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Pressure distortion of an artificial membrane and the effect of ligand/protein binding

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Fourier transform infrared spectroscopy was employed to study configurational changes induced in a model membrane exposed to very high (to 25 kbar) hydrostatic pressures. The model membrane consisted of 25% dimyristoyl-L-α-phosphatidylcholine (DMPC) and 12.5% human albumin suspended in 62.5% heavy water (D_2O). Clofibrate (10^{-2} M), which binds avidly to albumin, was used as a ligand. The suspension formed a lipid bilayer to which albumin was attached by hydrogen bonding of a side chain to a carbonyl group of DMPC. The protein structure remained mainly helical with some β-sheeting. Hydrogen bond strength within the protein was increased by lipid–protein interactions. Clofibrate bound to the lipid-albumin complex and reduced somewhat the β-sheet/α-helix ratio. Increasing pressure increased the intermolecular interactions between albumin and DMPC as indicated by the increasing intensity of a band near 1587 cm^{-1}. Clofibrate reduced this effect by preventing pressure-enhanced protein interactions with DMPC. The results indicate that model membrane physical chemistry is altered by pressure and this may have consequences for ligand–receptor interactions in biological systems.

Introduction

Previous work by one of us (P.T.T.W.) [1] showed that pressures in excess of 2 kilobars (1 kbar = 1000 × normal atmospheric pressure) caused the expulsion of a local anesthetic drug (tetracaine) both from an artificial, lipid membrane and from segments taken from the frog sciatic nerve or from lobster nerve. The distortion was observed in the C=O band of tetracaine and occurred only when cholesterol was present in the artificial lipid bilayer composed of either 1,2-dimyristoylphosphatidylcholine (DMPC) or 1,2-di-*O*-hexadecylphosphatidylcholine (DHPC) at physiological pH. Although these pressures are greatly in excess of any that occur on earth (the deepest ocean depth has a hydrostatic pressure of about 1.1 kbar), or that have been encountered by human subjects (about 0.06 kbar), the findings are relevant to our understanding of the effects of pressure on cell function. It is well-known that hydrostatic pressure can reverse the action of a general anesthetic. Tadpoles anesthetized with alcohol recovered swimming ability when exposed to about 0.063 kbar of hydrostatic pressure [2] and newts anesthetized with a variety of agents recovered the righting reflex when exposed to similar pressures [3]. Moreover, neurophysiological changes have accompanied, and sometimes restricted, deep ocean diving. The use of compressed air as the breathing gas is hazardous at depths exceeding 55 metres of seawater (msw) because of the development of Inert Gas Narcosis (IGN) or progressive sedation resulting from the anesthetic effect of high partial pressures of N_2 [4]. The replacement of air with He in the breathing mixture eliminated this problem but revealed another: the High Pressure Neurological Syndrome (HPNS) which occurs at depths exceeding 180 msw and is accompanied by tremor, motor incoordination, myoclonus and convulsions. HPNS is generally believed to be due to the effects of hydrostatic pressure per se [5]. Neurotransmission must be affected in both IGN and HPNS but the molecular site or sites of the disruption in transmission remain obscure.

The previous study [1] employed a relatively simple lipid bilayer model and a local anesthetic. It is now, however, accepted that local and general anesthetics probably exert their actions through different mecha-

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nisms and that the final event in the action of a general anesthetic may involve an effect on a protein component of the membrane [6]. For this reason, and because the abnormalities of neurotransmission alluded to above might involve changes in protein receptors, it is important to study the influence of pressure on ligand binding to a protein component of membrane. The present study attempts to do this by employing a ligand with a high affinity for human albumin and an artificial lipid/protein membrane that incorporates human albumin.

Materials and Methods

The following chemicals were employed: Human albumin Fraction V, dimyristoyl-L-α-phosphatidylcholine (DMPC) and ethyl *p*-chlorphenoxyisobutyrate (clofibrate), all from Sigma Chemical Co., and deuterium oxide (D_2O) from MSD Isotopes.

The membrane model was prepared by adding 250 mg of DMPC and 125 mg of albumin to 625 mg of D_2O. The mixture was then sonicated with a Kontex microultrasonic cell disrupter at about 20% of maximum power for 30 s at 22°C to produce a uniform opalescent dispersion. The sample was then divided into equal portions and clofibrate was dissolved in one portion to a final concentration of 10^{-2} M.

Fourier transform infrared spectroscopy (FTIR) was employed to study the effects of high hydrostatic pressure (to 25 kbar) on the configuration of the model membrane constituents and on ligand binding.

Small amounts of the homogeneous dispersions were placed at room temperature, together with powdered α-quartz, in the 0.37 mm diameter hole of a 0.23 mm thick stainless steel gasket mounted on a diamond anvil cell, as described previously [7]. Infrared spectra were measured at 28°C on a Digilab FTS-60 Fourier transform spectrophotometer with a liquid nitrogen cooled mercury cadmium telluride detector. The infrared beam was condensed by a sodium chloride lens system onto the pinhole of the diamond anvil cell. Typically, 512 scans (600–4000 cm^{-1}) were coadded to produce a spectrum with a resolution of 4 cm^{-1}. The total measuring time was about 5 min. Pressures were determined from the 695 cm^{-1} phonon band of α-quartz by using data reduction methods previously reported [7]. For all the figures of spectra the *Y*-scale is absorbance.

Results

Infrared amide I spectra (1600–1690 cm^{-1}) for albumin (A), albumin plus DMPC (B) and albumin plus DMPC and clofibrate (C) after band narrowing using Fourier self-deconvolution with an enhancement factor of 2 and a band width of 18 cm^{-1} [8] are shown in Fig. 1. The amide I band shape of albumin was not changed

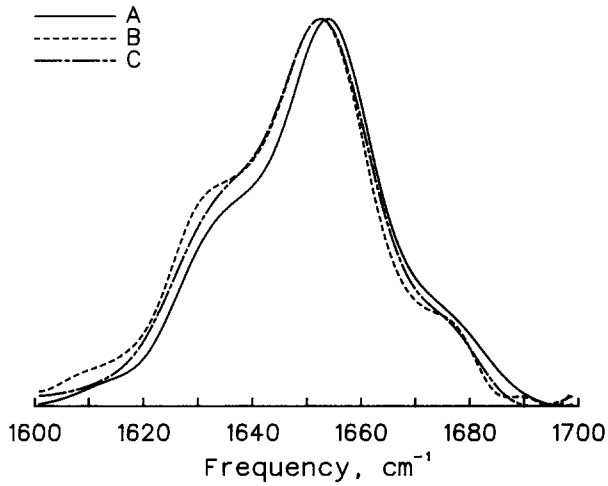

Fig. 1. Infrared spectra of the amide I band of albumin (A), albumin plus DMPC (B) and albumin plus DMPC and clofibrate (C) at atmospheric pressure.

significantly and only a slight change in the relative intensities of the component bands near 1635 and 1680 cm^{-1} and a approx. 2 cm^{-1} shift of the overall amide I band were induced by the presence of either DMPC or DMPC and clofibrate. The amide I band in the infrared spectra of polypeptides and proteins has been widely used for determination of the secondary structure, since the peak maximum of the amide I band locates at different frequencies for various specific types of secondary structure [9]. The peak position of the amide I band of albumin is at 1654 cm^{-1} and two shoulders are observed at 1635 and 1680 cm^{-1}. The component band at 1654 cm^{-1} is due to the amide I mode of the amide group segments in the α-helical conformation whereas those at 1635 and 1680 cm^{-1} are due to the amide I mode of the β-sheet segments in the protein (Ref. 9 and references therein). These fine features of the spectra in Fig. 1 indicate that the protein conformation is mainly α-helical with some β-sheet components. The amide I band is mainly due to the in-place C=O stretching vibration and its frequency is very sensitive to the hydrogen-bond strength on the C=O groups [9]. The overall frequency of the amide I band was decreased in the presence of the lipid, whether or not the ligand was present, indicating that the overall hydrogen bond strength in the protein is increased when it is associated with the DMPC matrix. The effect of clofibrate on the secondary structure of albumin was to reduce slightly the β-sheet/α-helix ratio as indicated by the change in the relative intensity of the β-sheet and α-helix components of this amide I band (Fig. 1).

Stacked spectra for 0–25 kbar of the DMPC plus albumin samples in the absence and presence of clofibrate are shown in Figs. 2 and 3, respectively, over the frequency range 1350–1800 cm^{-1}. A vibration of albumin side chains produced a band near 1587 cm^{-1}

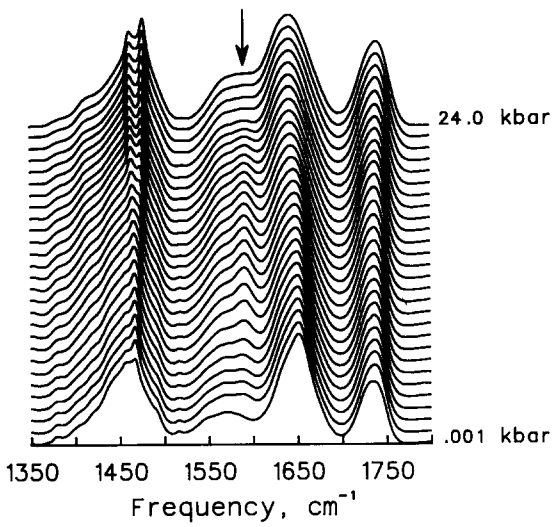

Fig. 2. Stacked contour plots of the infrared spectra of aqueous dispersions of DMPC plus albumin in the frequency region 1350–1800 cm^{-1} at increasing hydrostatic pressures.

(arrows). When clofibrate was absent, this band increased in intensity as hydrostatic pressure was increased and peak broadening occurred above 12 kbar (Fig. 2). The presence of clofibrate (Fig. 3) almost totally damped out the pressure-induced intensity of this side chain vibration. In Fig. 4A the vibration in this side chain band region is examined more closely (frequency 1585–1595 cm^{-1}). The frequencies of this band were measured from the third power derivative spectra with a breakpoint of 0.3 [10]. Vibrational frequency was generally greater in albumin plus DMPC than when clofibrate was included. The accuracy of the frequency measurements of this band is reduced above 12 kbar due to the broadening of this band.

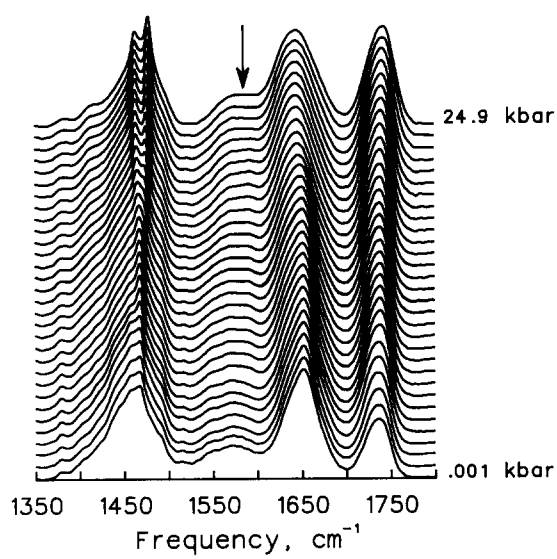

Fig. 3. Stacked contour plots of the infrared spectra of aqueous dispersions of DMPC plus albumin and clofibrate in the frequency region 1350–1800 cm^{-1} at increasing hydrostatic pressures.

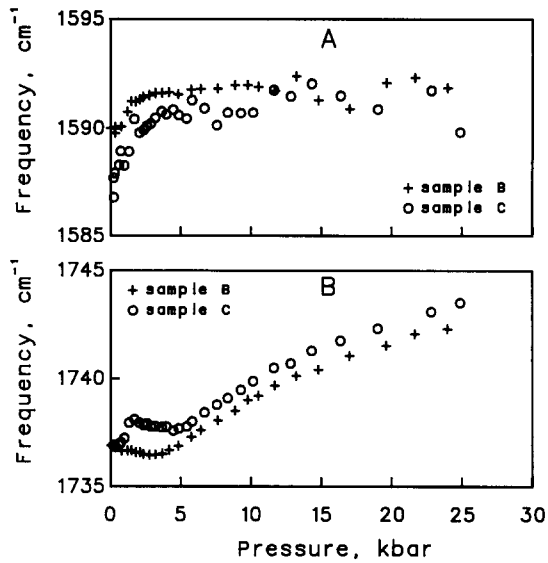

Fig. 4. Pressure dependences of frequencies of the side chain mode of albumin (A) and the C=O stretching mode of DMPC (B) for the samples of DMPC plus albumin (sample B) and DMPC plus albumin and clofibrate (sample C). Frequencies were obtained from third-order derivative spectra with a breakpoint of 0.3 for the side chain mode (A) and 0.2 for the C=O stretching mode (B).

Fig. 4B displays the pressure effects on the C=O stretching frequency of DMPC. In the presence of albumin alone, the C=O stretching frequency was lower relative to that when clofibrate was present in the pressure range 0–25 kbar. In both, however, frequency increased with pressure above 5 kbar. When clofibrate was present, the mixture behaved like a simple lipid bilayer with respect to C=O stretching frequency (not shown). Therefore, in the absence of clofibrate, the C=O groups of DMPC interact strongly with the protein molecules.

Fig. 5 shows the correlation field splitting pressure of the CH_2 bending mode of the acyl chains in DMPC.

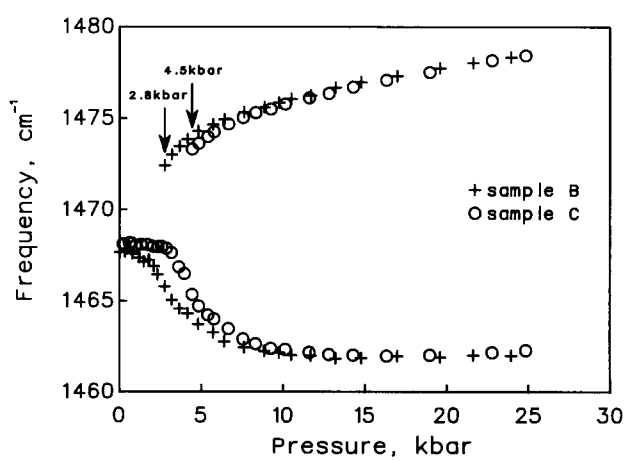

Fig. 5. Pressure dependences of frequencies of the CH_2 bending mode of DMPC in the presence of albumin (sample B) and albumin plus clofibrate (sample C). Frequencies were obtained from third-order derivative spectra with a breakpoint of 0.95.

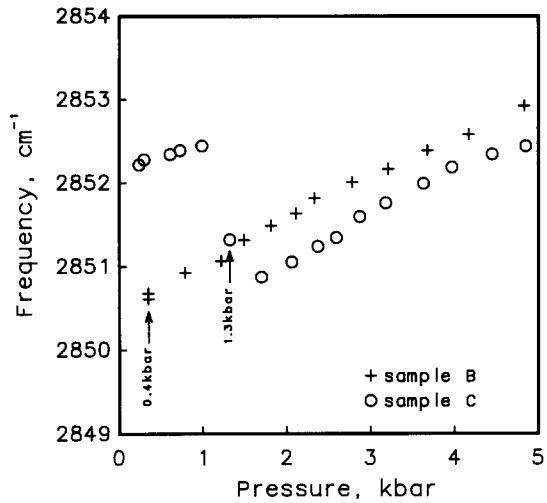

Fig. 6. Pressure dependences of frequencies of the symmetric CH_2 stretching mode of DMPC in the presence of albumin (sample B) and albumin plus clofibrate (sample C). Frequencies were obtained from original infrared spectra.

Correlation field splitting starts at 1.7 kbar higher in the presence of clofibrate. At lower pressure the interchain interactions in DMPC are weak due to the reorientational fluctuations of the chains. These reorientational fluctuations can be damped by pressure, which results in stronger interchain interactions and the appearance of correlation field splitting. Therefore, the 1.7 kbar increase in the correlation field splitting pressure indicates that orientational disorder due to fluctuations in the DMPC acyl chains is larger in the presence of clofibrate due to the breakage in the strong interactions between the protein and the C=O groups of DMPC by the binding of clofibrate to albumin. The liquid-crystal to gel-phase transition pressure of aqueous DMPC bilayers is 0.15 kbar [11,12] and is below 0.4 kbar in the presence of albumin. However, when clofibrate is bound to albumin in DMPC, the liquid crystal to gel phase transition pressure of DMPC becomes 1.3 kbar as shown by the discontinuities in the pressure dependences of the frequencies of all the vibrational modes, for instance the symmetric CH_2 stretching mode of the acyl chains (Fig. 6). This indicates that the liquid crystalline phase of DMPC is more disordered in the presence of the drug as a result of this cleavage of the lipid-protein binding.

Discussion

In these experiments the albumin-DMPC artificial membrane assumed a lipid bilayer configuration with attachment of the protein. In the absence of clofibrate the application of kbar pressures increased the vibrational intensity of the albumin side chain mode. The increase in this intensity is due to the increase in the transition moment of the normal vibration of the side chains arising from the enhancement of intermolecular interactions by external pressure. The effect of pressure on the sample is to shorten the intermolecular distance and thus increase intermolecular interactions. Since the pressure effect on the intensity of this side chain band was not observed in the pure albumin sample (Fig. 7) and only observed in the sample of albumin in DMPC, the intermolecular interactions which cause an increase in the intensity of the 1585 cm^{-1} band must be those between the side chains of albumin molecules and DMPC molecules. The broadening of this band above 12 kbar indicates that the orientation of the side chains becomes more disordered at high pressure. Clofibrate bound to the protein side chains and prevented this pressure-induced change in the vibrational intensity by blocking the intermolecular interactions between albumin and DMPC. The C=O stretching frequency was decreased in the presence of albumin but returned to that of pure lipid DMPC bilayers (not shown) when clofibrate was added. Since hydrogen bonding of C=O groups causes a reduction in the C=O stretching frequency [11], this would suggest that albumin binds to the C=O of DMPC via hydrogen bonds. On the other hand, it is also possible that the presence of albumin makes the C=O groups more accessible to the water solvent, which can also cause a decrease in the C=O stretching frequency [13]. The observations that clofibrate increased the correlation field splitting pressure of the CH_2 bending mode of the acyl chain of DMPC as well as the liquid-crystal to gel-phase transition pressure support the contention that clofibrate caused DMPC to become more disordered by disrupting the stabilizing effect of lipid-protein bonding.

Clofibrate is employed to reduce elevated circulating plasma triacylglycerol levels in patients deemed to be at risk for atherosclerosis and it binds avidly to plasma albumin [14]. Albumin obviously is not a protein found in plasma membranes, nor are its binding sites true

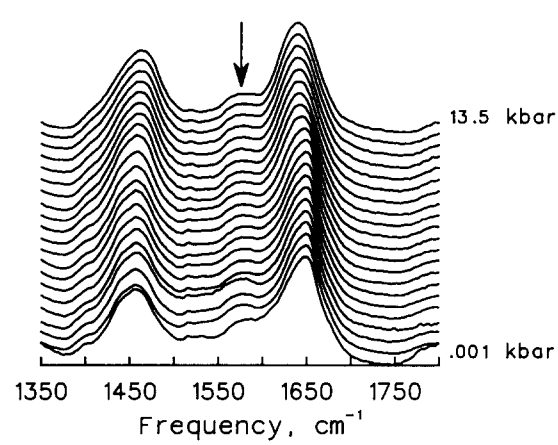

Fig. 7. Stacked contour plots of the infrared spectra of an aqueous solution of albumin (12.5% by wt.) in the frequency region 1350–1800 cm^{-1} at increasing hydrostatic pressure.

drug receptors. Despite these drawbacks, the model system used in this study has some advantages. Albumin has over 200 functional groups per molecule that accept many drugs, mostly weakly acidic ones, as well as some hormones such as thyroxine [15]. Because of this large number of binding sites, the potential for molecular distortion is great enough to cause detectable vibrational perturbations by FTIR. Preliminary (unpublished) studies suggest that this may not be so for more specific receptor proteins which are assumed to have a single active site per molecule. Like true receptors, however, albumin's bind sites give up their ligands more or less readily (depending on the nature of the ligand).

Pressure disturbances of protein function have been shown by others. In previous studies it has been demonstrated that a proteoglycolipid with the binding characteristics of the acetylcholine neuroreceptor had reduced affinity for its ligand (acetylcholine) at pressures as low as 0.08 kbar [16]. Studies with the luciferin-luciferase system obtained either from bacteria or from firefly lanterns, indicated that the activity of this pure protein enzyme system was inhibited by anesthetics and that this effect was reversed by pressure (5 kbar) [17]. In their studies, however, a lipid component was lacking and our findings suggest that there is an important interrelationship amongst the lipid, the protein and the ligand, and that a pressure-induced effect on one component is reflected in alterations in the others.

Our findings may have relevance for the understanding of pressure- and inert gas-induced changes in neurophysiological function and the pharmacological response to centrally acting drugs, including anesthetics. Neurotransmitters and centrally-acting drugs that mimick or antagonize their action, all work on receptors located on membrane proteins. The question of the site of action of general anesthetics is still open, but protein involvement is not out of the question. The original observation of Myer and Overton that the potency of general anesthetics increased in parallel with the oil solubility led to the conclusion that anesthetics worked by perturbing the lipid of membrane but more recent work has suggested that the site of action may be within a hydrophobic pocket of protein [18], which could be located either at the lipid/protein interface or the water/protein interface. Whatever the site of action, the observations that hydrostatic pressure can reverse the effects of general anesthetics in experimental animals and N_2 can antagonize HPNS in divers [19] provide additional stimuli for the study of the pressure effects in lipid-protein membrane models.

Although the pressures used in this study are beyond the range encountered by living organisms, it is important to note that the pressure effect on the model membrane is a continuum and that it was detectable at or below 1 kbar. Indeed, the liquid-crystal to gel-phase transition of DMPC in the presence of albumin occurred at 0.4 kbar, and in its absence at 0.15 kbar, which is not far beyond those pressures that have been experienced by human subjects. Thus there is a strong likelihood that more subtle but physiologically-significant changes may occur at pressures encountered by divers, and certainly by deep-sea organisms.

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