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STUDIES ON THE COMPLEX FORMED BETWEEN GLUCAGON AND DICAPRYLPHOSPHATIDYLCHOLINE

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The interaction between glucagon and dicaprylphosphatidylcholine (DCPC) was studied by fluorescence, circular dichroism and calorimetry, as well as by ^{1}H- and ^{31}P-nuclear magnetic resonance. The water-soluble lipid-protein complex was also characterized by gel filtration and ultracentrifugation. The complex appeared to be monodisperse by sedimentation equilibrium measurements, with a molecular weight of $(4.55 \pm 0.57) \cdot 10^4$. This complex contained approximately 7 molecules of glucagon and 35 molecules of phospholipid. Proton-decoupled ^{31}P-NMR spectra of the phospholipid in the lipid-protein complex display narrower resonances than those of sonicated vesicles of DCPC, and ^{1}H-^{31}P coupling could be detected in proton coupled spectra. These NMR results, together with gel-filtration results, suggest that glucagon 'solubilizes' phospholipid aggregates, forming a lipid-protein complex which is smaller than sonicated preparations of DCPC. ^{1}H-NMR resonance of both the methionine methyl group (met-27) and the aromatic envelope of glucagon are broadened by the phospholipid, indicating that the C-terminal region and the aromatic residues are involved in the interaction with the phospholipid. Nuclear magnetic resonance titrations of the imidazole ring C(2) and C(4) protons of the histidine residue of glucagon show that DCPC lowers the pK of the imidazole. The alterations caused by the phospholipid in the far and near ultraviolet CD spectra of glucagon reflect, respectively, the increased helix content of the hormone and the fact that the aromatic residues are located in a more structured environment. The phospholipid also alters the fluorescence properties of glucagon, shifting the fluorescence emission maximum of the hormone to shorter wavelength, and enhancing its relative intensity. This suggests that the fluorophore is experiencing a more hydrophobic environment in the presence of the lipid. Binding of glucagon to the phospholipid was analysed by Scatchard plots of the enhancement of fluorescence caused by the phospholipid and showed that the equilibrium binding constants of glucagon to DCPC are $(4.4 \pm 0.5) \cdot 10^4$ M^{-1} and $(7.5 \pm 0.5) \cdot 10^4$ M^{-1}, at 15°C and 25°C, respectively. The average number of moles of phospholipid bound per mole of glucagon is 4.4 ± 0.6. The isothermal enthalpy of reaction of glucagon with DCPC is -20.5 kcal/mol of glucagon at 25°C and -32.5 kcal/mol of glucagon at 15°C. The observed enthalpies can arise from glucagon-induced cyrstallization of the phospholipid, from the non-covalent interactions between the peptide and lipid as well as from the lipid-induced conformational change in the protein. These results demonstrate that, unlike the complexes formed between glucagon and phospholipids which form more stable bilayers, the complex formed between glucagon and DCPC is stable over a wide range of temperatures, including temperatures well above the phase transition.

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Abbreviations: DCPC, 1,2-dicapryl-*sn*-3-glycerophosphocholine; Pipes, piperazine-*N*,*N*′-bis(ethanesulfonic acid); *C*, concentration of glucagon-DCPC complex; *r*, distance from the center of the rotor in the analytical centrifuge.

Introduction

Glucagon is a 29-amino acid polypeptide hormone of known sequence [1]. The hormone can interact with bilayer-forming phospholipids [2–4] as well as with detergents [5–8], inducing a conformational change in the hormone. The ability to interact with phospholipids is a common feature of a number of peptide hormones about the same size as glucagon, which have regularly spaced hydrophobic amino acids at every third or fourth residue [9]. The interaction of glucagon with phospholipids is particularly sensitive to phase transition phenomena [3,10,11], with the peptide dissociating from the lipid above the phase transition temperature. The complex between glucagon and phospholipid in the gel state appears to be kinetically but not thermodynamically stable [12,13]. In the present study, we examine the complex formed between glucagon and dicaprylphosphatidylcholine (DCPC), a phospholipid with short acyl chains and weaker hydrophobic lipid-lipid interactions. We find this complex to be stable over a wide range of temperatures well above the temperature of the phospholipid phase transition. The conformation of glucagon in the complex, as well as the size, stoichiometry and thermodynamics of formation of the glucagon-DCPC complex is compared with complexes between glucagon and phospholipids forming more stable bilayers or between glucagon and detergents.

Materials

Crystalline bovine-porcine glucagon was purchased from Elanco Corp. and used without further purification. The purity of this preparation has previously been analyzed [14]. Dicaprylphosphatidylcholine was purchased from Calbiochem Co. and Avanti Polar Lipid Inc., and their purity was checked by thin-layer chromatography (chloroform/methanol/water, 65 : 35 : 5) on silica gel H and visualized with iodine vapor. The fatty content was analyzed by gas-liquid chromatography. Lysophosphatidylcholine was detected with iodine vapor as a minor contaminant of the product from Calbiochem. No impurity was detected in the product from Avanti. Control experiments showed that the spectroscopic properties of the lipid-protein complex are similar with either phospholipid sample. Deuterium oxide was purchased from Stohler Isotopic Chemicals. Twice-distilled water was used throughout. All other reagents were analytical grade.

Methods

Preparation of the glucagon-DCPC complex. A suspension of glucagon crystals in 20 mM Pipes/1 mM EDTA/150 mM NaCl/0.02 mg/ml NaN_3 at pH 7.4 (Pipes buffer), was added to a vortexed dispersion of DCPC to give a final molar ratio of 5–7 phospholipids per protein. These suspensions were then vortexed for 1–2 min and warmed to 50°C. The pH of the resulting solution was readjusted, when necessary, to pH 7.4 and the undissolved material was removed by centrifugation in a Sorvall centrifuge at 15000 rpm in a SS-34 rotor at 20°C for 20 min. For the gel-filtration experiments, the lipid-protein complex was prepared by adding a suspension of glucagon to sonicated vesicles of DCPC. Similar results were obtained when vortexed dispersions of DCPC were used instead of sonicated vesicles. The resulting supernatant contained a homogeneous population of low-molecular-weight lipoprotein complex (see below). The concentration of free glucagon was determined using $E^{1\ mg/ml}_{1\ cm} = 2.38$ [15]; and the concentration of glucagon in solutions of the complex was determined using $E^{1\ mg/ml}_{1\ cm} = 2.12$ [2]. The protein determination in the gel-filtration experiments was carried out by a modification of the method of Lowry et al. [16], using as a standard a glucagon solution, the concentration of which was determined spectrophotometrically. Turbidity caused by undissolved lipid was eliminated by adding 0.5% sodium dodecyl sulfate to the samples. The lipid concentration was determined by the method of Bartlett [17], after ashing the samples with perchloric acid. In the absence of DCPC at pH 7.4, only 0.10 mg/ml of glucagon is detected in a saturated solution of the hormone. However, up to 15 mg/ml of glucagon can be detected in the supernatant in the presence of phospholipid. Glucagon is known to form fibrils and gels at high concentrations in acid and basic solutions [18–20]. However, far-ultraviolet CD spectra of the DCPC-glucagon complex showed

that those processes do not occur in the presence of the phospholipid.

The determination of the lipid-to-protein molar ratio in the complex. This was carried out by solubilizing increasing amounts of phospholipid with a fixed amount of glucagon (0.5 mg/ml). Up to 20 mol phospholipid are solubilized per mol glucagon. However, it has been observed that a stable solution of the complex is obtained only at a low lipid-to-protein molar ratio (approx. 5). Apparently, an excess of lipid can be solubilized by glucagon in freshly prepared solution. The storage of these solutions at room temperature causes slow and variable precipitation of both glucagon and phospholipid.

Preparation of sonicated vesicles. Vesicles of DCPC were prepared by ultrasonic irradiation of a suspension of phospholipid at room temperature for 20 min, under nitrogen atmosphere, in a Bransonic 12 bath-type sonicator. The turbidity of the dispersion disappeared rapidly with ultrasonic irradiation, and the resulting transparent solution was centrifuged in a Sorvall centrifuge at 20000 rpm using a SS-34 rotor at 15°C for 15 min, to remove large particles. The size and uniformity of the preparations were not critically evaluated.

Ultracentrifugation. The determination of the molecular weight of the lipid-protein complex was carried out by sedimentation equilibrium using a Beckman Model E analytical ultracentrifuge, equipped with a photoelectric scanner. All measurements were carried out at 14000 rpm at 20°C. Double-sector cells and an An-G rotor were used. The glucagon concentration was followed by measuring the ultraviolet absorption at 278 nm. The number of glucagon molecules in the complex were determined according to the procedure described by Reynolds and Tanford [21], which allows the determination of the molecular weight of the protein moiety in the lipid-protein complex without knowledge of the amount of lipid bound. According to the following equation [22]:

$$M_p(1-\phi\rho) = M_p\left[(1-\bar{v}_p\rho)+\delta_L(1-\bar{v}_L\rho)\right] \qquad (1)$$

where M_P is the molecular weight of the protein, ϕ is the apparent partial specific volume of the lipid-protein complex, $\bar{v}_p$ is the partial specific volume of the protein, ρ is the solvent density, δ_L is the amount of phospholipid bound (in g per g protein) and $\bar{v}_L$ is the partial specific volume of the phospholipid. The method is based on the adjustment of the solvent density, by addition of ^2H_2O, to the density of the bound phospholipid. The samples with different densities were prepared by diluting small aliquots of concentrated solutions of lipoprotein complex into a buffer containing varying amounts of ^2H_2O. The partial specific volume of glucagon is 0.708 cm^3/g, calculated from the amino acid composition [23], and the partial specific volume of DCPC is 0.927 cm^3/g [21].

Gel filtration. Sepharose 6B was repeatedly washed and suspended in 0.1 M ammonium acetate (pH 7.4)/0.2 mg/ml of sodium azide. The gel was packed into a 1.5×30 cm column and equilibrated at room temperature under 80 cm of hydrostatic pressure. 1 ml of 15 mg/ml of sonicated DCPC vesicles was passed through the column to saturate the phospholipid binding sites [24].

Density. The apparent partial specific volume (ϕ) of the lipid-protein complex and densities (ρ) of solutions used for ultracentrifugation were measured using an A. Paar precision densimeter, Model DMA 60 with two DMA 602 measuring cells at 20°C, maintained at contant temperature by circulating fluid from a Neslab RTE-4 bath thermostatically controlled. A more detailed description of the apparatus has been presented by Epand and Epand [25].

Circular dichroism. CD measurements were performed with a Cary Model 61. Quartz cells with pathlengths of 0.05 and 1.0 mm were used to record the spectra in the far-ultraviolet and a 10 mm cell was used for measurements in the near-ultraviolet. The CD data were expressed as the mean residue ellipticity [θ] using 120.2 as the calculated mean residue weight of glucagon.

Nuclear magnetic resonance. ^{1}H-NMR spectra were obtained on a Bruker WP80 Fourier transform spectrometer operating at 80 MHz. Typically, 100 (90°) pulses were required to achieve adequate signal to noise and transforms involving 4K (real) datum points were used throughout. ^2H_2O was used as an internal lock and chemical shifts are in parts per million (ppm) relative to an internal H^2HO reference, which was taken as having a chemical shift of 4.70 ppm with respect to

tetramethylsilane (external reference). All samples were prepared in 99.8% ^2H_2O (Stohler Isotopic Chemicals). The pH of the solutions was measured with a Radiometer pH meter, model 25, with a Markson Science Inc. No. 7.14 electrode. The pH was calibrated every few readings with standard buffers in the range being measured. The pH of the samples was adjusted with small amounts of 1 M NaO^2H or 1 M ^2HCl introduced with a 5 μl SMI micro/pettor syringe. The pH of the solutions was measured before and after reading a spectrum, the values generally agreed within 0.02 pH units. The reported values of p^2H are pH meter readings and are uncorrected for isotope effects. The values of the ionization constants of the imidazole ring and the amino group of histidine amide and of the histidine residue of glucagon were determined by the curve-fitting procedure for nuclear magnetic resonance titrations developed by Shrager et al. [26]. ^{31}P-NMR spectra were obtained on a Bruker WH90 Fourier transform spectrometer, operating at 36.43 MHz. Normally, 5000 (90°) pulses were required to achieve adequate signal-to-noise and transforms involving 8K (real) data points were used. ^{31}P chemical shifts are in ppm with respect to an external H_3PO_4 (85%). The samples were prepared in Pipes buffer with 20% of ^2H_2O that was used as an internal lock. All NMR measurements were carried out at 32 ± 1°C, and a Bruker B-ST 100/700 temperature controller was used to maintain the temperature of the samples.

Fluorescence. A Perkin-Elmer Model MPF-44 fluorimeter was used to measure spectra in the ratio mode, using an excitation wavelength of 295 nm. At this wavelength, the main absorbing species is the tryptophan residue at position 25 of the amino acid sequence of glucagon. The binding of phospholipid to glucagon was studied by following the enhancement of fluorescence intensity of the tryptophan residue of glucagon caused by DCPC (see Results), by addition of small aliquots of phospholipid into a solution of free glucagon, according to the procedure described by Epand and Epand [11]. The excitation wavelength was 295 nm and the emission was measured at 340 nm setting the slit widths at 4 nm and response time at 0.3 s. The data were anlysed by Scatchard plots (ν/[P] vs. ν, where ν is the average mole ratio of protein bound to lipid and [P] is the concentration of free protein in solution). The intercept of the linear regression of the plots gives ν, which is reported as its reciprocal, and the reciprocal of the slope gives the binding constant. The analysis assumes that the fraction of protein bound to lipid is equal to the factional increase of the fluorescence intensity. It is also assumed that the system is in equilibrium and that the binding sites are identical and independent [11]. The linearity of the Scatchard plots supports the use of this analysis.

Batch microcalorimetry. The isothermal enthalpy of reaction of glucagon solutions with suspensions of DCPC was measured on a LKB 10700-2 batch microcalorimeter. The instrument was calibrated both electrically and by means of the reaction of NaOH with HCl [27]. Each reaction mixture was taken through three mixing cycles, and the heat of reaction was corrected for the frictional heat of mixing after completion of the reaction. Generally, 4 ml of glucagon solution were used and the final volume after mixing was 6 ml. The heat of dilution of glucagon and DCPC were measured separately, and were negligible compared to the heat of reaction. Either glucagon solution or buffer was used in the reference cells for the measurements.

Results

Properties of dicaprylphosphatidylcholine

Small unilamellar vesicles of DCPC are eluted in the void volume of a column packed with Sepharose 6B (for comparison with the glucagon-DCPC complex see Fig. 1). The ^{31}P-NMR spectra of a DCPC suspension and of sonicated vesicles are similar to those obtained for homologs of phosphatidylcholine with longer hydrocarbon chains [28,29]. The former shows a broad resonance with a low-magnetic-field shoulder (not shown) and the latter a narrow 'high-resolution' ^{31}P-NMR resonance (Fig. 2B). These results suggest that DCPC forms high-molecular-weight aggregates and organizes into bilayer structures. The study of dynamic and structural properties of aggregates formed by DCPC will be discussed in a forthcoming paper.

Characterization of the shape and size of the lipid-protein complex

The lipid-protein complex was characterized by gel filtration and ultracentrifugation. Sonicated vesicles of DCPC were eluted in the void volume of a Sepharose 6B column. When a mixture of glucagon-DCPC at low lipid-to-protein molar ratio (approx. 10) is applied to the column, both components are eluted near the bed volume (see Fig. 1 for comparison). If a mixture with a higher lipid-to-protein molar ratio (approx. 20) is applied to the column, the phospholipid is eluted both in the void volume as well as in the bed volume together with glucagon (Fig. 1A). In the region where glucagon cochromatographs with phospholipid, the lipid-to-protein molar ratio varies from 10 to 2, with a plateau around 6. When a more concentrated preparation of lipid-protein complex is applied to the column, the elution profile shows that complexes of higher molecular weight and different stoichiometry can be formed (Fig. 1B).

The apparent partial specific volume of the lipid-protein complex determined by density measurements is 0.817 ± 0.005 cm^3/g. The analysis of the sedimentation equilibrium results gave a linear plot of log C vs. r^2 (C being the protein concentration and r the distance from the centre of the rotor), suggesting the presence of a single complex with a molecular weight of $(4.55 \pm 0.57) \cdot 10^4$. Fig. 3 shows that apparent values of $M_p(1 - \rho\phi)$ as a function of solvent density. The density at which $1 - \bar{v}\rho$ is equal to zero for DCPC is indicated by an arrow. At this point, the apparent value of $M_p(1 - \phi\rho)$ is 6000 ± 200 and the molecular weight of the protein moiety is $(2.53 \pm 0.06) \cdot 10^4$, corresponding to 7.3 ± 0.3 molecules of glucagon. The stoichiometry of the lipid-protein complex is approx. 7 molecules of glucagon and 35 molecules of phospholipid. The initial lipid-to-protein molar ratio used in all ultracentrifugation experiments was around 6. However, when higher lipid-to-protein molar ratios were used, $d\log C/dr^2$ was not constant.

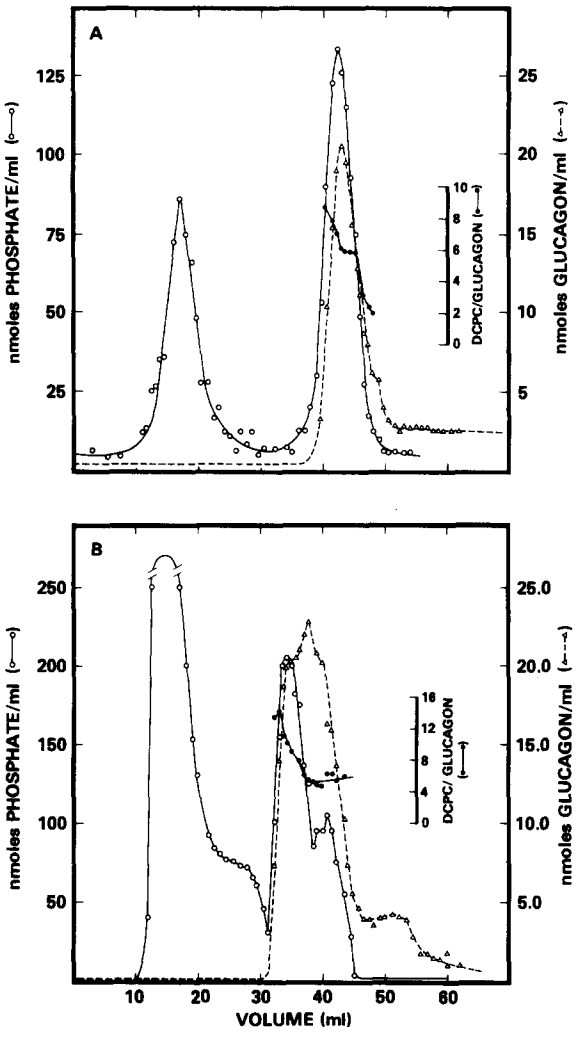

Fig. 1. Elution profile of glucagon-DCPC complex on Sepharose 6B at room temperature. Samples were prepared by solubilizing 0.25 mg (A) and 1.25 mg (B) of glucagon with 1.0 ml of a solution of sonicated lipid, containing 1.0 mg and 5.0 mg of DCPC, respectively. The 1.5×30 cm column was eluted with 0.10 M ammonium acetate (pH 7.4) at room temperature at approx. 10 ml/h. Protein (△) was determined by the Lowry procedure as described under Methods. Phospholipid (○) was determined by the method of Bartlett [17]. The inserted ordinate (●) shows the stoichiometry of the peak where glucagon cochromatographs with the phospholipid. The void volume of the column is 15 ml and the column volume is 43 ml.

Interaction between glucagon and DCPC

^{31}P-NMR was used to monitor the structural changes of the phospholipid upon interaction with glucagon. The proton-decoupled ^{31}P-NMR spectrum of the complex (Fig. 2B) is narrower than that of sonicated vesicles (Fig. 2A). In the proton-coupled spectrum for the phospholipid in the complex ^{1}H-^{31}P coupling is observed (Fig. 2D).

The specific regions of glucagon that are di-

rectly interacting with DCPC were studied by ^{1}H-NMR. The general effects of the phospholipid on the ^{1}H-NMR spectra of glucagon (Fig. 4 and Table I) are the broadening of the proton resonances of the peptide and lowering of the ionization constant of the imidazole ring of the histidine residue of glucagon. The former effect is most clearly seen with the resonance of the methionine methyl group at around 2 ppm [8], and the aromatic envelope of glucagon. Some broadening may also occur as a result of the increase in molecular weight from 3500 for glucagon to 45 500 for the glucagon-DCPC complex. Table I shows the pK values of the imidazole ring (pK_1) and of the terminal amino group (pK_2) determined from the pH-dependence of the chemical shift of the C(2) and C(4) protons of histidine [27]. DCPC lowers the pK of the imidazole ring, while the pK of the α-amino group is not altered greatly.

The effect of phopsholipid on the conformation of the hormone was monitored by circular dichroism. The far-ultraviolet CD spectra of dilute aqueous solutions of glucagon (Fig. 5A) indicated the presence of little helical structure, in agreement with the results previously reported [30,31]. However, in the presence of DCPC the helical content of glucagon increases, as evidenced by the appearance of a double minimum at 222 and 208 nm (Fig. 5A). The variation of the magnitude of $[\theta]_{222}$ as a function of the DCPC/glucagon molar ratio (Fig. 5B) suggests that a few molecules of phospholipid cause most of the conformational change in glucagon. The near-ultraviolet CD spectrum of glucagon was used to follow changes in the environment of the aromatic side-chains (Fig. 5C). The observed spectrum appears to be an intermediate between that observed at 6°C and at 20°C for glucagon in the presence of dimyristoyl-

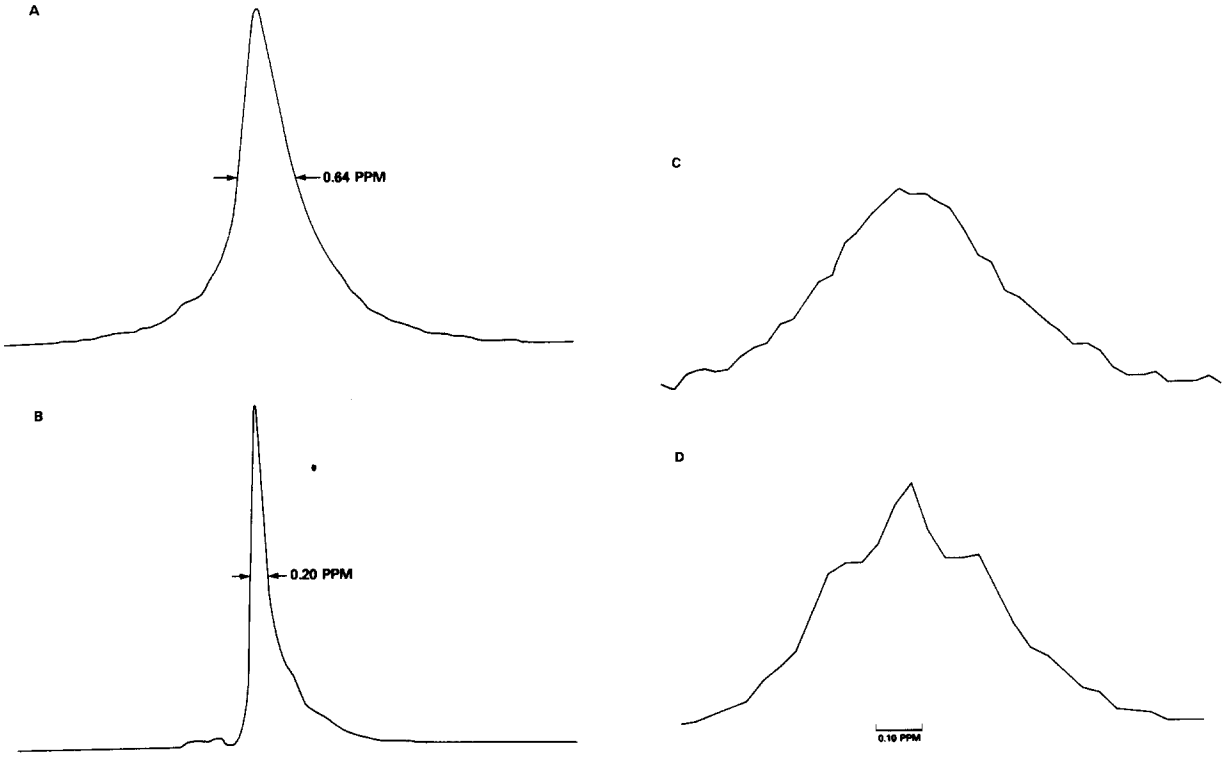

Fig. 2. Proton-decoupled (A and B) and proton-coupled (C and D) 36.4 MHz ^{31}P-NMR spectra of DCPC (A and C) sonicated lipid (DCPC, 25 mg/ml). (B and D) glucagon-DCPC complex (glucagon, 11.7 mg/ml, DCPC, 14.2 mg/ml). The spectral widths were 6000 Hz (A and B) and 60 Hz (C and D). The chemical shifts are given in ppm relative to 85% (v/v) H_3PO_4 (external reference). The samples were prepared in Pipes buffer at pH 7.4 with 20% ^2H_2O and the measurements were carried out at 33°C.

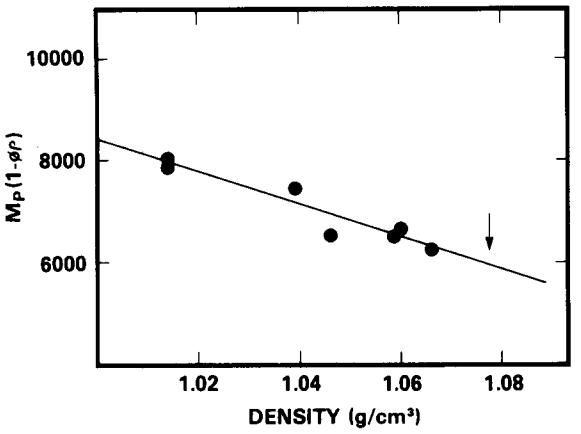

Fig. 3. Density perturbation sedimentation equilibrium for glucagon-DCPC complex. The apparent values of $M_P\ (1-\phi\rho)$ are plotted as a function of density. The samples were prepared by diluting small aliquots of concentrated solution of lipid-protein complex into Pipes buffer containing varying amounts of ^2H_2O. The concentration of glucagon in the experiments was around 0.12 mg/ml. The lipid-to-protein molar ratio varied from 5 to 7, and all measurements were carried out at 20°C. M_P is the molecular weight of the lipid-protein component and ϕ is the apparent partial specific volume of the bound protein. The arrow indicates the value of $\rho = 1/\bar{v}$ for DCPC.

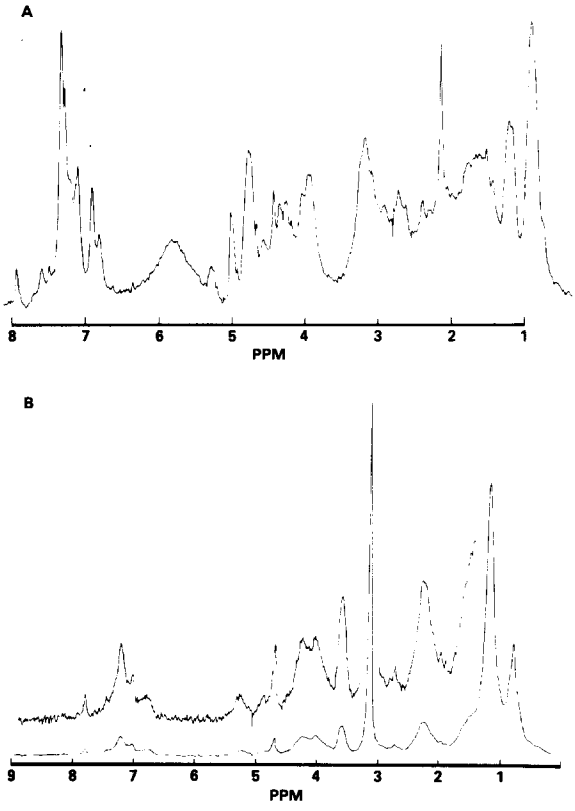

Fig. 4. ^{1}H-NMR spectra at 80 MHz of (A) glucagon, 6.5 mg/ml in 6 M deuterated urea, p^{2}H 6.85, at 33°C; and (B) glucagon-DCPC complex: glucagon, 5.75 mg/ml, DCPC, 5.0 mg/ml, p^{2}H 7.49, at 33°C. Samples were prepared in ^2H_2O. Spectra are presented at two different gains to present both lipid and peptide resonance bands.

phosphatidylcholine [2]. The spectrum is different from that of glucagon in the absence of lipid.

The wavelength of maximum fluorescence emission of glucagon is 352 nm in aqueous solution. In the presence of DCPC, the maximum emission is shifted to shorter wavelength, and there is an enhancement of the relative fluorescence intensity (Fig. 6). The equilibrium constant for binding of phospholipid to glucagon determined by Scatchard analysis of the enhancement of fluorescence is $(4.4 \pm 0.5) \cdot 10^4\ M^{-1}$ and $(7.5 \pm 0.5) \cdot 10^4\ M^{-1}$ at 15°C and 25°C, respectively. The average number of moles of DCPC bound per mole of glucagon is

TABLE I

pK VALUES FOR THE IMIDAZOLE (pK_1) AND AMINO GROUP (pK_2) OF THE N-TERMINAL HISTIDINE RESIDUE OF GLUCAGON IN THE PRESENCE OF DCPC OR 6 M DEUTERATED UREA, AND FOR HISTIDINAMIDE IN ^2H_2O

1.5 mM glucagon; 8 mM DCPC; 10 mM histidinamide. Samples prepared in ^2H_2O, pH adjusted with 1 M ^{2}HCl or 1 M NaO^{2}H, p^{2}H values are taken as uncorrected pH meter reading, pK values obtained from a curve-fitting procedure [26]. n.d., not determined.

	C(2)		C(4)	
	pK_1	pK_2	pK_1	pK_2
Glucagon-DCPC complex in ^2H_2O	5.08 ± 0.04	7.43 ± 0.10	4.92 ± 0.05	7.53 ± 0.08
Glucagon in 6 M urea-d_4	5.35 ± 0.13	7.53 ± 0.40	n.d.	n.d.
Histidinamide in ^2H_2O	5.24	7.57	5.23	7.49

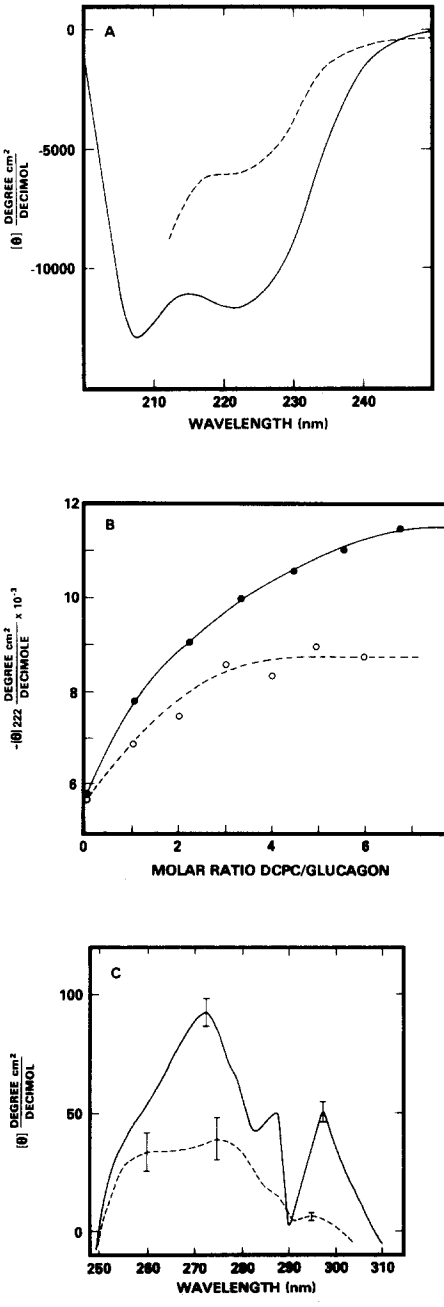

Fig. 5. Circular dichroism spectra of glucagon at 20°C. (A) Far-ultraviolet CD: — — —, glucagon, 0.093 mg/ml; ———, glucagon, 3.1 mg/ml, DCPC, 2.7 mg/ml. (B) $[\theta]_{222}$ of glucagon as a function of DCPC/glucagon molar ratio. Glucagon 0.091 mg/ml: O— — —O, 25°C; ●———●, 15°C. (C) Near-ultraviolet CD: — — —, glucagon, 0.105 mg/ml; ———, glucagon, 0.98 mg/ml, DCPC, 1.15 mg/ml. Samples were prepared in Pipes buffer. Error bars are estimated from noise level of three accumulated scans.

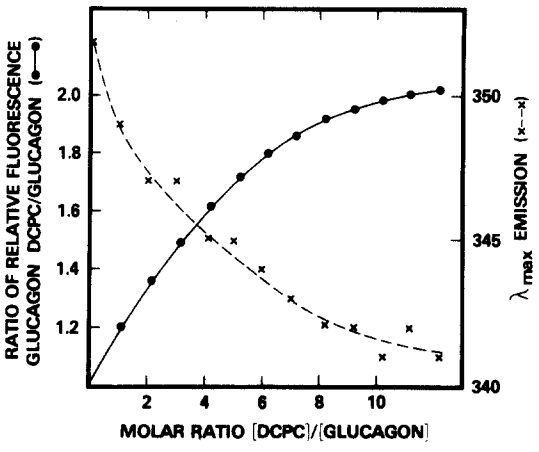

Fig. 6. Fluorescence properties of the tryptophan residue of glucagon in the presence of DCPC as a function of lipid-to-protein molar ratio at 20°C. ●———●, Ratio of fluorescence intensity measured at 340 nm; x— — —x, wavelength of maximum emission. Excitation wavelength 295 nm. Glucagon, 0.11 mg/ml dissolved in Pipes buffer (pH 7.4).

4.4 ± 0.6. These results are an average of three independent determinations, and the resulting Scatchard plots fitted well to a straight line, with correlation coefficients varying from 0.99 to 0.97.

The isothermal enthalpy of reaction of glucagon with DCPC at 15°C and at 25°C as a function of DCPC-to-glucagon molar ratio is shown in Fig. 7. The total enthalpy change is -32.5 kcal/mol of

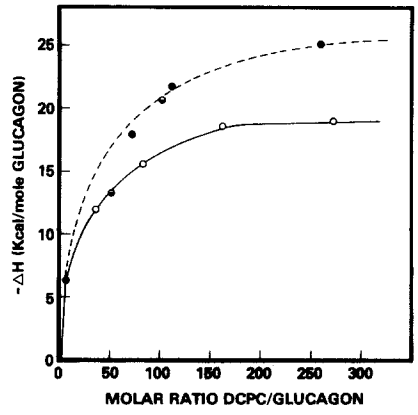

Fig. 7. Isothermal enthalpy of reaction of glucagon with DCPC as measured by batch microcalorimeter as a function of the lipid-to-protein molar ratio. The samples were prepared in Pipes buffer. Glucagon, 40 μM (◒), 29 μM (O) and 14 μM (●), at 15°C (— — —) and 25°C (———).

TABLE II

THERMODYNAMIC PARAMETERS FOR THE REACTION OF GLUCAGON WITH DCPC AT 15°C AND 25°C

Temperature (°C)	15°C	25°C
$\Delta H°$ (kcal/mol glucagon) [a]	-32.5 ± 2.5	-20.5 ± 0.5
$\Delta G°$ (kcal/mol glucagon) [b]	-6.16 ± 0.07	-6.69 ± 0.06
$\Delta S°$ (e.u./mol glucagon) [c]	-91 ± 6	-46 ± 2
ΔC_p (kcal/K^{-1} per mol glucagon) [d]	1.2 ± 0.2	

[a] Calorimetric measurements; [b,c,d] calculated from
$\Delta G° = -RT \ln K$, $\Delta G° = \Delta H° - T\Delta S°$, $\Delta C_p = \frac{\Delta H°_{25} - \Delta H°_{15}}{\Delta T}$, respectively.

glucagon and -20.5 kcal/mol of glucagon at 15°C and 25°C, respectively. The reaction is an exothermic process. The fact that the observed heat changes occurred over a period of a few minutes demonstrates that the reaction occurs rapidly. It was not possible to describe the dependence of the measured isothermal ΔH on the lipid-to-glucagon ratio (Fig. 7) by any simple equilibrium process. It is possible that the binding of the first few lipid molecules is a more highly exothermic process, because it includes most of the conformational changes induced in glucagon and most of the lipid-peptide interactions (see Fig. 5B) compared with the addition of subsequent lipid molecules. Table II shows the thermodynamic parameters for the reaction of glucagon with DCPC at 15°C and 25°C. For these calculations it was assumed that the system is in equilibrium, and that the equilibrium binding constant determined by fluorescence measurements is equal to that measured by calorimetry. The latter assumption is based on the results obtained for the binding studies of dimyristoylphosphatidylcholine to glucagon [11,32].

Discussion

The data reported in this paper show that glucagon interacts with DCPC, forming a water-soluble complex with a specific size and stoichiometry. Gel-filtration experiments show that the Stokes' radius of sonicated vesicles of DCPC is decreased when glucagon is added and a low-molecular-weight complex with a low lipid-to-protein molar ratio is formed. Sedimentation equilibrium experiments show that the complex has a molecular weight of $(4.55 \pm 0.57) \cdot 10^4$ and contains, approximately, 7 molecules of glucagon and 35 molecules of DCPC. The complex formed between glucagon and DCPC does not resemble those formed between the hormone and dimyristoylphosphatidylcholine [4] or dodecylphosphocholine [8]. The former contains 32 molecules of glucacon and 1810 molecules of phospholipid with a molecular weight of $1.14 \cdot 10^6$, and the latter contains 1 molecule of glucagon and 40 molecules of dodecylphosphocholine, with a molecular weight of 17000.

The interaction between glucagon and DCPC results in a conformation changes of the peptide, and alterations in the structural properties of the phospholipid. ^{31}P-NMR was used to monitor the changes in the dynamic and structural properties of DCPC upon interaction with glucagon. The proton-decoupled ^{31}P-NMR spectrum of the phospholipid in the lipid-protein complex (Fig. 2B) shows a narrower resonance than that of sonicated vesicles of DCPC (Fig. 2A). In the absence of decoupling, splitting is observed in the ^{31}P resonance as a result of ^{1}H-^{31}P spin coupling (Fig. 2D). These results, in agreement with results from gel filtration and ultracentrifugation, suggest that glucagon solubilizes DCPC aggregates, forming a lipid-protein complex with a molecular weight lower than that of sonicated vesicles of DCPC. The results also suggest that upon solubilization of DCPC aggregates by glucagon, the phospholipid is no longer organized into an ordinary bilayer structure, considering the small number of phospholipid molecules per particle.

Circular dichroism, ^{1}H-NMR and fluorescence were used to monitor conformational changes of the hormone upon interaction with the phos-

pholipid. DCPC alters the far and near ultraviolet CD spectrum of glucagon (Fig. 5). The enhancement of the helical content of the hormone in the presence of DCPC is not specific for this phospholipid, but similar alterations of the far-ultraviolet CD spectra were observed in the presence of other phospholipids such as dimyristoylphosphatidylcholine [2] and diheptanoylphosphatidylcholine [8], as well as in the presence of the detergents dodecylphosphocholine [8], doecylmethylamine oxide [8], dodecyl(oxyethylene)-7,8-glycol [8] and sodium dodecyl sulfate [7]. These results indicate that the change which occurs in the far-ultraviolet CD spectrum of the hormone does not depend on the molecular structure of the amphiphile or the size or stoichiometry of the lipid-protein complex which is formed.

At low pH, glucagon fragments containing residues 1–17 as well as 19–29 are more helical in the presence of sodium dodecyl sulfate [33], while only the carboxyl terminal fragment retains this helicity at higher pH [7]. These results, combined with calculations of the overall hydrophobicity of the fragments [33] suggest that the carboxyl-terminal half of the molecule is more embedded in a lipid, while the amino-terminal half of glucagon may be more capable of entering and leaving a hydrophobic matrix with small changes of conditions. The glucagon molecule must apparently be intact in order to form a complex with dimyristoylphosphatidylcholine [33]. Alterations of the near-ultraviolet CD spectra of glucagon (Fig. 5C) indicate that changes in the environment of the aromatic residues caused by the presence of DCPC are qualitatively similar to those caused by dimyristoylphosphatidylcholine [2], suggesting that similar structural features of glucagon are responsible for the ability of this peptide to interact with both of these phospholipids.

^{1}H-NMR measurements show the DCPC, in general, broadens the resonances of the hormone (Fig. 4). The resonance of glucagon in the region 0–4 ppm overlaps those of the phospholipid, and the sharp resonance line of the methionine methyl group (Fig. 4A) is too broad to be observed in the presence of DCPC. A well-resolved and detailed analysis of the ^{1}H-NMR spectrum of glucagon in the presence of perdeuterated detergent demonstrated changes in the chemical shift, and broadening of glucagon resonances [8]. The former were attributed to conformational changes of the peptide, and the latter are due to decreased motion of the particular proton, and/or increased size of glucagon-DCPC complex. The ^{1}H-NMR results suggest that the aromatic residues, and the C-terminal region of glucagon are involved in the interaction with DCPC, as well as with dodecylphosphocholine [8]. Bösch et al. [8] suggest that the histidine residue of glucagon is highly mobile and located in the external region of the glucagon-containing dodecylphosphocholine micelle with the pK of the histidine imidazole being 5.8. The C(2) proton resonances of the imidazole ring of the histidine residue of glucagon (around 7.8 ppm) in the presence and absence of DCPC (Fig. 4) is relatively sharp, suggesting that the histidine residue is mobile compared with many other groups on the peptide. The pK value of both the imidazole group and the α-NH_2 group of histidine for glucagon in urea (Table I) or for *S*-methylglucagon [34] are close to those found for histidinamide, indicating that these groups are exposed to the aqueous environment in the free peptide. Phospholipid is able to lower the pK of the imidazole ring, while not greatly altering the pK of the amino group (Table I). These results suggest that the histidine residue is located in the lipid-protein complex/aqueous phase interface, or that it is in a region with net positive charge. A model has been proposed in which the polypeptide backbone is oriented roughly parallel to the surface in the mixed-glucagon dodecylphosphocholine micelle with the polar side-chains oriented toward the interface and the nonpolar side-chains into the interior of the micelle [35]. In this model, the C(2) and C(4) protons of histidine and the ϵ-CH_2 and δ-CH_2 groups of lysine 12 and arginine 17, 18, respectively, are located in the mixed micelle surface. If glucagon has similar orientation when complexed with DCPC, these positively charged groups of lysine and arginine could cause a lowering of the pK of the imidazole. The lower imidazole pK could also result from a perturbation caused by the overall charge of the lipid-protein complex, which is greater at lower pH, thus explaining the larger effect on the pK of the imidazole ring compared with the α-NH_2 group (Table I).

The alterations in the fluorescence properties of the tryptophan residue of glucagon (Fig. 6) suggest that the fluorophore is experiencing a more hydrophobic environment. Similar effects on fluorescence properties of glucagon have been observed with hexadecyltrimethylammonium bromide [5], lysophosphatdylcholine [6], dimyristoylphosphatidylcholine [3], dodecylphosphocholine and dodecyldimethylamine oxide [8]. It has been reported, based on fluorescence experiments [8], that glucagon interacts more strongly with a detergent micelle than with a detergent monomer. Compared with detergents, more hydrophobic amphipathic molecules such as DCPC cause a major change in the fluorescence properties of the hormone with the binding of only a few molecules of amphiphile. The equilibrium constants for the binding of DCPC to glucagon are $(4.4 \pm 0.5) \cdot 10^4$ M^{-1} and $(7.5 \pm 0.5) \cdot 10^4$ M^{-1} at 15°C and 25°C, respectively. These binding constants are close to the values obtained for dimyristoyl- and dilauroylphosphatidylcholine [11]); however, the average number of moles of phospholipid bound per mol of glucagon decreases considerably with the shortening of the length of the fatty acid chains of the phospholipid, being around 20, 10 and 5 for dimyristoyl, dilauroyl- and dicaprylphosphatidylcholine, respectively (Ref. 11 and this work).

The exothermic enthalpies of reaction of glucagon with DCPC -32.5 and -20.5 kcal/mol of glucagon at 15°C and 25°C, respectively, are small when compared with that caused by glucagon-induced crystallization of dimyristoylphosphatidylcholine [32]. However, the low magnitude of the heat of reaction of glucagon with DCPC, the low lipid-to-protein molar ratio determined for the complex and the fact that all measurements were carried out well above the reported phase transition temperature of -8.5°C for DCPC [36], suggest that lipid-protein interactions and peptide conformational change are also likely to make contributions to the overall measured enthalpy.

The self-association of glucagon results in a conformational change in which the helix content is increased to about 30% [37]. This association process is presumed to involve hydrophobic interactions among amino acid side-chains, as is found in the crystal structure [38]. The self-association process has an exothermic enthalpy of -10 kcal/mol of glucagon [39,40]. A similar increase in helix content occurs when glucagon binds to phospholipids [2] and therefore one would expect a similar contribution to the overall enthalpy from this process. In addition, two other processes which are likely to contribute to the overall enthalpy change are the glucagon-induced alteration of the phase transition of up to 20 molecules of DCPC/glucagon molecule (see Results) and non-convalent lipid-protein interactions, including hydrophobic and Van der Waals interactions as well as hydrogen bond formation [41].

Glucagon is a relatively small peptide, available in pure form, which reacts rapidly with phospholipids [11]. For this reason, it has been used as a well-defined model system for the study of lipid-peptide interactions (Refs. 32, 42, 43 and reference therein). Furthermore, the nature of the interaction of glucagon with phospholipids may be similar to that which occurs between some serum apolipoproteins and phospholipids [44] and it may also be a component of the binding of certain peptide hormones to their receptor [9].

The nature of the lipid or detergent has relatively little effect on the equilibrium constant for the binding of glucagon to these substances. The affinity constants for the binding of glucagon to DCPC, dilauroylphosphatidylcholine [11] or dimyristoylphosphatidylcholine [11] are all about 10^5 M^{-1}, while the binding constant for lysophosphatidylcholine is somewhat larger, $2 \cdot 10^6$ M^{-1} [6]. The spectral properties of glucagon as measured by fluorescence or circular dichroism are also similar when the peptide is embedded in one of several phospholipids (Refs. 2, 3, 8 and this work) or detergent [7,8,33] complexes.

However, the number of lipid-molecules bound per glucagon varies for each individual lipid, with the number of lipids decreasing in going to phospholipids with shorter chains. The size and stoichiometry of the complexes also vary. Phospholipids which form stable bilayers, such as dimyristoylphosphatidylcholine, form the largest particles, with a molecular weight of $1.4 \cdot 10^6$ [4] and containing 32 molecules of glucagon. In contrast, detergent micelles incorporate only one molecule of glucagon [8]. The complexes formed with DCPC have intermediate properties. Their size is closer to that of the detergent micelles [8]

but seven glucagon molecules are incorporated into each complex with DCPC. This suggests that glucagon may have more of a tendency to self-associate in a phospholipid environment than in detergent micelles. There is little effect of temperature on the association of glucagon with DCPC, unlike the case of the longer-chain phospholipids [3]. Thus, presumably as a result of the relatively weak lipid-lipid interactions in DCPC, this phospholipid behaves with glucagon in a manner intermediate between that of phosphatidylcholine molecules containing longer-chain fatty acids and micelle-forming detergents.

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