Interaction of Partially Structured States of Acidic Fibroblast Growth Factor with Phospholipid Membranes

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ABSTRACT: Although acidic fibroblast growth factor (aFGF) lacks a conventional signal sequence, it is often found complexed to sulfated proteoglycans on the external surface of cells. The protein also forms a "molten globule"-like state at neutral pH and physiological temperatures as well as at acidic pH in the presence of physiological ionic strength or moderate quantities of polyanions. These states display a marked tendency to aggregate. Such observations suggest that related partially structured states might be involved in the membrane translocation of aFGF. To explore this hypothesis, we examined the interaction of this growth factor with lipid vesicles as well as the effect of such surfaces on the structure of the protein. We find that these states interact with negatively charged but not neutral phosholipid unilammelar vesicles at acidic pH, inducing bilayer disruption. The rate of leakage of a liposome-entrapped fluorescent probe is proportional to the logarithm of the aFGF concentration, suggesting competition between protein self-association and membrane binding. Liposome leakage can be also induced at neutral pH by partial unfolding of aFGF at or above physiological temperature in contrast to most control proteins. The importance of partially folded hydrophobic surfaces in aFGF self-association and membrane binding is further suggested by the fact that thermally unfolded aFGF does not aggregate, in contrast to states observed at intermediate temperatures or transiently during unfolding at high temperatures. In contrast to heparin, a polyanion which stabilizes the native structure of aFGF, negatively charged phospholipid membranes appear to enhance the disruption of aFGF tertiary structure at submicellar concentrations of sodium dodecyl sulfate but stabilize the remaining secondary structure. Thus negatively charged lipid bilayers appear to interact with partially structured states of aFGF by preferential binding of both its apolar and charged surfaces to complementary regions of the lipid bilayer. Such interactions may play a role in the membrane translocation of this growth factor.

Acidic fibroblast growth factor (aFGF;[1] FGF-1) is a 16-kDa protein possessing mitogenic, angiogenic, and chemotactic stimulatory activities [reviewed by Burgess and Maciag (1989)]. The three-dimensional structure of the bovine form of the protein has been determined by X-ray crystallography and the human form by NMR (Pineda-Lucena et al., 1994) and consists of 12 antiparallel β-strands with pseudo 3-fold symmetry (Zhu et al., 1991). In the absence of stabilizing polyanions, aFGF is significantly unfolded at physiological temperatures (>30 °C) (Copeland et al., 1991; Dabora et al., 1991) and requires polyanions such as heparin to maintain its native structure and biological activity (Gospodarowicz & Cheng, 1986; Volkin et al., 1993). At low pH, the presence of small amounts of polyanionic ligands or moderate amounts of salts induces formation of partially folded states in aFGF, characterized by high secondary but low tertiary structure content. At neutral pH, intermediate amounts of chaotropic agents or slightly elevated temperature (>30 °C) impose similar "molten globule"-like conformational states which display noncooperative unfolding transitions (Mach et al., 1993). Unlike the native and fully unfolded states, these partially structured conformations exhibit very low solubility, resulting in irreversible aggregation of aFGF. This tendency to aggregate as well as the ability of the protein to bind amphiphilic dyes (e.g., ANS) suggest that partially folded states of aFGF may have the potential to interact with lipid bilayers.

"Molten globule" states have been implicated in membrane interactions of several proteins [e.g., Bychkova et al. (1988) and van der Goot et al. (1991)]. The membrane insertion and translocation of the majority of proteins appears to be mediated by a hydrophobic signal peptide which is subsequently enzymatically cleaved to yield the mature functional protein. Signal peptides also generally possess several positive charges proximate to an apolar region arranged in such a manner that positively charged and apolar faces are created by α-helical secondary structure [for reviews, see Kaiser and Kezdy (1987), Attardi and Schatz (1988), Pfanner and Neupert (1990), and Glick and Schatz (1991)]. Although aFGF lacks a conventional signal sequence, it has been shown to translocate through biological membranes when fused with the A-fragment of diptheria toxin (Wiedlocha et al., 1992). The requirement of low pH and blockage of translocation by stabilizing polyanions suggests the possible involvement of "molten globule" states of aFGF in this process. Furthermore, the ability of the aFGF−toxin conjugate to stimulate DNA synthesis in the absence of functional aFGF membrane receptors is consistent with such a mode of transport (Wiedlocha et al., 1994). In the present study, we characterize the interaction of aFGF with phos-

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[1] Abbreviations: aFGF, acidic fibroblast growth factor; ANS, 8-aniline-1-naphthalenesulfonate; DOPG, dioleoylphosphatidylglycerol; DOPC, dioleoylphosphatidylcholine.

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pholipid vesicles in an exploration of this hypothesis. We find that aFGF-induced disruption of membrane structural integrity coincides with the formation of partially structured states at physiological temperature (>30 °C) possessing the characteristics of "molten globules". These states are formed at low pH (<4) or at physiological temperature (>30 °C). The data indicate that the presence of negatively charged lipids (e.g., DOPG) is necessary for the interaction. In addition, we find that the structural changes in aFGF induced by the presence of submicellar concentrations of SDS at pH 7 and ambient temperature (20 °C) also produce membrane-interactive states.

EXPERIMENTAL PROCEDURES

Materials. Recombinant human aFGF (the amino truncated 141 amino acid form of $M_r \sim 15\,900$) was prepared from transformed *Escherichia coli* as previously described (Thomas et al., 1984; Linemeyer et al., 1987, Volkin et al., 1993). Dioleoylphosphatidylglycerol (DOPG) and dioleoylphosphatidylcholine (DOPC) were purchased from Avanti Polar Lipids (Alabaster, AL). All control proteins were obtained from Sigma. The fluorescent dyes 5,6-carboxyfluorescein and pyrenyldodecanoic acid were obtained from Molecular Probes, Inc. (Eugene, OR). Ten millimolar sodium phosphate, 120 mM sodium chloride buffer was used throughout, and pH was adjusted with 1 N hydrochloric acid.

Liposome Preparation. Unilammelar vesicles were prepared by drying 0.4 mL aliquots of lipid in chloroform (10−25 mg/mL) under vacuum using a Rotavapor R-114 rotary evaporator (Buchi, Switzerland). Buffered suspensions with or without 0.1 M 5,6-carboxyfluorescein were prepared and sonicated until translucent in a bath-type sonicator (Laboratory Supplies, Hickville, NY). The dye containing buffer surrounding the entrapped volumes was removed employing 5 mL Presto desalting columns (Pierce, Rockford, IL)

Fluorescence Measurements. Two milliliters of buffer was placed in a 2 mL 1 × 1 cm quartz cuvette, and small aliquots (typically 20 μL) of a liposome suspension were introduced. After 60 s aFGF was added (typically 20 μL at a concentration of 1 mg/mL). Fluorescence emission was then monitored continuously for 5 min at 520 nm using 470 nm excitation. Intrinsic tryptophan fluorescence measurements were performed using liposomes without carboxyfluorescein employing excitation at 285 nm and emission monitored from 300 to 500 nm with 10 nm bandwidths. The fluorescence of pyrenyl dodecanoic acid was detected using the same spectral parameters. Quenching of intrinsic tryptophan fluorescence was assessed by titration of 2 mL of an aFGF/liposome solution with 20 μL aliquots of 1 M acrylamide or potassium iodide.

Circular Dichroism (CD). CD measurements were performed with a Jasco J-720 spectropolarimeter. A rectangular 1 mm path cuvette and protein concentrations of 10−50 μM were employed. The temperature was controlled with a circulating water bath connected to a jacketed cuvette. CD spectra were collected for lipid suspensions in the absence of protein and subtracted from protein spectra to produce final corrected spectra.

Light Scattering. Light scattering measurements were performed with a Malvern 4700 spectrometer (Malvern Instruments, Malvern, England) equipped with a 5 W argon laser (Spectra Physics) operating at 488 nm and a photomultiplier detector capable of multiangle measurements. The temperature of the sample was maintained at 25 °C. Cumulant analysis of the autocorrelation function of the variations of the scattered light intensity arising from the Brownian motions of vesicles in solution yielded average diffusion coefficients (Koppel, 1972) and a hydrodynamic diameter calculated from the Stokes-Einstein equation.

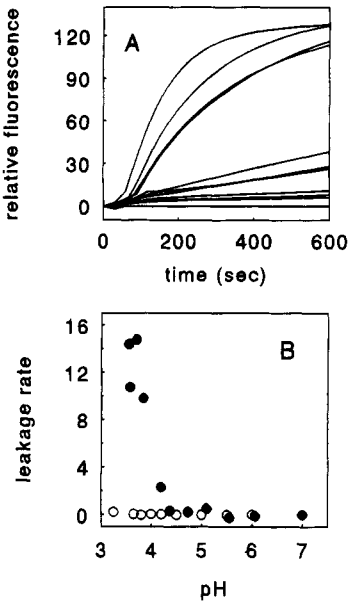

FIGURE 1: pH dependence of aFGF-induced fluorescent probe leakage rates. (A) Kinetics of 5,6-carboxyfluorescein release from liposomes at various pH values. The vesicles were composed of 25% DOPG and 75% DOPC. Acidic FGF (1 mg/mL) was added at 60 s. The lines correspond to the following pH values (from top to bottom): 3.57, 3.55, 3.70, 3.84, 4.20, 4.37, 4.73, 5.10, 5.55, 6.06, and 7.00. (B) Relative leakage rates of liposomes containing 25% DOPG, 75% DOPC (closed circles), and 100% DOPC (open circles). The temperature was 20 °C.

UV Spectrophotometry. The heat-induced aggregation of aFGF was monitored as the turbidity at 300 nm using a diode-array Hewlett-Packard 8450A spectrophotometer and jacketed cuvettes connected to a circulating water bath. Protein was initially unfolded by a 2-h incubation in 2 M guanidine-HCl, 20 μL aliquots were added to the buffer preincubated at a given temperature, and 10 s data acquisition times were employed.

RESULTS

The potential interaction of aFGF with model lipid vesicles was initially assessed by detecting the protein induced leakage of a liposome encapsulated fluorescent probe. This procedure is based on the observation that entrapped carboxyfluorescein is self-quenched at high concentrations (e.g., 100 mM) but becomes highly fluorescent upon release and subsequent dilution in the surrounding medium. Figure 1 illustrates the pH dependence of such aFGF-induced leakage rates. Dramatic increases in the rate of release of the dye are observed at pH values below 4.5 in vesicles containing 25% DOPG and 75% DOPC at 20 °C (Figure 1B). The rates at pH values below 3 could not be reliably measured due to the quenching of fluorescein fluorescence at reduced pH. In contrast to DOPG-containing vesicles, neutral vesicles consisting of 100% DOPC do not exhibit any leakage upon addition of aFGF (Figure 1B).

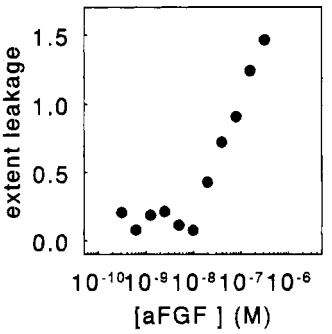

FIGURE 2: Dependence of the leakage rates of the fluorescent probe (Figure 1) from liposomes composed of 25% DOPG/75% DOPC on aFGF concentration. The temperature was 20 °C and pH 3.25.

At pH 3.3, the rate of leakage increases linearly with the logarithm of the protein concentration at higher aFGF concentrations (Figure 2). The linear portion of this data can be extrapolated to zero leakage at 10 nM, which can be considered the minimum amount of protein necessary to induce leakage. This also suggests that a critical number of membrane-associated aFGF molecules is necessary to cause membrane alterations. In a single-hit type reaction, in which one molecule or molecular complex rapidly inserts into a lipid bilayer, a linear relationship between leakage rate and inducer concentration is often observed (Wendt, 1970; Bruggemann & Kayalar, 1986; Levinthal et al., 1991). Assuming that one lipid molecule occupies about 0.7 nm^2 of vesicle surface (Small, 1986) and knowing the hydrodynamic dimensions of the vesicles of this study obtained from dynamic light scattering measurements (~70 nm diameter), the molar concentration of the vesicles under the conditions of the above experiment (30 mM lipid) is estimated to be approximately 1.4 nM. Thus, at an aFGF concentration of 10 nM, the protein to liposome ratio is approximately 7:1. Since aFGF does not appear to manifest simple single-hit kinetics, presumably at least partially due to the competition between self-association and liposome binding, this ratio cannot be reliably used to estimate the probability of functional insertion (e.g., resulting in channel formation) upon interaction with the membrane (Mel & Stroud, 1993).

To estimate the maximum number of aFGF molecules that can interact with a single liposome, aFGF solutions at fixed concentration were titrated with liposomes, and both the relative intrinsic fluorescence intensity increase (due to the change in the microenvironment of the single aFGF tryptophan residue upon interaction with the membrane) and the change in light scattering intensity (reflecting the increase in the average molecular weight of aFGF-induced aggregated vesicles) were measured. Both the aFGF fluorescence intensity and vesicle size changes cease at a lipid/aFGF molar ratio of approximately 30:1 (Figure 3). Assuming about 22 000 lipids/vesicle based on their size, this corresponds to about 730 aFGF molecules associated with each vesicle. Each protein molecule is then calculated to occupy an area of about 21 nm^2, which corresponds to a square of dimension 4.6 nm. This is a reasonable value considering that aFGF in its native globular state (Zhu et al., 1991) has a hydrodynamic diameter of approximately 3.7 nm, as measured by dynamic light scattering. Since any unfolding of a protein would be expected to increase its dimensions, these data suggest that the surface of the liposomes may be saturated with at least

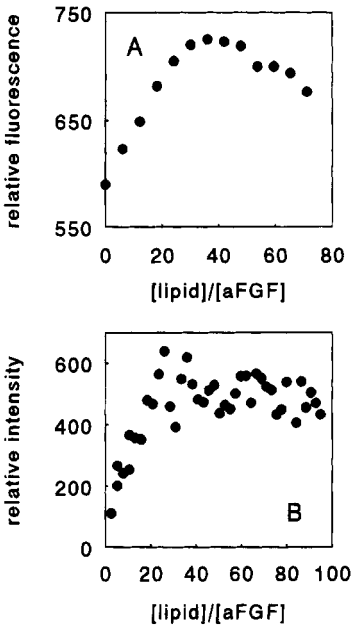

FIGURE 3: Stoichiometry of the aFGF/liposome interaction. (A) The relative fluorescence intensity from the intrinsic aromatic residues of aFGF as a function of lipid/aFGF molar ratio. (B) The scattering light intensity as a function of lipid/aFGF ratio. The temperature was 20 °C.

partially unfolded aFGF molecules, although uncertainities in this calculation must temper this conclusion.

The fluorescence of native aFGF is characterized by a tyrosine maximum at 305 nm due to extensive quenching of the proteins single tryptophan fluorescence (Copeland et al., 1990). Perturbation of the structure of aFGF causes this quenching to be relieved and a more typical tryptophan-dominated fluorescence spectrum to appear with emission in the 340−350 nm region (Mach et al., 1994). The fluorescence intensity of unfolded or partially folded aFGF increases about 2-fold, and the peak maximum shifts to ca. 10 nm lower wavelengths upon interaction with phospholipid membranes (Figure 4A). Such increases in intensity are often associated with the transfer of indoles to more apolar microenvironments like those existing in membrane interiors. Alternatively, the observed change could be the result of a conformational change affecting the local protein matrix environment of the aFGF Trp or its distance from a quenching moiety. To differentiate between these possibilities, aFGF/liposome mixtures were titrated with the neutral quenching agent acrylamide and the charged compound potassium iodide. The results are shown as Stern−Volmer plots (the ratio of the initial fluorescence intensity to the intensity obtained in the presence of the quencher versus the quencher concentration) in Figure 4B. *N*-Acetyl-L-tryptophanamide was used to assess the changes which would be expected for a fully solvent-exposed indole side chain. The single tryptophan side chain of aFGF appears to be substantially shielded from the solvent even in the absence of any stabilizing ligand with about 30% solvent exposure estimated by comparison to the tryptophanamide model. The addition of liposomes (25% DOPG, 75% DOPC) results in further shielding to about 10% solvent exposure. This effect is even more pronounced for liposomes composed of 100% DOPG (~5% exposed). These data could be due to either actual burial of the indole side chain in the bilayer or the result of a conformational change or reduction in confor-

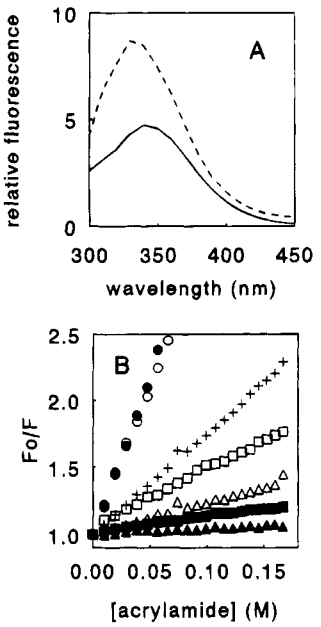

FIGURE 4: Fluorescence emission spectra of aFGF alone (solid line) and in the presence of liposomes (dashed line) at pH 2.35. The liposomes were composed of 25% PG, 75% PG. (B) Stern–Volmer plots of aFGF fluorescence quenching by acrylamide with no additions (crosses), in the presence of 100% DOPG liposomes at 50 °C (open squares), 25% DOPG/75% DOPC liposomes (open triangles), 100% DOPG liposomes (filled squares), and 100× excess heparin (filled triangles). The temperature was 20 °C. Mixtures of *N*-acetyl-L-tryptophanamide in the absence (open circles) and presence of liposomes (filled circles) were also employed to obtain reference curves corresponding to 100% solvent exposed residues.

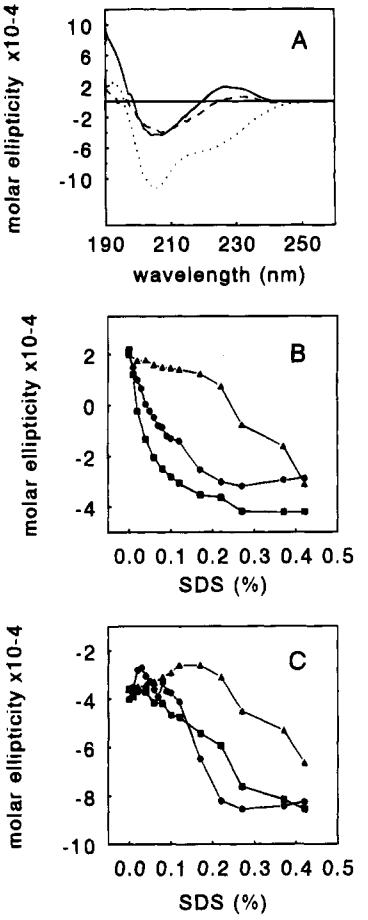

FIGURE 5: Circular dichroism spectra of aFGF in the presence of various amounts of SDS. (A) CD spectra of aFGF in the absence of SDS at neutral pH (solid line), in the presence of submicellar (0.05%, cmc = 0.2%) concentrations of SDS (dashed line) and in the presence of an excess of SDS (0.4%) (dotted line). (B) Molar ellipticity at 225 nm (primarily tertiary structure-dependent) as a function of SDS cencentration; aFGF alone (circles), aFGF in the presence of 25%DOPG/75% DOPC liposomes (squares), aFGF in the presence of 3× excess of heparin (triangles). (C) Molar ellipticity at 205 nm (primarily secondary structure-dependent). Symbols as in panel B.

mational flexibility upon binding of the protein to the membrane surface. Significantly, the presence of an excess of heparin results in even greater shielding than that observed during liposomal interaction. Increases in average solvent accessibility are also observed at pH 7 and elevated temperature (50 °C) presumably as a consequence of increased thermal unfolding of aFGF (not illustrated). In contrast to the aggregation seen at low pH, interaction of aFGF with liposomes at neutral pH and high temperature does not result in extensive aggregation of the vesicles as judged from both static and dynamic light scattering measurements (not illustrated).

To further probe the nature of the interaction of aFGF with membranes, we attempted to measure energy transfer between the protein's tryptophan residue and pyrenyl dodecanoic acid, a lipid analogue often employed as a depth marker (Freire et al., 1983; Michangeli et al., 1990; Collins & Cha, 1994). Since this probe incorporates directly into lipid bilayers when added to aqueous solutions, an energy transfer from tryptophan to pyrene can occur if the tryptophan is also embedded sufficiently deeply [e.g., Collins and Cha (1994)]. No energy transfer in the form of acceptor emission or donor quenching was observed in any experiment, suggesting that the single aFGF tryptophan residue is located at or near the bilayer surface (not illustrated).

Circular dichroism measurements of aFGF/liposome mixtures were complicated by artifacts often encountered with aggregating systems (e.g., absorption flattening). Spectra similar to those observed previously, however, in the absence of liposomes at low pH (Mach et al., 1993) were obtained (not illustrated), suggesting the presence of extensive β-sheet secondary structure. Amide I′ FTIR spectra of these same aFGF solutions at low pH were also dominated by peaks associated with β-structure at 1628, 1637, and 1678 cm^{-1} as well as contributions from turns at 1662, 1669, and 1690 cm^{-1} (Mach et al., 1993), further consistent with a retention of the majority of the protein's secondary structure.

To further probe potential effects of lipid bilayers on aFGF structure, CD spectra of aFGF were obtained in the presence of submicellar and micellar concentrations of SDS. Many peptides assume organized structure under such conditions (Zhong & Johnson, 1992; Waterhous & Johnson, 1994). CD spectra collected at various SDS concentrations (Figure 5) show that, at micellar concentration of SDS (>0.1%), aFGF assumes a highly disordered (i.e., "unfolded") conformation (Mach et al., 1993), while a "molten-globule"-like spectrum (high secondary structure content) is observed at submicellar concentrations at ambient temperature (20 °C). Lower concentrations of SDS are needed to disrupt the tertiary structure of aFGF if negatively charged membranes are present. In contrast, the complete unfolding of the secondary structure of aFGF at micellar concentrations appears to be hindered by their presence. A similar trend is observed when the shape and intensity of aFGF's fluorescence emission

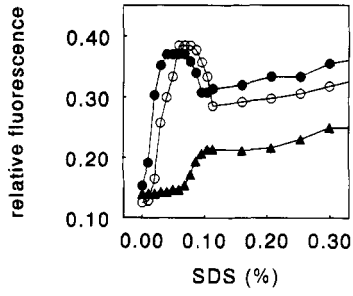

FIGURE 6: Intrinsic aromatic amino acid fluorescence intensity as a function of SDS concentration of aFGF alone (open circles), in the presence of 25% DOPG/75% DOPC liposomes (filled circles) and in the presence of a 3× excess of heparin (filled triangles).

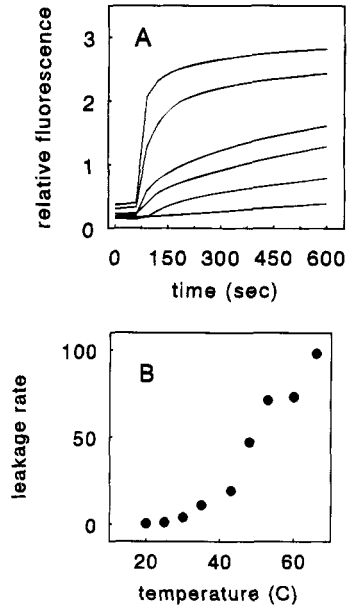

FIGURE 7: Kinetics of fluorescent probe leakage of 100% DOPG liposomes as a function of temperature at pH 7.0. (A) Relative fluorescence intensity of 5,6-carboxyfluorescien released from liposomes upon addition of aFGF at 60 s. The lines correspond to the following temperatures (from top to bottom): 66, 60, 48, 43, 30, and 20 °C. (B) The leakage rates observed in panel A plotted as a function of temperature.

spectra are analyzed (Figure 6) with extensive tertiary structure disruption observed at submicellar SDS concentration. The presence of liposomes facilitate these spectral changes suggesting that the lipids may have effects similar to those induced by SDS in the initial destabilization of the tertiary structure. The presence of heparin completely abolishes these changes, and micellar concentrations are now needed to perturb the structure of aFGF.

To assess the extent of interaction of thermally perturbed aFGF with lipid bilayers, rates of carboxyfluorescein leakage as a function of temperature were measured under physiological-like conditions (120 mM NaCl, pH 7). The results obtained using 100% DOPG vesicles are shown in Figure 7A. The observed enhanced leakage of fluorescein at elevated temperatures may be associated with at least partial unfolding of aFGF and subsequent interaction of these species with the membranes, since it has been established by several spectral methods that the structure of aFGF is initially perturbed at $T > 30$ °C, the temperature near which leakage appears to be initiated in this system (Figure 7B) (Dabora et al., 1990). At higher temperatures ($T > 50$ °C) where aFGF unfolding is more rapid, the majority of the

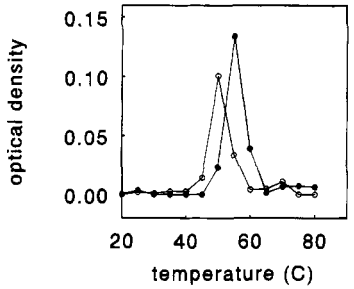

FIGURE 8: aFGF heat aggregation as a function of temperature in the absence (open circles) and presence of 25% DOPG/75% DOPC liposomes (filled circles). The protein was first unfolded in 2 M guanidine-HCl and then diluted 40-fold into 10 mM sodium phosphate and 120 mM sodium chloride, pH 7, at the indicated temperatures. The turbidity at 300 nm was measured after 5 min.

liposome's contents is released within 12 min. The slower leakage rates observed at lower temperature may also be at least partially due to the reduced rates of aFGF unfolding, which in turn could result from the stabilizing effect of the negatively charged phospholipids on aFGF structure. To test the specificity of this phenomenon, five control proteins (lysozyme, bovine serum albumin, α-chymotrypsin, papain, and ribonuclease A) were examined for their ability to release liposomally entrapped dye at 37 °C and neutral pH (not illustrated). The only case in which leakage could be detected was that of lysozyme at low ionic strength (10 mM sodium phosphate, pH 7.0). We presume this to be due to the extreme basic nature of this protein which results in extensive interaction with the negatively charged liposome surface.

To test whether fully unfolded or partially folded (e.g., "molten globule") states of aFGF are the species interacting with the lipid bilayers, the extent of aggregation of actively refolding aFGF in the presence and the absence of liposomes was measured as a function of temperature (Figure 8). The protein was initially unfolded by a chaotropic agent (2 M guanidine-HCl) to prevent aggregation of the transient "molten globule" states that appear during the unfolding process (Mach et al., 1993). It was then diluted under folding conditions at the indicated temperatures. Aggregation is observed only in a relatively narrow temperature range with maximum aggregation between 50 and 55 °C. Aggregation occurs at temperatures about 5 °C higher in the presence of lipid, again suggesting a stabilizing effect upon the protein. No aggregation is observed at temperatures above 65 °C (a temperature at which unfolded protein does not refold). When native aFGF is exposed to temperatures above 45 but below 65 °C, however, samples aggregate implying involvement of unfolding intermediates in the aggregation process.

DISCUSSION

Acidic FGF is found in both the cytoplasm and nucleus of certain cells as well as complexed to proteoglycans on their external surface. This growth factor lacks a conventional signal sequence, raising the question of its mode of transport across biological membranes. One interesting possibility is that the ability of the protein to be induced into "molten-globule"-like states by physiological conditions may be involved in translocation events (Mach et al., 1994). In the absence of stabilizing polyanions at neutral pH and moderate temperatures (e.g., $T > 30$ °C) or at low pH in the presence of anions including certain phospholipids, the

protein manifests little evidence of stable tertiary structure although its secondary structure remains essentially intact. These states are significantly apolar as evidenced by the binding of hydrophobic dyes and display a marked tendency to aggregate. In this work, we show that such states are also able to interact with lipid bilayers as evidenced by their ability to induce the release of encapsulated dyes from negatively charged liposomes as well as cause aggregation and fusion of the same vesicles.

Several experiments hint at the nature of the interaction of aFGF with these lipid bilayers. The leakage of the fluorescent probe carboxyfluorescein at a threshold near pH 4.5 parallels the cell membrane translocation of an aFGF−diphtheria toxin conjugate. A dramatic increase in the normally quenched tryptohan fluorescence in this same pH range suggested significant disruption of aFGF native structure during translocation of this conjugate (Wiedlocha et al., 1992). In contrast, aFGF in its native, polyanion-stabilized compact state appears to be unable to penetrate lipid bilayers. It seems probable, however, that in the absence of high affinity polyanions and at low ionic strength and low temperatures which preserve native structure, aFGF can bind to liposomes by the virtue of their high negative charge density. It has previously been shown that aFGF can bind to wide variety of polyanions and that charge density appears to be the major determinant of affinity (Volkin et al., 1993). Nevertheless, the growth factor must associate more tightly with cell surface proteoglycans, such as heparan sulfate than with phospholipids, since the former are found to be the primary storage depot of the protein on cell surfaces (Vlodavsky et al., 1991).

Several events occur upon lowering the pH of a protein solution near or below the protonation point of aspartic and glutamic acid side chains. The disappearance of compensating negative charges increases the repulsion between positively charged side chains, such as lysine and arginine. This effect is especially pronounced when the ionic strength is lowered. Under these conditions, aFGF is very soluble and manifests a CD spectrum characteristic of a randomly coiled polypeptide (Mach et al., 1993). Addition of counterions, however, neutralizes long-range charge repulsion between side chains and allows the formation of a partially folded state. At neutral pH, ambient temperature, and low ionic strength aFGF is significantly unfolded (not illustrated), presumably due to the same repulsive forces. In addition, the disappearance of negative charge increases the net positive charge and, consequently, the affinity for negatively charged phospholipid surfaces (Kim et al., 1991). Upon binding, the well defined positively charged patches on the surface of aFGF presumably directly interact with the lipid bilayer surface while some of the more apolar regions of the protein may insert at least partially into the bilayer. By analogy, both positively charged and apolar residues of phospholipase A2 appear to be involved in this proteins interaction with membranes (Scott et al., 1990). Aggregation of such partially embedded protein molecules within the lipid bilayer may then result in membrane disruption and the leakage of any encapsulated contents.

In micelles and lipid bilayers composed of either neutral or charged amphiphiles, even if the bulk pH changes by a large amount, dissociable groups on the surface are buffered by the surrounding lipid heads (Scarlata & Rosenberg, 1990). After initial ionization, other phosphate protons in bilayers can stabilize the negatively charged surface through an extensive hydrogen-bonding network in which the protons are delocalized over two phosphate groups (Eibl, 1983). Consequently, stabilization of the protonated state of groups by the negatively charged surface occurs. For example, insertion of potentially negatively charged 6-decanoylnapthol or 3-hexadecanoylcoumarin into SDS micelles shifts the probes dissociation constant upward by 2 pH units (Scarlata & Rosenberg, 1990). This suggests that, as a protein approaches a negatively charged bilayer surface, the acidic groups of the protein may lose their charge promoting protein unfolding and membrane insertion. At elevated temperatures, where the formation of the "molten globule" state can be achieved without low pH, naturally occurring basic patches (e.g., residues 100−128) of aFGF (Zhu et al., 1993; Chavan et al., 1994; Pineda-Lucena et al., 1994) together with the apolar and protonation inducing properties of the bilayer may induce insertion. The importance of negative charge density appears to be more crucial at neutral than at low pH (Figures 1 and 8). This is presumably due to the presence of negatively charged groups remaining on aFGF upon membrane binding under these conditions.

Unlike many of the channel-forming membrane proteins, which are able to functionally insert in a monomeric or limited oligomeric state, aFGF's interaction with membranes appears to be in competition with self-association, as judged from the logarithmic dependence of liposome leakage rates on aFGF concentration. One of the best studied water-soluble proteins capable of membrane insertion and ion channel formation, colicin A, manifests single-hit insertion kinetics (Mel & Stroud, 1993). Its three-dimensional structure in aqueous solution has been determined (Parker et al., 1989, 1992) and is composed of a bundle of 10 helices arranged in three layers. The kinetics of insertion of colicin A into lipid bilayers at low pH also correlates with the appearance of a "molten globule" state (van der Goot et al., 1991). After insertion, the protein apparently remains in this conformation (Muga et al., 1993). There is good evidence that most of the protein resides at the surface of the bilayer with only helices 8 and 9 inserted into the bilayer's apolar interior (Massotte et al., 1993). The area at the bilayer surface occupied by the bound colicin A appears to be larger than the cross-sectional area of the native compact solution form as a consequence of this conformational change. A similar result can be deduced from the average lipid bilayer area occupied by one aFGF molecule under saturating conditions, estimated to be about 21 nm^2, or a square of dimension 4.6 nm. Native aFGF has a hydrodynamic diameter of approximately 3.7 nm (equivalent to a cross-sectional area of about 11 nm^2), assuming spherical geometry, implying the possibility of significant expansion of the bound growth factor, although these estimates are sufficiently crude that no definitive conclusion should be drawn.

There are major changes in the fluorescence emission of the protein's single tryptophan residue upon interaction with liposomes implying transfer of at least this residue to a more apolar environment. Fluorescence quenching experiments also detect a decrease in solvent accessibility of this side chain, although binding of heparin to aFGF in solution causes an even greater loss in accessibility (but no change in overall intrinsic fluorescence properties). The precise nature of this conformational change is difficult to ascertain at this point. One possibility is that the planar lipid bilayer may force the

protein into a somewhat more extended conformation, resulting in greater tryptophan exposure. The lack of energy transfer between the tryptophan residue and pyrenyl dodecanoic acid embedded in the bilayer interior is consistent with some Trp solvent exposure of the liposome-bound aFGF. Use of the protein's single indole group as a probe of overall protein structure, however, could be misleading in this regard. Unlike numerous natural and synthetic peptides that form amphiphilic α-helices in the presence of lipid membranes, aFGF bound to liposomes appears to maintain its predominantly β-strand structure (Mach et al., 1993). Although it is not possible to perform definitive CD measurements under exactly the same conditions as the dye release experiments, the observed distorted spectra as well as more interpretable FTIR studies argue that substantial β-type secondary structure remains in the liposome-associated protein. These results are supported by more unambiguous CD and fluorescence studies in SDS which clearly show that the anionic lipid bound protein possesses extensive secondary structure but dramatically decreased tertiary contacts (as evidenced by the loss of the 228 nm CD band and large increases in the 350/300 nm fluorescence ratio). Another possibility is that a segment of the polypeptide chain of aFGF become extruded from the major body of the protein and serves as a membrane interaction vehicle like the situation observed with the influenza hemagluttinin (Bullough et al., 1994).

Many of the proteins and peptides known to insert into lipid bilayers require acidic conditions for their functional activity (e.g., Sandvig & Olsnes, 1988; Kono et al., 1990; Levy-Mintz, & Kielian, 1991; Stegmann et al., 1991; Sanyal et al., 1993; Collins & Cha, 1994). Similarly, a disruption of native structure and formation of a "molten globular" state facilitated by low pH was previously shown to be a prerequisite for membrane insertion and translocation of aFGF conjugated to the A-fragment of diptheria toxin (Wiedlocha et al., 1992). In this study, we demonstrate that at neutral pH thermally induced "molten-globular" states of aFGF are also capable of functional membrane insertion. This may represent a rather general phenomenon. For example, conformational changes of influenza hemagglutinin related to fusion can also be triggered by high temperature at neutral pH (Ruigrok et al., 1986). In these cases, hydrogen ions are thought to act only to enable the conformational changes and not to directly participate in membrane insertion events per se. The requirement for the presence of negatively charged lipids for aFGF to insert into the bilayer at neutral pH appears to be more important than at lower pH, presumably as a consequence of a less favorable charge balance.

Of some interest is the question of whether there exists a correlation between the aggregation tendencies of partially folded states and membrane insertion propensity. A simplistic consideration of the forces involved in protein folding would suggest that minimization of unfavorable entropic effects by shielding the apolar regions from water might indeed be a common feature involved in the formation of "molten globules" and lipid bilayer insertion. In particular, in this work we show that thermally unfolded aFGF does not aggregate. To demonstrate this, the concentrated protein unfolded by a chaotropic agent was introduced into bulk solutions at various temperatures. Only over a relatively narrow range of temperatures (45 to 55 °C) could aggregation be observed. This is exactly the temperature range over which the protein's fluorescence properties indicate disruption of tertiary structure but far-UV CD spectra imply that a significant amount of secondary structure still persists (Mach et al., 1993). These, of course, are the hallmarks of "molten globule" states, which have previously been linked to heat-induced aggregation (Chrunyk et al., 1993; Fischer et al., 1993). The temperature-dependent aggregation profile observed in this work reinforces the idea that intermediate rather than high (i.e., unfolding) temperatures are responsible for most "heat denaturation" of proteins. Acidic FGF and perhaps most water soluble proteins are probably very soluble at temperatures high enough to induce more extensively unfolded states. In a limited predenaturing temperature region, however, partially unfolded (tertiary structure-disrupted) states appear, and the apolar surfaces of these molten-globule states induce aggregation. Since the size of the aggregates frequently reach the submicron level, the unfavorable surface-to-volume ratio and possibly polypeptide chain entanglement cause the apparent irreversibility that is usually observed.

In conclusion, these results suggest the following simple model: the strong electrostatic attraction between the negatively charged phospholipid vesicles and positively charged regions of aFGF are responsible for the formation of an initial complex between the two entities. Unlike the interaction with heparin and other related polyanions, the binding of aFGF to liposomes does not seem to induce the formation of native structure. Rather, the liposomes appear to stabilize intermediate "molten globule" states of aFGF presumably through apolar interactions between protein and lipid side chains. This does not, however, seem to involve extensive penetration of the majority of the protein into the lipid bilayer as evidenced by energy transfer results. Most simply, one can envision penetration of perhaps one or a few elements of the protein's secondary structure into the bilayer in much the same way that a number of fusion and pore-forming proteins mediate their physiological activity (e.g., Doms et al., 1985; Novick & Hoekstra, 1988; Stegmann et al., 1991; Bullough et al., 1994; Yu et al., 1994). Thus, the lipid bilayers can be viewed as simply stabilizing one or a few substates of the rather dynamic MG states. It is not clear how this initial interaction might lead to actual translocation. This process may be facilitated *in vivo* by other (perhaps chaperone-like) proteins although it is possible that the phenomenon described in this work can itself lead to translocation competent species in appropriate environments. It must be emphasized, however, that a role for such states in the transport of this protein remain hypothetical.

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