The thermodependence of the activity of integral enzymes in liver plasma membranes

Evidence consistent with a functionally asymmetric lipid bilayer

Anthony D. Whetton†, Axel Johannsson, Sally R. Wilson*, Alan V. Wallace* and Miles D. Houslay*

Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, †Patterson Laboratories, Christie Hospital and Holt Radium Institute, Manchester M20 and **Department of Biochemistry, UMIST, PO Box 88, Manchester M60 1QD, England*

Received 3 May 1982

Lipid phase separation *Na^+/K^+-ATPase* *Phosphodiesterases* *Liver plasma membranes* *Membrane asymmetry* *Spin-labelled fatty acid*

1. INTRODUCTION

The characterisation of thermotropic lipid phase separations has proved to be a useful tool for identification of lipid–protein interactions in biological membranes. In many instances well-defined lipid phase separations have been identified and shown to give rise to breaks in the Arrhenius plots of the activity of membrane enzymes and transport processes at the temperature at which the lipid phase separation occurs. Such studies have been used both to determine the importance of the lipid environment in regulating the activity of integral membrane proteins and to obtain information on the vertical position of these proteins within the membrane (see, e.g., [1–8]).

In rat liver plasma membranes there occurs a well-defined lipid phase separation, with a high temperature onset at ~ 28°C and a low temperature onset at ~ 19°C, which has been identified using a variety of different physical techniques [9–15]. The high temperature onset of this thermotropic lipid phase separation is believed to be due to the formation of quasi-crystalline clusters (QCC) in the bilayer, whilst the low temperature onset reflects the aggregation of such clusters to form solid, crystalline phase (S) lipid which co-exists with fluid (F)-phase domains [10,11]. This lipid phase separation may be exclusively localised to those lipids occurring in the external half of the bilayer [9,12,16]. Indeed, the phospholipids in rat liver plasma membranes exhibited a marked chemical asymmetry with the acidic phospholipids occurring almost exclusively in the internal (cytosol-facing) half of the bilayer [17]. This study examines the thermodependence of the activity of a number of asymmetrically orientated integral membrane enzymes and shows that whilst some are influenced by the lipid phase separation, others are not.

Address correspondence and reprint requests to M.D.H at UMIST

2. METHODS

Liver plasma membranes, free of contaminating subcellular organelles, were prepared from 200–300 g male Sprague Dawley rats as in [18]. All enzyme assays were performed as in [2] except for the following. The *p*-nitrophenyl phosphatase (PNPase) assay was carried out in 40 nM glycine–NaOH buffer, final pH 9.0 (at all temperatures), 2.5 mM $MgSO_4$, 0.1 mM EDTA and 4.0 mM *p*-nitrophenyl phosphate in the absence and presence of 100 mM KCl. The change in absorption at 412 nm was measured. In every case initial rates were determined from linear time-courses and assay pH was kept constant. Arrhenius plots were analysed using a least squares minimalisation process [2], to determine the break points, if any.

Total membrane lipids were extracted by estab-

Published by Elsevier Biomedical Press
00145793/82/0000–0000/$2.75

lished procedures [12,14,19] and used to prepare multilamellar liposomes by shaking 6.6 mg dried lipid extract with 3 ml Tris–HCl (pH 7.2) under a nitrogen atmosphere at 19°C for 2 h. Alternatively unilamellar liposomes were prepared [20] by sonicating the above suspension for 10–15 min (to optical clarity) under a nitrogen atmosphere at 19°C. The state of the vesicles was ascertained by chromatography and negative-stain electron microscopy [20].

Electron spin resonance (ESR) procedures were done as in [10,11,15]. Briefly the *N*-oxyl-4′,4′-dimethyloxazolidine derivative of 5-ketostearic acid, I [3,12] was used as a spin probe at experimentally determined 'low probe' concentrations which were 9 μg/mg membrane protein for the liver plasma membranes and 4 μg probe/mg lipid for the liposomes. The outer and inner hyperfine splittings, $2T_{\parallel}$ and $2T_{\perp}$, were used to calculate the order parameter S ($T_{\parallel}$) and the polarity corrected order parameter S. Arrhenius plots were constructed using S, S ($T_{\parallel}$) and $2T_{\parallel}$, which have been shown to be useful parameters for following changes in fluidity in these membranes (see [21]). A Varian E9 EPR spectrometer was used on 2.0 G modulation amplitude, 0.30 s time constant, $\leq 5 \times 10^3$ receiver gain, 10 mW microwave power, 9.24 Gc microwave frequency all set on a scan range of 100 G run on a scan time of 4 min.

The spin probe I [3,12] was obtained from Syva Co. (Palo Alto CA). Radiochemicals were from the Radiochemical Centre (Amersham, Bucks). Pyruvate kinase, lactate dehydrogenase, phosphoenolpyruvate creatine phosphokinase, NADH, cyclic 3′,5′-AMP and ATP were from Boehringer (Lewes, Sussex). All other biochemicals were from Sigma (Kingston-upon-Thames, Surrey). Ouabain and all other chemicals, which were AnalaR grade, were from BDH (Poole, Dorset).

3. RESULTS

Arrhenius plots of the activity of Na^+/K^+-ATPase, the K^+-stimulated PNPase and alkaline phosphodiesterase I were all biphasic, demonstrating a single well-defined break which occurred at ~28–30°C. In contrast Arrhenius plots of the activity of the integral, low affinity cyclic AMP phosphodiesterase yielded a linear plot (fig.1, table 1).

Arrhenius plots of the polarity corrected order parameter, S, exhibited breaks or discontinuities using both liposome preparations (fig.2). With the multilamellar vesicles there was no evidence for a sharply defined lipid phase separation. Instead, small changes in slope at 37°C and 5°C implied that, at most, an ill-defined, very broad and shallow lipid phase separation might occur in these vesicles. This is in marked contrast to the unilamellar liposome preparation where the two well-defined onsets occurred at ~30°C and 21°C, respectively (fig.2) and native membranes with onsets at 28°C and

Table 1

Enzyme	Break point, °C (no. obs.)	Activation energy (kJ/mol) Above break	Below break
Na^+/K^+-ATPase	28.6 ± 1.7 (4)	16.7 ± 6.3	116.0 ± 18.5
K^+-stimulated PNPase	28.2 ± 0.3 (3)	21.2 ± 8.0	81.6 ± 39.5
Alkaline phosphodiesterase I	30.6 ± 1.2 (4)	18.3 ± 3.6	39.3 ± 10.1
Integral cyclic AMP phosphodiesterase	Linear (5)	48.7 ± 10.5	

Break points were assigned by a least squares minimalisation procedure [2] using different membrane preparations. The results shown are the mean of those determined ± SD

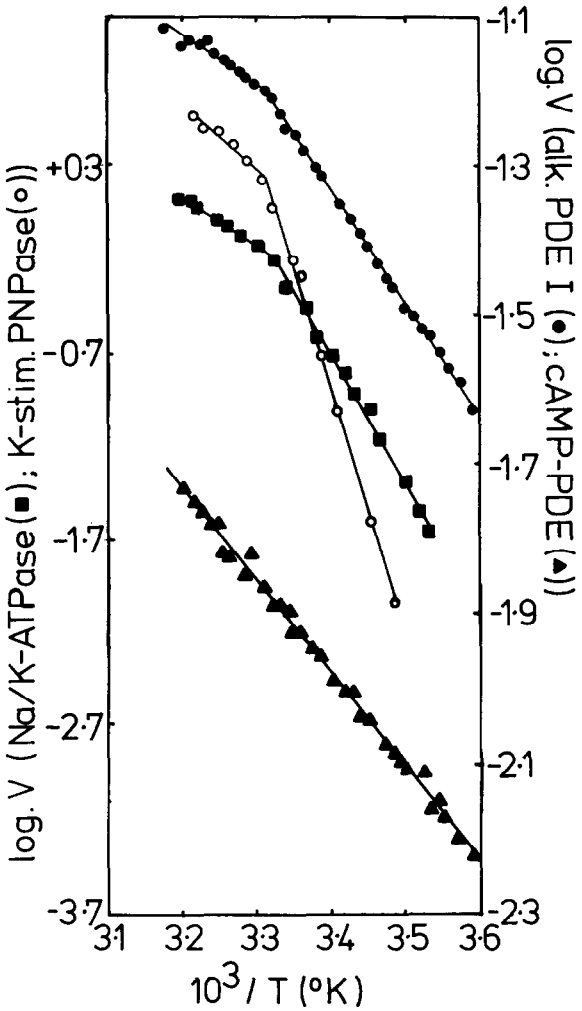

Fig.1. Enzymes from rat liver plasma membranes yielding biphasic Arrhenius plots of their activity: Na^+/K^+-stimulated, Mg^{2+}-dependent ATPase (■); K^+-stimulated PNPase (○); alkaline phosphodiesterase I (●) and the integral cyclic AMP phosphodiesterase (▲).

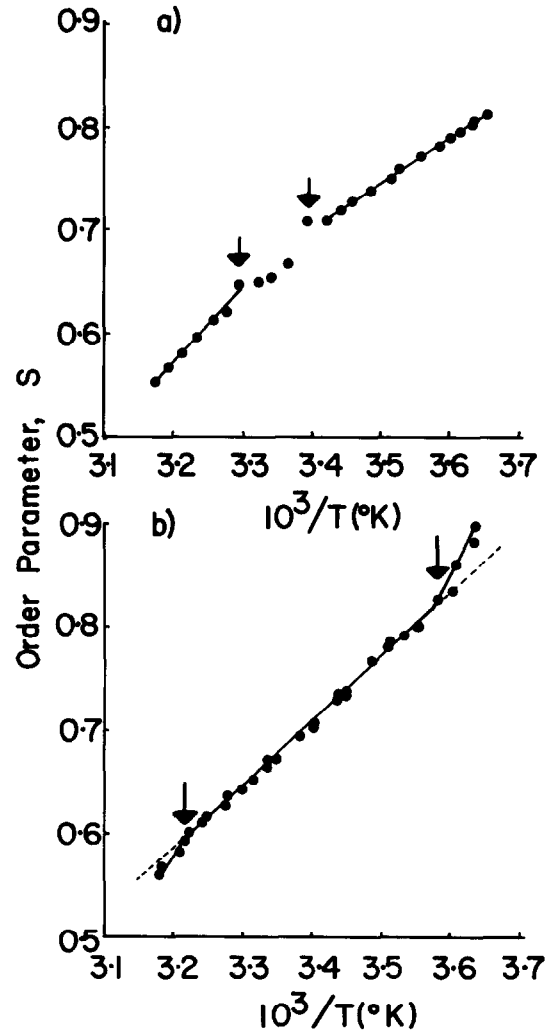

Fig.2. Arrhenius plots of the order parameter, S for spin-labelled lipid extracts from liver plasma membranes. The temperature dependence of the polarity corrected order parameter, S for extracted lipids spin-labelled with I [3,12] is shown for both unilamellar (a) and multilamellar (b) liposomes. Lipid phase separations (arrows) were identified as in [10,11]. This data shows typical experiments.

19°C [11]. Identical results could be obtained from Arrhenius plots of $2T_{\parallel}$ and the order parameter $S(T_{\parallel})$.

4. DISCUSSION

Rat liver plasma membranes exhibit a single, well-defined lipid phase separation with a high temperature onset at ~ 28°C and a low temperature onset at ~ 19°C. This has been identified using ESR techniques [10–12,15], differential scanning calorimetry [14] and other, independent physical techniques [9,13,14]. In a wide variety of membranes, Arrhenius plots of the activity of integral proteins with their functional, globular domains inserted into the bilayer exhibit breaks at temperatures which correspond to those at which lipid phase

separations occur [1–9]. Here (fig.1) we show that the Arrhenius plots of the activity of Na^+/K^+-ATPase and K^+-stimulated PNPase, a transmembrane enzyme [22], exhibits a break at ~28°C as does that for alkaline phosphodiesterase I, an ectoenzyme [23]. On the other hand, Arrhenius plots of the activity of the integral [24], low affinity cyclic AMP phosphodiesterase, which has its active site exposed at the cytosol surface of the membrane [25,26], are linear even though the activity of this enzyme does respond to changes in the fluidity of its membrane environment [11]. In [9,27] the ectoenzyme, 5′-nucleotidase exhibited a break in its Arrhenius plot at ~28°C. Furthermore Arrhenius plots of the activity of adenylate cyclase when fully stimulated by glucagon under conditions where the receptor forms a transmembrane complex with the enzyme, and presumably the guanine nucleotide regulatory protein, also exhibits a break at ~28°C [16,28,29]. However, when adenylate cyclase which is exposed at the cytosol surface of the membrane is fully stimulated with fluoride or guanine nucleotides, and is not interacting with the glucagon receptor, then, Arrhenius plots of its activity are linear [16,28,29]. These results are all consistent with our hypothesis [12] that the lipid phase separation occurring in rat liver plasma membranes, with its high temperature onset at 28°C, is localised to the external half of the bilayer. Thus Arrhenius plots of the activity of proteins that have functional, globular domains which penetrate the external half of the bilayer, such as transmembrane proteins and ectoenzymes, will exhibit a break at 28°C. However Arrhenius plots of the activities of these proteins whose functional, globular domains are localised to the cytosol half of the bilayer will not exhibit such a break. Indeed, in the case of rat liver plasma membranes they will be linear as no other lipid phase separation occurs in these membranes over 2–40°C [9–16]. Such an hypothesis demands that the phospholipids in the bilayer exhibit a marked chemical asymmetry and this has been clearly demonstrated [17]. Here the acidic phospholipids and phosphatidyl ethanolamine are found to predominate in the cytosol half of the bilayer.

The high temperature onset of the lipid phase separation is believed to be due to the formation of quasi-crystalline clusters which are intermediate in density to solid and fluid phase lipid. The low temperature onset reflecting aggregation of these clusters to form a solid lipid domain within a fluid lipid pool [10,11]. It is thus not too surprising that only the high temperature onset influences enzyme activity as proteins preferentially inhibit fluid lipid domains [30,31], whose size, composition and physical properties will alter upon cluster formation [7]. However, such parameters, and hence enzyme activity, would be unaffected by the re-arrangement or aggregation of such clusters to form a solid array within the fluid lipid matrix [11].

Similar arguments have been used to rationalise the breaks observed in Arrhenius plots of the activity of all of these enzymes in liver plasma membranes from both control and hibernating hamsters [2]. The enzymes fell into identical groups, although in hamster liver plasma membranes distinct lipid phase separations were attributed to both halves of the bilayer. Thus Arrhenius plots of the activity of both fluoride/guanine nucleotide-stimulated adenylate cyclase and also the integral cyclic AMP phosphodiesterase exhibited identical breaks, presumably reflecting a lipid phase separation occurring in the inner half of the bilayer.

If the lipid phase separation occurring between 28–19°C in these membranes is restricted to the external half of the bilayer, then the extraction of the lipids from the membrane should lead to their scrambling with the subsequent loss of the lipid phase separation. This does indeed appear to be the case, as the thermodependence of the order parameters of the spin-labelled fatty acid when incorporated into multilamellar vesicles, which would be expected to exhibit a random distribution of lipids [33,34], have no obvious lipid phase separation (fig.2). However, such an observation is in marked contrast to the lipid phase separation occurring at 28–19°C in native membranes [11] and indeed to unilamellar vesicles, made from the extracted lipids, which exhibit a well-defined lipid phase separation occurring over 30–21°C (fig.2). The result obtained using unilamellar vesicles is of some significance as the distribution of the various headgroup phospholipids between the inner and outer halves of the bilayer would not be expected to be random as in multilamellar liposomes. It has been clearly demonstrated [33,34] that the rigid constraints on packing of phospholipids in unilamellar vesicles causes a spontaneous asymmetry to ensue which is determined by the size of the headgroup of the phospholipids present. In mixtures of phospho-

lipids such as are obtained from liver plasma membranes [17], then, phosphatidyl choline and sphingomyelin would be expected to pack preferentially in the outer half of the bilayer, whereas phosphatidyl serine and phosphatidyl ethanolamine should predominate in the inner half of the bilayer. It is not unreasonable to expect that such a vertical asymmetry would mimic closely that shown to exist in native liver plasma membranes [17], which on our hypothesis gives rise to lipid phase separation at 28–19°C in the outer half of the bilayer.

Such experiments provide support for our contention that the lipid phase separation occurring between 28–19°C in liver plasma membranes is restricted to the external half of the bilayer. On the basis of the chemical asymmetry of these membranes, where the acidic phospholipids are found almost exclusively at the cytosol surface of the membrane [17] one might predict that charged fluidising agents would preferentially perturb one or other half of the bilayer. Consistent with this are our observations of the selective effects of drugs of opposite charge on both 5′-nucleotidase activity [35] and the various ligand-stimulated states of adenylate cyclase [36,37].

We would like to propose that the known chemical asymmetry of the bilayer of plasma membranes in general (see [38–41]) is reflected in a similar fluidity asymmetry. It may be possible to assess this, the degree of penetration of integral proteins into the bilayer, and the vertical asymmetry of integral proteins, both by observations of Arrhenius plots of their activity and by the sensivity of their activity to changes in membrane fluidity. The ability of a cell to exert independent control of each half of the bilayer, as has been shown in hamster liver plasma membranes [2] may allow it to modulate selectively the activity of asymmetrically orientated integral proteins.

ACKNOWLEDGEMENTS

This work was funded by grants to M.D.H. from the MRC, SRC, NATO (RG 218.80) and California Metabolic Research Foundation. We would like to thank Dr L.M. Gordon for helpful discussion and the Wellcome Trust for a travel grant (to M.D.H.). A.V.W. thanks the SRC for a research studentship.

REFERENCES

[1] Gennis, R.B. and Jonas, A. (1977) Annu. Rev. Biophys. Bioeng. 6, 195–238.
[2] Houslay, M.D. and Palmer, R.W. (1978) Biochem. J. 174, 909–919.
[3] Kimelberg, H.K. (1977) in: Dynamic Aspects of Cell Surface Organisation (Poste, G. and Nicolson, G.L. eds) Cell Surface Rev. vol. 3, pp. 205–293, Elsevier Biomedical, Amsterdam, New York.
[4] Melchior, D.L. and Stein, J.M. (1976) Annu. Rev. Biophys. Bioeng. 5, 205–238.
[5] Rimon, G., Hanski, E. and Levitzki, A. (1980) Biochemistry 19, 4451–4460.
[6] Sandermann, K. (1978) Biochim. Biophys. Acta 515, 209–237.
[7] Lee, A.G. (1975) Prog. Biophys. Mol. Biol. 29, 3–56.
[8] Silvius, J.R. and McElhaney, R.N. (1981) J. Theor. Biol. 88, 135–152.
[9] Dipple, I. and Houslay, M.D. (1978) Biochem. J. 174, 179–190.
[10] Gordon, L.M., Sauerheber, R.D. and Esgate, J.A. (1978) J. Supramolec. Struct. 9, 299–326.
[11] Gordon, L.M., Sauerheber, R.D. Esgate, J.A., Dipple, I., Marchmont, R.J. and Houslay, M.D. (1980) J. Biol. Chem. 255, 4519–4527.
[12] Houslay, M.D., Hesketh, T.R., Smith, G.A., Warren, G.B. and Metcalfe, J.C. (1976) Biochim. Biophys. Acta 436, 495–504.
[13] Hui, S.W. and Parsons, D.F. (1976) Cancer Res. 36, 1918–1922.
[14] Livingstone, C.J. and Schachter, D. (1980) J. Biol. Chem. 255, 10902–10908.
[15] Sauerheber, R.D., Gordon, L.M., Crosland, R.D. and Kuwahara, M.D. (1977) J. Membr. Biol. 31, 131–169.
[16] Houslay, M.D., Metcalfe, J.C., Warren, G.B., Hesketh, T.R. and Smith, G.A. (1976) Biochim. Biophys. Acta 436, 489–494.
[17] Higgins, J.A. and Evans, W.H. (1978) Biochem. J. 174, 563–567.
[18] Marchmont, R.J., Ayad, S.R. and Houslay, M.D. (1981) Biochem. J. 195, 645–652.
[19] Folch, J., Lees, M. and Sloane-Stanley, G.H. (1975) J. Biol. Chem. 226, 497–509.
[20] Backer, J.M. and Dawidowicz, E.A. (1979) Biochim. Biophys. Acta 551, 260–270.
[21] Houslay, M.D. and Gordon, L.M. (1982) in: Curr. Top. Membr. Transp. Membrane Receptors (Kleinzeller, A. and Martin, B.R. eds.) vol.1, Academic Press, New York, in press.
[22] Kyte, J. (1974) J. Biol. Chem. 249, 3652–3658.
[23] Evans, W.H. (1974) Nature 250, 391–394.
[24] Marchmont, R.J. and Houslay, M.D. (1980) Biochem. J. 187, 381–392.

[25] Westwood, S.J., Luzio, J.P., Flockhart, D.A. and Siddle, K. (1979) Biochim. Biophys. Acta 583, 454–466.
[26] Houslay, M.D., Wallace, A.V., Marchmont, R.J., Martin, B.R. and Heyworth, C.M. (1982) Adv. Cyclic Nucl. Res. 15, in press.
[27] Stanley, K.K. and Luzio, J.P. (1978) Biochim. Biophys. Acta 514, 198–205.
[28] Rene, E., Pecker, F., Stengel, D. and Hanoune, J. (1978) J. Biol. Chem. 253, 838–841.
[29] Houslay, M.D., Dipple, I. and Elliott, K.R.F. (1980) Biochem. J. 186, 649–658.
[30] 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.
[31] Wunderlich, F., Ronai, A., Speth, V., Seelig, J. and Blume, A. (1975) Biochemistry 14, 3730–3735.
[32] Cullis, P.R., De Kruijff, B., McGrath, A.E., Morgan, C.G. and Radda, G.K. (1976) Nobel Found. Symp. 34, 389–405.
[33] Berden, J.A., Barker, R.W. and Radda, G.K. (1975) Biochim. Biophys. Acta 375, 186–208.
[34] Buckland, B.R., Radda, G.K. and Wakefield, C.M. (1981) Biochim. Biophys. Acta 643, 363–375.
[35] Dipple, I., Gordon, L.M. and Houslay, M.D. (1982) J. Biol. Chem. 257, 1811–1815.
[36] Houslay, M.D., Dipple, I. and Gordon, L.M. (1981) Biochem. J. 197, 675–681.
[37] Houslay, M.D., Dipple, I., Rawal, S., Sauerheber, R.D., Esgate, J.A. and Gordon, L.M. (1980) Biochem. J. 190, 131–137.
[38] Etemadi, A.H. (1980) Biochim. Biophys. Acta 604, 347–422.
[39] Etemadi, A.H. (1980) Biochim. Biophys. Acta 604, 423–422.
[40] Op den Kamp, J.A.F. (1979) Annu. Rev. Biochem. 48, 47–71.
[41] Rothman, J.E. and Lenard, J. (1977) Science 195, 743–753.