Lipid Solvation of Cytochrome *c* Oxidase. Deuterium, Nitrogen-14, and Phosphorus-31 Nuclear Magnetic Resonance Studies on the Phosphocholine Head Group and on Cis-Unsaturated Fatty Acyl Chains†

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ABSTRACT: The interaction of cytochrome *c* oxidase (from beef heart) with the polar groups and cis double bonds of unsaturated phosphatidylcholine bilayers was investigated by ^2H, ^{14}N, and ^{31}P nuclear magnetic resonance (NMR). Cytochrome *c* oxidase was reconstituted into bilayers composed of a single synthetic lipid, namely, either 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) or 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC). Due to their low gel-to-liquid-crystal phase transition temperatures (POPC, −5 °C; DOPC, −22 °C), both lipids support enzyme function at low temperatures while the NMR spectra retain the characteristics of a fluid lipid bilayer. POPC was selectively deuterated at the two methylene segments of the choline moiety, while DOPC was deuterated at the cis double bond of both fatty acyl chains. The interaction of the lipid polar group with cytochrome *c* oxidase was investigated for four different segments of the phosphocholine moiety, namely, the quaternary ammonium group, the two methylene segments, and the phosphate group, yielding a rather detailed picture of the movement of a phospholipid head group in a protein-containing membrane. The anisotropy of the segmental movements was characterized by the residual ^2H and ^{14}N quadrupole splittings and the phosphate chemical shielding anisotropy, respectively. For all segments investigated the segmental fluctuations in the reconstituted membrane resembled closely those observed in the pure lipid bilayer even when a large percentage of the lipid molecules was in direct contact with the protein surface. The rate of segmental fluctuations was determined by measuring spin–lattice (T_1) relaxation times. Both the ^2H T_1's of the choline segments and the ^{31}P T_1 of the phosphate group were rather similar with and without protein. The ^{31}P T_1 relaxation time of pure POPC bilayers exhibited a minimum at 15 °C, whereas this minimum was shifted to 25 °C for the reconstituted cytochrome *c* oxidase/POPC membrane. This rather small shift disproves earlier suggestions of a strong immobilization of the phosphate groups by cytochrome *c* oxidase. Taken together, the anisotropy parameters as well as the T_1 relaxation times provide no evidence for any strong polar interaction between the phosphocholine group and cytochrome *c* oxidase, neither in terms of a conformational change of the head group nor in terms of a significant immobilization of individual segments. The only noticeable difference between the NMR spectra of reconstituted membranes and pure lipid bilayers was a distinct line broadening in the presence of protein. This line broadening probably arises from slower motions, but its molecular origin is not well understood at present. Similar conclusions hold true for the nonpolar interactions in the interior of the lipid bilayer. The quadrupole splittings and the T_1 relaxation times of the deuterated cis double bonds were only little changed in the presence of protein, indicating that the average orientation and the fluctuations of the cis double bonds were not influenced significantly by cytochrome *c* oxidase. On the other hand, the intrinsic line width of the ^2H NMR spectra was again larger in reconstituted membranes than in pure DOPC bilayers.

Cytochrome *c* oxidase is an intrinsic membrane protein which catalyzes the transfer of electrons from cytochrome *c* to molecular oxygen. The beef heart enzyme can be isolated and purified in almost lipid-free form (Yu et al., 1975; Wei & King, 1981; Robinson et al., 1980), and its function can be reconstituted by incorporation into a variety of lipid matrices (Racker & Kandrach, 1973; Yu et al., 1975; Eytan et al., 1976; Vik & Capaldi, 1977). The interaction of cytochrome *c* oxidase with the surrounding lipids has been investigated extensively by spin-label electron paramagnetic resonance (EPR)[1] [Jost et al., 1973; cf. Marsh & Watts (1982) for a review and discussion of these and later results]. The EPR experiments revealed two motionally distinct lipid populations, and the term "boundary lipid" was coined for the motionally slower component. However, subsequent ^2H and ^{31}P NMR studies failed to detect the presence of two lipid populations; all lipids in the reconstituted membrane exhibited a very similar motional behavior (Oldfield et al., 1978; Seelig & Seelig, 1978; Kang et al., 1979, 1981; Rice et al., 1979; Paddy et al., 1981). This apparent discrepancy can be resolved by assuming that the lipids are in rapid exchange between the "boundary layer" and the bulk lipid phase (Jost & Griffith, 1980; Paddy et al., 1981; Seelig et al., 1982). If the exchange rate is of the order of 10^7 Hz and if the motion of the spin-label on the protein surface is slowed down by a factor of 2–3, this would lead to two distinct EPR spectra for the two lipid populations and, at the same time, create a single, time-averaged NMR spectrum (Birrell et al., 1982). Unfortunately, no direct measurement of the exchange rate is available to date.

The role of boundary lipid could be likened to that of hydration water. It has been demonstrated mainly by NMR studies that the motion of water on a protein or membrane surface is quite rapid (Berendsen, 1975; Borah & Bryant, 1982; Finer & Darke, 1974). Moreover, there is a fast exchange between water bound in different "hydration shells" and bulk

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[1] Abbreviations: EPR, electron paramagnetic resonance; NMR, nuclear magnetic resonance; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)-aminomethane; $NaDodSO_4$, sodium dodecyl sulfate; BSA, bovine serum albumin.

0006-2960/83/0422-1474$01.50/0

water. On the other hand, the motional freedom of the water molecules in the immediate vicinity of a macromolecule or a membrane surface is more restricted compared to that of bulk water even though the question of the extent of motional anisotropy is largely unresolved [for a review, see Marthur-de Vrē (1979)]. Hence, if the solvation of membrane-bound proteins by lipids is visualized in an analogous manner, the immediate question arises to which extent the properties of the boundary lipid are similar to or different from those of the bulk lipid. Obviously, the analogy between hydration and lipid solvation cannot be stretched too far. A lipid is a much more complex molecule than water, and differential interactions might be possible at the level of the polar groups, the glycerol backbone, and the fatty acyl chains.

The bulk of the previous reconstitution experiments was directed toward an understanding of the fatty acyl chains, and the reporter group was generally attached at a saturated fatty acyl chain. This work represents the first detailed NMR study of the interaction of a phospholipid polar group with cytochrome *c* oxidase. This was achieved by reconstituting the protein into bilayers of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) selectively deuterated at either methylene segment of the choline residue. The structural and dynamic features of the lipid–protein interaction were inferred by measuring the residual deuterium quadrupole splittings and the T_1 relaxation times of the labeled segments. In addition, the phosphocholine head group was also characterized by ^{14}N and ^{31}P NMR, leading to a rather complete picture for the main segments of the polar group.

Cytochrome *c* oxidase was further incorporated into an unsaturated bilayer with deuterium labels at the cis double bonds, namely, 1,2-di[9,10-$^2H_2$]oleoyl-*sn*-glycero-3-phosphocholine (DOPC). Thus it became possible to monitor the infuence of cytochrome *c* oxidase on the chain motion of cis-unsaturated fatty acyl chains. Special attention was given to a thorough biochemical characterization of the reconstitution process in order to ensure a functionally active cytochrome *c* oxidase in a membrane containing very little residual detergent. When unsaturated phosphatidylcholines were used for cytochrome *c* oxidase reconstitution, it was possible to conduct all experiments at low temperatures in a fluid lipid bilayer, thus retaining almost full enzymic activity of cytochrome *c* oxidase over the whole measuring period.

Materials and Methods

For simplification of the discussion the following notation for the head-group segments is introduced:

$$PO\text{—}\underset{\alpha}{CH_2}\text{—}\underset{\beta}{CH_2}\text{—}\underset{\gamma}{N(CH_3)_3}$$

Lipid Synthesis. α- and β-deuterated 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholines were synthesized according to Gally et al. (1975) and incubated with phospholipase A_2 from *Crotalus atrox* to yield the corresponding lysophosphatidylcholines (Chakrabarti & Khorana, 1975). The lyso compounds were reacted with oleic acid anhydride to yield POPC (Gupta et al., 1977) and purified on silica gel. 1,2-Di[9,10-$^2H_2$]oleoyl-*sn*-glycero-3-phosphocholine was prepared by reacylation of *sn*-glycero-3-phosphocholine with oleic acid anhydride (Seelig & Waespe-Šarčevič, 1978). Nondeuterated lipids were purchased from Fluka, Buchs, Switzerland, and from R. Berchtold, Bern, Switzerland.

Lipid Dispersions. For the NMR measurements, the lipids were dispersed by vortexing in excess (~50% w/w) deuterium-depleted water (CEA, Saclay, France) or 50 mM Tris buffer, pH 7.4, and 1 mM EDTA (made from deuterium-depleted water), forming large multilayered liposomes. For ^{31}P T_1 relaxation measurements buffer containing EDTA was essential; in all other cases EDTA (and buffer) could be omitted with no observable difference.

Preparation of Cytochrome c Oxidase. Lipid-depleted cytochrome *c* oxidase was prepared by the method of Yu et al. (1975) with some modifications. Beef heart mitochondria, prepared on a large scale as described by Blair (1967) and stored in 0.1 M H_3BO_3/0.1 M Na_2HPO_4 (1/1), pH 7.4, and −70 °C, were diluted to 20 mg of protein/mL with 0.1 M borate/phosphate buffer. To this suspension was added 20% (w/v) sodium cholate, pH 7.8, to a final concentration of about 0.5% (w/v). The optimal cholate concentration for the separation of red cytochromes from green cytochromes was determined for each mitochondrial preparation separately and was in the range of 0.4–0.5%. If submitochondrial particles (20 mg/mL) were used instead of Blair mitochondria, the optimal cholate concentration was close to 1%.

The ammonium sulfate fractionations were carried out as described by Yu et al. (1975) with the exception that often 50 mM Tris-HCl, pH 7.4, was used instead of phosphate buffer and that small ammonium sulfate increments were added in the second fractionation in order to get subfractions of higher purity. Usually fractions containing 10–12 nmol of heme *a*/mg of protein were obtained by this procedure. The phospholipid content of these preparations was about 20–30 μg/mg of protein or 10 mol of organic phosphate/mol of cytochrome *c* oxidase (5 mol/mol heme *a*). The spectroscopically determined activity in 0.5% (w/v) Tween 80 and 50 mM phosphate at 25 °C was 2–4 μmol of cytochrome *c* oxidized per min^{-1} (nmol of heme *a*)$^{-1}$ (depending on whether or not the sample had been frozen once before the measurement). In the absence of Tween 80 the activity of the lipid-deficient oxidase was 0.05 ± 0.02 μmol of cytochrome *c* min^{-1} (nmol of heme *a*)$^{-1}$. A third cycle of ammonium sulfate fractionation neither improved purity nor reduced the phospholipid content any further. If submitochondrial particles were used instead of crude mitochondria, we obtained enzyme preparations of comparable quality. They still contained about 5 mol of P/mol of heme *a*.

Protein was measured by the method of Lowry et al. (1951) with BSA as a standard. Purity of cytochrome *c* oxidase was checked on a 15% $NaDodSO_4$–polyacrylamide gel and showed no major contaminating bands. Reduced minus oxidized difference spectra were run in the α-band region (500–650 nm), and heme *a* was determined from the absorption difference (reduced − oxidized) at 605 nm by using a millimolar extinction coefficient of 12.0 (Kuboyama et al., 1972). Organic phosphate was measured by the method of Ames & Dubin (1960). Activities were determined spectroscopically following the oxidation of excess (0.6 mg/mL) dithionite-reduced cytochrome *c* (horse heart, Sigma type III) in 50 mM phosphate buffer, pH 7.4 at 25 °C. A millimolar extinction coefficient $(redox)_{550nm}$ of 19.2 was used.

Reconstitution of Cytochrome c Oxidase. Since a substantial loss (up to 50%) in activity was observed when cytochrome *c* oxidase was frozen and thawed once,[2] reconstitution was performed routinely immediately after isolation. The reconstitution of cytochrome *c* oxidase with the different synthetic phospholipids was accomplished by cholate dialysis (Racker & Kandrach, 1973). A 50–80-mg sample of phos-

[2] Even when cooled rapidly with liquid nitrogen and dissolved in a small volume of 0.25 M sucrose buffer.

pholipid in 50 mL of 50 mM Tris buffer, pH 7.4, containing 4 mg/mL cholate (final) was vortexed until a clear solution had formed and then cooled on ice. Concentrated cytochrome *c* oxidase (20–40 mg/mL) was added to yield the desired lipid-to-protein ratio (0.3–0.5 mg/mg). Finally, the concentration of cytochrome *c* oxidase was adjusted to 2 mg/mL by addition of Tris buffer. Activity was restored immediately as shown by $\sim 10^5$-fold dilution into the cholate-free assay medium. Oxidation rates of 2.5–3.5 μmol of cytochrome *c* min^{-1} (nmol of heme *a*)$^{-1}$ were typical at 25 °C.

The lipid-replenished cytochrome *c* oxidase solution was dialyzed for 48–60 h against five to six changes of 50 mM Tris buffer, pH 7.4, in order to remove sodium cholate. After dialysis the membranes were centrifuged for 30 min in a Beckman rotor 35 at 27 000 rpm (85000*g*) and taken up in about 15 mL of 50 mM Tris, 1 mM EDTA, and deuterium-depleted buffer, pH 7.4. Negative-stain electron microscopy revealed large membrane fragments of about 1-μm diameter. On an analytical 10–60% (w/w) sucrose gradient the membranes equilibrated as a single band at approximately 40% sucrose, depending on the lipid-to-protein ratio. For NMR measurements the samples were spun down in a Beckman Ti50 rotor at maximal speed (150000*g*) for 2 h. The tight pellet [containing about 70–80% (w/w) water] was transferred into a NMR sample tube. Cholic acid from Serva, Heidelberg, was used without further recrystallization. [^{3}H]Cholic acid (16 Ci/nmol) was purchased from New England Nuclear, Boston, MA.

NMR Measurements. All measurements were carried out on a Bruker-Spectrospin CXP-300 spectrometer equipped with a high-power proton decoupler. The ^{2}H NMR spectra were measured at 46.06 MHz by using the quadrupole echo technique (Davis et al., 1976). Pulses (90°) of 3.5–4 μs were used with an echo pulse separation of 30–40 μs. The spectra were recorded with quadrature phase detection, and the spectral width was varied between 100 and 500 kHz. Deuterium T_1 relaxation times were measured by the pulse sequence 180_x–τ–90_x–τ_e–90_y–τ_e, where τ_e is the constant quadrupole echo pulse spacing. In all ^{2}H NMR experiments a phase alternating sequence was employed to reduce coherent noise and phase errors.

The ^{31}P NMR spectra were recorded at 121.4 MHz with inverse gated proton decoupling. Usually 2-μs (~50°) pulses were used with a recycle time of 1 s. Phosphorus T_1 relaxation times were measured by the 180°–τ–90° sequence with or without decoupling.

^{14}N NMR spectra were obtained at 21.68 MHz by the quadrupole echo technique. The 90° pulses were 9.5 μs and the echo pulse spacing 180 μs with a relaxation delay of 150 ms. The ^{14}N NMR spectra were taken on resonance with quadrature detection.

Results

Cholate Dialysis. The time course of cholate removal by dialysis was monitored in two reconstitution experiments with [^{3}H]cholate. The activity of cytochrome *c* oxidase was measured simultaneously, and both sets of data for the same experiment are displayed in Figure 1. The cholate concentration decreased from its initial value of 5 mg/mL to 130 μg/mL after 21.5 h of dialysis. At the same time, the protein concentration decreased slightly from 3.8 to 2.4 mg/mL. Figure 1 therefore represents the measurements in terms of milligrams of cholate per milligram of protein. The decay of the radioactivity followed a single exponential with a half-time of $t_{1/2}$ = 4.3 h. The cholate concentration after 21.5 h of dialysis was reduced to 4% of its starting value.

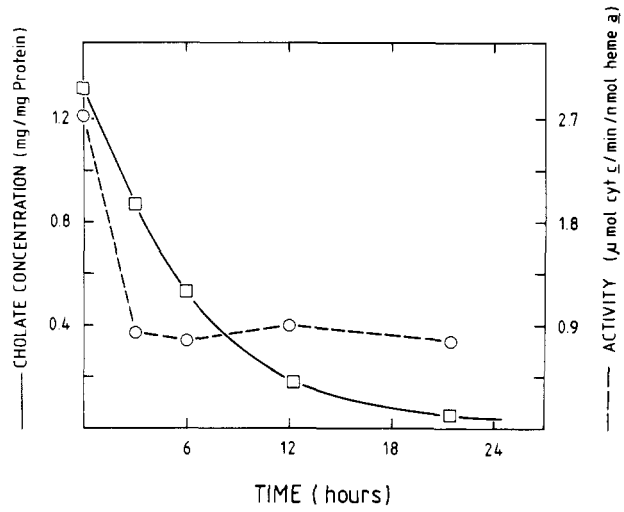

FIGURE 1: Time course of cholate dialysis. A mixture of 19.1 mg of cytochrome *c* oxidase, 10 mg of DOPC, and 25 mg of [^{3}H]cholate with a specific activity of 3 μCi/mg in 5 mL of 50 mM phosphate buffer, pH 7.4, was dialyzed against 50 mM phosphate buffer, pH 7.4, at 4 °C. Samples were taken out of the dialysis bag at indicated times and measured for radioactivity, cytochrome *c* oxidase activity, and protein concentration. (□) Cholate concentration. (○) Cytochrome *c* oxidase activity.

For determination of the fraction of cholate associated with the lipid phase, the membranes of the experiment shown in Figure 1 were spun down after 21.5 h of dialysis. The residual cholate content of the supernatant was 68 μg/mL whereas that of the pellet was 2.02 μg/mg of wet pellet (all measurements were corrected for background counts). Since the water content of the pellet was 81.5 wt % as determined by drying under high vacuum, the amount of cholate associated with the membrane was calculated to be 10.6 μg/mg of dry membrane. With a lipid-to-protein ratio of 0.52 (w/w) this leaves a residual membrane-bound cholate concentration of 16.5 μg of cholate/mg of protein. In the second dialysis experiment with [^{3}H]cholate the dialysis was extended to 48 h, and the residual cholate content was 0.78 μg of cholate/mg of protein, again with a lipid-to-protein ratio of 0.5 (w/w). For the NMR measurements the samples were usually dialyzed for 48–60 h.

The enzyme activity, measured after 10^5-fold dilution, decreased rapidly within the first few hours of dialysis approaching a more or less constant value of 0.9 μmol of cytochrome *c* oxidized min^{-1} (nmol of heme *a*)$^{-1}$. However, most of the later (large scale) preparations yielded a more stable enzyme, oxidizing 1.2 μmol of cytochrome *c* min^{-1} (nmol of heme *a*)$^{-1}$ at 25 °C even after 60 h of dialysis. The initial loss in activity is paralleled by an increased turbidity in the dialysis bag due to membrane formation. Whether the loss in activity is due to irreversible denaturation or to the formation of some inactive aggregates within the membrane at these low lipid-to-protein ratios is not known at present. However, the addition of 0.5% (w/v) Tween 80 did not change the observed values. This result suggests that denaturation rather than aggregation is the cause of the observed loss in activity. Respiratory control cannot be responsible for the lower activities, since it should be abolished by treatment with detergent and since at these low lipid/protein ratios no closed vesicles were formed as evidenced by electron microscopy. No significant variation of the enzyme activity was observed with the different phosphatidylcholines used in this study.

Cytochrome c Oxidase Membranes Reconstituted with Head-Group Deuterated POPC. ^{2}H NMR spectra of lipid

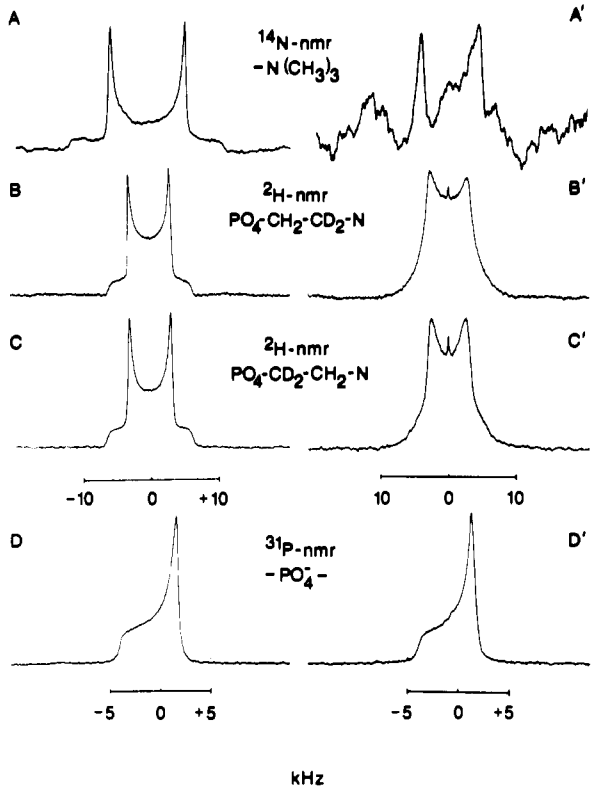

FIGURE 2: ^{14}N, ^{2}H, and ^{31}P NMR spectra of pure POPC liposomes (A–D) and cytochrome *c* oxidase reconstituted with POPC (A′–D′) at 15 °C. (A) ^{14}N NMR: 250 mg of POPC dispersed in water, 90 000 transients, $\Delta\nu_Q$ = 11.0 kHz. (A′) ^{14}N NMR: cytochrome *c* oxidase reconstituted with [β-$^{2}H_2$]POPC, lipid-to-protein ratio, L/P = 0.33 (w/w), ~50 mg of lipid, 525 000 transients, $\Delta\nu_Q$ = 8.8 kHz. (B) ^{2}H NMR: 57 mg of [β-$^{2}H_2$]POPC dispersed in water, 7000 scans, $\Delta\nu_Q$ = 6.05 kHz. (B′) ^{2}H NMR: cytochrome *c* oxidase reconstituted with [β-$^{2}H_2$]POPC, same sample as in (A′), 26 000 scans, $\Delta\nu_Q$ = 5.5 kHz. (C) ^{2}H NMR: 55 mg of [α-$^{2}H_2$]POPC dispersed in water, 20 000 transients, $\Delta\nu_Q$ = 6.20 kHz. (C′) ^{2}H NMR: cytochrome *c* oxidase reconstituted with [α-$^{2}H_2$]POPC, lipid-to-protein ratio, L/P = 0.37 (w/w), ~45 mg of lipid, 27 000 transients $\Delta\nu_Q$ = 5.1 kHz. (D) ^{31}P NMR: 80 mg of POPC dispersed in 50 mM Tris and 1 mM EDTA, pH 7.4, 2000 scans, $\Delta\sigma$ = −49.4 ppm. (D′) ^{31}P NMR: cytochrome *c* oxidase reconstituted with [α-$^{2}H_2$]POPC, same sample as in (C′), 3000 scans, $\Delta\sigma$ = −49.0 ppm.

bilayers of α- and β-deuterated POPC with and without protein (lipid-to-protein ratio of 0.3–0.4 w/w) are shown in Figure 2. All spectra were recorded at 15 °C which is well above the phase transition temperature of POPC (T_c = −5 °C). The pure lipid dispersions exhibit the typical line shapes of non-oriented, fluid lipid bilayers with well-resolved 0° edges of the so-called "powder pattern" (Seelig, 1977). The corresponding spectra of the cytochrome *c* oxidase containing membranes are also characteristic of fluid lipid bilayers, but the spectra are distinctly broadened compared to pure POPC bilayers. This is particularly noticeable for the 0° edges which are almost smoothed out beyond detection. In contrast, the residual quadrupole splittings, $\Delta\nu_Q$, were only slightly affected by the incorporation of cytochrome *c* oxidase. $\Delta\nu_Q$ was determined by measuring the separation between the two most intense peaks in the powder pattern and was related to the deuterium order parameter, S_{CD}, according to

$$\Delta\nu_Q = (3/4)(e^2qQ/h)S_{CD} \quad (1)$$

Here e^2qQ/h is the static quadrupole constant ($\simeq$170 kHz for a methylene group), and S_{CD} describes the average angular excursions of the C–D bond around the bilayer normal. The temperature dependence of the quadrupole splittings with and without protein is shown in Figure 3. The quadrupole

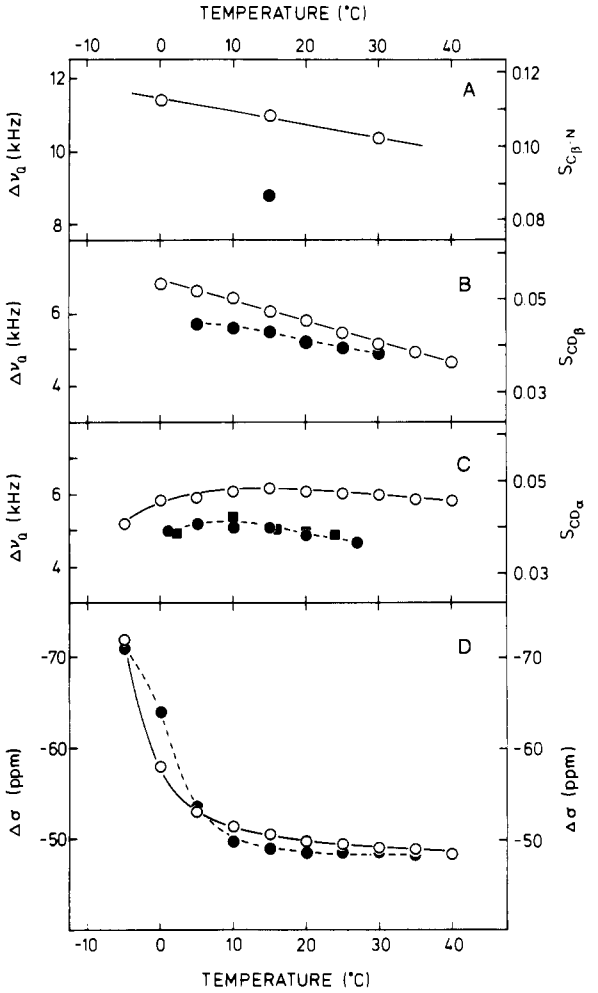

FIGURE 3: Temperature dependence of the quadrupole splittings, $\Delta\nu_Q$, and the chemical shielding anisotropies, $\Delta\sigma$, of cytochrome *c* oxidase reconstituted with POPC. (A) ^{14}N NMR: POPC (○) and cytochrome *c* oxidase reconstituted with [β-$^{2}H_2$]POPC, L/P = 0.3 (w/w) (●). (B) ^{2}H NMR: [β-$^{2}H_2$]POPC (○) and cytochrome *c* oxidase reconstituted with [β-$^{2}H_2$]POPC, L/P = 0.33 (w/w) (●). (C) ^{2}H NMR: [α-$^{2}H_2$]POPC (○) and cytochrome *c* oxidase reconstituted with [α-$^{2}H_2$]POPC, L/P = 0.37 (w/w) (●) and L/P = 0.55 (w/w) (■). (D) ^{31}P NMR: POPC (○) and cytochrome *c* oxidase reconstituted with [α-$^{2}H_2$]POPC, L/P = 0.37 (w/w) (●).

splittings of both head-group segments are smaller with protein than without it, and this effect is more pronounced for the α segment than for the β segment. On the other hand, the difference in $\Delta\nu_Q$ between pure POPC and cytochrome *c* oxidase/POPC increases for the α segment but decreases for the β segment as the temperature is raised.

In addition to the methylene segments, the properties of the quaternary ammonium and phosphate groups were measured by ^{14}N and ^{31}P NMR, respectively, and representative results are also displayed in Figure 2. The ^{14}N NMR spectra exhibit qualitatively the same line shape as the ^{2}H NMR spectra since ^{14}N is also an I = 1 nucleus with a quadrupolar moment. Unfortunately, the static quadrupole moment of the $-CH_2N-(CH_3)_3$ group is not accurately known at present. The best estimate is probably $e^2qQ/h \simeq$ 135 kHz as determined from model calculations using the deuterium order parameter of the γ segment (Siminovitch et al., 1980; Rothgeb & Oldfield, 1981). The changes observed for the ^{14}N NMR spectra are qualitatively similar to those obtained by ^{2}H NMR. The reconstituted membranes give rise to smaller quadrupole splittings (cf. Figure 3) and increased intrinsic line width. This result is in agreement with ^{14}N NMR studies on cytochrome

Table I: Deuterium T_1 Relaxation Times (at 46.1 MHz) and Derived Correlation Times τ_c for Cytochrome *c* Oxidase Reconstituted with Unsaturated Phosphatidylcholines

		pure lipid		reconstituted cytochrome *c* oxidase	
	T (°C)	T_1 (ms)	τ_c (ns)[a]	T_1 (ms)	τ_c (ns)[a]
[9,10-$^2H_2$]DOPC[b]	−10	6.3	0.36		
(lipid/protein	−5			5.6	0.41
ratio 0.32 w/w)	0	7.0	0.33		
	5			6.7	0.34
	12	9.2	0.25		
	15			8.8	0.26
	28	15.8	0.15	11.9	0.20
	40	21.7	0.11		
[α-$^2H_2$]POPC	5	8.2	0.28	8.5	0.27
(lipid/protein	15	11.8	0.20	11.3	0.20
ratio 0.37 w/w)	25	16.4	0.14	13.9	0.17
[β-$^2H_2$]POPC	5	9.2	0.25	8.4	0.27
(lipid/protein	15	13.1	0.18	11.0	0.21
ratio 0.33 w/w)	25	18.1	0.13	13.9	0.17

[a] Calculated according to eq 3. [b] Within experimental error all three quadrupole splittings have the same relaxation time T_1. τ_c was calculated for the largest splitting [cf. also Seelig et al. (1981) Table I].

c oxidase/1,2-dimyristoyl-*sn*-glycero-3-phosphocholine membranes (Rothgeb & Oldfield, 1981).

The line shape of the ^{31}P NMR spectra is determined by the averaging of the chemical shielding tensor of the phosphate group (cf. Seelig, 1978). The ^{31}P NMR spectra shown in Figure 2 also exhibit the characteristic signature of fluid lipid bilayers. In contrast to the ^2H and ^{14}N NMR spectra there is little or no obvious difference in the intrinsic line width with and without protein. The separation of the edges of the ^{31}P NMR spectrum, i.e., the distance between the weak low-field shoulder and the intense high-field peak, defines the residual chemical shielding anisotropy $\Delta\sigma$. Due to the asymmetry of the static phosphorus shielding (Kohler & Klein, 1976; Griffin, 1976) the molecular interpretation of $\Delta\sigma$ requires two independent order parameters (Niederberger & Seelig, 1976). The variation of the phosphorus chemical shielding anisotropy $\Delta\sigma$ with temperature in reconstituted cytochrome *c* oxidase/POPC membranes is summarized in Figure 3.

Deuterium T_1 relaxation times were measured for pure POPC bilayers as well as for reconstituted membranes in order to compare the rate of head-group movement (cf. Table I). The recovery of the magnetization after a 180° pulse followed a single exponential. The temperature dependence of the T_1 relaxation times of α- and β-CD_2-POPC with and without protein is represented as Arrhenius plots in Figure 4. Straight lines were observed under all experimental conditions with activation energies of the order of 4–6 kcal/mol (cf. Table II). The activation energies were always smaller in the reconstituted systems. In general, the effect of protein on the head-group T_1 relaxation times was small, inducing a reduction of T_1 between 0 and 20%.

The temperature dependence of the T_1 relaxation time is characteristic for the fast correlation time regime with $\omega_0\tau_c \ll 1$ (ω_0 = Larmor precessional frequency). With the simplifying assumption that the segment motion can be described by a single correlation time τ_c it follows (Brown et al., 1979)[3]

$$\frac{1}{T_1} = \frac{3\pi^2}{2}\left(\frac{e^2qQ}{h}\right)^2\left(1 + \frac{1}{2}S_{CD} - \frac{3}{2}S_{CD}^2\right)\tau_c \qquad (2)$$

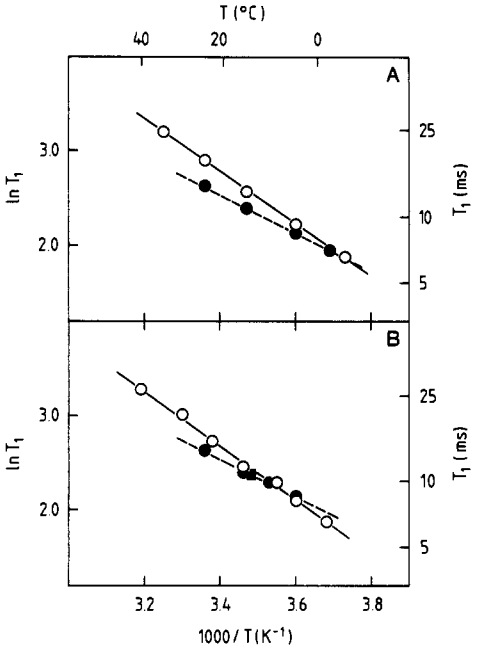

FIGURE 4: Variation of the deuterium T_1 relaxation time (at 46.1 MHz) with temperature. (A) Pure [β-$^2H_2$]POPC liposomes dispersed in water (○), E_a = 5.5 kcal/mol. Cytochrome *c* oxidase reconstituted with [β-$^2H_2$]POPC, L/P = 0.33 (w/w) (●), E_a = 4.1 kcal/mol. (B) Pure [α-$^2H_2$]POPC liposomes dispersed in water (○), E_a = 5.8 kcal/mol. Cytochrome *c* oxidase reconstituted with [α-$^2H_2$]POPC, L/P = 0.37 (w/w) (●) and L/P = 0.55 (w/w) (■), E_a = 4.0 kcal/mol.

Table II: Activation Energies As Derived from the Temperature Dependence of the T_1 Relaxation Times

	pure lipid (kcal/mol)	reconstituted cytochrome *c* oxidase (kcal/mol)
[α-$^2H_2$]POPC	5.8	4.0
[β-$^2H_2$]POPC	5.4	4.1
[PO_4]DOPC[b]	4.1	ND[d]
[9,10-$^2H_2$]DOPC[b]	4.7	3.8
chain-CD_2-segments of DPPC[c]	3.5 ± 0.3	ND

[a] Calculated from ^2H and ^{31}P T_1 relaxation time measurements. [b] From Seelig et al. (1981). [c] From Brown et al. (1979), mean of five different positions in the fatty acyl chains. [d] ND, not determined.

The correlation times τ_c calculated with eq 2 are included in Table I.

The same samples were also used for ^{31}P T_1 relaxation time measurements. To a first approximation the whole powder pattern was found to be determined by a single T_1 time. Figure 5 shows the variation of the phosphorus T_1's with temperature indicating distinct relaxation time minima with and without protein. For pure POPC this minimum occurred at about 15 °C, whereas for the reconstituted cytochrome *c* oxidase/POPC system the minimum was shifted to 25 °C. However, above about 30 °C much shorter T_1 relaxation times were measured. This effect was irreversible and is ascribed to a partial dena-

[3] The rotational isomerizations and torsional oscillations of the methylene segments are characterized by an anisotropic diffusion tensor. In addition, the molecule as a whole may undergo some reorientations with rather long correlation times. The T_1 relaxation times are determined essentially by those motions which have rates close to the Larmor precessional frequency while the slower motions have no effect on T_1. This justifies the assumption of a single correlation time. More complicated models assuming a distribution of correlation times have also been suggested (Brown, 1982).

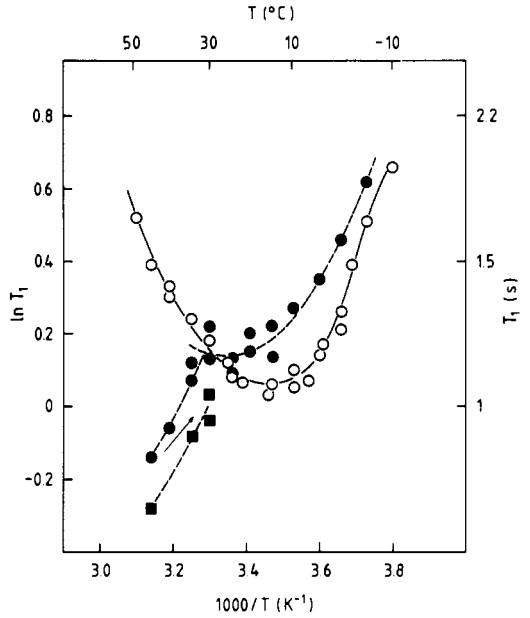

FIGURE 5: Variation of the phosphorus T_1 relaxation time (at 121.4 MHz) with temperature. (○) Pure POPC dispersed in 50 mM Tris buffer, pH 7.4, containing 1 mM EDTA. (●) Cytochrome *c* oxidase reconstituted with [α-$^2H_2$]POPC in 50 mM Tris, pH 7.4, and 10 mM EDTA (same sample as in Figure 2C',D'). The higher temperatures (>30 °C) were measured last. (■) Same sample as above, but resuspended and washed in additional 10 mM EDTA after the measurement at 45 °C.

turation of cytochrome *c* oxidase, resulting in an exposure of the paramagnetic heme groups with a concomitant increase in the relaxation rate $R = 1/T_1$. This was verified by isolating heme a_3 from cytochrome *c* oxidase and adding it to pure POPC bilayers. A dramatic shortening of the phosphorus T_1 relaxation time was observed (L. Tamm and J. Seelig, unpublished results). The preparation also lost most of its electron transfer activity, which decreased from about 0.8 to 0.1 μmol of cytochrome *c* min^{-1} (nmol of heme *a*)$^{-1}$ in the course of these last high temperature measurements.

Cytochrome c Oxidase Reconstituted with [9,10-$^2H_2$]DOPC. The ^2H NMR spectrum of multilamellar DOPC liposomes deuterated at the 9,10 positions of both oleic acyl chains consists of three overlapping "powder-type" spectra as illustrated in Figure 6A. The splittings of the two deuterons at position 9 are identical (14.2 kHz at 5 °C), whereas the two deuterons at position 10 have splittings that are well separated (7.7 kHz for the *sn*-1 chain deuteron and 2.6 kHz for the *sn*-2 chain deuteron). This indicates that the two fatty acyl chains although chemically identical are motionally inequivalent. The spectrum of [9,10-$^2H_2$]DOPC therefore provides a spectral "fingerprint" for the average lipid conformation in the hydrophobic region. The assignment of the splittings was made with lecithins in which only one oleic acyl chain was deuterated (Seelig & Waespe-Šarčevič, 1978; Seelig et al., 1981).

Figure 6B shows a ^2H NMR spectrum of cytochrome *c* oxidase reconstituted with [9,10-$^2H_2$]DOPC at a lipid-to-protein ratio of 0.32 mg/mg corresponding to ~80 lipids per molecule cytochrome *c* oxidase. Except for an increase in line width this spectrum is quite similar to that of the pure lipid bilayer; the variation of the quadrupole splittings with temperature is displayed in Figure 7. The deuterium spin–lattice relaxation times of pure [9,10-$^2H_2$]DOPC liposomes and reconstituted cytochrome *c* oxidase membranes were also measured. In either case the recovery of the magnetization followed a single exponential at all temperatures, and some numerical values are summarized in Table I. The T_1 relaxation

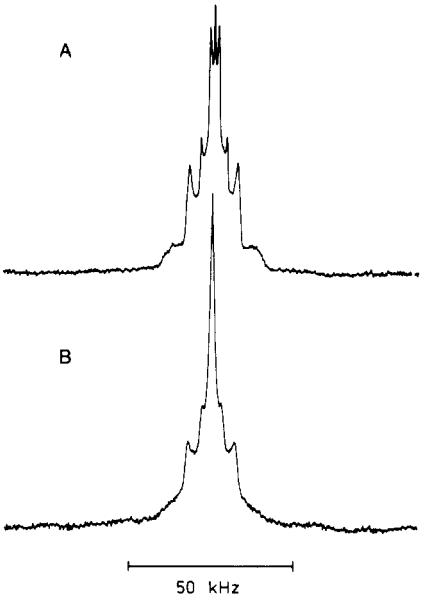

FIGURE 6: ^2H NMR spectra at 46.1 MHz of cytochrome *c* oxidase reconstituted with [9,10-$^2H_2$]DOPC at 5 °C. (A) Pure lipid dispersed in water, 120 mg, 1500 scans. The quadrupole splittings are 14.2, 7.7, and 2.6 kHz. (B) Reconstituted cytochrome *c* oxidase, lipid-to-protein ratio, L/P = 0.32 (w/w), ~50 mg of lipid, 30 000 scans. The two resolved quadrupole splittings are 14.4 and 5.5 kHz.

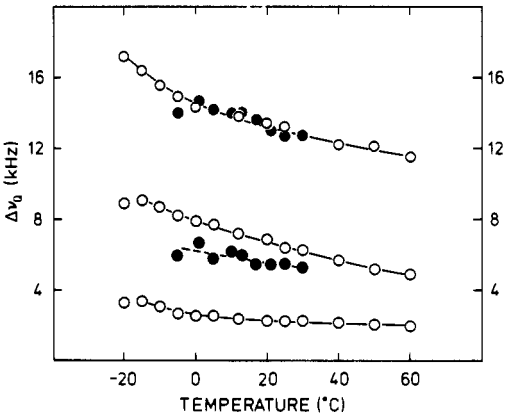

FIGURE 7: Temperature dependence of the quadrupole splittings, $\Delta\nu_Q$, of cytochrome *c* oxidase reconstituted with [9,10-$^2H_2$]DOPC. (○) Pure lipid liposomes dispersed in water. (●) Reconstituted cytochrome *c* oxidase, L/P = 0.32 (w/w).

times increase with increasing temperature, indicating that the motion is again in the fast correlation time limit. Above 5 °C Arrhenius plots of the relaxation data yield straight lines (Figure 8) with activation energies of 4.7 kcal/mol for the pure lipid bilayer and 3.8 kcal/mol for reconstituted cytochrome *c* oxidase.

Discussion

Biochemical Characterization of the Reconstituted Cytochrome c Oxidase Membranes. Using the procedure of Yu et al. (1975) we routinely obtained cytochrome *c* oxidase fractions that contained 8–10 mol of organic phosphate/mol of cytochrome *c* oxidase. This is 4–5 times more than Yu et al. (1975) described in their original paper for the "lipid-depleted" cytochrome *c* oxidase. This lipid content could not be reduced any further by introducing a third fractionation step or by using submitochondrial particles instead of whole mitochondria as the starting material. More recently, Wei & King (1981) reported much higher residual phospholipid contents, ranging from about 40 mol of P/mol of heme aa_3 after two fractionation cycles down to about 6 mol of P/mol

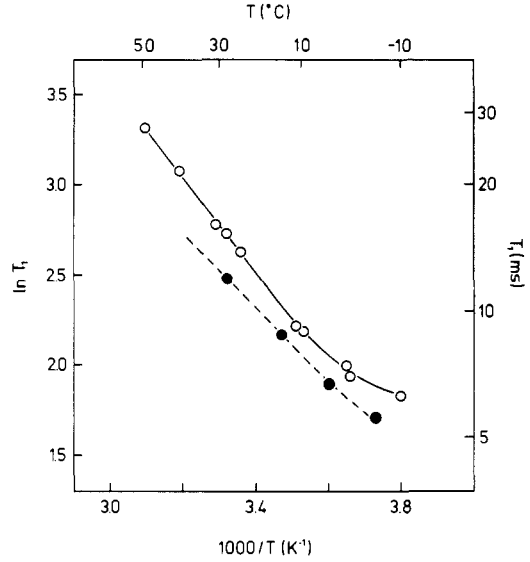

FIGURE 8: Variation of the deuterium T_1 relaxation times (at 46.1 MHz) with temperature. (○) Pure [9,10-$^2H_2$]DOPC liposomes dispersed in water. The activation energy, E_a, determined from the slope in the high temperature region is 4.7 kcal/mol. (●) Cytochrome *c* oxidase reconstituted with [9,10-$^2H_2$]DOPC L/P = 0.32 (w/w). E_a = 3.8 kcal/mol.

of heme aa_3 after six cycles, although they used the same procedure as in 1975. According to these authors, the origin of this discrepancy is not known.

In two dialysis experiments with [^{3}H]cholate we determined the residual cholate content in the membrane. After 48 h of dialysis the molar ratios of phospholipid/cholate/cytochrome *c* oxidase were found to be 120/0.4/1. Hence it is safe to conclude that the cholate contents of the reconstituted membranes after 48–60 h of dialysis (the standard conditions for the NMR samples) were negligible. These results are in good agreement with those obtained by other laboratories (Kagawa et al., 1973; Vik & Capaldi, 1977).

Rice et al. (1979) have claimed that after 36 and 54 h of dialysis the cholate content of the solution was still 2.8 and 1.4%, respectively (w/w based on total mass of cholate plus lipid plus protein). This is in contrast to our results and to those of others (cf. above). After 46 h of dialysis the residual cholate content of the solution was 2.94 μg/mg of protein which is equivalent to only 0.2% in the units used by Rice et al. (1979). The origin of this discrepancy remains unclear. However, even this lower figure is misleading since it does not take into account the distribution of cholate between the aqueous phase and the membrane. Because of the large excess of aqueous phase, only 0.84 μg of cholate/mg of protein is actually bound to the membrane. This is less than 1 mol of cholate/2 mol of cytochrome *c* oxidase.

Lipid–Protein Interaction. Cytochrome *c* oxidase is a typical integral membrane protein spanning the inner mitochondrial membrane [for reviews, cf. Azzi & Casey (1979) and Azzi (1980)]. From two-dimensional crystals of the enzyme prepared with deoxycholate the approximate shape of the molecule was derived (Fuller et al., 1979; Deatherage et al., 1982). The monomer is roughly 110 Å long and resembles a lopsided "Y". By contrast, the thickness of a fluid POPC bilayer is only about 45 Å as determined by the separation of the choline dipoles across the bilayer (Büldt et al., 1979). Thus from the dimensions and the location of the protein one may expect hydrophobic interactions with the fatty acyl chains as well as polar interactions with the phosphocholine groups. However, the principal conclusion that must be drawn from the present NMR studies is that neither the conformation of the phosphocholine head group nor the orientation of the cis double bonds is influenced to any significant extent by cytochrome *c* oxidase. Because of the low lipid-to-protein ratios employed in this investigation (~0.32–0.37 w/w) at least 50% of the phospholipid molecules should be in direct contact with the protein surface, yet the corresponding NMR spectra of the pure lipid bilayers and the reconstituted cytochrome *c* oxidase membranes are remarkably similar.[4] This is even more surprising since it has been demonstrated that the polar head groups are quite sensitive to external agents. Addition of metal ions or local anesthetics to DPPC and POPC bilayers can change the quadrupole splittings of the α and β segments by as much as 6–10 kHz (Brown & Seelig, 1977; Akutsu & Seelig, 1981; Boulanger et al., 1981; Browning & Akutsu, 1982; C. Altenbach and J. Seelig, unpublished results). These changes are brought about by only a weak binding of the metal ions to the phosphocholine groups,[5] suggesting that the interaction of the protein surface with the polar head groups is even less specific. As an aside it should be noted that the phosphate group and the N-terminal methyl group are much less sensitive to structural changes as judged from ion binding studies (Akutsu & Seelig, 1981) and hence are also less suited to monitor lipid–protein structural interactions.

Since cytochrome *c* oxidase spans the lipid bilayer, the motional freedom of the fatty acyl chains could be restricted simply for geometric reasons. No such phenomenon is observed. Even the tilt angle of the cis double bond which can be derived from the ratio of the quadrupole splittings (Seelig & Waespe-Šarčevič, 1978) remains practically unchanged, in agreement with earlier observations on reconstituted sarcoplasmic reticulum membranes (Seelig et al., 1981). In contrast, the addition of cholesterol has a dramatic effect on the cis double bonds; at a DOPC/cholesterol 1/1 molar ratio the experimentally observed quadrupole splittings are 24.5, 9.2, and 6.4 kHz (at 30 °C) compared to pure DOPC bilayers with splittings of 13.0, 6.4, and 2.4 kHz (H. U. Gally and J. Seelig, unpublished results). The quadrupole splittings increase *linearly* with cholesterol concentration (up to ca. 30 mol %), and a similar behavior has been observed for mixtures of cholesterol with saturated phosphatidylcholines at temperatures well above the phase transition (e.g., Haberkorn et al., 1977; Jacobs & Oldfield, 1979; Rance et al., 1982). This argues against specific hydrophobic forces between the fatty acyl chains and cholesterol and suggests that the unique shape of cholesterol is the dominant ordering factor. The rodlike steroid frame restricts the rotational isomerizations around the carbon–carbon single bonds of the fatty acyl chains and also limits the angular excursions of the cis double bond [for a review on structure–function relationships of cholesterol see Bloch (1982)]. In contrast, the outer surface of cytochrome *c* oxidase must be pictured as rather rough and irregular in order to account for the lack of any change in the hydrocarbon chains (Seelig & Seelig, 1978, 1980; Bloom, 1979; Oldfield et al., 1978).

These conclusions are further substantiated by the T_1 relaxation times observed for the head group and the fatty acyl chains. Let us first consider the movement of the phosphate segment which gives rise to a characteristic minimum in the T_1 relaxation time. Phosphorus T_1 relaxation minima have

[4] The ^2H and ^{31}P NMR intensities account for at least 90% of the total phospholipid.

[5] The binding constants of Ca^{2+} to bilayers of DPPC (at 59 °C) and POPC (at 40 °C) are 19 M^{-1} and 4 M^{-1}, respectively (Akutsu & Seelig, 1981; C. Altenbach and J. Seelig, unpublished results).

now been observed for synthetic lipid bilayers such as DOPC (Seelig et al., 1981) and POPC (this work) as well as for reconstituted sarcoplasmic reticulum membranes (Seelig et al., 1981) and the phospholipid microdomains of a crystalline lipoprotein (Banaszak & Seelig, 1982). From basic NMR theory it follows that the correlation time, τ_c, of the phosphate group at the T_1 minimum equals $\tau_c \simeq 0.7/\omega_0 = 0.93$ ns ($\omega_0 = 2\pi \times 121.4$ MHz) (cf. Seelig et al., 1981). In the presence of cytochrome *c* oxidase the reorientation rate of the phosphate group is only slightly reduced as indicated by the shift of the relaxation minimum from 15 °C for pure POPC to 25 °C for the reconstituted membrane. These results are in contrast to those reported by Rajan et al. (1981), who observed that the addition of cytochrome *c* oxidase significantly decreased the phosphorus spin–lattice relaxation time (from 660 ms for the pure lipid to 170 ms for the reconstituted membrane) and who suggested an "immobilization" of the phospholipid head group due to protein–lipid (polar) interaction. However, this conclusion was based on a single T_1 experiment carried out at 30 °C. Figure 5 demonstrates that at this temperature cytochrome *c* oxidase may already be denatured (depending on the prehistory of the sample), leading to artificially short T_1 relaxation times. The present more extensive set of phosphorus T_1 relaxation time measurements provides no evidence that the protein causes any large immobilization of the lipid phosphate segments. This is further supported by the deuterium spin–lattice relaxation times. The T_1 relaxation times of the α and β segment of the phosphocholine head group as well as those of the cis double bond are reduced by at most 20% upon incorporation of protein. Since the motions are in the fast correlation time regime, the increase in the correlation time τ_c according to eq 2 is of the same order. Thus all segments reorientation rates, at least those in the vicinity of the Larmor precessional frequency of 46 MHz, are only slightly reduced by the presence of cytochrome *c* oxidase.

Taken together the structural and dynamic data presented here suggest a perfect fluidlike match between the outer surface of cytochrome *c* oxidase and the surrounding lipids (cf. Bloom, 1979). The NMR studies on the various head-group segments provide no evidence for a motionally restricted boundary lipid neither in terms of a separate NMR signal nor in terms of significant changes in the head-group structure and dynamics. The same conclusion applies to the cis double bond and is thus consistent with earlier studies on saturated fatty acyl chains.

Nevertheless, as a quantitative refinement of this picture three differences between pure lipid bilayers and reconstituted cytochrome *c* oxidase membranes should be mentioned. The most significant of these is a change in the intrinsic line width of the resonance lines composing the powder pattern. Inspection of the ^{2}H NMR spectra (Figures 2 and 6) reveals that the line shapes of the reconstituted membranes are broadened compared to the pure lipid bilayers. In particular, the outer edges of the powder pattern are much better resolved for pure lipid bilayers. This broadening is also reflected in the ^{14}N NMR spectra but not in the ^{31}P NMR spectra. A similar spectral broadening was also observed for specifically deuterated and perdeuterated fatty acyl chains in bilayers with cytochrome *c* oxidase (Rice & Oldfield, 1979; Paddy et al., 1981), rhodopsin (Deese et al., 1981; Bienvenue et al., 1982), or cholesterol (Taylor et al., 1982; Blume & Griffin, 1982). The question arises as to the molecular origin of the line broadening. (1) It should be pointed out that the decay of a quadrupole echo has a rather complex theoretical behavior (Woessner et al., 1969). In particular, if some slow motions occur which have correlation times of the order of the reciprocal of the rigid lattice quadrupole interaction (~170 kHz), echo distortions and loss of echo intensity will be observed (Spiess & Sillescu, 1981; Blume et al., 1982). (2) Qualitatively similar spectral changes have been obtained by NMR line-shape calculations for anisotropic diffusion when the rotation rate around one principal diffusion axis was slowed down from 10^8 to 10^7 s^{-1} [Meirovitch & Freed, 1979, Figure 1 (a)]. (3) It has also been suggested that the line broadening is caused by the exchange between motionally restricted lipid on the protein surface and bulk lipid with an exchange rate of 10^6–10^7 s^{-1} (Paddy et al., 1981). (4) In the case of rhodopsin/1,2-dimyristoylphosphatidylcholine vesicles it has been demonstrated that the presence of proteins does not modify the distribution of quadrupole splittings but does produce both homogeneous (T_{2e}) and inhomogeneous line broadening (line width). The latter was related to a heterogeneity of protein concentrations (Bienvenue et al., 1982). Evidently, a consistent molecular interpretation of T_{2e} and line-shape effects must await further experimental and theoretical work.

The second effect to be noted is the reduction in the ^{2}H and ^{14}N NMR quadrupole splittings upon incorporation of cytochrome *c* oxidase. However, these reductions may only be apparent and could be caused by an increase in the intrinsic line width (cf. Paddy et al., 1981). Line-shape calculations on α- and β-deuterated POPC assuming a very simple line-shape model (Seelig, 1977) demonstrate that it is possible to account for most of the reduction in the residual quadrupole splitting by simply increasing the intrinsic line width. However, even if the quantitative differences in $\Delta\nu_Q$ are real they are not significant enough to affect the above conclusion that the interaction of the phosphocholine head group with the surface of cytochrome *c* oxidase is extremely weak.

The third difference, finally, concerns the T_1 relaxation times and the activation energies. The ^{2}H T_1 relaxation times were found to be 0–20% shorter in the presence of cytochrome *c* oxidase, and the activation energies were also reduced for all segments investigated. Let us then assume that the lipids in immediate contact with the protein surface exhibit the relaxation time T_{1P} and those free in the bulk lipid the relaxation time T_{1f}. If there is a rapid exchange between the two states, the experimentally observed relaxation rate ($1/T_1$) is given by the weighted average

$$\frac{1}{T_1} = \frac{X_f}{T_{1f}} + \frac{X_P}{T_{1P}} \tag{3}$$

Here X_f and X_P are the weight fraction of the two types of lipids. About 55 phospholipids (0.2 mg of lipid/mg of protein) are needed to form a single shell around cytochrome *c* oxidase (Jost & Griffith, 1980). For the cytochrome *c* oxidase/[β-$^2H_2$]POPC system (lipid-to-protein ratio 0.33 w/w; Table I) one calculates $X_P = 0.61$ and $X_f = 0.39$, leading to $T_{1P} = 12.1$ ms for the boundary lipid as compared to $T_{1f} = 18.1$ ms for the free lipid (at 25 °C). Hence, it can be concluded that even those head-group segments which are in immediate contact with the protein are only a little slowed down in their reorientation rate.

This result may be compared with the interaction of the polar head groups with water as reflected in the relaxation time of water. If ^2H_2O is mixed with DPPC (at 50 wt %), the deuterium T_1 relaxation decreases from 640 ms for pure water to 420 ms in the presence of lipid (at 45 °C; F. Borle, unpublished results). From X-ray diffraction studies it is known that DPPC can take up at most 36% of its weight as interstitial water, the rest remaining bulk water (Ruocco & Shipley,

1982). Under the assumption of a single relaxation time T_{1L} for the interstitial water an analogous reasoning as described above immediately leads to T_{1L} = 370 ms. At 36 wt % interstitial water each phospholipid head group is surrounded by about 26 water molecules. However, the first hydration shell of the phosphocholine head group will probably comprise less than this number for spatial reasons. It can thus be expected that the water molecules in the first hydration shell are even more restricted in their motional freedom and give rise to an even shorter relaxation time than 370 ms. Hence, on the basis of these T_1 experiments one is tempted to conclude that the interaction between the phosphocholine head group and water is "stronger" (corresponding to larger motional restrictions) than the interaction between the same lipid head group and membrane-bound cytochrome *c* oxidase.

Acknowledgments

We thank P. Ganz and R. Jenni for the competent synthesis of selectively deuterated POPC and DOPC derivatives.

Registry No. POPC, 26853-31-6; DOPC, 4235-95-4; cytochrome oxidase, 9001-16-5.

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Photochemical Cross-Linking of Translation Initiation Factor 3 to *Escherichia coli* 50S Ribosomal Subunits†

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ABSTRACT: Translation initiation factor 3 (IF-3) was bound noncovalently to *Escherichia coli* 50S ribosomal subunits. Irradiation of such complexes with near-ultraviolet light (>285 nm) resulted in covalent attachment of initiation factor 3 to the 50S subunit. Photo-cross-linking attained its maximum level of 40% of that which was noncovalently bound after 90 min of irradiation. Cross-linking was abolished in the presence of either 0.5 M NH_4Cl or 0.25 mM aurintricarboxylic acid, indicating that specific binding of initiation factor 3 to the ribosome was a prerequisite for subsequent covalent attachment. Further analysis showed that all the IF-3 was covalently bound to a small number of 50S subunit proteins. The major cross-linked proteins were identified as L2, L7/L12, L11, and L27 by immunochemical techniques. These results are discussed in light of the proposed mechanism for IF-3 function.

Initiation of the synthesis of a peptide chain on ribosomes in *Escherichia coli* requires the participation of three protein factors (Grunberg-Manago & Gros, 1977; Revel, 1977). One of these, initiation factor 3 (IF-3),[1] serves two functions—it ensures the availability of free 30S subunits by preferentially binding to the free subunit and is required for the functional binding of mRNA to these subunits. A variety of studies have been interpreted as indicating that IF-3 carries out its function as an antiassociation factor (rather than a dissociation factor) by interacting solely with 30S subunits, thereby shifting the 70S $\rightleftarrows$ 30S + 50S equilibrium in favor of dissociation (Godefroy-Colburn et al., 1975; Grunberg-Manago & Gros, 1977; Chaires et al., 1981). Other biophysical evidence, however, is incompatible with this model and is more consistent with a 70S·IF-3 complex as an intermediate in initiation (Chaires et al., 1979; Goss et al., 1980a,b, 1982). In addition, there have been a number of studies which demonstrated a direct interaction between IF-3 and 70S couples. Thus, IF-3 was found to be required for efficient poly(U)-dependent polyphenylalanine synthesis at 18 mM Mg^{2+}, a concentration which does not favor ribosome dissociation (Schiff et al., 1974). Indeed, Hawley et al. (1974) have cross-linked IF-3 to both 50S subunits and 70S ribosomes by treatment of the appropriate complexes with dimethylsuberimidate, and periodate oxidation and reduction of 70S ribosome–IF-3 or 50S subunit–IF-3 mixtures resulted in covalent attachment of the IF-3 to the 3′ end of 23S RNA (Van Duin et al., 1976). These findings led Van Duin et al. (1976) to propose that IF-3 functions by disrupting complementary base pairs between 16S and 23S RNA which are at least partially responsible for 70S couple formation. Since the mRNA–16S RNA interaction occurs in the same region of the 16S RNA (Shine & Dalgarno, 1974; Steitz & Jakes, 1975), this single IF-3 binding event could explain its apparently dual function.

Previously, we reported the covalent cross-linking of IF-3 to its binding site on 30S subunits by irradiation of the complex with near-ultraviolet light (MacKeen et al., 1980). The target ribosomal proteins were identified immunochemically. Here we describe a similar cross-linking reaction between IF-3 and 50S subunits and present the identification of 50S ribosomal proteins which are in the neighborhood of the IF-3 binding site.

Experimental Procedures

Preparation of Ribosomes and Initiation Factor 3. The 70S ribosomes and IF-3 were prepared as described by Hershey

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[1] Abbreviations: IF, translation initiation factor; poly(U), poly(uridylic acid); Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate; RNase, ribonuclease; TP50, total protein extracted from the 50S ribosomal subunit; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

0006-2960/83/0422-1483$01.50/0