Biochimica et Biophysica Acta, 942 (1988) 341–352
Elsevier

BBA 74040

Lipid–protein interactions. The mitochondrial complex III-phosphatidylcholine-water system

J.M. Valpuesta, F.M. Goñi, A. Alonso, J.L.R. Arrondo and J.M. Macarulla

Department of Biochemistry, Faculty of Science, University of the Basque Country, Bilbao (Spain)

(Received 30 November 1987)
(Revised manuscript received 31 March 1988)

Key words: Lipid–protein interaction; Mitochondria; Phosphatidylcholine bilayer; Complex III; Electron microscopy; Differential scanning calorimetry; (Bovine heart)

Bovine heart mitochondrial complex III (ubiquinol–cytochrome-*c* reductase) has been reconstituted into phosphatidylcholine bilayers and the effect of varying lipid/protein ratios on the structure and function of the protein has been examined. Electron microscopy, differential scanning calorimetry and Arrhenius plots of enzyme activity provide evidence that the protein is incorporated in an active conformation into pure phosphatidylcholine bilayers. At low lipid/protein ratios (e.g. 80:1 molar ratio) the protein exists in the form of aggregates. As the lipid proportion is increased, electron microscopy reveals the gradual formation of lipid bilayers; structures with the appearance of closed vesicles are seen at or above 300:1 phospholipid/protein molar ratios. Changes in enzyme activity as a function of lipid contents reveal a progressive increase in activity as more lipid is added, with a tendency to reach a saturation point. From the experimental data, a kinetic model is proposed, according to which the protein has an indefinite number of unspecific, independent and identical binding sites for phospholipids, the latter acting as essential enzyme activators. Varying lipid/protein ratios induce structural changes in complex III; visible spectra indicate changes in the polarity of the heme group environment, while Fourier-transform infrared spectroscopy suggests a change in the secondary structure of the protein as the lipid proportion is increased.

Introduction

A large amount of studies have been carried out on lipid–protein interactions, especially on the alteration of fluidity and order of the lipid bilayer produced by the presence of protein [1–6]. The influence of the lipids on the protein has not been studied in such detail, and only indirect data are available. No significant structural changes have been detected after qualitative or quantitative modification of membrane lipid components; however, some functional data are known. In sarcoplasmic reticulum ATPase reconstituted with pure lipids, breaks in the Arrhenius plots of enzyme activity have been related to the melting of the lipids forming the proteoliposomes [7,8]. Discontinuities have also been detected in Arrhenius plots in systems containing natural lipids [9]. Another point under study is the influence of the

Abbreviations: DMPC, dymiristoylphosphatidylcholine, DPPC, dipalmitoylphosphatidylcholine; DSC, differential scanning calorimetry; FT-IR, Fourier transform infrared; ΔH, enthalpy change of the phase transition; Mops, 4-morpholinepropanesulphonic acid; PC, phosphatidylcholine; T_c, onset transition temperature; T_m, mid-point transition temperature.

Correspondence: F.M. Goñi, Department of Biochemistry, Faculty of Science, University of the Basque Country, P.O. Box 644, 48080 Bilbao, Spain.

lipid/protein ratio on the protein structure and function. The enzyme activities of various membrane-bound proteins have been assayed as a function of the lipid/protein ratio [7,10] and it has been shown that the activity decreases slowly or even remains constant as the proportion of lipid is decreased, until, at a certain lipid/protein ratio, the activity is suddenly abolished. That lipid/protein ratio seems to correspond to the minimum number of lipid molecules surrounding the protein [2].

Many membrane-bound proteins have been used in the study of lipid–protein interactions. However, data on mitochondrial complex III or ubiquinol–cytochrome-*c* reductase (EC 1.10.2.2) are scarce. Nelson and Fleischer [11] showed that this protein, when incorporated into phosphatidylcholine bilayers, could not support good respiratory control ratios, and deduced that pure phosphatidylcholine bilayers were unable to accommodate the protein. In our previous studies we have characterized [12] the size and shape of complex III-phospholipid-detergent mixed micelles, the final product of protein purification. The effects of detergent removal on the protein structure and function have also been examined [13]. This paper deals with the reconstitution of the complex III in phosphatidylcholine bilayers and the effects of lipids on the protein, according to functional and structural studies.

Materials and Methods

Complex III was isolated from bovine heart mitochondria according to Engel et al. [14] using the nonionic detergent Tritin X-100 as the solubilizing agent. The enzyme is purified in the form of protein-detergent-phospholipid mixed micelles. A molecular weight of 270 000 was assumed for the complex III monomer [12]. Protein reconstitution, with egg PC purified according to Singleton et al. [15], was carried out as follows: 0.25 ml of the enzyme solution (6 mg protein/ml) were mixed with 1 ml of sonicated liposomes (100 mg/ml) and 60 μl sodium cholate (Sigma) (100 mg/ml). The solution was stirred for 30 min at 4° C and then precipitated with 35% ammonium sulfate. The suspension was then centrifuged at 105 000 $\times$ *g* for 30 min in a Beckman 50Ti rotor at 4° C. The pellet was resuspended in 20 mM Mops (pH 7.2), and introduced in a 20–70% sucrose gradient to remove the detergents, cholate or Triton X-100. The sample was centrifuged at 270 000 $\times$ *g* for 4 h at 4° C in a Beckman SW 50.1 rotor. The protein-lipid aggregate was isolated as a single band and washed twice in 20 mM Mops (pH 7.2), centrifuging at 105 000 $\times$ *g* at 4° C. The resulting pellet was virtually free of detergent (less than 1 : 1 detergent/protein molar ratio) according to isotopic dilution assays using [^{14}C]cholate (Amersham) and ^{3}H-labeled Triton X-100 (New England Nuclear). The lipid/protein molar ratio of preparations obtained as above was approximately 98 : 1. When higher lipid/protein molar ratios were required, the amount of lipid was increased and that of cholate decreased; the opposite changes lead to lower lipid/protein molar ratios. Lipid reconstitution with DMPC and DPPC (Fluka A.G.) was carried out according to Knowles et al. [10]. The purity of the reconstitution, checked by gas-liquid chromatography, was of 99%. Samples were transmethylated, as described by Morrison and Smith [16] and introduced in a Carlo Erba Fractovap 2350 gas-chromatograph equipped with a Xpectrix DEGS column.

For negative-staining electron microscopy, the samples were transferred on top of collodion-coated grids; after 3 min, the excess liquid was removed and a drop of 2% uranyl acetate was added. After another 3 min, the excess stain was also removed and the grids were dried at room temperature and examined under a JEOL JEM 100 C electron microscope at 80 kV.

For freeze-fracture electron microscopy, samples were sedimented, imbibed in 33% glycerol and then equilibrated at the required temperature prior to transferrings droplets on to gold-nickel specimen holder plates resting on a metal block at the desired quenching temperature. After 5 min equilibration, the samples were rapidly plunged into partially solidified liquid N_2 and kept under liquid N_2 until fracturing in a Balzers BAF 301 device. Fracturing was carried out at a pressure of approx. $2 \cdot 10^{-6}$ Torr and at -100° C with etching for 30 s. The exposed faces were shadowed with platinum-carbon from a 45° angle, then coated with carbon perpendicular to the specimen stage. Replicas were floated off on water, kept on

sodium hypochlorite solution for 1–2 h, washed in water, picked up on untreated 400-mesh copper grids and examined in a JEOL JEM 100 °C electron microscope at 80 kV.

Calorimetric data were obtained using a Perkin-Elmer DSC2 differential scanning calorimeter. A scanning rate of 1.25 C°/min and a sensitivity of 1 mcal/s were used. Samples were hermetically sealed in Perkin-Elmer aluminium 'volatile' sample pans. The instrument was calibrated with cyclohexane and indium standards. The areas of the peaks were determined by weighing paper cut-outs of the peaks. At least three heating and cooling cycles were performed for each sample, and results from the first run were neglected. The phospholipid contents of the pans were determined after the measurements as inorganic phosphate after perchloric acid digestion, according to Bartlett [17]. The combined error of ΔH estimations by this method, calculated from repeated measurements of pure DMPC and DPPC, was estimated at about $\pm 5\%$.

Enzyme activities were assayed according to Barbero et al. [18]. To 1 ml reaction medium (40 mM NaN_3, 0.1% bovine serum albumin, 1 mM EDTA, 50 mM phosphate bufer (pH 7.4)) 10 μl of the lipoprotein suspension was added. The enzyme was incubated in the reaction medium at 37 °C for 1 min, a cytochrome *c* solution (1 mM in 10 mM phosphate buffer (pH 7.4)) was added to a 70 μM final concentration, and, after stirring, the reaction was recorded in a dual-beam spectrophotometer against a blank containing every reagent except the enzyme. The blank accounts for nonenzymatic reduction of cytochrome *c* by duroquinol. Under these conditions, enzyme activity was inhibited to less than 5% the original values, in the presence of antimycin A (Sigma) (2 μg/ml). Differential visible spectroscopy (reduced minus oxidized) was carried out adding 50 μg protein to each cuvette. Sodium dithionite was used as a reducing agent. Enzyme assays and visible spectra recordings were carried out in a double-beam UV 5260 Beckman spectrophotometer.

Samples for Fourier transform infrared spectroscopy (20 mg protein/ml) in H_2O buffer were introduced in a Beckman FH-01CFT thermostatted cell equipped with CaF_2 windows; the pathlength used was 7 μm; typically, 1024 spectra were collected, averaged, apodized with a Happ-Genzel function and Fourier-transformed with a resolution better than 2 cm^{-1}. Buffer contribution was subtracted from spectra of proteins in solution by increasing the factor of a pure buffer spectrum until a straight line was no longer maintained between 1900 and 1300 cm^{-1}. The same subtraction factor was obtained when the water band centered around 2150 cm^{-1} was abolished. A Nicolet 10 MX Fourier transform IR spectrometer was used in the experiments.

Protein was assayed according to Lowry et al. [19] with some modifications; lipid phosphorus was determined according to Bartlett [17].

Results

Electron microscopy

Complex III-PC mixtures in aqueous dispersions were examined by electron microscopy, both with negative-staining and after freeze-fracturing. Various different situations are seen (Fig. 1). At low lipid/protein ratio (e.g. the 80 : 1 mixture) no vesicles are seen; actually, there is no evidence for a bilayer to exist. Rather, phospholipids and proteins seem to occur in the form of aggregates [13]. However, when the phospholipid/protein ratio increases from 80 : 1 to about 300 : 1 (Figs. 1A–1C), the progressive formation of a bilayer can be seen, until the negative stain reveals the usual structure of multilamellar proteoliposomes obtained by detergent-reconstitution procedures (Fig. 1D, 1000 : 1, lipid/protein ratio). Freeze-fracture replicas also show, in the case of the 80 : 1 mixture, a protein aggregation due to the lack of lipid [13]. In the case of the 1000 : 1 mixture (Fig. 1E), quasi-spherical fracture surfaces are seen, containing particles about 20 min in diameter, embedded in the lipid bilayer; these particles, also observed in the 80 : 1 mixtures are referable to complex III. The electron microscopic observations suggest that, when lipid/protein ratios are high enough to support the formation of bilayers and vesicles, complex III is incorporated into the lipid matrix.

Differential scanning calorimetry

As a further test for the integration of complex III into pure PC bilayers, some reconstitution

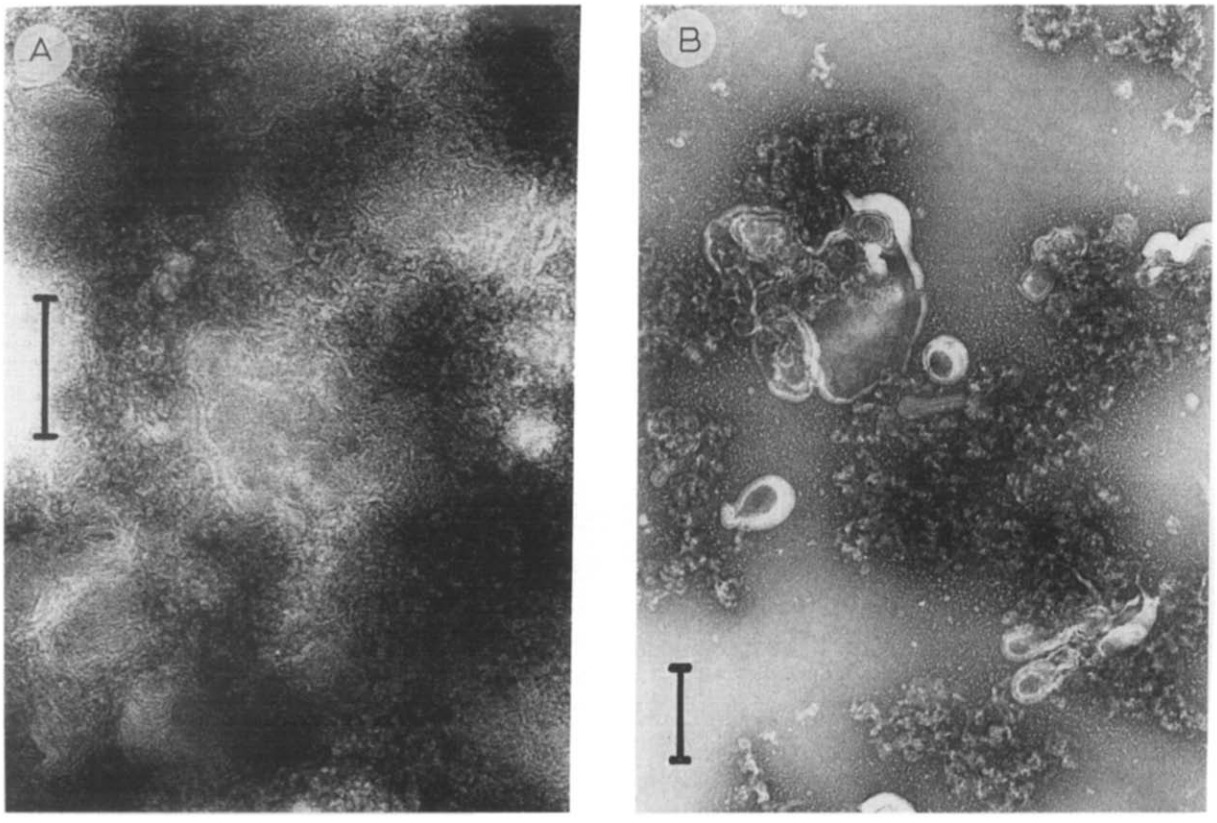

Fig. 1. Electron micrographs of complex III-phosphatidylcholine mixtures. (A) negative staining 150 : 1 lipid/protein molar ratio, (B) 225 : 1 lipid/protein molar ratio, (C) negative staining, 305 : 1 lipid/protein molar ratio, (D) negative staining, 1000 : 1 lipid/protein molar ratio, (E) freeze-fracture, 1000 : 1 lipid/protein molar ratio. Bar = 0.2 μm.

experiments were performed using DMPC or DPPC, and DSC measurements were carried out on the pure lipids and lipid-protein mixtures in excess water. Results are shown in Fig. 2. In the presence of the protein, DSC endotherms become wider, suggesting a decrease in transition cooperativity, and the pretransition is lost. Furthermore, there are no significant changes in the mid-point temperature of the main transition (T_m) as shown previously for integral proteins [3,21]. However, the enthalpy change of the phase transition (ΔH) is increased in the presence of low concentrations of complex III, e.g. the presence of protein at a 2000 : 1 phospholipid/complex III mole ratio increases ΔH by 12% (average of two measurements), with respect to the pure DPPC (ΔH of the pure lipid is 8.3 and 6.7 kcal/mol DPPC and DMPC, respectively). This is in contrast to the behaviour of other integral membrane proteins [20,21]. The fact that, although complex III is a membrane-bound protein, 70% of its mass is located outside the membrane can explain this behaviour, complex III acting both as an intrinsic and as an extrinsic protein [22] from the point of view of DSC. Increased protein concentrations give rise to mixtures that behave like most other intrinsic proteins [3], i.e. the transition enthalpy is considerably decreased. This is the case of the 400 : 1 sample in Fig. 2 ($\Delta H = 0.8$ kcal/mol DMPC); a complex endotherm is observed with this sample, perhaps because of the presence of 'bulk' and 'trapped' lipid [8] in the bilayers in the gel state. Samples with lipid : protein ratios lower than 400 : 1 did not produce measurable endotherms under our conditions.

Enzyme activity measurements

Enzyme activity was measured at 37 °C, in the absence of uncouplers, for a variety of lipid-protein mixtures, as indicated under Methods. The

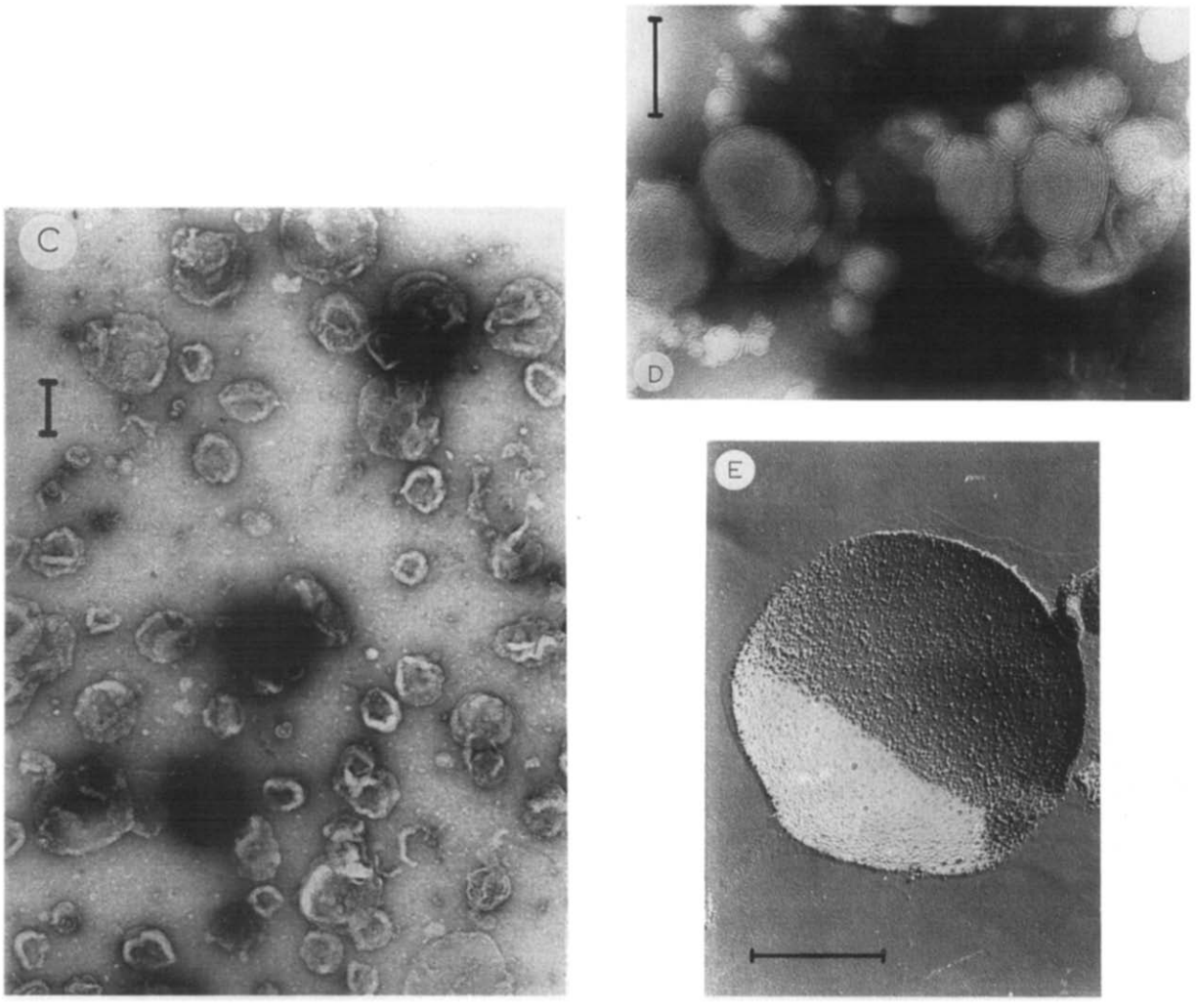

Fig. 1 (continued).

activities of mixtures containing between 32 and 560 phospholipids per protein molecule are shown in Fig. 3A. The specific activity increase hyperbolically with increasing lipid/protein ratios, so that ratios above 400 : 1 scarcely lead to an increase in specific enzyme activity. Actually a decrease is observed (data not shown) at very high lipid/protein ratios (above 1000 : 1), probably due to difficulties in the access of substrates across the bilayers [23].

When double-reciprocal Lineweaver-Burk plots are constructed for mixtures containing various phospholipid/protein ratios, the various straight lines intercept each other at a single point (Fig. 3B). This kinetic behaviour fits the proposed model (see Appendix), in which lipid molecules seem to act as essential enzyme activators [24] of the enzyme. From the primary and secondary plots of the experimental data, the activation parameters can be obtained. From our data we obtain V_{max} = 4.2 U/mg protein; K_S (cytochrome c) = $1.7 \cdot 10^{-5}$ M and αK_L (egg yolk PC) = $1.4 \cdot 10^{-6}$ M, α (with a value of 0.03) being the constant that relates the affinity towards the substrate between the inactive conformation (without lipid) and the active conformation. This means that the active conformation has about 30-times more affinity towards the substrate than the inactive conformation. The

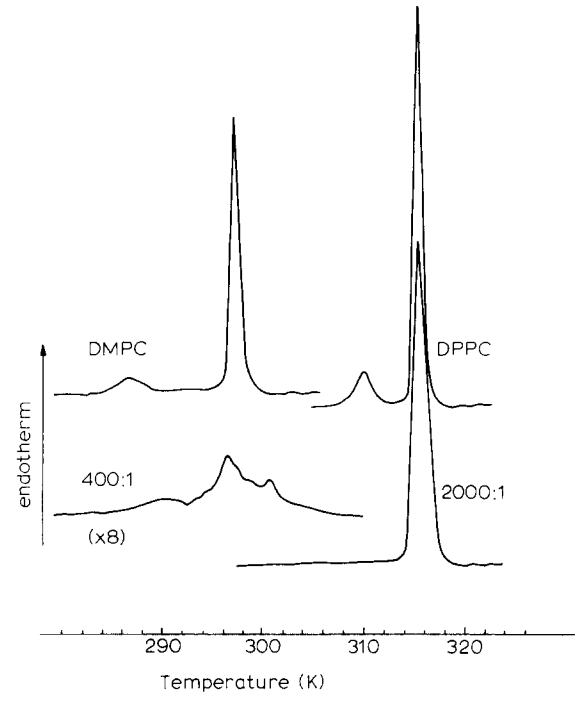

Fig. 2. DSC thermograms (heating curves) of pure phospholipids (upper curves) and phospholipid-complex III mixtures (lower curves). The figures on each curve correspond to phospholipid : protein molar ratios. All samples contained about 1 mg phospholipid; the signal corresponding to the 400 : 1 DMPC mixture has been amplified 8-fold.

model also assumes independent binding sites. A hyperbolic curve may be constructed from these kinetic values, that fits very well the experimental data (Fig. 3A, continuous line); according to it, 90% activation would be achieved at a 1870 : 1 lipid/protein ratio.

The effect of temperature on enzyme activity may be conveniently expressed in the form of Arrhenius plots. Our results with complex III reconstituted with diffferent phosphatidylcholines, at various phospholipid/protein molar ratios, are shown in Fig. 4. Enzyme activity does not vary linearly with temperature, rather discontinuities or 'breaks' are seen in the Arrhenius plots. In general, the thermal behaviour is very similar irrespective of lipid/protein ratio, but the temperatures at which breaks occur differ with the nature of the phospholipid acyl chains. In the case of egg-yolk PC, a discontinuity is always seen near 28 °C; with DMPC, it occurs at about 25 °C, while with DPPC two distinct breaks are seen, respectively, at 19 and 38 °C.

Protein structural studies

Changes in protein structure due to variation in the lipid/protein ratio were explored by visible and infrared spectroscopy. Differential (reduced minus oxidized) absorption spectroscopy of the

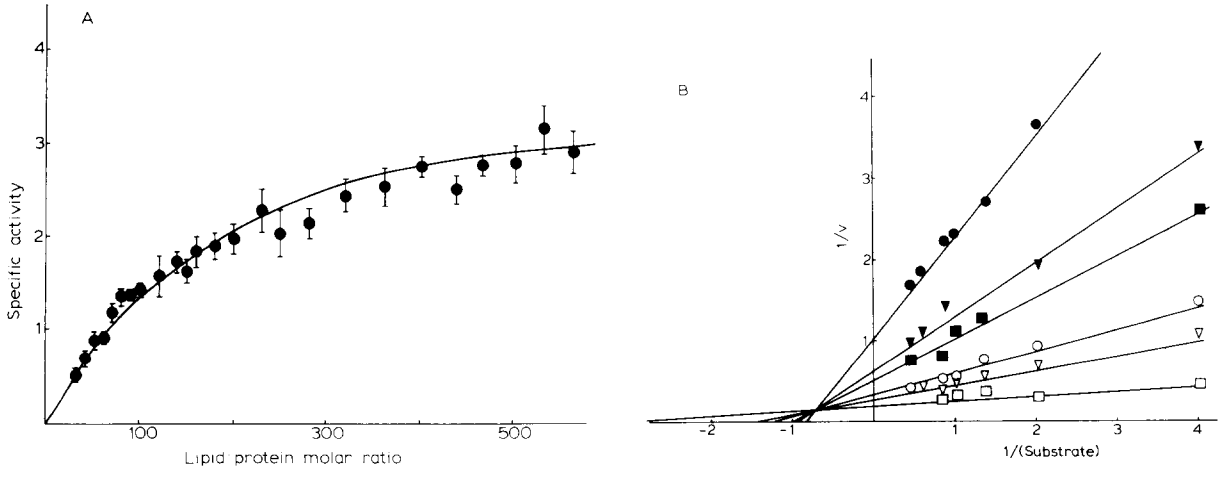

Fig. 3. (A) The effect of varying the egg PC/complex III molar ratio on the enzymic activity of the protein. Specific activity is given as μmol cytochrome *c* reduced/min per mg protein (initial rates). Assay as indicated under Methods. Average of 2–5 experiments. (B) Lineweaver-Burk plots of various samples with different egg PC/complex III molar ratios. The concentration of cytochrome *c* (substrate) is varied. 1/(substrate) is expressed as $mmol^{-1} \cdot l$. Lipid/protein ratios are 40 : 1 (●); 70 : 1 (▼); 90 : 1 (■); 180 : 1 (○); 230 : 1 (▽) and 530 : 1 (□).

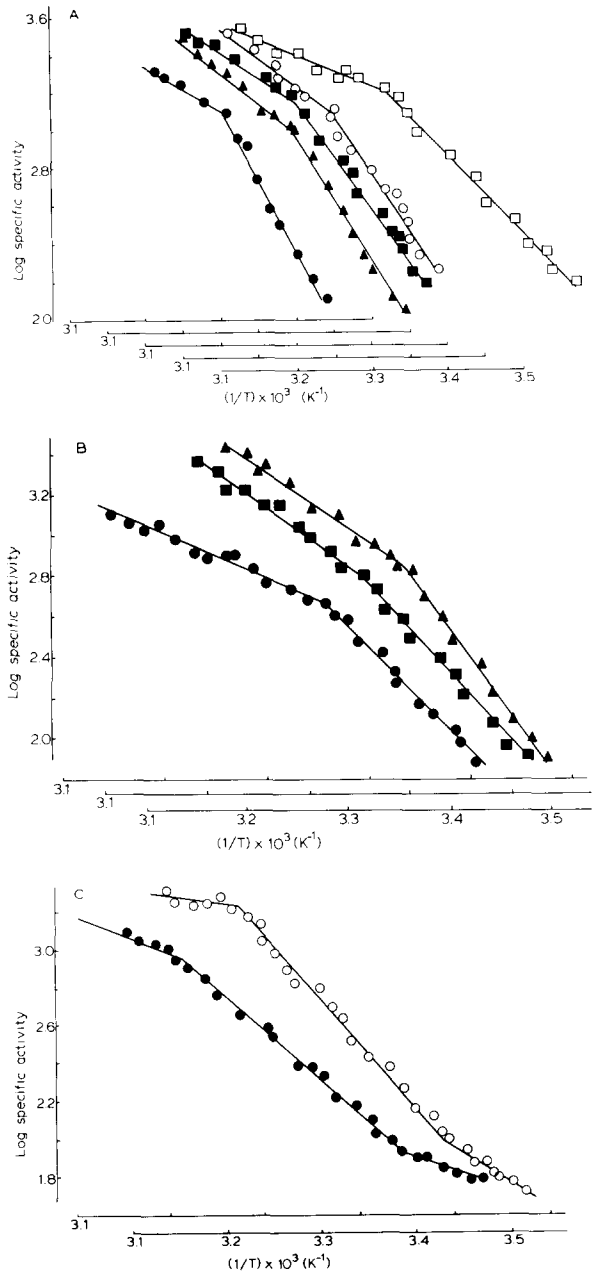

Fig. 4. (A) Arrhenius plots of the enzymic activity of various egg PC-complex III mixtures at lipid/protein molar ratios: 60:1 (●); 80:1 (▲); 280:1 (■); 500:1 (○) and 2000:1 (□). Specific activity is expressed as mmol cytochrome *c* reduced/min per mg protein. (B) Arrhenius plots of the enzymic activity of various DMPC-complex III mixtures. Lipid/protein molar ratios: 80:1 (●); 160:1 (■) and 240:1 (▲). Specific activity units as in (A). (C) Arrhenius plots of the enzymic activity of various DPPC-complex III mixtures. Lipid/protein molar ratios: 80:1 (●) and 1000:1 (○). Specific activity units as in (A).

various complex III-phosphatidylcholine mixtures in aqueous dispersion reveals that the Soret band of cytochromes bc_1 is shifted towards lower wavelengths with increasing lipid/protein ratios (Fig. 5), thus suggesting that conformational changes do occur in complex III as a result of changes in the lipid environment, at least at lipid/protein ratios below 400:1.

Complex III-PC-water systems were also examined by Fourier transform infrared spectroscopy, especially in the 1500–1800 cm^{-1} region, corresponding to the amide I and II region. Fig. 6A shows the spectra of a lipid-poor (80:1, curve 1) and a lipid-rich (1000:1, curve 2) mixture. In the spectrum corresponding to the 1000:1 lipid/protein ratio there is a peak at 1734 cm^{-1}, related to the stretching vibration of the carbonyl groups from phospholipid fatty acyl esters. This peak is not so strong in the case of the 80:1 lipid/protein sample, due to the smaller number of phospholipid molecules. In the 80:1 lipid/protein sample, the maximum of the amide I band is located at 1650 cm^{-1}, with a clear shoulder at 1635 cm^{-1} and a smaller feature at 1665 cm^{-1}, corresponding, respectively, to α-helix conformation, with contributions from antiparallel β-sheet structure and turns [25,26]. Peaks at 1546 cm^{-1} and 1515 cm^{-1} can also be observed, corresponding to the amide II and to C-C stretching of the

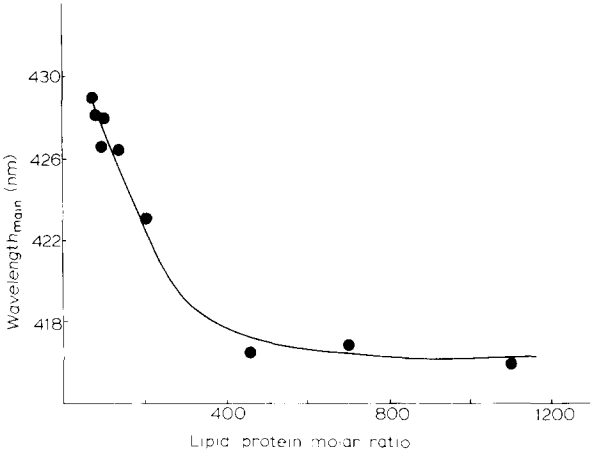

Fig. 5. The variation of the maximum wavelength of the Soret band of different egg PC-Complex III samples, as a function of lipid/protein molar ratio. Differential visible spectra (reduced minus oxidized) of the corresponding egg PC-Complex III mixtures were recorded in each case.

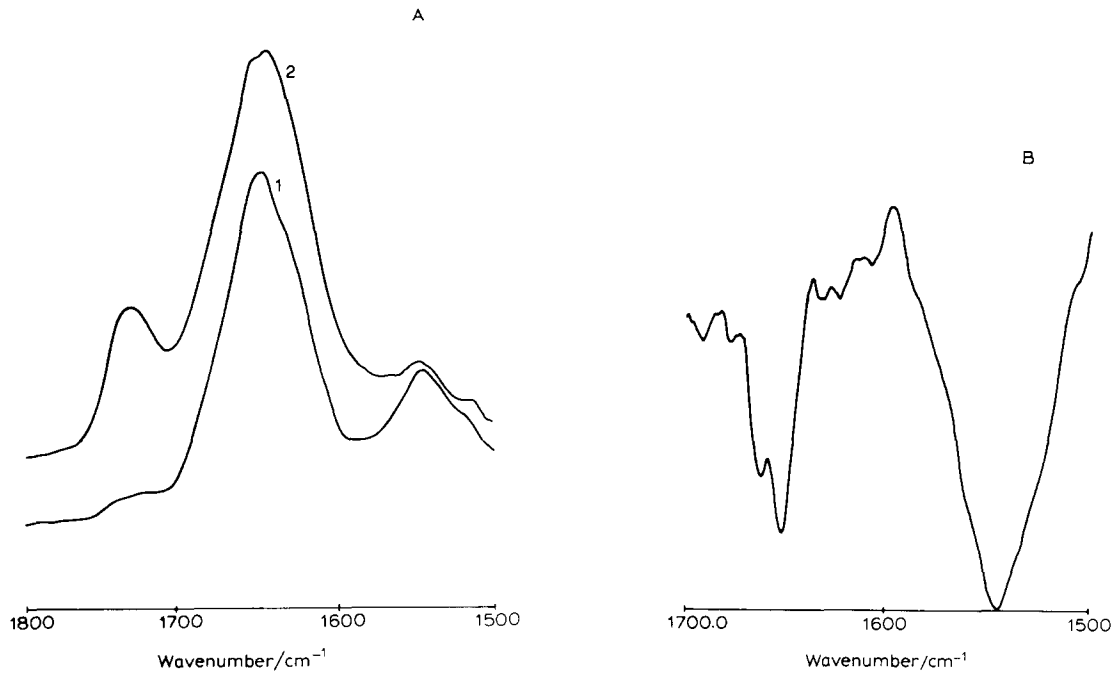

Fig. 6. (A) FT-IR spectra in the amide region (1500–1800 cm^{-1}) of two different egg PC-complex III mixtures in H_2O medium: 80:1 (curve 1) and 1000:1 (curve 2). 1204 scans. The buffer spectrum has been subtracted from the spectrum corresponding to the sample in buffer suspension, as indicated in the Methods. (B) Differential FT-IR spectrum in the amide region (1500–1800 cm^{-1}): 1000:1 minus 80:1 preparations. The spectra were adjusted to the same baseline and the difference factor was obtained by keeping a straight line of about zero absorbance in the region 1800–2000 cm^{-1}.

tyrosinyl ring, respectively [27]. When the lipid/protein ratio is increased, a clear change occurs in both the amide I and II regions. Apart from the increased intensity of the phospholipid carbonyl band, the amide I shows two differences: the main peak is now located at 1648 cm^{-1}, with another maximum at 1654 cm^{-1}, and the width of the amide I band contour (measured at half-height) has increased from 62 to 71 cm^{-1}. The relative intensity of the amide II band has decreased, and, consequently, the tyrosinyl vibration is more clearly seen, and a peak at 1580 cm^{-1} (corresponding to aminoacyl carboxylates and arginine) [28] can be observed. The amide I modifications could be due to changes in the relative intensities of the different vibrations overlapping in the band. In order to ascertain this hypothesis, a difference spectrum (1000:1 minus 80:1 samples) was obtained, and is shown in Fig. 6B. Only the 1700–1500 cm^{-1} region is shown, since the big difference in carbonyl intensity would mask possible differences in the adjacent spectral regions. The difference spectrum shows minima at 1665, 1650 and 1545 cm^{-1}, which is consistent with a decrease in the intensity of the 1650 cm^{-1} band, attributed to α-helical structure, and a decrease in the turns. The difference in amide II band intensity observed in Fig. 6A is also more clearly seen in the difference spectrum.

Discussion

The mitochondrial complex III-phosphatidylcholine-water system offers a good opportunity to examine some of the problems still unsolved in the field of lipid–protein interactions. We shall discuss briefly the structure of lipid-poor systems, the role of lipids in the activity of membrane enzymes and their influence on intrinsic protein structure. However, a preliminary question, namely whether complex III is incorporated into phosphatidylcholine bilayers, must be examined first.

Nelson and Fleischer [11] suggested, mainly based on measurements of respiratory control

ratios of complex III reconstituted with different phospholipids and phospholipid mixtures, that the protein could not be inserted correctly into pure PC bilayers. Whether complex III-PC proteoliposomes constitute a functional respiratory chain subunit or not is a debatable question, especially since we have preliminary evidence that FCCP and CCCP have effects other than uncoupling on reconstituted complex III (data not shown). Apart from that, when enough lipid is available, complex III can be incorporated into PC bilayers very much like other integral proteins. This conclusion is supported by various lines of evidence that have been presented in the Results. First, the presence of particles in the fracture faces of PC vesicles (Fig. 1E); these particles are usually interpreted as integral proteins, and similar images were used by Nelson and Fleischer [11] as a proof for the incorporation of complex III into phosphatidylcholine-phosphatidylethanolamine bilayers. Second, the thermal behaviour of complex III-saturated PC systems (Fig. 2): it has been shown in many cases that integral proteins abolish the pretransition, decrease the cooperativity and enthalpy change of the main transition and leave almost unchanged the midpoint transition temperature (T_m) [3,20,29]. This is precisely what we observed in our case. Third, the Arrhenius plots of enzyme activity of complex III-DMPC of DPPC systems (Fig. 4); discontinuities or 'breaks' are seen very near the main transition temperature (T_c) in both cases: DMPC, break at 25°C, $T_c = 23°C$; DPPC, break at 38°C, $T_c = 41°C$. Above these temperatures, the activation energies decrease, which is consistent with a facilitation of protein movement due to lipid melting. Indeed, the integral protein Ca^{2+}-ATPase from sarcoplasmic reticulum, when incorporated into DPPC vesicles, has a break at about 38°C [7] while when inserted into DMPC bilayers, the discontinuity appears at 25°C [30]. We consider that these data constitute unequivocal proof of the physical incorporation of complex III into PC bilayers. In the case of complex III-DPPC systems, the Arrhenius plots show another break at 19°C; the meaning of this break may be the same than that of the discontinuity seen at 30°C in Ca^{2+}-ATPase-DPPC systems [7,8]. The latter was interpreted either as melting of annular lipids [7] or as melting of lipids trapped in protein-rich patches [8]. Protein-rich patches are seen by freeze-fracture electron microscopy in complex III-DPPC systems quenched from 4°C (data not shown); it is possible that, in this case, patches melt at 19°C. Arrhenius plots of complex III reconstituted in egg-yolk PC always show a discontinuity near 28°C. The origin of such a feature is not clear. Houslay and Stanley [31] have discussed various possible explanations for this kind of breaks at temperatures far apart from the transition temperature of the lipid. One possibility could be that some change in water structure occurs near 30°C [32]. However, the possibility that the Arrhenius breaks are due primarily to the protein, the transition temperature being modulated by the nature of the lipid, cannot be excluded either.

The aim of membrane protein reconstitution consists of reproducing as faithfully as possible, albeit in a simple model, the situation in cell membranes. However, in practice, many experiments are described in which lipid/protein ratios are very low, with the implicit assumption that membranous systems are formed as soon as the detergents used in the reconstitution procedure are removed. We have described in detail the structure of the systems obtained by removing the surfactant from complex III-phospholipid-Triton X-100 mixed micelles at low lipid levels [3]. Green [33] has also described the properties of partly delipidated Ca^{2+}-ATPase; it is interesting to note the structural similarity of his preparations with ours [13]. In the present study, we show additional proof that, at low lipid/complex III ratios, no vesicles are formed, and freeze-fracture fails to detect any lipid surfaces; Fig. 1 is eloquent in this respect. It should be noted that, when enzyme activity is considered (Figs. 3 and 4) there is no clear-cut difference between both situations. Even from the structural point of view, a gradual change appears to occur, as more lipid is available, from lipid-protein aggregates to bilayers (Fig. 1). According to our observations, bilayers are clearly present when 300 or more PC molecules are available per protein molecule.

The role of lipids in the activity of membrane enzymes has been extensively studied [3,4,34–36]. It is generally accepted that enzymes require a fluid lipid environment in order to display their

catalytic activities; however, the minimum amount of lipid required to maintain the enzyme activity has been the object of some controversy. Hesketh et al. [7] pointed out the necessity of at least 30 phospholipid molecules, i.e. the minimum amount required to surround the protein with one layer of phospholipids, to maintain the activity of sarcoplasmic reticulum Ca^{2+}-ATPase; it was shown that the specific activity remained constant for lipid/protein ratios above 30 : 1, but decreased dramatically below that figure, becoming zero at a lipid/protein ratio of about 15 : 1. A similar behaviour was later found by Knowles et al. [10] for cytochrome oxidase. The finding by Dean and Tanford [36] that nonionic detergents could restore the enzyme activity of totally delipidated Ca^{2+}-ATPase preparations confirmed the previous assumption that there was no specific requirement for phospholipids, but rather a need for some protein regions to be kept in a hydrophobic environment. The behaviour of Complex III towards the minimum lipid requirements seem to be different from the above proteins, since there is no minimal value of lipid/protein ratio below which the enzyme becomes inactive; rather, specific activity increases regularly from virtually zero to, at least, 600 phospholipids per protein molecule (Fig. 3A). We have found that the best model to fit the experimental values consists of treating the phospholipids as essential enzyme activators (see Appendix), considering that the enzyme activity is zero in the total absence of phospholipids. The model assumes that the enzyme has an indefinite number of unspecific, independent and identical binding sites. The model has the advantage of interpreting the experimental results in terms of well-known kinetic equations [24]; to the authors' knowledge, no other proposed model of lipid binding explains adequately the data. Among the drawbacks, we should note that phospholipids are usually considered as the solvent for the hydrophobic parts of the protein molecule, a conceptually different role from that of 'activators' (although a given molecule may well perform both functions). Also it is obvious, from the size of the complex III molecule that no more than 100 moles phospholipid may be arranged in a single layer around the protein (result estimated from Perkins and Weiss [38]); thus phospholipids in excess of 100 molecules are 'activating' the molecule without all of them being simultaneously in contact with it, which is untenable from the point of view of classical enzyme activation, but rather points again to the role of phospholipids as solvents. A final word of caution concerning the role of lipids; the expression 'binding sites' is used here in a non-rigorous sense, because there is no proof that complex III has binding sites for phospholipid as it has for, say, cytochrome *c* or ubiquinol. For the same reason, we prefer to speak of lipid-protein mixtures, or systems, rather than complexes, in order to avoid confusion with the concept of stoichiometric complexes, as it is commonly used in chemistry.

The way in which intrinsic proteins perturb the lipid bilayer is quite well known [4]. Complex III seems to act in this respect very much like other integral proteins, as judged from the experimental data (Figs. 1 and 2). Much less is known, however, of the way in which phospholipids modulate protein conformation. Since complex III remains active within a large range of phospholipid/protein ratios, this system is a good candidate to explore protein conformational changes as a function of the lipid fraction present. The results in Figs. 5 and 6 demonstrate the existence of such changes, that can also be related to enzyme activity. In Fig. 5 a shift in the Soret band of the bc_1 cytochromes to lower wavelengths occurs as the lipid/protein ratio decreases. This means that a decrease in the polarity of the environment produces changes in the heme region of the protein. Experiments carried out with samples of complex III in 10% aqueous ethanol (data not shown) promote the same type of shift, which confirms the previous explanation. Using FT-IR spectroscopy, structural changes in complex III have been observed by varying the lipid/protein ratio. These changes consist mainly of an increase in the width of the amide I band contour and a decrease in the intensity of the 1650 and 1665 cm^{-1} band components. The former can be due to differences in the relative intensities of bands overlapping in the broad amide I signal; a change in bandwidth is thus suggestive of a protein conformational change. Changes in conformation (increase in peptide β-structure) have also been observed when detergent is removed from complex III-phospholipid-

Triton X-100 mixed micelles [13]. Vibrations assigned to polypeptide turns are known to contribute to the amide I band; in particular, the 1665 cm^{-1} vibration has been attributed to such turns [39]. Since they are located mainly in the outer part of the protein, they may be particularly sensitive to changes in the protein environment. A decrease in the 1650 cm^{-1} band is most easily interpreted in terms of a diminished proportion of α-helical structure [25]. Simultaneous changes in the proportion of α-helical structures and β-turns have been observed when proteins are transfered to a different environment [40].

Appendix

Complex III activation by phospholipids

Let us assume that complex III is inactive in the absence of lipids; let us also suppose that phospholipid molecules (L) act as essential activators for enzyme activity, in such a way that their presence induce in the enzyme conformational changes leading to the active conformation [24].

If the enzyme (E)-substrate (S) binding constants with and without lipid (K_S and αK_S) are related through an additional constant α, in such a way that the substrate binds with different affinity to the different enzyme conformations, then

$$\begin{array}{lll} E + S \overset{K_S}{\rightarrow} ES & & \\ + \quad + & & \text{where } V = k[ELS] \\ L \quad L & & \text{and } V_{max} = k[E]_T \\ \downarrow K_L \quad \downarrow \alpha K_L & & \\ EL + S \overset{\alpha K_S}{\rightarrow} ELS \overset{k}{\rightarrow} EL + P & & \end{array}$$

$$\frac{V}{V_{max}} = \frac{S}{S(1 + \alpha K_L/L + \alpha K_S(1 + K_L/L)}$$

This equation can be represented in the double-reciprocal form:

$$\frac{1}{V} = \frac{\alpha K_S}{V_{max}}(1 + K_L/L)\frac{1}{S} + (1 + \alpha K_L/L)\frac{1}{V_{max}}$$

These equations are developed for the binding of just one phospholipid molecule, but we have assumed that the n protein sites for phospholipid binding are identical and independent. Thus, the n sites behave kinetically as if they were only one [34], and the above equation can be applied to our system.

Acknowledgments

The authors are grateful to Dr. J.L. Carrascosa and Mr. P. González for their help with the freeze-fracture electron micrographs, and to Professor G.M. Guzmán and Dr. J.J. Iruin, for making available to them their DSC instrument. The authors are also indebted to Dr. J.L. Serra for his advice on enzyme kinetics. The photographic artwork of Miss C. Otamendi is gratefully acknowledged. This work was supported in part by grant No. 992/84 from CAICYT and grant No. X-86.047 of the Basque Government.

References

1 Träuble, M. and Sackmann, E. (1972) J. Am. Chem. Soc. 94, 4499–4510.
2 Jost, P.C., Griffith, O.H., Capaldi, R.A. and VanderKooi, G. (1973) Proc. Natl. Acad. Sci. USA 70, 480–484.
3 Chapman, D., Gómez-Fernández, J.C. and Goñi, F.M. (1979) FEBS Lett. 98, 211–223.
4 Chapman, D., Gómez-Fernández, J.C. and Goñi, F.M. (1982) Trends Biochem. Sci. 7, 67–70.
5 Gómez-Fernández, J.C., Goñi, F.M., Bach, D., Restall, C.J. and Chapman, D. (1980) Biochim. Biophys. Acta 598, 507–516.
6 Watts, A. and De Pont, J.J.H.H.M. (eds.) (1985, 1986) Progress in Protein-Lipid Interactions (2 vols.), Elsevier, Amsterdam.
7 Hesketh, T.R., Smith, G.A., Houslay, M.D., Mc Gill, K.A., Birsdall, N.J.M., Metcalfe, J.C. and Warren, G.B. (1976) Biochemistry 15, 4145–4151.
8 Hoffman, W., Sarzala, G., Gómez-Fernández, J.C., Goñi, F.M., Restall, C.J., Chapman, D., Heppeler, G. and Kreutz, W. (1980) J. Mol. Biol. 141, 119–132.
9 Raison, J.K. and McMurchie, E.G. (1974) Biochim. Biophys. Acta 363, 135–140.
10 Knowles, P.F., Watts, A. and Marsh, D. (1979) Biochemistry 18, 4480–4487.
11 Nelson, B.D. and Fleischer, S. (1981) Biochem. J. 194, 783–787.
12 Valpuesta, J.M., Goñi, F.M., Katime, I. and Macarulla, J.M. (1985) Biochim. Biophys. Acta 807, 96–99.
13 Valpuesta, J.M., Arrondo, J.L.R., Barbero, M.C., Pons, M. and Goñi, F.M. (1986) J. Biol. Chem. 261, 6578–6584.
14 Engel, W.D., Schägger, H. and Von Jagow, G. (1980) Biochim. Biophys. Acta 592, 211–222.
15 Singleton, W.S., Gray, M.S., Brown, M.L. and White, J.C. (1965) J. Am. Oil Chem. Soc. 92, 52–53.
16 Morrison, W.R. and Smith, L.M. (1964) J. Lipid Res. 5, 600–605.

17 Bartlett, G.R. (1959) J. Biol. Chem. 234, 466–468.
18 Barbero, M.C., Valpuesta, J.M., Rial, E., Gurtubay, J.I.G., Goñi, F.M. and Macarulla, J.M. (1984) Arch. Biochem. Biophys. 228, 560–568.
19 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
20 Papahadjopoulos, D., Poste, G. and Schaeffer, B.E. (1979) FEBS Lett. 98, 211–223.
21 Chapman, D., Urbina, J. and Keough, K.M. (1974) J. Biol. Chem. 249, 2512–2518.
22 Weiss, H., Perkins, S.J. and Leonard, K. (1983) in Structure and Function of Membrane Proteins (Quagliariello, E. and Palmieri, F., eds.), pp. 215–222, Elsevier, Amsterdam.
23 Schneider, H., Semasters, J.J., Höchli, M. and Hackenbrock, C.R. (1980) J. Biol. Chem. 255, 3748–3756.
24 Segel, I.H. (1975) Enzyme Kinetics, Wiley-Interscience, New York.
25 Timasheff, S.N., Susi, H. and Steven, L. (1967) J. Biol. Chem. 242, 5467–5472.
26 Frazer, R.D.B. and Suzuki, E. (1970) in Physical Principles and Techniques of Protein Chemistry (Leach, S.J., ed.), pp. 213, 232, Academic Press, New York.
27 Rothschild, K.J., Sanches, R. and Clark, N.A. (1982) Methods Enzymol. 82, 696–714.
28 Chirgadze, Yu.N., Fedorov, O.V. and Trushina, N.P. (1975) Biopolymers 14, 679–694.
29 Cortijo, M., Alonso, A., Gómez-Fernández, J.C. and Chapman, D. (1982) J. Mol. Biol. 157, 597–618.
30 Teruel, J.A., Delicado, E.G., Carmona, F.G., Cánovas, F.G. and Gómez, J.C. (1985) An. Vet. (Murcia) 1, 123–131.
31 Houslay, M.D. and Stanley, K.K. (1982) Dynamics of Biological Membranes, Wiley and Sons, New York.
32 Drost-Hansen, W. (1977) in Chemistry of the Cell Interface, part B (Brown, H.D., ed.), pp. 87–92, Academic Press, New York.
33 Green, N.M. (1975) in Calcium Transport in Contraction and Secretion (Carafoli, E., ed.), pp. 329–348, North Holland Publishing Company, Amsterdam.
34 Sandermann, H.J. (1982) Eur. J. Biochem. 127, 123–128.
35 Sandermann, H.J. (1983) Trends Biochem. Sci. 8, 408–411.
36 Dean, W.L. and Tanford, C. (1977) J. Biol. Chem. 252, 3351–3353.
37 McElhaney, R.N. (1986) Biochim. Biophys. Acta 864, 361–421.
38 Perkins, S.J. and Weiss, H. (1983) J. Mol. Biol. 168, 847–866.
39 Krimm, S. and Bandekar, J. (1980) Biopolymers 19, 1–29.
40 Susi, H. and Byler, D.M. (1983) Biochem. Biophys. Res. Commun. 115, 391–397.