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

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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.

TABLE I
RELATIVE LABELING OF SUBUNITS OF CYTOCHROME *c* OXIDASE BY HYDROPHOBIC REAGENTS

Reagent	Enzyme state	Subunit number						
		I	II	III	IV	V	VI	VII(s)
PLI	vesicles	0.64	0.19	1.00	0.14	>0.05	>0.05	2.27
PLII	vesicles	0.41	0.16	1.00	0.22	>0.05	>0.05	3.76
AD*	vesicles	0.36	0.10	1.00	0.18	>0.05	>0.05	0.73
AD	0.1% Tween 80	0.40	0.08	1.00	0.12	>0.05	>0.05	0.33
AD	1.0% SDS	1.07	1.23	1.00	2.56	1.53	1.40	5.49

*AD, adamantane diazirine. SDS, sodium dodecyl sulfate. Relative labeling was determined by summing counts per minute in each subunit, dividing by the molecular weight of the polypeptide, and setting labeling of subunit III equal to 1.00.

More direct evidence concerning which subunits are within the lipid bilayer and a part of the M domains has been sought in experiments with hydrophobic labeling reagents. Beef heart cytochrome *c* oxidase has been reacted with two different arylazidophospholipids [1-palmitoyl-2-(2-azido-4-nitrobenzoyl)-*sn*-glycero-3-[^{3}H] phosphocholine (PLI) and 1-myristoyl-2-amino [*N*-2-nitro-4-azidophenyl]lauroyl)-*sn*-glycero-3-[^{14}C]phosphocholine (PLII)] and [^{3}H] adamantane diazirine, using both vesicular and detergent-dispersed preparations of enzyme (7, 8). Table I lists the relative reactivity of the different subunits with these photoaffinity labeling reagents. Subunits I and III were most heavily labeled by all of the hydrophobic reagents. There was also labeling of subunits II, IV and VII.

The different subunits of beef heart cytochrome *c* oxidase have been sequenced either from the DNA (subunits I, II, and III) or by sequencing of the protein directly (II, IV, V, VII Ser, VII Ile) (e.g., 9, 10). Fragmentation studies are now in progress to locate the sites of reaction of arylazidophospholipid and adamantane diazirine within the sequences of individual subunits. Studies to date have localized the hydrophobic probes to fragment 1–81 in subunit II and fragment 72–123 in subunit IV. These segments contain two and one hydrophobic stretches, respectively, out of a total of 14–16 in the entire cytochrome *c* oxidase molecule.

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