Biochimica et Biophysica Acta, 1021 (1990) 27–32
Elsevier

BBAMEM 74675

Effects of cholesterol on the interaction of Ca^{2+}-ATPase with 1-palmitoyl-2-oleoylphosphatidylethanolamine. An FTIR study

Jhyshyong Yang, Gloria L. Anderle and Richard Mendelsohn

Department of Chemistry, Rutgers University, Newark College of Arts and Sciences, Newark, NJ (U.S.A.)

(Received 26 June 1989)

Key words: ATPase, Ca^{2+}-; Enzyme–phospholipid interaction; 1-Palmitoyl-2-oleoylphosphatidylethanolamine; Cholesterol; Lipid vesicle; Fourier transform infrared spectroscopy; DSC

Ca^{2+}-ATPase from rabbit skeletal muscle has been isolated, purified, and reconstituted into vesicles containing binary mixtures of 1-palmitoyl-2-oleoylphosphatidylethanolamine (POPE)/cholesterol. Fourier transform infrared spectroscopy (FTIR) was used to investigate the effect of protein on the thermotropic behavior of POPE in these reconstituted ternary complexes. The CH_2 symmetric stretching modes of the phospholipid acyl chains near 2850 cm^{-1} served as an index of the melting process. The thermotropic transition of the POPE component in a 103:12:1 (POPE/cholesterol/Ca^{2+}-ATPase) complex was shifted to lower temperatures compared with a protein-free binary lipid mixture of the same relative proportions. When combined with differential scanning calorimetric (DSC) data for the binary (POPE/cholesterol) lipid systems, this observation suggests that Ca^{2+}-ATPase preferentially sequesters 15–35 molecules of POPE from the lipid mixture and therefore excludes cholesterol from its immediate environment. Higher levels of cholesterol in ternary complexes progressively eliminate the cooperative POPE melting event.

Introduction

Although the normal role of cholesterol in biological membranes is not well understood, the sterol modulates the function of many membrane proteins, including the acetylcholine receptor [1], Na^+/K^+-ATPase [2], and Ca^{2+}-ATPase [3]. A variety of biochemical and biophysical studies have been undertaken to elucidate the physical basis by which cholesterol functions in this context. Ca^{2+}-ATPase from rabbit skeletal muscle has probably been the protein most widely studied for this purpose. In an early investigation, Warren et al. [4] showed that cholesterol did not alter the ATPase activity of this protein in reconstituted systems. A similar result was later obtained for coupled sarcoplasmic reticulum vesicles [5]. Simmonds et al. [6] have demonstrated that the effect of sterols on the activity of the ATPase depends markedly on the particular phospholipids present. For example, both dibromocholesterol and cholesterol nearly quadrupled the activity of ATPase reconstituted with dimyristoylphosphatidylcholine, but decreased the activity of a brominated PC-ATPase complex. More recently, Cheng et al. [3] observed that in reconstituted systems containing mixtures of PC and PE, the coupling efficiency of Ca^{2+}-ATPase-containing liposomes increased with increasing cholesterol content and was more pronounced in lipid mixtures containing high PE levels. These widely varying effects of cholesterol are difficult to reconcile simply in terms of the ability of this sterol to alter membrane fluidity [7,8]. Thus, other physical origins for the observed effects have been sought. Simmonds et al. [6,9] have utilized a fluorescence spectroscopy approach to measure the relative binding of cholesterol and phospholipid to Ca^{2+}-ATPase and suggested that cholesterol is effectively excluded from the vicinity of protein. Additional fluorescence and ESR spectroscopic measurements [3] showed the phospholipid acyl chains to become increasingly ordered with slower rates of motion in the presence of cholesterol. The disadvantage of the fluorescence and ESR spectroscopic techniques is their requirement for the use of probe molecules, which sample mainly the local environment of the probe and have, in addition, the potential to perturb the system under investigation [10]. It therefore seemed worthwhile to

Abbreviations: FTIR, Fourier transform infrared spectroscopy; POPE, 1-palmitoyl-2-oleoylphosphatidylethanolamine; DSC, differential scanning calorimetry; PC, phosphatidylcholine; PE, phosphatidylethanolamine; ESR, electron paramagnetic resonance.

Correspondence: R. Mendelsohn, Department of Chemistry, Rutgers University, Newark College of Arts and Science, 73 Warren Street, Newark, NH 07102, U.S.A.

examine the effect of cholesterol on phospholipid/protein interaction using structural techniques possessing less potential for structural alteration.

FTIR spectroscopy is widely used as a non-perturbing probe of phospholipid acyl chain order in reconstituted lipid-protein complexes [11–13]. The symmetric CH_2 stretching modes of the acyl chains absorb in a spectral region (approx. 2850 cm^{-1}) relatively free from interference from other membrane components. The frequency of this band can be monitored with high precision (about 0.02 cm^{-1}), and serves as a useful index of the physical state of the acyl chains. In previous studies from this laboratory [14–16] we have investigated the effects of Ca^{2+}-ATPase on the thermotropic properties of various phospholipid classes and binary phospholipid mixtures. The current FTIR study was undertaken in conjunction with DSC measurements, to determine the ability of this protein to preferentially exclude or select cholesterol for its immediate environment.

Experimental

(A) Sample preparation and characterization

Purified sarcoplasmic reticulum was prepared as previously described [14]. The native lipids were exchanged through a deoxycholate-mediated lipid exchange protocol due to Warren et al. [17] as modified by Hidalgo et al. [18]. Vesicles were assayed for lipid acyl chain lengths by gas chromatography of their methylated acyl chains. The latter were transesterified as previously described [16].

Protein levels were determined by the method of Lowry et al. [19], lipid concentrations were determined from the lipid phosphorus determination of Chen et al. [20] and cholesterol was determined by the method of Zlatkis et al. [21]. ATPase activities were measured with the coupled enzyme assay of Warren et al. [22].

(B) FTIR spectroscopy

Samples for spectroscopic examination were suspended in Tris-maleate buffer (20 mM, pH 7.4), and placed in a 6 or 12 μM Harrick cell equipped with BaF_2 windows mounted in a thermal jacket. Cell temperature was monitored with a thermocouple placed close to the point where the IR beam passed through the sample. FTIR spectra were obtained with a Mattson Instruments Sirius 100 spectrophotometer. Scanning conditions were as previously described [16]. Peak positions of the CH_2 vibrational modes were determined with a three point parabolic routine applied to the solvent-subtracted, baseline-leveled spectra.

(C) DSC

DSC endotherms were obtained in a Micro Cal, Inc. MC-1 unit. Sample volumes were 0.70 ml containing 3–5 mg of the POPE/cholesterol mixture desired in a Tris-maleate buffer at pH 7.4. The same volume of buffer was used in the reference cell. Samples were heated at about 36 C°/h following 2 h equilibration time in the instrument. The gradual nature of the endotherm onset temperatures, especially for the gel → liquid crystal phase transition at moderate cholesterol levels made it difficult to estimate their absolute values with a precision better than 0.5–1.0 C°. Transition onset (and completion) temperatures were taken for sharp transitions as the points of intersection of the tangents to the leading (or trailing) edges of the endotherms and the baselines. For broad transitions, the points of baseline departure were selected. For the gel → liquid crystal transition of POPE/cholesterol mixtures, most of the transitions were sufficiently sharp so that the first method could be employed. The procedures used are similar to those described by Davis et al. [23].

Results

(A) Control systems: POPE/cholesterol binary mixtures

Differential scanning calorimetry (DSC) traces for pure POPE, and 95:5, 90:10, and 80:20 POPE/cholesterol mixtures are shown in Fig. 1. The data are in excellent accord with experiments reported by Epand and Bottega [24]. The gel → liquid crystal transition, which occurs near 25 °C for pure POPE, is rendered progressively less cooperative, appears with lower onset, completion and mid-point temperatures, and has re-

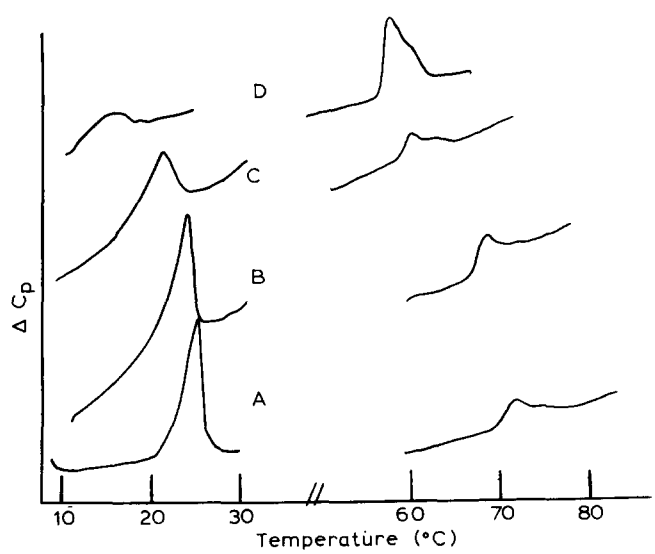

Fig. 1. DSC endotherms for POPE/cholesterol mixtures: A, 100:0 (w/w); B, 95:5; C, 90:10; D, 80:20. The lower temperature endotherm for each composition is the gel → liquid crystal transition. The higher temperature endotherm is the bilayer- → hexagonal phase transition. For samples A, B, and C, the gel-liquid crystal phase transition heat capacity sensitivity is one-fourth that of the bilayer → hexagonal transition. For sample D, the two transitions are examined under conditions of the same sensitivity.

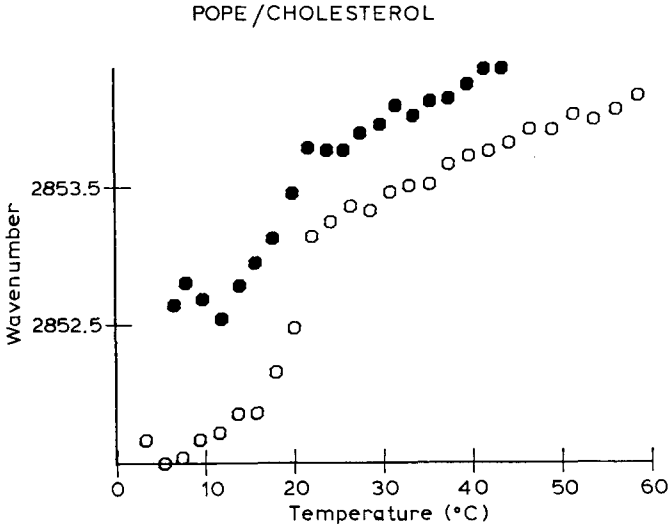

Fig. 2. FTIR melting curves for binary mixtures of POPE/cholesterol, constructed from the CH_2 symmetric stretching mode near 2850 cm^{-1} of the POPE acyl chains. Cholesterol vibrations at the levels of steroid used here do not interfere with the POPE bands. POPE/cholesterol 6.5 : 1 (mole ratio), •; POPE/cholesterol 9 : 1 (mole ratio), o.

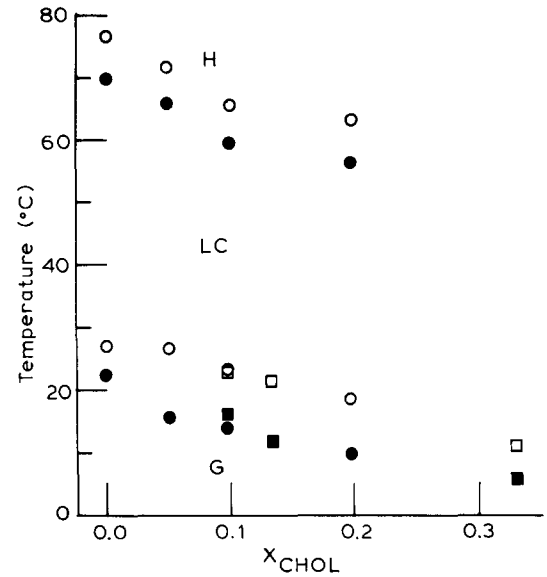

Fig. 3. Partial phase diagram for the POPE/cholesterol binary system. Points obtained from the onset and completion temperatures of DSC scans are shown as circles and those from FTIR as squares (filled = onset, unfilled = completion temperatures). G, LC, and H represent stability regions for the gel, liquid crystal and hexagonal phases, respectively.

duced enthalpy as cholesterol is added from 0 to 20 mol%. The bilayer → hexagonal phase transition, observed from 69.7 → 76.7 °C for pure POPE (Fig. 1), is also reduced in temperature as cholesterol is added. The bilayer destabilization properties of cholesterol in this system are thus directly evident from these experiments and are consistent with the observations of Cullis et al. [25]. The two components noted for this transition have been analyzed in detail elsewhere [24].

Melting curves for 9 : 1 and 6.5 : 1 mole ratio POPE/cholesterol mixtures as constructed from the temperature dependence of the 2850 cm^{-1} CH_2 symmetric stretching modes of the FTIR spectra are plotted in Fig. 2. As discussed elsewhere [13], although this parameter increases by only 2–4 cm^{-1} during lipid thermotropic events, it can be measured with a precision of 0.02 cm^{-1}, and thus provides a very useful index of acyl chain melting. FTIR is particularly helpful in situations where the melting events are not highly cooperative, hence difficult or impossible to detect with DSC. The temperature range shown in Fig. 2 includes only the gel → liquid crystal transition. The bilayer → hexagonal transition could not be reproducibly identified from its effect on this IR spectral parameter. In any case, only the gel → liquid crystal transition is of interest in connection with the effect of Ca^{2+}-ATPase on this binary lipid mixture, as the thermal denaturation of Ca^{2+}-ATPase occurs at lower temperatures than the bilayer → hexagonal interconversion. A partial phase diagram for the POPE/cholesterol system from 0 to 33 mol% cholesterol, as constructed from the set of measured onset and completion temperatures determined both by DSC and FTIR, is shown in Fig. 3. The data points for the FTIR-determined melting process are in good accord with the interpolated calorimetric experiments. The use of FTIR to study the thermotropic properties under experimental conditions where insufficient material is available for DSC is thus justified. We have thus used FTIR to probe the POPE/cholesterol/Ca^{2+}-ATPase ternary systems.

TABLE I

Characterization of POPE/cholesterol/Ca^{2+}-ATPase complexes

Sample	PL [a]/Chol/Ca^{2+}-ATPase (mole ratio)	ATPase activity (I.U./mg)	Acyl chain: number of C=C bonds			
			16:0	18:0	18:1	18:2
A	101 : 15 : 1	2.62	0.49	0.06	0.40	0.04
B	103 : 12 : 1	2.33	0.52	0.03	0.42	0.03
C	91 : 27 : 1	0	0.47	0.19	0.34	0.00
Purified SR (typical values) 3–10			0.32	0.09	0.23	0.36

[a] Total phospholipid.

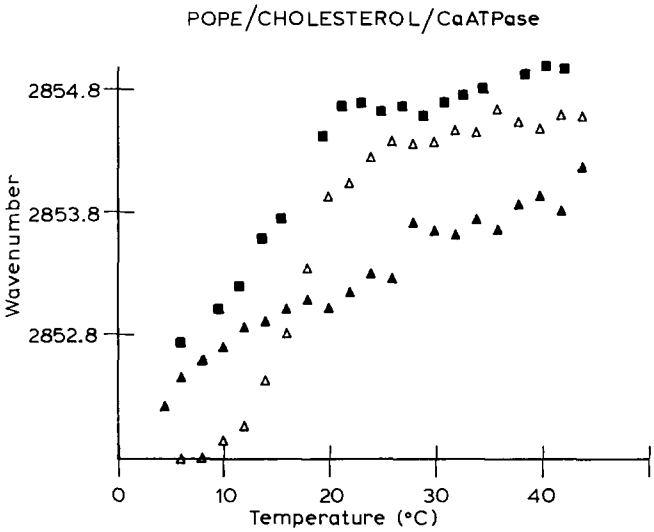

Fig. 4. FTIR melting curves for POPE/cholesterol/Ca^{2+}-ATPase constructed from the CH_2 symmetric stretching mode of POPE: POPE/cholesterol/Ca^{2+}-ATPase (mole ratio) 101 : 15 : 1, ■; 103 : 12 : 1, △; 91 : 27 : 1, ▲.

(B) POPE / cholesterol / Ca^{2+}-ATPase ternary complexes

The biochemical characterizations of the ternary complexes whose FTIR melting profiles were determined are summarized in Table I. ATPase activity levels are lower than those obtained in earlier studies in the absence of cholesterol [16]. At levels of cholesterol greater than about 20% of the total lipid, no activity was noted for any of several samples. The deoxycholate-mediated exchange protocol has been successful, as judged by the relatively high proportions of C16 : 0 and C18 : 1 and the relatively low levels of C18 : 2 in the ternary systems, compared with the purified native SR vesicles.

FTIR melting curves for three ternary complexes are plotted in Fig. 4. The 103 : 12 : 1 (POPE/cholesterol/Ca^{2+}-ATPase) complex has onset and completion temperatures of 12 and 20 °C respectively, while a 101 : 15 : 1 complex has a completion temperature of about 19–20 °C with no defined onset temperature above 5 °C, the lowest temperature used in the current work. Finally, the 91 : 27 : 1 complex shows only a monotonic increase in the CH_2 stretching frequency between 4 and 44 °C. Complexes were never heated above 45 °C because of the well-established thermal denaturation of the protein which begins close to that temperature (Ref. 14, and Mendelsohn, R. and Anderle, G., unpublished data).

Discussion

The observation (Table I) that increasing levels of membrane cholesterol in systems containing PE reduce the ATPase activity of the protein compared with native SR vesicles is in good accord with the studies of Cheng et al. [3]. These workers also observe a cholesterol-induced enhancement of the Ca^{2+} transport function of the Ca-ATPase, and suggest that it is related to the bilayer-destabilizing properties of the cholesterol molecule as revealed by DSC and by ^{31}P-NMR (Fig. 2 and Ref. 3).

FTIR melting curves for the binary lipid mixtures (Fig. 2) and ternary complexes (Fig. 4) coupled with the phase diagram (Fig. 3) can be used to gain a more quantitative insight into the relative tendencies of cholesterol compared with POPE to occupy sites in the immediate vicinity of the Ca^{2+}-ATPase. The premise of the analysis is as follows: If POPE is excluded from the protein environment compared with cholesterol, then insertion of protein into a cholesterol/POPE binary lipid mixtures should produce a bulk lipid environment with domains relatively enriched in phospholipid. These should have increased melting transition temperatures (onset, midpoint and completion) compared with the binary lipid mixture. If, in contrast, cholesterol is excluded from the protein environment, then addition of Ca^{2+}-ATPase should cause a reduction in the POPE transition temperatures since bulk lipid domains would become relatively enriched in cholesterol which tends (Fig. 2) to reduce T_m as measured by FTIR.

Previous studies of POPE melting in a 50 : 1 POPE/Ca^{2+}-ATPase complex [16] show a residual phase transition with an onset temperature of about 3–4 °C and a completion temperature of 18–19 °C. A 100 : 1 binary complex would thus be expected to have a residual phase transition with an even higher onset temperature. In the current study the 101 : 15 : 1 complex has its onset of melting below about 4–5 °C. Thus addition of cholesterol has clearly not resulted in sterol being preferentially located in the vicinity of protein, an effect which would have increased T_m for the phospholipid.

Semi-quantitative evaluation of the role of cholesterol in the ternary system comes from a study of the POPE/cholesterol phase diagram, as replotted in expanded form in Fig. 5, along with the onset and completion temperatures for the 103 : 12 : 1 ternary complex. Cholesterol induces a reduction in both the onset and completion temperatures for POPE melting which is adequately described by simple linear regression equations over the cholesterol mole fraction range 0.05 to 0.33, as follows:

Onset temperature:

$$T_{onset} = -36.45 X_{chol} + 17.81$$

Completion temperature:

$$T_{comp} = -54.08 X_{chol} + 29.02$$

These lines are included in Fig. 5.

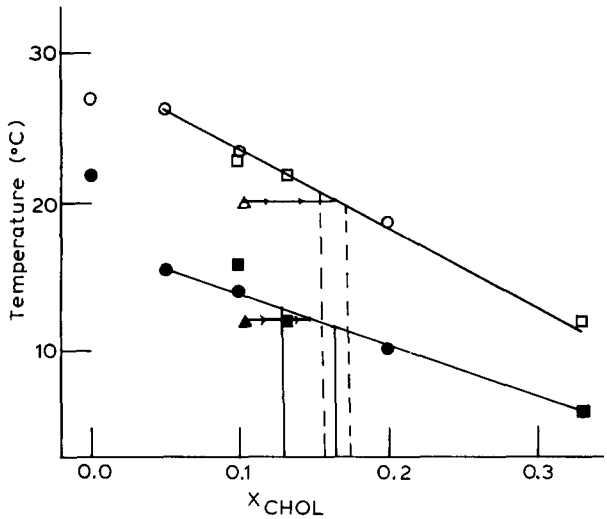

Fig. 5. Enlarged scale for the gel-liquid crystal phase transition region of the POPE/cholesterol phase diagram. Data points for binary mixtures are shown as circles (DSC data, filled = onset, open = completion) or squares (FTIR data, filled = onset, open = completion). Least-squares lines (see text) for the onset and completion temperatures over the mole fraction range 0.05–0.33 are included. also included are the onset (▲) and completion (△) temperatures for a 103:12:1 POPE/cholesterol/Ca^{2+}-ATPase ternary system. As discussed in the text, horizontal lines connecting the ternary system points to the least-squares lines are drawn, and propagation of error analysis is performed to determine the composition range of those residual lipids in the ternary mixture still able to undergo a phase transition in the presence of protein. Vertical lines (dashed from the onset temperature, solid from the completion temperature) indicate the 90% confidence limits for the range of compositions.

The onset and completion temperatures for the 103:12:1 ternary complex lie significantly below the line of least squares describing the melting of the binary mixture of lipids. Thus, we consider that the effect of protein insertion has been to produce a melting event comparable to a binary mixture of POPE/cholesterol containing a greater proportion of cholesterol than in the ternary complex. A simple propagation of errors analysis can be used to estimate the number of POPE molecules thus sequestered by the protein. If the data points for the ternary complex are shifted along the x-axis to intersect with the least-squares line (see Fig. 5) so as to determine the apparent mole fraction of cholesterol mixed with those (non-sequestered) POPE molecules that can undergo a phase transition, the following results are obtained:

$X_1 = 0.148 \pm 0.016$ (90% confidence limits)

$X_2 = 0.166 \pm 0.009$ (90% confidence limits)

where X_1 and X_2 are determined from the onset and completion temperatures, respectively. Back calculation then yields about 15–35 molecules of POPE/protein not able to undergo a transition, and assumed to be in the vicinity of the protein. This value agrees rather well with the initial experimental results expounded by Metcalfe and co-workers [26] that 20–30 molecules of phospholipid are required in order for Ca^{2+}-ATPase to function, and that these molecules form an annulus in the immediate vicinity of protein.

The physical properties of this putative annulus have been quite controversial. The current experiments shed some light on this issue. A comparison of Figs. 2 and 4 shows that addition of protein to POPE/cholesterol mixtures of constant composition (either 6.5:1 or 9:1) results in a substantial increase in the acyl CH_2 frequency at all temperatures. The measured frequency is an average over all the phospholipid molecules and suggests that, from the FTIR point of view, the phospholipid molecules are substantially more conformationally disordered than in the protein-free systems.

Since, as shown above, cholesterol is excluded from the vicinity of protein, we may gain further insight as to the conformation of the POPE in the vicinity of protein. In the protein containing systems, the POPE/cholesterol ratio in the non-protein regions is lower than the protein-free systems, as some of the POPE is removed from the cholesterol-rich areas. Cholesterol is known to order phospholipid liquid crystal phases, so that the observation of a disordered overall phospholipid in the ternary complex must reflect the fact that the phospholipid in the vicinity of the protein is substantially more disordered than in the lipid binary mixtures.

The motional characteristics (dynamic behavior) of POPE cannot be determined from the current type of FTIR experiment. Thus the current data are consistent with a lipid-protein model invoking phospholipids containing gauche rotamers at particular sites that have formed to complement the protein surface.

Higher levels of sterol (Fig. 4) lead to a residual POPE melting process progressively broadened to the point where for the 91:27:1 sample only a monotonic increase of the IR spectral parameter with temperature is observed. Consistent with the discussion above, high levels of cholesterol are seen to order the phospholipid liquid crystal phase.

The current conclusion that cholesterol is effectively excluded from the vicinity of protein, is in accord with the work of Simmonds et al. [6,9], who used a fluorescence quenching technique to measure relative binding constants of hydrophobic compounds to the phospholipid/protein interface. The loss of ATPase activity observed at high cholesterol levels, suggests that once cholesterol is forced into the protein environment, its presence is deleterious to enzyme function. The structural origin of this phenomenon may be related to the bilayer-destabilizing properties of cholesterol (Figs. 1 and 3) or to as yet unrecognized features of the enzyme/lipid interface.

Acknowledgments

This work was supported by the US Public Health Service through grant GM-29864 (R.M.). Additional funds were supplied from the Busch bequest to Rutgers University. We thank Professor P. Huskey for discussions concerning the statistical analysis.

References

1 Criada, M., Eibl, H. and Barrantes, F.J. (1982) Biochemistry 21, 3622–3629.
2 Yeagle, P. (1983) Biochim. Biophys. Acta 727, 39–44.
3 Cheng, K.-H., Lepock, J.R., Hui, S.W. and Yeagle, P.L. (1986) J. Biol. Chem. 261, 5081–5087.
4 Warren, G.B., Houslay, M.D., Metcalfe, J.C. and Birdsall, N.J.M. (1975) Nature 255, 684–687.
5 Johannsson, A., Keightley, C.A., Smith, G.A. and Metcalfe, J.C. (1981) Biochem. J. 196, 505–511.
6 Simmonds, A.C., East, J.M., Jones, O.T., Rooney, E.K., McWhirter, J. and Lee, A.G. (1982) Biochim. Biophys. Acta 693, 396–406.
7 Johannsson, A., Keightley, C.A., Smith, G.A., Richards, C.D., Hesketh, T.R. and Metcalfe, J.C. (1981) J. Biol. Chem. 256, 1643–1650.
8 East, J.M., Jones, O.T., Simmonds, A.C. and Lee, A.G. (1984) J. Biol. Chem. 259, 8070–8071.
9 Simmonds, A.C., Rooney, E.K. and Lee, A.G. (1984) Biochemistry 23, 1432–1441.
10 Taylor, M.O. and Smith, I.C.P. (1980) Biochim. Biophys. Acta 599, 140–149.
11 Surewicz, W.K., Epand, R.M., Orlowski, R.C. and Mantsch, H.H. (1987) Biochim. Biophys. Acta 899, 307–310.
12 Mitchell, M.L. and Dluhy, R.A. (1988) J. Am. Chem. Soc. 110, 712–718.
13 Mendelsohn, R. and Mantsch, H.H. (1986) in Progress in Protein–Lipid Interactions, Vol. 2 (Watts, A. and De Pont, J.J.H.H.M., eds.), pp. 103–146, Elsevier, Amsterdam.
14 Mendelsohn, R., Anderle, G., Jaworsky, M., Mantsch, H.H. and Dluhy, R.A. (1984) Biochim. Biophys. Acta 775, 215–224.
15 Jaworsky, M. and Mendelsohn, R. (1985) Biochemistry 24, 3422–3428.
16 Anderle, G. and Mendelsohn, R. (1986) Biochemistry 25, 2174–2179.
17 Warren, G.B., Toon, P.A., Birdsall, N.J.M., Lee, A.G. and Metcalfe, J.C. (1974) FEBS Lett. 41, 122–124.
18 Hidalgo, C., Ikemoto, N. and Gergely, J. (1976) J. Biol. Chem. 251, 4224–4232.
19 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
20 Chen, P.S., Toribara, T.Y. and Warner, H. (1956) Anal. Chem. 28, 1756–1758.
21 Zlatkis, A., Zak, B. and Boyle, A.J. (1953) J. Lab. Clin. Med. 41, 486–490.
22 Warren, G.B., Toon, P.A., Birdsall, N.J.M., Lee, A.G. and Metcalfe, J.C. (1974) Biochemistry 13, 5501–5507.
23 Davis, P.J., Coolbear, K.P. and Keough, K.M.W. (1980) Can. J. Biochem. 58, 851–858.
24 Epand, R.M. and Bottega, R. (1987) Biochemistry 26, 1820–1825.
25 Cullis, P.R., Van Dijck, P.W.M., De Kruijff, B. and De Gier, J. (1978) Biochim. Biophys. Acta 513, 21–30.
26 Hesketh, T.R., Smith, G.A., Houslay, M.D., McGill, K.A., Birdsall, N.J.M., Metcalfe, J.C. and Warren, G.B. (1976) Biochemistry 15, 4145–4151.