Spectra and Physical Properties of Liposomes and Monolayers of Polymerizable Phospholipids Containing Diacetylene Groups in One or Both Acyl Chains†

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ABSTRACT: Phosphatidylcholine (PC) molecules have been synthesized containing a diacetylene group in one (mixed-chain PC) or both acyl chains (identical-chain PC). Upon UV irradiation they form high molecular weight colored polymers. Under identical irradiation conditions the maximum absorption in the visible region of polymerized aqueous dispersions extracted into chloroform–methanol is far greater for mixed-chain PC than for identical-chain PC. Under certain conditions irradiation of identical-chain PC results in polymer which is insoluble in organic solvents. Mixed-chain PCs do not form insoluble polymer. The visible spectra of both classes of PC consist of two bands which vary with temperature. At all temperatures for identical-chain PC and at temperatures above 9 °C for C_{23} mixed-chain PC and 26 °C for C_{27} mixed-chain PC the thermochromism is fully reversible. When mixed-chain PCs are allowed to warm after polymerization at low temperatures, an irreversible shift in the longer wavelength band is observed. This shift is correlated with a change from a sharp positive band in the CD of C_{27} mixed-chain PC aqueous dispersions at 4 °C to a broad negative band at 25 °C. The thermochromism may be related to energetic barriers to rearrangement of the polymer crystal lattice. The differences in solubility, visible spectra, and thermochromism of polymerized mixed-chain and identical-chain PC favor a model in which short linear segments of polymer are interconnected through the glycerol backbone of the lipid in identical-chain PC while long linear regions are found in mixed-chain PC. The physical properties of polymerizable phosphatidylcholines have been examined at the air–water interface and in bulk aqueous dispersions. There is a phase transition in aqueous dispersions of both lipids and in monolayers of identical-chain PC. Monolayers of mixed-chain PC are not stable at low temperature. The phase transition temperatures (T_c) for mixed-chain PC aqueous dispersions are lower than for identical-chain PC with equivalent-length diacetylenic acyl chains. The T_c of polymerizable PC increases with diacetylenic acyl chain length, but not as rapidly as for saturated PC. Above T_c, the order parameter of nitroxide stearic acid spin labels decreases as the nitroxide group is placed closer to the terminal methyl group of the polymerizable PC. The order parameter of spin labels in polymerizable PC is smaller than in saturated PC. Polymerization of the dispersions does not affect the mobility of the spin labels. As expected for liposomes, aqueous dispersions before and after polymerization shrink when exposed to hypertonic solutions of sucrose. Lamellar structures are evident in freeze–fractured and negatively stained liposomes examined under the electron microscope. Liposomes of polymerizable PC are more permeable to glycerol than DMPC liposomes above T_c before and after polymerization, although polymerization reduced the permeability. Polymerization markedly enhances the stability of liposomes to precipitation.

In order to probe the molecular structure and dynamics of biological membranes, lipid bilayers, and lipid–protein recombinants, photoactivable groups have been attached to lipid molecules (Chakrabarti & Khorana, 1975; Johnston et al., 1980a; Hub et al., 1980; Stoffel et al., 1982). The presence of the photoactivable group allows the lipid molecules to be made reactive without disturbing the structure of a membrane of which they may be the whole or form a part. Upon activation they may link to molecules of their own type, other lipids, or protein if they are a part of a reconstituted or natural biomembrane. We have synthesized phosphatidylcholines which contain diacetylene in both acyl chains (Johnston et al., 1980a,b). Upon irradiation, diacetylenes link together and form a polymer backbone made up of conjugated single and multiple carbon bonds. Cooled below the temperature at which their hydrocarbon chains crystallize, diacetylenic phosphatidylcholine liposomes and multilayers undergo extensive photopolymerization (Johnston et al., 1980a,b). The polymer formed has both electronic absorption and circular dichroism bands in the visible region of the spectrum (Pons et al., 1982).

Polymeric liposomes, multilayers, and lipid–protein recombinants may have several uses: the first as stable carriers suitable for drug delivery, the second in the formation of stable sheets on solid supports where a defined head group such as phosphocholine is exposed on the surface (Albrecht et al., 1982; McLean et al., 1982), and the third in studies of protein–lipid interactions. Such applications require reactive photoactivable groups and the production of polymers of high molecular weight. The reactivity and extent to which phosphatidylcholines containing diacetylene groups in both acyl chains polymerize is less than the fatty acids from which they are derived.

Here we report the synthesis of several new phosphatidylcholines containing diacetylene groups in their acyl chains. Mixed-chain PC[1] have a fully saturated hydrocarbon chain on the *sn*-1 position of the glycerophosphocholine moiety and a diacetylene-containing chain on the *sn*-2 position. The polymerization and spectra of these lipids are compared with those of phosphatidylcholines which have diacetylene in both hydrocarbon chains (identical-chain PC). The physical properties of aqueous dispersions of these lipids before and

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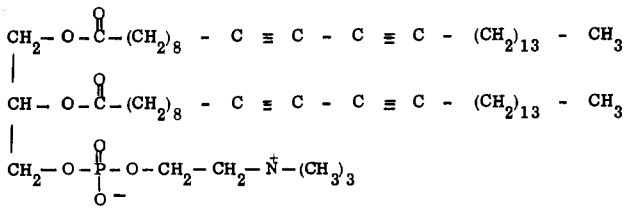

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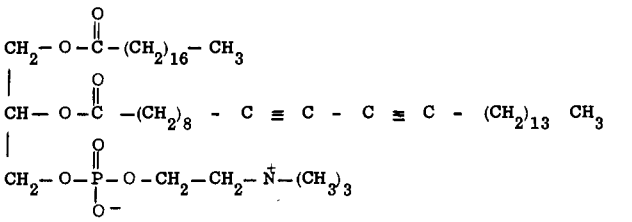

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FIGURE 1: Structures of polymerizable phosphatidylcholines. (A) C_{27} identical-chain PC. (B) C_{27} mixed-chain PC.

after polymerization are also examined.

Experimental Procedures

Materials. Dipalmitoylphosphatidylcholine was obtained from Fluka (puriss grade), stearoyllysophosphatidylcholine from Larodan (Sweden), palmitoyllysophosphatidylcholine from Sigma (London), and egg yolk lysophosphatidylcholine (70 palmitoyl:30 stearoyl, mol/mol, by gas–liquid chromatography) from Sigma (London). All other reagents were analytical or spectrophotometric grade. Water was first distilled and then passed through a Milli Q filtration system (Millipore, London). The nitroxide spin labels used were obtained from Syva (Palo Alto, CA).

Synthesis and Purification of Phosphatidylcholines (PC). Two classes of PC containing diacetylene groups in their acyl chains were synthesized (Figure 1): (a) identical-chain PC, containing diacetylene groups in both acyl chains, and (b) mixed-chain PC, with one diacetylene-containing acyl chain and one fully saturated acyl chain. The number of carbons (n) in the diacetylene-containing acyl chain will be designated by C_n. In this work the lipids have acyl chains with n values of 23 and 27. Synthesis of identical-chain PC has been described previously as has synthesis of the diacetylene-containing fatty acids (Johnston et al., 1980a). The mixed-chain PC was synthesized by the method of Gupta et al. (1977) to minimize acyl group migration.

All lipids gave a single spot when run on thin-layer chromatography plates with chloroform–methanol–water (65:25:4 v/v). The R_f values for the diacetylene PC were similar to those of a highly pure sample of dipalmitoylphosphatidylcholine (DPPC). The infrared spectra of the lipids were also identical with those of DPPC. Ultraviolet spectra of lipid solutions had the characteristic absorption bands of the diacetylene group, and extinction coefficients derived from these spectra were identical with those obtained with the fatty acids ($2.45 \times 10^2 M^{-1}$ cm^{-1} at 254 nm in hexane–ethanol, 90:10 v/v; cf. Johnston et al., 1980a).

Preparation of Aqueous Dispersions. A solution of PC in chloroform was dried onto the walls of a test tube, under a N_2 stream. An appropriate volume of deionized water was added to give a concentration of 2 or 10 mg/mL. The tube was heated to a temperature above the phase transition temperature of the PC and vortexed vigorously. In some cases the dispersion was sonicated with a probe sonicator (Kerry's Ultrasonics Ltd.). Sonication was carried out under an N_2 atmosphere, and measurements of the ultraviolet absorption of dispersions showed that there was no loss of diacetylene linkages.

Polymerization of Aqueous Dispersions. Dispersions were polymerized by placing them 5 cm from a high-intensity ultraviolet lamp (Mineralight R-52, Ultra-violet Products, Inc., San Gabriel, CA). The samples in 1-mm path-length quartz UV cells were kept at 4 °C by immersing the cells in ice–water. Prior to irradiation, lipid dispersions were thoroughly purged with N_2. Mixed-chain PC synthesized from either palmitoyllysophosphatidylcholine or stearoyllysophosphatidylcholine did not polymerize well under any conditions, giving a weak color. Mixed-chain PC synthesized from egg yolk lysophosphatidylcholine polymerized readily. This may be the result of differences in isomeric purity of the parent lyso compound. In all polymerization studies, mixed-chain PC synthesized from egg yolk lysophosphatidylcholine has been used.

Spectral Measurements. Visible and ultraviolet spectra of monomer and polymers in solutions and dispersions were recorded on a Unicam SP8-100 spectrophotometer. Circular dichroism spectra of dispersions were recorded on a JASCO J40 CS instrument. Both instruments were equipped with facilities which enabled the temperature of solutions and dispersions to be measured and controlled.

Differential Scanning Calorimetry (DSC). Aqueous dispersions of lipid for calorimetry were prepared as described above. Samples were sealed in Al pans and analyzed on a Perkin-Elmer DSC2. A scan rate of 5 °C/min was used with liquid N_2 as coolant. Runs were repeated on at least two samples. Temperatures were calibrated with cyclohexane and naphthalene. The phase transition temperatures of the lipids are defined according to Chapman et al. (1967).

Monolayers. Pressure-area isotherms of the phospholipids were recorded by using the film balance described previously (Albrecht et al., 1982). The phospholipid monolayer was spread on the subphase from a 9:1 v/v hexane–ethanol mixture containing approximtely 1 mg of PC/mL. Films were compressed at 0.14 nm^2 $molecule^{-1}$ min^{-1}, and pressures were continuously recorded with a Wilhelmy plate device coupled to a chart recorder.

Electron Spin Resonance Spectroscopy. Spectra were recorded on a Varian E9 spectrometer operating at 9 GHz. The temperature was maintained to ±1 °C with a regulated hot air blower and measured with a thermistor placed alongside the sample capillary. The samples were irradiated in these capillaries or in quartz cuvettes prior to transfer to the capillary tube.

[1] Abbreviations: PC, phosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; C_{23} identical-chain PC, 1,2-bis(10,12-tricosadiynoyl)-*sn*-glycero-3-phosphocholine; C_{27} identical-chain PC, 1,2-bis-(10,12-septacosadiynoyl)-*sn*-glycero-3-phosphocholine; C_{23} mixed-chain PC, mixture (approximately 70:30, mol/mol) of 1-palmitoyl-2-(10,12-tricosadiynoyl)-*sn*-glycero-3-phosphocholine and 1-stearoyl-2-(10,12-tricosadiynoyl)-*sn*-glycero-3-phosphocholine; C_{27} mixed-chain PC, mixture (approximately 70:30, mol/mol) of 1-palmitoyl-2-(10,12-septacosadiynoyl)-*sn*-glycero-3-phosphocholine and 1-stearoyl-2-(10,12-septacosadiynoyl)-*sn*-glycero-3-phosphocholine; 16-stearic acid spin-label, 2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxy; 12-stearic acid spin-label, 2-(10-carboxydecyl)-2-hexyl-4,4-dimethyl-3-oxazolidinyloxy; 7-stearic acid spin-label, 2-(5-carboxypentyl)-4,4-dimethyl-2-undecyl-3-oxazolidinyloxy; 12-nitroxide methyl stearate, 2-(10-carboxydecyl)-2-hexyl-4,4-dimethyl-3-oxazolidinyloxy methyl ester; Tempo, 2,2,6,6-tetramethylpiperidinyl-1-oxy; T_c, gel to liquid-crystalline phase transition temperature; DSC, differential scanning calorimetry; CD, circular dichroic; ESR, electron spin resonance.

Table I: Polymerization of Phosphatidylcholines Containing Diacetylene Groups in One or Both Acyl Chains[a]

lipid	dispersion OD_{420}[b]	polymer OD_{460} [cm^{-1} (M acyl chain)$^{-1}$][c]
C_{23} same-chain PC	0.35	90
C_{27} same-chain PC	0.4	130
C_{23} mixed-chain PC	0.5	620
C_{27} mixed-chain PC	0.35	680

[a] Aqueous dispersions of the indicated lipids were prepared and polymerized as described under Experimental Procedures. The C_{27} same-chain PC was irradiated at 20 °C. All other lipids were irradiated at 4 °C. [b] Absorbance at 420 nm of aqueous dispersions before polymerization. [c] Absorbance of polymer extracted from irradiated aqueous dispersions into chloroform–methanol, 2:1 v/v.

Spectrophotometric Determination of Permeability. The permeability of aqueous dispersions of the lipid was measured by the technique of Cohen & Bangham (1972) as modified by Hargreaves & Deamer (1978). The initial absorbance of 3 mL of a dispersion in distilled water was adjusted to 0.6–0.8 by varying the measured wavelength between 400 and 800 nm. Then, 0.2 mL of a 2 M solution of glycerol or sucrose was rapidly injected into the cuvette. Time-dependent changes in absorbance following mixing were recorded continuously. The rate of decrease in absorbance immediately following the absorbance maximum (i.e., see Figure 7) has been taken as proportional to the vesicle permeability (Cohen & Bangham, 1972). No change in absorbance was recorded in control experiments where 0.2 mL of distilled water was injected into the cuvette.

Electron Microscopy. Dispersions for electron microscopy were prepared as described previously in distilled water for negative staining or in 30% glycerol for freeze–fracture. Samples were negatively stained with 2% potassium phosphotungstate or prepared for freeze–fracture by using standard techniques.

Analytical Procedures. Concentration of the diacetylene-containing PC solutions in organic solvents was measured spectrophotometrically by using the extinction coefficient of the pure diacetylenic fatty acid at 254 nm. In a number of cases concentrations were confirmed by phosphorus analysis (Bartlett, 1959).

Results

Polymerization of Aqueous Dispersions. Upon irradiation of aqueous dispersions of diacetylene-containing PC with UV light, high molecular weight colored polymers are formed (Johnston et al., 1980a). The intensity of the color produced is related to the amount of conversion of monomeric PC to polymer within the liposome and depends on three experimental factors. (1) *Irradiation time and intensity.* With the Mineralight R-52 UV lamp held 5 cm from the dispersions, maximum color is obtained after 3–5-min irradiation. Considerably longer irradiation times (20 min) result in a reduction of color. (2) *Temperature.* The optimum temperature for polymerization of all of the lipids, except for the C_{27} identical-chain PC, is 4 °C. The C_{27} identical-chain PC gives higher absorbances in the visible region when polymerized at 20 °C. (3) *Method of preparation of aqueous dispersions.* Vigorously mixed or briefly sonicated (above T_c) dispersions gave maximum color yields. In the case of phospholipids with diacetylene in both acyl chains, the solubility of the polymer formed was found to depend on the concentration of their dispersions. At low concentrations (2 mg/mL) most of the polymer was insoluble in chloroform–methanol mixtures. At concentrations of 10 mg/mL all polymer formed dissolved. We have previously observed similar differences in solubility when microcrystals of these phospholipids are irradiated in compressed KBr disks (Pons et al., 1982). In this case the longer the irradiation period the higher the proportion of insoluble polymer. Unlike compressed KBr disks which are clear, aqueous dispersions of lipid are cloudy. Since opacity in dispersions increases with concentration, lipid in the concentrated dispersions will be exposed to less UV radiation, a situation corresponding to the irradiation of KBr disks for short periods.

The color yield of the mixed-chain and identical-chain PC has been determined under carefully controlled conditions to obviate these difficulties. In all cases dispersions were prepared by vigorously vortexing 10 mg of lipid/mL and irradiating for 3–5 min at optimum temperatures (see above). The polymer was then extracted into chloroform–methanol (2:1 v/v) and its maximum absorbance measured (Table I). The opacity of the original dispersion was monitored by the OD_{420}. Absorbance of organic solutions of polymer at 460 nm is directly related to the extent of conversion of diacetylene groups to high molecular weight linear polymer. The color yield of mixed-chain PC is far greater than that of identical-chain PC, but little difference may be observed within each lipid class. The OD_{460} [cm^{-1} (M acyl chain)$^{-1}$] of the mixed-chain PC is similar to the extinction coefficient of pure polydiacetylene published previously [cf. 650 cm^{-1} (M acyl chain)$^{-1}$] (Johnston et al., 1980a)]. Corresponding to the greater color yield of the mixed-chain PC polymer, its organic solution is highly viscous when compared with the organic solution of identical-chain PC polymer and does not pour readily out of a test tube.

Visible Spectra. Figure 2 shows that diacetylenic PC polymers are thermochromic in aqueous dispersion. At low temperatures, the spectra of aqueous dispersions of mixed- and identical-chain PC are bimodal, possessing a main peak and a shoulder. As the mixed-chain lipid dispersions are allowed to warm from 4 °C, at a discrete temperature an irreversible shift in the longer wavelength absorption takes place (Figure 2A). This shift occurs at approximately 9 °C for C_{23} mixed-chain PC (Figure 2A) and at 26 °C for C_{27} mixed-chain PC (data not shown). Further shifts in spectra to shorter wavelength at higher temperatures are less pronounced and are fully reversible on cooling. Polymerized dispersions of PC with diacetylene in both acyl chains exhibit small shifts to shorter wavelengths with increasing temperature which are similar to those observed for mixed-chain PC at higher temperatures, and these shifts are fully reversible (Figure 2B). The longer wavelength absorption (~525 nm) disappears at 50 °C in identical-chain PC but remains in mixed-chain PC at the same temperature (cf. Figure 2). These shifts in absorption maxima result in the conversion of polymerized aqueous dispersions from red at low temperatures to red-yellow, for mixed-chain PC, or yellow, for identical-chain PC, at high temperature.

A shift to shorter wavelengths on heating is also observed for diacetylene PC polymers in methanol. At high temperatures (65 °C) little difference is evident between identical-chain PC and mixed-chain PC polymers. A shift to shorter wavelengths occurs at lower temperatures for identical-chain PC than mixed-chain PC. No irreversible shift in the longer wavelengh absorption as observed in aqueous dispersions is found in methanol solutions of polymer. All changes are completely reversible in methanol where the polymer chains

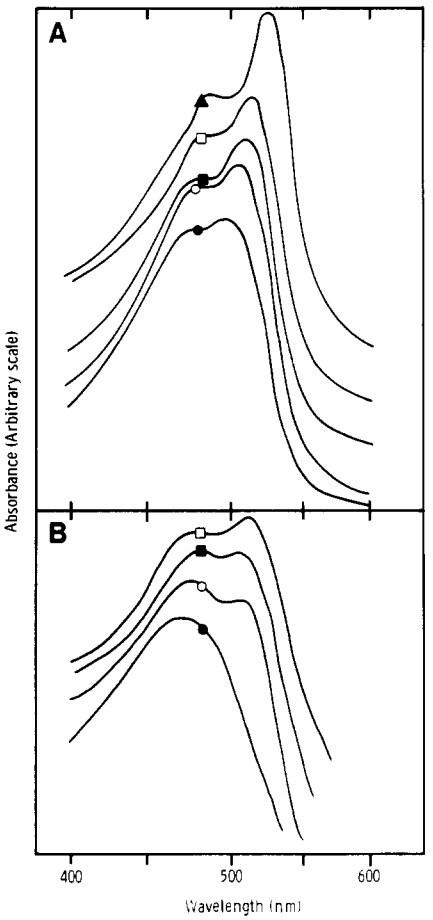

FIGURE 2: Visible spectra of phosphatidylcholines containing diacetylene groups in one or both acyl chains irradiated as aqueous dispersions as described under Experimental Procedures and Results. (A) C_{23} mixed-chain PC dispersions irradiated at 4 °C and spectra recorded at (●) 50, (○) 19, (■) 13, (□) 11, and (▲) 8 °C. (B) C_{23} identical-chain PC dispersions irradiated at 4 °C and spectra recorded at (●) 50, (○) 35, (■) 25, and (□) 10 °C.

are more free to rotate than in aqueous dispersions (Pons et al., 1982).

CD Spectra. As a polymer class, polydiacetylenes are not usually optically active. We have previously shown that optical activity arises when the chiral glycerophosphocholine is one of the substituents on the polymer chain (Pons et al., 1982). Figure 3 gives the CD spectrum of a polymerized dispersion of C_{27} mixed-chain PC at two temperatures. At 4 °C the spectrum consists of two superimposed bands: a broad positive–negative band which crosses the origin at 485 nm and a sharp positive band with a maximum at 525 nm. Irreversible changes in the circular dichroism spectra occur when the dispersion is allowed to warm. The positive–negative band is replaced by a broad negative band, and the sharp positive band observed at 4 °C reverses sign. The wavelength at which the sharp band is at a maximum is similar to that of the longer wavelength absorption band in the visible spectrum (cf. Figure 2A). The reversal in sign of the CD band is thus associated with the irreversible shift in the visible spectrum which also occurs on warming.

Differential Scanning Calorimetry. Diacetylenic lipids undergo thermotropic phase transitions similar in character to those of phospholipids with fully saturated acyl chains. The phase transition temperatures of the diacetylene-containing lipids are given in Table II (cf. Johnston et al., 1980a). For both classes of lipid, those containing diacetylene groups in both acyl chains and those containing a diacetylene group in one acyl chain, the phase transition temperatures of the

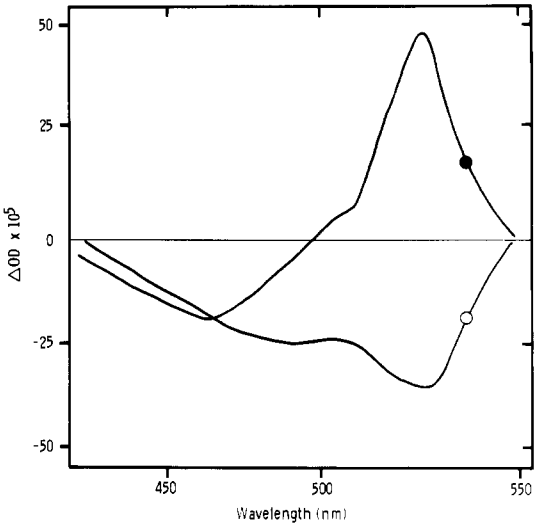

FIGURE 3: Circular dichroic spectra of polymerized C_{27} mixed-chain PC aqueous dispersions. Dispersions were prepared and polymerized at 4 °C as described under Experimental Procedures. Circular dichroic spectra were recorded at (●) 4 and (○) 25 °C. *G* factor (ΔOD/OD) at 525 nm = 10^{-3}.

Table II: Gel–Liquid-Crystalline Phase Transition Temperatures of Aqueous Dispersions of Diacetylene-Containing Phosphatidylcholines[a]

lipid class[b]	chain length of diacetylene-containing acyl chain	phase transition temperature (°C)
same-chain PC	C_{23}	38
	C_{27}	60
mixed-chain PC	C_{23}[c]	23
	C_{23}[d]	20
	C_{27}[d]	33

[a] Phase transition temperatures measured from DSC heating curves as described under Experimental Procedures. [b] Lipid classes are defined under Experimental Procedures. [c] Synthesized from stearoyllysophosphatidylcholine. [d] Synthesized from egg yolk lysophosphatidylcholine.

aqueous dispersions increase with increasing diacetylene-containing acyl chain length. For the identical-chain PC the increase in T_c per methylene unit is ~6 °C (cf. Johnston et al., 1980a). The phase transition temperatures of the mixed-chain PC are approximately 18 °C lower than those of their identical-chain counterparts. The phase transition temperature of the C_{23} mixed-chain PC synthesized from stearoyllysophosphatidylcholine is somewhat higher than that for the C_{23} mixed-chain PC synthesized from egg yolk lysophosphatidylcholine (palmitoyl:stearoyl, 70:30 by gas–liquid chromatography). It is evident that this increase is the result of an increase in the average length of the saturated acyl chain.

Monolayers at the Air–Water Interface. Mixed-chain phosphatidylcholines form stable monolayers at the air–water interface at room temperature. At lower temperatures (<10 °C) the monolayers are not stable: when the barrier is stopped during compression, the surface pressure of the monolayer decays rapidly (>1 dyn cm^{-1} min^{-1}) to close to zero pressure. The room temperature isotherm of a mixed-chain PC is given in Figure 4A. Similar isotherms were recorded for the other mixed-chain PCs at room temperature.

Identical-chain PCs form stable monolayers at pressures up to 35–50 dyn cm^{-1}. This pressure corresponds to the equilibrium spreading pressure of the crystal above T_c [cf. Phillips

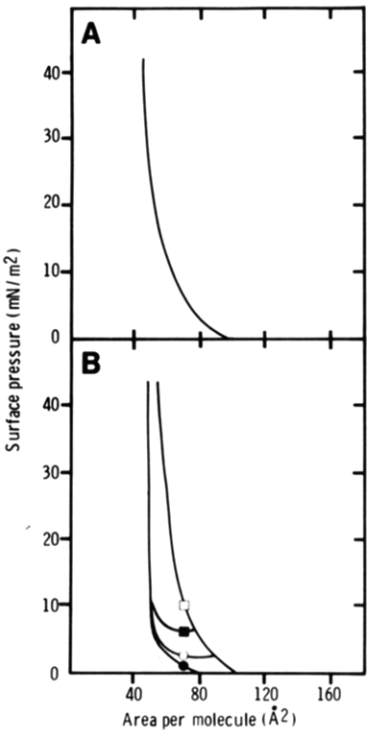

FIGURE 4: Pressure–area isotherms of monolayers of diacetylene-containing phosphatidylcholines. Isotherms were recorded as described under Experimental Procedures. (A) C_{23} mixed-chain PC at room temperature. (B) C_{23} identical-chain PC at (●) 20.8, (○) 22.8, (■) 25.3, and (□) 38 °C.

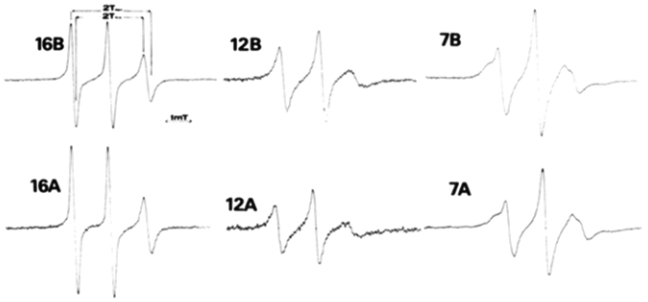

FIGURE 5: Representative electron spin resonance spectra for stearic acid spin-labels in C_{27} mixed-chain PC at 45 °C before and after irradiation at 4 °C. (B) Before irradiation. (A) After irradiation. 16A and 16B, 16-stearic acid spin-label; 12A and 12B, 12-stearic acid spin-label; 7A and 7B, 7-stearic acid spin-label.

& Hauser (1974) for DMPC]. The C_{27} identical-chain PC is fully condensed at all temperatures below 35 °C (data not shown). The C_{23} identical-chain (Figure 4B) exhibits a liquid expanded–liquid condensed phase transition at temperatures between 21 and 37 °C. The shape of the C_{23} identical-chain PC pressure-area isotherms is similar to that previously published for C_{25} identical-chain PC (Albrecht et al., 1982) but is fully condensed at lower temperatures, corresponding to its lower gel–liquid-crystalline phase transition temperature (T_c) in aqueous dispersions. The limiting area of the liquid-condensed monolayer of all of the identical-chain PC is 52 Å^2/molecule (cf. Hupfer et al., 1981). The lateral compressibility of the monolayer in the liquid-condensed region is 0.0023 cm dyn^{-2} at 20.8 °C [cf. Albrecht et al. (1978) for DPPC].

Electron Spin Resonance Spectra. The electron spin resonance spectra for stearic acid spin-labels containing the nitroxide group at carbon 16, 12, or 7 incorporated into mixed-chain PC liposomes are given in Figure 5. The splittings $2T_{max}$ and $2T_{min}$ were measured from these spectra, and hence, the order parameter, S, was calculated following

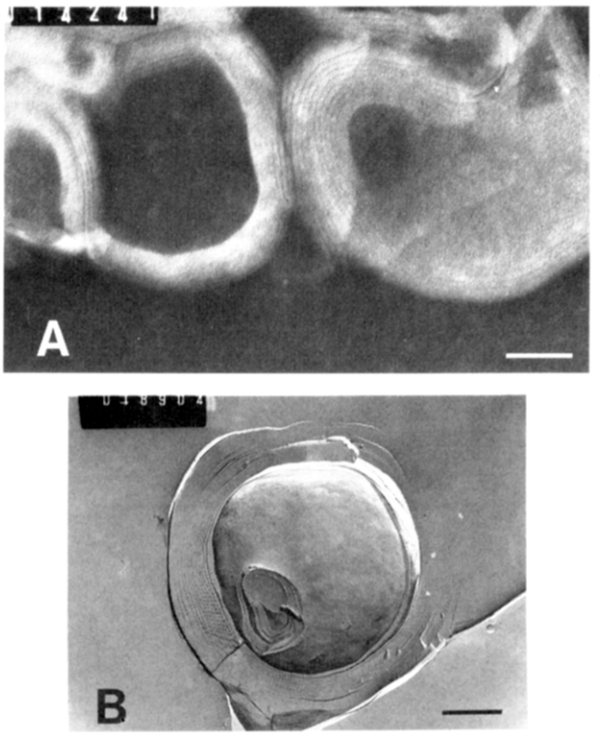

FIGURE 6: Electron micrographs of polymerized phosphatidylcholine liposomes. Aqueous dispersions of C_{27} mixed-chain PC were prepared and irradiated as described under Experimental Procedures. (A) Negatively stained dispersions. The bar is 0.1 μm. (B) Freeze–fractured dispersions. The bar is 1 μm.

the procedure of Jost & Griffith (1976) by taking $T_{\parallel}$ as equal to T_{max} and calculating $T_{\perp}$ from T_{min} after Gaffney (1976). The order parameters for mixed-chain PC liposomes show that no significant changes in spin-label mobility are produced by short periods of irradiation. No evidence for an ESR signal from polymerized liposomes without spin-label was found (cf. Bloor, 1976).

When these experiments were repeated with identical-chain PC, the stearic acid spin-label spectrum was dominated by the signal from spin-label in an aqueous environment as a result of the insolublility of the stearic acid probe in the bilayer. On irradiation the nitroxide free radical was destroyed. Tempo added to an aqueous dispersion of identical-chain PC also did not incorporate to a significant extent into the unpolymerized bilayer at temperatures above the phase transition temperature of the liquid (partition function ~ 0.05). However, when a methyl stearate spin-label was used, the molecule partitioned into the bilayer phase. With the methyl stearate label in C_{23} identical-chain PC liposomes, a sudden decrease in the order parameter was observed at close to 37 °C (data not shown), corresponding to the phase transition temperature measured by DSC (Table II) and in monolayers (Figure 4B). Following irradiation of the identical-chain PC dispersions under the same conditions as for mixed-chain PC, the ESR spectrum was unchanged (data not shown). Irradiation of egg PC vesicles containing the same probe molecule for short periods did not result in any significant alteration of the ESR spectrum (data not shown). Long-term irradiation of egg PC or identical-chain PC liposomes containing 12-nitroxide methyl stearate destroyed the ESR signal. The short periods of irradiation for both classes of lipid resulted in some polymerization of the acyl chains since colored suspensions were produced.

Electron Microscopy. Representative electron micrographs of polymerized aqueous dispersions of a diacetylene-containing

Table III: Permeability of Aqueous Dispersions of Diacetylene-Containing Phosphatidylcholines before and after Polymerization[a]

lipid	before	after
C_{23} same-chain PC	1.43	0.64
C_{27} mixed-chain PC	>10	0.43
DMPC	0.20	0.20

[a] Permeabilities were measured as described under Experimental Procedures and in the legend to Figure 2. Values are the average of two determinations at 50 °C and are expressed as OD min^{-1}.

PC are shown in Figure 6. The overall shape of the dispersion particles is spherical, and bilayer lamellae are distinct in both negatively stained and freeze–fractured samples. Similar results are obtained before and after polymerization for PC containing diacetylene in both acyl chains where the acyl chains are 23 carbons or greater and for PC which have one saturated and one diacetylene-containing acyl chain. The bilayer repeat distance measured on negatively stained samples is 75–80 Å [cf. 70 Å for egg PC vesicles (Bangham & Horne, 1964)]. Above the transition temperature of the lipid, smooth fracture faces and extended smooth lamellae are evident in freeze–fractured samples (Figure 6B).

Permeability of Aqueous Dispersions. Bangham et al. (1967) have shown that liposomes of egg yolk PC shrink when exposed to hypertonic solutions of sucrose resulting in an increase in visible light absorbance. Similar increases in absorbance are seen when sucrose is added to dispersions of both classes of diacetylene-containing phosphatidylcholine or to DMPC vesicles. Polymerization of the lipids with UV radiation before injection of the sucrose solution has no effect on the absorbance–time curves (Figure 7).

The rate of decrease in absorbance immediately following the absorbance maximum due to osmotic shrinkage of liposomes is related to the permeability of the liposomes to added solute (Cohen & Bangham, 1972). By use of this method (Figure 7), the permeabilities of DMPC, identical-chain PC, and mixed-chain PC liposomes to glycerol before and after irradiation were measured (Table III). For the mixed-chain PC shown in Figure 7 glycerol permeability before polymerization was greater than could be measured. The permeability of identical-chain PC to glycerol before irradiation was also considerably greater than that for DMPC liposomes. All of the liposomes which were impermeable to sucrose were permeable to glycerol at temperatures (50 °C) above their gel–liquid-crystalline phase transition temperatures (23 °C or DMPC; Ladbrooke et al., 1968; Table II). Ultraviolet irradiation had no effect on the permeability of DMPC liposomes to glycerol but markedly reduced the permeability of the diacetylene-containing lipids.

Liposome Stability. Aqueous dispersions of C_{27} identical-chain PC and mixed-chain PC were probe sonicated to clarity above T_c under an N_2 stream. The time for appearance of a precipitate was followed by visual inspection. At 50 °C lipid from both irradiated and unirradiated dispersions precipitated within 1 h. While the rate of precipitation of unirradiated dispersions was temperature independent, irradiated dispersions became more stable as the temperature was reduced. At 20 °C precipitation only became apparent after 24 h and at 4 °C after 7 days. Identical results were obtained for both lipids.

Discussion

Nature of the Links Formed between Lipid Molecules. The reactivity of a PC molecule containing a diacetylene group within only one hydrocarbon chain (mixed-chain PC) is increased in comparison to a PC molecule containing a diacetylene group in both acyl chains (identical-chain PC; Table I). When only one hydrocarbon chain contains diacetylene, there is only one way in which lipid molecules can link, i.e., to two other lipid molecules. If both chains contain diacetylene, several structures are possible; i.e., linking can also occur between the diacetylenes in the 1 and 2 acyl chains of the same lipid or adjacent lipids. The possibility for reaction between 1 and 2 acyl chains is the most obvious difference between identical- and mixed-chain lipids. This reaction may hinder chain growth and be the reason for the lower conversions obtained with lipid which has diacetylene in both acyl chains.

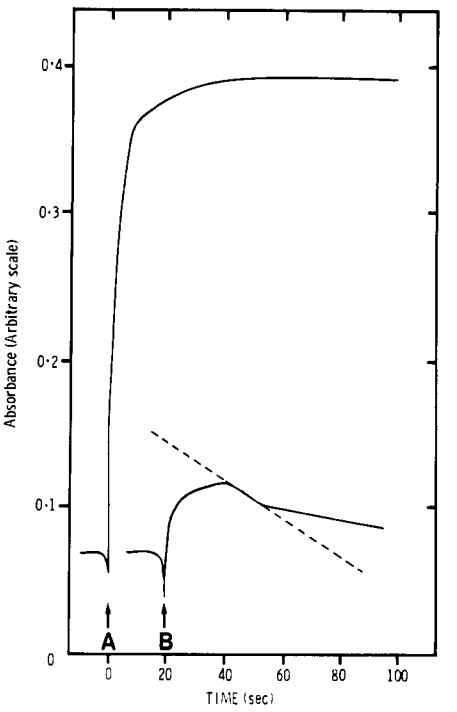

FIGURE 7: Permeability of polymerized C_{27} mixed-chain PC aqueous dispersions to sucrose (A) or glycerol (B) at 50 °C. Absorbance of an irradiated aqueous dispersion of C_{27} mixed-chain PC was measured continuously as described under Experimental Procedures. The nonelectrolyte was injected into the cuvette at the arrow. The absolute value of the slope of the dashed line was used to calculate the permeability to glycerol in Table III.

Exposure of identical-chain PC to large doses of radiation leads to the formation of an insoluble polymer. Such insolubility and the fact that mixed-chain PC polymers are always soluble indicate that this polymer has a cross-linked structure. Phosphatidylcholines with diacetylene in both acyl chains can potentially link to four other lipid molecules (Figure 8). The formation of a small number of linkages of this type would be sufficient to produce a polymer network where linear polymer chains are interconnected through the glycerol backbone of a PC molecule.

Thermochromism of Polymerized Phosphatidylcholines. The thermochromism in visible spectra and the circular dichroism of polymers derived from phosphatidylcholines with diacetylene in both acyl chains have been discussed at length in previous papers (Johnston et al., 1980a; Pons et al., 1982). Here we report thermochromism in the visible and circular dichroic spectra of linear polymers derived from mixed-chain PC. The absorption of light by the polydiacetylene chain is a complex phenomenon. The visible spectrum of the chain has two peaks (Figure 2). For identical-chain PC we have shown that different optical transitions are associated with each

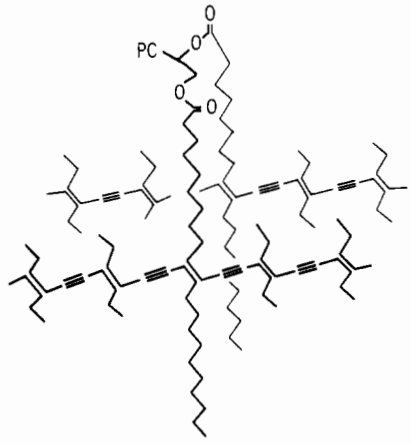

FIGURE 8: Schematic diagram of cross bridges formed between linear polymer through the glycerol of an identical-chain PC. The vertical chain segments represent part of the acyl chain of neighboring molecules. No attempt has been made to show precise bond angles in the head-group region.

peak, that each peak has its own characteristic CD spectrum, and that these transitions have diffferent temperature dependences (Pons et al., 1982). This leads to thermochromism, the polymers changing from red to yellow as the temperature is raised. Considerable understanding of the physical changes underlying the thermochromism of polydiacetylenes has been gained by applying a potential well model (Patel et al., 1978). In this model, the wavelength of maximum absorbance is considered as being determined by the length of overlapping p orbitals of the multiple bond system. Any change which causes twisting about chain single bonds and a reduction in overlap (such as increasing temperature) increases the energy of the excited state and shifts the band maximum to shorter wavelength.

Temperature change induces two different types of spectral shift, reversible and irreversible. Irreversible shifts occur in the solid state and are thought to be caused by polymer relaxing from the monomer crystal lattice into its own characteristic lattice. This process evidently causes some twisting of the polymer chain and a decrease in p-orbital overlap (Tieke & Bloor, 1979). Reversible changes in spectra take place at higher temperatures in a much more fluid environment.

Identical-chain PC polymers exhibit reversible thermochromism, and changes in the spectrum occur at lower temperatures than that of the mixed-chain PC (cf. Figure 2). This is probably the result of a lower molecular weight of the linear polymer in identical-chain PC. Polymers of lower molecular weight will require less energy to change their conformation. Rearrangement of their crystal structure during polymerization at low temperatures may occur, resulting in the formation of the most stable crystal structure. On the other hand, long linear regions of polymer as may be found in mixed-chain PC will require more energy to change their conformation. Thus, sufficient energy may not be available at low temperatures to allow rearrangement to the most stable crystal structure. When the polymer is heated, a rearrangement may take place and the most stable polymer structure form. This polymer then shows fully reversible thermochromism and a visible spectrum much like that of the identical-chain PC polymer. Apparently such temperature-dependent rearrangements occur only in lipid bilayers and are sensitive to the length of the PC acyl chain. Longer chain diacetylene PC polymers rearrange at higher temperatures (cf. 26 °C for C_{27} mixed-chain PC and 9 °C for C_{23} mixed-chain PC). These temperatures are approximately 10 °C below the T_c of the liposomes (see below).

In a previous report (Pons et al., 1982) we considered the means by which the symmetrical polydiacetylene chain might exhibit optical activity. It was concluded that the chiral glycerophosphocholine group caused the polymer chains to adopt an asymmetric packing of predominantly one twist sense. The irreversible change which occurs in the visible spectrum of the C_{27} mixed-chain PC is associated with the reversal in sign of the CD spectrum. Evidently the rearrangement of the crystal lattice involved causes a switch from an asymmetric packing of one twist sense to its mirror image.

The reversal in sign of the CD spectrum of aqueous dispersions of polymerized C_{27} mixed-chain PC has not been observed for other polymerized PCs we have investigated (Pons et al., 1982). The CD spectrum of identical-chain PC is dominated by a negative band at all temperatures which is similar to the room temperature negative band observed for C_{27} mixed-chain PC (Figure 3). This supports our suggestion that slight warming of C_{27} mixed-chain PC polymerized dispersions results in a rearrangement to a more stable polymer packing which is similar to that for identical-chain PC. In contrast, heating C_{23} mixed-chain PC polymerized dispersions to high temperatures (>50 °C) does not cause a reversal in the sign of the CD spectrum. At all experimentally accessible temperatures the CD spectrum is dominated by a sharp positive band. Heating the sample simply shifts the positive band to shorter wavelengths. A negative CD signal may be obtained for C_{23} mixed-chain PC by extracting the polymer into solvent, dissolving an excess of saturated PC in its solution and then redispersing this mixture in water (M. Pons, D. S. Johnston, and D. Chapman, unpublished results). Evidently, this drastic treatment results in a polymer conformation similar to that adopted by identical-chain PC and by C_{27} mixed-chain PC at room temperature. As a number of metastable states would be expected for PC polymer restricted to the confines of a lipid bilayer, it is not surprising that three such states can be observed in visible and CD spectra. The energetic barriers required to cross from one state to another may be considerable, as in the case of C_{23} mixed-chain PC polymerized dispersions.

The differences in polymerized mixed-chain and identical-chain PC favor a model in which long linear segments of polymer are found in mixed-chain PC and shorter regions are found in identical-chain PC. The higher molecular weight of insoluble identical-chain PC polymer is the result of a cross bridging of the linear polymer regions through the glycerol backbone of the PC.

Physical Properties of Diacetylene-Containing Phospholipids. A phase transition similar to the gel–liquid-crystalline phase transition of saturated phosphatidylcholines (Chapman et al., 1967) is observed for diacetylene-containing PC before polymerization by differential scanning calorimetry (Table II) and electron spin resonance of aqueous dispersions. A liquid-expanded to liquid-condensed transition (Gaines, 1966) is evident in pressure-area isotherms of identical-chain PC monolayers (Figure 4B). The phase transition temperatures measured by each of these techniques do not differ for any one PC molecular species. The order parameter of incorporated spin-labels containing nitroxide free radicals decreases as the radical is placed closer to the terminal methyl group (Figure 5), similar to the behavior of spin-labels incorporated in DPPC above T_c (Hubbell & McConnell, 1971). The order parameters indicate that stearic acid spin-labels in saturated PC bilayers (cf. Hubbell & McConnell, 1971) are more restricted in their motion than in diacetylene PC bilayers.

For identical-chain PC, the increase in T_c is only ~6 °C per methylene group (Table II; Johnston et al., 1980a) com-

pared to saturated PC where the increase is ~9 °C per methylene group (Ladbrooke et al., 1968). Evidently, the bulky diacetylene group reduces the interaction energy between the alkyl groups lying past the diacetylene. This gives rise to lower transition temperatures and enthalpies (Johnston et al., 1980a) than expected for equivalent-length saturated chains.

Replacement of the long diacetylene-containing acyl chain with a saturated acyl chain (mixed-chain PC) disrupts further the acyl chain packing of the lipid and, thereby, decreases the T_c below that found for identical-chain PC containing equivalent-length diacetylenic acyl chains (Table II). This disruption in acyl chain packing is also evident in the inability of mixed-chain PC to form a stable lipid monolayer at the air–water interface at low temperatures.

The molecular areas of same-chain PC spread at the air–water interface in the condensed phase are somewhat greater than the comparable areas observed for saturated PC (Phillips & Chapman, 1968). This is the direct result of the introduction of the rodlike diacetylene group in the middle of the acyl chains of the lipid. This group has no effect on the compressibility of the liquid-condensed monolayer.

In aqueous dispersions the diacetylene-containing lipids form closed lamellar structures (liposomes). Although X-ray diffraction cannot be used with these lipids, evidence for a lamellar structure is given by the presence of a phase transition due to the melting of the acyl chains, the vesicular and lamellar appearance of the dispersions by negative-stain electron microscopy (Stoeckenius, 1962), the smooth fracture faces evident in freeze–fractured samples (Deamer et al., 1970), and the behavior of the dispersions as osmometers when placed in sucrose solutions (Bangham et al., 1967).

Structure of Aqueous Dispersions of Polymerized Liposomes. Following UV irradiation of the diacetylene-containing phospholipid liposomes, the diacetylene groups link together forming polymer-containing aqueous dispersions (Johnston et al., 1980a). Freeze–fracture and negative-stain electron microscopy and the impermeability of the polymerized dispersions to sucrose provide good evidence that the dispersions remain as lamellar vesicles. The appearance of the liposomes before and after polymerization and their osmotic behavior in the presence of sucrose are identical.

However, polymerization influences liposome permeability and stability. The high permeability to glycerol of diacetylene-containing PC liposomes before polymerization (as compared with DMPC liposomes) may be the result of increased polarity in the bilayer center owing to the presence of triple bonds in the lipid acyl chains. Following polymerization this enhanced permeability is reduced, corresponding to the conversion of triple bonds to less polar double bonds.

The absence of any change in the electron spin resonance spectra of nitroxide methyl esters and stearic acid spin-labels in polymerized PC may be attributed to one of two mechanisms: (1) the spin-label is excluded from the polymerized regions of the bilayer or (2) the polymerized acyl chains form an open structure in which the spin-label is free to rotate. The latter mechanism is favored by the observation that the mobility of a spin-label is greater in diacetylene PC than in saturated PC above T_c. The absence of any change in the ESR spectra after polymerization is in contrast to the immobilization of spin-label observed when the unpolymerized lipid is cooled below T_c. Long-term irradiation destroys the nitroxide group. Due to background fluoresence of the polymer, such changes could not be monitored with diphenylhexatriene.

Polymerization of diacetylene-containing phospholipid dispersions enhances their stability when stored at 4 or 20 °C. This enhanced stability may arise from a barrier to aggregation of the acyl chains following their polymerization. Such processes would be expected to proceed more slowly at lower temperatures.

Acknowledgments

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Registry No. C_{23} identical-chain PC, 76078-28-9; C_{27} identical-chain PC, 85407-26-7; 1-palmitoyl-2-(10,12-tricosadiynoyl)-*sn*-glycero-3-phosphocholine, 84271-00-1; 1-steoroyl-2-(10,12-tricosadiynoyl)-*sn*-glycero-3-phosphocholine, 84270-98-4; 1-palmitoyl-2-(10,12-septacosadiynoyl)-*sn*-glycero-3-phosphocholine, 85407-27-8; 1-stearoyl-2-(10,12-septacosadiynoyl)-*sn*-glycero-3-phosphocholine, 85407-28-9.

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Resonance Raman Spectra of Copper(II)-Substituted Liver Alcohol Dehydrogenase: A Type 1 Copper Analogue†

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ABSTRACT: Liver alcohol dehydrogenase (LADH) with copper in place of the catalytic zinc has recently been proposed to contain a type 1 site analogous to that in "blue" copper proteins. Resonance Raman spectra for the copper-substituted enzyme, Cu(II)·LADH, and its binary complexes with reduced nicotinamide adenine dinucleotide (NADH) and pyrazole support this viewpoint. These spectra have two dominant features: a sharp peak at ~415 cm^{-1}, which is believed to be associated with vibration of the single histidine ligand, and a broader, asymmetric band at ~350 cm^{-1}, whose components are assigned predominantly to vibrational modes of the two cysteinate ligands. The high frequency of these transitions, which is reminiscent of the blue copper proteins, is ascribed to the tetrahedral nature of the metal site that produces unusually short Cu–S bonds and coupled vibrational modes. Solvent exchange with $H_2{}^{18}O$ reveals no contribution to the resonance Raman spectrum of the water molecule, which is a metal ligand in free Cu(II)·LADH; however, the spectrum of the binary complex with pyrazole has several new peaks attributable, in part, to pyrazole ligation. The strong similarity among the vibrational spectra demonstrates that the Cu(II) environment in alcohol dehydrogenase maintains its near-tetrahedral geometry in the various enzyme derivatives. The resonance Raman spectrum of Ni(II)·LADH is close to that of Cu(II)·LADH and suggests a similar tetrahedral site. The Raman spectra presented here together with available optical and EPR data indicate that Cu(II)·LADH belongs to the type 1 copper classification and, thus, can provide new insights into this unusual coordination geometry.

Alcohol dehydrogenase from horse liver is a dimeric molecule wherein each subunit contains two zinc ions. The metal-site structures of these ions are known from X-ray crystallography and include a structural zinc that is coordinated to four cysteinates and a catalytic zinc that is coordinated to two cysteinates, one histidine, and one water molecule (Eklund et al., 1976). Recently, Maret and co-workers (Maret et al., 1980) prepared a derivative of liver alcohol dehydrogenase (LADH)[1] in which the catalytic zinc was substituted by copper. Characterization of this cupric protein by optical and EPR measurements (Maret et al., 1980, 1981) showed Cu(II)·LADH to be strikingly similar to the "blue" copper proteins such as azurin, plastocyanin, and stellacyanin and the type 1 sites of several multicopper oxidases such as laccase and ascorbate oxidase (Fee, 1975). The optical absorption spectrum of Cu(II)·LADH at 296 K has an intense band at 620 nm ($\epsilon \simeq 4000$ M^{-1} cm^{-1}), and the EPR spectrum at 100 K exhibits rhombic character with $g_\parallel = 2.21$ and $A_\parallel = 0.005$ cm^{-1} (Maret et al., 1983).[2] The anomalously small hyperfine coupling constant and the intense electronic absorption of the type 1 site have been explained as arising from thiolate coordination of Cu(II) in a distorted tetrahedral environment with the characteristic absorption at ≃600 nm being assigned to cysteinate sulfur → Cu(II) charge transfer (Gray & Solomon, 1981). Thus, it is of interest to learn whether similar structural parameters are present in Cu(II)·LADH.

Resonance Raman spectroscopy appears to be a valuable technique for probing the structure and bonding of the type 1 site chromophore. All of the blue copper proteins that have been investigated show the following common features in their resonance Raman spectra: (i) three or more strong bands between 350 and 450 cm^{-1} that are associated with vibrations of the copper chromophore; (ii) intensity enhancement of these vibrations via resonance with the ≃600-nm LMCT band; and (iii) two nearly constant frequency vibrational modes at ≃750 and ≃260 cm^{-1} (Miskowski et al., 1975; Siiman et al., 1976; Ferris et al., 1979). The unexpectedly high frequencies of the Cu(II)–ligand vibrations (when compared with inorganic Cu(II) complexes) can be explained by the shortness of the Cu–S(Cys) bond and the tetrahedral character of the copper binding site that have been observed in the crystal structure of plastocyanin (Freeman, 1981). A normal coordinate

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[1] Abbreviations: LADH, liver alcohol dehydrogenase; EPR, electron paramagnetic resonance; NADH, reduced nicotinamide adenine dinucleotide; Tes, 2-[[tris(hydroxymethyl)methyl]amino]-1-ethanesulfonic acid; LMCT, ligand to metal charge transfer; ^1H NMR, proton nuclear magnetic resonance. Metal ion derivatives of LADH, M(II)·LADH, used throughout this paper refer to metal substitution solely in the catalytic site.

[2] Maret et al. (1980) initially reported values for $g_\parallel$ and $A_\parallel$ of 2.25 and 0.003 cm^{-1}, respectively, from an enzyme preparation having ≃60% site-specific replacement of Cu(II); the present values are a redetermination for Cu(II)·LADH of significantly higher copper content in the active site (≃80%).

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