THE SPECIFICITY AND AFFINITY OF PHOSPHOLIPIDS FOR CYTOCHROME *c* OXIDASE

NEAL C. ROBINSON
Department of Biochemistry, The University of Texas Health Science Center at San Antonio, San Antonio, Texas 78284 U.S.A.

Beef heart cytochrome *c* oxidase has been shown to have a boundary layer of ~40 phospholipids (PL's) per heme aa_3 complex as determined by the immobilization of spin-labeled lipid probes (1, 2). We have been able to subdivide these boundary layer PL's into two main subclasses based upon their ability to exchange with nonionic detergents, e.g., Triton X-100 (TX), Tween 80 or Tween 20, and to measure the effect of this exchange upon the activity of the resulting PL- depleted complexes (3). Most of the boundary layer PL's are not essential for the full activity of the complex since the molecular activity of cytochrome *c* oxidase is unaltered after exchange of all but about four of the PL's by the nonionic detergent Tween 80. The remaining tightly bound PL's are entirely diphosphatidylglycerol. These few DPG molecules are essential for full activity because their removal results in decreased activity which can be reconstituted by exogenous DPG but not by exogenous phosphatidylcholine (PC) or phosphatidylethanolamine (PE)(3). This report describes an attempt to define the functional PL specificity of the about four essential high affinity sites on cytochrome *c* oxidase by measuring the effectiveness of a variety of PL's in restoring the full activity of the enzyme that had 40–60% of these essential DPG's removed by detergent exchange.

METHODS

Cytochrome *c* oxidase was depleted of all of its boundary layer PL's with the exception of 1.8 mol DPG/heme aa_3 complex by the TX-glycerol gradient method of Robinson et al. (3). This preparation had 60–65% of the electron transport activity of cytochrome *c* oxidase containing 4–40 mol PL/heme aa_3 complex when assayed in Tween 80 because of the partial removal of the 3–4 essential DPG. Full activity could be reconstituted by exogenous bovine DPG. All enzyme activities were measured spectrophotometrically by dilution of a 5-μl aliquot of the enzyme in 1% TX (2.3×10^{-7} M heme aa_3 and 0–300 μM exogenous PL) into an assay cuvette at 25.0°C containing 0.7 mL of 30 μM reduced cytochrome *c*, 0.5% Tween 80, and 0.01 M sodium phosphate buffer at pH 7.0. Molecular activities were calculated from the first order rate constants as described by Vanneste et al. (4). The PL content of cytochrome *c* oxidase before and after the addition of exogenous PL's was determined by the phosphorous assay of Chen et al. (5) after: (*a*) extraction of PL; (*b*) removal of the extracted TX from PL by thin-layer chromatograph in acetone; (*c*) digestion of the silica gel scraped from the plate with percholoric acid.

RESULTS AND DISCUSSION

The head group specificity of the essential high affinity PL sites of cytochrome *c* oxidase was determined by measuring the increase in the molecular activity of the lipid depleted complex in Tween 80 after its incubation with various PL's dissolved in 1% TX (Fig. 1 *A*). This assay could be used to assess the functionally competent binding of PL's to the vacant high affinity sites on the enzyme because the activity of cytochrome *c* oxidase in Tween 80 is proportional to the DPG occupancy of these sites, e.g., enzyme with ~60% of the essential DPG remaining had 60–65% of the activity of enzyme containing all of the essential DPG. Of the PL's tested only DPG was effective

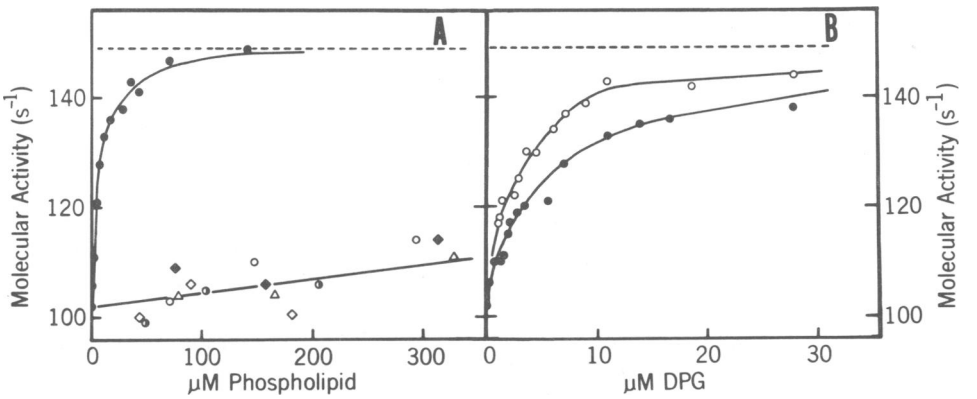

FIGURE 1 The effect of exogenous PL's upon the activity of lipid-depleted cytochrome *c* oxidase. All PL-enzyme incubations and determinations of molecular activities were performed as described in Methods. The dashed line in each panel represents the molecular activity of cytochrome *c* oxidase prior to delipidation. *A*, 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, (Δ) PG, and (◇) PA, respectively, prior to assaying the PL-TX-cytochrome *c* oxidase complex in 0.5% Tween 80. *B*, Cytochrome *c* oxidase was incubated in 1% TX, pH 8.1 buffer with (●) bovine DPG or (○) bacterial DPG prior to assaying the complex as described for Panel *A*. Each data point was the average of 4–8 separate activity measurements.

in restoring the initial molecular activity of cytochrome *c* oxidase. Other PL's, even those having a net negative charge, i.e., phosphatidylglycerol (PG), phosphatidylserine (DS), and phosphatidic acid (PA), were unable to stimulate the activity more than 5–10%. One possible explanation for the apparent specificity for DPG is its unique structure: two phosphatidic acids joined by a central glycerol molecule. This structure might permit DPG to form a bridge between two separate hydrophobic sites which none of the other PL's would be able to do.

Another structural difference between DPG and the other PL's is its high percentage of linoleic acid (bovine DPG contains 90% $C_{18:2}$ fatty acyl chains). To assess the importance of the high proportion of linoleic acid upon the restoration of full activity, the effectiveness of bacterial DPG was measured and compared with bovine DPG (bacterial DPG contains 32% $C_{16:0}$, 38% $C_{18:1}$, 0% $C_{18:2}$, and 30% cyclopropane fatty acids). Either type of DPG was capable of stimulating the activity of lipid depleted cytochrome *c* oxidase see Fig. 1*B*. Analysis of this data using double reciprocal plots allowed an estimation of the apparent binding affinities of these two types of DPG ($K_{d_{app}}$ = 5 μM for bovine DPG, $K_{d_{app}}$ = 2 μM for bacterial DPG). These values indicate a remarkably high affinity of the lipid depleted complex for either type of DPG, considering that there were 650 TX micelles present for every TX-cytochrome *c* oxidase complex during the PL incubations. In fact, only an eight-fold molar excess of bacterial DPG per heme aa_3 complex was sufficient to achieve one-half maximal stimulation of activity (a 20-fold molar excess of bovine DPG was required). This corresponds to a 50% reconstitution of full activity, i.e., 50% of the vacant high affinity sites filled with exogenous DPG, when only 1.3% of the TX micelles contain a DPG molecule. Clearly, the exogenous DPG has a higher affinity for the cytochrome *c* oxidase complex than it has for the TX micelles.

We are now measuring the binding of PL's to lipid depleted cytochrome *c* oxidase rather than just measuring the functional restoration of full activity in order to distinguish between PL's that are able to bind but are unable to meet the functional requirements and PL's that are unable to bind. To date, we know that restoration of full activity occurs when 2.1 mol of exogenous DPG reassociate per mol of heme aa_3 if the complex is incubated with 65 μM DPG in the presence of 1% TX (total DPG = 3.9 mol/heme aa_3), and that PC does not bind at all to the lipid depleted complex when it is incubated with 130 μM PC in the presence of 1% TX. We are now in the process of synthesizing radioactively labeled PL's to assess the binding of all of these PL's directly.

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CYTOCHROME *c* OXIDASE-LIPID INTERFACE FROM THE PROTEIN SIDE

G. GEORGEVICH AND R. A. CAPALDI

Institute of Molecular Biology and Department of Biology, University of Oregon, Eugene, Oregon 97403 U.S.A.

Cytochrome *c* oxidase, the terminal member of the electron transport chain, is an intrinsic part of the mitochondrial inner membrane. Electron microscopy and image reconstruction studies of two-dimensional crystals of the beef heart enzyme reveal a Y-shaped protein (1, 2). The stalk of the Y or the C domain is located outside the lipid bilayer, extending ~ 50 Å from the cytoplasmic face of the inner membrane. The arms of the Y, or two M domains, each span the lipid bilayer, extending a short distance from the matrix side of the inner membrane. Each M domain is ~ 20 Å in diameter and these two domains are separated in the bilayer by ~ 40 Å center to center (1, 2).

Beef heart cytochrome *c* oxidase is composed of at least seven different subunits (3, 4). The largest subunits (I–III) are coded for on mitochondrial DNA while the rest are made in the cytoplasm (5). Labeling studies using the water-soluble, membrane-impermeant, protein-modifying reagents [^{35}S]diazobenzenesulfonate and [^{35}S]NAP taurine have shown that subunits II and III constitute a major portion of the C domain (6). Both of these subunits are labeled by [^{35}S]DABS from the matrix side of the mitochondrial inner membrane (6), indicating that both must span the lipid bilayer and contribute a portion of the M domains.