Molecular Interactions in the Model Lipoprotein Complex Formed between Glucagon and Dimyristoylglycerophosphocholine†

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ABSTRACT: Glucagon forms water-soluble complexes with dimyristoylglycerophosphocholine below the phase transition temperature of the lipid. The peptide has no effect on the proton spin–lattice relaxation times of the lipid but causes a marked broadening of the line width of the terminal methyl and methylene resonances of the lipid. These results are interpreted as arising from a decrease in the rate of lateral diffusion of the lipid caused by the presence of glucagon. The thermal-transition temperature of the lipid is found to be unaltered by glucagon as monitored with the fluorescent probe, pyrene, or with differential scanning calorimetry. The latter technique also indicates that there is no change in the enthalpy of this transition, although some broadening is detected. Thus, there is no major alteration in the bilayer structure of the lipid caused by the presence of glucagon. Ultraviolet difference spectra indicate that the tyrosine and tryptophan residues of glucagon enter a more hydrophobic environment in the lipoprotein complex. A change in structure involving the aromatic chromophores of the peptide is also observed by the appearance of Cotton effects of large magnitude in the near ultraviolet. This circular dichroism property changes more abruptly below the "premelt" temperature of the lipid than above it. A discontinuity in the temperature dependence of the difference spectrum is also observed at this temperature. These results indicate a conformational change of the peptide at the premelt temperature which does not involve a major structural change, as the far-ultraviolet Cotton effects of glucagon show little change with temperature and they do not exhibit a discontinuity at 15 °C. Thus, in addition to the marked increase in the helix content of glucagon which occurs upon its association with dimyristoylglycerophosphocholine only at temperatures below the major phase transition, there is also a detectable conformational change of the peptide at the "premelt" temperature of the lipid.

Glucagon has an amino acid sequence which would allow it to form amphipathic helices (Epand et al., 1977) and such structures have been observed in the crystalline form of the hormone (Blundell et al., 1976). It has been suggested that the ability of a protein to form amphipathic helices is related to its ability to form complexes with lipids. Interaction of lipids with proteins capable of forming amphipathic helices has been demonstrated for the human (Assmann and Brewer, 1974) and porcine (Andrews et al., 1976) high-density lipoproteins, a lipoprotein from the outer membrane of *Escherichia coli* (Inouye, 1974), the apolipoprotein C-III (Pownall et al., 1974; Novosad et al., 1976), and amyloid A (Segrest et al., 1976). Glucagon is a particularly interesting example of a protein containing amphipathic helices which can form complexes with lipids. This is due to the small size of the hormone which provides the first example of a whole peptide chain capable of amphipathic helix formation. In addition, glucagon has the unusual property of associating more strongly with lecithin when it is in the gel state compared with the liquid-crystal state (Epand et al., 1977).

Materials and Methods

Materials

Crystalline bovine–porcine glucagon was purchased from the Elanco Corp. and used without further purification. We have previously analyzed the purity of this preparation (Epand et al., 1977). Dimyristoylglycerophosphocholine (Sigma Chemical Co.) showed only one spot when visualized with iodine vapors after thin-layer chromatography (chloroform–methanol–water, 65:25:4) on silica gel H even with sample applications of 500 μg of lipid. This material was therefore used without further purification. To free the deuterium oxide (Stohler Isotope Chemicals) from possible impurities of paramagnetic metal ions it was distilled in an all-glass apparatus which had previously soaked in a solution of ethylenediaminetetraacetate and washed.

Methods

Preparation of the Glucagon–Dimyristoylglycerophosphocholine Complex. Dimyristoylgiycerophosphocholine (25 mg) was dissolved in a solution of chloroform–methanol (2:1 volume ratio). 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. Glucagon (10 mg) was suspended at 40 °C in 10 mL of 0.1 M ammonium acetate solution, pH 7.4, and rapidly added to the dried lipid film and vortexed at 40 °C for 1–2 min. The pH of the resulting suspension was readjusted when necessary to 7.4, the suspension was cooled in an ice bath, and undissolved material was removed by centrifugation at 40 000 rpm for 1 h at 5 °C in an SW 50.1 rotor of a Beckman Model L-2 preparative ultracentrifuge. Occasionally, lower speeds were used with similar results. The resulting supernatant contained more than 95% of the added lipid in the form of uniform, soluble complexes with glucagon having a molar ratio of lipid to glucagon of 35:1, a molecular weight of 1.7×10^6, and dimensions of 70×270 Å (Jones et al., 1977, and manuscript in preparation). If glucagon is omitted from the procedure less than 0.01 mg/mL lipid is detected in the supernatant. The concentration of glucagon solubilized in these complexes by this procedure is approximately 0.5 mg/mL. In the absence of lipid, only 0.02 mg/mL of peptide is solubilized when a suspension of glucagon is centrifuged under similar conditions.

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Therefore, only a relatively small percentage of free glucagon should be present in solutions of the complex. Preliminary data show that the complex is formed between pH 6 and 9 and ionic strength 0.02 and 1.3 M. Yield seems to be increased if the mixture is taken at least once to the phase transition before the centrifugation at 5 °C. The concentration of glucagon in solutions of the complex was determined using $E_{1cm}^{1mg/mL} = 2.12$ (this work, see UV difference spectra) and in solutions of free glucagon using $E_{1cm}^{1mg/mL} = 2.38$ (Gratzer et al., 1967). Optical density readings for the complex were corrected for scattering contributions by extrapolation of log OD. vs log λ plots constructed from at least ten points in the range 330–380 nm (Cancellievi et al., 1974). The correlation coefficients of such plots always exceeded 0.99. The procedure does not account for small wavelength-dependent refractive-index changes which would effect the *correction* by a few percent and therefore have a negligible effect on the precision of the corrected optical density due to the chromophores. Lipid concentrations were determined by the method of Bartlett (1959) after perchloric acid ashing of the samples. For measurements which required higher concentrations of the complex, such as the differential scanning calorimetry and the proton spin-lattice relaxation time measurements by nuclear magnetic resonance (NMR), the supernatants were dried by lyophilization, redissolved in a smaller volume, and recentrifuged at 20 °C, below the phase-transition temperature of the lipid where the complex is soluble. No significant differences in NMR spectra, see below, were observed between lyophilized and "fresh" complex, although the latter required severalfold more accumulations to achieve comparable signal to noise enhancement. Sedimentation data and EM studies also showed no significant difference between "fresh" and lyophilized complex (Jones and Epand, in preparation). The process of lyophilization is therefore not likely to have caused major changes in the molecular structure of the lipid–protein complex.

Preparation of Unilamellar Vesicles. The vesicles were prepared by sonication of a suspension of dimyristoylglycerophosphocholine at 40 °C for 1 h under nitrogen in a Bransonic 12 bath-type sonicator. The resulting transparent solution was centrifuged to remove any larger vesicles. The size uniformity of this preparation was not critically evaluated. The physical properties of uni- and multilamellar vesicles of the lipid have been reported by many authors. For the T_1 measurements, the lipid suspension was passed through a column of Chelex 100 (Bio-Rad Corp.) contained in a Pasteur pipet to further remove any traces of paramagnetic impurities and was purged with nitrogen to remove dissolved oxygen.

Nuclear Magnetic Resonance. Proton spectra were obtained from a Bruker WH90 Fourier transform spectrometer operating at 90 MHz. Normally, 36 (90°) pulses were required to provide adequate signal to noise enhancement and transforms involving 8K (real) data points were employed. A Bruker B-ST 100/700 temperature controller was used to maintain sample temperature within ±1 °C. Sample pH was adjusted with a small amount of NaOD or DCl to give a pH meter reading of 7.2 ± 0.2. Areas of resonance lines were measured with respect to an external standard contained in a coaxial capillary. Spin–lattice relaxation time measurements (T_1) were made using the 180°–τ–90° pulse sequence. T_1 values were calculated from plots of ln $(1 - A/A_\infty)$ vs. τ where the relative values of A and A_∞ are the peak heights at time τ and $\tau > 5T_1$, respectively. Each plot contained at least 8 points.

Pyrene Excimer Fluorescence. The monomer (I_m) and excimer (I_E) emission of pyrene was measured as previously described (Epand et al., 1977). More precise measurements were obtained from clear solutions of the complex below the phase-transition temperature than from turbid suspension at higher temperatures or from lipid alone.

Pyrene (5.5 μg) and dimyristoylglycerophosphocholine (1.1 mg) were deposited as a film on the walls of glass test tubes and a solution of 0.01 M potassium phosphate buffer, pH 7.4, containing varying amounts of glucagon was added to these lipid films above the phase-transition temperature with rapid vortexing.

Differential Scanning Calorimetry. These experiments were performed with a Perkin-Elmer DSC-2 scanning calorimeter. A heating rate of 1 °C/min was employed to achieve maximum resolution but which necessitated the use of the most sensitive detector range (0.1 mcal s^{-1}). Ten to fifteen microliters of solution of lipid at a concentration of 40–50 mg/mL (with or without glucagon) in 0.1 M ammonium acetate, pH 7.4, was sealed in aluminum sample pans. The lipid alone was sonicated the previous day and probably contains a distribution of vesicle sizes. Vesicle size is known to affect both sharpness and temperature of transitions measured by calorimetry (Suurkuusk et al., 1976). There was no change in the trace upon repeated scanning as has been observed in other systems (Tall and Small, 1977), and cooling scans, when performed, appeared to be the mirror image of heating scans. After completion of the scans, the sample pans were prized open in 1 mL of 1% sodium dodecyl sulfate and sonicated to ensure solution of the contents. This solution was then analyzed for phosphate (Bartlett, 1959) after perchloric acid digestion at 180 °C. Areas under the transition peaks were measured with a planimeter, converted to millicalories from which value the enthalpy of the transition was calculated in kcal/mol.

Circular Dichroism (CD). These measurements were performed with a Cary, Model 61, spectropolarimeter. The temperature of the sample was maintained by means of a thermostated cell holder and monitored by a thermistor probe in the sample. One of the helical forms of the peptide (Contaxis and Epand, 1974) was prepared by dissolving glucagon in a solution of 7 volumes of propylene glycol and 3 volumes of 0.1 M ammonium acetate, pH 7.4.

Ultraviolet Difference Spectra. Ultraviolet absorption spectra, difference spectra, and thermal difference spectra were measured in a Cary 118 spectrophotometer. Difference spectra in the presence and absence of dimyristoylglycerophosphocholine were calculated, after correction of the lipid containing samples for light scattering (see above), from the mean of three individual complex preparations and two separate glucagon solutions. Scan rates of 0.02 nm/s were used with a period of 5 s. Baselines were obtained before and after individual spectra and were superimposable within the residual noise level. The sample temperature was maintained by the thermostated cell holder and the reference solution was maintained by the use of a jacketed cell connected to a water bath whose temperature was maintained to ±0.002 °C. The temperatures of the solutions were continuously monitored (±0.1 °C) with thermistor probes immersed in the solution. For the thermal difference spectra, the 0–0.05 optical density units full scale was used. The baseline obtained with buffer in both cells was indistinguishable from that obtained with a solution of complex in both cells. The spectra are uncorrected for thermal expansion which amounts to only 0.2% for the 6 to 20 °C temperature range. The difference in extinction coefficients for glucagon in the absence and presence of the lipid was determined in the following manner: the optical density at 278 nm of three glucagon solutions and three independently prepared solutions of complex was measured. The solutions were made 1% in sodium dodecyl sulfate and the optical densities rerecorded after brief

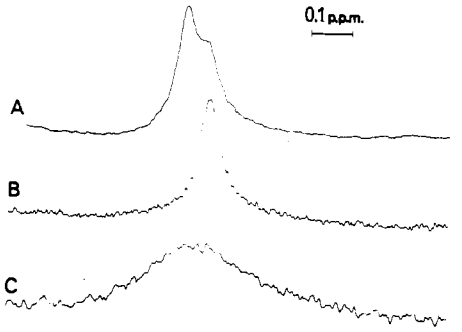

FIGURE 1: Effect of Mn^{2+} on the NMR spectra of the quaternary ammonium protons of dimyristoylglycerophosphocholine and its complex with glucagon: ^2H_2O, 20 °C, pD 7.4, 100 scans. (A) Dimyristoylglycerophosphocholine (7 mM), sonicated dispersion. (B) Same as A in the presence of $MnCl_2$ (200 μM). (C) Dimyristoylglycerophosphocholine–glucagon complex (3.5 mM in lipid). When $MnCl_2$ (200 μM) was added to this solution no signal was observed in this region of the spectrum.

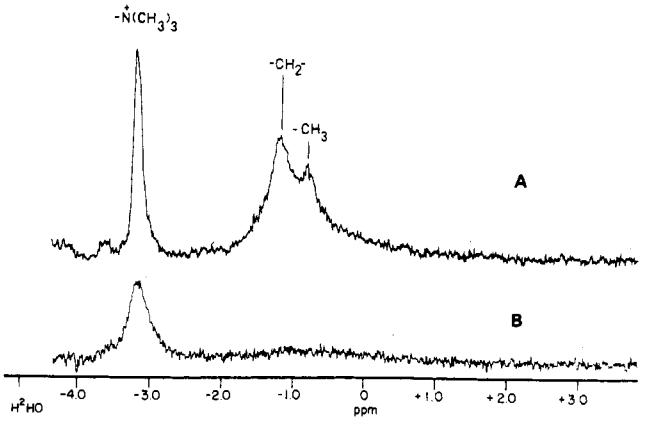

FIGURE 2: Effect of glucagon on the proton NMR spectra of dimyristoylglycerophosphocholine: ^2H_2O, 20 °C, pD 7.4, 3.5 mM lipid. (A) Sonicated dispersion of lipid. (B) Lipoprotein complex.

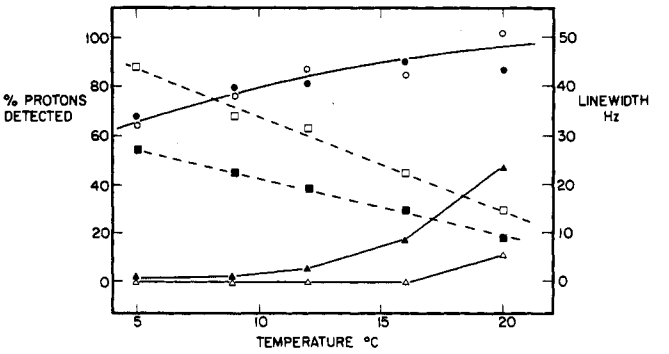

FIGURE 3: Effect of glucagon on the fraction of protons detected by NMR (solid lines) and on the line width of the NMR signal from the quaternary ammonium methyl protons (dashed lines) at various temperatures: ^2H_2O, pD 7.4. One-hundred percent detection was taken for the quaternary ammonium group at 30 °C (see, for example, Chan et al. (1973)). (○, ●) Quaternary ammonium protons detected; (△, ▲) terminal methyl and methylene protons detected; (□, ■) line width of quaternary ammonium peak. Open symbols refer to glucagon–dimyristoylglycerophosphocholine complex and closed symbols to a sonicated dispersion of dimyristoylglycerophosphocholine.

TABLE I: Proton Spin-Lattice Relaxation Times for Sonicated Dimyristoylglycerophosphocholine and for a Soluble Complex of Glucagon with Dimyristoylglycerophosphocholine at 20 °C and pD 7.4.[a]

Sample	T_1 (s^{-1}) for $N^+(CH_3)_3$	$-(CH_2)_n-$	Terminal CH_3
Vesicles	0.217 ± 0.005	0.27 ± 0.01	0.302 ± 0.007
Glucagon complex	0.221 ± 0.009	0.31 ± 0.03	Nd[b]

[a] Lipid concentration = 30 mM. [b] Nd, not determined.

sonication of the solutions. Assuming that the tenfold molar excess of sodium dodecyl sulfate over dimyristoylglycerophosphocholine in the resulting solution swamps the effect of the dimyristoylglycerophosphocholine on the optical density at 278 nm, this method yields a value of $E_{1cm}^{1mg/mL} = 2.12$ for glucagon in the complex if a value of 2.38 is assumed for glucagon in aqueous solution (Gratzer et al., 1967).

Results

The inner and outer layers of lecithin in unilamellar vesicles have sufficiently different chemical shifts for the quaternary ammonium methyl groups to be resolvable as two peaks in proton NMR spectra. The outer layer can be further distinguished by its susceptibility to effects of paramagnetic ions in the solution external to the vesicles (Bystrov et al., 1971). We have reproduced this result with sonicated unilamellar vesicles of dimyristoylglycerophosphocholine which show two peaks for the quaternary ammonium methyl groups in the absence of Mn^{2+}, the larger of the two peaks presumably being due to the larger external surface of the vesicle (Figure 1A). This latter peak is removed by paramagnetic broadening in the presence of Mn^{2+} (Figure 1B). By contrast, in the presence of glucagon only one resonance line is observed for these protons (Figure 1C) and all of the lipid is affected by Mn^{2+} resulting in the loss of this resonance line. This demonstrates that in the presence of glucagon the lipid is either no longer in vesicular form or that the vesicle has a greatly enhanced permeability to paramagnetic ions. We believe the latter explanation to be less likely because no time dependence of signal area was observed for spectra taken immediately after addition of the paramagnetic ion. Further studies on the shape and size of this lipoprotein complex show that it is an oblate ellipsoid which is not thick enough to accommodate two bilayers and consequently must be regarded as a disk of bilayer and not a vesicle (Jones and Epand, in preparation).

The major effect of glucagon on the NMR spectra of dimyristoylglycerophosphocholine is to broaden the resonance lines, particularly those of the methylene envelope and of the terminal methyl groups of the fatty acid (Figure 2). In the presence of glucagon these resonances are only observed at 20 °C (Figure 3). The quaternary ammonium methyl resonances are observable down to at least 5 °C and the area of this peak is independent of the presence of glucagon (Figure 3). The line width of this peak at half height is somewhat greater in the presence of glucagon than in its absence (Figure 3). Above the phase-transition temperature of the lipid, 24 °C, the complex precipitates and high-resolution NMR spectra are only observable at these temperatures after sonication, at which time they are indistinguishable from the spectra obtained from the lipid alone. The lipid requires sonication to exhibit high-resolution NMR spectra because it is liberated from the lipoprotein complex as high-molecular-weight aggregates which appear as a turbid suspension. This process is completely reversible and the lipid redissolves when the suspension is cooled below 23 °C. At concentrations where the glucagon remains soluble, it can be separated from the lipid by centrifugation above the phase-transition temperature. Results of measurements of the spin-lattice relaxation of the lipid at 20 °C in the presence and absence of glucagon are given in Table I.

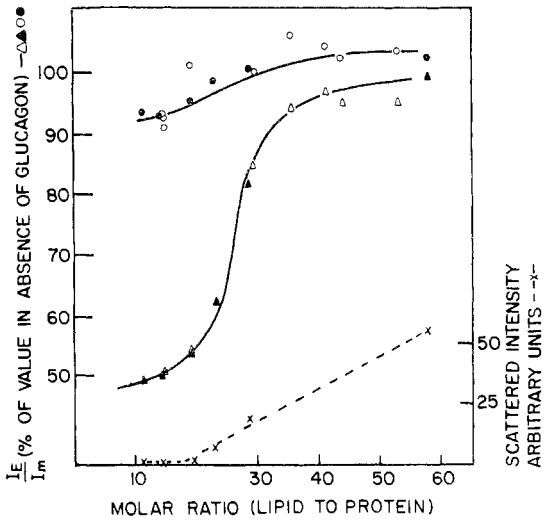

FIGURE 4: Effect of the molar ratio of dimyristoylglycerophosphocholine to glucagon on the relative magnitudes of pyrene (5.4 μM) excimer (I_E) and monomer (I_m) fluorescence emission and on the intensity of light scattering at 550 nm. All values are expressed as percent of that found for preparations of multilamellar vesicles of the lipid in the absence of glucagon: 0.01 M potassium phosphate buffer, pH 7.4, 300 μM lipid; I_E/I_m at 20 °C (○-●) and at 6 °C (△-▲); light scattering at 20 °C (XXX). Open and filled symbols represent results from two independent experiments.

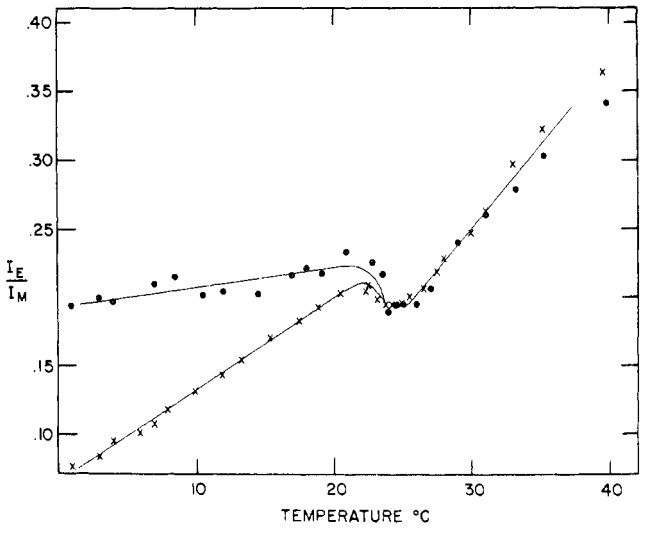

FIGURE 5: Effect of temperature on the relative magnitudes of pyrene (5.4 μM) excimer (I_E) and monomer (I_m) fluorescence in multilamellar vesicles of dimyristoylglycerophosphocholine (300 μM) (●) and in a solution of 300 μM dimyristoylglycerophosphocholine containing 20 μM glucagon (X).

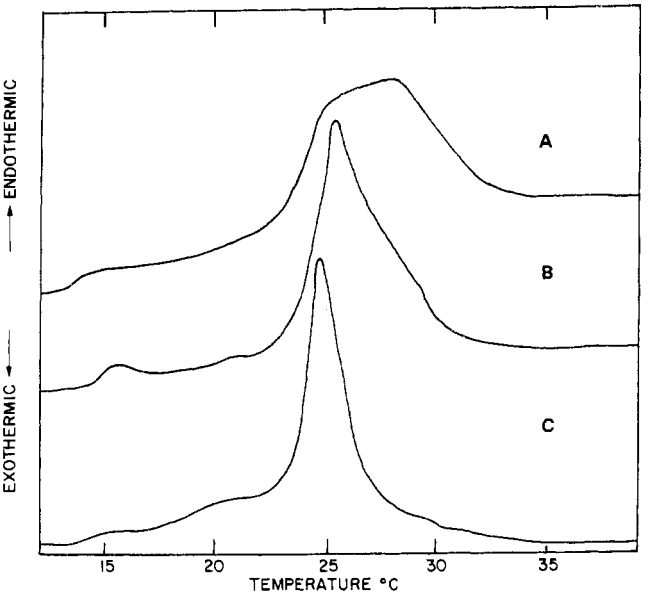

FIGURE 6: Variation of excess specific heat with temperature on heating. Dimyristoylglycerophosphocholine alone (curve c) and admixed with glucagon at 55:1 (curve b) and 35:1 (curve a) molar ratios of lipid to protein. Sample pans contained approximately 1 mg of lipid.

We have previously shown (Epand et al., 1977) that the fluorescent properties of pyrene, a probe which is sensitive to the state of the lipid bilayer (Soutar et al., 1974), are affected only by high concentrations of glucagon when embedded in bilayers of dimyristoylglycerophosphocholine. We find that the change in the fluorescence properties of pyrene occurs over a narrow range of ratios of dimyristoylglycerophosphocholine to glucagon (Figure 4) and the effect of glucagon is greater at lower temperatures (Figures 4 and 5). The transition to higher values of the I_E/I_m ratio is accompanied by the appearance of visually observable turbidity and is demonstrated by the increased light scattering measured in the fluorimeter at 550 nm (Figure 4). The glucagon remains associated with the lipid at high lipid to peptide ratios, as shown by the fact that the fluorescence enhancement of glucagon is maintained under these conditions (Epand et al., 1977) and the glucagon is readily sedimentable with the lipid at high lipid to peptide ratios (unpublished observations). The cooperative nature of the lipid binding to the glucagon complex is indicated by a Hill coefficient of 7.3 at 6 °C.

Differential scanning calorimetry demonstrates that glucagon broadens the calorimetric transition of the lipid without greatly affecting the temperature at which the transition is initiated on heating (Figure 6). The transition enthalpy measured from the scans is 5 ± 0.5 kcal/mol and is unaffected by the presence of glucagon. The value agrees favorably with recent estimates of the transition enthalpy for multilamellar suspensions of dimyristoylglycerophosphocholine (Mabrey and Sturtevant, 1976).

Changes in the environment of the aromatic side chains of glucagon caused by association of the peptide with lipid can be observed by ultraviolet difference spectroscopy (Figure 7) and by the CD (Figure 8). The temperature dependence of the near ultraviolet Cotton effects (Figure 9) was followed in detail for the 292-nm negative band and at several temperatures for the other bands. Although the spectra are noisy due to the weak rotational strengths of the transitions, the temperature profile exhibits a marked discontinuity at approximately 13 °C. The α-helical band at 222 nm (Figure 10) shows no such discontinuity in the temperature dependence (inset, Figure 10). Temperature changes can also generate a difference spectrum (Figure 11). The magnitude of this difference spectrum at 287 nm shows a small discontinuity at about 14 °C (inset, Figure 11). Changes in the light-scattering properties of the lipoprotein complex with temperature are not responsible for the discontinuity in the CD and difference spectrum observed at 13–15 °C. The right angle light scattering of the glucagon–lipid complex was measured at 350 nm in the fluorimeter. It varies only 8% over the temperature range of 5 to 21 °C and shows no discontinuity with temperature.

Discussion

NMR relaxation measurements have been extensively used to gain information about molecular motions within biological

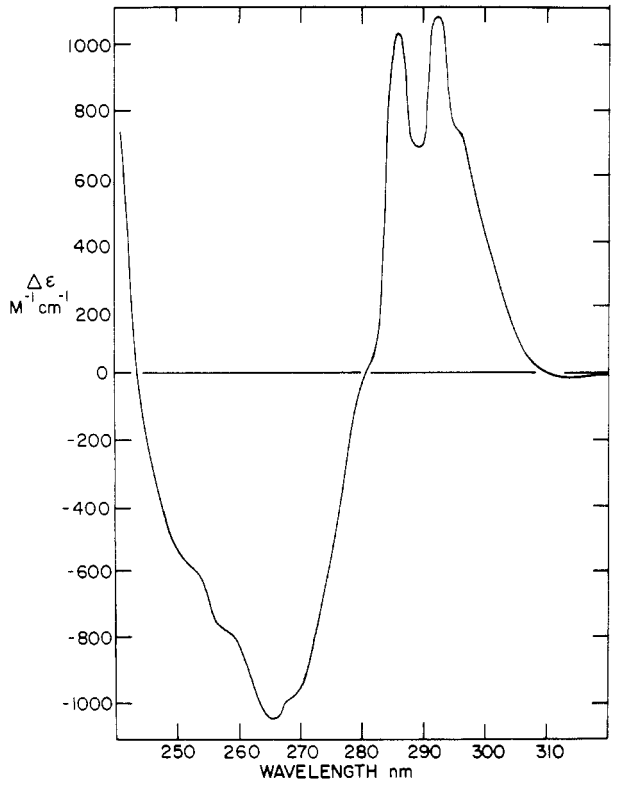

FIGURE 7: Ultraviolet difference spectra for dimyristoylglycerophosphocholine–glucagon complex vs. glucagon alone as reference: 0.1 M ammonium acetate, pH 7.4, 20 °C.

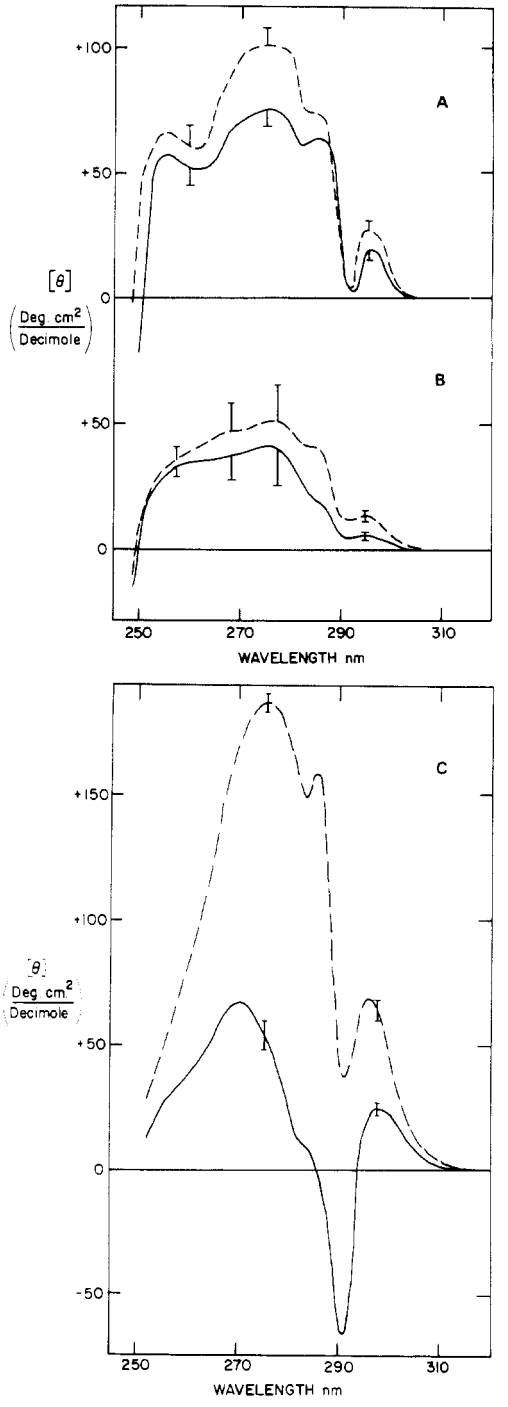

FIGURE 8: Circular dichroism of glucagon. Dashed curves, 6 °C; solid curves, 20 °C. Error bars are estimated from the noise level of at least three accumulated scans. (A) Seven volumes of propylene glycol and 3 volumes of 0.1 M ammonium acetate, pH 7.4; (B) 0.1 M ammonium acetate, pH 7.4; (C) complex of glucagon with dimyristoylglycerophosphocholine in 0.1 M ammonium acetate, pH 7.4.

membranes (Lee et al., 1974). It has been found that lateral diffusion of lipids in vesicles is the most significant intermolecular mechanism contributing to proton T_2 relaxation of terminal methyl groups and that line-width measurements from proton spectra can provide a lower limit for the rate of lateral diffusion (Lee et al., 1973). Our results demonstrate that glucagon drastically increases the line widths of dimyristoylglycerophosphocholine (Figures 2 and 3) without affecting the spin-lattice relaxation time (Table I). The line-broadening effect of glucagon is greatest on the methylene and terminal methyl protons which become broadened beyond detection at lower temperature (Figure 3). As the glucagon–lipid complex has a molecular weight similar to sonicated lipid vesicles and has dimensions of approximately 70 × 270 Å (Jones and Epand, manuscript in preparation), its overall tumbling rate should be rapid enough not to lead to line-width broadening for the anisotropic motion of lipids in bilayers (Finer, 1974). The terminal methyl group is the one whose relaxation would be expected to be most affected by lateral diffusion (Lee et al., 1973). Kroon et al. (1976), using protonated lecithin embedded in bilayers of deuterated lecithin, have confirmed that T_1 relaxation is dominated by intramolecular motions. They also point out the difficulties of obtaining a value for the lateral diffusion constant of lipid from line-width data. They do demonstrate however, using a 100 MHz NMR instrument, that at lower temperatures intermolecular relaxation mechanisms contribute to line-width broadening. In their study they analyzed the relaxation mechanisms of the methylene protons. In the case of the more mobile terminal methyl protons, T_1 should be affected even more exclusively by intramolecular mechanisms, while line broadening would be determined by intermolecular mechanisms such as lateral diffusion. We therefore conclude that the primary effect of glucagon is to restrict the lateral diffusion of lipid in the complex, without affecting intramolecular motion of the lipid. Alamethicin (Hauser et al., 1970) as well as glucagon increase the line-width of resonances from the lipid, while high density lipoprotein decreases it (Andrews et al., 1976) and rhodopsin has no effect on line-width but it affects the spin-lattice relaxation time of some of the lipid (Brown et al., 1976). Thus, there appear to be several mechanisms by which proteins can complex with lipids and influence their motional properties.

Results using pyrene as a fluorescence probe indicate that glucagon is able to alter the properties of the lipid only at

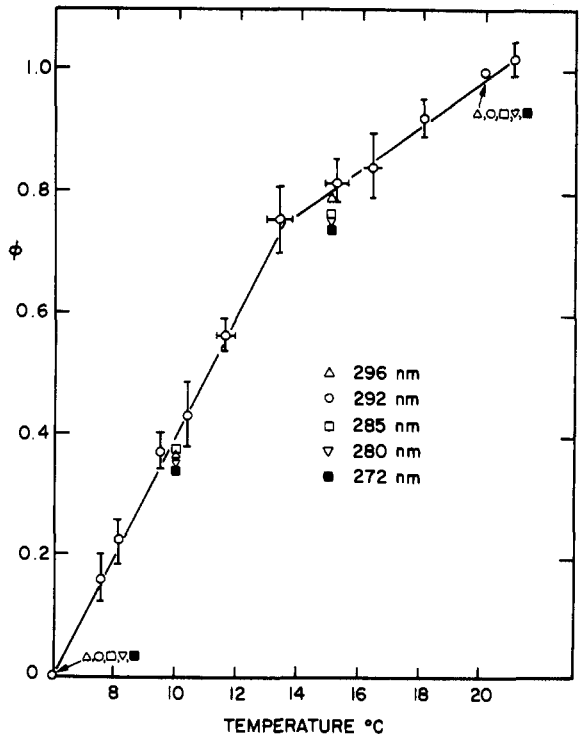

FIGURE 9: Effect of temperature on the fractional change in the magnitude of the circular dichroism of glucagon-dimyristoylglycerophosphocholine. ϕ is the fractional change at temperature T defined as $|[\theta]_T - [\theta]_6/[\theta]_6 - [\theta]_{20}|$, where $[\theta]_T$, $[\theta]_6$, and $[\theta]_{20}$ are the mean residue ellipticities at temperatures T, 6, and 20 °C, respectively. Error bars are estimated from the noise level of recordings at constant wavelength and from the variation of temperature during the recording which was continued for about 5 min for each point. Fractional changes of ellipticity at 6 and 20 °C are 0 and 1, respectively, by definition: 0.1 M ammonium acetate, pH 7.4. Ellipticities measured at 296 (△), 292 (○), 285 (□), 280 (▽) and 272 nm (■).

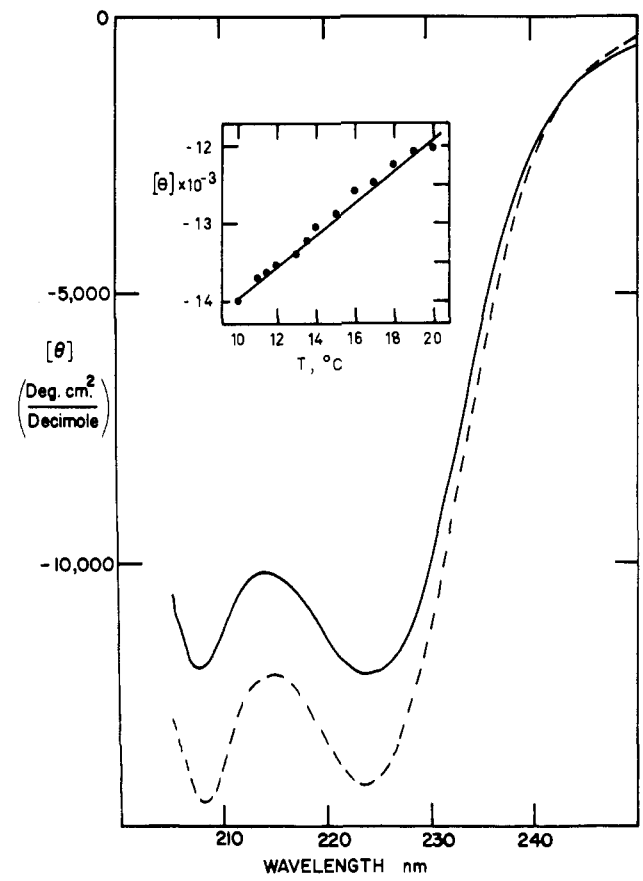

FIGURE 10: Circular dichroism of glucagon-dimyristoylglycerophosphocholine. Dashed curve, 6 °C, solid curve, 20 °C: 0.1 M ammonium acetate, pH 7.4. Inset: Variation of mean residue ellipticity at 222 nm with temperature.

temperatures below the phase transition (Figure 5). Glucagon can decrease pyrene excimer formation either by increasing the viscosity of the bilayer or by increasing the volume available for the pyrene to dissolve in. We favor the latter explanation, since T_1 studies indicate that there is no change in the viscosity of the lipid and by simply increasing the lipid concentration (Figure 4) the effect of glucagon can be abruptly diminished concomitant with the appearance of large particles and turbidity. Such behavior would not be expected if glucagon were decreasing the intramolecular motions of its surrounding lipid. It is likely that, because of the small dimensions of the glucagon-dimyristoylglycerophosphocholine complex (Jones et al., 1977), at least some of the lipid molecules are arrayed with a small radius of curvature as is found in unilamellar vesicles. The difference in pyrene fluorescence in the presence and absence of glucagon is greater when comparing the lipoprotein complex with multilamellar vesicles (Figure 5) than with unilamellar vesicles (cf. Figure 7 in Epand et al., 1977). Both our results as well as those using 1,6-diphenyl-1,3,5-hexatriene (Lentz et al., 1976) indicate that the properties of fluorescent probes in unilamellar vesicles in the gel state are more affected by changes of temperature than multilamellar vesicles. Thus, the properties of the lipid in the glucagon complex are similar to those in unilamellar vesicles of lipid alone, although the arrangement of lipid in association with glucagon is not in vesicular form as shown by its susceptibility to paramagnetic ion broadening (Figure 1). This susceptibility has also been observed for lipid in the high-density lipoprotein (Andrews et al., 1976). Our pyrene results are similar to those found for

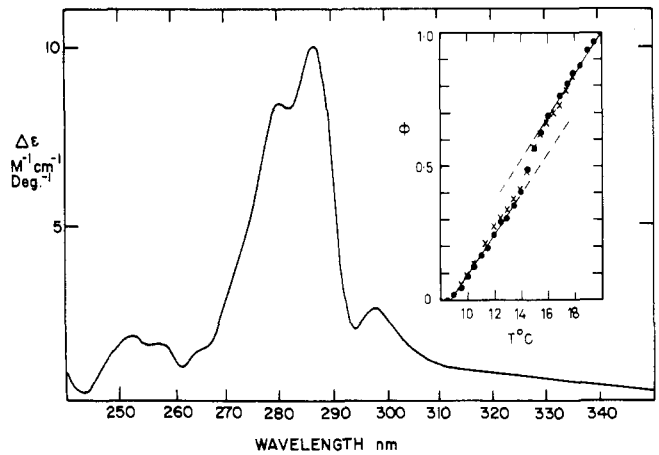

FIGURE 11: Thermal perturbation difference spectra of glucagon-dimyristoylglycerophosphocholine: 0.1 M ammonium acetate, pH 7.4. Reference 20 °C, sample 6 °C. Inset: temperature dependence of $\Delta\epsilon$ at 287 nm. ϕ is the fractional change at temperature T defined as $\epsilon_T - \epsilon_{8.5}/\epsilon_{20} - \epsilon_{8.5}$, where ϵ_T, ϵ_{20}, and $\epsilon_{8.5}$ are the molar extinction coefficients at 287 nm at temperatures T, 20, and 8.5 °C, respectively. The two different symbols represent values obtained from two independent experiments.

pyrene fluorescence in complexes of apolipoprotein C-III with dimyristoylglycerophosphocholine before the complex was heated above the phase-transition temperature (Novosad et al., 1976). In the case of the apolipoprotein C-III irreversible changes occurred when the complex was warmed above the

phase-transition temperature, while with glucagon the thermal changes are completely reversible. In the presence of higher concentrations of lipid it appears that the glucagon–lipid complex is incorporated into larger structures, exhibiting greatly enhanced light scattering, in which the structure of the lipid, as revealed by the pyrene probe, is similar to multilamellar vesicles and is not altered by the presence of glucagon.

The enthalpy change for the lipid-phase transition is independent of the presence of glucagon, indicating that the peptide does not grossly alter the bilayer structure of the lipid, despite the fact that the lipid is no longer in vesicular form. Glucagon dissociates from the complex at temperatures above the phase-transition temperature of the lipid but this is a slow process with a half-time of several minutes (Epand et al., 1977, and unpublished observations). During the time of approximately 10 min used to span the phase transition of the glucagon complex, a fraction of the peptide would be expected to be released. The enthalpy change corresponding to this dissociation is not observed, probably because it is too broad to be distinguished from a sloping baseline.

The temperature profile for the lipid transition in the presence of glucagon is different when measured with pyrene excimer fluorescence and when measured with differential scanning calorimetry. In the latter case, glucagon appears to broaden the transition and shift it to somewhat higher temperatures, although there is still a component melting at 24 °C (Figure 6), while in the former case the temperature and sharpness of the transition are unaltered by the peptide (Figure 5). In the case of the calorimetric measurements, the scan corresponding to a 35:1 molar ratio of lipid to glucagon is the same composition as that found for the solubilized glucagon–dimyristoylglycerophosphocholine complex and the sample used for this scan was prepared by concentration of the solubilized complex by lyophilization. It thus appears that different processes are being monitored by the two techniques. The NMR results indicate that the major effect of glucagon is to lower the rate of lateral diffusion of the lipid while intramolecular motions are unaffected. The pyrene fluorescence in pure lipid systems has been shown to accurately reflect the phase transition as measured by differential scanning calorimetry (Soutar et al., 1974). It is expected that pyrene fluorescence would be mainly affected by the hydrophobic volume within the bilayer as well as the microviscosity as determined largely by intramolecular motions, neither of which are altered by glucagon above 23 °C. By contrast, lateral diffusion of the lipid may be a major factor in determining the calorimetric transition, as it would affect the translational energy of the lipid. The temperature and sharpness of the calorimetric transition resemble those for the change of glucagon fluorescence in the complex with temperature (Epand et al., 1977). Both processes are caused by the glucagon becoming less buried in the bilayer, leading to a loss of fluorescence enhancement of the tryptophan residue of glucagon and an increase in the lateral diffusion of the lipid which may be responsible for the observed calorimetric results. In addition, some broadening of the calorimetric transition may result because thermodynamic equilibrium is not attained at each temperature with the scanning rate of 1 °C/min which was employed.

The difference spectrum (Figure 7) indicates that the tryptophan and tyrosine residues of glucagon are entering a more hydrophobic environment when interacting with lipid. This is shown by the red-shifted bands with maxima at 292 and 286 nm characteristic of tryptophan and tyrosine, respectively (Herskovits and Sorensen, 1968; Bello, 1970). The 292-nm band may also have contributions from tyrosine, as the difference spectrum of *p*-cresol shifts to this wavelength when incorporated into a hydrophobic solvent capable of hydrogen bonding (Filippi et al., 1976), but part must also be due to tryptophan, as it extends above 300 nm and the fluorescence emission from tryptophan also indicates that this residue is in a more hydrophobic environment in the presence of lipid (Epand et al., 1977). The large magnitude of the difference spectra at 300 nm suggests that there may be a weak band in this wavelength region. This is supported by the existence of a band at 295 nm in the CD spectra of the hormone which shifts to 297 nm in the presence of lipid (Figure 8) and a band at 298 nm in the thermal difference spectrum (Figure 11). There is a weak band at 298 nm in tryptophan containing peptides due to the 0–0 transition of the ^1L_a band (Strickland et al., 1969). It has been suggested that the magnitude of this band in difference spectra is enhanced by an increase in the negative charge near the indole group (Ananthanarayanan and Bigelow, 1969). The closest charged groups, in the primary structure, to the tryptophan at position 25 are the aspartic acid and the terminal threonine residues at positions 21 and 29, respectively (Bromer et al., 1957). These charged residues may be expected to come into closer proximity to the tryptophan when the glucagon adopts a highly α-helical conformation as it does in the presence of lipid (Epand et al., 1977), thus giving rise to the high-wavelength shoulder in the difference spectrum.

Changes in the environment of the aromatic chromophores are also demonstrated by changes in the near-ultraviolet CD in the presence of lipid (Figure 8). The CD, in the presence of lipid, does not resemble that of the glucagon monomer (Figure 8) nor of more highly α-helical forms of glucagon, such as that of glucagon in the associated state or in 2-chloroethanol (Gratzer and Beaven, 1969) or in the presence of 70% propylene glycol (Figure 8). These results suggest that the state of glucagon in the lipoprotein complex does not closely resemble that of the hormone in the crystalline state. The larger magnitude of the near-ultraviolet CD of glucagon at 6 °C in the presence of lipid (Figure 8C) suggests that there is a decreased molecular motion of the aromatic side chains under these conditions (Strickland et al., 1969).

A more detailed analysis of the temperature dependence of the near-ultraviolet Cotton effects of glucagon in the presence of lipid suggests that the change is biphasic, being more rapid below about 14 °C (Figure 9). This temperature corresponds to that for the lower calorimetric transition observed for dimyristoylglycerophosphocholine (Mabrey and Sturtevant, 1976). These results thus suggest that the conformational flexibility of the aromatic side chains of glucagon in the lipoprotein complex decreases with decreasing temperature and that the degree of rigidity increases more rapidly below the "premelt" temperature of the lipid. This temperature effect on the conformation of glucagon in association with lipid is a subtle one and does not involve a major rearrangement of the polypeptide chain. This is indicated by the far-ultraviolet Cotton effects (Figure 10) which show relatively little change with temperature and the rate of change of $[\theta]_{222}$ with temperature is linear (inset, Figure 10).

Thermal difference spectra of proteins and model chromophores have been recently analyzed by Nicola and Leach (1976). The magnitudes of our thermal difference spectra are of the same order as that found for model peptides and are larger than would be expected for aromatic chromophores buried in the interior of a protein. However, part of our difference spectra may be caused by a minor conformational change of the protein with temperature which would lead to a decreased exposure of the chromophores at lower temperature. No comparable effect would be expected from rigid

globular proteins or small model peptides. It should also be noted that the magnitude of the thermal difference spectra between 6 and 20 °C is only about 15% of the difference spectrum observed for glucagon in the presence and absence of lipid (Figure 7). Such a phenomonon would explain the positive sign of the band at 298 nm and the low magnitude of the band at 292 nm which are different from those observed in model peptides or globular proteins (Nicola and Leach, 1976) or from glucagon itself (Gratzer et al., 1968). The discontinuity of the magnitude of the thermal difference spectra with temperature (inset, Figure 11) confirms that a conformational transition of the peptide contributes to the observed thermal difference spectrum. No such deviation from a linear dependence on temperature is observed for model peptides or globular proteins (Nicola and Leach, 1976). The discontinuity occurs in the region of the premelt temperature of the lipid and confirms the results from the CD that the conformation of the peptide is sensitive to this transition of the lipid. Whether the observed changes are direct consequences of lipid–protein interaction or modulation of protein–protein interactions cannot be determined by these techniques.

We can thus conclude that glucagon is incorporated into a more hydrophobic environment when interacting with dimyristoylglycerophosphocholine in the gel state. This is accompanied by a marked enhancement of helix content. The conformation of glucagon is sensitive not only to the major thermal transition of the lipid at 24 °C but changes in the near ultraviolet CD are also observed at the "premelt" transition of 15 °C. The effects of glucagon on the properties of the lipid by contrast are smaller. The properties of pyrene around the transition temperature are unaltered as are the NMR spin-lattice relaxation times and transition enthalpy. The main effect which is observed is a broadening of the NMR line width of the lipid.

There are many similarities between the interactions of glucagon and of high-density lipoproteins with lecithin. Both glucagon (Jones et al., 1977) and the apo-A1 protein from high-density lipoprotein (Tall and Small, 1977) can solubilize lipid in a form which appears similar by electron microscopy. Glucagon (Epand et al., 1977) and high-density lipoprotein (Andrews et al., 1976) enhance their helical contents when interacting with lipid. All of the phospholipid head groups in the complexes of both proteins are susceptible to NMR line broadening by paramagnetic ions (this work and Andrews et al. (1976)). The main differences between the two systems are that the interaction of glucagon is dependent on the state of the lipid (Epand et al., 1977), the effects of the protein on the NMR line widths of the lipid (this work and Andrews et al. (1976)) are in opposite directions, and that the enthalpy of the phase transition of the lipid, which is little affected by glucagon (Figure 6), is greatly diminished in the high-density lipoprotein. Part of these differences may arise because of the existence of regions in the high-density lipoprotein which do not form amphipathic helices. A model has been suggested for the interaction of the high-density lipoprotein with dimyristoylglycerophosphocholine (Andrews et al., 1976) in which the amphipathic helix lies parallel to the plane of the lipid bilayer at the level of the glycerol moiety. This model could readily explain how glucagon could interfere with the lateral diffusion of lipids without greatly affecting their intramolecular motions. Lateral diffusion of lipids in biological membranes has been found to be at least an order of magnitude slower than in pure phospholipid bilayers (Schlessinger et al., 1977). The interaction of some membrane proteins with lipid, in a manner similar to that described in this paper for glucagon, could contribute to this effect.

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References

Ananthanarayanan, V. S., and Bigelow, C. C. (1969), *Biochemistry 8,* 3717–3723.

Andrews, A. L., Atkinson, D., Barratt, M. D., Finer, E. G., Hauser, H., Henry, R., Leslie, R. B., Owens, N. L., Phillips, M. C., and Robertson, R. N. (1976), *Eur. J. Biochem. 64,* 549–563.

Assmann, G., and Brewer, H. B., Jr. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 1534–1538.

Bartlett, G. R. (1959), *J. Biol. Chem. 234,* 466–468.

Bello, J. (1970), *Biochemistry 9,* 3562–3568.

Blundell, T. L., Dockerill, S., Sasaki, K., Tickle, I. J., and Wood, S. P. (1976), *Metabolism 25,* 1331–1336.

Bromer, W. W., Sinn, L., and Behrens, O. K. (1957), *J. Am. Chem. Soc. 79,* 2801–2805.

Brown, M. F., Miljanich, G. P., Franklin, L. K., and Dratz, E. A. (1976), *FEBS Lett. 70,* 56–60.

Bystrov, V. F., Dubrovina, N. I., Barsukov, L. I., and Bergelson, L. D. (1971), *Chem. Phys. Lipids 6,* 343–350.

Cancellieri, A., Frontali, C., and Gratton, E. (1974), *Biopolymers 13,* 735–743.

Chan, S. I., Sheetz, M. P., Seiter, C. H. A., Feigenson, G. W., Hsu, M., Lau, A., and Yau, A. (1973), *Ann. N.Y. Acad. Sci. 222,* 499–522.

Contaxis, C. C., and Epand, R. M. (1974), *Can. J. Biochem. 52,* 456–468.

Epand, R. M., Jones, A. J. S., and Schreier, S. (1977), *Biochim. Biophys. Acta 491,* 296–304.

Filippi, B., Borin, G., Moroder, L., and Marchiori, F. (1976), *Biochim. Biophys. Acta 454,* 514–523.

Finer, E. G. (1974), *J. Magn. Reson. 13,* 76–86.

Gratzer, W. B., and Beaven, G. H. (1969), *J. Biol. Chem. 244,* 6675–6679.

Gratzer, W. B., Beaven, G. H., and Bailey, E. (1967), *Biochem. Biophys. Res. Commun. 28,* 914–919.

Gratzer, W. B., Beaven, G. H., Rattle, H. W. E., and Bradbury, E. M. (1968), *Eur. J. Biochem. 3,* 276–283.

Hauser, H., Finer, E. G., and Chapman, D. (1970), *J. Mol. Biol. 53,* 419–433.

Herskovitis, T. T., and Sorensen, M. (1968), *Biochemistry 7,* 2523–2532.

Inouye, M. I. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 2396–2400.

Jones, A. J. S., Epand, R. M., and Vail, W. J. (1977), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 36,* 707.

Kroon, P. A., Kainosho, M., and Chan, S. I. (1976), *Biochim. Biophys. Acta 433,* 282–293.

Lee, A. G., Birdsall, N. J. M., and Metcalfe, J. C/ (1973), *Biochemistry 12,* 1650–1659.

Lee, A. G., Birdsall, N. J. M., and Metcalfe, J. C. (1974), *Methods Membr. Biol. 2,* 1–156.

Lentz, B. R., Barenholz, Y., and Thompson, T. E. (1976), *Biochemistry 15,* 4521–4528.

Mabrey, S., and Sturtevant, J. M. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 3862–3866.

Nicola, N. A., and Leach, S. J. (1976), *Int. J. Peptide Protein Res. 8,* 393–415.

Novosad, Z., Knapp, R. D., Gotto, A. M., Pownall, H. J., and

Morrisett, J. D. (1976), *Biochemistry 15,* 3176-3183.
Pownall, H. J., Morrisett, J. D., Sparrow, J. T., and Gotto, A. M. (1974), *Biochem. Biophys. Res. Commun. 60,* 779-786.
Schlessinger, J., Axelrod, D., Koppel, D. E., Webb, W. W., and Elson, E. L. (1977), *Science 195,* 307-309.
Segrest, J. P., Pownall, H. J., Jackson, R. L., Glenner, G. G., and Pollack, P. S. (1976), *Biochemistry 15,* 3187-3191.
Soutar, A. K., Pownall, H. J., Hu, A. S., and Smith, L. C. (1974), *Biochemistry 13,* 2828-2836.
Strickland, E. H., Horwitz, J., and Billups, C. (1969), *Biochemistry 8,* 3205-3213.
Suurkuusk, J., Lentz, B. R., Barenholz, Y., Biltonen, R. L., and Thompson, T. E. (1976), *Biochemistry 15,* 1393-1401.
Tall, A. R., and Small, D. M. (1977), *Nature (London) 265,* 163-164.

Ribonucleotide Reductase from *Escherichia coli.* Identification of Allosteric Effector Sites by Chromatography on Immobilized Effectors†

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ABSTRACT: Ribonucleotide reductase is responsible for the production of deoxyribonucleotides by catalyzing the reduction of ribonucleoside diphosphates. The enzyme is allosterically regulated in a complex way by the nucleoside triphosphates, ATP, dTTP, dGTP, dCTP, and dATP. Ribonucleotide reductase consists of two nonidentical subunits, proteins B1 and B2. Both substrates and allosteric effectors bind exclusively to B1. Binding of protein B1 to dTTP or dATP covalently coupled to Sepharose and elution with concentration gradients of the different nucleoside triphosphate effectors gave information about (1) the arrangement of the effector binding sites on protein B1 and (2) the affinity of the effectors for these sites. Protein B1 thus has two classes of effector binding sites. One class binds all effectors, as demonstrated by elution of the protein from dTTP-Sepharose with dATP, dGTP, ATP, or dCTP. The second class binds only dATP or ATP, since dATP and ATP were the only nucleotides which eluted protein B1 from dATP-Sepharose. These results confirm earlier data obtained by dialysis binding experiments. The eluting concentrations obtained for the different nucleoside triphosphates in experiments with dTTP-Sepharose could be used to calculate unknown dissociation constants for protein B1-effector binary complexes. This was possible, since a plot of the eluting concentrations vs. known dissociation constants was linear.

Ribonucleotide reductase from *Escherichia coli* (EC 1.17.4.1) is an allosteric enzyme which catalyzes the reduction of ribonucleoside diphosphates to the corresponding deoxyribonucleotides (Reichard, 1968). The same enzyme reduces all four common ribonucleoside diphosphates and the allosteric effectors are nucleoside triphosphates.

The allosteric regulation controls both the general activity and the substrate specificity of the enzyme (Larsson and Reichard, 1966a,b). Thus ATP and low concentrations (~1 μM) of dATP stimulate the reduction of CDP and UDP, while dGTP stimulates the reduction of ADP and GDP. Reduction of all four substrates is stimulated by dTTP. High concentrations of dATP (>10 μM) inhibit the enzyme.

Ribonucleotide reductase consists of two nonidentical subunits, protein B1 and protein B2. The separated subunits are inactive, but recombine in the presence of Mg^{2+} ions to form a 1:1 complex, the active enzyme molecule (Thelander, 1973). Protein B2 contains iron and an organic free radical essential for activity (Brown et al., 1969b; Ehrenberg and Reichard, 1972). Protein B1 binds substrates and allosteric effectors, as demonstrated by binding experiments with dialysis techniques (von Döbeln and Reichard, 1976; Brown and Reichard, 1969). In addition, B1 contains redox active dithiols, which supply the electrons necessary for the reduction (Thelander, 1974).

This paper describes the characterization of the allosteric effector binding sites on protein B1 by affinity chromatography on Sepharose columns containing covalently coupled dTTP or dATP.

Experimental Procedure

Materials. Unlabeled nucleotides (at least 97% pure) were obtained from Calbiochem AG or Sigma and tritium-labeled nucleotides were from Amersham. They were used without further purification. α-^{32}P-labeled dGTP and β-^{32}P-labeled ATP were synthesized in this laboratory (Pigiet et al., 1974). Protein B1 (sp act. 400-450 units/mg) was a gift from Dr. Britt-Marie Sjöberg and had been prepared from a λ-lysogenic *Escherichia coli* strain overproducing ribonucleotide reductase (Eriksson et al., 1977). Two different preparations, which were 80-90% pure as determined by gel electrophoresis in the presence of sodium dodecyl sulfate, were used.

Sepharose 4B with covalently coupled dTTP or dATP was kindly provided by Dr. Lars Thelander and had been synthesized according to the method described by Berglund and Eckstein (1972). This involved synthesis of the *p*-aminophenyl esters of the γ-phosphate of dTTP or dATP and coupling of these derivatives to Sepharose via the amino group. The *p*-aminophenyl esters of the nucleoside triphosphates had allosteric effects similar to those of the corresponding unmodified

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