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SMALL-ANGLE X-RAY SCATTERING STUDIES OF CYTOCHROME *c*–PHOSPHOLIPID COMPLEXES

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SUMMARY

Cytochrome *c* in water and isooctane-soluble lipid–protein complexes formed between cytochrome *c* and a mixture of natural phospholipids (phosphatidylcholine/phosphatidylserine) has been studied by small-angle X-ray scattering methods. In the concentration range corresponding to 0.04%–1.00% cytochrome *c* in isooctane, two distinct types of isooctane-soluble complex with radii of gyration of 66 Å and 87 Å are observed, whilst in water, cytochrome *c* has a radius of gyration of 12–14 Å. The large radii of gyration and sedimentation coefficients suggested by ultracentrifugation studies show that the complexes in isooctane cannot consist simply of 20:1 or 30:1 lipid–protein monomeric units, but molecular aggregation occurs (particle weight > 1000000).

INTRODUCTION

The validity of the DANIELLI–DAVSON[1,2] bilayer model of membrane structure has been seriously questioned in the last few years[3,4]. Biochemical studies, largely confined to mitochondrial membranes[5], and physical techniques, such as optical rotatory dispersion, infrared spectroscopy, and nuclear magnetic resonance, applied to a wide variety of membrane types[6–10], have suggested that protein is a much more integral part of the membrane structure than is implied by this model.

In order to reduce some of the inherent difficulties in studying natural membrane systems, well-characterised model complexes of phospholipids with various proteins are being studied and may provide information on the mode of interaction and the overall lipoprotein structures produced. Several groups of workers[11–14] have studied the formation of such complexes between well-defined proteins and phospholipids. The stoichiometry and the predominantly electrostatic or hydrophobic binding forces appear dependent upon the type of protein and phospholipid involved and the conditions of formation. In particular, DAS AND CRANE[13] and DAS, HAAK AND CRANE[14] have described the formation of isooctane-soluble complexes between the basic protein cytochrome *c* and phospholipids. Apart from optical rotatory dispersion studies by ULMER *et al.*[15] and ULMER[16], and a preliminary study using the ESR spin-labelling technique[17], no physical studies appear to have been used to further characterise these model lipoproteins.

Small-angle X-ray scattering has found many applications in the study of

biomacromolecules, such as proteins, nucleic acids, viruses *etc.*, in solution (for reviews see KRATKY[18], BEEMAN *et al.*[19]) and more recently in the study of interactions between molecules in solution[20]. As part of an integrated physical study of model lipoproteins, we have used this technique to obtain an idea of the size and shape of the isooctane-soluble phospholipid–cytochrome *c* complexes.

MATERIALS AND METHODS

Materials

Horse heart cytochrome *c* (Sigma type VI) was used without further purification. Egg yolk lecithin was prepared essentially according to the method of SINGLETON *et al.*[21], with minor modifications. Final purification was achieved by chromatography on alumina (Woelm, neutral) using benzene–methanol (9:1, v/v) as eluting solvent.

Ox-brain phosphatidylserine was obtained commercially (Koch Light, Colnbrook, Bucks.). The major impurity appeared to be phosphatidylethanolamine. The fatty acid distribution of one sample was determined by gas–liquid chromatographic analysis and was similar to that reported by PAPAHADJOPOULOS AND MILLER[22]. Thus the ratio of stearic to oleic acid was 1.33 compared with 1.32 found by these authors. The presence of significant amounts of the polyunsaturated acids (*e.g.*, 20:4) indicates that no appreciable oxidation had occurred.

Isooctane (2,2,4-trimethylpentane) (Hopkins and Williams) was of spectroscopic quality and used without further purification. Ethanol (Burroughs, absolute) and other materials used were of reagent quality.

Complex formation and analysis

Cytochrome *c*–phospholipid complexes were formed in water and extracted into isooctane essentially as described by DAS AND CRANE[13]. A typical procedure was as follows: 2 ml of 1 % egg yolk lecithin, and 1 ml of 1 % phosphatidylserine dispersed in distilled, deionized water or dilute phosphate buffer (0.01 M, pH 7.0) were diluted to 15 ml. 10 ml of 0.2 % cytochrome *c* were added, and the mixture vigorously shaken in a 100-ml volumetric flask for a few minutes. 10 ml of absolute ethanol and 10 ml of isooctane were added, and the mixture vigorously shaken again. The isooctane layer, now containing most of the complex, was separated from the aqueous layer by centrifugation in a clinical centrifuge at 5000 rev./min, care being taken not to include any insoluble interfacial material which sometimes formed. Following DAS AND CRANE[13], the cytochrome *c* concentration was determined by measuring the absorbance at an isosbestic point for the oxidised and reduced forms (410 mμ) of a 0.2-ml aliquot, diluted as necessary, of the complex in isooctane. 1 ml of the isooctane solution was assayed for lipid phosphorus, using the method of CHEN, TORIBARA AND WARNER[23] and a conversion factor of 25 to convert grams lipid phosphorus to moles of phospholipid. In this study, only the total lipid phosphorus was determined, and the stoichiometry of the complexes is expressed in terms of molecules of phospholipid per molecule of cytochrome *c*.

Solutions of the complexes were concentrated by evaporation of the isooctane with a stream of nitrogen.

Ultracentrifugal studies of the phospholipid–cytochrome *c* complexes in isooctane were performed by DR. L. IRONS of this laboratory. Protein concentrations of the

range 0.1–0.5 % were used. Experiments were carried out using a Spinco Model E ultracentrifuge equipped with phase-plate Schlieren optics and RTIC unit. Sedimentation-velocity experiments were carried out at room temperature (18–23°) in 12-mm or 30-mm single-sector aluminium centrepiece cells at rotor speeds of 29 500 rev./min or 25 980 rev./min.

Sedimentation coefficients were calculated from the rate of movement of the maximum ordinate of the Schlieren curve and were corrected to values in water at 20° by the usual methods[24]. The viscosity of the isooctane was determined in a capillary viscometer of the Ostwald type. The viscosity at 20° was 0.571 cP.

Small-angle X-ray scattering technique

The small-angle X-ray scattering measurements were performed with a Kratky camera adapted to a highly stabilized Philips PW 1010 generator. Further stability was obtained by air conditioning the laboratory and by controlling the temperature of the anode-cooling water. A standard copper target, focal area 10 mm × 1 mm, was operated at 40 kV and 20 mA.

The unique collimation system, described in detail by KRATKY and co-workers[25–27] was modified in order to produce an 'infinitely' long primary beam. A diaphragm was placed in the tube shield between the tube window and the entrance slit, giving the crossing-beam geometry[28] necessary to produce an 'infinitely' long beam (4 cm) at the recording plane. Entrance slits 8–400 μ wide were available, but most of the measurements described here were obtained using an entrance slit width of 40 μ and a corresponding counter tube slit width of 83 μ. The distance between the sample cell and the recording plane was 205 mm.

The scattered radiation was detected by a xenon-filled proportional counter and a transistorised Philips PW 1310 counting chain. Monochromatisation of the radiation was achieved by use of a nickel β filter in conjuction with the proportional counter and pulse-height discrimination. The position of the counter, determining the scattering angle 2θ, was controlled by a programmed step-scanner, and counting times of at least 10 min per point were made. The scattered intensity was determined on an absolute scale (*i.e.*, the ratio of the scattered intensity to that of the incident beam) by means of a calibrated polyethylene (Lupolen 1811M) sample[29,30]. In order to correct the scattering curves for the effect of the line-focus collimation system, a 'desmearing' correction program[31,32] for both finite and infinite beams based on the Guinier and Fournet method is now operational on an IBM 360/40 computer.

Samples were mounted in thin-walled (1 mm diameter) capillary tubes, and the scattering curves derived by subtraction of the solvent from the solution scattering curves.

RESULTS

Table I lists the cytochrome *c* concentration and the complex stoichiometry (molecules phospholipid per molecule cytochrome *c*) for a number of sample preparations. The analysis indicated that the average stoichiometry of the complexes was 20:1 or 30:1, an essentially similar result to that found by other workers[13,15]. Using mixed beef heart phospholipids as the interacting phospholipid, they observed phospholipid to protein ratios of 22:1 and 32:1.

Fig. 1 shows a sedimentation-velocity ultracentrifugation plate of a 30:1 complex in isooctane. An estimation of the particle weight in isooctane by the Archibald method gave a value of about $1.3 \cdot 10^6$ for a complex in which the phospholipid: protein ratio was 29:1. A more detailed description of the ultracentrifugation studies will appear in a separate paper.

In order to derive some molecular parameters for cytochrome *c* itself, small-angle scattering curves for the protein, concentration 0.5 % to 5.0%, in both water

TABLE I

PHOSPHOLIPID: PROTEIN RATIOS OF ISOOCTANE-SOLUBLE COMPLEXES

Phospholipid–cytochrome *c* complexes were formed in water and extracted into isooctane, as described in the text. Cytochrome *c* was determined spectrophotometrically at 410 mμ and lipid phosphorus by the method of CHEN, TORIBARA AND WARNER[23].

Sample No.	*Cytochrome c concn. (mg/ml)*	*Complex stoichiometry (molecules phospholipid per molecule protein)*
A3-1	2.27	18
A3-2	9.08	18
A3-3	4.54	18
A3-4	2.0	18
A4-1	0.68	30
A4-2	0.405	30
A4-3	1.64	30
A7-1	0.91	33
A7-2	7.1	33
A9-1 (1:1 Lec:Ser)	1.08	20.5
A10	0.67	22
A11	0.82	19.5
A12-1	0.68	26
A12-2	1.43	26
A13-1	3.16	29.5
A13-2	1.06	29.5
A13-3	10.0	29.5
A13-4	6.8	29.5

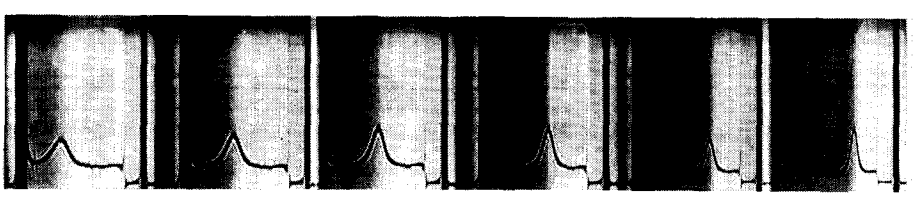

Fig. 1. Analytical ultracentrifugal pattern of a phospholipid–cytochrome *c* complex (30:1) in isooctane, run at 29500 rev./min in a Model E Spinco ultracentrifuge at approx. 20°. Photographs taken at 4-min intervals.

and 0.15 M NaCl were determined. (Since cytochrome *c* is insoluble in isooctane, no information was obtainable for the protein alone in this solvent.) Fig. 2 shows a scattering curve at three concentrations before collimation correction ($\tilde{I}(h)$ *versus* h), and Fig. 3 shows the corresponding Guinier plots. For a 5 % solution of the protein in

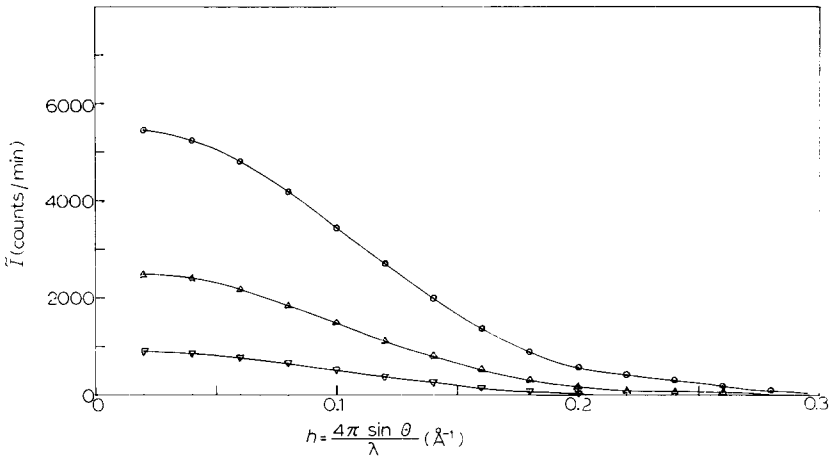

Fig. 2. Small-angle scattering curves of cytochrome *c* in 0.15 M NaCl. Cytochrome *c* concn. 1 % (▽), 3 % (△) and 5 % (⊙). $\tilde{I}$: intensity with collimation error.

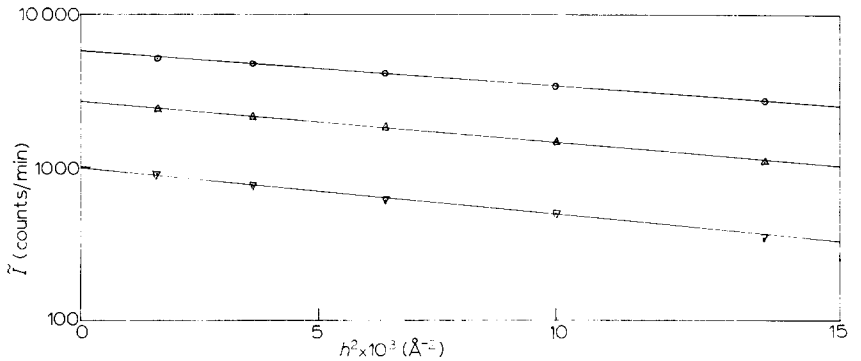

Fig. 3. Guinier plots (log $\tilde{I}$ *versus* h^2) of scattering curves of cytochrome *c* in 0.15 M NaCl. Cytochrome *c* concn. 1 % (▽), 3 % (△) and 5 % (⊙).

0.15 M NaCl, the radius of gyration, $\tilde{R}_g$, derived from the linear region of the Guinier plot using Eqn. 1

$$\tilde{R}_g = \sqrt{\frac{3}{\log e}\left|\frac{d \log \tilde{I}}{dh^2}\right|_{h \to 0}} \qquad (1)$$

was 12.5 Å. The radius of gyration of the protein had a small concentration dependence; the radius of gyration corresponding to zero concentration, R_{g_0}, was 14.5 Å.

Scattering curves of the complexes were determined for the concentration range 0.04–1.00 % cytochrome *c* in isooctane. Fig. 4a shows a typical scattering curve. The effect of desmearing is shown in Fig. 4b. The Guinier plots (log $\tilde{I}$ *versus* h^2) for 'smeared' scattering curves are shown in Fig. 5. Two groups of lines with different slopes occur.

When the radius of gyration is plotted as a function of concentration (Fig. 6), two separate linear plots are obtained. Extrapolation to zero concentration yields radii of gyration 66 Å and 87 Å, respectively. Desmearing the scattering curves has little effect on the radius of gyration, *e.g.*, $R_{g(\text{smeared})} = 64.0$ Å, $R_{g(\text{desmeared})} = 62.5$ Å.

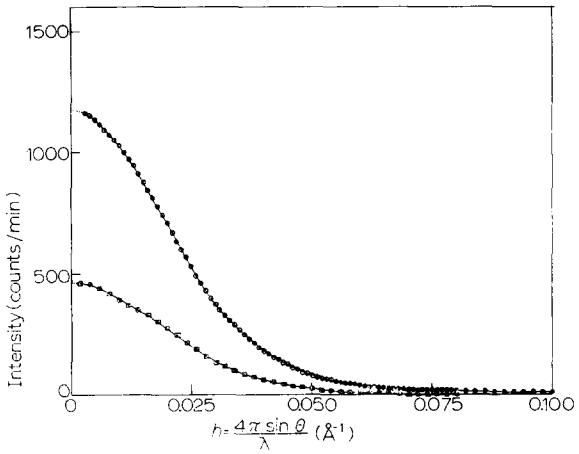

Fig. 4. Small-angle scattering curve of a phospholipid–cytochrome *c* complex (30:1) in isooctane (concentration equivalent to 0.12% cytochrome *c*). ⊙, "smeared" scattering curve $I(h)$; ⊡, "desmeared" scattering curve $I(h)$.

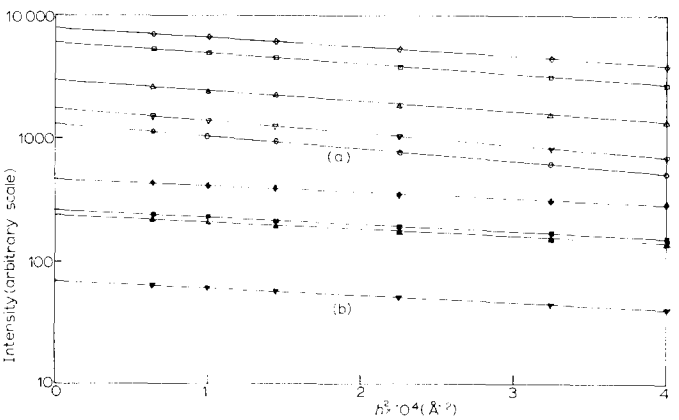

Fig. 5. Guinier plots (log $\tilde{I}$ *versus* h^2) of phospholipid–cytochrome *c* complexes in isooctane: (a) 20:1 complexess (b) 30:1 complexes. Symbols correspond to different sample concentrations.

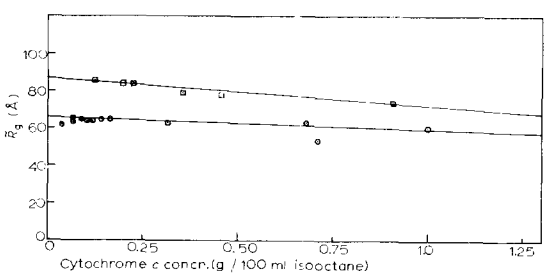

Fig. 6. Plot of radius of gyration of the complexes ($\tilde{R}_g$) as a function of cytochrome *c* concentration. ⊡, 20:1 complexes; ⊙, 30:1 complexes.

To determine the shape of the scattering particles comparisons were made between the desmeared experimental scattering curves corresponding to the two different radii of gyration and the theoretical curves for different particle shapes. The best agreement for the 30:1 complex corresponds to a prolate ellipsoid of axial ratio $v = 2.0$. No satisfactory shape has been deduced for the 20:1 complex.

DISCUSSION

The small-angle X-ray scattering data for cytochrome *c* obtained during this study have relevance to the size of the complexes and are of considerable intrinsic interest. The radius of gyration obtained for cytochrome *c* in aqueous buffer systems was approx. 12–14 Å. On the basis of the X-ray single-crystal structure analysis of horse heart ferricytochrome *c*, DICKERSON *et al.*[34,35] suggest that the molecule is roughly spherical with a diameter of 31 Å. The radius of gyration of a sphere of radius 15.5 Å, calculated according to the equation $R^2 = 3/5\ r^2$, is 12.0 Å, in excellent agreement with our value of 12–14 Å, determined from the scattering curves for the molecule in solution. The agreement between these figures suggests that there is unlikely to be any major conformational change when the cytochrome *c* molecule is dissolved in water.

The analytical data (Table I) show that two distinct complexes with total phospholipid:protein ratios of 30:1 and 20:1 are formed. These complexes are fairly homogeneous within a given sample as shown by the analytical ultracentrifuge data, *e.g.*, Fig. 1. The linearity of the Guinier plots over a large range of the scattering curves (see Fig. 5) is consistent both with this homogeneity and also with a reasonably uniform electron-density distribution within the scattering particle.

The small-angle scattering data in Fig. 6 also show the existence of two distinct complexes; the gradual decrease in radius of gyration for the two complexes with increasing concentration can be ascribed to inter-particle interference. There appears to be a correlation between the complex stoichiometry and the radius of gyration, suggesting $\tilde{R}_g = 87$ Å for the 20:1 complex and $\tilde{R}_g = 66$ Å for the 30:1 complex. These two radii of gyration are much greater than the value for cytochrome *c* itself and demonstrate directly that the phospholipid–protein complexes in isooctane must contain many protein and associated lipid molecules. This is consistent with the behaviour of the complexes in the analytical ultracentrifuge.

To be consistent with the large radii of gyration and the ultracentrifuge behaviour, aggregated structures must be envisaged. Since 'monomeric units' of one protein molecule to 20 or 30 lipids would have molecular weights of about 27 000 or 35 000, respectively, these aggregates must contain 30 or 40 proteins with their associated lipids.

From a comparison of a typical scattering curve with theoretical scattering curves the particle shape of the 30:1 complex may be prolate ellipsoidal of axial ratio $v = 2.0$.

Using the relationship

$$R_g = \sqrt{\frac{2 + v^2}{5}} \cdot a \qquad (2)$$

an ellipsoid of semi-axes 58 Å, 58 Å and 116 Å and a volume 17 000 000 Å^3 is obtained. This is an adequate volume to accommodate the 30 or 40 protein molecules with their

associated lipid. Spherical particles with diameters deduced from the radii of gyration can similarly accommodate this number of molecules. Nothing can be said at this stage to account for the observation that the 20:1 complex has a greater radius of gyration than the 30:1 complex.

Highly purified lecithin does not form a complex with cytochrome *c* (ref. 14), and although phosphatidylserine alone reacts electrostatically with cytochrome *c* (*cf.* cardiolipin or phosphatidylinositol–cytochrome *c* complexes[13]), the complex is not extractable into isooctane. Coupled with the observation that optimal complex formation and extractability into isooctane require a certain ratio of neutral to acidic lipid, this suggests that both electrostatic and nonpolar interactions are involved in stabilizing the complex.

The total number of phospholipid molecules bound per cytochrome *c* molecule correlated in one case with the number of positively charged groups (20) and in the other with the sum of the positively and negatively charged groups (32) of the protein, leading DAS, HAAK AND CRANE[14] to imply a predominantly electrostatic bonding. They have established that the acidic lipid binds to the external basic sites, and suggest lecithin is bound such that the complexes become soluble in isooctane. A recent report[17] from this laboratory using the ESR spin-label technique supported the electrostatic nature of the binding forces. In this work it was considered that these complexes were composed of a single protein molecule surrounded by the appropriate number of phospholipid molecules, but our present data suggest that this may not be the case.

The precise mode of interaction of the lecithin in these complexes is uncertain. It raises the question whether lecithin in the presence of acidic lipid can bind electrostatically to protein or whether nonpolar interaction occurs. Changes in the ratio of lecithin to acidic lipid could be of importance in determining the spatial distribution of charges presented to the cytochrome *c* in the initial interaction.

Since the charged and hydrophobic residues occur in separate and discrete clusters in the cytochrome *c* molecule, MARGOLIASH AND SCHEJTER[36] also raise the possibility of both electrostatic and hydrophobic bonding in these complexes. Because of the possibility of both types of forces being involved, together with the possibility of protein–protein interactions in isooctane, detailed descriptions of the structures of the complexes are not possible. However, a close-packed core containing all the protein molecules surrounded by a lipid shell seems an unlikely structure unless the sites of interaction are very asymmetrically arranged over the protein surface.

Another possibility is a structure comprised of an inner lipid core, a protein shell, and an outer lipid envelope. In this structure, all the lipid polar groups would be able to interact with charged sites anywhere on the protein surface. In both of these structures, the hydrophobic shell required for solubility in the isooctane would be present. If, in fact, regions of different electron density do occur within the scattering particle, for example between a lipid core and a protein shell, an X-ray scattering study involving variation of the electron density of the solvent should prove helpful.

A major determinant of the final structures of the isooctane-soluble complexes may be the structure of the original water-insoluble complex. The latter structures may in their turn be determined by the phase behaviour of the lipids in water. Experiments to investigate this possibility and to define the complex structures in greater detail are in progress.

Biological relevance

WIDMER AND CRANE[37] extracted an enzymatically active isooctane-soluble form of cytochrome *c* from the electron transport particle of beef heart mitochondria. In mitochondria the lipid composition is approx. cardiolipin 20 %, ethanolamine phospholipid 30 %, and choline phospholipid 40 % (ref. 38), so that a suitable balance of neutral to acidic lipids for interactions similar to that found for the model complexes, may be present.

Conclusions

(a) The radius of gyration of cytochrome *c* in water is 12–14 Å.

(b) Two phospholipid–protein complexes formed from mixtures of phosphatidylserine and phosphatidylcholine with cytochrome *c* and having lipid:protein ratios 20:1 and 30:1 are observed.

(c) The radii of gyration of the two complexes are 87 Å and 66 Å, respectively.

(d) The data show that aggregated lipid–protein systems, instead of simple 'monomeric units', occur in isooctane.

(e) Further work on this and related systems should lead to a better understanding of these model complexes and help to assess their relevance to natural lipid–protein systems.

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