase; therefore, all of the molecular activities were measured at the maximal rate of cytochrome *c* oxidation.

[3] The stimulation of activity by exogenous DPG is smaller than many of the earlier studies on the PL specificity of lipid-depleted cytochrome *c* oxidase (Brierley & Merola, 1962; Awasthi et al., 1971; Yu et al., 1975, 1979). This difference results from the method used to assess the activity of the PL-depleted enzyme. The activities shown in Figure 1 were measured relative to the activity of the completely solubilized enzyme assayed in Tween 80. This detergent meets the bulk boundary layer requirements of the enzyme and permitted us to investigate the PL specificity of only the essential DPG sites. In previous studies, the activities were usually measured relative to either a PL- and detergent-depleted complex that was highly aggregated or the activity of the complex assayed in a detergent unable to satisfy the boundary layer requirements of the complex, e.g., cholate, deoxycholate, TX, or octyl glucoside.

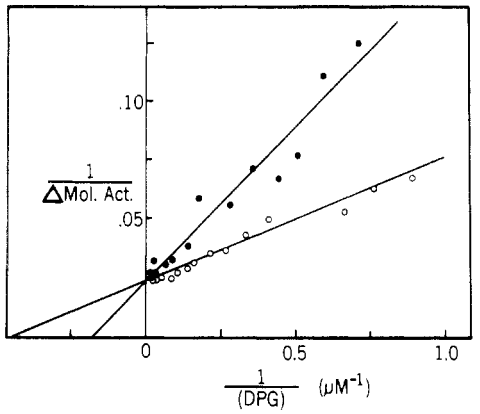

FIGURE 2: A double-reciprocal plot of the regeneration of activity of lipid-depleted cytochrome *c* oxidase by exogenous DPG. The change in molecular activity (ΔMol. Act.) is the observed activity after incubation with the appropriate concentration of either (●) bovine DPG or (○) bacterial DPG minus the activity prior to incubation with either PL. The data points are the average of four to eight separate activity measurements.

structural feature that is necessary for the generation of full activity by exogenous PL's is the unique overall structure of DPG, not its unusually high content of linoleic acid.

Affinity of DPG for Lipid-Depleted Cytochrome c Oxidase. Because bovine DPG and bacterial DPG each elicits a hyperbolic type of stimulation in the activity of the lipid-depleted cytochrome *c* oxidase, the assay system appears to measure the binding affinity of functionally important saturable sites on the surface of cytochrome *c* oxidase. For evaluation of the relative binding affinities of bovine and bacterial DPG to lipid-depleted cytochrome *c* oxidase in 1% TX, the hyperbolic data were linearized by plotting a double-reciprocal plot (Figure 2). The apparent dissociation constants ($K_{d,app}$) were evaluated from the two intercepts on the abscissa. Both types of DPG exhibited high affinity for the functionally important sites on the enzyme considering that 1% TX, i.e., 150 μM TX micelles, was present and could compete with cytochrome *c* oxidase for the exogenous DPG ($K_{d,app}$ = 2 μM for bacterial DPG; $K_{d,app}$ = 5 μM for bovine DPG). Evidently, the differences in the fatty acid composition between bacterial DPG and bovine DPG are not sufficient to substantially alter their binding affinity to the delipidated enzyme. The high affinity of the enzyme for DPG is evident from these data since only an 8-fold molar excess of bacterial DPG per heme aa_3 complex was sufficient to achieve half-maximal stimulation (a 20-fold molar excess was required by bovine DPG). This corresponds to only 1 DPG molecule per 70 TX micelles for bacterial DPG, i.e., only 1.3% of the TX micelles contain a DPG molecule when 50% of the essential high-affinity sites on cytochrome *c* oxidase are occupied by DPG. (With bovine DPG, 4% of the TX micelles contain a dissolved DPG at 50% saturation of the cytochrome *c* oxidase sites.)

Once the essential sites were saturated with either type of DPG, the maximal activity of the complex was the same as evidenced by the identical ordinate intercepts for the two sets of data in Figure 2. This value for the maximal velocity of the regenerated cytochrome *c* oxidase was within 3% of the initial activity of the complex prior to TX delipidation. Clearly, a high percentage of linoleic acid is not essential for maximal activity since the palmitic, oleic, and cyclopropane fatty acids of bacterial DPG can meet the functional requirements at the high-affinity sites of the complex once the sites are saturated. The key structural feature of PL's capable of binding to the high-affinity sites and regeneration of activity must be either the unique structure of DPG with its four hydrocarbon chains

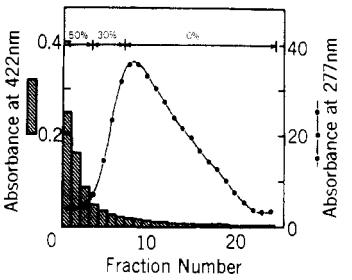

FIGURE 3: Separation of excess TX and DPG from cytochrome *c* oxidase after regeneration of full activity by exogenous DPG using discontinuous glycerol gradient centrifugation in TX. 2.5×10^{-7} M lipid-depleted cytochrome *c* oxidase (molecular activity = 112 s^{-1}) was incubated with 65 μM bovine DPG in 1% TX, pH 8.1 buffer (total volume of solution was 40 mL) for 2 h. Equal volumes of the incubation solution were layered on top of each of two discontinuous glycerol gradients composed of 3.5 mL of 50% glycerol and 4.0 mL of 30% glycerol each containing 0.2% TX, pH 8.1 buffer and centrifuged for 16 h at 45 000 rpm at 5 °C with a 50.2 Ti rotor. After centrifugation, 1.2-mL fractions were collected from the bottom of the tube by using a long needle and a peristaltic pump set at 1.2 mL/min. The cytochrome *c* oxidase concentrations in each fraction was determined by measuring the A_{422} (▣); the TX concentration of each fraction, and presumably the excess unbound DPG, was determined by measuring the A_{277} (●).

or the high fluidity of the hydrocarbon tails that is the common feature of both types of DPG.

Isolation of DPG-Reactivated Cytochrome c Oxidase. The DPG-reactivated, phospholipid-depleted cytochrome *c* oxidase was reisolated from the TX–DPG incubation mixture in order (1) to measure the total binding of exogenous DPG to the lipid-depleted complex and (2) to prove that regeneration of activity was due to reassociation of DPG with the high-affinity sites on the complex. A similar reisolation of the complex after incubation with PC was performed and used as a control (1) to determine the amount of binding of a PL that could not reactivate the complex and (2) to assess the effects of the reisolation procedure upon the enzymatic activity of enzyme that had not been reactivated by DPG. After incubation of 2.2 mg of delipidated cytochrome *c* oxidase [0.05 mg of protein/mL with 100 μg/mL DPG or PC in 1% TX (65 μM DPG; 130 μM PC)], the protein–PL–TX complex was reisolated by centrifugation through a discontinuous glycerol gradient. This gradient served the purpose of removing excess unbound PL and also of concentrating the enzyme. The separation of the DPG reactivated complex from the excess DPG–TX micelles is shown in Figure 3. (The separation obtained after incubation of the complex with PC was identical with that shown for DPG.) The 50% glycerol layer at the bottom of the gradient prevented the pelleting of the cytochrome *c* oxidase on the bottom of the centrifuge tube and avoided the loss of enzymatic activity due to protein aggregation; the 30% glycerol layer allowed the cytochrome *c* oxidase to sediment but greatly retarded the sedimentation of TX and excess DPG, thereby facilitating the separation of the complex from the excess unbound DPG. The bottom two layers of the gradient contained 0.2% TX rather than 1% TX to avoid reextraction of the bound DPG during the reisolation of the complex. By use of this procedure, a nearly complete separation of excess TX and PL from the cytochrome *c* oxidase complex was possible (refer to Figure 3).

The activity and amount of bound PL were determined for both the reisolated DPG and PC complexes. Table I compares the results of these determinations with the values obtained for the delipidated complex prior to its incubation with PL and for the delipidated complex after incubation with either DPG or PC but prior to reisolation of the complex by glycerol

Table I: Activity of Lipid-Depleted Cytochrome *c* Oxidase before and after Removal of Unbound Exogenous PL by Glycerol Gradient Centrifugation

sample	before glycerol gradient centrifugation: molecular act. (s^{-1})	before glycerol gradient centrifugation: g-atoms of P/mol of heme aa_3	after glycerol gradient centrifugation: molecular act. (s^{-1})	after glycerol gradient centrifugation: g-atoms of P/mol of heme aa_3
lipid-depleted cyt *c* oxidase (no additions)	112	3.4		
lipid-depleted cyt *c* oxidase + DPG [a]	150		142	7.6
lipid-depleted cyt *c* oxidase + PC [b]	114		104	3.0

[a] 2.5×10^{7} M enzyme was incubated with 65 μM bovine DPG in 1% TX, pH 8.1 buffer. [b] 2.5×10^{-7} M enzyme was incubated with 130 μM PC in 1% TX, pH 8.1 buffer.

gradient centrifugation. The activities obtained after reisolation of each complex were very similar to the activities observed for each complex before excess PL was removed: the DPG complex exhibited nearly complete regeneration of activity, and the PC complex exhibited activity very similar to the activity of the complex prior to the addition of PL. Reisolation of each complex showed a slight decrease of activity, but the ratio of the activity of the DPG complex to the activity of the PC complex was 1.35 before and after reisolation by glycerol gradient centrifugation in TX. Clearly, the excess PL that was present in the incubation mixture did not influence the activity after dilution into the Tween 80 assay solution, indicating that the data collected by this method, i.e., the data in Figures 1 and 2, accurately reflected the formation of a protein–PL complex in the TX solution. Phospholipid analysis of the isolated complex after each PL incubation indicated that the amount of bound PL approximately doubled after incubation with DPG (ca. 4 mol of DPG was now bound to the enzyme) while the amount of bound PL after incubation with PC did not change. Evidently DPG, but not PC, is capable of reassociating with the essential high-affinity sites of cytochrome *c* oxidase in the presence of 1% TX which probably explains the regeneration of full activity by DPG but the lack of stimulation of activity by PC.

Discussion

The results described in this communication are the first reported measurements of the binding affinity of DPG to the essential high-affinity DPG sites of cytochrome *c* oxidase. These results provide clear evidence for the functional specificity of DPG at these sites and for its high binding affinity. By incubating the lipid-depleted enzyme with various PL's in the presence of 1% TX followed by the assay of the complex in Tween 80, we have been able to make the following conclusions concerning the binding of phospholipids to the essential high-affinity sites on cytochrome *c* oxidase.

First, cytochrome *c* oxidase has a very high affinity for DPG at the essential PL sites; the apparent dissociation constant for bovine DPG is 5 μM even in the presence of 1% TX. It should be noted, however, that this value is a measure of the relative affinity of DPG for the high-affinity sites since the process being followed in our experiments is an exchange reaction between micelle-bound DPG and protein-bound DPG. If TX were bound to the high-affinity sites prior to DPG addition, the absolute value of this constant would be affected since TX would be competing with the exogenous DPG. Therefore, the relative dissociation constant that we measure does not represent the absolute affinity of the enzyme for DPG; it should, however, more closely approximate the binding of DPG to the enzyme when it is embedded in the inner mitochondrial membrane since in that case the association of DPG with the high-affinity sites would also be an exchange reaction between DPG dissolved in the fluid phospholipid bilayer and DPG bound to the enzyme.

Second, the high-affinity PL sites of cytochrome *c* oxidase have a definite functional specificity for DPG. None of the other PL's tested (PE, PC, PG, PS, or PA) were able to stimulate the activity of the lipid-depleted enzyme more than 5–10% by our method. Therefore, of the major PL's found in the inner mitochondrial membrane, only DPG is capable of restoring full activity to the lipid-depleted enzyme when it is assayed in Tween 80. These results are similar to those of Yu et al. (1975), who found that DPG stimulated the activity of PL-depleted cytochrome *c* oxidase much more than either PC or PE when the enzyme was assayed in Emasol-1130.

Third, the overall structure of DPG, not the negative charge on this PL, appears to be the factor controlling its functional specificity for the high-affinity sites; neither PG, PS, or PA, all of which are negatively charged, was able to stimulate activity. Whether these other negatively charged PL's are able to bind to the high-affinity sites in the presence of 1% TX, but are incapable of affecting the electron transport activity, is not presently known since only the stimulation of the enzymatic activity of lipid-depleted cytochrome *c* oxidase, not the true binding of these PL's, was measured. However, PC was found not to reassociate with the high-affinity sites in the presence of 1% TX (refer to Table I), which suggests that PE, PG, PS, and PA may not reassociate with these sites.

Fourth, the fatty acid composition of DPG may slightly affect its binding to these high-affinity sites, but it is not the controlling structural feature necessary for the regeneration of full activity. This is evident from the data presented in Figure 2 which show that bacterial DPG containing a completely different fatty acid composition from bovine DPG had only a slightly different functional affinity for the essential sites than bovine DPG ($K_{d,app}$ = 2 μM for bacterial DPG; $K_{d,app}$ = 5 μM for bovine DPG). Clearly, the high percentage of linoleic acid found in bovine DPG, but not found in bacterial DPG, is not responsible for the functional binding of this PL to cytochrome *c* oxidase.

Fifth, in the presence of 1% TX, DPG can reassociate only with the essential high-affinity sites of cytochrome *c* oxidase, the low-affinity sites being blocked by the TX in the incubation mixture (refer to Table I). After incubation of the lipid-depleted enzyme with DPG and reisolation of the resulting reconstituted complex in the presence of TX, the DPG content increased from 1.8 mol of DPG bound/complex to 3.8 mol of DPG/complex. This value is very close to our previous estimate of 3 tightly bound DPG mol/complex that are necessary for full activity (Robinson et al., 1980).

Finally, it should be noted that Watts et al. (1978) have reported that yeast cytochrome *c* oxidase does not have an absolute requirement for DPG at the high-affinity sites since they achieved 47% activity after complete exchange of exogenous PC for endogenous DPG using a cholate-mediated exchange method. Their result is not necessarily in disagreement with our findings since we had two-thirds of the initial activity remaining after removal of approximately half of the essential DPG; thus, we potentially could have some

residual activity remaining in Tween 80 after removal of all of the high-affinity DPG. It is also possible that the bovine enzyme and the yeast enzyme are not equivalent since Vik & Capaldi (1977) have tried this same exchange experiment with the bovine enzyme and reported that exchange did not occur.

The conclusions reached in this study together with the earlier results of Vik & Capaldi (1977) and Robinson & Capaldi (1977) on the specificity of the low-affinity boundary layer sites of cytochrome *c* oxidase enable us to specify the PL requirements of this electron transport complex. The high-affinity sites must be occupied by DPG in order to achieve maximal activity. Once this requirement is satisfied, the activity of the complex becomes dependent only upon the hydrocarbon environment present at the boundary layer of the complex. Electron transport activity is about twice as great when the phospholipids or detergents at the boundary layer contain unsaturated hydrocarbon tails, e.g., C18:1, than it is when the bound amphiphiles contain saturated tails, e.g., C16:0. Although hydrocarbon fluidity at the boundary layer appears to greatly influence the activity of the complex, little head group specificity exists at these low-affinity amphiphile sites. Approximately the same activity is observed for the complex when the boundary layer is composed of asolectin, a crude mixture of soybean PL's, or PC, PE, or lyso-PC containing C18:1 tails. Even synthetic detergents such as Tween 80 or Brij 96 which also contain C18:1 tails have almost the same ability to maintain full activity.

With an understanding of the PL and/or detergent requirements of cytochrome *c* oxidase, it will be much easier to compare and understand the significance of the wide variety of results that have been obtained for this complex after various PL or detergent treatments. This is particularly true of the various delipidated cytochrome *c* oxidase complexes that have been prepared. It now remains to be established how these PL requirements correlate with the structural organization of the protein in this multisubunit complex, but by use of the methods described in this paper for the reversible removal and reassociation of PL's with both the high- and low-affinity sites, this question can now be addressed.

Acknowledgments

I am grateful to Fredlein Strey and Linda Talbert for their excellent technical assistance during the course of this work and to Dr. LeAnn Robinson for her invaluable help in writing this manuscript.

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