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Fluorescence Studies of Possible Protein–Protein Interactions in Model Lipoprotein Complexes

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Summary

The structure of model lipoprotein complexes, extracted from an aqueous phase into isooctane, has been investigated using a fluorescence technique. The technique is based on the transfer of excitation energy from one protein (or DNS-labelled protein) to a second protein containing a fluorescence quencher, such as a haem group. The results obtained with model complexes in isooctane are consistent with a structure comprised of an inner protein core, and an outer layer of phospholipids.

Introduction

Several examples of model lipoprotein complexes which can be extracted or solubilized in organic solvents[1–3] have now been studied. The stoichiometries of the interactions have often been clearly established,[2–4] but little is known about their structures. The most extensively studied interaction is that between cytochrome *c* and phsopholipid mixtures containing an acidic phospholipid. Insoluble complexes having principally lamellar structures,[5–6] are formed in water, but providing the proportions of cytochrome *c*, acidic phospholipid and neutral phospholipid in the complexes fall within certain limits, they can be completely extracted as a lipoprotein complex into isooctane.[4] Such isooctane-soluble complexes are large, the size depending on their stoichiometry;[7,8] the lower the lipid to protein ratio, the larger the system. This suggests that both in water and isooctane, protein–protein interactions as well as lipid–lipid and lipid–protein interactions may play an important role. Similar protein–protein interactions may be structurally important in serum and membrane lipoproteins.

Fluorescence techniques, based on the use of suitable probe molecules, have in general been mainly applied to the study of protein and lipid:protein interactions in water.[6,9,10,11] Specifically the use of the non-covalently linked fluorophore 1-anilinonaphthalene-8-sulphonate has been employed recently to prove the structure of membranes,[10] and model lipoprotein complexes similar to those studied here.[6] We have examined the potential of the energy transfer technique for studying possible protein–protein interactions in model lipoproteins in isooctane solution. Lipoprotein complexes containing cytochrome *c*, should be particularly amenable to this approach as the cytochrome *c* is a fluorescence quencher (by virtue of the haem group). Furthermore it can also be replaced by lysozyme, covalently labelled with a fluorescent chromophore, without affecting the stoichiometry of the system.[4] Thus the intensity of the fluorescence

emission of a chromophore attached to lysozyme in a lipoprotein complex containing both lysozyme and cytochrome *c* would be expected to be critically dependent on the structure of the complex. We have measured the quenching of the fluorescence emission of both the chromophore and the intrinsic fluorescence of lysozyme as the proportion of cytochrome *c* in the complex with phospholipids in isooctane was varied and have interpreted the data in terms of possible structures.

Materials and Methods

Materials

All reagents were A.R. grade and were used without further purification, 2,2,4-trimethylpentane (isooctane) was of spectroscopic grade (Hopkin and Williams). Dimethylaminonaphthalene-5-sulphonyl chloride (DNS) was obtained from Nutritional Biochemicals Limited. Cytochrome *c* (horse heart type VI) and lysozyme were obtained from Sigma Chemical Company. Phosphatidylcholine (PC) was prepared from hens egg yolk and phosphatidylserine (PS) from ox brain as described previously.[4]

Preparation and Analysis of Isooctane Soluble Complexes

The procedure was essentially that of Das *et al.*[1] and has been described elsewhere.[4] The composition of the phospholipid mixture used to extract the complexes into isooctane was 1 part of PS to 3 parts of PC (w/w). The ratio of total phospholipid to protein was 2·2:1 for cytochrome *c* and 1·9:1 for lysozyme, conditions giving optimal extraction of complexes with lipid:protein ratios of approximately 36:1.[4] Complexes formed and extracted into isooctane under these conditions have been found to be homogeneous by ultracentrifugation[8] and X-ray scattering.[7] Complex preparation was routinely performed at 10°C to help reduce experimental variability, also noted by Lesslauer *et al.*[3]

The final concentration of cytochrome *c* in the extracted complexes was determined by absorption at 410 nm. The phospholipid composition was not routinely measured but was occasionally checked to verify that the stoichiometry was of the correct order.

Preparation of Lysozyme-DNS Conjugates

Lysozyme-DNS conjugates were prepared according to the method of Weber[12] and freed from excess dye to Sephadex gel chromatography. The degree of labelling was determined according to Weber[12] and was varied by adding unlabelled lysozyme.

Fluorescence Instrumentation

Fluorescence measurements were made with an Aminco-Bowman spectrophotofluorometer with slits in the excitation and emission beams giving a band pass of 12 nm. The protein fluorescence at 330 nm was excited at 285 nm and the DNS fluorescence at 520 nm was excited by 285 and 330 nm radiation, the 520 nm emission being monitored with an Ilford 624 filter in the emission beam. The temperature of the cell housing was controlled at 25°C by circulating water from a thermostatted water bath, and the samples in the cuvette were stirred magnetically. The sensitivity and flexibility of the equipment was greatly increased by replacing the standard photometer with a Vibron 63B electrometer (Electronic Instruments Limited, Richmond, Surrey).

Fluorescence Titrations

A 0·001% solution of lysozyme in water or of lysozyme-phospholipid complex in isooctane, was titrated with aliquots of the appropriate, concentrated cytochrome *c* solution or cytochrome *c*—phospholipid complex in isooctane from a microlitre syringe. The fluorescence intensity at the selected wavelength was measured after each addition. An alternative series of titration experiments was performed where instead of preparing two *separate* lipoprotein complexes of cytochrome *c* and lysozyme-DNS with phospholipids and adding one complex to the other, *integral* lipoprotein complexes containing both cytochrome *c* and lysozyme-DNS and phospholipids were prepared. Required amounts of phospholipid were added to 25 ml volumetric flasks containing 500 μg of lysozyme-DNS and from 25 to 1500 μg of cytochrome *c*. The aqueous volume was adjusted to 7 ml and, using the standard procedure, extracted with 2·5 ml of isooctane. The fluorescence intensities of 1:10 dilutions of these extracts were then measured at the appropriate wavelengths.

Results

The change in the fluorescence emission intensity of DNA bound to lysozyme (at a concentration of 0·001%) in water was measured as a function of added cytochrome *c*. There was very little change in intensity up to an addition of 100 μg cytochrome *c* showing that in this concentration range, collisional quenching arising from Brownian motion can be ignored.

An example of each type of fluorescence intensity titration curve for the lysozyme-phospholipid, cytochrome *c*-phospholipid complexes, and alternatively for the lysozyme phospholipid-cytochrome *c* lipoprotein complexes in isooctane is shown in Fig. 1. The fluorescence emission of DNS at 520 nm was excited at the absorption maximum of the dye at 345 nm. The upper curve in Fig. 1 is a titration of a lysozyme-DNS phospholipid complex by a *separate* cytochrome *c*: phospholipid complex and the lower curve is a titration of lysozyme-DNS by cytochrome *c* as a single, *integral* phospholipid complex. Similar titration curves were obtained by monitoring the fluorescence emission intensity of DNS at 520 nm excited at the absorption maximum of lysozyme at 285 nm. In this case the DNS is excited primarily by resonance transfer of energy from lysozyme, although in more quantitative studies allowance for the direct excitation will be necessary. As the extraction of cytochrome *c*, measured by the absorption at 410 nm, was found to be 100% in nearly all cases, the curves for the *integral* complexes have been plotted on the assumption of 100% extraction of the lipoprotein complexes.

The upper curve in Fig. 1 confirms that in isooctane, the quenching observed in the lower curve cannot arise simply from Brownian motion. Approximately 90% quenching of the DNS fluorescence was obtained by preparing integral complexes from equal weights of lysozyme-DNS and cytochrome *c* (Fig. 1).

Titration curves obtained by monitoring the intrinsic emission intensity of lysozyme at 330 nm excited at 285 nm showed the same general features as Fig. 1. However, only 60% quenching was obtained by preparing *integral* complexes from equal weights of lysozyme and cytochrome *c*. Even at high cytochrome *c* levels, approximately 35% of the original intensity was observed (cf. 2–3% of the original DNS emission intensity, Fig. 1).

The stoichiometry of the lysozyme-cytochrome *c* interaction was estimated from the lower curve of Fig. 1. Tangents to the initial and final slopes of the titration curve were

drawn, the value of the intercept projected onto the abscissa was taken as the weight of cytochrome *c* reacting with 50 μg lysozyme. This was converted into a molar ratio and a series of experiments yielded a value of 3·0 ± 0·5. The variation arises mainly from the difficulties of constructing the tangent to the initial slope. This tangent is critically dependent on the intensities of the samples containing very small amounts of cytochrome *c*, and is therefore susceptible to small variations in the degree of extraction of complexes. Any variations due to change in degree of labelling of the lysozyme (varied from 0·25 to 0·60 molecules DNS per molecule lysozyme) were masked by this variation.

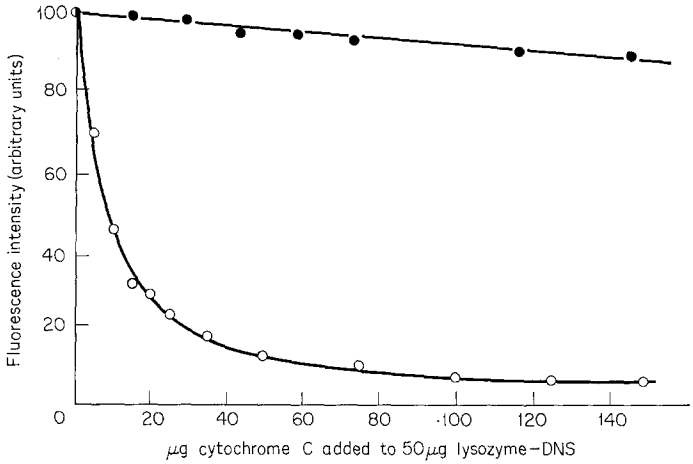

Figure 1. Fluorescence titration curves of 50 μg of lysozyme-DNS by cytochrome *c* as phospholipid complexes in isooctane. Excitation 345 nm, emission 520 nm. ●——●, separate complexes; o——o, integral complexes.

Discussion

Resonance transfer of electronic excitation energy from one part of a molecule to another, or from one chromophore on one protein molecule to another located on a different protein, has been shown by Förster[13] to depend inversely on the sixth power of their separation. Although it is theoretically possible to calculate the separation of the donor and acceptor groups by quantifying the energy transfer, in practice it has proved possible only for simple rigid systems.[14] Energy transfer experiments may, however, provide stoichiometric information on protein–protein interactions.

Since we have examined the complexes at low concentrations where collisional quenching by Brownian motion is negligible (upper curve Fig. 1), the almost 100% quenching of the DNS fluorescence and the 60% quenching of the lysozyme fluorescence by cytochrome *c* in the *integral* complex is strong evidence favouring direct protein–protein associations. The 60% quenching of the lysozyme fluorescence is very similar to that obtained by Edwards and Criddle for the binding of cytochrome *c* and myoglobin to "mitochondrial structural protein"[15,16] and by Wasemiller *et al.* for the binding of haemoglobin to erythrocyte structural protein.[17] In these cases the stoichiometries were 1:1 and 2:1 respectively. The difference in the degree of quenching of the lysozyme fluorescence as opposed to the DNS fluorescence must arise from the different location

of the DNS group and the tryptophan groups. Some of the tryptophan groups may be buried within the lysozyme molecule and are unable, therefore, to approach the haem group in cytochrome *c* sufficiently closely for energy transfer and fluorescence quenching to occur. DNS is bound to lysozyme via accessible NH_2 groups, located on the exterior of the protein, and can participate in energy transfer to the haem group.

The term "stoichiometry" in the case of the lipoprotein complexes in isooctane discussed here is probably best interpreted as a statistical measure of the number of nearest neighbours to each protein molecule. Ultracentrifugation[8] and low angle X-ray scattering studies[7] showed that these complexes in isooctane have a high molecular weight (approximately 1×10^6) and large size and that each complex contained some 20–40 protein molecules plus associated phospholipid.

The hydrocarbon chains of some or all of the phospholipids must be located at the surface of the complex to account for the solubility in isooctane. Assuming that the proteins largely retain their globular structure, there are two broad types of structure that can be envisaged. Firstly, an assembly of discrete lipoprotein subunits. Such a model implies, however, a minimum separation between protein molecules never less than twice the thickness of the lipid layer surrounding each protein molecule, i.e., *ca.* 55–60 Å, the dimensions of a phospholipid bilayer. It is extremely unlikely that a virtual 100% quenching of the DNS fluorescence could occur via energy transfer over such distances. A second type of structure, compatible with the fluorescence results, is one having a protein core surrounded by a phospholipid shell. An assembly of globular proteins constituting the core could conceivably have a symmetry and precise nearest neighbour frequency and a more precise estimate of this number by the fluorescence quenching technique would provide valuable information in elucidating the structure.

Cytochrome *c* and lysozyme do not normally associate in water alone. Addition of phospholipid can, by converting them into a lipoprotein complex, induce them to associate. Similarly a lipid induced association may well have an important function within other naturally occurring lipoprotein systems, e.g., serum lipoproteins and membranes. The cell membrane lipids may, for example, be able to induce protein–protein interactions not normally found in the cell sap or in free solution. It may be possible to apply the technique outlined above to study insoluble lipoportein systems by building up multilayers of lipid and protein on a suitable inert support. Non-haem proteins might also be studied if a suitable quenching group can be bound to one protein.

Conclusion

The results of the present work suggest that protein–protein interactions occur in isooctane-soluble lipoprotein complexes. The fluorescence technique used could be refined to give more precise data and has potential as a tool for studying protein–protein interactions in other systems. The results also suggest that phospholipids may induce protein–protein associations not found in free solution.

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