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Labeling of Bovine Heart Cytochrome *c* Oxidase with Analogues of Phospholipids. Synthesis and Reactivity of a New Cardiolipin Benzaldehyde Probe†

Andreas Kuppe, Randall J. Mrsny, Masato Shimizu, Sharbil J. Firsan, John F. W. Keana, and O. Hayes Griffith*
Institute of Molecular Biology and Department of Chemistry, University of Oregon, Eugene, Oregon 97403
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ABSTRACT: The syntheses of two new radioactive probes derived from cardiolipin and phosphatidylcholine are reported. These probes are derivatives of natural lipids and contain an amine-specific benzaldehyde in the head-group region. This functional group allows a choice of timing of the reaction (e.g., after equilibration and detergent removal) because an irreversible covalent bond is formed only upon the addition of reducing agent. These probes, as well as a benzaldehyde analogue of phosphatidic acid, and a water-soluble benzaldehyde reagent were covalently attached to bovine heart cytochrome *c* oxidase. After reconstitution into vesicles, the lipid–benzaldehyde probes selectively incorporated into the smaller polypeptides of the enzyme, while the remaining subunits (I–IV) exhibited little incorporation of label. The accessibility of amine groups labeled under the conditions used here was independent of the structural and charge differences between the benzaldehyde probes. This suggests that all three lipid probes react with polypeptides of the cytochrome *c* oxidase complex at general contact sites for membrane phospholipids. A water-soluble benzaldehyde reagent predominantly labeled subunits IV, Va, and Vb and polypeptides of VII–VIII. A comparison of these results facilitates a more refined view of the disposition of polypeptides of cytochrome *c* oxidase in respect to the lipid and aqueous phases.

Cytochrome *c* oxidase (ferrocytochrome *c*:oxygen oxidoreductase; EC 1.9.3.1) is the terminal enzyme of the electron transport chain in all eukaryotes and in some prokaryotes. It is a multimeric protein complex that accepts electrons from reduced cytochrome *c* and transfers them to molecular oxygen to form water. In eukaryotes, the three largest subunits are encoded by the mitochondrial genome and have been implicated in binding of the prosthetic groups (two heme irons and two copper ions) and proton pumping activity. The remaining subunits are coded for by the nuclear genome and may be involved in regulatory function of the enzyme complex. Some

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nuclear coded subunits appear to vary in composition from tissue to tissue (Kadenbach et al., 1983b; Takamiya et al., 1986).

One of the most extensively studied forms of cytochrome *c* oxidase is that obtained from bovine heart. It is currently envisioned to be comprised of 12 or 13 polypeptides (Kadenbach et al., 1983a), 12 of which have been sequenced (Buse, 1984). From these primary sequences, a number of lysine residues have been projected to reside at the lipid–protein interface and possibly interact with negatively charged phospholipids (Buse & Steffens, 1978). Proper evaluation of such potential interactions has not yet been possible due to a lack of structural information. Electron diffraction studies of two-dimensional crystals (Fuller et al., 1979) suggest the beef heart cytochrome *c* oxidase monomer to be a Y-shaped, integral membrane protein with two domains (M1, M2) protruding into the matrix space and the stalk of the Y extending far out into the C-space between the two mitochondrial membranes. These data provide a general idea of the shape of the protein but do not resolve individual polypeptides of the enzyme.

In vivo, eukaryotic cytochrome *c* oxidase resides in the inner mitochondrial membrane. Of the lipids present in this membrane, cardiolipin [diphosphatidylglycerol (DPG)[1]] and phosphatidylcholine (PC) constitute a major fraction (Stoffel & Schiefer, 1968). Cardiolipin, found almost exclusively in the inner mitochondrial membrane and in bacterial membranes, is an acidic phospholipid with two phosphatidyl groups linked via a single glycerol moiety and was the subject of considerable interest in studies on cytochrome *c* oxidase (Awasthi et al., 1971; Fry et al., 1980; Robinson et al., 1980; Powell et al., 1985; Robinson & Wiginton, 1985). This lipid was suggested to influence the catalytic activity of several mitochondrial proteins [see review in Lambeth (1985)] and might serve as a recognition site on the inner mitochondrial membrane for lipid–protein interactions (Müller et al., 1985).

Chemical labeling has proven useful for the description of the topology of intrinsic membrane proteins relative to the lipid bilayer. For example, the protein–lipid interface of cytochrome *c* oxidase has been examined with photoreactive arylazidophospholipids (Bisson et al., 1979; Griffith & Jost, 1979; Prochaska et al., 1980; Griffith et al., 1986). Upon photoactivation of the arylazido lipids, arylnitrenes are formed that insert rather nonspecifically into polypeptide stretches of individual subunits of the enzyme complex close to the probe. A more specific form of labeling was recently introduced by McMillen et al. (1986), who synthesized a radioactive benzaldehyde-containing analogue of phosphatidic acid (PA–ba) and used this probe to label free amino groups of bovine heart cytochrome *c* oxidase accessible from the lipid bilayer.

In the present study we report the syntheses of two new amine-specific lipid probes (see Figure 1): diphosphatidylglycerol–benzaldehyde (DPG–ba) and phosphatidylcholine–benzaldehyde (PC–ba). We investigated the accessibility of amino groups of bovine heart cytochrome *c* oxidase to the lipid analogues PA–ba, DPG–ba, and PC–ba and a water-soluble benzaldehyde reagent, methyl 4-[^{3}H]formylphenyl phosphate (or MP–ba, methylphosphobenzaldehyde) on high-resolution SDS–polyacrylamide gels, which separated the unlabeled enzyme into 12 or 13 polypeptides. Use of the lipid probes allows for the examination of the effects of phospholipid head-group charge and acyl chain structure on polypeptide labeling patterns. Comparison of the labeling results of lipid probes and the water-soluble benzaldehyde yields information on the arrangement of subunits with respect to the bilayer and aqueous phases and the distribution of free amines over the protein complex.

Experimental Procedures

Synthesis of Benzaldehyde Labels. Synthetic routes are outlined in Figure 2. The procedures were first worked out with nonradiolabeled materials. After satisfactory chromatographic and spectral analyses of key intermediates and final products were obtained, the syntheses were repeated with the radiolabeled compounds.

4-Formylphenyl Isocyanate (**5**). For protection of the formyl group, *p*-nitrobenzaldehyde (**4**) (15.1 g, 0.1 mol) was converted into the corresponding dimethyl acetal by using dimethyl sulfate (25.2 g, 0.2 mol; freshly distilled) and methanol as described by Schmitz (1958). Distillation afforded *p*-nitrobenzaldehyde dimethyl acetal (17.3 g, 87%) as a pale yellow oil: bp 130–131 °C (3 mmHg); IR ($CHCl_3$) 1525 and 1350 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 3.30 (s, 6 H, 2 OCH_3), 5.50 [s, 1 H, $CH(OCH_3)_2$], 7.65 (d, J = 9.1, 2 H, aromatic H's), 8.23 (d, J = 9.1, 2 H, aromatic H's).

The nitroacetal (5.07 g, 25.7 mmol) was dissolved in 50 mL of absolute ethanol and reduced over W_7 Raney Ni (about 1 mL of settled catalyst) in a Parr low-pressure hydrogenator for 21 h at 40 psi and 25 °C. The reaction mixture was filtered through a Celite pad and washed with ethanol, and the solvent was removed under reduced pressure. Rapid short-path distillation using base-washed glassware afforded pure *p*-aminobenzaldehyde dimethyl acetal (3.8 g, 88%) as a pale yellow oil: bp 85–86 °C (0.25 mmHg); ^{1}H NMR ($CDCl_3$) δ 3.29 (s, 6 H, 2 OCH_3), 3.71 (br s, 2 H, NH_2), 5.28 [s, 1 H, $CH(OCH_3)_2$], 6.63 (d, J = 8.7, 2 H, aromatic H's), 7.20 (d, J = 8.7, 2 H, aromatic H's). This aminoacetal decomposed slowly to an orange solid at 25 °C under nitrogen but could be stored for several months at −20 °C under nitrogen.

The isocyanate **5** was obtained by dropwise addition of a solution of *p*-aminobenzaldehyde dimethyl acetal (172.0 mg, 1.03 mmol) in dry AcOEt (4 mL, distilled from CaH_2) to a cold (ice bath), stirred solution of excess phosgene (10.3 mmol) in toluene (8.2 mL, Fluka) and dry AcOEt (4 mL). The resulting suspension was refluxed for 1 h in a dry ice–acetone condenser to avoid loss of phosgene. Gravity filtration of the dark red suspension and evaporation of the filtrate afforded crude *p*-formylphenyl isocyanate (110 mg) as a moist, pale orange solid: IR (Nujol) 2260 and 1705 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 7.25 (d, J = 8.3, 2 H, aromatic H's), 7.86 (d, J = 8.3, 2 H, aromatic H's), 9.96 (s, 1 H, HCO). Isocyanate **5** is not stable at 25 °C even under high vacuum and must be stored in a freezer under N_2. Freshly prepared **5** was always used in subsequent steps.

1,3-Bis[(1,2-di[^{3}H]acyl-3-sn-glycero)phospho]glycerol Dimethyl Ester (**2**). The disodium salt of DPG (**1**) (Sigma; from bovine heart), which contains predominantly (87%) linoleic acid (Powell & Marsh, 1985), was converted into the dimethyl ester as previously described (Keana et al., 1986). Catalytic hydrogenation of DPG dimethyl ester with tritium

[1] Abbreviations: DOPC, 1,2-dioleoyl-3-*sn*-phosphatidylcholine; NMR, nuclear magnetic resonance; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; SHE, 250 mM sucrose, 50 mM HEPES, and 1 mM EDTA at pH 7.4; 1% SHEC, 1% cholic acid (w/v) in SHE at pH 7.4; cm, complex multiplet; br, broadened peak; d, doublet; t, triplet; s, singlet; *J*, spin–spin coupling constant in hertz; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PA, phosphatidic acid; DPG, diphosphatidylglycerol; BA, benzaldehyde; MP–ba, sodium methyl 4-[^{3}H]formylphenyl phosphate; TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)-aminomethane.

gas was carried out in an apparatus consisting of two two-neck, round-bottom flasks. Dry 2-propanol (0.5 mL) and platinum oxide (40.0 mg) were introduced into the reactor flask, 50% aqueous acetic acid (0.15 mL) was charged into the generator flask, and the apparatus was flushed with nitrogen. A solution of sodium borohydride (9.45 mg, 0.25 mmol) in 0.15 mL of 1 M aqueous sodium hydroxide was injected into the generator flask over a 3-min period, and the hydrogen gas was flushed out of the apparatus with nitrogen after 15 min. A solution of DPG dimethyl ester (81.0 mg, 0.055 mmol) in 1.5 mL of 2-propanol was injected into the reactor flask, and a solution of sodium borotritide (25 mCi, 0.018 mmol; ICN) and sodium borohydride (8.78 mg, 0.232 mmol) in 0.15 mL of 1 M aqueous NaOH was added dropwise to the generator flask over 4 min. After 2 h at 25 °C, the apparatus was purged with nitrogen and excess tritium gas was absorbed into a solution of cyclohexene (152.0 mg, 1.85 mmol), 10% Pd–C (30 mg), and ethanol (2 mL) contained in a trap that was connected to the reactor vessel with a stainless steel needle. The contents of the reactor were filtered through a Celite pad (250 mg, prewashed with 5 mL of 2-propanol, 5 mL of ethanol, and 10 mL of 50% ethanol–ether), and the pad was rinsed with 2-propanol (4 mL), 50% ethanol–ether (20 mL), and ethanol (5 mL). The combined filtrate was evaporated to dryness affording **2** (80.0 mg, 98%) as a white powder. The ^{1}H NMR spectrum of unlabeled **2** lacked the signals from the olefinic and allylic protons found in the spectrum of the starting material, suggesting complete reduction of the acyl chains: ^{1}H NMR ($CDCl_3$) δ 0.88 (t, J = 6.6, CH_3), 1.26 (br s, CH_2), 1.61 (m, CH_2CH_2COO), 2.31 (t, J = 7.9, CH_2COO), 2.34 (t, J = 7.7, CH_2COO), 3.79 (d, J = 11.3, P–OCH_3), 3.80 (d, J = 11.3, P–OCH_3), 4.05–4.28 (m), 4.33 (dd, J_1 = 4.0, J_2 = 12.1, H_AH_BOCO), 5.18–5.30 (m, CHOCO).

Disodium 2-[(4-Formylphenyl)carbamoyl]-1,3-bis[(1,2-di[^{3}H]acyl-3-sn-glycero)phospho]glycerol (**3**) (*DPG–ba*). All of **2** (80.0 mg, 0.054 mmol), freshly prepared *p*-formylphenyl isocyanate (55.1 mg, 0.375 mmol), and dry pyridine (1.8 mL) were stirred for 75 min at 25 °C. Evaporation of the solvent under reduced pressure resulted in a yellow residue that was purified by preparative TLC (ether–methanol, 97:3). The dimethyl ester of **3** was isolated as a pale yellow oil (44.0 mg, 50%) and was heated at reflux for 20 min in the presence of sodium iodide (10.1 mg, 0.067 mmol) in 2.25 mL of dry methyl ethyl ketone (MEK). Cooling the reaction mixture to 0 °C precipitated a pale yellow solid, which was collected by centrifugation and rinsed with two 0.25-mL portions of MEK. The yield of the disodium salt was 40.9 mg (92%), and its specific radioactivity was 13.2 mCi/mmol. This product cochromatographed with an unlabeled sample prepared for spectral analysis.

1-[1-^{14}C]Palmitoyl-2-[(4-formylphenyl)carbamoyl]-sn-glycero-3-phosphocholine (*PC–ba*). Dry, unlabeled 1-lyso-2-palmitoylphosphatidylcholine (6.0 mg, 0.012 mmol; Sigma) and 4-(dimethylamino)pyridine (1.9 mg, 0.015 mmol; Aldrich) were combined with a 1:1 toluene–ethanol solution (1.0 mL) of L-1-lyso-2-[1-^{14}C]palmitoylphosphatidylcholine (0.45 mg, 0.00091 mmol; with a specific radioactivity of 55.0 mCi/mmol, New England Nuclear). The solvent was removed completely with a stream of nitrogen, and the residual white solid was further dried under reduced pressure for 1 h. Crude isocyanate **5** was washed with three 2-mL portions of pentane, and the pentane washes were combined and evaporated under reduced pressure. The residue was dissolved in 2 mL of deuteriated chloroform, and the amount of **5** in solution was determined from the ^{1}H NMR spectrum against 1,2-dichloroethane as an internal standard. This solution, containing 15.3 mg of **5** (0.104 mmol), was added to the dried phosphatidylcholine, and the volume of the resulting solution was reduced to about 0.5 mL with a stream of nitrogen. After 3 h of stirring under N_2 at 25 °C, the solution was applied along the 10-cm edge of a 10 × 20 cm analytical TLC plate (Analtech, SiO_2 GF, 250 m), which had been preeluted with $CHCl_3$–MeOH–NH_4OH (65:25:5), $CHCl_3$–MeOH (2:1), and $CHCl_3$ and air-dried. The plate was developed in $CHCl_3$–MeOH–NH_4OH (65:25:5) and visualized with UV light (254 nm). An intense band at R_f = 0.30 was scraped off the plate and extracted 5 times with 2-mL portions of $CHCl_3$–MeOH (2:1). The combined extracts were evaporated to dryness with a nitrogen stream, the residual solid was transferred to a tared flask with five 0.3-mL portions of dry chloroform, and the solvent was removed completely with a stream of nitrogen. This yielded 2.1 mg (25%) of radiolabeled aldehyde carbamate (specific radioactivity 2.4 mCi/mmol) as an almost colorless solid that cochromatographed with similarly prepared unlabeled material. The ^{1}H NMR ($CDCl_3$) spectrum of unlabeled PC–ba exhibited the following resonances: δ 0.91 (t, J = 6.3, 3 H, CH_3CH_2), 1.21 (s, 10 H, 5 CH_2), 1.27 (s, 14 H, 7 CH_2), 1.53 (unresolved m, 2 H, CH_3CH_2), 2.26 (t, J = 7.6, 2 H, CH_2COO), 3.27 (s, 6 H, 2 NCH_3), 3.32 (s, 3 H, NCH_3), 3.82 (br s, 2 H), 4.05 (br s, 2 H), 4.33 (br m, 4 H), 5.24 (br s, 1 H, CHO), 7.81 (s, 4 H, aromatic H's), 9.87 [s, 1 H, HC(=O)], 9.94 (br s, 1 H, NH); the spectrum exhibited a water peak at δ 2.15 (br s). Satisfactory C, H, and N analysis was obtained for the unlabeled compound.

Materials and Assays. Cytochrome *c* oxidase was prepared by fractional ammonium sulfate precipitation of beef heart submitochondrial particles after the method of Yu et al. (1975) with subsequent cytochrome *c* affinity chromatography (Thompson & Ferguson-Miller, 1983) in 1% (w/v) cholate, 250 mM sucrose, 50 mM HEPES, and 1 mM Na_2EDTA (1% SHEC buffer) at pH 7.4. The final preparation, containing 11.5 nmol of heme *a*/mg of protein and having an activity of 129 nmol of O_2 s^{-1} (nmol of heme *a*)$^{-1}$, was stored in this buffer at 10.1 mg/mL at −20 °C. Labeled samples of cytochrome *c* oxidase do not show activity, presumably due to the poisoning with cyanide released from the reducing agent ($NaCNBH_3$). Analysis demonstrated 12–13 mol of lipid phosphorus/mol of protein, which was comprised of 3–3.5 DPG, 3 PE, 1.5–2 PC, and less than two other lipids per molecule of the enzyme. L-α-Dioleoylphosphatidylcholine (99% pure) was from Sigma. Specially pure SDS was obtained from BDH, and ultrapure urea was from Schwarz/Mann. Acrylamide, *N,N'*-methylenebis(acrylamide), and Coomassie Brilliant Blue R-250 were from Eastman Kodak. Cholic acid (Sigma) was recrystallized as described by Silvius et al. (1984) prior to use. Special enzyme grade ammonium sulfate and sucrose were procured from Schwarz/Mann. EDTA was from Matheson Coleman and Bell. All other chemicals were of reagent grade or better, purchased from commercial sources.

Protein was determined by the method of Smith et al. (1985) using bovine serum albumin (Miles Pentex fraction V, fatty acid poor) as a standard. Heme *a* was determined by the method of Yonetani (1961). Phospholipid was extracted by the method of Bligh and Dyer (1959) in the presence of ammonium hydroxide (Awasthi et al., 1971) and quantitated as described by Mrsny et al. (1986) after digestion at 130 °C overnight. $NaH_2PO_4 \cdot H_2O$ (J. T. Baker) was used as the standard for phosphate analysis. Characterization of endogenous phospholipids was performed by two-dimensional thin-layer chromatography on silica gel G (Merck) analytical

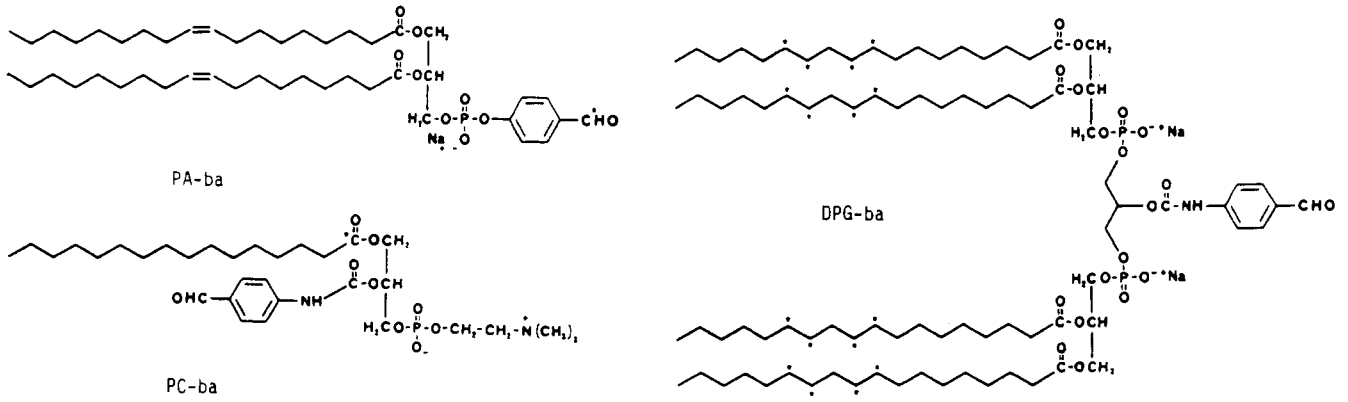


FIGURE 1: Structures of benzaldehyde-containing lipid probes used in this study. Radioactive atoms are indicated by a star.

plates using the solvent system of Parsons and Patton (1967). Cytochrome *c* oxidase activities were measured polarographically in the presence of 0.5% Tween 80 as described by Vik and Capaldi (1980).

Covalent Labeling of Cytochrome c Oxidase. Labeling of cytochrome *c* oxidase following reconstitution into vesicles of DOPC was performed as described previously (McMillen et al., 1986) except that the buffer used was SHE, pH 7.4 (250 mM sucrose, 50 mM Hepes, 1 mM Na_2EDTA) and labeling was performed at a protein concentration of about 3 mg/mL. Labeling of cholate-solubilized cytochrome *c* oxidase was done in 1% SHEC (1% cholic acid, w/v, in SHE, pH 7.4) at a protein concentration of 6 mg/mL. Label dissolved in chloroform was dried in a small Potter-type glass homogenizer under a stream of argon and for at least 2 h in a high vacuum before addition of 1% SHEC, pH 7.4, containing protein at the desired concentration. Following homogenization, carefully avoiding the formation of bubbles, the samples were incubated with constant stirring for 30 min at 4 °C and reacted in 100 mM sodium cyanoborohydride and 10 mM hydroxylysine, analogous to experiments where cytochrome *c* oxidase was reconstituted into vesicles. Samples of labeled cytochrome *c* oxidase were stored frozen at −20 °C before gel electrophoresis.

SDS–Polyacrylamide Gel Electrophoresis. Slab gels of up to 23 cm in length were prepared essentially as described previously (Kadenbach et al., 1983a). These contained final concentrations of 16.2% acrylamide, 0.43% bis(acrylamide), 6 M urea, and 0.1% SDS at pH 8.6 and 7.2% acrylamide, 0.19% bis(acrylamide), 6 M urea, and 0.1% SDS at pH 6.2 in the separating and stacking gels, respectively. The electrode buffer consisted of 192 mM glycine, 25 mM Tris base, and 0.1% SDS at pH 8.6. Labeled protein samples were dissociated by addition of an equal volume of a buffer of 16 M urea and 10% SDS in 0.5 M Tris-HCl (pH 6.2). Gels were run at 150 V (constant) and stained with 0.5% Coomassie Blue R-250 in methanol–water–glacial acetic acid (5:5:1 by volume) at room temperature with gentle shaking. Destaining was achieved by shaking in 2-propanol–water–glacial acetic acid (1:8:1 by volume). Destained gels were swollen in water and photographed. Gel scans were obtained from the photographs with a Zeineh soft laser scanning densitometer (SL-504-XL). Lanes were cut out from the gel with a razor blade, and a Hoeffer gel slicer was used to obtain 1-mm slices. These were digested in 1 mL of 30% hydrogen peroxide at 70 °C overnight. Aquassure (7 mL, New England Nuclear) was added, and radioactivity was measured in a Beckman LS 7000 liquid scintillation counter.

RESULTS

Syntheses of Benzaldehyde Labels. Syntheses of DPG–ba and PC–ba involved attachment of a benzaldehyde group to a secondary alcohol group (Figure 1). Recognizing that phenyl isocyanates react rapidly with most primary and secondary alcohols to give carbamates, *p*-formylphenyl isocyanate (**5**) was synthesized. The two phosphate groups of DPG were blocked by methylation, and the resulting dimethyl ester was reacted with the isocyanate (Figure 2). The dimethyl ester was chosen since the alcohol group of DPG itself shows a low reactivity toward acylation. For the preparation of a tritium-labeled DPG benzaldehyde we had planned to follow a route that paralleled our earlier synthesis of PA–[^{3}H]ba (McMillen et al., 1986). This would have involved reduction of the benzaldehyde dimethyl ester with sodium boro[^{3}H]-hydride and regeneration of the aldehyde by oxidation of the resulting labeled benzyl alcohol. However, in practice runs the DPG benzaldehyde dimethyl ester could not be cleanly reduced to the corresponding benzyl alcohol with sodium borohydride, even though a model carbamate, *N*-(isopropoxycarbonyl)-*p*-formylphenylalanine, gave the corresponding benzyl alcohol in high yield under similar conditions. Tritium was therefore introduced by catalytic reduction with ^{3}H–H generated from sodium boro[^{3}H]hydride and aqueous acid. The reduction was carried out on the DPG dimethyl ester since

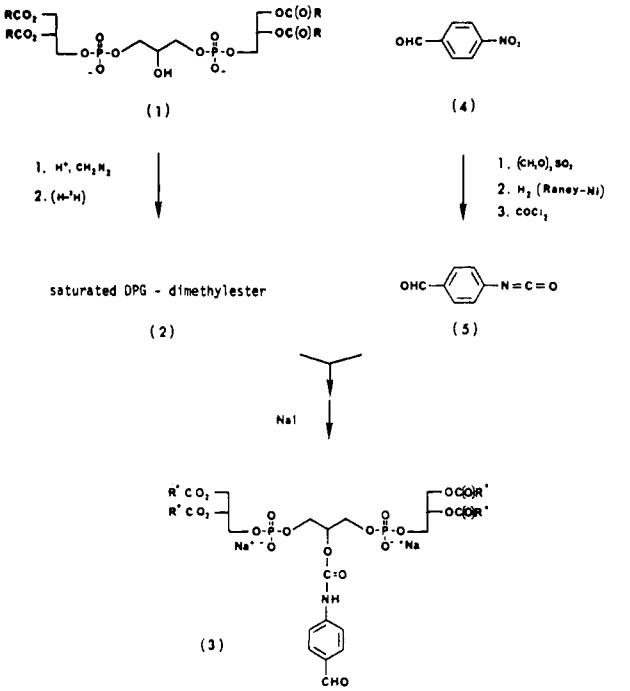


FIGURE 2: Overview of the synthetic route followed to obtain DPG–ba. R represents lipid chains of natural cardiolipin and R* the saturated hydrocarbon tails after reduction. See Experimental Procedures for details of syntheses.

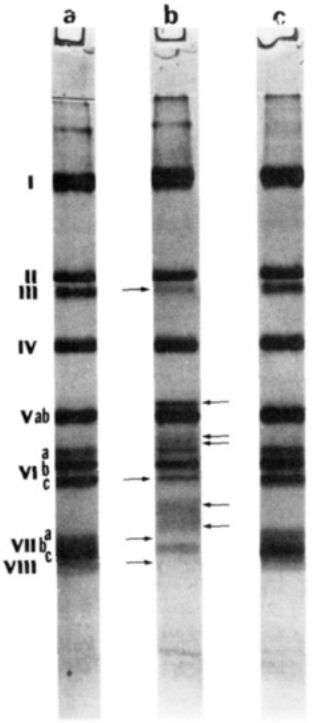


FIGURE 3: High-resolution SDS–PAGE demonstrating separation of polypeptides of bovine heart cytochrome *c* oxidase before (a) and after (b and c) exposure to lipid probes. Cytochrome *c* oxidase, solubilized in 1% cholate, was labeled with DPG–ba (b). Lane c shows a control that had hydroxylysine and sodium cyanoborohydride added at the same time and did not incorporate radiolabel. Formation of covalent adduct(s) with radiolabels was found to change the electrophoretic mobility of cytochrome *c* oxidase polypeptide bands in SDS–PAGE (compare lanes a and b). Similar effects on the mobility of labeled polypeptide bands were observed following reaction with PA–ba or PC–ba (data not shown). Coomassie Blue stained bands removed by labeling are indicated by arrows pointing to the right. New bands that appear following labeling (i.e., not observed in control samples) are indicated by arrows pointing to the left. The subunit nomenclature is that of Kadenbach (see text).

DPG itself was sparingly soluble and tended to coat the catalyst surface, causing inactivation. Freshly prepared isocyanate **5** reacted smoothly with DPG dimethyl ester in pyridine, affording the corresponding carbamate in high yield. The blocked phosphate groups of DPG were then demethylated with sodium iodide (Keana et al., 1986) to give the corresponding DPG carbamate **3** (DPG–ba) in which the aldehyde group was intact. 1-Lyso-2[^{14}C]palmitoylphosphatidylcholine was similarly reacted with **5**, leading to PC–ba. Here protective methylation of the phosphate group was not necessary. Our results suggest that the isocyanate **5**, used in both these syntheses, may prove to be a versatile reagent for the attachment of a benzaldehyde group to a nucleophilic site on a biomolecule.

Labeling of Cytochrome c Oxidase. Protein was reacted with the benzaldehyde probes PA–ba, DPG–ba, PC–ba, or MP–ba as described under Experimental Procedures. The Schiff bases formed between the label and amino groups of cytochrome *c* oxidase were reduced by the addition of cyanoborohydride, and subsequently the reaction mixture was quenched with hydroxylysine. The labeling conditions employed allowed for efficient incorporation (0.5–3 mol label/mol of protein) of all three lipid analogues. A high-resolution SDS–PAGE system (Figure 3) that allowed for separation of the protein complex into 12 or 13 subunits was used to determine the distribution of attached label. The gel system used was that of Kadenbach, slightly modified to provide for better resolution of subunits VIa–c. The subunit nomenclature used for the modified gel system corresponds to that of Kadenbach et al. (1983a). This was verified by comparative runs of labeled samples in the two gel systems. After sodium cyanoborohydride treatment, the radiolabels comigrate with the protein bands in SDS–PAGE, indicating covalent attachment of the lipid probes. No label was found associated with protein bands when sodium cyanoborohydride was omitted or when the quencher (hydroxylysine) was added to the reaction mixture concomitant with reducing agent (see Figure 3, lane c). The attachment of the probes to a subunit causes an increase in the apparent molecular weight, resulting in a changed electrophoretic mobility of that polypeptide as shown in lane b of Figure 3. The reduction in staining intensity of certain bands was interpreted as removal of a fraction of the respective polypeptide from the unlabeled position due to the incorporation of the lipid probes. Comparable effects have been observed by others using different labeling reagents (Kornblatt & Lake, 1980; Jarausch & Kadenbach, 1985a). We found the following polypeptides to be reduced in staining intensity after labeling: III, VIc, VIIa–c, and VIII. Coincident with the reduction in staining intensity of some polypeptides, new bands are observed in Coomassie Blue staining upon incorporation of the radioprobes (Figure 3, lane b), which are not present in unlabeled controls. A quantitative correlation of the Coomassie Blue staining of the bands that change intensities during labeling cannot be established since binding of the dye is influenced by the presence of positive charges in the protein (Leffak, 1983). The extent of removal and appearance of bands was dependent on the total amount of label incorporated into protein. New bands in Coomassie Blue staining always coincided with a peak in the distribution of radioactivity.

The assignment of labeled subunits was based on the observed reduction in staining intensity (Coomassie Blue) upon labeling (Figure 3) and the labeling patterns as they are represented in Figure 4.

The enzyme complex, reconstituted into vesicles of DOPC by cholate dialysis, showed preferential incorporation of either PA–ba (Figure 4B) or DPG–ba (Figure 4D) into the smaller subunits (V–VIII), while only small amounts of these labels were found to be associated with subunits I–IV. Changes in the subunit pattern of labeled cytochrome *c* oxidase and radiolabel distribution suggest that polypeptides V (probably Vb, see below) and VIc and probably all of the polypeptides of VII–VIII are readily labeled from the confines of the bilayer. The reduction of staining intensity (Coomassie Blue) correlates with the amount of label incorporated (data not shown). Labeling of subunits VIa and VIb is not clear, since the band intensities observed for these polypeptides were unaltered from unlabeled controls. The counts associated with these bands might arise from a covalent adduct of VIc and attached probe molecule migrating in position of VIa or VIb. We observed a loss of much of subunit III during the labeling procedure. This polypeptide is well-known to readily aggregate during biochemical manipulations and to stay behind on top of the gels during SDS–PAGE (Robinson & Wiginton, 1985). However, if this polypeptide was labeled here, then radioactivity should be detected slightly shifted to higher molecular weight from the unlabeled position of subunit III or on top of the gel. Only background radioactivity was observed at these locations. Thus, we conclude that subunit III is probably not labeled by the benzaldehydes.

Cytochrome *c* oxidase labeled in the presence of 1% cholate (770 mol of cholate/mol of protein) with either PA–ba (Figure

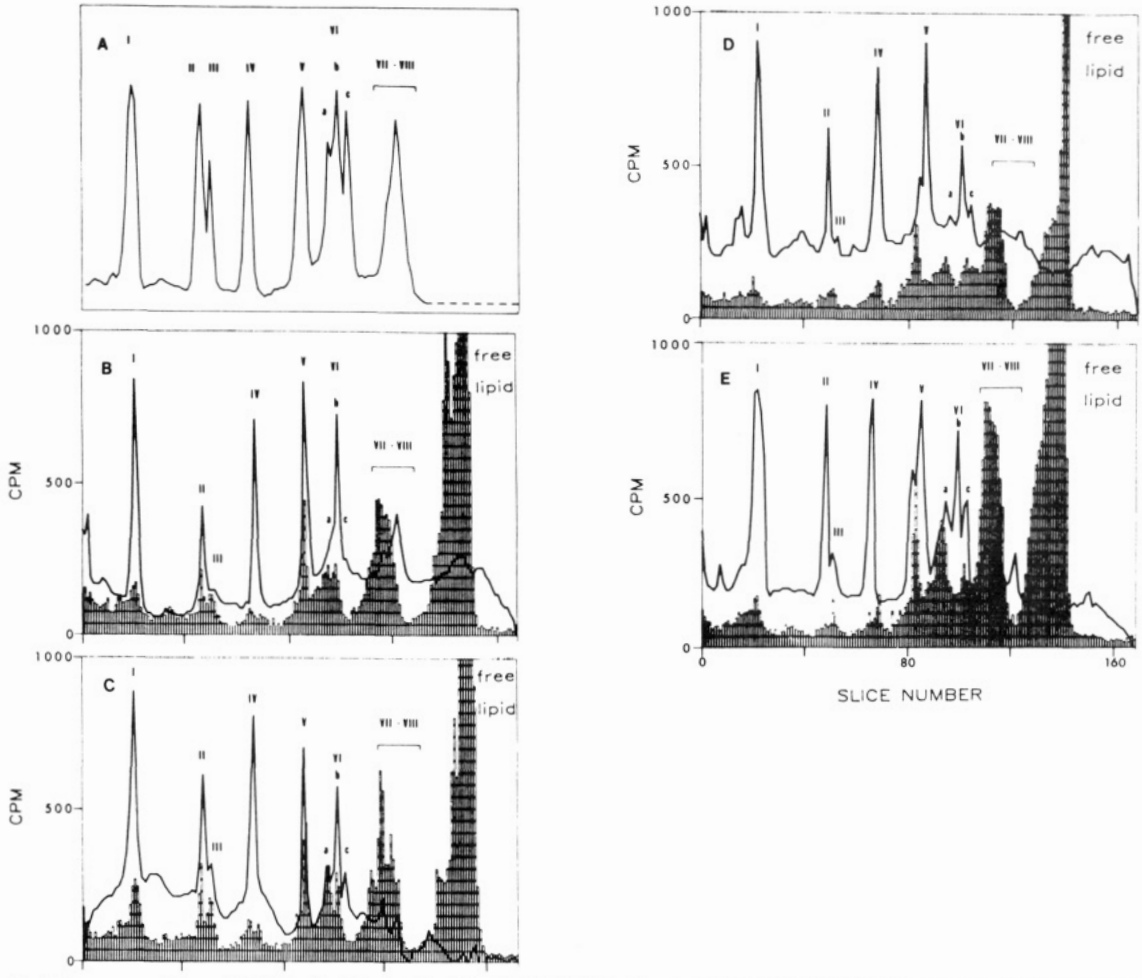


FIGURE 4: Cytochrome *c* oxidase labeled with PA–ba (B, C) or DPG–ba (D, E). A gel scan of an untreated control is shown in (A). Labeling was performed following reconstitution into vesicles of DOPC (B, D) or in 1% cholate (C, E) as described under Experimental Procedures. Note the low staining intensity (Coomassie Blue) in position of subunits VIc and VII–VIII. The peaks in radiolabel distribution appear shifted from the gel scan toward higher apparent molecular weight (i.e., to the left), due to attachment of radiolabeled lipid to the protein. Molar ratios of label:phospholipid:protein were 7:122:1 (B) and 5.4:151:1 (D). Label:detergent:protein ratios for (C) and (E) were 7.6:770:1 and 10.4:770:1, respectively. Calculated ratios of bound label:protein were (B) 2.1:1, (C) 1.8:1, (D) 1.6:1, and (E) 1.4:1. In each plot the line is the optical densitometer tracing of a photograph of a Coomassie Blue stained SDS–polyacrylamide gel of cytochrome *c* oxidase, and the superimposed bar graph represents the distribution of the radioactive label. The direction of migration is to the right. Label–hydroxylysine adducts clearly migrate ahead of protein subunits on these gels and are indicated as "free lipid". Peaks in the gel scan are identified with Roman numerals (I–VIII).

4C) or DPG–ba (Figure 4E) also demonstrated preferential reaction of the benzaldehyde analogues with the smaller subunits of the oxidase complex, V–VIII. No assessment concerning the availability of amino groups on subunits VIa and VIb for the benzaldehyde analogues could be made for reasons stated above. Labeling with PA–ba under these conditions resulted in some incorporation of radiolabel into subunits I, II, and III. There appears to be less reaction of DPG–ba with these polypeptides. Some of our gels of cholate-solubilized cytochrome *c* oxidase labeled with PA–ba separated subunits Va and Vb sufficiently to show a single peak in the distribution of radioactivity in this part of the gel. This peak was slightly shifted from the position of polypeptide Vb toward higher apparent molecular weight, suggesting labeling of this subunit.

In addition, we labeled cytochrome *c* oxidase with the zwitterionic PC–ba to test the influence of the probe charge on the observed labeling patterns (Figure 5). The distribution of PC–ba radioactivity over the gel and the effects of the probe on the migration of subunits closely resemble results of reconstituted cytochrome *c* oxidase labeled with the negatively charged PA–ba and DPG–ba, suggesting that the reactive benzaldehyde group and not the head-group charge dominates the interaction with the protein.

Reaction of cytochrome *c* oxidase with the water-soluble benzaldehyde (MP–ba) resulted in a labeling pattern markedly different from those obtained for the lipid benzaldehyde probes (Figure 6). Subunits IV, Va, and Vb and polypeptides within VII–VIII are most heavily labeled with this molecule, while there is only low incorporation into subunits I–III and VIa–c. The attachment of MP–ba to polypeptides does not cause appreciable shifts in the electrophoretic mobility of the labeled polypeptide, presumably due to the lower molecular weight compared to the lipid benzaldehydes. This fact permits assignments to be based on Figure 6 alone.

Discussion

Chemical labeling has proven especially useful in the characterization of the topology of membrane proteins such as bovine heart cytochrome *c* oxidase (Malatesta et al., 1983). McMillen et al. (1986) recently introduced the use of benzaldehyde-containing lipid analogues for the amine-specific labeling of membrane proteins at the lipid–protein interface. These probes were designed to selectively interact with polypeptide lysine residues and N-terminal amino acids that reside in the proximity of phospholipid head groups. After recon-

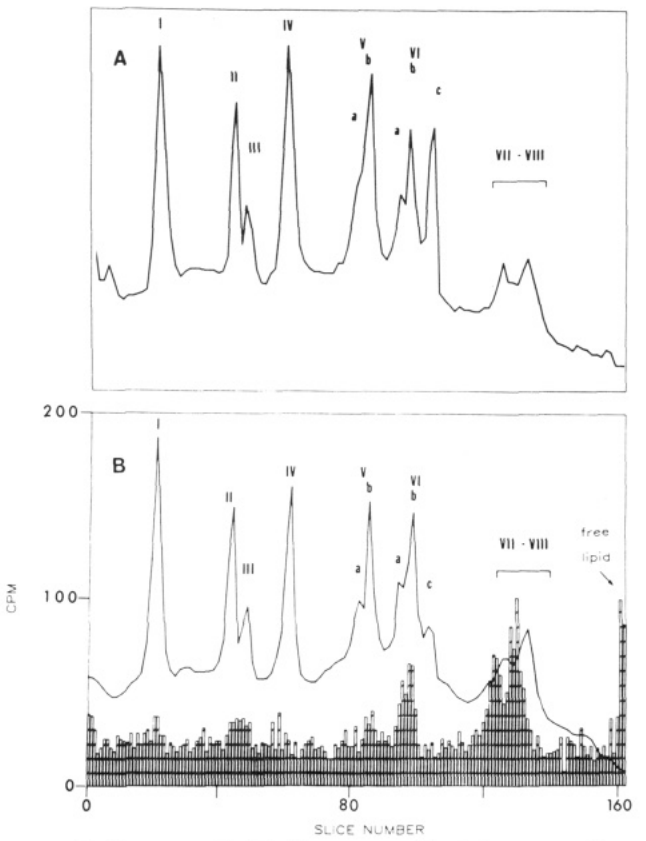


FIGURE 5: Representative labeling pattern of cytochrome *c* oxidase reacted with PC–ba: (A) untreated control; (B) DOPC-reconstituted cytochrome *c* oxidase reacted at molar ratios of label:phospholipid:protein of 27:305:1. Line and bar graphs are as in Figure 4. The apparent higher fluctuation of background than that observed with PA–ba and DPG–ba and the low total counts are due to the low specific radioactivity of PC–ba.

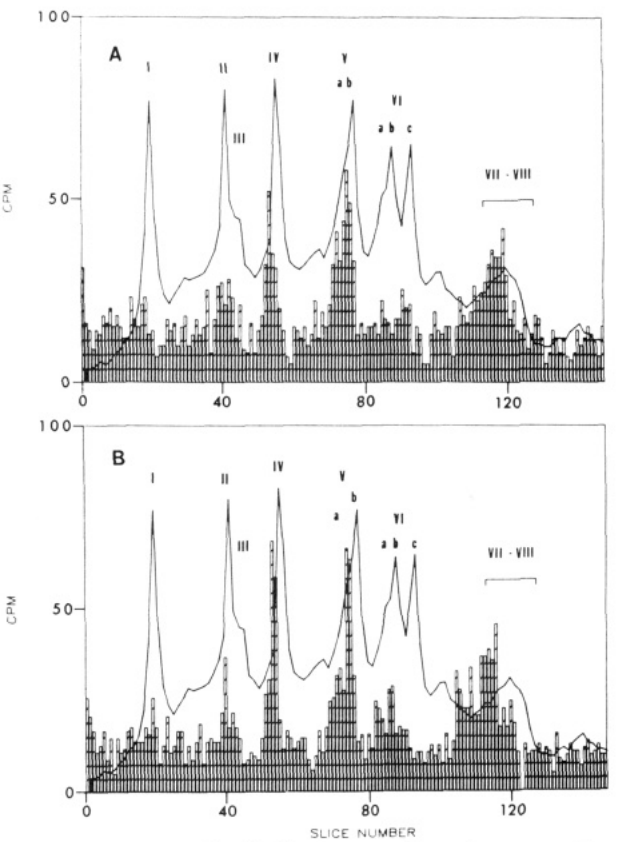
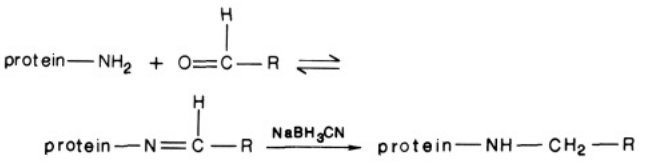


FIGURE 6: Representative labeling pattern of cytochrome *c* oxidase reacted with the water-soluble benzaldehyde (MP–ba) after reconstitution into vesicles (A) or in 1% cholate (B). Molar ratios of label:phospholipid (detergent):protein are (A) 76.5:100:1 and (B) 38:770:1. No band shifts upon incorporation of the label were observed. The low specific radioactivity of MP–ba caused the total incorporation to be low. Line and bar graphs are as in Figure 4.

stitution into vesicles or detergent micelles and equilibration with protein, these probes can be permanently attached to protein amines by reductive amination:



In the present study we report the syntheses of two new benzaldehyde-containing lipid probes, which are derivatives of diphosphatidylglycerol (DPG–ba) and phosphatidylcholine (PC–ba). These molecules, together with a benzaldehyde analogue of phosphatidic acid (PA–ba) and a water-soluble benzaldehyde (MP–ba) reported previously (McMillen et al., 1986), were reacted with accessible amines of bovine heart cytochrome *c* oxidase reconstituted in lipid bilayers or solubilized in detergent. The three phospholipid–benzaldehyde probes differ in their charge and structure and were designed to retain much of the character of their native phospholipid counterparts (Figure 1). The head-group regions of PA–ba and DPG–ba are negatively charged at neutral pH. PC–ba, which carries the reactive benzaldehyde in the *sn*-2 side chain rather than in the head-group region, is zwitterionic. High-resolution gel electrophoresis was used to resolve all 12 or 13 subunits of this multimeric enzyme complex and to examine the distribution of radioactivity over individual polypeptides. We observed that labeled subunits moved slightly to positions of higher apparent molecular weight due to the incorporation of label. We conclude that it is not sufficient to make assignments from the radioactive lipid label distributions (e.g., Figures 4 and 5) alone because of these shifts and the reduction in some band intensities. The assignments of labeled polypeptides reported here are therefore based on the examination of the distribution of radiolabel over the gels combined with an interpretation of the observed changes in subunit staining (e.g., Figure 3). There are no appreciable band shifts with the water-soluble benzaldehyde label (MP–ba).

All three benzaldehyde–lipid probes used reacted most readily with subunits V and VIc and probably all of the polypeptides of VII–VIII of bovine heart cytochrome *c* oxidase labeled after reconstitution into lipid vesicles of DOPC. This suggests that the interactions between benzaldehyde groups and free amines of the enzyme were dominated by the accessibility of the amines to the benzaldehyde group and that charge or hydrocarbon tail structure of the probes did not limit the reaction. Reaction of the benzaldehyde–lipid probes with bovine heart cytochrome *c* oxidase does not appear to be confined to specific sites for acidic lipids, but rather occurs from general lipid binding sites.

The strategy in these experiments was to reconstitute the benzaldehyde probes with lipid-depleted cytochrome *c* oxidase. The number of lipids surrounding this large protein complex is on the order of 50 (Jost et al., 1973; Knowles et al., 1979). The preparation used here contained 12–13 mol of lipid phosphorus. Removal of lipids below this number is known to increase the probability of irreversible aggregation of several subunits. Thus, for this initial test of the cardiolipin label, we stripped off most of the lipid, obtaining a preparation where

Table I: Labeling Patterns of Cytochrome *c* Oxidase with Probes of Different Structure

polypeptide	M_r[a]	transmembrane segments[a]	no. of lysines[a]	stoichiometry	lipid–benzaldehydes[b]	arylazido lipid[c]	water-soluble benzaldehyde[b]
I	56993	several	9	1	+	+++	+
II	26049	2	6	1	+	0	+
III	29918	several	3	1	+	+++	+
IV	17153	1	18	1	0	+	+++
Va	12436	none	7	1	+[d]	+	++
Vb	10670	none	6	1	++[d]		+++
VIa	9419	1	3	1	–		+
VIb	10068	none	6	1	–	+	+
VIc	8480	1	8	1	+++		+
VIIa	6244	1	5	1			
VIIb				1	+++	+++	+++
VIIc	5441	1	4	1			
VIII	4962	1	5	(2)			

[a] Deduced from amino acid sequence or mitochondrial DNA sequence [see review in Buse et al. (1983)]. [b] Present study. [c] Results of Griffith et al. (1986) using an arylazido lipid with the reactive group in the lipid head group. Other probes that carry the arylazido group in place of one of the fatty acid chains gave similar results, except for an increased incorporation of probes into subunit II (Bisson et al., 1979; Prochaska et al., 1980). [d] From labeling of cholate-solubilized cytochrome *c* oxidase (see Results).

there is general agreement the enzyme is stable. No attempt was made to remove the one or two tightly bound cardiolipin molecules since there is a controversy in the literature over the appropriate procedures for accomplishing this (Robinson & Wiginton, 1985; Powell et al., 1985). We show that the new labels attached at many sites and characterized these by SDS–PAGE. This parallels the approach used for the arylazido labels to which we compared our results.

Cholate-solubilized cytochrome *c* oxidase was also labeled with PA–ba and DPG–ba in the presence of 1% cholate (770 mol of detergent/mol of protein) to probe the structure of the mixed micelle of protein–detergent–lipid label. The distribution of radiolabel closely resembles that obtained with DOPC-reconstituted cytochrome *c* oxidase, except for a small increase in relative incorporation of PA–ba into subunits I–III. The similarity of the labeling patterns, however, supports the notion that solubilization of integral membrane proteins by nondenaturing detergents, such as cholate, leads to substitution of membrane lipids by detergent molecules with retention of the native structure of the complex.

Table I compares the labeling patterns of cytochrome *c* oxidase obtained with the benzaldehyde probes and an arylazido lipid that carries the reactive group in the lipid headgroup region (Griffith et al., 1986). While the benzaldehyde probes are designed to react with exposed free amino groups (e.g., lysine side chains and the N-termini), the arylazido probes facilitate relatively nonspecific attachment to polypeptides and lipids in the vicinity of the probe (Robson et al., 1982) and identify lipid-accessible parts of the polypeptide complex. The location of a subunit within the protein oligomer gives rise to certain structural features, such as the accessibility to bilayer lipids, the presence of free amines near the lipid head group, and the exposure of free amines to the aqueous phase, that can be probed with the collection of labels shown in Table I.

We find that none of the mitochondrially coded subunits (I–III) present a significant number of amines to the benzaldehyde reagents (lipid and water-soluble benzaldehydes), while parts of polypeptides I and III are accessible to photoreactive lipid probes (Bisson et al., 1979; Griffith & Jost, 1979; Prochaska et al., 1980; Griffith et al., 1986). Most of the free amines of these two subunits appear, therefore, to be located in the interior of the protein complex or involved in interactions (i.e., "salt bridges") that reduce their reactivity with the benzaldehyde probes.

Polypeptide IV contains the highest number of free amino groups of all the subunits of bovine heart cytochrome *c* oxidase. The results of labeling shown in Table I suggest that this polypeptide is partially shielded by other subunits in the region of membrane passage but that it protrudes beyond these subunits into the aqueous phase. The two lysine residues at the end of the single putative membrane-spanning segment of polypeptide IV (Lys-79 and Lys-100) are not significantly labeled by the lipid–benzaldehydes and might be involved in close contacts to membrane-spanning segments of other subunits, as was suggested earlier by Wikström et al. (1985) for subunit II.

The amino acid sequence of subunit Vb does not reveal a hydrophobic segment of sufficient length to span the bilayer. Our results indicate that this polypeptide reacts with both lipid–benzaldehydes and water-soluble benzaldehydes. This suggests that subunit Vb might be located at the periphery of the protein oligomer of cytochrome *c* oxidase, also involved in close contact with the membrane. This is in agreement with the model of Jarausch and Kadenbach (1985a,b).

The experiments presented here demonstrate that the benzaldehyde probes PA–ba, DPG–ba, PC–ba, and MP–ba efficiently incorporate into polypeptides of cytochrome *c* oxidase. The different labeling patterns obtained with lipid–benzaldehydes and the water-soluble MP–ba suggest that the reactivity of protein amines is determined by their location on the protein oligomer. The three lipid–benzaldehydes used appeared to label the same polypeptides of the enzyme, irrespective of differences in charge and hydrocarbon tail structure. We conclude that the interaction between the benzaldehyde probes and protein amines is dominated by the reactive benzaldehyde moiety and that the lipid labels sample amine-displaying general contact sites of the protein for membrane lipids. The comparison of the reactivity of subunits of this enzyme with lipid–benzaldehydes or water-soluble benzaldehydes or the photoactivatable arylazido lipids provides structural information on the disposition of individual subunits with respect to the aqueous and bilayer phase. This information is complementary to cross-linking and antibody-mapping experiments in that it defines the surface of the enzyme that is in contact with the membrane. The bilayer-inserting part of any membrane protein is not easily accessible to many kinds of reagents and will not yield to high-resolution analysis by X-ray diffraction due to the high conformational flexibility of lipids and detergent molecules bound in the interface between membrane and protein. Our experiments provide data that will have to be accommodated by current models of the three-dimensional structure of bovine heart cytochrome *c* oxidase and the subunit arrangement within the oligomer.

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