Calcium Binding and Translocation Properties of Glucagon and Its Fragments†

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ABSTRACT: Earlier studies have indicated that the N- and C-terminal regions of glucagon are functionally and structurally different. We have sought to understand this distinction in terms of the interaction of glucagon and its N- and C-terminal fragments with Ca^{2+}, Mg^{2+}, and Zn^{2+} in a nonpolar milieu. CD spectral data, in 98% (v/v) trifluoroethanol in water, reveal two binding sites for Ca^{2+} and Mg^{2+} and one site for Zn^{2+} in the intact hormone as well as in the C-terminal 19–29 fragment. The 1–6 fragment did not bind Zn^{2+} and formed a 2:1 peptide–Ca^{2+} or –Mg^{2+} complex. With glucagon and the 19–29 fragment, cation binding caused changes in the peptide's helix content. Fluorescence spectral changes involving Trp-25 in the 19–29 fragment and Trp-25 and Tyr-10 and/or Tyr-13 in glucagon were seen on Ca^{2+} binding to one of the two sites, while Zn^{2+} binding produced no change in fluorescence. The spectral data suggest that Ca^{2+} and Zn^{2+} binding sites (with K_d in the micromolar range in 98% trifluoroethanol) are distinct and are contained in the C–terminal domain of glucagon. Glucagon and the 19–29 fragment, but not the 1–6 fragment, caused an influx of Ca^{2+} (as monitored by spectral changes in arsenazo III) in unilamellar vesicles made of dimyristoyllecithin. Leakage of vesicle contents induced by the 19–29 fragment was minimal but was significant (≈10%) in the case of glucagon. The transport data suggest an interaction of the C-terminal domain of glucagon with Ca^{2+} at the lipid–water interface. This, we suggest, may be important in dictating the bioactive conformation of the hormone and its interaction with the membrane receptor.

Glucagon is a highly conserved polypeptide hormone of 29 amino acids secreted by the pancreatic α-cells (Tager, 1984). Along with insulin, it plays an important role in glucose homeostasis. The initial event in glucagon is its interaction with plasma membrane receptor(s). This subsequently brings about changes in intracellular levels of cAMP (Campbell, 1983) as well as Ca^{2+} (Wakelam et al., 1986). A large number of studies have been carried out on chemically modified glucagon and synthetic peptide analogues of the hormone to determine the amino acid residues required for both receptor binding and signal transduction (Hruby et al., 1986; Zechel et al., 1991; Murphy et al., 1987; Unson et al., 1989a,b, 1991). The results of these studies, taken in conjunction with the highly conserved nature of glucagon (Tager, 1984), indicate that the entire hormone molecule is required for its function (Hruby et al., 1986). Within the hormone, there appears to be a spatial separation of the receptor binding function, presumably located in the C-terminal region, from the signal-transducing function located near the N-terminal part (Lin et al., 1975; Bregman et al., 1980; Hruby et al., 1986). Additional functional distinction between the N- and C-terminal regions of glucagon has been brought to light by some recent studies. Merrifield and co-workers (Unson et al., 1989a,b, 1991) have shown that certain chemical modifications in the N-terminal region enable glucagon to activate the inositol phosphate-mediated rise in Ca^{2+} in preference to the cyclic AMP pathway (Unson et al., 1989a,b). Blache et al. (1990) have demonstrated a novel physiological role for the 11-residue fragment at the C-terminus (residues 19–29) which is 1000-fold more potent than glucagon as an inhibitor of the liver plasma membrane calcium pump (Blache et al., 1990). In the context of the above results, several physicochemical studies have been carried out to understand the structure of glucagon and its N- and C-terminal domains (Unson et al., 1991; Zechel et al., 1991; Chou & Fasman, 1975; Carrey & Epand, 1983). The consensus structure of glucagon emerging from these studies is one where the N-terminal segment containing residues 1–6 is a flexible random coil while the C-terminal segment 17–29 has an ordered secondary structure, most likely an α-helix in dilute solutions (Chou & Fasman, 1975). The details of the central region between residues 10 and 17 are less clear, although there is an indication of the presence of a β-turn (Chou & Fasman, 1975).

The aim of the present study is to examine the conformational characteristics of glucagon and its N- and C-terminal fragments in terms of their interaction with cations in a nonpolar milieu. In an earlier study, we have shown that insulin can interact with Ca^{2+} at a lipid membrane and that there is a marked difference between the A- and B-subunits of this hormone in their interaction with this cation (Brimble & Ananthanarayanan, 1992). In the case of glucagon, there have been indications from earlier studies that metal ion binding may be important in glucagon function (Epand, 1982). Lipson et al (1988) proposed that divalent cations may be involved in the binding of glucagon to its receptor and in the latter's interaction with regulatory proteins. Morand et al. (1988) found that extracellular Ca^{2+} is required for maximal cAMP production induced by glucagon. These data, along with the fact that glucagon acts in conjunction with insulin in regulating glucose homeostasis and that the latter hormone interacts with metal ions (Hill et al., 1991; Brimble & Ananthanarayanan, 1992), prompted us to carry out a detailed investigation of the metal binding properties of glucagon and

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[1] Abbreviations: TFE, trifluoroethanol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; PC, phosphatidylcholine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid, disodium salt; DPX, *p*-xylenebis(pyridinium bromide); SDS, sodium dodecyl sulfate; LUV, large unilamellar vesicle(s); MLV, multilamellar vesicle(s); CD, circular dichroism.

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its N-terminal 1–6 fragment and the C-terminal 19–29 fragment in the membrane mimetic solvent trifluoroethanol (TFE)[1] used in earlier studies on secretin (Gronenborn et al., 1987). The results obtained from CD and fluorescence studies show that all of these peptides bind Ca^{2+} in 98:2 (v/v) TFE/water solution. Both the intact hormone and the 19–29 fragment, but not the 1–6 fragment, bind Zn^{2+} in a similar manner. Ion transport studies on the former two peptides using model liposomes reveal that both can transport Ca^{2+} across the lipid bilayer. Leakage assays indicate that glucagon causes a significant perturbation of the bilayer, whereas the 19–29 fragment does not.

MATERIALS AND METHODS

Materials

The following chemicals were purchased: Mops, Hepes, Sephadex G-75 resin, valinomycin, melittin, arsenazo III, safranine O, TFE, EDTA, and EGTA from Sigma Chemical Co. (St. Louis, MO); glucagon and glucagon 1–6 fragment from Schweizer-Hall (South Plainfield, NJ); glucagon 19–29 fragment from Bachem (Torrance, CA); Triton X-100 from Pierce Chemical Co. (Rockford, IL); egg PC and DMPC from Avanti Polar Lipids (Birmingham, AL); fura-2, ANTS, and DPX from Molecular Probes (Eugene, OR); $CaCl_2$, $MgCl_2$, and $ZnCl_2$ from Fisher Scientific (Unionville, Ontario, Canada). All other reagents were of analytical grade.

Methods

All solutions were made with Millipore-filtered deionized water to ensure minimal Ca^{2+} levels. Concentrations of glucagon solutions were determined from absorbance (at pH 7.2) using an extinction coefficient of 8289 $M^{-1}\cdot cm^{-1}$ at 278 nm (Moran et al., 1977). Concentrations of solutions of the N- and C-terminal peptide fragments were determined using, respectively, extinction coefficients of 197 M^{-1} cm^{-1}, at 257 nm, for the phenylalanyl residue and 5600 M^{-1} cm^{-1}, at 280 nm, for the tryptophyl residue (Creighton, 1984). Purity of the peptides was verified by either gel-filtration HPLC using a Beckman Ultraspherogel TSK-2000 column and/or SDS–polyacrylamide gel electrophoresis using 22% acrylamide. For glucagon, the electrophoresis resolved two bands, a major one at 3500 daltons and a minor one at 10 000 daltons; the latter is likely due to the residual glucagon trimer found to occur even in dilute solutions (Gratzer et al., 1972). No attempt was made to separate the minor component (estimated to be <10% of the total peptide) off the monomeric material.

Preparation of Liposomes. LUV containing trapped arsenazo III were made as described previously (Brimble & Ananthanarayanan, 1992; Sokolove & Kester, 1989). Briefly, dried DMPC films were treated with 20 mM Mops buffer (pH 7.2) containing 125 mM KCl and 3.6 mM arsenazo III and were vortexed to obtain MLV. These vesicles were then subjected to 10 repetitions of freezing and thawing. The resulting suspension was extruded through a 0.1-μm polycarbonate membrane filter using a commercial extruder (Lipex Biomembranes, Vancouver, British Columbia). The LUV thus obtained were then passed through a 30 × 1 cm Sephadex G-75 column to remove the exterior arsenazo III. Lipid concentration was determined from inorganic phosphate measured according to Ames (1966).

Cation Translocation Measurements. The change in absorbance at 650–700 nm of the trapped arsenazo III arising as a result of peptide-mediated Ca^{2+} influx into the DMPC LUV was monitored at different time intervals (Brimble & Ananthanarayanan, 1992; Sokolove & Kester, 1989). Absorbance measurements were recorded on a Perkin-Elmer Lambda 6 UV/Vis spectrophotometer using a 1-nm slit width and a scan speed of 300 nm/min. Temperature was controlled at 37 ± 1 °C during the absorbance measurements using a Lauda Model RC 6 refrigerated liquid circulator. The sample and reference cuvettes initially contained 700 μL of the Mops KCl buffer, 150 μL of the LUV suspension (equivalent to 3.5 μmol of lipid), and 30 μL of 100 mM $CaCl_2$ (final Ca^{2+} concentration 3.5 mM). An aliquot of the stock solution of the hormonal peptide was then added to the sample cuvette to give the required final concentration, and the same volume of solvent was added to the reference cuvette. A zero base line was electronically established at this time, and difference spectra were recorded between 400 and 700 nm at specified time intervals. EDTA at a final concentration of 5 mM was added at the end of the experiment to estimate the amount of dye which had leaked out (Sokolove & Kester, 1989). In a parallel experiment, the absorption spectra of the reference solution were obtained corresponding to the initial and final time intervals of the difference spectral experiment so as to ascertain that dye leakage in the control vesicles was not excessive (as happens occasionally in poor vesicle preparations). The difference absorbance change (ΔOD) at any given time interval was converted to the internal Ca^{2+} concentration using the procedure of Sokolove and Kester (1989). This, in turn, was converted to the amount of Ca^{2+} translocated as described previously (Brimble & Ananthanarayanan, 1992).

Liposome Stability. The perturbation, if any, of the lipid bilayer of the LUV caused by the hormonal peptides was assessed in two ways (Brimble & Ananthanarayanan, 1992). The first involved the measurement of the peptide-induced leakage of the fluorescent dye ANTS trapped inside the vesicle (Ellens et al., 1985). The dye is nonfluorescent inside the vesicles due to the presence of the cotrapped quencher DPX. Any leak of the trapped ANTS would register as an increase of the dye fluorescence (caused by the absence of quenching) (Ellens et al., 1985). The second method made use of the response of the membrane potential-sensitive dye safranine O to the presence of the peptides outside the vesicles (Woolley et al., 1987). For the ANTS method, LUV were made containing 12.5 mM ANTS, 45 mM DPX, 10 mM Mops, and 68 mM NaCl at pH 7.4. Untrapped ANTS and DPX were removed by passing the vesicle solution through a Sephadex G-75 column. The LUV (3.5 μmol of lipid) were then suspended in an external buffer containing 10 mM Mops, 0.1 mM EDTA, and 150 mM NaCl at pH 7.4 (final volume 800 μL). When the fluorescence of ANTS monitored at 545 nm using excitation at 384 nm remained steady, the hormone peptide was added to yield a final concentratin of 30 μM, and the fluorescence signal was allowed to stabilize again. An aliquot of $CaCl_2$ was subsequently added to give a final Ca^{2+} concentration of 3.5 mM. Addition of Triton X-100 [10 μL of a 10% (v/v) solution] caused vesicle disruption, and the resulting fluorescence signal was used as the 100% reference value for quantitative estimation of membrane leakage caused by peptides.

For the safranine O leakage assay, DMPC LUV were made as described above in the presence of 5 mM Hepes and 100 mM KCl at pH 7.2. Vesicles were then passed through a 30 × 1 cm Sephadex G-75 column equilibrated with 5 mM Hepes (pH 7.2), 100 mM NaCl, and 100 μM KCl, creating a large negative K^+ gradient across the membrane. Safranine O and valinomycin were added as previously described (Brimble & Ananthanarayanan, 1992) after 10-min incubation in the

presence of either 30 μM glucagon or the 19–29 peptide fragment; in the control experiment, the peptide was absent. Any leakage of the K^+ due to membrane perturbation caused by the hormone's presence would be observed as a decrease in the membrane potential-dependent safranine O fluorescence upon addition of valinomycin (Woolley et al., 1987).

Circular Dichroism. CD spectra were recorded at 22 ± 1 °C on a computer-controlled Jasco J-600 spectropolarimeter using a 1-mm quartz cell. Peptide concentrations were typically between 40 and 120 μM. Stock solutions were made by dissolving the hormonal peptides in Millipore-filtered water and diluting the solution with TFE to obtain a final TFE concentration of 98% by volume (referred to hereafter as 98% TFE). Scans were averaged 8 times at a scan speed of 20 nm/min. Stock solutions of metal chloride salts were prepared in 98% TFE. For metal ion titrations, aliquots of this solution (1–2 μL) were added to the solution of the peptide (180 μL) in the cell, mixed thoroughly, and recorded as described above. Dilution of the peptide over the titration was below 5%. The mean residue molar ellipticity, $[\theta]$, was expressed in degrees centimeters squared per decimole.

Fluorescence. Fluorescence spectra were recorded at 22 ± 1 °C on a computer-controlled Perkin-Elmer LS 50 fluorometer. Peptide concentrations were typically around 50 μM. Samples were prepared, and titrations with the cations were carried out as described above. Excitation was set at 270 nm and emission at 300–400 nm to monitor tryptophan and tyrosine fluorescence for glucagon and the 19–29 fragment. Spectra were deconvolved into the individual components by using an iterative curve-fitting program. The initial spectrum of the peptide in the absence of metal ion was fitted without any preset parameters. Once this fit was completed using two components, the band widths, shapes, and peak positions were fixed, allowing only the heights to change. This approach met with difficulty as the broad, longer wavelength peak clearly began to blue-shift at higher titrations. In these cases, the peak positions were also allowed to adjust, but bandwidths and shapes remained fixed. Excitation spectra were also recorded between 230 and 320 nm for the intact polypeptide by monitoring the emission at 346 nm for tryptophan. Light scattering of the glucagon solution was estimated by selecting an excitation wavelength of 425 nm and measuring the fluorescence emission at 428 nm. Intrinsic emission from the peptide fluorophores was insignificant at this excitation wavelength.

Absorbance. For monitoring metal binding, absorption spectra were recorded at 22 ± 1 °C as described earlier using a scan speed of 100 nm/min. Spectral scans were made between 230 and 350 nm upon cation addition, and the difference spectrum between the cation-added sample solution and the reference buffer was corrected for dilution of the peptide.

RESULTS

CD Studies. Earlier studies have shown that glucagon assumes a random structure in water at low concentrations and that addition of TFE to the aqueous solution induces an α-helical structure similar to the effect of detergents and micelles (Wu & Yang, 1980; Epand et al., 1977; Pasta et al., 1988; Robinson & Blakeney, 1982). Addition of Ca^{2+} to the helical hormone (at a concentration of about 50 μM) in 98% TFE caused significant changes to the CD signal as shown in Figure 1. The negative ellipticity at 222 nm, which is characteristic of the α-helix, increases slightly upon addition of Ca^{2+} up to about equimolar concentration, but upon further

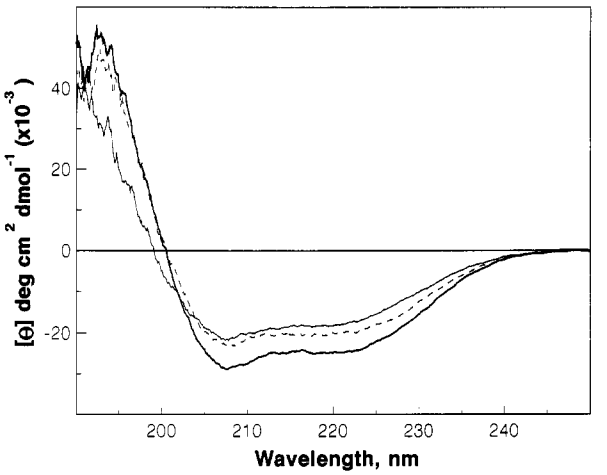

FIGURE 1: CD spectra of glucagon (44 μM) at 22 ± 1 °C in 98% TFE: (thick solid curve) free peptide; (dashed curve) peptide with equimolar $ZnCl_2$; (thin solid curve) peptide with 2 molar excess of $CaCl_2$.

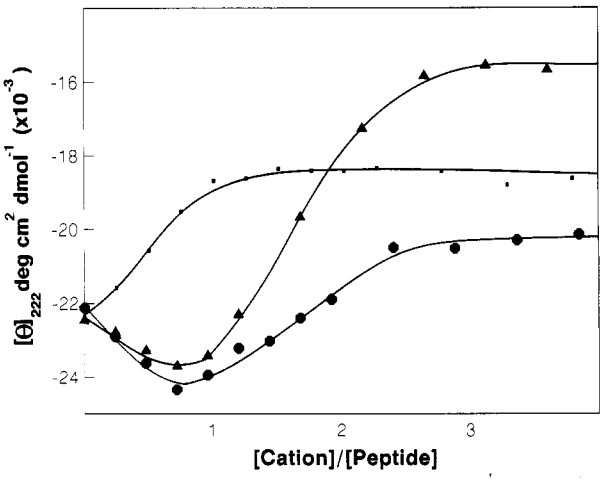

FIGURE 2: Binding isotherms in 98% TFE at 22 ± 1 °C for glucagon–cation complexes: Ca^{2+}; (●) Mg^{2+}; (■) Zn^{2+}. Glucagon concentration: 44 μM. $[\theta]_{222}$ is peptide ellipticity at 222 nm.

Ca^{2+} addition, the ellipticity becomes more positive, saturating at a ratio of about 2.2:1 Ca^{2+}:peptide mole ratio (Figure 2). The shape of the titration curve suggests cooperativity between the binding sites, but the complexity of the changes made any attempt at quantitative analysis difficult. Addition of Mg^{2+} induced similar qualitative changes but quantitatively had less of an effect than Ca^{2+} (Figure 2). Zn^{2+} addition has a much different effect, however. Addition of the ion causes a decrease in negative ellipticity, saturating at a mole ratio of 1:1. Addition of KCl had no effect on the CD of the hormone while addition of EDTA reversed the changes seen with divalent ions.

The C-terminal 19–29 fragment was also investigated with CD to compare its structure with the intact hormone. Like the parent hormone, this peptide was also α-helical in 98% TFE (Figure 3), compared to the random structure observed in water. Interestingly, addition of the various cations to the peptide in 98% TFE produced very similar effects to those observed with intact glucagon. The binding curves for Ca^{2+}, Mg^{2+}, and Zn^{2+} are shown in the Figure 3 inset. They suggest that the binding sites observed for Ca^{2+} and Zn^{2+} in theintact hormone are located on the C-terminal segment.

The N-terminal 1–6 fragment exhibited a CD spectrum quite different from those of the hormone or the C-terminal peptide fragment (Figure 4). Titration with Ca^{2+} and Zn^{2+}

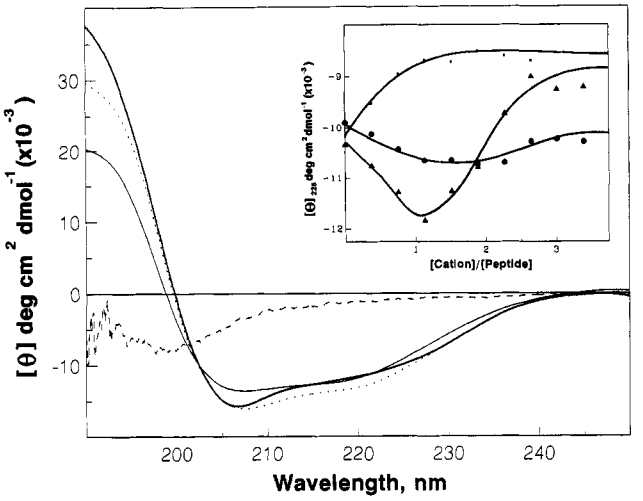

FIGURE 3: CD spectra of the C-terminal 19–29 fragment of glucagon (51 μM) at 22 ± 1 °C in water and in 98‰ TFE; (thick solid curve) free peptide; (dotted curve) peptide with equimolar $CaCl_2$; (thin solid curve) peptide with 2 molar excess of $CaCl_2$. Inset: Binding isotherms: (▲) Ca^{2+}; (●) Mg^{2+}; and (■) Zn^{2+}.

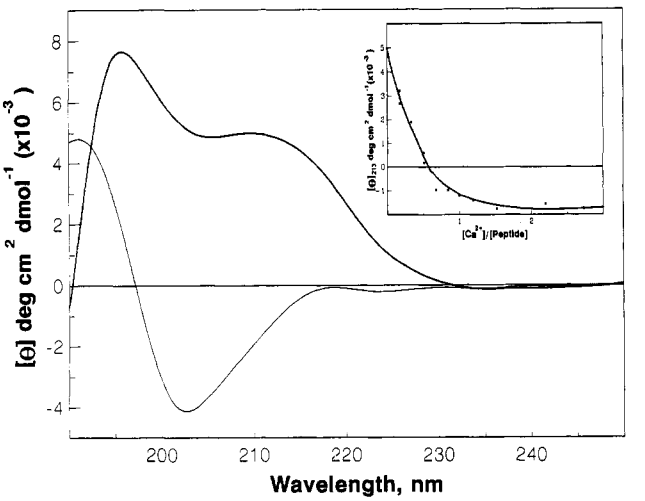

FIGURE 4: CD spectra of the N-terminal 1–6 fragment of glucagon (132 μM) at 22 ± 1 °C in 98% TFE: (thick solid curve) free peptide; (thin solid curve) peptide with equimolar $CaCl_2$. Inset: Ca^{2+} binding isotherm.

salts revealed dramatic changes in the CD spectrum as shown in Figure 4. This change was saturable with an end point near a Ca^{2+} to peptide ratio of about 0.6 (Figure 4, inset). Addition of Mg^{2+} to the fragment yielded similar but smaller changes (data not shown). Interestingly, addition of either Zn^{2+} or K^+ had no effect on the CD spectrum of the N-terminal fragment.

Fluorescence. To further investigate the binding of the various cations to glucagon and its fragments, changes in the fluorescence of the two tyrosine residues (Tyr-10 and Tyr-13) and the single tryptophan residue (Trp-25) were monitored. The single phenylalanine in the 1–6 fragment (Phe-6) was too weakly fluorescent to be useful in the binding assay. In 98% TFE, glucagon exhibited two emission peaks at 301 and 346 nm (with excitation set at 270 nm) (Figure 5A,B) corresponding, respectively, to tyrosine and tryptophan residues (Eisinger et al., 1969). Addition of Ca^{2+} caused an increase in the fluorescence of the longer wavelength peak, saturating at a ratio of 1:1 (Figure 5C and Figure 6). There was, however, a drastic decrease of fluorescence beyond this ratio (Figure 6). We also monitored changes in the tyrosine emission spectrum caused by Ca^{2+} addition. Due to the large overlap between the dominant tryptophan peak and the much smaller tyrosine peak, we had to deconvolute the composite fluorescence spectra into its individual components as described under Methods. As shown in Figure 5C, addition of Ca^{2+} resulted in a dramatic quenching of tyrosine fluorescence which was complete at a Ca^{2+}:glucagon mole ratio of 1:1.

Titration of glucagon with Zn^{2+} showed no change in the fluorescence spectrum (Figure 6). Even addition of Zn^{2+} up to an equimolar ratio prior to titration with Ca^{2+} had no effect on the change in fluorescence induced by Ca^{2+} (Figure 6). This suggests that the Ca^{2+} and Zn^{2+} binding sites may be located at different parts of the glucagon molecule.

On titration with Ca^{2+}, the fluorescence emission spectrum of the 19–29 fragment, which contains a tryptophan residue (Trp–25) but no tyrosine residue, showed qualitatively similar changes as the parent hormone with saturation occurring at a ratio of 1:1, followed by dramatic quenching (data not shown). As with the parent hormone, titration with Zn^{2+} had no effect either on its own or in parallel with Ca^{2+} (data not shown).

Absorbance. Addition of either Ca^{2+}, Mg^{2+}, or Zn^{2+} caused a change in the absorbance spectrum of glucagon in 98% TFE, the change being largest for Ca^{2+} (Figure 7). Difference spectra between the cation-added sample and the free peptide revealed two peaks at 284 and 291 nm, corresponding, respectively, to the absorption bands of the tyrosine and tryptophan residues. A similar difference spectrum has been observed on treating glucagon with DMPC (Epand et al., 1977). Compared to Ca^{2+} and Mg^{2+}, Zn^{2+} caused a larger perturbation of the tyrosine component than the tryptophan component (Figure 7).

Ca^{2+} Transport. As in a parallel study on substance P (Ananthanarayanan & Orlicky, 1992), we attempted to relate Ca^{2+} binding by glucagon and its N- and C-terminal fragments in an organic solvent (TFE) to the interaction of this cation with the hormonal peptides at the lipid–water interphase. Toward this aim, the ability of glucagon and its fragment to transport Ca^{2+} across a lipid bilayer was tested using LUV made from DMPC which contained the Ca^{2+}-sensitive dye arsenazo III. Experiments done previously with this system yielded the expected stoichiometries for A23187 and ionomycin with Ca^{2+} (Brimble & Ananthanarayanan, 1992). Figure 8 shows typical results from experiments with glucagon and its 19–29 fragment. Substantial spectral changes were seen with both glucagon and the 19–29 fragment after 10 min whereas very little change was observed with the 1–6 fragment (data not shown). However, addition of EDTA at the end of the run showed that a significant proportion of the transport observed in the presence of glucagon was due to leakage of arsenazo III out of the vesicles. The 19–29 fragment, on the other hand, showed very little leakage of the dye (Figure 8). With both glucagon and the 19–29 fragment, Ca^{2+} transport was negligible below the phase transition temperature of DMPC (≈23 °C), suggesting that the peptide may act as a carrier ionophore rather than forming a channel (Grasso et al., 1991). The initial rates of Ca^{2+} transport by the 19–29 fragment were determined by measuring the slopes of the initial linear portions of ΔOD versus time plots. A plot of the logarithm of the initial rate versus logarithm of the peptide concentration, shown in Figure 9, yielded a slope close to unity (average slope 1.07 ± 0.08). This would indicate the ion-transporting species has a stoichiometry of 1 Ca^{2+} to 1 peptide (Blau & Weissmann, 1988). A similar analysis was not possible with the intact hormone due to the relatively larger degree of leakage.

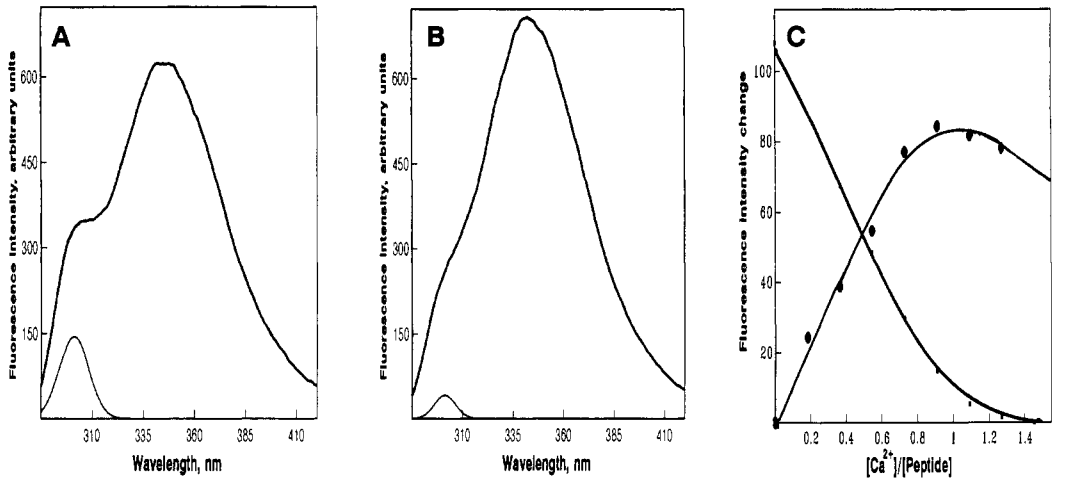

FIGURE 5: Fluorescence emission spectra and analysis at 22 ± 1 °C of glucagon (44 μM) in 98% TFE (excitation, 270 nm). (A) (Thick solid curve) emission spectra of free peptide; (thin solid curve) contribution from the tyrosine residue. (B) (Thick curve) emission spectra of the peptide with equimolar $CaCl_2$; (thin curve) contribution from tyrosine. (C) Ca^{2+} binding isotherms obtained by using the individual tryptophan (●) and tyrosine (■) contributions to the observed emission spectra. The latter were calculated as described under Methods.

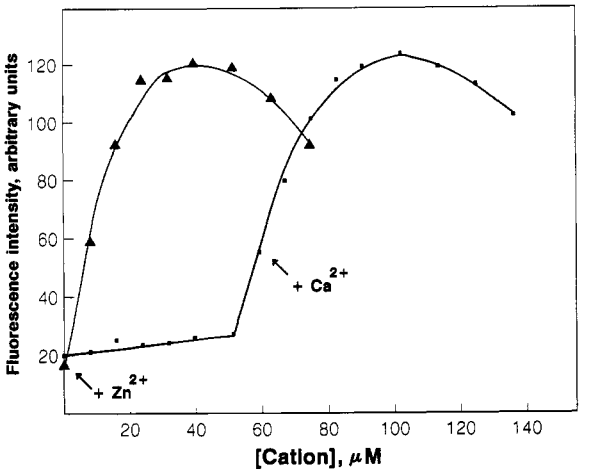

FIGURE 6: Changes in tryptophan fluorescence (270-nm excitation, 346-nm emission) of glucagon (44 μM) at 22 ± 1 °C in the presence of Ca^{2+}, Zn^{2+}, or both. (▲) Effect of Ca^{2+} alone; (■) effect of Zn^{2+} followed by Ca^{2+}. The points of addition of the cations are indicated by the arrows.

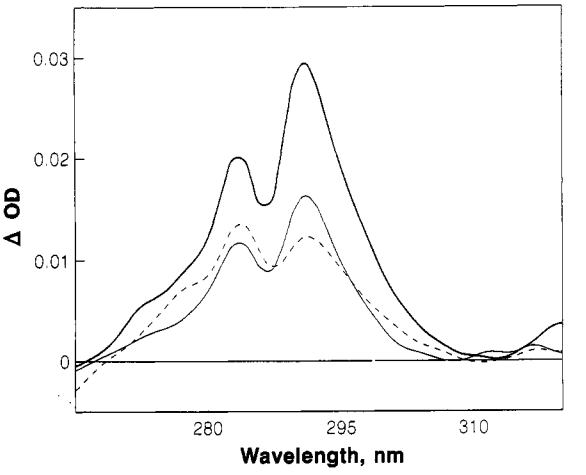

FIGURE 7: Difference absorbance spectra of glucagon in 98% TFE at 22 ± 1 °C in the presence of equimolar amounts of (thick curve) Ca^{2+}, (thin curve) Mg^{2+}, and (dashed curve) Zn^{2+}. The sample cuvette contained 44 μM glucagon and the appropriate metal salt while the reference cuvette contained the same concentration of glucagon without the salt.

The ability of glucagon and its fragments to induce leakage of vesicle contents was tested using the ANTS/DPX assay as described previously (Ellens et al., 1985). The results indicate that glucagon induced leakage of about 10% of its contents, compared to about 1% and 0% for the 19–29 and 1–6 fragments, respectively (Figure 10). Tests for the effect of the hormone peptides on the leakage of K^+ ions as monitored by the safranine O assay (Brimble & Ananthanarayanan, 1992) also showed that glucagon induced a much larger leakage of K^+ (causing the membrane potential to diminish) while the leakage caused by the 19–29 fragment was minimal (Figure 10).

DISCUSSION

Several studies have demonstrated the requirement for extracellular Ca^{2+} in the action of some of the key hormones involved in glucose metabolism, namely, insulin (Williams et al., 1990), glucagon (Lipson et al., 1988; Morand et al., 1988), and vasoactive intestinal peptide (Saito et al., 1992), the latter peptide showing significant amino acid sequence similarity to glucagon. This would imply that extracellular Ca^{2+} may interact with the hormone and/or the plasma membrane receptor. X-ray and NMR data have shown that insulin binds both Ca^{2+} and Zn^{2+} in aqueous solution (Hill et al., 1991). In addition, the insulin receptor appears to have a Ca^{2+} binding site (Williams et al., 1990). In the case of glucagon, an earlier study carried out by Epand (1982) found that Tb^{3+} ions bind to glucagon in an aqueous medium and that they may be readily displaced by Zn^{2+} but only weakly by Ca^{2+}. Saturatable binding of Tb^{3+} (and, by extrapolation, Zn^{2+}) required, however, a relatively large excess (>25 molar equiv) of the cation (Epand, 1982), indicating relatively weak binding affinity in water which is normally due to the hydration of the cation (Sussmann & Weinstein, 1989). As the glucagon-producing α-cells (Weitzel et al., 1956) and insulin-producing pancreatic β-cells have high levels of Zn^{2+}, it was suggested that Zn^{2+} binding may also play a role in the storage of glucagon (Epand, 1982).

Since Ca^{2+} is present in millimolar amounts in the extracellular space, and since glucagon exhibits an affinity, albeit very weak, to Ca^{2+} in aqueous solution (Epand, 1982), we were interested in understanding the role, if any, of Ca^{2+} in the bioactive conformation of this hormone prevailing near the lipid–water interphase where it would interact with its receptors. In this study, we have examined the ability of

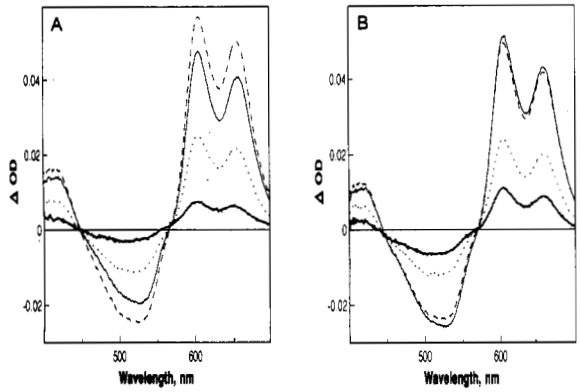

FIGURE 8: Ca^{2+} influx into DMPC LUV (3.5 μmol) measured using the arsenazo III technique at 37 ± 1 °C. The sample cuvette had LUV containing 3.6 mM arsenazo III incubated with 3.5 mM external $CaCl_2$ and 30 μM (24 nmol in 0.8 mL) of either (A) glucagon or (B) glucagon 19–29 fragment at pH 7.2. The reference cuvette had all the components except the peptide. Difference spectra were recorded (thick curve) 2 min, (dotted curve) 5 min, (dashed curve) 12 min, and (thin curve) after addition of EDTA (final concentration 5.0 mM) at 13 min.

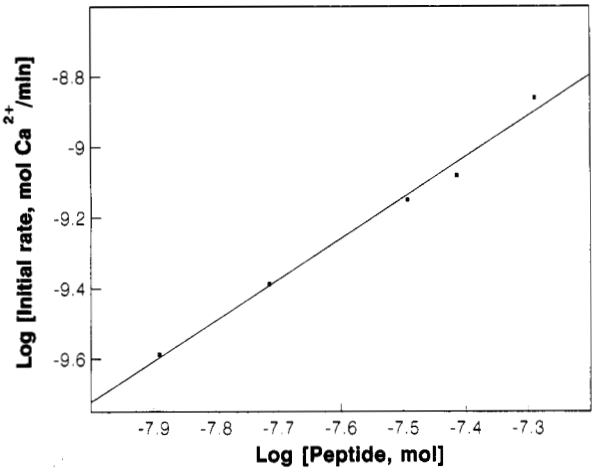

FIGURE 9: Plot of the logarithm of the initial rate of Ca^{2+} influx into DMPC LUV mediated by the 19–29 fragment as a function of the logarithm of the peptide concentration.

glucagon to bind Ca^{2+} and Zn^{2+} (and, for comparison with Ca^{2+}, Mg^{2+}) in the membrane mimetic solvent TFE using CD and fluorescence spectroscopy. Since the N- and C-terminal regions of the hormone have been shown to be distinct in their contribution to the overall function of glucagon (see the introduction), we have also investigated the interaction with cations of peptide fragments representing these two regions.

Analysis of Spectral Data. The development of an α-helical structure for both glucagon and the 19–29 fragment in 98% TFE, in contrast to a random structure in water (Figures 1 and 3), is similar to that observed when glucagon interacts with a variety of lipids (Epand et al., 1977; Pasta et al., 1988; Robinson & Blakeney, 1982). This would justify the use of the nonpolar solvent (98% TFE) in our cation binding experiments. A similar argument for the use of aqueous TFE has been made by Gronenborn et al. (1987) in their NMR study of the conformation of secretin (which is partially homologous to glucagon) and by Peggion and his colleagues (Peggion et al., 1984) in their studies of Ca^{2+} and Mg^{2+} binding by gastrin analogues. Addition of cations to glucagon and its N- and C-terminal fragments elicits different responses depending on the nature of the peptide and the cation as illustrated by the CD and fluorescence data (Figures 1–6). While glucagon and the 19–29 peptide fragment bind both Zn^{2+} and Ca^{2+}, the N-terminal 1–6 fragment binds only Ca^{2+} but not Zn^{2+}. (The effect of Mg^{2+} is similar to, but smaller than, that of Ca^{2+} and will not be discussed further. Absence of binding of K^+ indicates the peptides' specificity to divalent cations.) The close similarity in the cation binding behavior of glucagon and the C-terminal fragment suggests that the metal ion binding sites in the intact hormone are situated in the C-terminal part of the molecule. In this context, the observed Ca^{2+} binding by the isolated 1–6 fragment to glucagon–Ca^{2+} interaction is intriguing. It may be noted, however, that the CD spectrum of the 1–6 peptide in 98% TFE is quite unusual in that it does not correspond to any known secondary structure or the random coil whereas a longer (1–17) fragment showed the α-helical CD spectrum in micelles (Carrey & Epand, 1983). We therefore suggest that the interaction of this peptide with Ca^{2+} may not be relevant to understanding the behavior of the intact hormone. With glucagon and the 19–29 fragment, we observed major differences in the spectral changes and stoichiometries between the binding of Ca^{2+} and Mg^{2+} on the one hand and Zn^{2+} on the other (Figures 2 and 3). These peptides show a 1:1 stoichiometry with Zn^{2+} but a 2:1 (Ca^{2+}/peptide) binding with Ca^{2+}. The observation of two isodichroic points in the Ca^{2+} titration, spectra, in contrast to one in the Zn^{2+} spectra, indicates two types of Ca^{2+} complexes presumably corresponding to the 1:1 and 2:1 stoichiometries. Since the only histidine residue, a common coordinator of Zn^{2+} (Dodson et al., 1980), is located at the amino terminus of this peptide and since it is the 19–29 and not the 1–6 peptide fragment that binds Zn^{2+}, the earlier observation by Carrey and Epand (1983) on the requirement of the histidine residue for Zn^{2+} binding by glucagon in an aqueous solution would seem to be due to tertiary interactions in the hormone that involve this residue (Korn & Ottensmeyer, 1983).

Analysis of the spectral changes caused by Ca^{2+} and Zn^{2+} binding to glucagon and the 19–29 fragment provides some interesting insights into the accompanying molecular structural changes. The CD data in Figure 2 show that cation binding to the above peptides causes small, but significant, changes in the α-helical content of these peptides. Since these changes (as well as the tertiary structural changes monitored by fluorescence) occurring in the 19–29 fragment are similar in magnitude to those in the whole hormone (Figures 2, 3, and 5), any structural changes in the 1–18 region of glucagon caused by cation binding should be relatively minor. The structural changes caused by Zn^{2+} binding are different from those produced by the first phase of Ca^{2+} binding (i.e., up to 1:1 cation:peptide ratio). The latter increases the helical content of the peptide and enhances Trp-25 fluorescence while the former decreases the helical content and does not alter the fluorescence. The CD spectral changes produced by 2 molar equiv of Ca^{2+} are unaffected by the addition of 4 molar equiv of Zn^{2+} (data not shown). It would therefore appear that Ca^{2+} is capable of displacing Zn^{2+} from its binding site on glucagon. The dissociation constant (in 98% TFE) for the Zn^{2+} binding site, estimated using the method of Reuben (1973), is 1 μM. In the absence of a direct estimate of the K_d for Ca^{2+}, we infer it to be lower than 1 μM in the above solvent at either of the two binding sites.

The fluorescence data on glucagon and the 19–29 peptide fragment show that Zn^{2+} binding does not alter the spectral properties of tyrosine and tryptophan residues while Ca^{2+} binding at the first of the two sites does. The isosbestic point observed in the fluorescence spectra in Figure 6 up to a 1:1

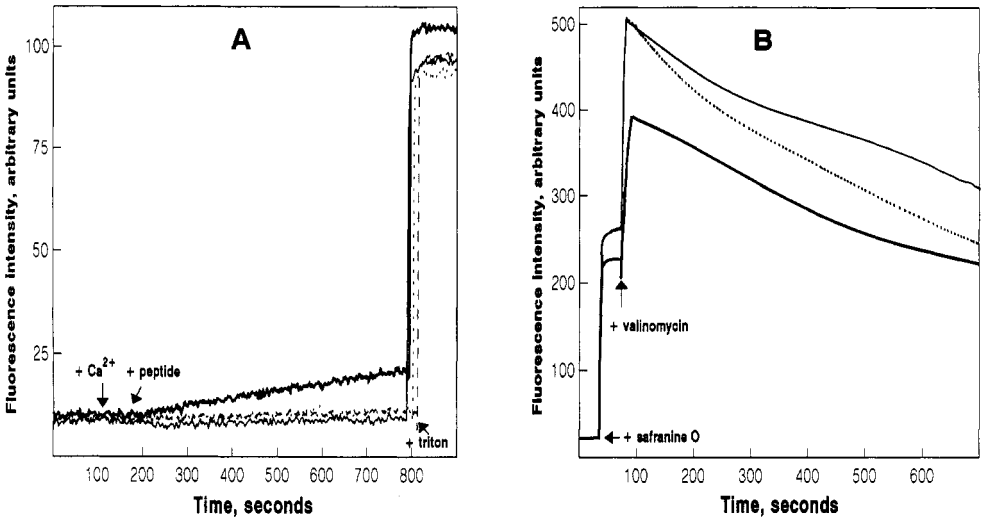

FIGURE 10: Tests for leakage of solutes across DMPC LUV (3.5 μmol) mediated by glucagon and its 19–29 fragment. (A) ANTS/DPX method: Vesicles contained 12.5 mM ANTS and 45 mM DPX (pH 7.4). Excitation, 384 nm; emission, 545 nm. Peptides, Ca^{2+}, and Triton X-100 added as indicated by the arrows: (thin solid curve) control; (dotted curve) 19–29 fragment; (dashed curve) 1–6 fragment; (thick solid curve) glucagon. Final peptide concentration: 30 μM. The fluorescence increase caused by adding 10 μL of 10% Triton X-100 was taken as 100% leakage. (B) Safranine O method: DMPC LUV were made with a K^+ concentration gradient as described under Methods. LUV were preincubated for 10 min with a 30 μM aliquot of either glucagon (thick curve) or the glucagon 19–29 fragment (dotted curve) before addition of safranine O (final concentration 1.4 μM) and valinomycin (final concentration 8.5 μM) at points indicated by the arrows. A control experiment (thin solid curve) was also carried out in the absence of peptide. The fluorescence emission of safranine O was monitored at 581 nm (excitation, 522 nm).

Ca^{2+}:peptide ratio indicates that the predominant species present are the free and Ca^{2+}-complexed peptide. Higher Ca^{2+}:peptide ratios resulted in a shift away from this isosbestic point.

The complementary increase in tryptophan fluorescence and decrease in tyrosine fluorescence seen in the intact hormone (Figure 6) suggested the possibility that Ca^{2+} binding induces an energy transfer from tyrosine to tryptophan (Eisinger, 1969). However, the similarity of the effect of Ca^{2+} on the 19–29 fragment which contains no tyrosine and on the intact hormone implied that fluorescence energy transfer in the intact hormone is negligible. We further verified this is to be true by calculating the extent of energy transfer (by comparing the fractional absorption of tryptophan to the relative quantum yield of tryptophan in glucagon) (Eisinger, 1969). An alternative explanation for the data in Figure 6 may lie in the conversion of tyrosine to tyrosinate ion which would reduce its fluorescence emission (Szabo et al., 1978). The pK for this conversion decreases from 10.3 in the ground state to around 4 or lower in the excited state (Lakowicz, 1983). Ionization to the tyrosinate form is aided by hydrogen bond formation with proton acceptors such as aspartic acid (Lakowicz, 1983; Szabo et al., 1978). An abnormal pK was reported for Tyr-10 by Frank and Pekar (1974), suggesting its involvement in hydrogen-bonding, possibly with the aspartic acid residue at position 21 which is spatially proximal to Tyr-10 in the model of glucagon proposed by Korn and Ottensmeyer (1983). That tyrosinate formation in the excited state brought about by Ca^{2+} binding is not simply due to an electrostatic effect is demonstrated by the fact that Zn^{2+} had no effect on the hormone's fluorescence.

The decrease in the tryptophan fluorescence seen with both glucagon and the 19–29 peptide fragment at Ca^{2+} concentrations above 1:1 mole ratio may not be due to a quenching effect of Ca^{2+} since quenching is usually observed only when paramagnetic ions (Steiner & Kirby, 1969) or iodide (Lehrer, 1971) is present. Measurement of light scattering by the samples at the concentrations of Ca^{2+} used in the titration studies showed that it was too low (<35% increase) to cause a reduction in fluorescence since no such reduction was observed in samples showing similar or higher light scattering caused by the addition of excess Zn^{2+}. Both glucagon and the 19–29 peptide show an increase in helix content up to about 1 mol equiv of Ca^{2+} beyond which the helicity decreases (Figures 2 and 3). We suggest that at the lower concentrations when the peptide takes up the enhanced helical structure, Phe-22 is brought closer to Trp-25 than in the free peptide where, according to the Korn and Ottensmeyer model (1983), the benzyl ring of Phe-22 is already close to and stacked against the indole ring of Trp-25. This structural change may, either by energy transfer or by other mechanisms, lead to an increase of Trp fluorescence. However, at the higher Ca^{2+} concentrations beyond a 1:1 mole ratio, the unfolding of the helix would cause separation of the Phe and Trp residues and leads to the observed decrease in the Trp fluorescence. In this context, Zn binding, which causes a decrease in helix content (Figures 2 and 3), would be expected not to significantly alter the Trp fluorescence as is found to be the case (Figure 6).

Additional information on the nature of the Ca^{2+} and Zn^{2+} binding sites is obtained from spectral data on glucagon in an aqueous buffer (0.1 M Hepes, pH 6.9) where Epand (1982) found Zn^{2+} to decrease Tb^{3+} fluorescence more effectively than Ca^{2+}. However, since Zn^{2+} causes only <30% reduction in Tb^{3+} fluorescence [Figure 3 in Epand (1982)], an additional binding site for Tb^{3+}, not displaceable by Zn^{2+}, is indicated. CD data in the above buffer (Epand, 1982; and our data not shown) show that Tb^{3+} (1 mM) causes a large increase in the helix content of glucagon (30 μM) while Ca^{2+} (100 mM) causes only a marginal increase [Figure 4 in Epand (1982)]. Interestingly, Zn^{2+} (1 mM) produces a substantial increase in the hormone's helix content which is significantly larger than that caused by Ca^{2+} but smaller than that induced by Tb^{3+} (data not shown). In an attempt to further link the data collected in water and TFE, we have carried out the titration of glucagon with Tb^{3+} in 97% (v/v) TFE (higher TFE concentrations caused solubility problems for terbium chloride). The Tb^{3+}-induced CD spectral changes (data not shown) were qualitatively similar to those seen with Ca^{2+}

addition (Figure 2), the helical content increasing initially and decreasing at higher Tb^{3+} concentrations with a saturation at 2 Tb^{3+} per glucagon. Taken together, the above data indicate that the cation binding sites of glucagon in 98% TFE might be the same as those in water; the lack of significant spectral changes on Ca^{2+} addition in water, in contrast to Tb^{3+} [which has been used as a Ca^{2+} mimic (Evans, 1983)] or Zn^{2+} is very likely due to the relatively high degree of hydration of the Ca^{2+} ion (Sussman & Weinstein, 1989).

On the basis of the above observations, we would like to propose the following model for cation binding to glucagon (at the C-terminal domain) in the nonaqueous environment. Assuming two binding sites for Ca^{2+} and one for Zn^{2+}, the binding of the first Ca^{2+} (in 98% TFE) to a relatively high-affinity site would result in an increase in peptide helicity and an enhancement of the fluorescence characteristics of Trp-25. The binding of the second Ca^{2+} occurs at the site where Zn^{2+} binds and leads to a decrease in helicity. While occupation of the second site does not per se affect the Trp-25 fluorescence, it leads to the reversal of the fluorescence enhancement caused by Ca^{2+} binding at the first site. Further, Ca^{2+} is capable of displacing Zn^{2+} from its binding site, indicating its higher affinity over Zn^{2+} for this site. We propose that one of the two cation binding sites in glucagon resides in the region 19–22 (Ala–Asn–Asp–Phe). This site would be available for Ca^{2+} and Zn^{2+} (and Tb^{3+}) and does not elicit significant fluorescence changes. The other binding site, available to Ca^{2+} (and Tb^{3+}) but not to Zn^{2+}, is likely to encompass the region containing Trp-25 which produces substantial fluorescence changes. (The partial quenching of Tb^{3+} fluorescence in water by Zn^{2+} might arise by severance of energy transfer to Tb^{3+} from Phe-21.)

The following structural and functional data on glucagon and its analogues are worth consideration in the context of the conclusions drawn from our cation–binding data. Experimental (Epand, 1971, 1983; Korn, 1978; Saski et al., 1975) and theoretical (Korn & Ottensmeyer, 1983) studies show glucagon to have a relatively compact structure made up of two domains that are separated by the arginine residues at positions 17 and 18 (Carrey & Epand, 1983). As mentioned earlier, this dichotomy seems also to influence glucagon action. Thus, the signal transduction and receptor binding functions of glucagon reside, respectively, in the N- and C-terminal domains. Interestingly, peptide fragments consisting of residues 1–17 and 19–29 (obtained by enzymatic cleavage of glucagon) lose most of their functional characteristics found in the intact hormone (Carrey & Epand, 1983). This underscores the importance of tertiary structural interactions between the two domains in the whole hormone that are not present in the isolated domain fragments. Our spectral data show that, in the nonaqueous medium, the C-terminal 19–29 domain retains most of the structural features as well as the cation binding ability of the intact hormone. However, it appears that tertiary interactions (such as those between Tyr-10 and Asp-21 inferred from our data and many other interactions suggested by the Korn–Ottensmeyer model) appear to be necessary for this fragment to exhibit the receptor binding function seen in intact glucagon. The recent observation that an endogenous 19–29 glucagon fragment inhibits the liver plasma membrane Ca^{2+} pump much more effectively than glucagon (Blache et al., 1990) shows that the tertiary interactions with the N-terminal domain are unnecessary (and, in fact, are impediments) for this particular function of the C-terminal domain. Since, according to our data, the Ca^{2+} binding capacity of glucagon is maintained in the 19–29 fragment, it is possible that this fragment would interact at the Ca^{2+} binding region of the Ca^{2+} pump (presumably in a hydrophobic environment) and inhibit the pump.

Analysis of Ca^{2+} Transport Data. The results of the cation transport experiments show that both glucagon and the 19–29 fragment are able to transport Ca^{2+} across the lipid bilayer in a model liposome (Figure 8). The fragment appears to transport Ca^{2+} as a 1:1 complex (Figure 9). A quantitative analysis could not be carried out on the intact polypeptide due to the relatively higher amount of leakage. This leakage is undoubtedly a result of glucagon interacting with DMPC to form a soluble complex (Epand et al., 1977), causing the vesicle to release its contents. Although this interaction is most pronounced in the gel state of the lipid (Epand et al., 1977), it persists even at 37 °C (Pasta et al., 1988). It should be noted, however, that the vesicle content leakage caused by glucagon is comparatively much less than its ionophoretic activity as evident from the results of the quantitative assays for leakage and membrane integrity (Figure 10). With the 19–29 fragment, contents leakage was negligible compared to ion transport. The safranine O data (Figure 10) show that the hormonal peptides do not transport K^+ ion but only cause its leakage in amounts expected from the other experiments.

The significance of Ca^{2+} transport by glucagon may be appreciated in the context of the hormone's interaction with Ca^{2+} in a nonaqueous medium discussed above and in light of similar observations made by us on insulin (Brimble & Ananthanarayanan, 1992) and substance P (Ananthanarayanan & Orlicky, 1992). The ability of glucagon to translocate Ca^{2+} across the lipid bilayer in the synthetic liposome used in this study implies, as in the case of well-known Ca^{2+} ionophores, the hormone's differential interaction with the cation in the polar aqueous solvent versus the nonpolar lipid milieu. The partitioning of the hormone into the lipid bilayer at the lipid–water interface apparently enhances the stability of its Ca^{2+} complex in comparison to that in the aqueous buffer. The Ca^{2+} translocation activity of glucagon is restricted to its C-terminal 19–29 domain in line with the conclusions derived from the equilibrium cation binding experiments. It is likely that the 1:1 Ca^{2+}/peptide complex suggested by the transport kinetic data to be the transporting species in the case of the 19–29 peptide fragment would represent the high-affinity binding site indicated by the CD and fluorescence spectral data in 98% TFE. It is also likely that a similar complex would represent the Ca^{2+}-transporting form of the intact hormone.

The localization of the Ca^{2+} binding site and Ca^{2+} translocation activity in one domain of a peptide hormone is also observed in the cases of insulin (Brimble & Ananthanarayanan, 1992), where this domain resides within the B-chain, and substance P (Ananthanarayanan & Orlicky, 1992), where it is the C-terminal region consisting of residues 7–11. Interestingly, the C-terminal segment (Phe-Val-Gln-Trp-Met) of glucagon shows significant similarity not only to the C-terminal end (Gln-Phe-Phe-Leu-Met) of substance P but also to those of bombesin, gastrin, Met-enkephalin, β-lipotropin, and gastrin releasing peptide. We might thus expect the latter peptides also to have their Ca^{2+}-interacting domains in their C-terminal ends. As in the case of substance P and insulin, the biological relevance of Ca^{2+} translocation by glucagon may be sought in terms of the ability of the hormone to penetrate the lipid bilayer with the bound Ca^{2+} ion. If the interaction of the hormone with its membrane receptor were to occur in the membranous milieu, we might speculate (Ananthanarayanan, 1991) that this interaction

involves the Ca^{2+}-bound form of the hormone whose structure would be different from those of the Ca^{2+}-free hormone observed in water (Sasaki et al., 1975) or in lipids (Braun et al., 1983). This proposal is consistent with the observed requirement of extracellular Ca^{2+} for glucagon action. Detailed studies on glucagon–receptor interaction in the absence and presence of Ca^{2+} are, however, needed to verify the proposal, particularly since glucagon can elicit either the cyclic AMP- or Ca^{2+}-mediated pathway for signal transduction (Wakelam et al., 1986).

The observed interaction of glucagon with Zn^{2+} in a nonaqueous medium may be relevant to the suggested role for Zn^{2+} in promoting aggregation of the hormone which would aid its packaging in the pancreatic islets (Epand, 1982). In view of the competition for the Zn^{2+} binding site by Ca^{2+} suggested by our results, and in view of the relatively high concentrations of both cations in the islets, it is possible that Ca^{2+} may also be involved in glucagon packaging. A similar situation might prevail in the case of insulin. It is interesting to note that several Ca^{2+} binding proteins, such as calcyclin (Filipek et al., 1990) and S-100 proteins (Ogoma et al., 1989), bind Zn^{2+} as well as distinct binding sites.

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