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PHYSICOCHEMICAL CHARACTERIZATION OF GLUCAGON-CONTAINING LIPID MICELLES

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Summary

Fluorescence studies showed that glucagon binds to a variety of micellar lipids. By means of ultracentrifugation and quasi-elastic light-scattering, it was found that stoichiometrically well defined complexes were formed between glucagon and perdeuterated dodecylphosphocholine micelles consisting of one glucagon molecule and approx. 40 detergent molecules. Well resolved ^{1}H-NMR spectra were obtained for glucagon in the deuterated micelles. Studies of nuclear Overhauser effects between individually assigned protons in different regions of the amino acid sequence indicated that micelle-bound glucagon adopts a well defined, predominantly extended conformation. Evidence obtained from circular dichroism indicates that the conformation of glucagon bound to various micellar lipids is largely independent of the type of lipid and, furthermore, appears to be very similar to that of glucagon bound to lipid bilayers.

Introduction

The lack of a purified receptor system and of methods for detailed conformational studies of membrane-bound proteins has led to efforts to characterize the glucagon-receptor interaction through studies of the conformational properties of the hormone. In previous studies, trimeric glucagon in crystals has been shown to have a high content of α-helix [1]. For self-aggregated glucagon in solution, evidence was presented for either α-helical [2,3] or β-sheet [4,5] conformations, and monomeric glucagon in solution has been shown to have a predominantly flexible, extended structure which includes at least on structured region with a conformation different from the crystal

conformation [6]. In the present paper, the nature of the interaction between glucagon and lipid/water interfaces and the conformation of glucagon when bound to such interfaces are investigated. Since glucagon is one of a number of polypeptides and proteins for which it has been proposed that interaction with lipid membranes involves formation of an amphipathic α-helix [7–9], glucagon should also provide a valid test of our recent proposal [10] that high-resolution ^{1}H-NMR of complexes formed between membrane proteins and fully deuterated micelles may provide a means of obtaining conformational information relevant to the functional properties of membrane-bound polypeptide chains. For the analysis of high-resolution ^{1}H-NMR data on polypeptide chains bound to deuterated lipid micelles, the size and stoichiometry of the micelle-polypeptide complexes have to be known. This report describes the characterization of glucagon-containing micelles with a variety of physico-chemical techniques and also includes an account of the NMR experiments used to outline the global features of the glucagon conformation in the micelles. These data provided the basis for the elucidation of more highly refined structures for selected fragments of the polypeptide chain, which will be described in a forthcoming publication.

Materials and Methods

Chemicals. Glucagon was purchased from Calbiochem and used without further purification. (Homoserine-27)-glucagon 1-27, prepared by CNBr cleavage according to the method of Epand [11], was a gift from Dr. A. Bundi. The syntheses of dodecyldimethylamine oxide [12], dodecylphosphocholine [12] and [$^2H_{38}$]dodecylphosphocholine [10] have been described previously. Dodecyl(oxyethylene)$_{7,8}$ glycol was a gift from Dr. M. Chabre and hexadecylphosphocholine and α-L-diheptanoylphosphatidylcholine were gifts from Dr. A.J. Slotboom.

Ultracentrifugation and light scattering. Unless otherwise stated, these measurements were all performed in 0.05 M phosphate buffer, pH 7.0, with $2 \cdot 10^{-2}$ M dodecylphosphocholine and $2 \cdot 10^{-4}$ M glucagon. The partial specific volume of glucagon, $\bar{\nu} = 0.708$ cm^3/g, was calculated from the amino acid composition [13].

Ultracentrifugation experiments were performed on an MSE Mark 2 analytical ultracentrifuge. Sedimentation equilibrium experiments were performed with double-sector cells using the ultraviolet absorption at 280 nm to follow the glucagon concentration. The stoichiometry of the glucagon-dodecylphosphocholine complexes was determined according to the method of Reynolds and Tanford [14]. An apparent absorption of the reference detergent solution caused by the optical system hindered the determination of the baseline, therefore the absorption of the glucagon-dodecylphosphocholine complex was measured against both buffer and detergent without glucagon in order to provide an estimate of the baseline error. Underfill, synthetic-boundary cells and schlieren optics were used for diffusion constant measurements and the data evaluated by using the height-area method [15]. The boundary was between a solution with $2 \cdot 10^{-2}$ M dodecylphosphocholine and a second solution with

$1 \cdot 10^{-3}$ M dodecylphosphocholine to avoid detergent concentrations below the critical micelle concentration.

Quasi-elastic light-scattering measurements utilized an argon laser (Spectra Physics) at 514.5 nm and a 96-channel correlator (Malvon). The autocorrelation function was fitted with a single exponential function [16]. For the light-scattering experiments and for ultracentrifugation experiments using schlieren optics, the detergent-to-glucagon ratio was chosen to provide sufficient detergent to bind all glucagon, but to avoid large excesses of detergent micelles without bound polypeptide.

Fluorescence spectroscopy. Fluorescence measurements were performed at 25°C on an Aminco-Bowman SPF fluorometer which automatically corrected for non-linearities in the optical system. The excitation wavelength was 280 nm. 3 ml of a $1 \cdot 10^{-6}$—$3 \cdot 10^{-6}$ M solution of glucagon or glucagon fragments in 0.05 M phosphate buffer at pH 7.0 were titrated with detergent by addition of aliquots of concentrated detergent solution.

Circular dichroism spectroscopy. Circular dichroism (CD) measurements were performed on a Jasco 3500 C instrument at room temperature. Quartz cells with pathlengths between 0.1 and 1 mm were used to keep the total absorbance below 2 units in the spectral region investigated. Glucagon-micelle complexes were prepared by direct dissolution of glucagon and lipid at the desired concentrations. No time-dependent variation of the CD spectra was observed.

NMR spectroscopy. 360 MHz Fourier transform ^{1}H-NMR spectra were recorded on a Bruker HX-360 instrument using internal ^2H_2O for the lock system. Chemical shifts are in parts per million (ppm) relative to an external reference of sodium 3-trimethylsilyl[2,2,3,3-$^2H_4$]propionate in aqueous solution at p^{2}H 7.0 [17]. An external reference was employed to avoid interactions between the reference compound and detergent micelles. Values of p^{2}H are pH meter readings uncorrected for isotope effects. In some cases, the free induction decays were multiplied with a phase-shifted sine bell to improve the spectral resolution [18,19]. Spin-decoupling difference spectra [20] and truncated driven nuclear Overhauser enhancement difference spectra [21,22] were recorded as previously described.

Results

Binding of glucagon to monomeric and micellar lipids

To test for interaction of glucagon and glucagon fragments with lipids, the relative intensity and wavelength of maximum emission for fluorescence of the single tryptophan residue at position 25 of the glucagon amino acid sequence were measured as a function of detergent concentration (Fig. 1). For glucagon and (homoserine-27)-glucagon 1-27 in the presence of dodecyldimethylamine oxide and for glucagon in the presence of dodecylphosphocholine, at most, small changes in the fluorescence intensity or emission maximum were observed below the critical micelle concentration of the detergent. Above the critical micelle concentration, increases in fluorescence intensity and a blue shift of approx. 10 nm of the emission maximum were observed (Fig. 1). We conclude that for the detergents used in this study, the presence

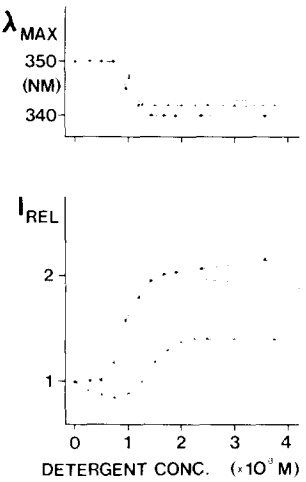

Fig. 1. Relative intensity and wavelength of maximum emission for the fluorescence of tryptophan-25 of glucagon and glucagon fragments as a function of detergent concentration. Measured with $1 \cdot 10^{-6}$—$3 \cdot 10^{-6}$ M peptide in 0.05 M phosphate buffer at pH 7.0 and 20°C. The excitation wavelength was 280 nm. •, glucagon plus dodecylphosphocholine; ▲, glucagon plus dodecyldimethylamine oxide; ○, (homoserine-27)-glucagon 1-27 plus dodecyldimethylamine oxide. The critical micelle concentrations are approx. $2 \cdot 10^{-3}$ M for dodecyldimethylamine oxide and approx. $1 \cdot 10^{-3}$ M for dodecylphosphocholine.

of detergent micelles is necessary to achieve binding of glucagon or the glucagon fragments to the detergent. Since binding of glucagon appeared to be essentially complete at detergent concentrations above the critical micelle concentration, these experiments also provided evidence that at the much higher polypeptide and detergent concentrations used for NMR experiments (see below), virtually all of the peptide must be bound to the detergent micelles.

CD spectra of glucagon bound to various different lipid micelles

To characterize the overall features of the conformation of glucagon bound to a lipid micelle and to determine whether the glucagon conformation is dependent on the nature of the lipid, CD spectra have been measured for glucagon in dilute aqueous solution and for glucagon bound to various types of detergent micelles or to phosphatidylcholine micelles (Fig. 2). As has been observed previously [3,23,24], glucagon in dilute aqueous solution shows a CD spectrum typical of a predominantly flexible, extended conformation (Fig. 2). This is consistent with NMR studies which indicated a flexible, extended conformation except near residues 22—26 where a local structure has been characterized [6]. Only minor variations in the CD spectra were observed for glucagon bound to the various lipids (Fig. 2) and similar spectra have been observed previously for glucagon bound to lysophosphatidylcholine micelles [25] or to dimyristoylphosphatidylcholine bilayers [9]. These results indicate that the CD spectrum of micelle-bound glucagon does not depend strongly on the type of lipid. It should be noted, in particular, that on the basis of CD spectra, glucagon appears to have a very similar confor-

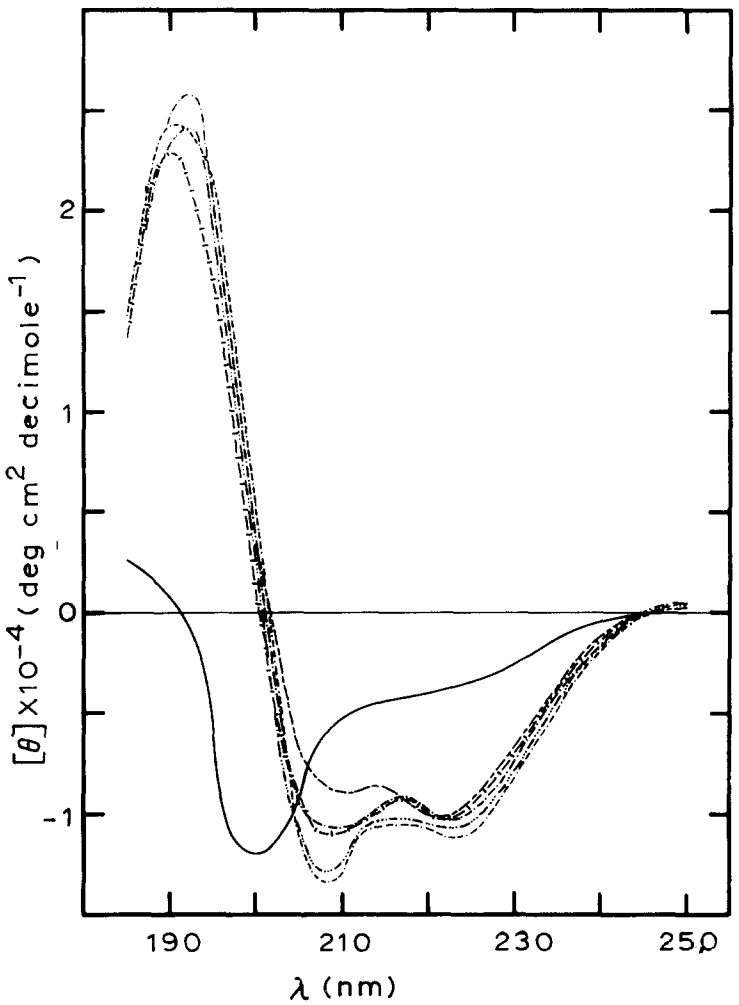

Fig. 2. CD spectra showing the mean residue molar ellipticities as a function of wavelength for glucagon and glucagon-lipid complexes at 23°C. ———, $1 \cdot 10^{-4}$ M glucagon at pH 11.0 in H_2O. Glucagon-lipid complexes were measured in 0.05 M phosphate buffer, pH 7.0. —·—·—, $1 \cdot 10^{-4}$ M glucagon plus $1 \cdot 10^{-2}$ M dodecylphosphocholine; —|—|—, $2 \cdot 10^{-4}$ M glucagon plus $3 \cdot 10^{-2}$ M dodecyldimethylamine oxide; ------, $2 \cdot 10^{-4}$ M glucagon plus $2 \cdot 10^{-2}$ M hexadecylphosphocholine; —···—···, $2 \cdot 10^{-4}$ M glucagon plus $2 \cdot 10^{-2}$ M dodecyl(oxyethylene)$_{7,8}$ glycol; — — —, $6 \cdot 10^{-5}$ M glucagon plus $1 \cdot 10^{-2}$ M α-L-diheptanoyl-phosphatidylcholine.

mation when bound to detergent micelles (Fig. 2) or to phosphatidylcholine bilayers [9].

Size and stoichiometry of glucagon-containing dodecylphosphatidylcholine micelles

While glucagon monomers with a largely flexible extended conformation are the prevalent species in dilute aqueous solution [6], trimeric glucagon with a high content of α-helix was observed in alkaline solution at higher concentrations [26]. This raises the question as to the state of aggregation

TABLE I

PHYSICOCHEMICAL CHARACTERIZATION OF THE COMPLEXES FORMED BETWEEN GLUCAGON AND DODECYLPHOSPHOCHOLINE MICELLES

Parameter measured	Experimental method	Glucagon-dodecylphosphocholine complex	Dodecylphosphocholine micelles [12]
Diffusion constant (cm^2/s)	Analytical ultracentrifugation	$D_{uc}^{20} = 7.9 \cdot 10^{-7}$	$D_{uc}^{20} = 9.2 \cdot 10^{-7}$
	Quasi-elastic light scattering	$D_{ls}^{20} = 7.6 \cdot 10^{-7}$	$D_{ls}^{20} = 7.8 \cdot 10^{-7}$
		$D_{ls}^{20} = 6.7 \cdot 10^{-7}$ [a]	$D_{ls}^{20} = 6.9 \cdot 10^{-7}$ [a]
Particle weight	Analytical ultracentrifugation	peptide: 3200 ± 500 [b] detergent: 13 300 ± 6300 [c]	
Partial specific volume	Density determination	0.89 cm^3/g [d]	0.937 cm^3/g

[a] Measured with deuterated dodecylphosphocholine.
[b] By equilibrium ultracentrifugation in $H_2O/^2H_2O$ mixtures according to the method of Reynolds and Tanford [14].
[c] From the slope of the plot in Fig. 3.
[d] Calculated from Fig. 3 for $M_P(1 - \phi'\rho) = 0$.

of glucagon bound to lipids, where the polypeptide also appears to adopt a helical conformation. We have used equilibrium ultracentrifugation and measurements of diffusion constants by both ultracentrifugation and quasi-

TABLE II

SIZE, SHAPE AND STOICHIOMETRY OF THE COMPLEXES FORMED BETWEEN GLUCAGON AND DODECYLPHOSPHOCHOLINE MICELLES DETERMINED FROM THE EXPERIMENTS IN TABLE I

Structual parameter	Experimental basis (see Table I)	Glucagon-dodecylphosphocholine complex	Dodecylphosphocholine micelles [12]
Stoichiometry	particle weight	0.9 ± 0.15 peptide molecules and 38 ± 18 detergent molecules per micelle	56 ± 5 detergent molecules/micelle
Diameter of equivalent sphere * (Å)	D_{uc}^{20}	54	47
	D_{ls}^{20}	56	55
	D_{ls}^{20}	64 **	62 **
	Particle weight and partial specific volume	35 ± 3	39 ± 3
Maximum thickness of hydration shell * (Å)	D_{uc}^{20}, D_{ls}^{20} and particle weight	—	6 ± 2
Maximum asymmetry a/b of prolate ellipsoid *	D_{uc}^{20}, D_{ls}^{20} and particle weight	—	~6 *

* See Ref. 15 for the procedures used in these calculations. Maximum hydration was calculated assuming a spherial shape of the particle. Maximum assymmetry was calculated with the assumption that there is no hydration and that the shape of the particle is a prolate ellipsoid with axes a and b.
** Calculated for deuterated dodecylphosphocholine.

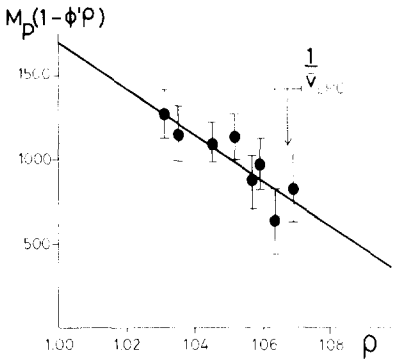

Fig. 3. Sedimentation equilibrium measurements using the method of Reynolds and Tanford [14] for a solution containing $2 \cdot 10^{-4}$ M glucagon and $2 \cdot 10^{-2}$ M dodecylphosphocholine in 0.05 M phosphate buffer at pH 7.0 and 20°C. The mean value, $M_P(1 - \phi'\rho)$, is plotted versus the solvent density, ρ, which was altered by mixing various proportions of H_2O and ^2H_2O. M_P is the molecular weight of the protein component of the complex and ϕ' is the volume increment/g protein. The arrow indicates the solvent density for which $\rho = 1/\bar{v}$ of the dodecylphosphocholine. Error limits for the value of M_P $(1 - \phi'\rho)$ at a given solvent density were calculated from the two different reference solutions used in the determination of the glucagon concentration by ultraviolet scanning (see Materials and Methods).

elastic light-scattering to characterize the size and stoichiometry of the complexes formed between glucagon and dodecylphosphocholine (Tables I and II). For comparison, Tables I and II also show data reported previously for dodecylphosphocholine micelles without bound polypeptide [12].

The state of aggregation of glucagon bound to dodecylphosphocholine micelles has been determined by equilibrium ultracentrifugation in $H_2O/^2H_2O$ mixtures [14]. This method is based on the principle that at a solvent density which matches that of the detergent, the apparent molecular weight of the complex is determined by the molecular weight of the protein component. Fig. 3 shows a plot of the apparent value of $M_P(1 - \phi'\rho)$ as a function of solvent density. The molecular weight obtained from these data indicates that monomeric glucagon is incorporated into the dodecylphosphocholine micelle (Tables I and II).

Comparison of the diffusion constants obtained by ultracentrifugation and by quasi-elastic light-scattering shows good agreement between these two methods (Table I). Since heterogeneous size distribution is manifested differently in the two methods [15], these studies also indicate that the glucagon-dodecylphosphocholine complexes have a narrow size distribution. Diameters of equivalent spheres have been calculated on the one hand from the diffusion constants using the Stokes-Einstein equation, and on the other, from the particle weight using the partial specific volume (Table II). Considering that the diameters determined from the diffusion constants include the hydration shell and would be systematically too large if the species were not spherical [15], the diameters of 54 and 56 Å determined from the two diffusion studies and of 35 ± 3 Å determined from the particle weight are in reasonable agreement.

The following approaches were used to determine the number of dodecylphosphocholine molecules per micelle. A relatively unreliable estimate of the

number of detergent molecules associated with each glucagon molecule can be obtained from the slope of the plot in Fig. 3 [14]. This yielded a value of 38 ± 18, as compared with 56 ± 5 detergent molecules for dodecylphosphocholine micelles alone (Table II). A more accurate value for the number of dodecylphosphocholine molecules/glucagon was then obtained by an NMR titration of glucagon with dodecylphosphocholine. Although 1 mM glucagon in 50 mM phosphate buffer at pH 7.0 and 37°C was highly insoluble, the polypeptide could be completely solubilized with a 15-fold molar excess of dodecylphosphocholine. As the ratio of dodecylphosphocholine to glucagon was increased further, small changes in the NMR chemical shifts of the polypeptide were observed up to a detergent-to-peptide ratio of approx. 40 : 1, at which the glucagon chemical shifts became independent of the detergent concentration. As we have shown in more detail elsewhere [12], the detergent-to-polypeptide ratio at which the chemical shifts of the polypeptide NMR resonances become independent of detergent concentration represents a lower limit for the number of detergent molecues per polypeptide chain in peptide-detergent complexes. We conclude that in the presence of excess detergent, the glucagon-dodecylphosphocholine complex contains one glucagon molecule and approx. 40 detergent molecules. This would be consistent with the observation that the size measured for the glucagon-micelle complex is rather similar to the size measured for dodecylphosphocholine micelles (Table II). Since a detergent-to-peptide ratio of 15 : 1 sufficed to solubilize fully glucagon, it appears likely that complexes containing more than one glucagon molecule are formed at low detergent-to-peptide ratios. The similarity of the glucagon NMR spectrum at detergent-to-peptide ratios of 15 : 1 and 50 : 1 would then indicate that peptide-peptide interactions are weak in complexes containing more than one glucagon molecule. Similar conclusions have been reached previously for binding of melittin to lipid micelles [12].

For detailed studies of the conformation of glucagon bound to a lipid/water interface, NMR measurements have been made of glucagon bound to fully deuterated dodecylphosphocholine micelles (see below). Measurement of diffusion constants by quasi-elastic light-scattering showed that deuteration of the detergent caused, at most, very slight changes in the size of dodecylphosphocholine micelles either with or without bound glucagon (Tables I and II). This indicates that glucagon also binds as a monomer to deuterated dodecylphosphocholine micelles. Furthermore, identical CD spectra were obtained for glucagon bound to either protonated or deuterated dodecylphosphocholine micelles. From the above physicochemical characterizations, we therefore conclude that in the samples used for the following NMR experiments, the glucagon is essentially fully bound to the detergent micelles, the glucagon conformation is very similar to that adopted when glucagon is bound to phosphatidylcholine bilayers and the size and stoichiometry of the glucagon-dodecylphosphocholine complex are well defined.

Resonance assignments in the ^{1}H-NMR spectrum of glucagon bound to deuterated dodecylphosphocholine micelles

Comparison of the ^{1}H-NMR spectra observed for monomeric glucagon in dilute aqueous solution (Fig. 4A) and for glucagon bound to fully deuterated

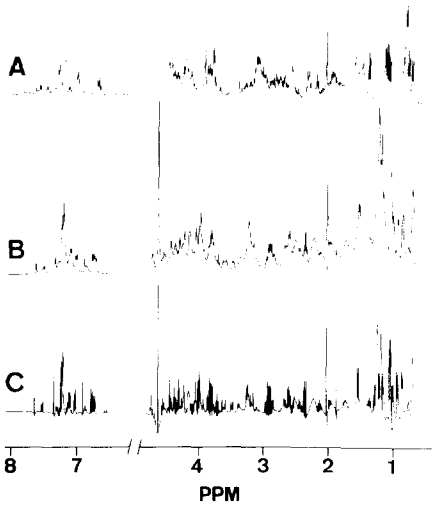

Fig. 4. 360 MHz ^{1}H-NMR spectra at p^{2}H 10.8 and 37°C of monomeric glucagon and glucagon bound to deuterated dodecylphosphocholine micelles. (A) $1 \cdot 10^{-4}$ M glucagon (monomeric). (B) $1.2 \cdot 10^{-3}$ M glucagon plus $6 \cdot 10^{-2}$ M [$^2H_{38}$]dodecylphosphocholine. (C) Same as B except that spectral resolution has been improved by applying a phase-shifted sine bell to the free induction decay [19].

dodecylphosphocholine micelles (Fig. 4B) shows that binding of glucagon to the micelles results in major changes in the observed chemical shifts and a general broadening of the resonances. The changes in chemical shifts are indicative of conformational changes upon binding of glucagon to the dodecylphosphocholine micelles. Qualitatively, the NMR observations indicate, in agreement with the above-mentioned CD data, that in dilute aqueous solution glucagon has a largely flexible, extended conformation [6], and that glucagon adopts a more ordered conformation upon binding to lipids. Despite the increase in line-widths for the NMR resonances of micelle-bound glucagon, which appear to be roughly consistent with the particle weight of 17 000 daltons determined by ultracentrifugation, Fig. 4C shows that excellent resolution of the micelle-bound glucagon resonances could be obtained by resolution-enhancement techniques. Consequently, spin systems corresponding to many different amino acid residues could be individually identified and, in part, assigned to specific locations in the amino acid sequence. The resonance assignments listed in Table III were obtained from the following experiments.

Difference spin-decoupling experiments [20] allowed identification of the following resonances: Leu-14 and Leu-26 γCH and $\delta_{1,2}CH_3$; Ala-19 αCH and βCH_3; Val-23 αCH, βCH and $\gamma_{1,2}CH_3$; Trp-25 indole ring protons; Tyr-10 and Tyr-13 aromatic ring protons; Thr-5, Thr-7 and Thr-29 βCH and γCH_3. Since the amino acid type is present only once in glucagon, the resonances of Ala-19, Val-23 and Trp-25 could also be assigned to the respective locations in the amino acid sequence.

Further resonance assignments came from the p^{2}H dependence. Between p^{2}H 4.0 and 12.5, the ^{1}H-NMR spectrum of micelle-bound glucagon was largely unchanged. The only marked spectral changes could be ascribed entirely to resonances from protons located near, in the covalent structure, to titra-

TABLE III

^{1}H-NMR CHEMICAL SHIFTS FOR THE ASSIGNED RESONANCES OF GLUCAGON BOUND TO PERDEUTERATED DODECYLPHOSPHOCHOLINE MICELLES

Measured in 0.05 M phosphate buffer at p^{2}H 7.0 and 37°C with $1 \cdot 10^{-3}$–$4 \cdot 10^{-3}$ M glucagon and a detergent-to-peptide molar ratio of 50 : 1. Chemical shifts are given relative to external sodium 3-trimethylsilyl[2,2,3,3-$^2H_4$]propionate at p^{2}H 7 [17].

Amino acid residue	Chemical shift (±0.01 ppm)			
	αCH	βCH	Others	
Ala-19	4.17	1.56		
Val-23	3.50	2.26	γCH_3	1.02
				1.21
Leu-14			γCH	1.80
			δCH_3	0.96
				0.88
Leu-26			γCH	1.58
			δCH_3	0.72
				0.72
Met-27			ϵCH_3	2.04
Thr I (5 or 7)		4.24	γCH_3	1.17
Thr II (5 or 7)		4.19	γCH_3	1.07
Thr-29		4.11	γCH_3	1.05
Lys-12			δCH_2	1.50
			ϵCH_2	2.80
His-1			ring C2H	7.81
			ring C4H	7.04
Phe-6			ring	(7.25)
Phe-22			ring	(7.25)
Tyr I (10 or 13)			ring C2, 6H	7.19
			ring C3, 5H	6.84
Tyr II (10 or 13)			ring C2, 6H	7.06
			ring C3, 5H	6.84
Trp-25	4.31	3.62	ring C2H	7.38
		3.32	ring C4H	7.34
			ring C5H	6.91
			ring C6H	7.13
			ring C7H	7.56

table groups. Consequently, the following resonances could be identified on the basis of the chemical shifts, multiplet structure and p^{2}H-dependent changes in chemical shifts: His-1 C2H and C4H; Lys-12 δCH_2 and ϵCH_2; Thr-29 βCH and γCH_3. For the aromatic ring protons of Tyr-10 and Tyr-13, differentiation between the C2,6H and C3,5H resonances (Table III) was made on the basis of the chemical shifts and the p^{2}H-dependent changes in chemical shifts. The following pK_a values were obtained from these experiments: His-1 imidazole ring, 5.8; Lys-12 ε-amino, 10.3; Thr-29 terminal carboxyl, 4.4. Because major changes in the ^{1}H-NMR spectrum occurred for p^{2}H $\gtrsim$ 12.5, which is indicative of considerable conformational change, only approximate pK_a values of 11.5 and 12.5 could be obtained for the two tyrosines.

Confirmation of the assignment of Thr-29 and an assignment for the ϵCH_3 of Met-27 were obtained from comparison of the ^{1}H-NMR spectra obtained for glucagon and for (homoserine-27)-glucagon 1-27 bound to dodecylphosphocholine micelles. Fig. 5 shows that the chemical modification resulted in removal from the ^{1}H-NMR spectrum of the threonine γCH_3 resonance at 1.05

ppm, which from its p^2H-dependent chemical shift was assigned to Thr-29. In addition, a sharp three-proton singlet at 2.04 ppm was lacking in the spectrum of (homoserine-27)-glucagon 1-27. The remaining methyl groups showed only very slight differences in chemical shift between native and modified glucagon (Fig. 5). Since spectral similarity between glucagon and (homoserine-27)-glucagon 1-27 was also observed in the other spectral regions, it appears that any conformational differences caused by the chemical modification are limited to the carboxy terminal regions of the two polypeptides. In view of this, the methyl resonances at 2.04 and 1.05 ppm in the spectrum of glucagon were assigned to Met-27 and Thr-29, respectively. Among the leucine δCH_3 resonances at 0.72, 0.88 and 0.96 ppm in the spectrum of micelle-bound glucagon, the two overlapping resonances at 0.72 ppm, which correspond to the same leucine residue, show small chemical shift changes in (homoserine-27)-glucagon 1-27 whereas the resonances at 0.88 and 0.96 ppm are unchanged (Fig. 5). On this basis, the two overlapping leucine δCH_3 resonances at 0.72 ppm and the two remaining leucine δCH_3 resonances at 0.88 and 0.96 ppm were tentatively assigned to Leu-26 and Leu-14, respectively.

Global features of the conformation of micelle-bound glucagon by nuclear Overhauser enhancement studies

Among the available NMR techniques, ^1H-^1H Overhauser effects are the most direct method for studies of through-space distances between pairs of hydrogen atoms in molecular structures [27,28]. However, when working with globular proteins of a size comparable to that of the glucagon-containing dodecylphosphocholine micelles, spin diffusion tends to mask the distance information contained in nuclear Overhauser effects [29,30]. This difficulty can be circumvented with special techniques which measure the build-up rates of the nuclear Overhauser enhancements [22,31]. One of these techniques is the recording of truncated driven nuclear Overhauser enhancement difference spectra [22], where the free induction decay is accumulated immediately after pre-irradiation of a selected resonance during a period which is short compared to the irradiation times needed to obtain a steady-state Overhauser effect [27]. For a medium-sized globular protein, selective truncated Overhauser effects between pairs of nearest neighbor hydrogen atoms are typically obtained with pre-irradiation times of approx. 0.2—0.5 s, whereas after longer times spin diffusion becomes dominant [22,32].

In contrast to the situation with globular proteins, for polypeptide chains bound to deuterated lipid micelles, highly selective truncated Overhauser effects are obtained with pre-irradiation times of several seconds [33]. This is illustrated in Fig. 6B where pre-irradiation of the leucine δCH_3 resonance at 0.72 ppm for 3 s is seen to cause Overhauser effects for Val-23, Trp-25, Met-27 and either Phe-6 or Phe-22 (Table III). Fig. 6C shows that when the C3,5 protons of the aromatic rings of Tyr-10 and Tyr-13 at 6.84 ppm were pre-irradiated for 4 s, Overhauser effects were observed for the C2,6 ring protons and the β-methylene protons of the two tyrosine residues. Additional weak effects are apparent in the α-proton region near 4.5 ppm. In the experiment of Fig. 6D, pre-irradiation of the C2H resonance of the imidazole ring of His-1 at 7.81 ppm for 7 s gave no significant Overhauser effects anywhere in the spectrum.

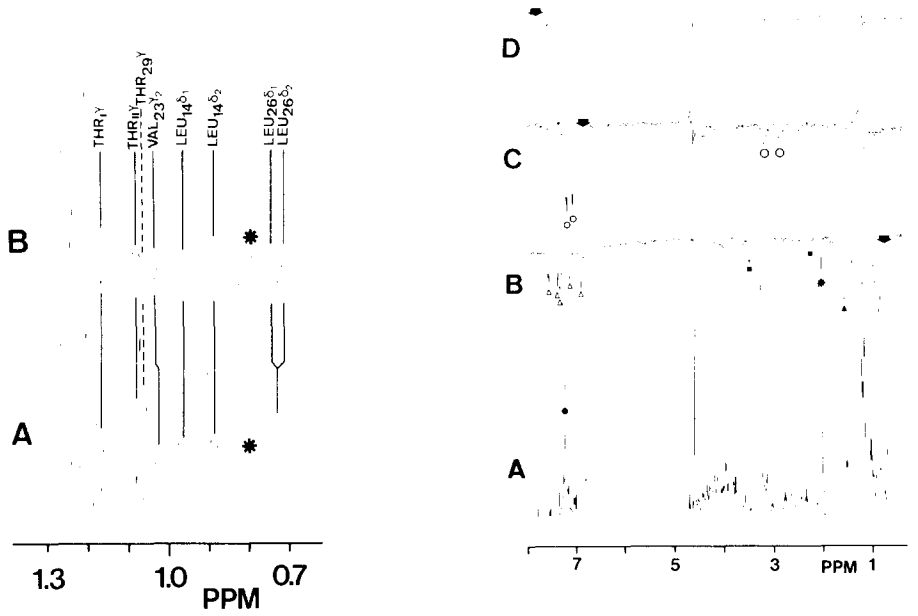

Fig. 5. High-field region from 0.5 to 1.5 ppm of the 360 MHz ^{1}H-NMR spectra in 0.05 M phosphate buffer at p^{2}H 7.0 and 37°C of glucagon and (homoserine-27)-glucagon 1-27 bound to deuterated dodecylphosphocholine micelles. (A) $2 \cdot 10^{-3}$ M glucagon plus 0.1 M [$^2H_{38}$]dodecylphosphocholine. (B) $2 \cdot 10^{-3}$ M (homoserine-27)-glucagon 1-27 plus 0.1 M [$^2H_{38}$]dodecylphosphocholine. The resonance assignments are described in the text. The asterisk denotes residual protons in the deuterated detergent. The spectral resolution was improved by multiplication of the free inducation decay with a phase-shifted sine bell [18,19].

Fig. 6. Truncated driven nuclear Overhauser enhancement difference spectra for glucagon bound to deuterated dodecylphosphocholine micelles. Recorded with $4 \cdot 10^{-3}$ M glucagon and 0.2 M [$^2H_{38}$]-dodecylphosphocholine in 0.05 M phosphate buffer at p^{2}H 7.0 and 37°C. (A) Normal spectrum of micelle-bound glucagon. (B—D) Truncated driven nuclear Overhauser enhancement difference spectra with a 16-fold vertical expansion compared to A. The arrows denote the resonances which were irradiated prior to data acquisition and the symbols denote assigned resonances for which nuclear Overhauser enhancements were observed. (B) Irradiation of Leu-26 $\delta_{1,2}CH_3$ for 3 s. ▲, Leu-26 γCH; ■, Val-23 αCH and βCH; ★, Met-27 ϵCH_3; △, Trp-25 indole ring protons; ●, Phe-22 aromatic ring protons. (C) Irradiation of Tyr-10 and Tyr-13 C3,5 aromatic ring protons for 4 s. ○, Tyr-10 and Tyr-13 C2,6 aromatic ring protons and β-methylene protons. (D) Irradiation of His-1 imidazole C2H for 7 s.

For structural interpretation of the selective Overhauser effects in micelle-bound glucagon, an important clue was obtained from the observations on the nuclear Overhauser effects between hydrogen atoms of the same amino acid residue (e.g., Fig. 6C). For all the assigned resonances listed in Table III, negative intra-residue Overhauser effects were detected, with the exceptions of His-1, where no effect was seen, Phe-6 and Phe-22, where the restricted spectral solution did not allow such experiments, and the ϵCH_3 line of Met-26, where no other resonance of the residue had been identified. The negative, intra-residue Overhauser effects show that the rotational mobility of the individual amino acid residues is largely restricted by the interactions with the lipid, so that the effective correlation time is probably mainly determined by the overall rotations of the micelle. Hence, the absence of cross-relaxation between hydrogen atoms bound to two different amino acid residues, each of

which shows negative intra-residue Overhauser effects, must arise because these protons are too far apart. As discussed in detail elsewhere [22,33], a proton-proton distance of 4—5 Å presents an effective barrier for the propagation of spin diffusion. Alternative explanations for the absence of such inter-residue Overhauser effects, e.g., quenching by paramagnetic species, are unlikely since the intra-residue Overhauser effects would also be quenched.

From a large number of experiments of the type shown in Fig. 6, qualitative information on the distances between the amino acid residues listed in Table III was obtained [34]. On the basis of the Overhauser effects, the residues for which one or more resonance lines had been assigned could be divided into three groups, i.e., His-1, Thr-5 and Thr-7 in the amino terminal region of the glucagon sequence; Tyr-10, Lys-12, Tyr-13 and Leu-14 in the central region; and Ala-19, Val-23, Trp-25, Leu-26, Met-27 and Thr-29 in the carboxy terminal region. When a resonance corresponding to one of the three groups of amino acid residues was pre-irradiated, the observed Overhauser effects corresponded to resonances assigned to other residues of the same group (or in some instances to unidentified resonances). In no case was there any evidence for Overhauser effects between amino acid residues in different groups. This shows that the three groups of amino acid residues are well separated and, hence, that glucagon does not adopt a compact, globular form in the micelles. The data would be compatible with either a predominantly extended overall molecular shape or with a loosely folded form where the three regions of the amino acid sequence are separated by deuterated lipid.

It was further observed that qualitatively different cross-relaxation patterns prevail in the amino terminal, central and carboxyl terminal regions of glucagon. For the amino terminal histidine, little or no Overhauser effect was observed (Fig. 6D), indicating that the amino terminus is highly mobile relative to the micelle, which might lead to 'nulling' of the Overhauser effect [27]. This region may be external to the micelle. In contrast, the strong selective intra-residue and inter-residue Overhauser effects (Fig. 6B) indicate that the carboxy terminal region of micelle-bound glucagon adopts a compact structure in which conformational mobility is strongly restricted by contacts between the amino acid side chains. Since all the assigned Overhauser effects seen in this region were between protons in the peptide fragment 19—27, the effects observed in these experiments for one phenylalanine residue were assigned to the spin system of Phe-22. In the central region, strong negative intra-residue Overhauser effects were seen (Fig. 6C), but little or no inter-residue effect could be detected. This indicates that this portion of the polypeptide chain adopts an extended form with the individual side chains well separated, yet is largely immobilized by interactions with the deuterated lipid.

Discussion

The data in this paper show that glucagon forms a complex of well defined size and stoichiometry with deuterated dodecylphosphocholine micelles (Tables I and II) and that a well resolved ^{1}H-NMR spectrum can be obtained for the micelle-bound glucagon (Fig. 4 and Table III). The following discussion concentrates on information on the nature of the glucagon-lipid interactions implied in the physico-chemical data on the complexes. In view of the CD

evidence that similar conformations prevail for glucagon bound to detergent micelles or to phospholipid bilayers, the conclusions from the work with micelles should be more generally applicable for glucagon bound to lipid bilayer membranes.

The following observations support the viewpoint that the interaction of glucagon with micellar detergents might best be described as a formation of mixed micelles by two different types of amphipathic species, an observation which was previously also made for binding of melittin to detergent micelles [12]. Both glucagon and the various detergents used are capable of forming self-aggregates independently of one another. The fluorescence experiments indicated that for dodecylphosphocholine, dodecyldimethylamine oxide (Fig. 1) and hexadecyltrimethylammonium bromide [35], monomeric glucagon and monomeric detergent molecules have, at most, weak interactions, but that glucagon binds strongly to micelles of these detergents. In the presence of excess dodecylphosphocholine, the detergent-glucagon complex has a particle size and number of detergent molecules quite similar to those observed for pure dodecylphosphocholine micelles (Table II), suggesting that glucagon may simply replace a few detergent molecules upon binding to the micelle. At low detergent-to-peptide ratios, glucagon-detergent complexes containing more than one glucagon molecule may be formed, which would also be compatible with the hypothesis of mixed micelle formation. For detergent micelles as diverse as dodecylphosphocholine, dodecyldimethylamine oxide, dodecyl(oxyethylene)$_{7,8}$glycol (Fig. 2) and hexadecyltrimethylammonium bromide [35], as well as for phospholipid micelles of lysophosphatidylcholine [25] or α-L-diheptanoylphosphatidylcholine (Fig. 2), the CD spectra of micelle-bound glucagon are very similar. This suggests that strong, specific interactions with detergent monomers are unlikely to play an important role either in binding glucagon to micelles or in determining the conformation of micelle-bound glucagon.

Whilst the covalent structures of the detergent molecules are inherently amphipathic, the CD and ^{1}H-NMR data indicate that glucagon attains an amphipathic character only by folding of the polypeptide chain into an appropriate conformation. The inherently amphipathic covalent structure of detergent and phospholipid molecules allows considerable conformational flexibility to be retained in micelles or bilayers, whereas the requirement of a folded conformation to attain an amphipathic character suggests a more definite conformation is to be expected for micelle-bound glucagon. NMR work to determine detailed features of the glucagon conformation in dodecylphosphocholine micelles is in progress, as well as experiments with paramagnetic probes to determine the location of the glucagon relative to the micelle surface [34].

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