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X-ray diffraction studies using a novel synthetic phospholipid

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Abstract

A novel brominated phospholipid has been designed and synthesised for future use in X-ray diffraction studies. It is an analogue of dipalmitoyl-phosphatidylcholine (DPPC), with the sn-2 chain terminal methyl group exchanged for a bromine atom. This bromine atom "label" has been incorporated into a phospholipid by substitution for a group of similar atomic radius, 1.85 versus 2.00 Å, thus creating a molecule which is sterically similar to its unlabelled analogue. The "bromolipid" has been studied using the swelling series method in conjunction with Patterson mapping in the gel phase at 20°C. It diffracts well to 12 orders in its pure form at 20°C and between 57% and 98% relative humidity. A combination of two phasing methods have allowed the diffraction patterns of bilayers incorporating the bromolipid to be phased unambiguously. We suggest that the bromolipid is excellently suited as a phasing agent for use in future isomorphous replacement and multiple anomalous diffraction (MAD) experiments.

Keywords: X-ray diffraction; Phospholipid; Bromolipid; Patterson mapping

1. Introduction

Brominated phospholipids have been utilised before in the study of lipid bilayers [1]. Synthetic bromolipids have been used in fluorescence, as well as X-ray diffraction, studies [2,3]. These studies have however focused on the production of a bromolipid, typically by the addition of two bromine atoms across a double bond, resulting in a phospholipid "isomorph" lacking a double bond, with two relatively large atoms sticking out from the lipid chain. Other methods of placing bromine in a bilayer include the approach of labelling a small molecule with bromine, which is then inserted into the bilayer [4].

The present study has set out to make and study a close structural isomorph of dipalmitoylphosphatidylcholine, which can be used as a phasing agent in isomorphous replacement studies. DPPC, a benchmark lipid, has been studied many times and is well characterised [5]. This bromolipid was designed so that it would have only one bromine per molecule, would not hinder chain packing by having been added across a double bond, and would have approximately the

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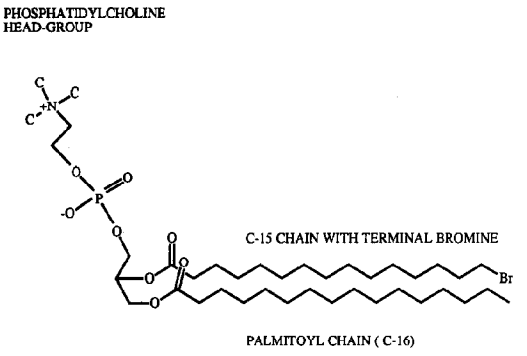


Fig. 1. The structure of the bromolipid used in this study. This shows the position of the bromine atom in the molecule, which replaces the sn-2 palmitoyl terminal methyl group of DPPC.

same molecular volume as DPPC. The optimum place to locate such a phasing agent "label" would be in the centre of the bilayer. This has been achieved by switching the terminal methyl of the sn-2 palmitoly chain of DPPC for a bromine atom, see fig. 1. The methyl group and bromine atom are of similar radius and so the substitution results in little difference in the overall volume of the two molecules and therefore similar packing in the membrane.

In this paper the fully hydrated structure of bromolipid has been studied, together with that for pure DPPC at 20°C. X-ray diffraction photographs of each have been collected to at least 12 orders, and swelling series have been created by repeating the experiments at varying sample humidity, using the method of Torbet and Wilkins, [6]. Diffraction spots have been phased using a combination of this swelling series method and by using one-dimensional Patterson maps to help phase ambiguous reflections. From these results electron density maps have been created showing the structure of bromolipid and DPPC to 4 Å.

2. Possible future uses of the bromolipid

2.1. Isomorphous replacement

This is a method, used extensively in protein crystalography for phasing, where a heavy atom is added into the sample as a "label". On introducing a known amount of heavy atoms into a system, one has a way of scaling the relative electron density scale into one of absolute density per unit cell. This is a great advantage over the swelling series which is always only on a relative scale. The use of heavy atom methods go back some time and have had limited success with biological membranes [7–9]. Phases have been determined for bilayer of fatty acids associated with a series of alkaline earth metals [10]. Franks et al. [11] have used halogenated cholesterol analogues in isomorphous experiments. They showed that the analogue could be isomorphously exchanged with cholesterol and this exchange be used to phase the signs of the lamellar reflections. This cholesterol labelled method works well, but obviously, only when one is studying a cholesterol containing system.

2.2. Multiple anomalous dispersion (MAD) experiments

Anomalous scattering as a method of phasing is still very new to protein crystalography [12,13], and has still yet to be used in membrane diffraction experiments. If possible this method would be the finest application for a phospholipid such as the bromolipid synthesised and studied as reported here.

MAD experiments have a major advantage over isomorphous replacement experiments. In a typical isomorphous replacement experiment one might study several samples, each with increasing amounts of heavy atom present, and hope for phase assignment to be successful, that there is no major structural change around the heavy atom. In the MAD experiment one need only study one sample with a percentage of label incorporated. The experiment is performed using X-rays, at energies either side of a heavy atom absorption edge, the difference being used as the phasing method, Hendrickson [14]. Therefore throughout the experiment there are no structural variations in the sample whatsoever, as it is the X-ray source and not the sample that is varied.

By using the swelling series as the phasing method to characterise this novel molecule, we

have shown that this molecule has great potential, in itself, as a phasing agent.

3. Materials and methods

1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) was purchased from Sigma Chemical Company Ltd. (Fancy Road, Poole, U.K.). The synthesis of the novel bromolipid shall be covered elsewhere. Both phospholipids were confirmed to be a single species by thin layer chromatography prior to use.

3.1. X-ray diffraction

Oriented bilayer stacks of phospholipid were prepared by pipetting 2 mg of sample, dissolved in chloroform, onto a curved glass slide of area 1 cm^2. The chloroform was then evaporated off by a stream of nitrogen, before the slide was placed in a vacuum for 2 h. The sample was then rehydrated over water, and reannealled at 70°C for 1 h. Samples were then held in a temperature controlled cell at 20°C. The relative humidity of the cell was varied from 57% to 98%, using varying salt solutions in a bath under the sample, for the swelling series experiments. The data were corrected for absorption, as described in Franks and Leib [15], and with the Lorentz factor. For the geometry used, this latter factor takes the form of h^2, where h is the order of diffraction.

Previous methods which attempt the determination of phases using only a single data set have been attempted. Luzzati et al. [16] used a pattern recognition approach. In this procedure all possible phase combinations are considered and the correct solution chosen on the basis of known or postulated properties of the electron density profile, such as levels of electron density and partial specific thicknesses of particular components. For a good pattern however, with a large number of orders, there are a large number of possibilities. Using only one data set also can allow in errors in data collection, that would stand out in a data series. This paper however uses a similar approach to this method for distinguishing ambiguous phases, i.e. the case where most phases are determined from the swelling series but one or two orders could be either phase. The properties of the electron density profile, in this case, coming from the Patterson maps.

3.2. Differential scanning calorimetry

The lipids, DPPC and bromolipid, were prepared by dissolving the weighed out mixtures in chloroform, drying down in a rotary evaporator, washing twice with acetone and again drying down to a mixed solid in a rotary evaporator. The dry phospholipid was then placed in a platinum pan and the mass of lipid weighed using a four point balance. An exact amount of water, 40 μl of water to 7 mg of lipid for water in excess experiments, were then measured into a sample pan, of volume 50 μl, by syringe. A lid was placed on top of the pan and sealed by cold welding using a Perkin Elmer sealing press. A reference pan containing only water was prepared in the same way as the sample pans. Samples were loaded into a Perkin Elmer DSC 7 machine with a Perkin Elmer Tac 7/7 instrument controller. Samples were then typically cycled from 10°C to 70°C, so as to anneal and homogenise the sample. The sample was then allowed to sit at 10°C for 30 minutes before an experimental run from 10°C to 70°C was recorded. A scan rate of 3.0°C per minute was used.

4. Results and discussion

The DSC results, table 1, compare the phase behaviour of two lipids, DPPC and bromolipid, calorimetrically. The results show that the phase behaviour of bromolipid is similar to that of

Table 1

DSC results for DPPC and bromolipid samples

Lipid	Main transition temperature (°C)	Pretransition temperature (°C)	Main transition enthalpy (J/g)	Main peak width at half height $\Delta T_{1/2}$ (°C)
DPPC	42.3	37.1	54.5	1.45
bromolipid	33.4	30.5	52.4	1.55

DPPC. Although the main phase transition temperature, for the bromolipid, occurs at ≈ 9°C lower than that found for DPPC, it melts with a similar enthalpy. The width of the melting peak, which is an indicator of the cooperativity of the event [17], is also similar for the two species. The bromolipid also has a pretransition peak, indicating it too forms the pretransition ripple phase, found for DPPC.

Scheme 1 shows a typical X-ray diffraction photograph of pure bromolipid, showing twelve orders of diffraction. Films were scanned and data corrected before a Patterson map of each data set was constructed. The Patterson maps show the interatomic distances of the major electron dense regions of the molecule in the bilayer without using any phase information whatsoever. An electron density graph constructed using data that is putatively phased should therefore show the same approximate interatomic distances. Because the Patterson map has no phase information involved in its construction it is a useful alternative test of accurate phasing.

Fig. 2 shows the Patterson map for pure bromolipid and pure DPPC, see also table 1. The graph for pure bromolipid naturally has more features to it, it having extra regions of high electron density, compared with DPPC. The bromines are close together giving rise to a peak from the origin out to ≈ 7 Å. The phosphate headgroup to bromine atom distance gives the peak at 18.5 Å. This partially masks the headgroup separation peak at ≈ 14 Å. Swelling series have been constructed for pure bromolipid and pure DPPC, figs. 3 and 4, using the method of Torbet and Wilkins [6]. Although ideally for phasing using the swelling series method, water lost

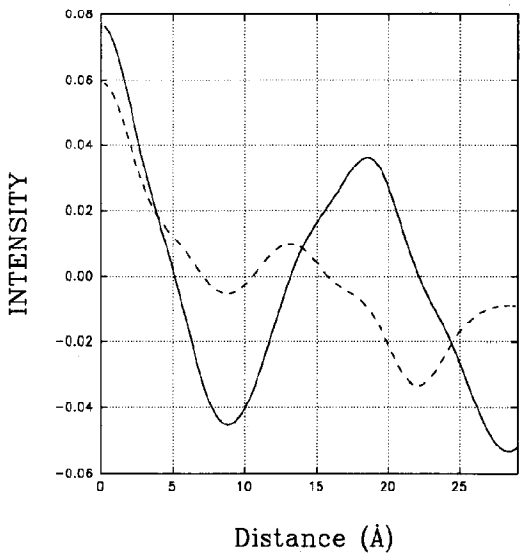
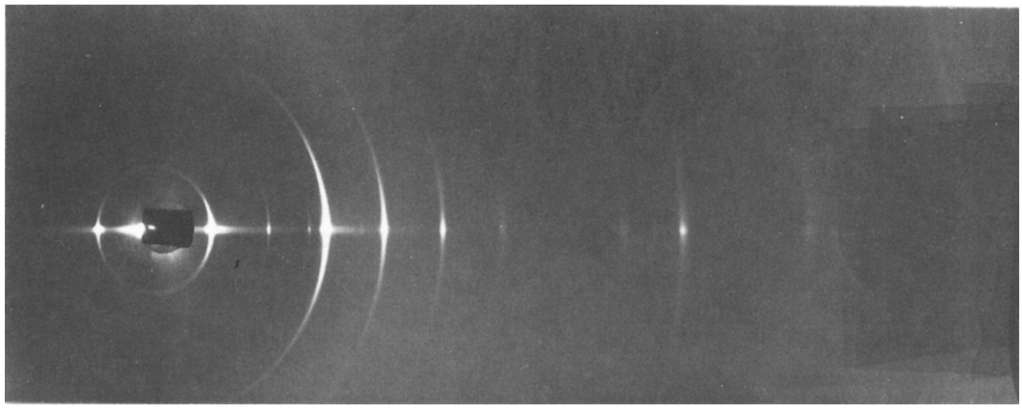


Fig. 2. One-dimensional Patterson maps of bromolipid (solid) and DPPC (dash), fully hydrated at 20°C. The bromolipid has increased intensity near the origin due to the closeness in space of the bromine atoms in the center of the bilayer. It also shows the bromine to phosphate distance as being about 18.5 Å.



Scheme 1.

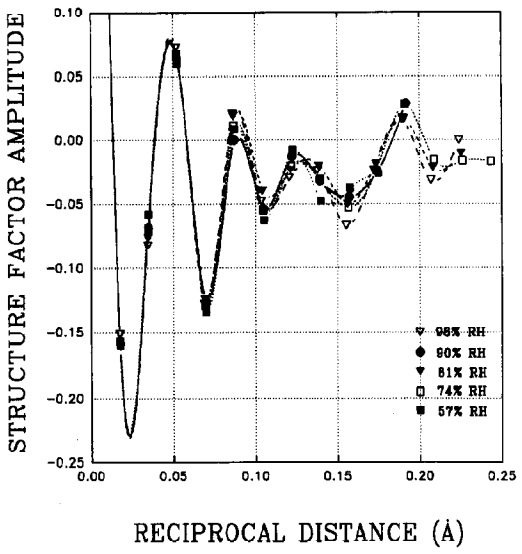


Fig. 3. Swelling series data for pure DPPC at 20°C. The humidity was varied from 57% to 98% using varying salt solutions. The corrected square roots of intensity are plotted against reciprocals of Bragg spacing.

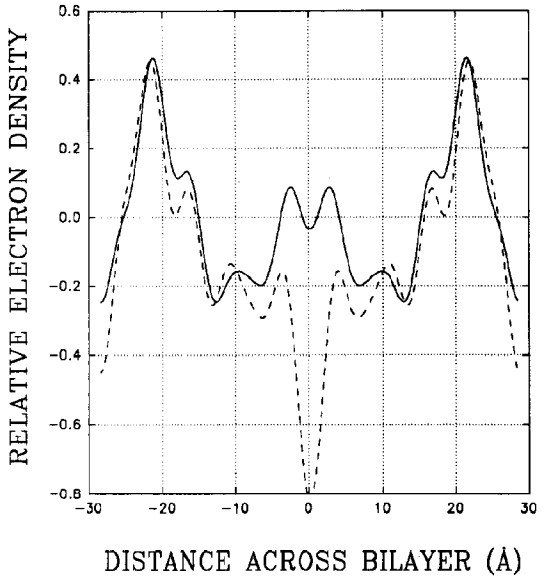


Fig. 5. Reconstructed electron density maps of bromolipid (solid) and DPPC (dash) using the phases from the swelling series, figs. 3 and 4. The maps clearly show the bromine atoms at the centre of the bilayer.

from the water layer is the only structural change in the sample, varying humidity has other known effects on the structure and thickness of the

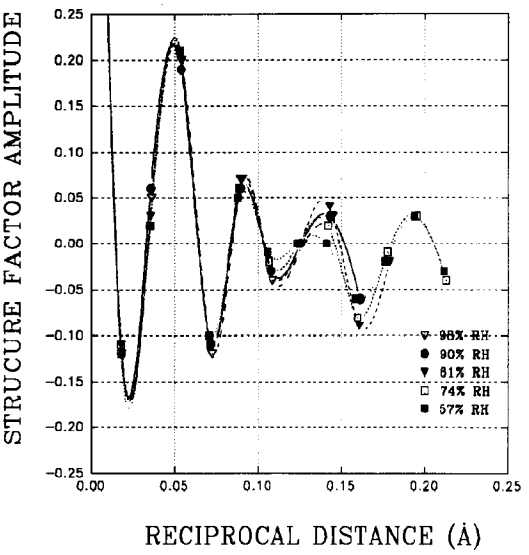


Fig. 4. Swelling series data for pure bromolipid at 20°C. The relative structure factors show a general displacement in the positive direction compared with those for pure DPPC.

bilayer. As the humidity decreases so does the chain tilt angle, which acts to increase the bilayer thickness [18,19]. At the same time water is removed from around the head group, and the N^+ end of the phosphocholine group moves closer to the hydrocarbon layer, acting to reduce the bilayer thickness [20]. In spite of this the swelling series method is still usable. The phases shown have been constructed by fitting data to continuous Fourier transforms, and comparison of putatively phased electron density graphs to Patterson maps. These show data collected out to fourteen orders in one case for DPPC and twelve orders for bromolipid. One can note the general tendency of the continuous transform of bromolipid being positively shifted, compared with DPPC, as one would expect for a large atom placed in the centre of the DPPC bilayer [21].

Fig. 5 shows the electron density graph of both pure bromolipid and DPPC each to twelve orders using the phases shown. From the electron density graph can be measured the same interatomic distances that give rise to maxima in the Patterson function. Table 2 summarises these distances,

Table 2

Inter-atomic distances inferred from Patterson functions versus those from phased electron density graphs

Phospholipid	Phosphate headgroup to headgroup distance	Phosphate headgroup to bromine distance
bromolipid		
Patterson	≈ 14 Å	≈ 18.5 Å
electron density map	13.6 Å	18.7 Å
DPPC		
Patterson	≈ 13.2 Å	
electron density map	13.0 Å	

as obtained from the Patterson function or electron density map. The correlation of the two are very good suggesting the right phases have been used in obtaining the electron density maps.

5. Summary

A novel structural isomorph of the benchmark phospholipid DPPC has been successfully synthesised for use in X-ray diffraction experiments. This bromolipid analogue of DPPC has a terminal bromine on the sn-2 lipid chain in place of a methyl group. This new molecule forms membrane bilayers very similar to those of pure DPPC and diffracts well to twelve orders. The diffraction patterns here have been successfully phased using the swelling series method and one-dimensional Pattersons. The resultant electron density maps show the two bromines close together in the centre of the bilayer structure.

We hope that in this paper we have prepared the way for future X-ray experiments where the bromolipid hopefully can be used as a phasing agent in isomorphous replacement experiments or in anomalous dispersion experiments which should be able to utilise the absorption edge of the bromine atom.

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