Stabilization of Granulocyte Colony-Stimulating Factor and Structurally Analogous Growth Factors by Anionic Phospholipids

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ABSTRACT: Recombinant granulocyte colony-stimulating factor (rhG-CSF) interacts with liposomes composed of the anionic phospholipid dioleoylphosphatidylglycerol (DOPG), and this interaction enhances the stability of the protein [Collins, D., & Cha, Y. (1994) *Biochemistry 33*, 4521−4526]. In the present studies, we have examined the interaction of rhG-CSF with phospholipids other than DOPG. Fluorescence spectroscopy of rhG-CSF with a variety of lipid vesicles demonstrated that rhG-CSF inserts into bilayers of anionic, but not zwitterionic, phospholipids. Isothermal titration calorimetry of the interaction between DMPG and rhG-CSF indicates that the binding is saturable and involves 10 lipids/rhG-CSF. Studies of phosphatidylglycerols with varying alkyl chain lengths determined that the stabilization of rhG-CSF by anionic phospholipids required a certain alkyl chain length; no stabilization was observed with lipids of shorter chain length. Also investigated was the stabilization of other growth factors, which are structurally similar to rhG-CSF, by anionic phospholipids. These proteins include recombinant porcine somatotropin (rpSt), recombinant human granulocyte−macrophage colony-stimulating factor (rhGM-CSF), recombinant human interleukin 4 (rhIL-4), and recombinant human interleukin 2 (rhIL-2). The helical secondary structure of the proteins was recoverable after heating and cooling in the presence of anionic phospholipids as observed by circular dichroism; the presence of zwitterionic lipids did not induce this effect. Results of these investigations concluded that a group of structurally similar proteins interact preferentially with anionic phospholipids and that the complexation of the growth factors with vesicles composed of anionic phospholipids improves the stability of the proteins under conditions where they normally denature.

Recombinant granulocyte colony-stimulating factor (rhG-CSF)[1] is a hematopoietic factor currently used in the treatment of chemotherapy-induced neutropenia. rhG-CSF stimulates neutrophil proliferation and activity and is a single-chain nonglycosylated protein of 18.5 kDa. The structures of several other growth factors have been shown to be quite similar in overall architecture to rhG-CSF despite little sequence homology (Hill et al., 1993). Included in this group of growth factors are recombinant forms of human granulocyte−macrophage colony-stimulating factor (rhGM-CSF), human interleukin 2 (rhIL-2), human interleukin 4 (rhIL-4), and porcine somatotropin (rpSt). All are single-chain polypeptides in the molecular mass range of 14.5−22 kDa. Their structures are shown schematically in Figure 1; all are composed of a four-α-helix bundle with up−up−down−down connectivity of the helices (Hill et al., 1993).

Protein−lipid interactions have been characterized in several systems including human plasma apolipoprotein A-I (Surewicz et al., 1986), human platelet P-235 (Heise et al., 1991), colicin A (Pattus et al., 1983; Lakey et al., 1991), apocytochrome *c* (Rietveld et al., 1983; Görrison et al., 1986), and transferrin receptor (Kurrle et al., 1990). Many of these proteins show a preference for anionic phospholipids as compared to zwitterionic phospholipids. In all cases, these proteins are either known lipid carrier proteins or bear at least one helix with sufficient hydrophobicity to insert into the lipid phase. While cytokines are not traditionally considered to be membrane-inserting proteins, interactions of cytokines with membranes have been reported (Collins & Cha, 1994; Yoshimura & Sone, 1987; Oku et al., 1987; Debs et al., 1989; Chu & Sharom, 1990, 1993). To our surprise, rhG-CSF exhibits dramatically enhanced stability

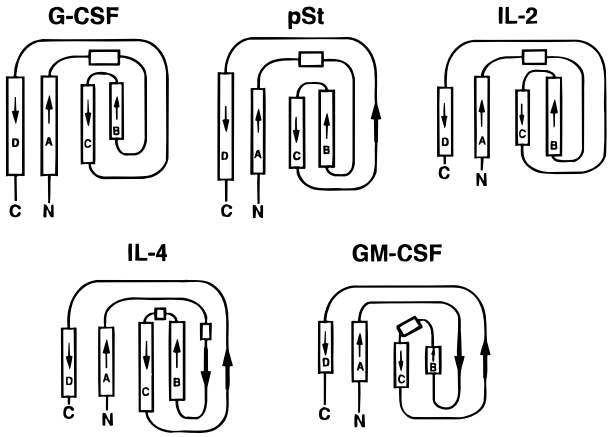

FIGURE 1: Connectivity diagrams of cytokines with similar architecture: four-α-helix bundles with up−up−down−down connectivity and two long crossover loops.

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[1] Abbreviations: rhG-CSF, recombinant human granulocyte colony-stimulating factor; rpSt, recombinant porcine somatotropin; rhGM-CSF, recombinant human granulocyte−macrophage colony-stimulating factor; rhIL-2, recombinant human interleukin 2; rhIL-4, recombinant human interleukin 4; DMPG, dimyristoylphosphatidylglycerol; DOPG, dioleoylphosphatidylglycerol; DPPG, dipalmitoylphosphatidylglycerol; DMPS, dimyristoylphosphatidylserine; DOPS, dioleoylphosphatidylserine; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; DSC, differential scanning calorimetry; CD, circular dichroism.

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after interacting with lipid vesicles composed of DOPG even after aggressive thermal stress (Collins & Cha, 1994). This enhanced stability has been attributed to the partial insertion of rhG-CSF into the lipid bilayer.

Because the nature of proteins as well as the lipid properties can affect the binding of proteins to lipids (Bergers et al., 1993), it is of interest to determine the interaction of rhG-CSF with phospholipids other than DOPG and to study the behavior of other growth factors with similar lipid vesicles. This paper extends the earlier investigations of the DOPG/rhG-CSF interaction to that of rhG-CSF and other phospholipids and describes the effects of anionic and zwitterionic phospholipids on a group of structurally similar growth factors.

EXPERIMENTAL PROCEDURES

Materials. rhG-CSF, rhGM-CSF, rhIL-2, rhIL-4, and rpSt were prepared by Amgen Inc. (Thousand Oaks, CA). All phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL).

Liposome Preparation. Sonicated unilamellar vesicles (SUV) were prepared by hydrating lipid powders or dry lipid films in water and then sonicating the preparations in a sonicator bath (Laboratory Supplies, Hicksville, NY) until optically clear.

Fluorescence Spectroscopy. The fluorescence behavior of rhG-CSF was measured using a PTI Alphascan fluorometer. The rhG-CSF samples were excited at 280 nm and the emission spectra were scanned from 285 to 420 nm. Samples were prepared by adding lipid vesicle suspensions to rhG-CSF. The final concentration of rhG-CSF was 0.2 mg/mL.

Circular Dichroism Spectroscopy. CD spectra were measured with a Jasco J-720 equipped with a Peltier-type thermostated cell holder and a magnetic stirrer. Spectra were acquired at 222 nm using a protein concentration of 80 μg/mL and a 1-cm path length cuvette. The molar ratio of lipid to protein was 150:1 in water, pH 6.0. Noise reduction of final curves was performed with software provided by Jasco.

Differential Scanning Calorimetry. Calorimetric scans of the proteins and protein/lipid complexes were measured with a Microcal MC-2 high-sensitivity differential scanning calorimeter (MicroCal, Inc., Northampton, MA). The sample cell volume was 1.2099 mL. Samples were prepared in water with a final protein concentration of 4.0 mg/mL. Samples were thoroughly degassed and equilibrated in the cell at 5 °C for 60 min before scanning; exact equilibration times were necessary to obtain reproducible lipid transition peaks. Scans were acquired at 90 °C/h. The data were stored and analyzed by software provided by MicroCal.

Isothermal Titration Calorimetry. Heats of reaction were measured with an Omega titration calorimeter (MicroCal, Inc., Northampton, MA). The calorimeter was calibrated electrically. Sample cell volume was 1.362 mL. Isothermal titration experiments were performed at 25 °C. rhG-CSF solutions and lipid suspensions were prepared in water. After thorough degassing, the protein solution (25 μM) was placed in the cell and the lipid suspension (7 mM) was placed in the 100-μL syringe. Injections of 2.5 μL of lipid were made at 5-min intervals with stirring at 100 rpm. The data were acquired, stored, and analyzed with software provided by MicroCal.

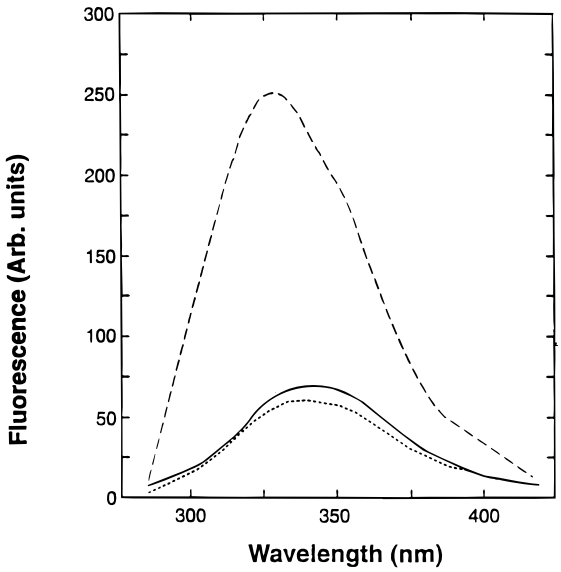

FIGURE 2: Fluorescence emission spectrum of rhG-CSF in the absence (solid line) and presence of DMPC vesicles (dotted line) or DMPG vesicles (dashed line). The concentration of rhG-CSF was 0.2 mg/mL and the lipid:rhG-CSF ratio was 100:1 (mol/mol).

Table 1: Effect of Phospholipid Vesicles on rhG-CSF Fluorescence

lipid	F/F_0[a]	emission maximum[b] (nm)
none	1.00	336
DMPG	3.36	329
DPPG	2.94	330
DOPG	3.22	329
DMPS	3.65	328
DOPS	2.47	331
DMPC	1.00	336
DPPC	1.00	336
DOPC	1.00	336

[a] Ratio of fluorescence in the presence of lipid (F) to fluorescence of rhG-CSF alone (F_0). [b] Determined from wavelength scans of samples as described in Experimental Procedures.

RESULTS

The tryptophan residues of rhG-CSF are sensitive to their local environment; changes in the environment are detectable by variations in fluorescence behavior (Collilns & Cha, 1994; Narhi et al., 1991). The fluorescence spectra of rhG-CSF alone and in the presence of DMPG and DMPC sonicated lipid vesicles are shown in Figure 2. rhG-CSF has an emission maximum at 336 nm in the absence of lipid vesicles. The presence of DMPC vesicles at 100:1 molar ratio of lipid to rhG-CSF does not affect the fluorescence of the protein (Figure 2). By contrast, a significant shift in emission maxima to 329 nm and a greater than 3-fold enhancement in fluorescence is observed when rhG-CSF is combined with DMPG vesicles at a molar ratio of lipid to protein of 100:1 (Figure 2). Table 1 describes the fluorescence behavior of rhG-CSF in the presence of sonicated vesicles composed of other phospholipids. The ratio of lipid to protein is 100:1 (molar) in all cases. The data obtained clearly show that the anionic phospholipids, such as phosphatidylglycerols, induce a significant change in the fluorescence behavior as observed by a change in intensity as well as a shift in the maximum emission wavelength. The zwitterionic lipids, such as phosphatidylcholines, induced no change in the fluorescence behavior of rhG-CSF. These data

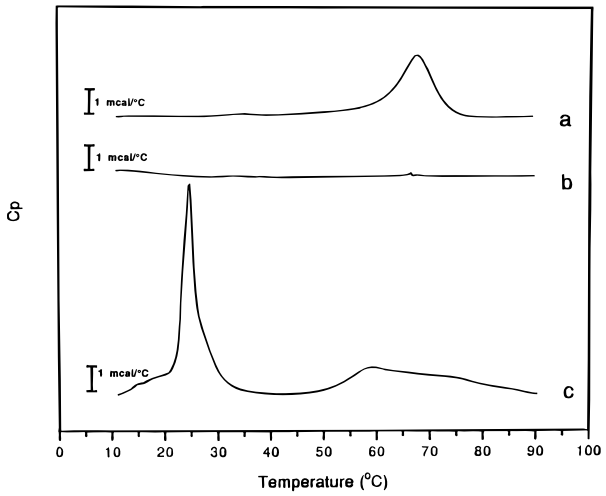

FIGURE 3: Differential scanning calorimetry thermograms of rhG-CSF (curve a), 20:1 DMPG:rhG-CSF (curve b) and 20:1 DMPC: rhG-CSF (curve c).

indicate that the tryptophans reside in a more hydrophobic environment in the presence of anionic phospholipids than they do in the native state or in the presence of zwitterionic lipids (Collins & Cha, 1994).

The interactions of rhG-CSF with vesicles composed of either DMPG or DMPC were also compared using DSC and isothermal titration calorimetry. The lipids DMPC and DMPG were chosen for direct comparison due to their equivalence in alkyl chain length and similarity in thermal transition behavior under these conditions. In the absence of lipid, rhG-CSF undergoes an endothermic melting transition in the range of 60−70 °C as measured by DSC (Figure 3). This melting transition is abolished in the presence of DMPG vesicles at a 20:1 molar ratio of lipid:protein (Figure 3). Interestingly, under these conditions, no melting transition for DMPG was seen, whereas DMPG itself shows a melting transition at 23 °C (data not shown). In contrast the DMPC vesicles had no effect on rhG-CSF melting by DSC (Figure 3). Likewise, rhG-CSF did not affect the gel−fluid transition of DMPC under these conditions. The data indicate that rhG-CSF interact strongly with DMPG, but not DMPC, vesicles.

This conclusion was confirmed by isothermal titration calorimetry. A stock solution of lipid vesicles at 7 mM total lipid was titrated into a 25 μM stock of rhG-CSF at 24.8 °C. Titration of DMPC into rhG-CSF yielded small exothermic peaks which did not vary in size with injection number (Figure 4). This indicates that the reaction is not saturable over the concentration range used in these experiments. The enthalpy of this constant exothermic reaction ($\Delta H = -0.33 \pm 0.09$ kcal/mol) may be due to a very weak interaction between DMPC and rhG-CSF as previously described for apolipoprotein A-1 and DMPC using this technique (Epand et al., 1990). In contrast to the data with DMPC, injection of DMPG into rhG-CSF resulted in heat absorption for the first several injections (Figure 5). The endothermic reaction was saturable as the molar ratio of DMPG to rhG-CSF was increased. The data indicate that under these conditions rhG-CSF binds to a cluster of 10 DMPG molecules with a binding constant of 4.3×10^5 M^{-1}. The thermodynamic parameters associated with the interaction of DMPG with rhG-CSF were determined to be $\Delta H =$ 5.42 kcal·mol^{-1}, $\Delta G = -7.69$ kcal·mol^{-1}, and $\Delta S = 44$ cal·mol^{-1}·deg^{-1} and indicate that the reaction is entropically driven.

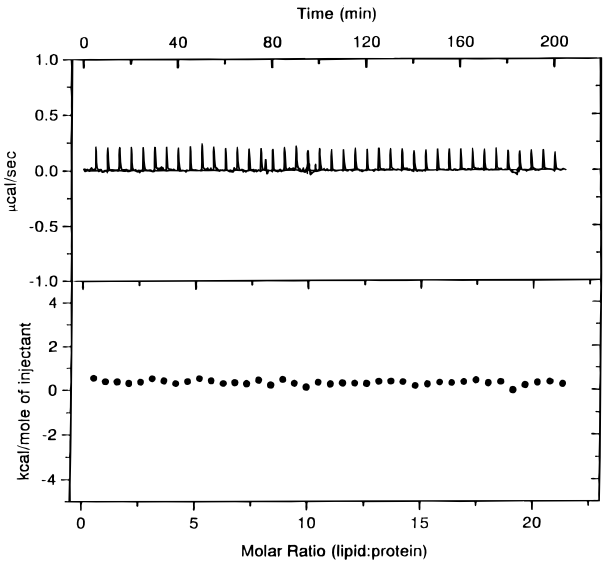

FIGURE 4: Isothermal titration at 24.8 °C of 40 injections of 2.5 μL each of 7 mM DMPC into 25 μM rhG-CSF. Injections are 5 min apart.

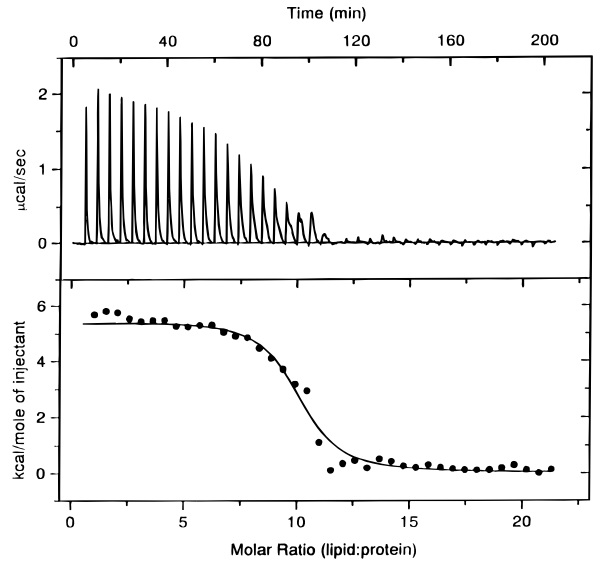

FIGURE 5: Isothermal titration at 24.8 °C of 40 injections of 2.5 μL each of 7 mM DMPG into 25 μM rhG-CSF. Injections are 5 min apart. The solid line in the bottom portion is the fitted binding curve.

Thermal denaturation of many proteins leads to changes in α-helicity which can be detected by circular dichroism. The helical secondary structures of rhG-CSF and structurally analogous growth factors and of their complexes with anionic and zwitterionic phospholipids were investigated by monitoring their ellipticity at 222 nm during temperature cycling. The samples were equilibrated at 10 °C, heated to 90 °C, and cooled back to 10 °C and the cycle was repeated. Figure 6 shows the behavior during thermal cycling of rhG-CSF alone, rhG-CSF in the presence of DMPC, and rhG-CSF in the presence of DMPG (150:1 lipid:protein). rhG-CSF itself denatures irreversibly as observed by an irreversible loss of α-helicity; DMPC does not aid in stabilization of rhG-CSF. Only the rhG-CSF/DMPG complex regains full secondary structure upon cooling. Figure 7 shows the CD during thermal cycling for complexes of DMPG with rhGM-CSF,

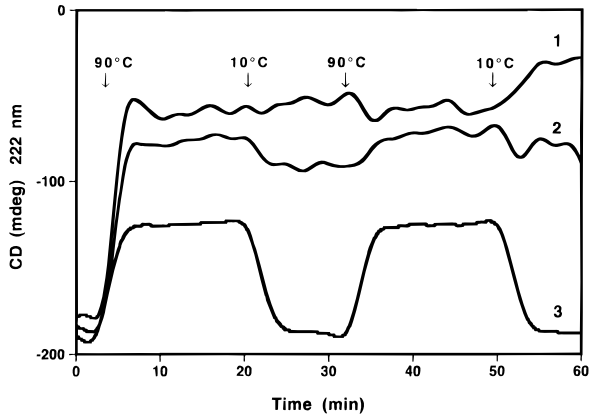

FIGURE 6: Effect of temperature cycling on the CD (222 nm) of rhG-CSF (curve 1), DMPC:rhG-CSF (150:1) (curve 2), and DMPG:rhG-CSF (150:1) (curve 3). The temperature of the thermostated cuvette was initially 10 °C; it was ramped to 90 °C, cooled to 10 °C, and repeated as indicated by the arrows. Concentration of rhG-CSF was 80 μg/mL, pH 6.0.

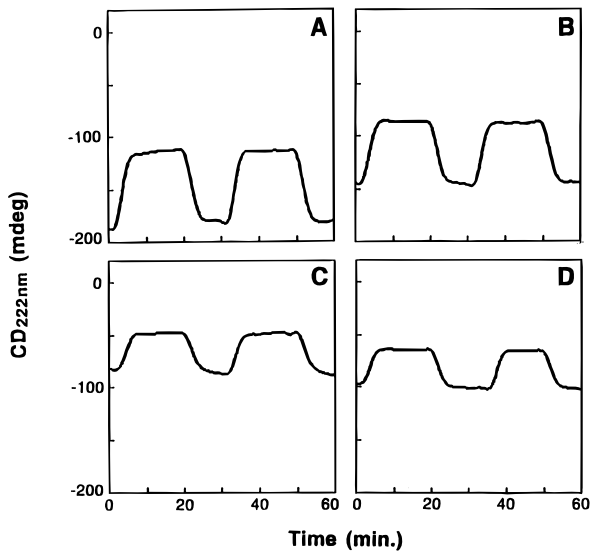

FIGURE 7: Effect of temperature cycling on the CD (222 nm) of rhIL-2 (A), rpSt (B), rhGM-CSF (C), and rhIL-4 (D). The temperature of the thermostated cuvette was ramped to 90 °C, cooled to 10 °C, and repeated as shown in Figure 6. Protein concentrations were 80 μg/mL, pH 6.0.

rhIL-4, rpSt, and rhIL-2. All display full recovery of ellipticity upon cooling, even after twice achieving temperatures of over 85 °C. Again, the presence of DMPC did not assist in recovery of the structure of these growth factors. Only rhGM-CSF showed substantial recovery of ellipticity after thermal cycling in the absence of lipid; however, successive cycles led to progressive loss of secondary structure. This was prevented in the presence of DMPG vesicles (Figure 7).

It was also of interest to examine the effect of lipid alkyl chain length on the stabilization of rhG-CSF. The stabilization of rhG-CSF by the different lipids was assayed using CD as described above. The final rhG-CSF concentration used in this experiment was 80 μg/mL, the final lipid:rhG-CSF molar ratio was 150:1, and the final lipid concentration was 0.65 mM. As shown in Figure 8, both DMPG (C_{14}) and dicaprylPG (C_{10}) fully protect rhG-CSF from thermally induced loss of secondary structure. By contrast, dioctanoylPG (C_8) and dicaproylPG (C_6) offer little or no protection to rhG-CSF. These data suggest that a certain lipid structure

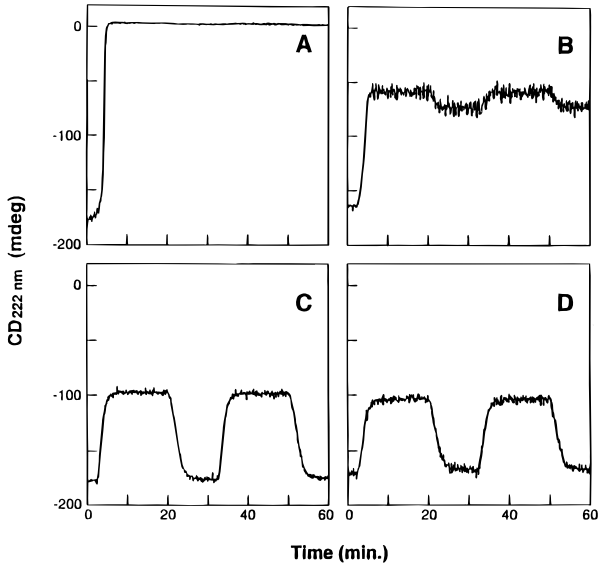

FIGURE 8: Effect of temperature cycling on rhG-CSF secondary structure in the presence of phospholipids of varying CMC: dicaproylPG (A), dioctanoylPG (B), dicaprylPG (C), and DMPG (D). The phospholipid:rhG-CSF ratio is 150:1 in all cases. The temperature of the thermostated cuvette was ramped to 90 °C, cooled to 10 °C, and repeated as described above.

or hydrophobicity may be required in order to fully stabilize rhG-CSF and that mere binding of anionic lipid monomers is not sufficient to provide stabilizing activity.

DISCUSSION

The present work enhances our understanding of the interactions between four-helix bundle cytokines and lipid membranes. From the data above it is clear that sonicated vesicles composed of negatively charged phospholipids interact with and stabilize cytokines. The data with lipids of varying alkyl chain length suggests that the stabilizing effect involves the interaction of proteins with organized lipid assemblies rather than lipid monomers in solution. Together with the requirement that the lipid be negatively charged, the data suggest that the interfacial properties of the lipid vesicles play a role in the interaction and stabilization of the proteins.

The interfacial pH of negatively charged liposomes has been shown to be more acidic than the bulk pH (Winiski et al., 1986; van der Goot et al., 1991). We have measured the interfacial pH of DMPG vesicles using the TNS assay (Winiski et al., 1986) and have found that the pH at the interface is 1.5 units lower than the bulk (data not shown). Therefore, a protein at the interfacial region of a DMPG vesicle would experience a pH of 4.5 when the pH of the bulk solution was 6.0. The cytokines used in the present studies have been shown to undergo conformational changes in response to pH. In particular, both rhIL-2 and rhG-CSF have been shown to form an "acid state" with loose or fluctuating tertiary structure and native or near-native secondary structure (Narhi et al., 1991; Dryden & Weir, 1991; Bychkova et al., 1988; Ohgushi & Wada, 1983; Ptitsyn et al., 1990). Acid states of proteins often show considerable hydrophobicity (Dryden & Weir, 1991; Bychkova et al., 1988; Ohgushi & Wada, 1983; Ptitsyn et al., 1990; Brems, 1988) and may participate in the membrane insertion of proteins. Therefore, formation of a partially folded cytokine, such as rhG-CSF or an analogous growth factor, could

involve exposure of previously sequestered hydrophobic regions which could interact with the phospholipid vesicles.

Our previous data suggest that for rhG-CSF the site of phospholipid interaction may be the crossover loop betwen the A and B helices, the so-called A−B loop (Figure 1). This loop contains a tryptophan residue (position 58) and seems to be involved in the acid-induced conformational changes of rhG-CSF (Narhi et al., 1991). In particular, rhG-CSF exhibits significant fluorescence changes in the pH range of 2.5−4.5, consistent with a loosening of the tertiary structure, but shows little or no change in α-helical content in the same pH range (Narhi et al., 1991). One tryptophan resides in the A−B loop (tryptophan 58) and the other tryptophan in the four-helix bundle (tryptophan 118). Since the fluorescence changes are not accompanied by changes in α-helicity, these changes must involve the region around tryptophan 58 in the A−B loop. The short 3_{10} helix in rhG-CSF may also be involved in the interaction. Helices of the 3_{10} class have been implicated as folding intermediates (Millhauser, 1995), and a slight unfolding of this small helix could have profound effects on the fluorescence of tryptophan 58 without significantly affecting the overall α-helicity of rhG-CSF. The other proteins used in this study also have long crossover loops between the major α-helices (Figure 1), and in many cases, these loops contain short 3_{10} helices.

In addition to the interfacial pH effect described above, the interaction and stabilization of proteins by anionic phospholipid vesicles may also involve electrostatic interactions between the proteins and the surface of the vesicles. In rhG-CSF, there are two histidine residues at either end of the 3_{10} helix within the A−B loop. These residues may be involved in the initial interaction, between rhG-CSF and anionic lipid vesicles. Unlike the case of rhG-CSF, the structures and locations of key residues in the other cytokines are not as well understood.

Our data are consistent with a model in which the first step in the interaction is primarily electrostatic, involving anionic lipid headgroups and cationic residues or regions on the protein. This electrostatic interaction sequesters the protein in the interfacial region of the lipid vesicles, where the protein experiences of pH roughly 1.5 units lower than the bulk pH. This more acidic environment leads to conformational changes and possibly the formation of intermediate folding states of the proteins. These intermediates are likely bound through partial insertion into the bilayer and stabilized by the lipid vesicles.

Intermediate folding states often have near-native secondary structure and loose tertiary structure and have been shown to be capable of forming multimers at moderate protein concentrations (Brems, 1988). These associated intermediates result from specific interaction of monomeric folding intermediates and can precipitate from solution under certain conditions (Brems, 1988). Solubilization of these associated intermediates can enhance the recovery of native protein during refolding (Brems, 1988). Our data suggest that anionic lipid vesicles may bind and solubilize acid state intermediates of the four-helix bundle cytokines. By binding and solubilizing these intermediates, the proteins are less likely to form higher order, insoluble aggregates and are more likely to be capable of refolding into their native states.

There is also the practical importance of stabilizing interactions between therapeutic cytokines and anionic phospholipids. The potential to formulate a protein in the presence of a simple phospholipid, such as DMPG, and to obtain a complex which is stable against the stresses of a pharmaceutical product (e.g., thermal, vibrational) could provide a useful tool for the protein formulator.

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