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Lipid–Protein Interactions in Cytochrome *c* Oxidase. A Comparison of Covalently Attached Phospholipid Photo-Spin-Label with Label Free To Diffuse in the Bilayer†

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ABSTRACT: The aim of this study was to clarify the possible origins of the motion-restricted electron spin resonance (ESR) spectral component observed in membranes. For this purpose, a phospholipid photo-spin-label was synthesized, characterized, and used to study lipid–protein interactions in beef heart cytochrome *c* oxidase. The probe was designed with a nitroaryl azide incorporated in the phospholipid head group, and a spin-label on the *sn*-2 side chain, and was radiolabeled. The resulting molecule, 1-palmitoyl-2-(14-proxyl[2-^{3}H]stearoyl)-*sn*-glycero-3-phospho-*N*-(4-azido-3-nitrophenyl)ethanolamine, was stable under subdued light and during the procedures required to reconstitute cytochrome *c* oxidase in phospholipid bilayers. Upon photolysis, the photo-spin-label reacted with the protein in high yields (50% attached). There was no detectable destruction of the spin-label. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of cytochrome *c* oxidase after reaction with the photo-spin-label showed highest levels of attachment to bands I, III, and VII, with some labeling of other bands. The labeling pattern demonstrated a distribution of attachment sites, which was needed for the spin-labeling studies. ESR spectra of the attached labels at 25 °C indicated a constant fraction of motion-restricted lipid chains, independent of the lipid to protein ratio. In contrast, a spin-labeled phosphatidylcholine and the prephotolyzed photo-spin-label, both free to diffuse in the bilayer, exhibited behavior in agreement with the multiple equilibria binding model. These results, as well as data obtained with membranes frozen at −196 °C, show how several situations that lead to a motion-restricted ESR line shape can be distinguished. This study provides additional evidence that the fraction of lipids normally in contact with protein, and not aggregation artifacts, accounts for the observed motion-restricted component of ESR spectra of reconstituted cytochrome *c* oxidase in phospholipid bilayers.

Many integral membrane proteins penetrate completely through the fluid phospholipid bilayer, thus creating an interface or boundary where the proteins and the surrounding lipids interact dynamically. This interface is responsible for the high solubility of proteins in membranes and is involved in the maintenance of the permeability barrier. Lipid–protein interactions may also modulate the function of some membrane proteins, influence the organization of membrane proteins

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through long-range forces, and provide part of the answer to the puzzling observation of the large variety of lipids found in membranes (Rouser et al., 1968; Wallach, 1975; Gennis & Jonas, 1977). Lipids at the interface of an integral membrane protein were first observed in cytochrome *c* oxidase reconstituted in vesicles by using electron spin resonance (ESR)[1] spectroscopy of lipid spin-labels (Jost et al., 1973; Griffith & Jost, 1973). Over the ensuing years, a number of general observations about lipid–protein interactions have been established by several laboratories: (a) To a good approximation, the lipids of membranes can be considered to be in one continuous bilayer with boundaries at the surface of each protein, or protein oligomer, that penetrates into it. Due to the high density of integral membrane proteins in many biolgical membranes, the combined total surface area of these boundaries is large, accounting for a significant fraction of the total lipid present. (b) When integral proteins are reconstituted into bilayers containing lipid spin-labels, a certain number of these labels become motion-restricted on the ESR time scale. There is an approximate correlation between this number and the hydrophobic surface area exposed to the bilayer, providing ample lipid is present. (c) The protein surface causes the adjacent lipid chains to be more spatially disordered (i.e., the flexible lipid chains conform to the irregular protein surface). (d) The perturbation by the protein surface of the lipid chain motion and order is very short ranged in the plane of the bilayer. (e) There is exchange of lipids between the boundary layer and the rest of the bilayer. (f) The distribution of lipids in the plane of the bilayer is not totally random; there is some selectivity by the proteins for different phospholipid head groups [for reviews, see Jost & Griffith (1980), Marsh & Watts (1982), Marsh (1985), and Devaux & Seigneuret (1985)].

In spite of the general agreement on the overall picture, some problems and controversies remain. Reasons for this include differences in sample preparations and difficulties in applying spectroscopic methods to studies of lipid–protein interactions. NMR, for example, is providing useful information, but the time scale is too long for direct detection of lipid in contact with protein (Seelig et al., 1982). In the spin-labeling method, considerable care must be exercised in the ESR spectral subtractions because line shapes can overlap and are sensitive to temperature and the lipid to protein ratio. Aside from these technical factors, a more fundamental problem is to take into account protein–protein interactions that undoubtedly occur in membranes. In the simplest case, random protein–protein interactions would only act to reduce the number of lipid–protein contacts and would have little effect on the ESR analysis which usually involves extrapolation from a range of lipid/protein ratios. Of more concern is lipid that may be sandwiched between two proteins or trapped between many proteins. These environments would also give rise to motion-restricted spectral components. One hypothesis is that the motion-restricted component observed in the ESR spectra in membranes and membrane model systems could be accounted for largely by labels trapped between aggregated proteins. It is this question we address here. An argument against this point of view is the observed stoichiometry between the number of motion-restricted lipids and the intramembranous perimeter

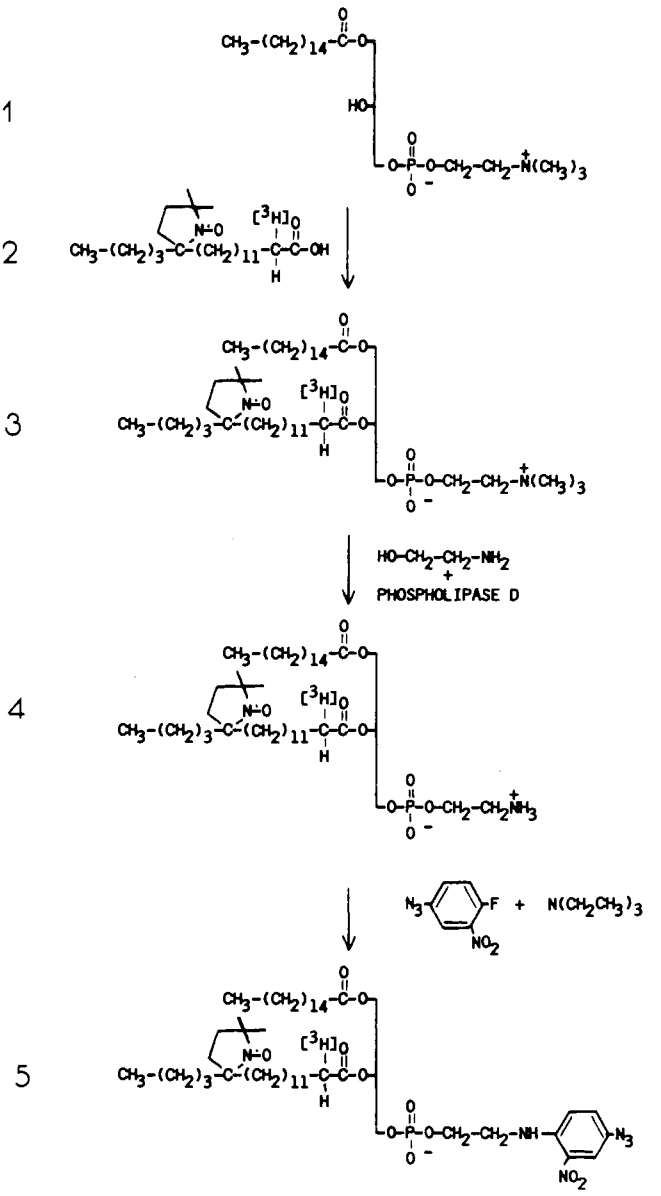

FIGURE 1: Reaction scheme for the synthesis of the photo-spin-label **5**. The final molecule is a triple-labeled (spin, tritium, and arylazido) phospholipid derivative.

of the proteins. Nevertheless, information about protein surfaces is far from complete. The question of the origins of the motion-restricted components can be approached another way: by introducing spin-labeled lipid covalently attached to the protein in one sample and spin-labeled lipids free to diffuse in the bilayer in another and observing the behavior of both as a function of the lipid to protein ratio. For this purpose, we selected an arylazido-photoactivated group since arylazidophospholipids have been the subject of considerable study (Chakrabarti & Khorana, 1975; Richards & Brunner, 1980) and are known to form covalent bonds with cytochrome *c* oxidase (Bisson et al., 1979; Griffith & Jost, 1979; Prochaska et al., 1980). We report here the synthesis of the phospholipid photo-spin-label **5** (Figure 1) and the results of a comparative ESR study of the covalently attached label **5** and a conventional phospholipid spin-label **3** free to diffuse between protein sites and the bilayer.

EXPERIMENTAL PROCEDURES

Synthesis of the Photo-Spin-Label 1-Palmitoyl-2-(14-proxylstearoyl)-sn-glycero-3-phospho-N-(4-azido-3-nitrophenyl)ethanolamine (**5**). The synthetic steps in Figure 1 were

[1] Abbreviations: DOPC, 1,2-dioleoyl-3-*sn*-phosphatidylcholine; ESR, electron spin resonance; proxyl, 2,2-dialkyl-5,5-dimethylpyrrolidine-*N*-oxyl; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SPE, 0.25 M sucrose, 50 mM phosphate, and 1 mM ethylenediaminetetraacetic acid; TLC, thin-layer chromatography; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

worked out and characterized on the nonradiolabeled compounds. Radiolabeled compounds were synthesized by the same procedures but were not individually characterized. The method of Boss et al. (1975) was adapted to esterify 1-palmitoyl-*sn*-glycero-3-phosphocholine (**1**) with 14-proxylstearic acid (**2**) (Keana et al., 1982) as follows. In a glovebox (dry nitrogen atmosphere), 1,1′-carbonyldiimidazole (Aldrich, 137 mg) was dissolved in dry chloroform (5 mL, freshly distilled from P_2O_5) and added to 322 mg of **2** (Keana & La Fleur, 1979). Gas evolution was observed immediately, and the solution was stirred for 1 h. A centrifuge tube containing 347 mg of **1** (Sigma, 98%, dried under high vacuum overnight) was transferred to the glovebox, and the solution of **2** and 1,1′-carbonyldiimidazole was added. The centrifuge tube was fitted with a nitrogen inlet and heated to 55 °C. After a few minutes, all of the lyso compound **1** had dissolved, and the chloroform was evaporated with nitrogen. The mixture was maintained at 55 °C for 4 days under nitrogen. Sublimed imidazole was removed and the remaining reaction mixture was taken up in chloroform (5 mL) with a few drops of water added to quench the remaining imidazole intermediate, **2**. The water was then removed azeotropically with several additions of carbon tetrachloride. The residue was dissolved in chloroform (5 mL) and applied to a 2 × 18 cm silica gel (Baker, 60–200 mesh) column that had been preeluted with 200 mL of chloroform/methanol (7/3 by volume) and 100 mL of chloroform. The column was eluted with another 100 mL of chloroform to remove unreacted **2**, followed by 50-mL portions of 5%, 10%, 15%, and 20% methanol in chloroform, and then 300 mL of 30% methanol in chloroform to remove 1-palmitoyl-2-(14-proxylstearoyl)-*sn*-glycero-3-phosphocholine (**3**) as a yellow band (yield, 452 mg of yellow semisolid, 75% based on **1**). Anal. Calcd for $C_{47}H_{92}N_2O_9P\cdot 4H_2O$: C, 60.27; H, 10.98; N, 2.99. Found: C, 59.86; H, 10.54; N, 2.66.

The methods of Dawson (1967) and Comfurius & Zwaal (1977) were adapted to achieve conversion of the choline head group of **3** to the ethanolamine head group; 100 mg of **3** was dissolved in 10 mL of dry ether and added to 17 mL of ethanolamine/0.5 M sodium acetate buffer, pH 5.5 (1/3 by volume), 1.7 mL of 1 M calcium chloride, and 25 mg of cabbage phospholipase D (Sigma). The flask was fitted with a reflux condenser and the solution stirred rapidly at 37 °C for 2 h. An aliquot was spotted on an analytical TLC plate (EM Reagents silica gel F-254) and the plate developed with chloroform/methanol/water (70/25/4 by volume) to determine the presence of unreacted **3**. After addition of a fresh portion of phospholipase D (25 mg) and stirring for another 2 h, the reaction was complete. Chloroform/methanol (1/1, by volume, 20 mL) was then added with stirring, the phases were separated by centrifugation, and the aqueous (upper) phase was extracted twice more with chloroform. The combined organic phases were extracted with a saturated sodium chloride solution, concentrated, and dried under high vacuum. The resulting yellow semisolid was taken up in chloroform (2 mL) and applied to a 1.5 × 40 cm column of CM-cellulose (Whatman CM-52, sodium salt form). The column was eluted with chloroform (195 mL) followed with increasing amounts of methanol in chloroform. Fractions containing 1-palmitoyl-2-(14-proxylstearoyl)-*sn*-glycero-3-phosphoethanolamine (**4**) were eluted with 9–15% methanol in chloroform and were further purified by preparative TLC over silica gel (Whatman PK-6 1000-μm plates) developed with chloroform/methanol/water (70/25/4). The combined yield of pure **4** was 56.9 mg (60%) as a yellow semisolid. The analytical sample was obtained by repeated azeotropic drying from dichloromethane followed by high vacuum. Anal. Calcd for $C_{44}H_{86}N_2O_9P\cdot {}^1/_3H_2O$: C, 64.12; H, 10.60; N, 3.40. Found: C, 64.21; H, 10.45; N, 3.35.

The photolabel was incorporated according to the procedure of Chakrabarti & Khorana (1975). **4** (11 mg), 4-fluoro-3-nitrophenyl azide (35 mg), triethylamine (0.1 mL), ethanol (0.2 mL), and chloroform (0.1 mL) were combined and stirred in the dark for 2 days at 40 °C. The resulting solution was aplied directly to a preparative TLC plate (Whatman PKF6) and developed with chloroform/methanol (4/1 by volume). The yellow band was removed, giving 9.0 mg (68%) of **5**. Anal. Calcd for $C_{50}H_{87}N_6O_4P\cdot 4H_2O$: C, 57.13; H, 9.11; N, 7.99. Found: C, 57.23; H, 8.89; N, 7.87.

The radioactive photo-spin-label **5** was prepared by the same procedure using tritiated compounds. A 32.6-mg sample of **1** was acylated with 23 mg of tritiated **2** (Keana & Boyd, 1981) to give 23.8 mg of radiolabeled **3**. This was enzymatically converted into radiolabeled **4** which in turn was combined with 4-fluoro-3-nitrophenyl azide to give the final phospholipid label, **5** (specific activity, 0.25 Ci/mmol).

Materials and Assays. Lipid-depleted beef heart cytochrome *c* oxidase was prepared by the method of Yu et al. (1975) and kindly supplied by Dr. Tsoo E. King. The enzyme preparation contained 10.3 nmol of heme *a* and 60 nmol of lipid phosphorus per milligram of protein. The cytochrome *c* oxidase, with an activity of 15 nmol of O_2 s^{-1} (nmol of heme *a*)$^{-1}$, was stored at 17.7 mg of protein/mL in 1% cholate, 0.25 M sucrose, 50 mM phosphate, and 1 mM EDTA (SPE), pH 7.4 at −196 °C. L-α-Dioleoylphosphatidylcholine (99% pure) and cholic acid (recrystallized twice from ethanol) were from Sigma. All other chemicals were obtained from commercial sources and were of reagent grade or better. Assays for protein and lipid phosphorus were as described earlier (Jost et al., 1977). Heme *a* was determined by the methods of Yonetani (1961) and Griffiths & Wharton (1961). Prior to the activity assay, the cytochrome *c* oxidase samples (400 μg of protein/mL) were homogenized in 50 mM phosphate buffer, pH 7.4, containing 26.6 mg of Emasol/mL, with 6.3 μg of lipid phosphorus (egg phosphatidylcholine) at 4 °C, followed by bath sonication for 5 min. Enzyme activity was then assayed polarographically by the method of Yonetani (1966) in 50 mM phosphate (pH 7.4), 20 μM reduced cytochrome *c* (horse heart, type VI, Sigma), 30 mM ascorbate, and 0.7 mM EDTA at 24 °C. Electron microscopy was performed with a Philips EM 300 transmission electron microscope. All specimens were negatively stained with phosphotungstate at pH 7.

SDS–Polyacrylamide Gel Electrophoresis. Disc gels contained 10% acrylamide, 0.66% bis(acrylamide), 8 M urea, 0.1 % SDS, and 100 mM phosphoric acid, adjusted to pH 6.8 with Tris base [essentially as described by Swank & Munkres (1971) and Eytan & Broza (1978)]. Cytochrome *c* oxidase samples (7 mg of protein/mL of SPE, pH 7.4) were dissociated by addition of an equal volume of a buffer containing 10% SDS, 8 M urea, 40 mM sodium phosphate, and 10% β-mercaptoethanol, pH 6.8, followed by heating at 37 °C for 1 h. A solution of 0.5% methyl green and 30% sucrose was added to the dissociated protein samples to final concentrations of 0.05% and 3%, respectively. Approximately 100 μg of protein was applied to each gel and then electrophoresed at 2 mA/gel for 1 h followed by 4 mA/gel for 6–7 h. Gels were stained with 0.25% Coomassie Blue R-250 (Eastman Kodak) in methanol/water/glacial acetic acid (5/4/1 by volume) at 60 °C for 30 min. Destaining was achieved at room temperature with gentle shaking in methanol/water/glacial acetic acid (5/4/1 by volume) in the presence of Bio-Rad analytical-grade

mixed-bed resin AG 501-X8 (20–50 mesh). Gels were swollen in 10% acetic acid and scanned at 550 nm with a Beckman DU spectrometer equipped with a Gilford linear-transport attachment. Radioactive gels, frozen on dry ice, were cut into 1-mm slices (Mickle gel slicer) and dissolved in 1 mL of 15% H_2O_2 at 70 °C overnight. Aquasol-2 (7 mL, New England Nuclear) was added, and radioactivity was measured in a Packard Tri-Carb liquid scintillation counter.

Covalent Labeling of Cytochrome c Oxidase with the Photo-Spin-Label **5**. Under subdued light, **5** (0.38 mg in benzene), DOPC (5.65 mg in chloroform), and [^{14}C]phosphatidylcholine (9.3 μg in ethanol, New England Nuclear, specific activity of 53 mCi/mmol) were mixed in a glass homogenizer, and the organic solvent was removed under a stream of nitrogen followed by aspiration for 30 min. The lipids were solubilized with homogenization in added 2% cholate/SPE, pH 7.4 (0.216 mL). All homogenization done in these experiments was gentle, without the introduction of air bubbles, using a smooth, glass, hand-held Wheaton homogenizer. Lipid-depleted cytochrome *c* oxidase (17 mg) in 0.6 mL of 1% cholate/SPE, pH 7.4, was added to the lipid mixture and the final cholate concentration adjusted to 2% with 10% cholate/SPE, pH 7.4. The sample was homogenized, incubated at room temperature for 1 h, and then dialyzed in a 75 000 molecular weight cutoff collodion bag (Schleicher & Schuell) in the dark at 6 °C against SPE, pH 8.0, with four 1-h changes, followed by dialysis overnight. The resulting vesicular sample (73 mol of DOPC and 4 mol of **5** per mole of cytochrome *c* oxidase, based on 10 nmol of heme *a*/mg of protein), kept in subdued light at 4 °C, was diluted to 2 mg of protein/mL and homogenized. The sample in the homogenizer was stirred while immersed in an ice bath, and the azide was photolyzed by a 5-min exposure with a Sylvania tungsten/halogen 1000-W lamp. During photolysis, the sample was placed 25 cm from the light source with a solution of 1 M sodium nitrite contained in a glass TLC tank (10 cm wide) positioned between the sample and the light source, allowing only light of wavelengths longer than 400 nm to illuminate the sample. The sample turned from golden-brown to rust-brown during the photolysis, with all apparent color changes complete after 2–3 min of irradiation. The enzyme activity of unlabeled cytochrome *c* oxidase remained unchanged when exposed to these photolysis conditions, but the activity of photolabeled cytochrome *c* oxidase was reduced significantly. For example, the activity was reduced to 30% of the original level when 50% of the lipid present was the photo-spin-label.

Removal of Noncovalently Attached Photo-Spin-Label **5** *for ESR*. Typically, the photolyzed sample was concentrated by pelleting at 15 000 rpm for 10 min in an RC-2B Sorvall centrifuge equipped with an SS-34 rotor. The protein pellet (about 16 mg) was solubilized in 2.5 mL of 2% cholate/SPE, pH 7.4. The protein solution was transferred to a homogenizer containing a dry film of DOPC (240 mol of added DOPC/mol of protein), homogenized, and allowed to equilibrate for 1 h at 37 °C. The protein was then centrifuged (Beckman L5-50 ultracentrifuge equipped with an SW41 rotor, 35 000 rpm, 4 °C, 16 h) through a 15–30% linear sucrose gradient (8 mL) containing 2% cholate onto a 0.5-mL 50% sucrose pad containing 2% cholate. Protein that banded at the 50% sucrose step retained 3 mol of **5** and 3 mol of DOPC per mole of protein, as calculated from the remaining [^{3}H]-**5** and [^{14}C]-phosphatidylcholine counts. The sample was diluted to 2% cholate and 15% sucrose, pH 7.4, homogenized with an additional 1.5 mg of DOPC/mg of protein, incubated for 30 min at room temperature, and delipidated on a second sucrose/cholate gradient. The cytochrome *c* oxidase with its attached photo-spin-label was then reconstituted with DOPC as follows. The delipidated protein (e.g., 3 mg in 1.3 mL of 15% sucrose and 2% cholate, pH 7.4) was homogenized with 3 mg of DOPC and incubated at room temperature for 30 min. The protein/lipid/cholate mixture was then centrifuged through a cholate-free sucrose density gradient (8 mL, 15–50% sucrose) with a 0.5-mL 50% sucrose pad (SW41 rotor at 35 000 rpm, 4 °C for 15 h). The sample was collected in a band from 42% to just above the 50% sucrose pad and homogenized. A single centrifugation step through a cholate-free sucrose gradient leaves on the order of 1–4 mol of cholate/100 mol of phospholipid, as determined in earlier experiments using ^{14}C-labeled cholate. A succeeding gradient removes cholate below the level of detection. The sucrose was reduced to 0.25 M by addition of buffer and the protein concentrated by pelleting in an SW50.1 rotor at 15 000 rpm for 30 min. While this step was performed to concentrate the sample for ESR, it had the added advantage of reducing any residual cholate present. The lipid/protein ratio of a typical sample was 0.15–0.2 mg of lipid/mg of cytochrome *c* oxidase with 2.2 mol of **5**/mol of cytochrome *c* oxidase. Within experimental error, the ratio of label to protein did not decrease with resolubilization of the reconstituted samples. In addition, no free label was recovered in the lipids of resolubilized samples, as judged by scintillation counting of the lipid/cholate mixture remaining at the top of the sucrose gradient. Reconstituted samples at higher lipid to protein ratios (e.g., 280 mol/mol) were obtained by the addition of different amounts of DOPC to the cholate-solubilized protein with its attached photo-spin-label, followed by dialysis to remove the cholate. These dialysis conditions remove cholate to less than 1 mol/200 mol of phospholipid, as judged from earlier experiments using [^{14}C]cholate (unpublished results). One sample at a very low lipid/protein ratio (13 mol/mol) was obtained by exhaustive dialysis of the cholate-solubilized protein without any added DOPC. To investigate the effect of any residual cholate on ESR line shapes, cholate was deliberately added back to one of the final samples. At concentrations of up to 4 mol of cholate/100 mol of phospholipid, no detectable change in the ESR spectra was observed. Thus, even if trace quantities of cholate remained, the ESR spectra would not be affected in these experiments.

Spin-Labeling with Probes Free To Diffuse in the Bilayer. Under subdued light, 50 μg of **5** in benzene and 4 mg of DOPC in chloroform were dried down in a vial under a stream of nitrogen gas followed by aspiration for 30 min. Lipid vesicles were formed by the addition of 0.2 mL of SPE, pH 8.0, with vortexing and bath sonication (Heat Systems, Ultrasonics, 40 W). The homogeneous lipid solution was photolyzed for 5 min, revortexed, and rephotolyzed. An aliquot was transferred to glass capillaries, and ESR spectra were recorded at temperatures ranging from −20 to 37 °C in order to obtain lipid reference spectra. The remaining lipid sample was solubilized in cholate (2% final concentration) by the addition of 10% cholate/SPE, pH 7.4. Depending on the desired final lipid to protein ratio, 1–4 mg of the prephotolyzed **5**/DOPC/cholate mixture was added to 3 mg of lipid-depleted cytochrome *c* oxidase in 2% cholate/SPE, pH 7.4, and mixed with homogenization. Vesicles were formed either by dialysis against SPE, pH 7.4 (four 30-min changes followed by overnight), at 6 °C or by 20-fold dilution of the 2% cholate-solubilized sample with cholate-free SPE, pH 7.4, followed by dialysis. The membrane samples were concentrated for ESR measurements by loose-pelleting at 18000*g* for 15 min at 4 °C.

Phospholipid vesicles without protein were prepared with the spin-labeled phosphatidylcholine **3** (no photoreactive group, see Figure 1) as follows: 4 mg of DOPC in chloroform was mixed in a vial with 40 μg of **3** and the solvent removed by a stream of nitrogen gas followed by aspiration for 30 min, 0.2 mL of SPE, pH 7.4, was added, and the mixture was vortexed to form lipid vesicles. Vesicles containing cytochrome *c* oxidase in DOPC bilayers with the lipid spin-label **3** were prepared by two methods: One method was reconstitution by dialysis of cholate-solubilized mixtures of the desired amounts of DOPC, **3**, and cytochrome *c* oxidase as described above. The other method was done as a control for the preparation of the protein samples containing covalently attached **5**. A mixture of DOPC, **3**, and cytochrome *c* oxidase (20, 0.68, and 17 mg, respectively) cosolubilized in 3.4 mL of 1% cholate/SPE, pH 7.4, was centrifuged (SW41, 22000 rpm, 16 h, 4 °C) through a 7-mL sucrose step containing 20% sucrose, 50 mM phosphate, and 1 mM EDTA, pH 8.0, onto a 60% sucrose pad. The protein band was retrieved and homogenized with buffer added to adjust the sucrose concentration to 0.25 M. The membrane sample (0.2 mg of lipid/mg of protein, 4 mg of protein/mL) was then photolyzed for 5 min under conditions described earlier. The vesicles were then solubilized in 2% cholate with 1 mg of added DOPC per milligram of protein, and the sample was delipidated by centrifugation through 6 mL of 20% sucrose/2% cholate. After retrieval of the sample, this procedure was repeated. To obtain reconstituted samples, the cholate-solubilized, delipidated protein (10 μg of phospholipid/mg of protein) was homogenized with the desired amounts of DOPC with 1 mol of **3**/mol of protein. The mixture was centrifuged (SW41 rotor, 22000 rpm, 16 h, 4 °C) into a sucrose density gradient (6 mL of 15–50% sucrose). The sample, which banded at 36% sucrose, was collected, diluted to SPE, pH 7.4, and concentrated by pelleting (SW50.1 rotor, 15000 rpm, 15 min, 4 °C).

Electron Spin Resonance Spectroscopy. ESR spectra were recorded on a Varian E-9 9.5-GHz spectrometer interfaced with a 32K Varian 620/L100 computer for spectral analysis. ESR instrument settings were as follows: microwave power, 5 mW; scan time, 8–16 min; scan range, 100 G; filter time constant, ≤0.2 s; modulation amplitude, ≤1 G. Sample temperature was maintained within ±0.2 °C and monitored with a Fluke digital thermometer and copper/constantan thermocouple located 1 cm above the sample in the ESR cavity. Spectral subtractions and data analysis were performed as described previously (Jost & Griffith, 1978; Silvius et al., 1984).

Results

Synthesis of the Photo-Spin-Label **5** *and Its Reaction with Cytochrome c Oxidase.* The simultaneous presence of the proxyl moiety containing the unpaired electron and the aryl-azido group did not interfere in the synthetic procedures, and the final molecule **5** was obtained in satisfactory yield. The product was stable by the criteria that neither the reactivity nor the ESR signal intensity of stock solutions in organic solvents decreased significantly over a period of months when kept in the dark at −20 °C.

The photo-spin-label was reconstituted with cytochrome *c* oxidase and photolyzed, and excess label and non-protein reaction products were removed as diagrammed in Figure 2. Electron micrographs showed that the photo-spin-labeled preparations were vesicular (Figure 3a) and had the same appearance as the unlabeled preparations. These vesicles were disrupted when solubilized in cholate (Figure 3b) and then, after several cholate washes and lipid addition, were re-formed

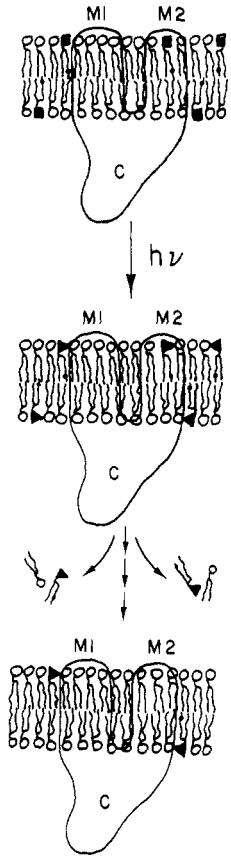

FIGURE 2: Diagram outlining the photolabeling experiment. The protein, phospholipid, and photo-spin-label are cosolubilized in the dark by using detergent. (Top) Detergent is removed by dialysis, leaving behind a reconstituted membrane with the photo-spin-label inserted correctly in the bilayer (square head groups). (Center) Upon exposure to light, the azido compound forms the reactive nitrene and covalently attaches (black triangles) to adjacent proteins and phospholipids. (Bottom) All labels not covalently attached to the protein are replaced by repeated detergent solubilization and delipidation steps, followed by reconstitution with fresh lipid, leaving a membrane with photo-spin-label covalently linked to the interfacial (boundary) region of the protein. C, M1, and M2 indicate the cytoplasmic and two matrix domains, respectively, of cytochrome *c* oxidase.

in the final cholate-free preparations (Figure 3c,d). The preparation on which the majority of ESR data were recorded had a molar ratio of 4/95/1 of photo-spin-label/DOPC/protein at the time of photolysis. After photolysis and removal of unreacted or unattached label, this sample contained 2–3 mol of photo-spin-label per mole of protein. Another sample had a labeling ratio of 4/120/1 (**5**/DOPC/protein) at the time of photolysis and yielded, after removal of unattached label, 3 mol of photo-spin-label bound per mole of protein. Several independent tests were performed to ensure that unattached photo-spin-label products were removed and replaced by unlabeled phospholipid during the cholate/phospholipid treatments. First, the radioactivity of **5** was monitored. The tritium counts decreased as excess photo-spin-label was removed, and then the radioactivity per milligram of protein reached a constant, indicating that the remaining label was not removable. Second, ESR spectra of the final cholate-solubilized, covalently labeled protein sample exhibited no detectable narrower three-line spectrum which is seen when unattached label is present at these high cholate to lipid ratios. (Care must be taken here because the ESR line shape of cholate-containing lipid preparations is dependent on the amount of phospholipid present and the cholate to lipid molar ratios.) The third test was provided by the introduction of a ^{14}C-labeled phosphatidylcholine (not spin-labeled or photolabeled) along with the

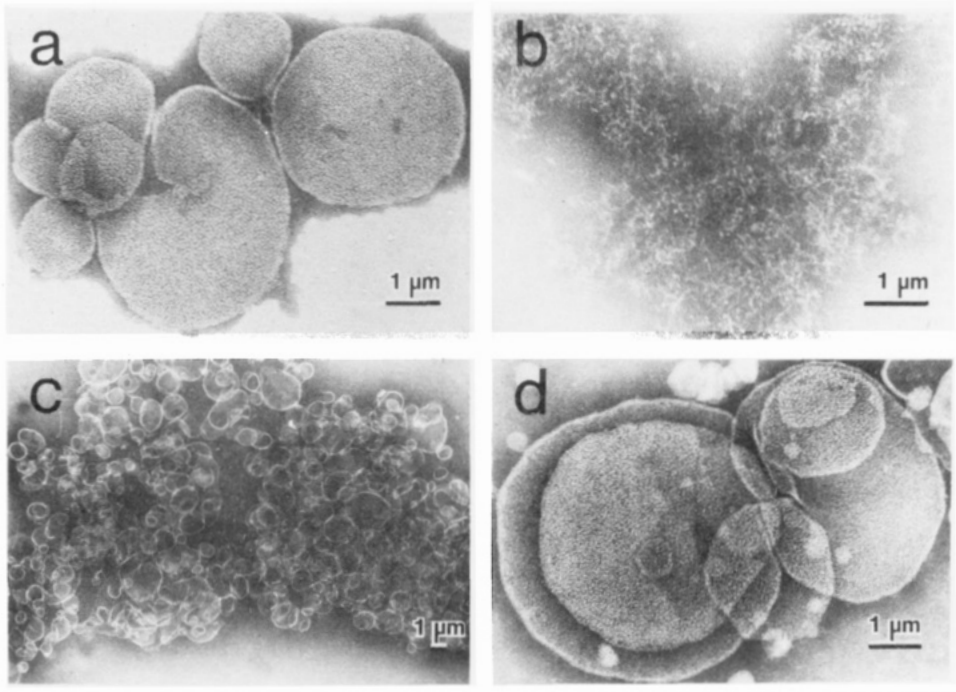

FIGURE 3: Electron micrographs of negatively stained cytochrome *c* oxidase preparations after sequential steps of the photolysis and reconstitution procedures outlined in the previous figure. (a) The initial photolyzed sample and (b) the same sample solubilized in cholate with added DOPC prior to delipidation by centrifugation. (c and d) The same photolyzed sample after removal of the unattached photo-spin-label and reconstitution at phospholipid/protein ratios of 62/1 and 240/1, respectively.

photo-spin-label in the original reaction vesicles. By monitoring the radioactivity during the subsequent steps of Figure 2, it was found that essentially all of the ^{14}C-labeled phospholipid was lost, as one would expect if the cholate/phospholipid treatments were replacing unattached lipids with fresh DOPC. A fourth criterion was the observation that resolubilization of the final reconstituted samples, with separation of labeled protein from the bulk lipid, left the label/protein ratio unchanged and no label was detected in the separated lipid fraction. Each of these tests is subject to some limitations, but taken together, they provide strong evidence that most if not all of the unattached photo-spin-labels were removed prior to obtaining the final reconstituted samples for the ESR experiments.

Polypeptide Distribution of Photo-Spin-Labels Covalently Attached to Cytochrome c Oxidase. Some information regarding the physical distribution of attached labels is obtained from comparison of the Coomassie Blue staining profiles of the protein and the radioactive counts of the lipid labels obtained from the same gels (Figure 4). The major radioactive peaks coincide with bands I, III, and VII, indicating that portions of these polypeptides are exposed to phospholipid head groups in the bilayer and have been labeled by **5**. There is minor labeling of band IV and some labeling in the region of bands V and VI. The lipid peak to the right in Figure 4 evidently represents photolabeled lipids released during the pregel dissociation treatment with urea, SDS, and β-mercaptoethanol, since several tests (see above) indicated that the unattached lipid was largely removed by the cholate/phospholipid exchange process.

We are unaware of any previous gel data using this or similar nitrene precursors in the phospholipid head group, but the labeling pattern of Figure 4 is consistent with the results of Bisson et al. (1979) and Prochaska et al. (1980). The nitrene precursor used by these authors was located in the *sn*-2 acyl chains of phosphatidylcholine, and the heaviest labeling was observed in bands I, III, and VII.

Electron Spin Resonance Spectroscopy. Figure 5 illustrates

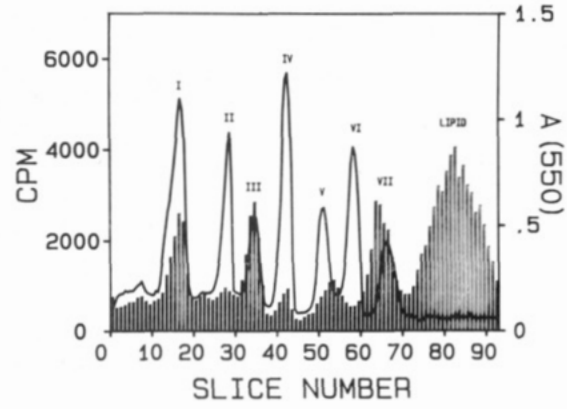

FIGURE 4: Polypeptide distribution of covalent attachment of the photo-spin-label **5** to cytochrome *c* oxidase as determined by SDS–polyacrylamide gel electrophoresis. (Solid line) Densitometric scan at 550 nm of the Coomassie blue stained gel. (Bar graph) Distribution of the tritium counts of **5**. Direction of migration is to the right. The sample placed on the gel contained 68 mol of DOPC and 2 mol of **5** per mole of protein. The counts in the free lipid peak (far right) are about 45% of the total counts and apparently represent lipid released under the dissociation conditions used during the preparation of the samples for SDS–PAGE.

ESR spectra of photo-spin-labeled cytochrome *c* oxidase at 25 °C as a function of the amount of added DOPC. Each experimental spectrum is a composite of two components; one that is motion-restricted and another that is bilayer-like. The motion-restricted line shape (Figure 5, bottom right) was derived by subtraction of the ESR spectrum of the 50 lipids/protein sample from the spectrum of the 70 lipids/protein sample using the pairwise subtraction technique described by Brotherus et al. (1980). This motion-restricted line shape was then incrementally subtracted from each of the experimental ESR spectra. The end point of each spectral titration yields the percent of motion-restricted component and the difference line shape (Figure 5, second and fourth columns). The difference line shapes in this case are bilayer line shapes. To ensure self-consistency, ESR line shapes of samples of phos-

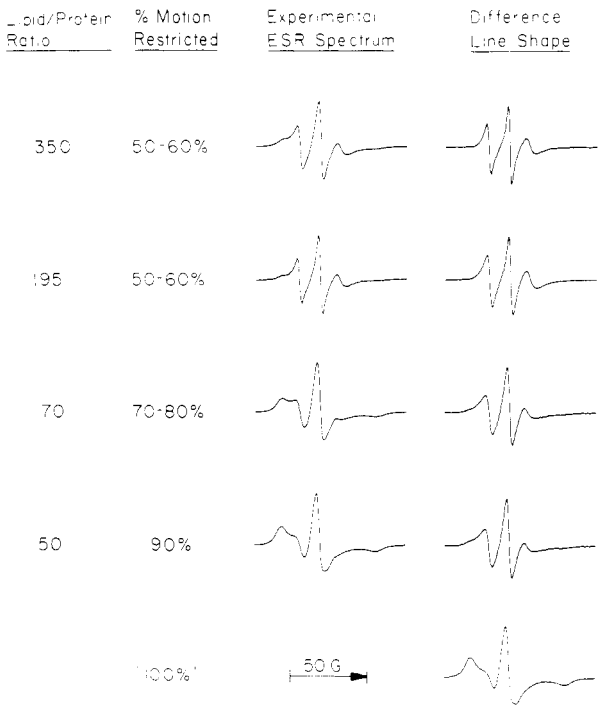

FIGURE 5: ESR data for the photo-spin-label **5** covalently attached to cytochrome *c* oxidase at 25 °C. (First column) The final phospholipid/protein ratio after removal of all noncovalently attached label and replacement with DOPC. (Second column) The percent of the spin-label in contact with the protein. (Third column) Experimental ESR spectra normalized to a constant center-line height. (Fourth column) Difference "bilayer" line shapes obtained by subtracting the reference motion-restricted line shape (bottom right) from each experimental ESR spectrum. Each of the line shapes in column 4 is faithfully reproduced by bilayer spectra obtained in the absence of protein but at temperatures below 25 °C [for examples, see Silvius et al. (1984)].

pholipid (DOPC) bilayers without protein that contained the prephotolyzed photo-spin-label **5** were obtained as a function of temperature, and it was confirmed that the difference spectra did match actual experimental bilayer spectra. The match generally occurs with bilayer spectra recorded at a temperature somewhat below the composite spectra since the presence of the protein broadens somewhat the bilayer line shape, as is well-known in the analysis of ESR spectra of lipid spin-labels (Marsh & Watts 1982; Silvius et al., 1984). As pointed out previously, it is important to avoid the presence of lipids in the gel phase, because these can give rise to a motion-restricted line shape that is very much like that of the boundary layer and otherwise might be averaged in, giving the appearance of larger amounts of lipids in contact with proteins (Griffith et al., 1982a).

The ratios of fluid bilayer to motion-restricted components of the attached photo-spin-label obtained from the ESR analysis are plotted as a function of the lipid/protein ratio in Figure 6. For comparison, a similar set of ESR data were recorded and analyzed for lipid spin-labels free to diffuse in the bilayer. The open squares are data for the phosphatidylcholine label **3**, and the open circles are data for the prephotolyzed photo-spin label **5**. The ESR line shapes for these labels are similar to those of **5** attached to cytochrome *c* oxidase, but the ratio of the spectral components as a function of the lipid/protein ratio clearly distinguishes the unattached from the attached labels.

Additional information obtained from measurements of the outermost splittings of motion-restricted ESR line shapes is plotted in Figure 7. A number of samples were examined, including one of very low lipid content (13 mol of DOPC/mol

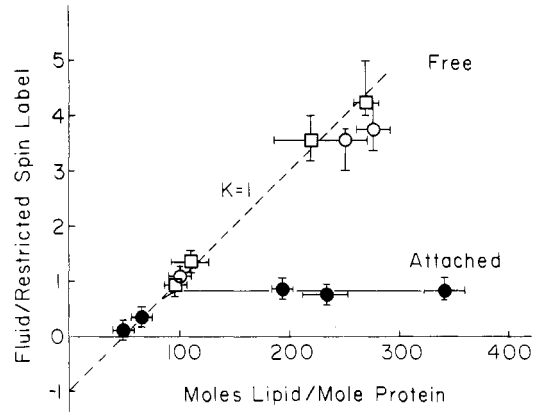

FIGURE 6: Comparison of the behavior of the covalently attached photo-spin-label **5** (closed circles) with labels free to diffuse in the bilayer (open squares and circles) as a function of the lipid/protein ratio at 25 °C. The open squares are data from the nonreactive phospholipid spin-label **3**, and the open circles were obtained by using the prephotolyzed **5**. Error bars indicate the average combined errors of the lipid and protein analyses and spectral data treatment.

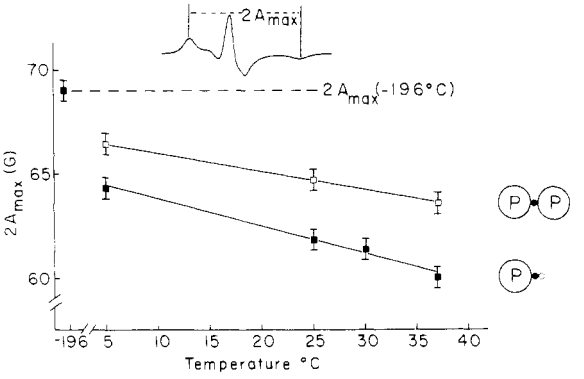

FIGURE 7: Temperature dependence of the outermost splitting of the motion-restricted component ($2A_{max}$) measured from the first-derivative ESR spectra of **5** covalently attached to cytochrome *c* oxidase. (Dashed reference line) Splitting observed at liquid nitrogen temperature. (Open squares) Data obtained with detergent-free samples containing only 13 mol of DOPC per mole of protein, i.e., aggregated lipid-deficient cytochrome *c* oxidase. (Closed squares) Data obtained for samples ranging from 50 to 350 mol of DOPC per mole of protein. The simplified drawing at the right is a view perpendicular to the bilayer diagramming two environments of the lipid spin-label (shaded small circles) among lipids (open small circle) and the larger proteins (P). There were approximately two to three photo-spin-labels per protein.

of protein, upper solid line of Figure 7. The other samples ranged from 50 to 350 mol of DOPC/mol of protein. Points measured from this latter group were the same within experimental error and are plotted as the lower line in Figure 7. At liquid nitrogen temperature, all the samples have the same $2A_{max}$ and this value (69 G) is drawn in as a dashed line for reference in Figure 7. In the absence of motion, $2A_{max} = 2A_{zz}$, the principle value of the hyperfine tensor corresponding to the magnetic field along the *z* molecular axis, parallel to the nitrogen 2p orbital containing the unpaired electron (Jost & Griffith, 1978). The principle values will vary with structure and therefore differ slightly for the proxyl moiety used here and the doxyl group which is also used in biophysical studies of membranes (Keana et al., 1976). The plots of Figure 7 show that lipid sandwiched between proteins can be distinguished from lipid in the boundary layer by measurements of the outermost splittings as a function of temperature.

Discussion

For the present study, the arylazido group was selected because it is unreactive until photolyzed, permitting the re-

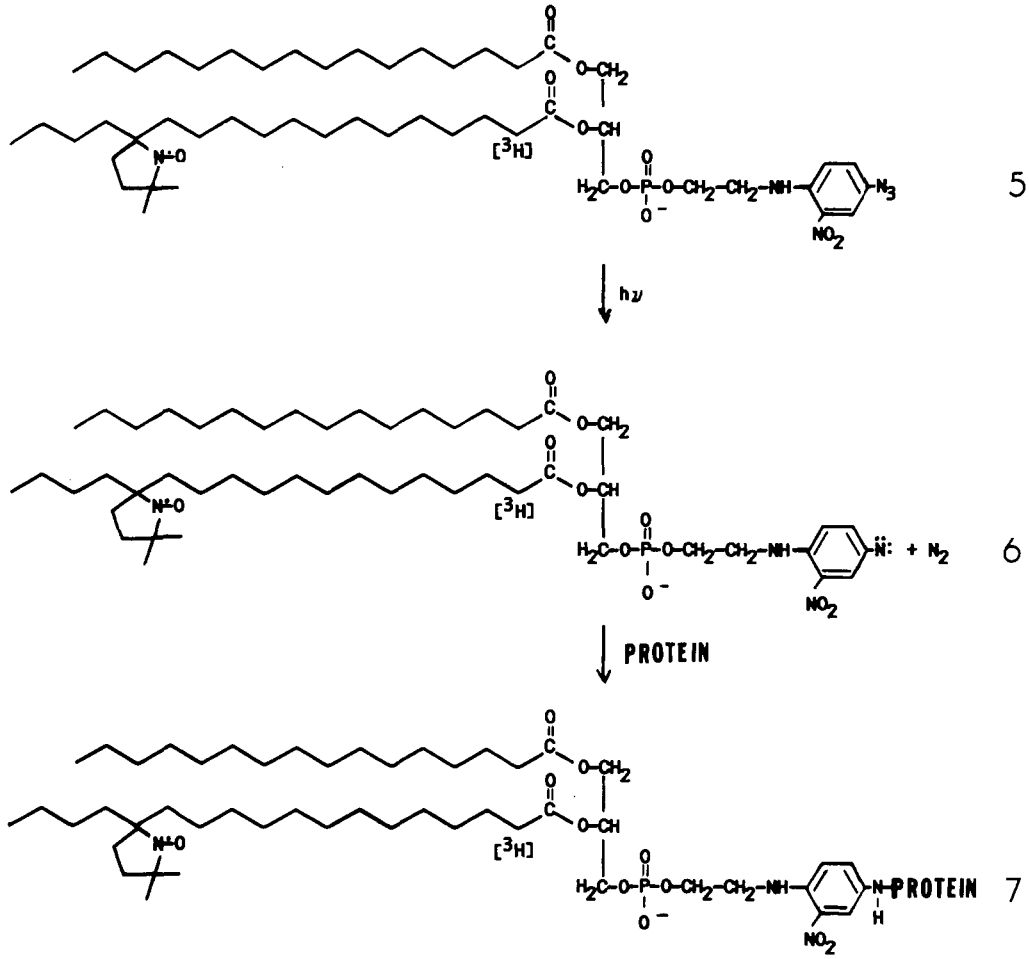

FIGURE 8: Diagrammatic representation of the reaction of the photo-spin-label **5** with cytochrome *c* oxidase. Upon photolysis, the reactive nitrene **6** is formed. Some of the nitrene then reacts and forms covalent bonds (**7**) with the amino acids exposed at the lipid–protein lamellar interface.

constitutions to be performed and detergent removed to form the stable bilayer configuration before covalent attachment takes place. The electron micrographs and ESR data support the idea that the photo-spin-label was situated in the bilayer, placing the arylazido group in the proper position to react with functional groups in the interfacial region. Once the intense visible light was switched on, the reaction proceeded according to the reaction scheme shown in Figure 8. The azido group is converted by photolysis to the nitrene which then reacts with nearby chemical groups by a variety of mechanisms that are at this point only partially understood. The nitrene can react with functional groups on the protein, phospholipids, other labels, or buffer by mechanisms such as insertion into C–H and N–H bonds, addition to double bonds, hydrogen abstraction, rearrangements, or reactions typical of electrophilic species (Bayley & Knowles, 1978a,b; Richards & Brunner, 1980; Robson et al., 1982). As pointed out by these authors, achieving a truly random pattern of attachment is probably not realistic. Nevertheless, given the spectrum of possible reactions, one would certainly expect attachment at a number of sites around the interfacial region, as is needed for the present study. A distribution of attachment sites on the protein is clearly indicated by the gel pattern of Figure 4.

Considering the variety of possible fates of the nitrene, the reaction with cytochrome *c* oxidase appears to be very efficient. For example, our earlier attempts at labeling had a molar ratio of spin-labeled arylazido phospholipid (nonradiolabeled) to unlabeled phospholipid of 1/1 in reconstituted membranes of cytochrome *c* oxidase. After photolysis, removal of the unattached photolabel, and reconstitution with excess unlabeled phospholipid, the ESR line shapes obtained were heavily broadened by spin-exchange, indicating that a number of spin-labeled photolabels had covalently attached to the protein (Griffith & Jost, 1979). A second experiment with a label/DOPC ratio of 1/6 still showed substantial exchange broadening. In a similar approach, a single hydrocarbon chain photo-spin-label was prepared and reacted with sarcoplasmic reticulum (Fellmann et al., 1980). This label may not be anchored appropriately in the bilayer, but their results also indicate a significant attachment to the protein.

For the present work, the amount of label was greatly reduced to avoid magnetic dipolar and exchange interactions between spins, and the photolabel was tritiated so that more quantitative information could be obtained. Even with the reduced labeling levels, the reaction is quite efficient. For example, photolysis of a reconstituted membrane sample containing 95 mol of unlabeled lipid (DOPC) and 4 mol of photo-spin-label per mol of cytochrome *c* oxidase yielded at least 50% attachment to the protein. This is even more remarkable when one takes into account the dilution of the photo-spin-label by the unlabeled lipids. At any instant in time, only about 50 phospholipids are in direct contact with the hydrophobic surfaces of cytochrome *c* oxidase, as discussed below. Assuming random distribution of the photo-spin-label and the unlabeled lipids in this sample, there are $50/(95 + 4) \times 4 = 2$ molecules of photo-spin-label in the boundary layer, and the other 2 molecules of photo-spin-label are at least one lipid layer away. Arylnitrenes have relatively long lifetimes

in solution, evidently in the range 10^{-3}–10^{-4} s (Reiser, 1968; Robson et al., 1982), whereas the exchange rate of lipids in fluid bilayers is on the order of 10^7 s^{-1} (Träuble & Sackmann, 1972; Devaux et al., 1973). The exchange rate of lipids between the boundary and the rest of the bilayer can approach the lateral diffusion rates of lipids. Thus, the reactive photo-spin-label can probably sample the protein surface and the lipid environments many times during its lifetime. The attachment of the photo-spin-label to the protein surface is clearly favored over internal rearrangements or reaction with lipids or buffer. A higher yield (14%) than expected for attachment of nitrenes to gramicidin A in phospholipid bilayers has also been reported (Richards & Brunner, 1980).

The central point of this study is addressed in the comparison of the behavior of the covalently attached photo-spin-label with that of a similar label free to diffuse in the plane of the phospholipid bilayer (Figure 6). At or below 40–50 mol of lipid per mole of protein, both the attached and unattached labels show only the line shape indicative of restricted molecular motion on the ESR time scale. This is consistent with the idea that at these low lipid/protein ratios, essentially all lipid present is in contact with protein. That is, the lipids first tend to coat the hydrophobic surfaces of cytochrome *c* oxidase in one homogeneous phase, rather than forming segregated pools of lipid bilayer and domains of pure protein. As the lipid content of the samples is increased, the proteins on average are spaced further apart. Analysis of the ESR data shows that the behavior of the attached and unattached lipid labels differs well beyond any possible experimental errors as the lipid/protein ratio is increased.

The unattached phospholipid spin-labels exchange between protein sites and positions where they are surrounded by lipids in the bilayer according to a set of multiple equilibria equations of the form

$$L^* + PL_{N-i}L^*_i \rightleftharpoons PL_{N-i-1}L^*_{i+1} + L \qquad (1)$$

where N is the number of independent sites on the hydrophobic surface of the protein. L, L*, and P are lipid, labeled lipid, and protein, respectively (Brotherus et al., 1981; Griffith et al., 1982b). Since the amount of labeled lipid (i.e., solute) in this study is small compared to the unlabeled lipid (solvent), we may use the simplified relationship

$$y = x/NK - 1/K \qquad (2)$$

to express the number of binding sites (N) and the binding constant (K) of the labeled lipid relative to that of the unlabeled lipid in terms of the two experimental quantities x (the lipid/protein ratio) and y (the ratio of mobile/motion-restricted ESR spectral components) (Brotherus et al., 1981). The term "binding sites" in the lipid–protein field does not necessarily imply the degree of specificity of geometry or high binding constants encountered in enzyme–substrate or protein–drug binding in aqueous solution. In membranes, the solvent system is the two-dimensional bilayer so that the molecules remain in a hydrophobic environment whether or not they contact the protein or diffuse away and are surrounded by other lipids. The lipids contacting the protein do not experience the large change in solvent environment that is a major factor in the binding of small molecules to proteins in aqueous solutions. Nevertheless, the equilibrium binding model is a convenient way to obtain thermodynamic information from spectroscopic data (Silvius et al., 1984; Marsh, 1985).

Analyzed by this method, the unattached phosphatidylcholine spin-label **3** gives $K = 1.0 \pm 0.1$ and $N = 47 \pm 5$ (dashed line, Figure 6). The K value of unity confirms that the presence of the spin-label moiety does not perturb the equilibrium, a fact that has now been established in several systems (Knowles et al., 1979; Brotherus et al., 1980; Marsh, 1985). The value of N is within the range of 40–55 obtained from earlier estimates of the number of lipids in the boundary layer (Jost et al., 1973, 1977; Marsh, 1985). It is also consistent with the available structural information. The three-dimensional structure of cytochrome *c* oxidase has recently been determined to a resolution of about 20 Å by a combination of electron microscopy and electron diffraction (Deatherage et al., 1982). There are three domains; the large C domain extending out of the cytoplasmic surface and the M_1 and M_2 domains extending through the bilayer to the matrix side as sketched in Figure 2. Although many details of the structure are lacking, and there are considerable uncertainties in estimating the number of lipids that could contact the protein, an examination of the structural data suggests an N value of about 40–45, an observation already noted by Marsh (1985). The question of whether cytochrome *c* oxidase exists as a dimer is not addressed by these data, since dimer formation may displace only a very small number of lipids at the points of contact. Unless the number of lipids displaced was large and the dimer dissociated into monomers over the range of lipid/protein ratios examined here, the change would not be detected.

The value of K for the prephotolyzed **5** is very nearly unity ($K \sim 1.3$). These measurements on the unattached lipids are important here primarily as controls, demonstrating that the labels did not inadvertently become trapped in nonexchangeable sites during the workup of the samples. However, the big effect occurred when the photo-spin-label was allowed to react with the protein. Approximately half of the attached labels exhibited the more characteristic motion-restricted ESR line shape, and the remaining half had more flexibility, approaching that of a bilayer. This range of motion is understandable when one considers that the nitrene can react at a variety of sites, for example, by insertion in bonds so that the lipid chains are directly at the hydrophobic surface where motion tends to be restricted. Alternatively, the large hydrophilic portions of the protein that extend into the aqueous phase may contact the top of the bilayer at points removed from where the hydrophobic region of the protein emerges from the bilayer. In this case, the attachment of the label could occur in places where the lipid tails do not contact the hydrophobic regions of the protein. This would permit considerable motion and may even allow lipids to occupy second-shell regions. For the motion-restricted fraction of the attached labels, K is essentially infinity at the hydrophobic protein boundary. The important result is the divergence between the two curves of Figure 6. Above N, the curve for the attached label is flat and does not curve upward as would be expected for lipids trapped between proteins as the proteins moved farther apart (Davoust et al., 1980; Pink et al., 1981). This horizontal curve also confirms that there is no significant amount of unattached label remaining in the preparation, since the latter would also cause an upward sloping curve as the lipid content increased. Conversely, the bilayer/motion-restricted ratio increases linearly with lipid in accordance with the multiple equilibria binding treatment. Both the attached and unattached spin-labels exhibit similar line shapes. It would be difficult to determine conclusively from a single sample whether the labels were free to diffuse or were attached or trapped lipids. However, analysis of the ratio of the fluid/motion-restricted spectral components as a function of the lipid/protein ratio clearly distinguishes these alternatives.

Another way to distinguish trapped lipid from the solvation layer of the hydrophobic surfaces (the boundary layer or first shell) of membrane proteins involves the analysis of the motion-restricted line shapes of these two lipid environments. This is inherently more difficult because the line shapes are similar. As the temperature decreases, the outermost splittings increase, and the spectrum approaches the type described by spectroscopists as an immobilized spectrum, a term that should be used sparingly in the biochemical literature because it can be misinterpreted to mean absolutely no motion of the lipid chains. That there is motion in both cases can be seen by comparing the experimental splittings of the motion-restricted spectra near room temperature to the value observed at liquid nitrogen temperature (dashed line, Figure 7) where most of the degrees of freedom have been frozen out except methyl group rotation and small-amplitude torsional oscillations. Besides showing that there is residual motion of lipids in contact with proteins, a fact that has been demonstrated previously by this laboratory and others [see reviews by Jost & Griffith (1980) and Marsh (1985)], Figure 7 demonstrates that there is a systematic difference between the overall splitting observed when lipid labels are artificially trapped in low lipid regions and when lipid labels are at the boundary between the bilayer and the hydrophobic surfaces of membrane proteins.

In summary, the current methods of incorporating lipid spin-labels into defined reconstituted systems and spectral analysis of the ESR data are providing a useful picture of events occurring at the lipid–protein interface. The results cannot be accounted for by aggregation artifacts. There are two compelling lines of evidence for this. One is the fit of the spin-labeling data to the multiple equilibrium binding model. The other is the very different behavior observed between the normal preparations and samples in which the lipid labels have been deliberately tethered to the interface by covalent attachment. In addition, differences between label environments can be detected by comparing the splittings of the motion-restricted spectra. These combined data also illustrate the importance of examining a range of lipid to protein ratios in studies of lipid–protein interactions.

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Registry No. **1**, 17364-16-8; **2**, 66978-86-7; **2** (labeled), 78387-58-3; **3**, 78709-94-1; **3** (labeled), 99618-02-7; **4**, 99618-01-6; **4** (labeled), 99618-03-8; **5**, 70764-33-9; **5** (labeled), 99631-59-1; cytochrome *c* oxidase, 9001-16-5.

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A Calcium-43 NMR Study of Calcium Binding to an Acidic Proline-Rich Phosphoprotein from Human Saliva†

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ABSTRACT: The ^{43}Ca NMR line width measured for Ca^{2+} bound to protein A, an acidic proline-rich salivary protein, is 1 order of magnitude narrower than has previously been observed for other proteins of similar molecular weight. The correlation times, quadrupole coupling constants, and chemical shifts estimated for Ca^{2+} ions bound to the intact protein ($M_r \sim 10\,000$) and its 30 amino acid residue long acidic N-terminal TX peptide were indistinguishable within experimental error. These results—as well as the outcome of ^1H NMR relaxation rate measurements—are indicative of extensive motions for the protein residues, which in turn give rise to a high degree of flexibility for the protein-bound Ca^{2+}. Ca^{2+} titration and pH-dependent measurements on protein A, the TX peptide, and the dephosphorylated TX peptide established the importance of the two phosphoserine residues in the binding of Ca^{2+}. Moreover, a comparison of the ^{43}Ca NMR parameters with those obtained for other Ca^{2+}-binding proteins suggests the presence of Ca^{2+}-binding sites of similar symmetry in all these proteins. No evidence was found for a proposed interaction between the highly acidic N-terminal and the weakly basic C-terminal regions of protein A. In contrast, the high pH inflection that was observed in the pH titration curve for the intact protein was also found for the phospho and dephospho TX peptides, thus suggesting that basic moieties in the N-terminal region rather than those in the C-terminal region may be responsible for this observation.

Proline-rich proteins make up roughly 70% of the total proteinaceous material in human saliva. They constitute a unique superfamily of proteins (Wong & Bennick, 1981), which are also present in salivary secretions from other species (Muenzer et al., 1979; Oppenheim et al., 1979) and in small amounts in tracheobronchial tissue (Warner & Azen, 1984). Approximately 30% of the salivary proteins are "acidic" proline-rich proteins, the major ones being proteins A and C. The amino acid sequence of these two proteins has been determined (Wong et al., 1979; Wong & Bennick, 1981). As is shown in Table I, the N-terminal 30 residues of protein A, which is known as the TX peptide and is obtained by tryptic cleavage, contains 13 of the protein's 15 negatively charged amino acid side chains. This includes the two phosphoserine residues that are present. Only one proline residue is found in this region. The remainder of the protein (residues 31–106) consists almost entirely of glutamine, glycine, and proline

Table I: A Comparison of the Amino Acid Composition of Protein A with That of the TX Peptide

residue type	residue	no. of residues: TX peptide	no. of residues: protein A
acidic	Asp	6	8
	Glu	5	5
	P-Ser	2	2
basic	Lys	0	1
	Arg	1	4
	His	0	2
uncharged polar	Asn	1	3
	Gln	2	23
	Glu[a]	1	1
	Gly	2	20
	Ser	1	3
nonpolar	Ala	0	1
	Val	3	3
	Leu	2	3
	Ile	2	2
	Pro	1	24
	Phe	1	1

[a] The N-terminal residue is pyroglutamate.

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