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STUDIES ON THE EFFECT OF THE LIPID PHASE TRANSITION ON THE INTERACTION OF GLUCAGON WITH DIMYRISTOYL GLYCEROPHOSPHOCHOLINE

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Summary

Glucagon is found to interact with dimyristoyl glycerophosphocholine both above and below the phase transition temperature of the lipid. Above the phase transition temperature the interaction is manifested by an increase in the rate of vesicle aggregation and by an increased permeability of unilamellar vesicles to Eu^{3+} and to $Fe(CN)_6^{3-}$. However, no stable lipoprotein complex can be detected by gel filtration. Below the phase transition glucagon can form stable complexes with dimyristoyl glycerophosphocholine vesicles but cannot rapidly rearrange these vesicles to disk-shaped particles until the phase transition temperature is approached. The energy of activation for the dissociation of glucagon from the disk-shaped lipoprotein particle is 29 kcal/mol at temperatures above 36°C but increases markedly at lower temperatures, as the region of the lipid phase transition is approached. This increase in energy of activation at lower temperatures is most probably due to the larger amount of energy required to rearrange gel-state lipid in the transition state and provides an explanation for the unusual kinetic stability of the glucagon-dimyristoyl glycerophosphocholine lipoprotein complex only at temperatures below the phase transition of the lipid.

Introduction

Recent studies on the effect of the phospholipid phase transition on protein-lipid interactions have confirmed that proteins interact preferentially with lipid in the liquid-crystal state and are excluded from domains of gel state lipid [1]. There are, however, several peptides [2], glucagon being the most noteable examples [3], which appear to interact more strongly with gel-state lipid. In

Abbreviation: TES, *N*-tris-(hydroxymethyl)methyl-2-aminoethane sulfonic acid.

this work we investigate the causes for the unusual stability of complexes of glucagon and dimyristoyl glycerophosphocholine below the major phase transition temperature of the lipid.

Materials and Methods

Dimyristoyl glycerophosphocholine (Sigma Chemical Co.) showed a single spot when visualized with iodine vapors after thin-layer chromatography. Glucagon was purchased from the Elanco Corp. and europium chloride from Fluka A.G. (puriss grade).

Preparation of unilamellar vesicles. Dimyristoyl glycerophosphocholine was dissolved in chloroform and the solvent was evaporated under a stream of nitrogen while vortexing so as to deposit a film of lipid on the walls of a glass test tube. Final traces of solvent were removed by drying for at least 1 h under high vacuum with a liquid nitrogen trap. The lipid was suspended in 0.1 M ammonium acetate or in ^2H_2O by vortexing at 40°C for 1 min. The resulting suspension was sonicated at 40°C for 1 h under nitrogen in a Bransonic 12 bath-type sonicator. The resulting transparent solution was centrifuged at 4°C for 2 h at 20 000 rev./min (approx. 48 000 $\times g$) in a Sorval centrifuge with an SS-34 rotor. The low temperature was chosen to minimize the partial specific volume of the lipid and only the supernate in the top portion of the centrifuge tube was withdrawn for subsequent use [4].

Preparation of the glucagon-dimyristoyl glycerophosphocholine lipoprotein complex. Solutions of glucagon (approx. 0.1 mg/ml) were prepared by centrifugation of suspensions of the hormone in 0.1 M ammonium acetate, pH 7.4, which had been briefly warmed to 40°C to accelerate dissolution. The centrifugation was done at room temperature using the top speed of a clinical centrifuge. An aliquot of this solution was added to a lipid film, prepared as described above, and vortexed at 40°C. The lipid was dissolved at a molar ratio of lipid to glucagon of approximately 30 : 1 by cooling the suspension in an ice bath. The lipoprotein particle was then saturated with lipid (except for the gel filtration experiments) by transferring this solution to a second lipid film, briefly warming to 40°C to suspend the lipid film with vortexing and recooling in an ice bath. Excess lipid was removed from the resulting suspension by centrifugation at 4°C.

Right-angle light scattering. The relative intensity of light scattered at 90° from solutions or suspensions containing dimyristoyl glycerophosphocholine was measured with a Perkin-Elmer MPF-44 fluorimeter in the ratio mode at 450 nm using 2-nm slit widths and a standard 1 cm pathlength sample cell.

Gel filtration of glucagon-lipid mixtures on Sephadex G-75. 2-ml samples of glucgon/lipid mixtures were applied to a 20 × 1.5 cm column equilibrated in an environmental chamber at the specified temperature. The column was eluted with 0.1 M ammonium acetate, pH 7.4, and 1.62 ml fractions (25 drops) were collected. Unilamellar lipid vesicles (not centrifuged) were passed through the column to saturate any phospholipid binding sites and then a sample of saturated glucagon solution was applied. The void volume was marked with lipid vesicles as well as dextran blue and the glucagon eluted in the column volume.

Effect of Eu^{3+} on the proton magnetic resonance (PMR) spectrum of the lipid. Small volumes of a solution of $EuCl_3$ in ^2H_2O were added to unilamellar vesicles in ^2H_2O, prepared as described above, after the pH of the solution was adjusted to 7. PMR spectra were obtained from a Bruker WH 90 fourier transform spectrometer operating at 90 MHz. Each spectrum was obtained with 4 (90°) pulses. A Bruker B-ST 100/700 Temperature Controller was used to maintain the sample temperature at 37 ± 1°C.

Reduction by ascorbate of ferricyanide entrapped in unilamellar lipid vesicles. Unilamellar vesicles were prepared by sonication of the lipid in the presence of 0.2 M potassium ferricyanide/0.02 M *N*-tris-(hydroxymethyl)-methyl-2-aminoethane sulfonic acid (TES)/1 mM ethylenediamine tetracetate (EDTA), pH 7.40. Ferricyanide entrapped in vesicles was isolated by passage of the sonicate through a 20 cm column (1.5 cm diameter) packed with Sephadex G-75 using 0.02 M TES/1 mM EDTA/0.3 M NaCl, pH 7.40, as eluant. The vesicles were collected in the void volume and centrifuged. 200 μl of the ferricyanide entrapped in vesicles were added to 1.0 ml of 5 mM ascorbic acid (made fresh daily, to avoid air oxidation)/0.02 M TES/1 mM EDTA/0.3 M NaCl, pH 7.40. The rate of reduction of the ferricyanide by ascorbate was monitored by measuring the decrease in absorbance at 420 nm using a Cary 118 double-beam spectrophotometer with 1 cm pathlength cells and a thermostated cell holder. The observed absorbance readings were corrected for light scattering by extrapolation of log *A*-log λ curves which were constructed from absorbance readings in the region 500—700 nm. The correlation coefficients of these plots were generally 0.99.

Rate of decay of enhanced tryptophan fluorescence from glucagon-dimyristoyl glycerophosphocholine lipoprotein particles. Fluorescence emission at 340 nm was measured as a function of time with a Perkin-Elmer MPF-44 spectrofluorimeter in the ratio mode using 1 cm pathlength cells, 295 nm excitation and 2 nm slit widths. Buffer (1.8 ml) was pre-equilibrated to the desired temperature. The solution of lipoprotein (0.2 ml) stored in an ice-bath between runs, was briefly warmed to room temperature and added to the large excess of buffer in the thermostated cell compartment. The initial fluorescence intensity from this solution was 1.9 times that of a solution of equivalent glucagon concentrations, after correcting for solvent background. No inner filter corrections were needed for the low glucagon concentrations used. At higher temperatures the decay was too rapid to measure the initial values and first order rate plots were constructed from data obtained from the earliest time possible after mixing (within 1 min). The final intensity reached after long times was identical to that for free glucagon. In general, the decay was followed over the disappearance of about 70% of the starting material and the decay fitted well to a single first-order rate process, with correlation coefficients for plots of log concentration versus time greater than 0.99. At 25 and 26°C the rate of decay was very slow and the process was followed for the disappearance of less than 10% of the starting material. It is possible that these rates are slower because of a back reaction of glucagon with lipid, i.e. a change in the position of equilibrium with temperature so that at the lower temperatures the equilibrium position is not completely toward dissociation. However, this possibility is made less likely because: (1) The initial free glucagon concentra-

tion is zero, so that there is no back reaction at zero time. (2) Within the time the reaction was monitored at the lower temperatures the rate of decay was approximately constant with no evidence for a slowing down of the reaction with time as would be expected if the position of equilibrium had been drastically shifted toward the lipoprotein particle. (3) Between 26 and 32°C the decay was monitored over a substantial fractional loss of starting material and these data already show the deviation from the Arrhenius plot which is continued at the lower temperature.

Glucagon concentration. This was determined spectrophotometrically using $E_{1\,cm}^{1\,mg/ml} = 2.38$ at 278 nm [5] after correction for light scattering, when required, by a plot of log A vs. log λ and by the Lowry assay [6] using glucagon as a standard and containing 0.5% sodium dodecyl sulfate (Pierce, Sequanal grade) to dissolve and lipid.

Phospholipid concentration. This was determined by measured of total phosphate by the procedure of Bartlett [7] after perchloric acid ashing at 180°C.

Results

Right angle light scattering has been used to monotor phase transitions in aqueous dispersion of unilamellar phospholipid vesicles [8]. With lipid alone a small increase in scattering intensity is observed at the phase transition temperature on cooling and this is largely reversible on reheating (Fig. 1A). The behavior of the glucagon/dimyristoyl glycerophosphocholine (Fig. 1B) is quite different however. The scattering intensity at the phase transition temperature for the cooling curve is over 100 times greater than that for the unilamellar vesicles and there is a very large difference between the heating and cooling

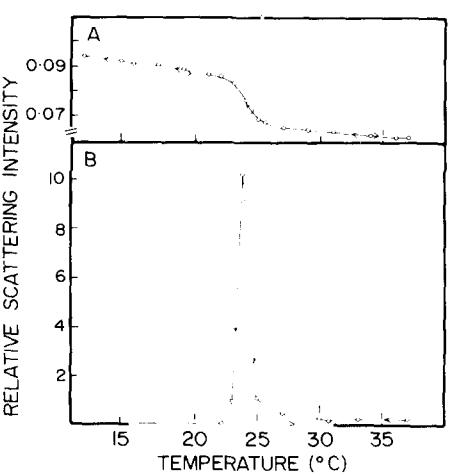

Fig. 1. Temperature dependence of intensity of light scattered at 90° from unilamellar vesicles of dimyristoyl glycerophosphocholine (A) or the glucagon-dimyristoyl glycerophosphocholine lipoprotein particle (B). Wavelength of incident light, 450 nm. Temperature sweep rate, approximately 1°/min (manually controlled). 0.1 M ammonium acetate, pH 7.40, 1.5 mM dimyristoyl glycerophosphocholine (A and B), 25 μM glucagon (B). Note difference in magnitude of ordinates. Arrows indicate direction of scan. Dotted curve is heating scan (where not superimposable).

scans for the lipoprotein. This observed hysteresis in the scattering curves can be repeated more than once with the same sample of lipoprotein.

Changes in right-angle light scattering were also measured after addition of the lipoprotein to unilamellar vesicles. Both the rate and the extent of increase of light scattering is much greater when the lipoprotein is added to vesicles at 24°C compared to when it is added to buffer at this temperature (Table I). This suggests that some of the vesicles are being incorporated into very large aggregates. The results also demonstrate that at 35°C glucagon can cause vesicle fusion and/or aggregation. At 17°C no increase in light scattering is observed although the final lipid to protein ratio is greater than that observed for the glucagon lipoprotein particle [9].

Although spectroscopic evidence indicated that glucagon interacted with phospholipid only in the gel state [3], the ability of the hormone to effect vesicle fusion above the phase transition temperature led us to re-examine the interaction by gel filtration as a function of temperature. A solution of the glucagon-dimyristoyl glycerophosphocholine lipoprotein complex, not saturated with lipid, was centrifuged at 20°C. The resulting solution was warmed to 37°C for 15 min to allow the lipoprotein to dissociate and then analyzed on a Sephadex G-75 column at 37, 24 or 20°C (Fig. 2 and Table II). In the case of the solution cooled to 24°C, a large amount of precipitate was removed by centrifugation in an Eppendorf Model 3200 at this temperature before the sample could be applied to the G-75 column. The precipitate was resuspended in buffer and centrifuged at 15°C. The resultant supernate from this centrifugation contained 30% of the lipid originally solubilized by the glucagon at a lipid to glucagon ratio of 46. Thus, significant amounts of both

TABLE I

EFFECT OF GLUCAGON OR ITS LIPOPROTEIN COMPLEX ON VESICLE FUSION AND AGGREGATION

After mixing at the indicated temperature the intensity of light scattered at 90° was measured as a function of time. 0.1 M ammonium acetate, pH 7.4. The final concentration of glucagon is 6.4 μM in all cases while that of the dimyristoyl glycerophosphocholine from the lipoprotien complex is 0.3 mM.

Temperature (°C)	Form of glucagon	Concentration of vesicles (mM)	Rate of increase in intensity of scattered light (%/min)
24	Lipoprotein complex (after incubation for 15 min at 35°C)	1.3	105
		0	67
24	Lipoprotein complex (stored in ice)	1.3	0.4
24	Glucagon solution	1.3	13
35	Lipoprotein complex (after incubation for 15 min at 35°C)	1.3	3
		0	0
35	Glucagon solution	1.3	3
17	Lipoprotein complex (stored in ice)	1.3	0
17	Glucagon solution	1.3	0

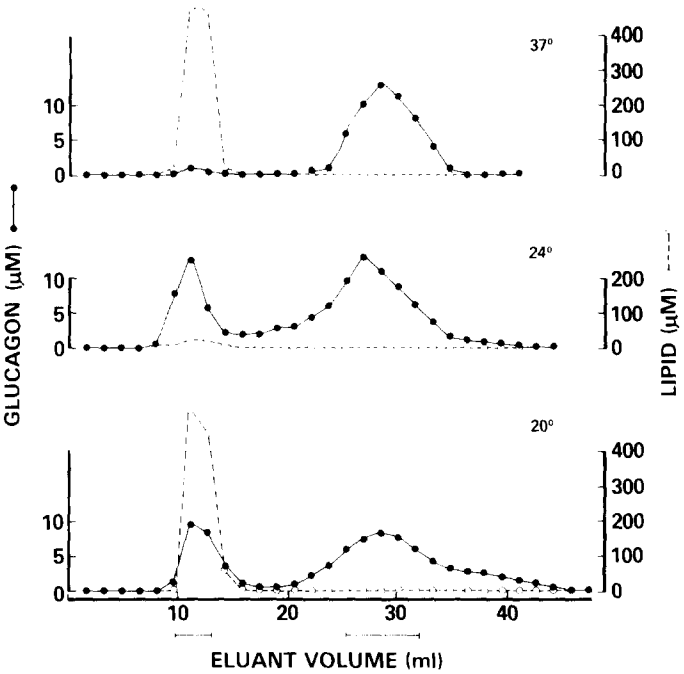

Fig. 2. Elution pattern for glucagon and lipid from a 20 × 1.5 cm column of Sephadex G-75. A solution of glucagon and dimyristoyl glycerophosphocholine was prepared by centrifugation at 20° C, heated to 37° C and analyzed by gel filtration at the temperature indicated in the figure. The 24° C sample represents the material which did not precipitate at this temperature. The bars at the bottom of the figure mark the void volume and column volume.

TABLE II

SEPHADEX G-75 CHROMATOGRAPHY OF GLUCAGON/DIMYRISTOYL GLYCEROPHOSPHOCHOLINE MIXTURES

0.1 M ammonium acetate, pH 7.4.

Temperature (° C)	Concentration of sample applied to column (μM)		% of Total recovered glucagon [b]		Phosphate/glucagon Molar ratio	
	Glucagon	Glucagon--solubilized lipid	Void volume	Column volume	Void volume	Column volume
20	30	800	50	50	53	0.25
24	35 [a]	185 [a]	20	80	5	0.1
37	25	900 Unilamellar vesicles	9	91	360	0.07
4	35	2000	76	24	106	n.d.
37	35	2000	6	94	800	n.d.

[a] After centrifugation to remove precipitate at 24° C.

[b] Total glucagon recovered averaged 103 ± 10% except for the run with vesicles at 4° C which was 70%, possibly because of some precipitation of glucagon on the column.

n.d., not determined.

glucagon and lipid are incorporated in the precipitate formed at 24°C. In addition, glucagon and unilamellar lipid vesicles were mixed at either 4 or 37°C and immediately passed through the Sephadex column at the same temperature (Table II). In all cases all of the lipid was recovered in the void volume and only traces of phosphate were associated with the glucagon which ran in the column volume. The estimates for the percent of glucagon in the void and column volume fractions are precise to only ±10% as the concentration of glucagon is low and the total amounts eluted for each peak are summed over several fractions.

Since glucagon can effect vesicle fusion above the transition temperature, presumably by exposing the hydrophobic regions of the bilayer, this process may also sufficiently rupture the vesicle so as to make it permeant to certain paramagnetic ions. It has already been demonstrated that below the phase transition temperature lipid head groups are completely exposed to Mn^{2+} by glucagon [10]. The paramagnetic ion Eu^{3+} is impermeant to phospholipid vesicles and its interaction with phospholipid head groups results in a shift of the PMR resonance line of the effected lipid to a new position. The PMR spectrum of unilamellar diacyl glycerophosphocholine vesicles is thus altered by having the resonance of the quaternary ammonium methyl protons split into two resolvable peaks with those correnspounding to the outer layer of the vesicle shifted to a new position [11]. In the case of dimyristoyl glycerophosphocholine incubated at 37°C with $2 \cdot 10^{-2}$ M $EuCl_3$ in the presence and absence of glucagon, the fraction of the initial quaternary ammonium peak which is shifted to a new position increases with increasing amounts of glucagon (Table III). In the absence of glucagon there are no time-dependent changes in the ratio of the peaks and as expected, somewhat more than half of the peak is shifted as the external layer of the bilayer contains more lipid molecules than the internal one. The value of the fraction of protons shifted in the absence of glucagon is comparable to that found previously with egg lecithin [11]. However, this figure is somewhat lower than the value of 0.76 reported for dimyristoyl glycerophosphocholine using paramagentic shift reagents with ^{31}P magnetic resonance spectroscopy [12]. The presence of some multilamellar structures in our preparation or some incompletely sealed vesicles in the ^{31}P work caused by more vigorous sonication could account for these differences.

TABLE III

EFFECT OF GLUCAGON ON FRACTION OF LIPID EXPOSED TO THE PARAMAGNETIC SHIFT REAGENT $EuCl_3$.

37°C, D_2O, pH meter reading 7, 20 mM $EuCl_3$, 5 mM dimyristoyl glycerophosphocholine.

Lipid/glucagon	Fraction of quaternary ammonium methyl protons undergoing Eu^{3+}-induced chemical shift
∞ *	0.55
830	0.58
280	0.65
165	0.68
110	0.84

* ∞, Absence of glucagon.

The point we wish to emphasize in the present work is the ability of glucagon to expose more lipid to the paramagentic shift reagent (Fig. 3). In the presence of glucagon, the lipid and peptide were mixed after both solutions had been brought to 37°C and the ratio of the two peaks was measured shortly after mixing. Subsequent change in the ratio of the peaks occurred very slowly and no attempt was made to quantitate this slower phase. The kinetics of this process may be complicated by glucagon-induced vesicle aggregation, although neither visual turbidity nor marked broadening of the spectra were observed. Serum lipoproteins are also permeable to paramagnetic ions [13].

Ferricyanide anion and ascorbic acid are also impermeant to phospholipid bilayers and they provide a convenient tool for monitoring the rupture of vesicles as the reduction of ferricyanide by ascorbate can be readily monitored spectrophotometrically [14]. In the absence of glucagon at 37°C, there was a very slow rate of reduction of entrapped ferricyanide by external ascorbate which followed first-order kinetics and was followed over 70% of the reaction. In the presence of glucagon, however, the kinetics were more complex. As in the case of the Eu^{3+} studies, there was a rapid burst followed by a slower phase which itself did not follow a first-order decay and never reached completion even after several hours. The scattering correction was larger for the runs containing glucagon and in contrast to the vesicles alone, its magnitude increased with time, because of glucagon-induced vesicle aggregation. In order to qualita-

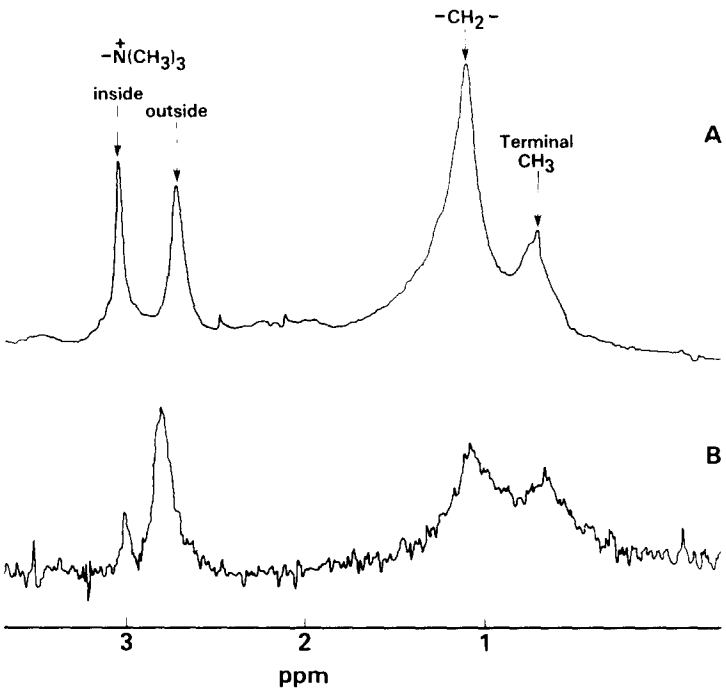

Fig. 3. Proton magnetic resonance spectra of a sonicated preparation of dimyristoyl glycerophosphocholine containing $EuCl_3$ and D_2O at pH 7, 37°C in the presence (B) and absence (A) of glucagon. Spectra are the average of 36 transients. A, 8 mM lipid, 0.07 M $EuCl_3$; B. 2.4 mM lipid, 0.02 M $EuCl_3$, 22 μM glucagon.

tively compare the rates of reduction of ferricyanide in the presence and absence of glucagon, we have summarized the data by listing the fraction of ferricyanide observed as soon as possible after mixing, as well as the rate constant for the disappearance of this ferricyanide obtained from the initial linear portion of the first-order rate plot (Table IV). The percent of ferricyanide initally detected is taken at an arbitrary time point (i.e. the 'initial' value) and should not be considered to have any quantitative significance at the lowest lipid to glucagon ratio at 23 and 37°C where the disappearance was most rapid. The glucagon was effective in making the vesicle permeant at or above the phase transition temperature. When glucagon in ascorbate was mixed below the phase transition temperature with ferricyanide entrapped in vesicles, no rapid reduction of the ferricyanide took place until the phase transition temperature was approached (Fig. 4).

The rate of decomposition of the glucagon-dimyristoyl glycerophosphocholine lipoprotein complex was measured by monitoring the rate of loss of fluorescence enhancement at a particular temperature. The first-order rate constants obtained from these experiments were plotted on an Arrhenius plot (Fig. 5). Above 36°C the energy of activation for the dissociation of glucagon from the lipoprotein particle is 28.8 ± 1.5 kcal/mol. At lower temperatures, however, the Arrhenius plot deviates markedly from a linear relationship and below 28°C the energy of activation is 1.6 (± 0.3) · 10^2 kcal/mol, an unusually high value for a chemical reaction. This energy of activation is still probably lower than that corresponding to the gel state, as the Arrhenius plot is not likely to be linear until below 24°C. However, because of the very long half-times for reaction at the low temperatures, data could not be obtained in this region. The entropy of activation is 0.28 ± 0.01 e.u. and 1.47 ± 0.01 e.u. for

TABLE IV

EFFECT OF GLUCAGON ON THE REACTION OF EXTERNAL ASCORBATE WITH FERRICYANIDE ENTRAPPED IN UNILAMELLAR VESICLES OF DIMYRISTOYL GLYCEROPHOSPHOCHOLINE

20 mM TES, 1 mM EDTA, 0.3 M NaCl, 1 mM dimyristoyl glycerophosphocholine.

Temperature (°C)	Lipid/glucagon Molar ratio	Initial [a] first order rate constant (s^{-1})	% of Ferricyanide initially detected after mixing
37	∞ [d]	$1.9 \cdot 10^{-4}$	100 [b]
	320	$5.8 \cdot 10^{-3}$	81
	160	$4.9 \cdot 10^{-3}$	56
	65	$1.9 \cdot 10^{-2}$	28 [a]
23	∞	0 [c]	100 [b]
	65	$1.3 \cdot 10^{-2}$	33 [a]
15	∞	0 [c]	100 [b]
	65	0 [c]	82

[a] See text.

[b] Solutions with glucagon compared with this value taken as 100%. There is a 10% loss of fericyanide initially after mixing with ascorbate and this fraction does not change with storage of the vesicles during the course of the experiment.

[c] Rate too slow to quantitate; much slower than at 37°C.

[d] ∞, Absence of glucagon.

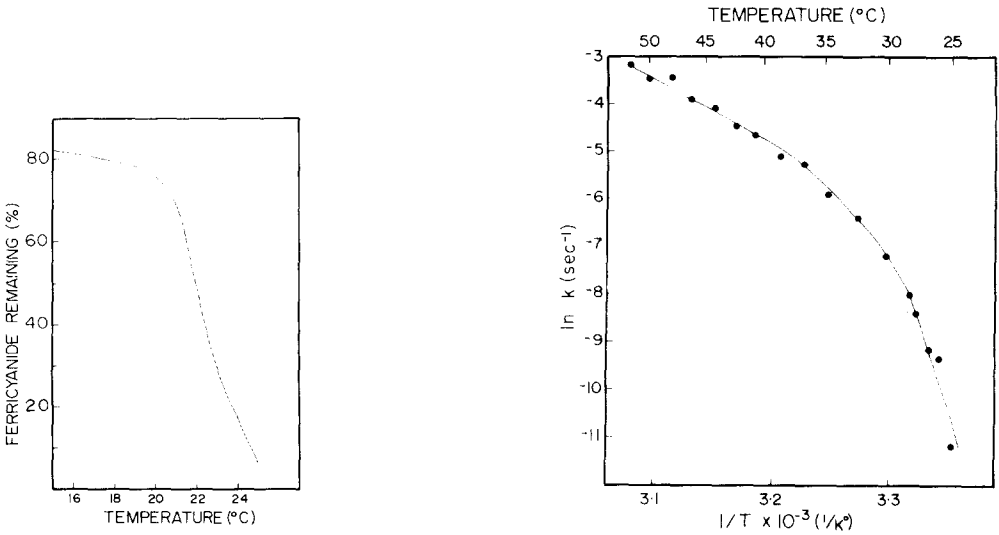

Fig. 4. Effect of increasing temperature on the fraction of ferricyanide entrapped in vesicles of dimyristoyl glycerophosphocholine which becomes exposed to external ascorbate in the presence of glucagon. 20 mM TES, 1 mM EDTA, 0.3 M NaCl, 1 mM dimyristoyl glycerophosphocholine, 15 μM glucagon. Temperature sweep rate approximately 0.5° C/min.

Fig. 5. Arrhenius plot for the rate of dissociation of the glucagon-dimyristoyl glycerophosphocholine lipoprotein complex. 0.1 M ammonium acetate, pH 7.4, 3.6 μM glucagon, 200 μM lipid.

the low and high temperature limits, respectively. The free energy of activation is thus due largely to the enthalpy term and, in fact, the entropy of activation is favorable. This may be due in part to a decreased structuring of the peptide in the transition state.

Discussion

Glucagon can interact with dimyristoyl glycerophosphocholine below the phase transition temperature to form disk-shaped particles [9] in which at least some of the glucagon must be at the rim of the disc to shield the acyl chains of the phospholipid (Fig. 6). When glucagon dissociates from the particle as the temperature is raised above the phase transition temperature [2], an unstable intermediate is formed with fatty acid chains exposed to water. This lipid must be shielded from the aqueous solvent either by interaction with glucagon to reform the lipoprotein particle, by intraparticle rearrangement of the lipid to a vesicular form or by interaction of the exposed acyl chains with other lipid structures to form high molecular weight aggregates. The latter path requires free lipid for the intermediate to interact with and would occur to a smaller extent on the heating scan where no free lipid is initially present when the lipoprotein particle begins to dissociate. In contrast, above the phase transition temperature most of the glucagon is dissociated from the lipid. As the phase transition temperature is approached from higher temperatures, a few lipoprotein particles begin to form in the presence of a large proportion of free lipid. As glucagon can readily dissociate from these particles above the phase

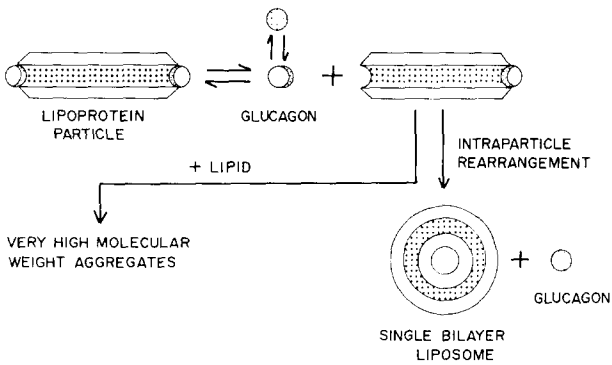

Fig. 6. Schematic representation of the decomposition of the glucagon-dimyristoyl glycerophosphocholine lipoprotein particle. Diagrams represent cross-sections of the disk-shaped lipoprotein (side view) and the spherically-shaped liposome. Hydrophobic areas are stippled. Glucagon is represented both as an amphipathic helix with the hydrophobic groups segregated to one zone and also as the free peptide in solution with the hydrophobic residues more evently distributed.

transition temperature, exposing the hydrophobic regions of the lipid in the presence of other lipid particles, fusion of lipid particles to form higher molecular weight aggregates will be more common and increased light scattering will result (Fig. 1). Such a process would also explain why light scattering increases to a greater extent when the lipoprotein dissociates in the presence of unilamellar vesicles. Below the phase transition temperature, however, these vesicles have no effect because the glucagon is not exchanging rapidly among lipid structures (Table I). In this simplified scheme, we do not wish to imply that these steps occur sequentially but, more likely, they occur in a concerted fashion.

Glucagon can also interact with phospholipid vesicles above the phase transition temperature, as demonstrated by its ability to induce vesicle fusion and to increase the permeability of the vesicle to Eu^{3+} (Fig. 3 and Table III) and $Fe(CN)_6^{3-}$ (Table IV). It does not, however, give rise to stable lipid complexes which can be separated by gel filtration (Fig. 2) nor are the spectral properties of glucagon greatly altered by lipid above the phase transition temperature [3]. In the gel filtration runs a major fraction of the glucagon is associated with lipid in a high molecular weight form only below the phase transition temperature of 24°C, while at 37°C only a trace of glucagon is associated with this void volume fraction. The small amount of glucagon eluting in the void volume at 37°C may arise from entrapment of the peptide due to lipid fusion and aggregation which we have shown to occur at this temperature in the presence of glucagon. There is also a trace of phosphate associated with the glucagon that runs in the column volume but its significance is uncertain. Because of the low glucagon concentrations in these solutions large sample volumes of 1.0 ml had to be taken for phosphate analysis and although blanks contained equal amounts of buffer, trace phosphate contamination could occur. Some phosphate contamination comes from the glucagon sample itself which was found to have 0.014 mol inorganic phosphate/mol glucagon. Thus, as expected for a diacyl phospholipid which forms stable bilayers, no binding of single phospholipid molecules by the hormone could be detected. Glucagon must, therefore,

associate and rapidly dissociate from vesicles at temperatures above the phase transition. This process is likely to leave an exposed rim of lipid on the vesicle, similar to that which is left after glucagon dissociates from a lipoprotein particle (Fig. 6). These beginnings of a conversion of the vesicle to a flat disk would leave the lipid in a state where it would be leaky and prone to aggregate as we have found.

Even when mixed below the phase transition temperature, glucagon can rapidly associate with vesicles as shown by gel filtration (Table II) and by the enhancement of glucagon fluorescence (unpublished observations). Nevertheless, glucagon cannot rapidly render the vesicle permeant to ferricyanide until the phase transition temperature is approached (Fig. 4), suggesting that the activation energy for the rearrangement of gel-state lipid by glucagon is too great to allow the formation of an exposed rim. It should be noted that the phase transition of unilamellar vesicles has been found to be broader and shifted to somewhat lower temperatures compared with multilamellar vesicles [12,15,16]. Similarly, apolipoprotein A-1 does not rapidly solubilize phospholipid in the gel state until the phase transition temperature is approached [17] and phospholipase A_2 does not rapidly act upon gel-state liposomes unless defects are present [18].

The greater energy required to rearrange the gel state also provides a ready explanation as to why glucagon forms stable complexes with lipid only below the phase transition temperature. When glucagon dissociates from the rim of the lipoprotein particle, the hydrophobic interior of the lipid bilayer is exposed to the aqueous environment and must be shielded by rearrangment of the lipid. This lipid rearrangement can occur more readily in the liquid crystalline state than in the more stable gel state. This results in a larger energy difference between the lipoprotein particle and the transition state for its dissociation below the phase transition temperature than above it. An alternative explanation is that the enthalpy of interaction of the peptide with lipid is greater in the gel state than the liquid crystalline state. This alternative is much less likely as there is no reason to expect glucagon to be different from what has been found for many other protein-lipid interactions which occur more readily with lipid in the liquid-crystalline state [1,19,20]. Thus, the energy of activation for the dissociation of a lipoprotein particle involves both the breaking of lipid-lipid interactions to allow for rearrangement of the bilayer to begin to cover exposed hydrophobic areas as well as the breaking of lipid-protein interactions. We conclude that the glucagon lipoprotein has unusual kinetic stability in the gel state as a result of the large activation energy required to rearrange lipid at these temperatures. This does not occur with lipoprotein particles formed with the serum apolipoproteins [21] presumably because of the larger energy of interaction between lipid and protein in this case so that the breaking of lipid-protein bonds makes a larger contribution to the activation energy for the dissociation of these particles resulting in their stability above the phase transition temperature. However, for proteolytic fragments of serum apolipoproteins, the phase transition temperature of the phospholipid or the cholesterol content of the partcle may determine the rate at which these peptides are transferred to cell membranes. The result of their interaction with cell membranes may be to damage them by increasing their permeability as occurs with

glucagon upon interaction with phospholipid vesicles in the liquid crystalline state. In particular, in the case of glucagon it may provide a mechanism for the peptide to escape from pancreatic storage granules by a means which is not under physiological control.

Acknowledgement

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