Liposomes as Formulation Excipients for Protein Pharmaceuticals: A Model Protein Study

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Purpose. The advent of recombinant DNA technology has made possible the pharmaceutical use of a wide range of proteins and peptides. However, the complex structure of proteins renders them susceptible to physical instabilities such as denaturation, aggregation and precipitation. We tested the hypothesis that partial unfolding and exposure of hydrophobic domains leads to physical instability, and investigated approaches to stabilize protein formulations.

Methods. KP6 β, an 81 amino acid killer toxin from *Ustilago maydis*, was used as a model protean. Circular dichroism and fluorescence spectroscopy were used to study the temperature dependent folding/unfolding characteristics of KP6 β. ANS (1,8 anilinonaphthalene sulfonate), a fluorescent probe that partitions into hydrophobic domains, was used to detect exposure of hydrophobic domains.

Results. As the temperature was elevated, near-UV CD indicated progressive loss of KP6 β tertiary structure, while far-UV CD indicated retention of secondary structure. Increasing exposure of hydrophobic domains was observed, as indicated by the penetration of ANS. At elevated temperatures (60°C), KP6 β conserved most secondary structural features. However, tertiary structure was disordered, suggesting the existence of a partially folded, structured intermediate state. Liposomes bound to partially unfolded structures and prevented the formation of aggregates.

Conclusions. Partial unfolding resulted in increased exposure of hydrophobic domains and aggregation of KP6 β, but with preservation of secondary structure. Liposomes interacted with the structured intermediate state, stabilizing the protein against aggregation. These results suggest a general formulation strategy for proteins, in which partially unfolded structures are stabilized by formulation excipients that act as molecular chaperones to avoid physical instability.

KEY WORDS: protein pharmaceuticals; liposomes; formulation excipient; molecular chaperones; structured intermediate states.

INTRODUCTION

Advances in protein engineering have led to the large scale production of proteins and peptides for pharmaceutical purposes. However, for many proteins, the preservation of higher order structure, such as secondary, tertiary and quaternary conformation, is necessary to retain activity. Proteins undergo physical and chemical instability, and these instabilities present unique difficulties in the production, formulation, and storage of protein pharmaceuticals (1,2). Denaturation, aggregation, and precipitation are frequent manifestations of physical instability.

To date, most approaches to the development of protein formulations appear to define the challenge broadly, e.g., as problem in freeze-drying or conventional chemical stabilization, rather than more specifically as one of protein folding and the retention of protein structural motifs. Particular excipients usually are selected to: (1) improve thermal stability (3,4), (2) as cryoprotectants (5,6), or (3) as antibacterial preservatives (7). Several groups have approached the problem with the rationale of choosing excipients to stabilize proteins against denaturation (3,4). However, the existence of transient, non-equilibrium structures or partially folded intermediates in the protein folding/unfolding pathway may nonetheless lead to the aggregation and precipitation of the proteins (1), and these states may occur without complete protein denaturation.

Because the process of denaturation is related to protein unfolding at the molecular level, our approach was to analyze protein unfolding in detail, and investigate the means to intervene in key steps in the process. We have chosen a yeast toxin as a model protein with which to investigate this problem. Certain protein toxins provide a good model system for formulation development, because their activity may require both the maintenance and triggering of specific and subtle conformational elements. KP6 β is one of the yeast killer toxins, secreted by the fungal pathogen *Ustilago maydis*. Killer toxins kill closely related organisms and are also potential candidates for the development of novel antifungal agents.

KP6 β provides several advantages as a model to understand molecular details associated with protein stability and intermediate structures. First, there are parallels between the molecular mechanism of KP6 β membrane insertion and the processes that lead to physical instability of protein pharmaceuticals; to exert the toxin activity, KP6 β spontaneously inserts into target membranes, accompanied by small conformational changes that may comprise partially unfolded states (8). Second, KP6 β has been expressed in homologous and heterologous systems, allowing production of large quantities of protein and designed mutations for physical studies (9,10). Third, detailed structural studies of KP6 β are underway (W. Duax, personal communication) that will aid in understanding the role of misfolding and kinetic or transient intermediates on protein stability. Fourth, the primary sequence of the KP6 β shows the presence of chemically labile groups, and site directed mutagenesis is possible in the KP6 β system, allowing more comprehensive future studies on structure-stability relationships.

In the present work, we have carried out biophysical studies to understand the folding/unfolding properties of KP6 β in detail, with specific experimental approaches designed to investigate the possible existence of intermediate structures and their relationship to aggregation. In addition, we examined the interaction of these structures with phospholipid vesicles (liposomes) to investigate whether exposed hydrophobic protein domains could bind to bilayer membranes, altering the protein refolding pathway. Based on these results, a formulation strategy is discussed for protein pharmaceuticals that form intermediate structures.

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ABBREVIATIONS: CD, circular dichroism; CCA, Convex Constraint Analysis; ePC, egg phosphatidylcholine; ANS, 1,8 anilinonaphthalene sulfonate.

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EXPERIMENTAL PROCEDURES

Materials

The *Ustilago maydis* strains used have been described elsewhere and the purification was performed as described previously (11). ANS (1-anilino-8-naphthalene sulfonate), a probe of hydrophobic domains (12–14), was purchased from Molecular Probes Inc. (Eugene OR). Lipids were obtained from Avanti Polar lipids and used without further purification.

Preparation of Liposomes

The required amount of egg phosphatidylcholine (ePC) was dissolved in chloroform and the solvent was removed using a rotary evaporator, depositing the lipid as a thin film on the walls of a round-bottomed flask. Multilamellar vesicles (MLV) were formed by dispersing the thin film in distilled water, with intermittent vortexing at 25°C. The resulting MLV were sonicated in a bath-type sonicator (Laboratory Systems, Inc.) to obtain Small Unilamellar Vesicles (SUV).

Circular Dichroism Experiments

CD spectra were acquired on a JASCO J500 spectropolarimeter calibrated with d10 camphor sulfonic acid. Temperature-dependent studies were performed using water-jacketed cell holders (Jasco, Inc.). The cell holders were kept at the desired temperature using a circulating water bath (NesLab RTE 110). Samples were scanned in the range of 240 to 190 nm for secondary structural analysis, and the protein concentration used was 30 μM. For near UV CD studies, spectra were acquired in the range of 350 to 250 nm, using a 10 mm quartz cuvette, and the protein concentration used was 40 μM.

The CD spectra of samples containing liposomes may be distorted as a result of light scattering. The contribution due to light scattering was corrected as follows: (1) spectra were acquired with small unilamellar vesicles in the absence of protein to determine the magnitude of the contribution; (2) CD spectra of the protein were corrected by subtracting the spectrum of liposomes alone; (3) the ellipticity values at 350 to 400 nm were monitored and used as a baseline that was subtracted from scans; (4) multiple scans were acquired and averaged to improve signal quality. The spectra thus obtained were invariant with the path length of the cuvette or position of the sample along the light path, indicating that the effect of scattering on the spectra was minimal.

Secondary Structure Analysis

Far UV CD spectra were analyzed by Convex Constraint analysis (CCA) (15), as described previously (13,14). The ellipticity values at each wavelength in the range of 240 to 190 nm were used for the calculation. The input data set consisted of a matrix of 30 proteins, and the spectra were deconvolved in 100 iterations.

Fluorescence Studies

Fluorescence spectra were acquired on an SLM 8000C spectrofluorometer (Urbana, IL). Emission spectra were acquired over the range of 300 to 400 nm, using a slit width of 4 nm on the excitation and emission paths. The excitation monochromator was set either at 265 or 290 nm, and a 295 nm long pass filter was used on the emission path to minimize scattering effects. Samples were maintained at the desired temperature using a water bath (Neslab RTE 110). Spectra were corrected through the use of an internal reference and further processed using software provided by the manufacturer.

Equilibrium Folding Analysis

A two-state unfolding model was used to analyze the equilibrium unfolding data. To compare the transitions detected by several methods, each unfolding curve was normalized to the apparent fraction of the unfolded form (F_{app}), using the relationship:

$$F_{app} = (Y_{obs} - Y_{nat})/(Y_{unf} - Y_{nat})$$

where Y_{obs} is the fluorescence intensity or the molar ellipticity (at 220 nm or 268 nm) at a given temperature, and Y_{unf} and Y_{nat} are the spectral values for unfolded and native structures, respectively. Y_{unf} and Y_{nat} are obtained by performing a linear regression analysis of the spectrum plateau region at high and low temperatures, respectively.

ANS Binding Studies

ANS (1-anilino-8-naphthalene sulfonate) was dissolved at high concentration in water and a small volume was added to a solution of 10 μM KP6 β, to a final probe concentration of 0.3 μM. The excitation wavelength was 380 nm and the emission was monitored at 482 nm. Correction for the inner filter effect was performed by appropriate procedures (16).

RESULTS

Conformation of KP6 β in the Native State

The far UV CD spectrum of KP6 β was acquired in water at 20°C (Fig. 1A) and was deconvolved to provide baseline information on secondary structural content (Fig. 1B) using Convex Constraint Analysis (CCA) (15). Several underlying assumptions were made in calculating the secondary structural content, as described by Manning (17). CCA estimated that KP6 β in water at 20°C consists of 18 ± 6% α helix, 56 ± 8.8% β sheet content (β turns, antiparallel β sheet, parallel β sheet), 14 ± 4.8% disulfide/aromatic and other contributions, and 12 ± 2.4% random coil/γ turn structure. Overall, the result of deconvolution suggested that the protein exists predominantly in a β sheet conformation, with detectable helical content. The deconvolved structural components were re-convolved (using the parameters described in Materials and Methods), and the resulting curve fit well (Fig. 1A) when analyzed as described by Perczell *et al.* (15).

CD spectra were acquired for a range of concentrations of KP6 β. For 10–100 μM protein, neither the molar ellipticity nor the calculated β content varied (data not shown). This observation, plus the sequence homology to KP6 α, suggest that the high β content observed was not the result of protein aggregation.

KP6 β has one tryptophan and six tyrosine residues. Energy transfer between Tyr and Trp (16) was measured for KP6 β in the native conformation (in water at 20°C) to provide qualitative

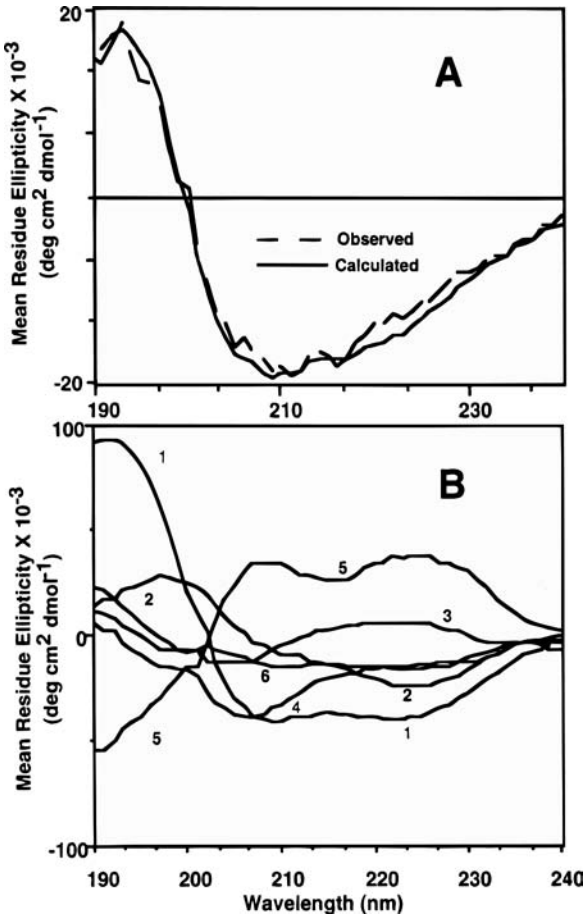

Fig. 1. Structural motif content of KP6 β killer toxin in water. The CD spectrum of subunit of KP6 β was acquired in water at 20°C, and was deconvolved using CCA into six pure circular dichroism component curves as described in Experimental Procedures: The concentration of the protein was 30 μM, and the path length of the quartz cuvette was 1 mm. (A) The CD spectrum of KP6 β in water ("Observed") and a spectrum calculated by re-convolution of the discrete structural contributions determined by CCA ("Calculated"). Spectra represent the average of three scans. (B) Results of deconvolution of the CD spectrum of KP6 β into structural motifs: (1) α helix; (2) anti parallel β sheet; (3) disulfide/aromatic/other non-peptidic contributions; (4) parallel β sheet/turns; (5) β turns; (6) random coil/γ turns.

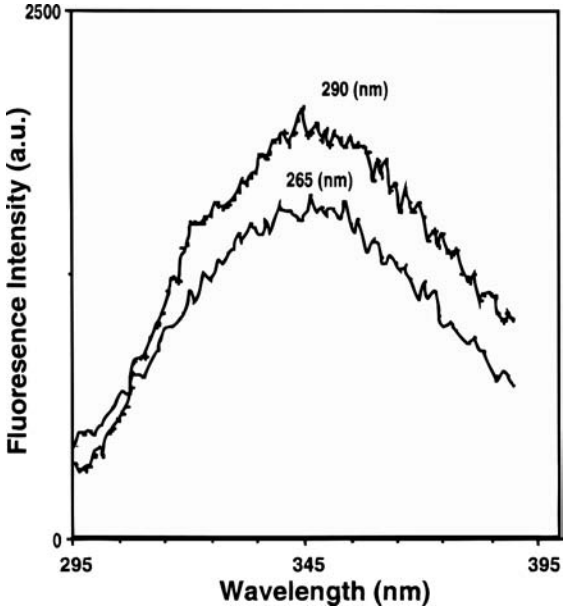

Fig. 2. Tyr-Trp resonance energy transfer in KP6 β. Fluorescence emission spectra of KP6 β in water were acquired at 20°C using excitation wavelengths of 265 nm and 290 nm. The emission and excitation slits were 4 nm and a 1 cm pathlength quartz cuvette was used for data acquisition. The concentration of the protein was 20 μM. A.U.: arbitrary units of fluorescence.

baseline information with which to detect changes in the global fold of the protein (Fig. 2). In spite of the greater number of Tyr residues, the emission spectrum of KP6 β was dominated by Trp fluorescence (peak maximum approx. 344 nm), with little observable Tyr emission at 305 nm. This observation is consistent with resonance energy transfer between Trp and Tyr, with a resultant quenching of Tyr fluorescence and concomitant enhancement of Trp fluorescence (16). Furthermore, peak maxima observed at approx. 344 nm suggests the partial shielding of Trp from water. Thus in the native, folded conformation of KP6 β, Trp-Tyr residues are spatially close. This global fold defining the tertiary structure is consistent with a calculated structure based on molecular modeling and dynamics studies (W. Duax, personal communication).

Thermal Denaturation Studies

Temperature-induced changes in KP6 β secondary structure were studied by acquiring far-UV CD spectra (240 nm to 190 nm), and the spectra were deconvolved using CCA to identify the types of structural domains likely to be involved in the underlying conformational changes. As described above (Fig 1), KP6 β at 20°C exists predominantly in β sheet conformation, with detectable helical content. Over the temperature range of 20–60°C, there were no changes in the far UV spectrum (Fig. 3A), indicating that the secondary structure of the protein was not altered. Above 60°C, the ellipticity at 220 nm decreased progressively with increasing temperature (data not shown).

Far UV spectral data was used to calculate F_{app}, the apparent fraction in the unfolded form, according to the method described in Experimental Procedures. Little change in F_{app} was observed at temperatures ≤ 60°C (Fig. 4), as would be expected from the data in Fig. 3A, indicating little change in secondary structure. Above 60°C, F_{app} increased over a broad temperature range, and a transition was observed (Fig. 4). These observations suggest a progressive loss of secondary structure at elevated temperature, with a midpoint of transition at approximately 76°C.

Fluorescence emission spectra of KP6 β were acquired over a range of temperatures to detect changes in Trp fluorescence that would indicate thermal denaturation of the tertiary structure. Over the range of 20–60°C, there was a large decrease in the intensity of the emission peak centered at 350 nm (Fig. 3B), as well as a slight red shift in the emission peak. These changes are consistent with exposure of Trp to a more hydrophilic environment as temperature increased, as would occur during protein unfolding.

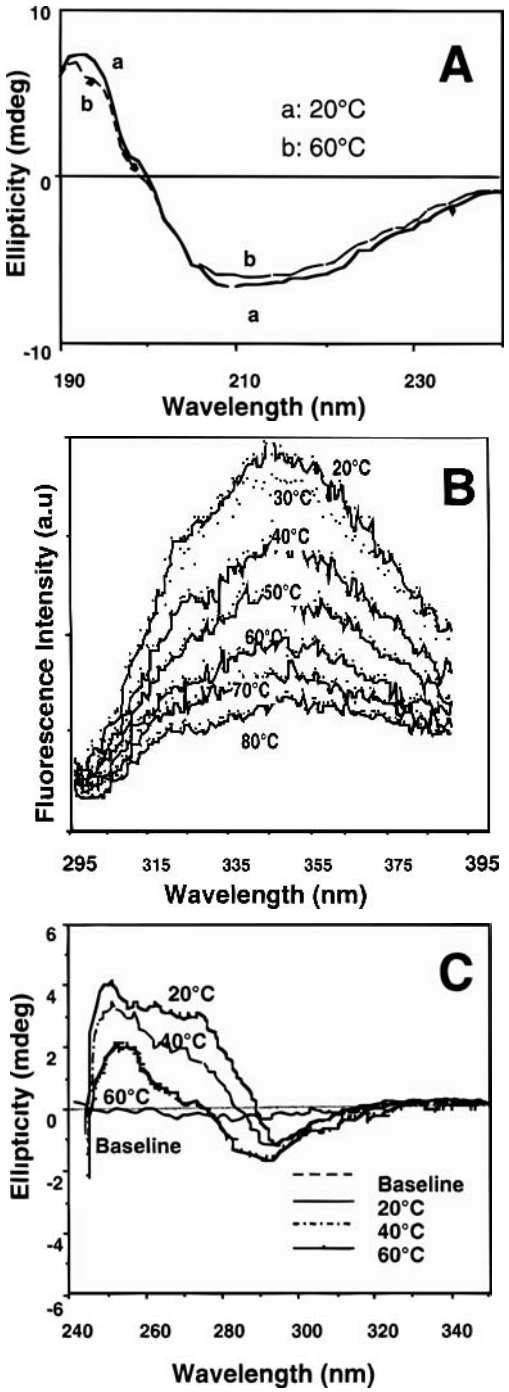

Fig. 3. Temperature dependence of secondary and tertiary structure of KP6 β. (A) The far-UV CD spectrum of KP6 β in water was acquired at (a) 20 °C and (b) 60 °C, over a range of 260 nm to 190 nm. The path length of the cuvette used was 1 mm, and the concentration of protein was 30 μM. (B) Trp fluorescence emission spectra of KP6 β in water were acquired over the temperature range of 20–60°C, in 10°C increments. The excitation wavelength was 290 nm, and the emission and excitation slits were 4 nm. The path length of the cuvette used was 1 mm, and the concentration of protein was 30 μM. A.U.: arbitrary units of fluorescence. (C) The near-UV CD spectrum of KP6 β in water was acquired over the temperature range of 20–60°C. The path length of the cuvette used was 10 mm and the concentration of the protein was 40 μM. The baseline indicates the signal derived from solvent alone.

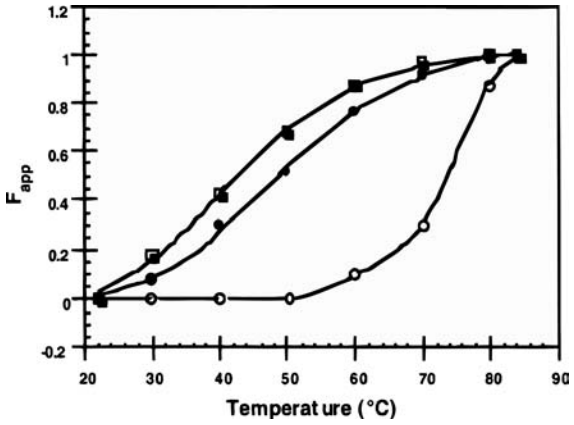

Fig. 4. Temperature-depended changes in secondary and tertiary structure of KP6 β. The temperature dependent changes in secondary and tertiary structure of KP6 β in water are compared by plotting ellipticity at 220.5 nm (open circles) and 268 nm (filled circles), and Trp fluorescence intensity at 348 nm (filled squares) as a function of temperature. Each data point is average of three experiments. F_{app}, the fraction of protein in the unfolded state, is calculated as described in Experimental Procedures. A.U.: arbitrary units of fluorescence.

The Trp fluorescence data was used to calculate the fraction of protein in the unfolded form. In this case, F_{app} indicates loss of tertiary structure. Between 30°C and 80°C, a progressive increase in F_{app} was observed, with the midpoint of this transition at approximately 45°C (Fig. 4).

An alternative hypothesis to explain the changes in Trp fluorescence observed with increasing temperature is deactivation of the fluorescence excited state as a function of temperature. In order to discriminate changes in tertiary structure from fluorescence artifacts, we investigated the temperature dependence of the near-UV CD spectrum; whereas the far-UV CD spectrum is indicative of secondary structure, the near-UV spectrum is dominated by effects of the specific orientation of the aromatic groups. Figure 3C shows the near-UV CD spectrum of KP6 β at various temperatures. At 20°C, a broad positive band was observed for the native protein. As the temperature increased to 60°C, the intensity of this band decreased, suggesting the loss of tertiary structure.

The near-UV CD spectrum was used to calculate the temperature dependence of the unfolding of tertiary structure. At lower temperatures (20–60°C), ellipticity at 268 nm decreased sharply (Fig. 3C) and therefore F_{app} increased drastically (Fig. 4). The midpoint of transition for the near UV CD spectrum was slightly greater (6°C) than that observed for Trp fluorescence, and occurred in the range of 45 to 50°C. In contrast, the transition detected by far UV CD was considerably higher, approx. 76°C. Such a difference in the temperature at which tertiary and secondary structural changes occurred suggest the existence of intermediate unfolded states (18).

Effects of Thermal Denaturation on the Exposure of Hydrophobic Domains

To determine whether KP6 β unfolding forms intermediates with exposed hydrophobic domains, we measured protein complexation with 1,8 anilinonaphthalene sulfonate (ANS), a

probe of hydrophobic domains (12–14). The fluorescence intensity of protein-bound ANS increased with increasing temperature, and displayed a biphasic character (Fig. 5). Over the temperature range of 20–60°C, there was a progressive increase in the exposure of hydrophobic domains. More drastic unfolding of the protein was observed at a higher temperature, with an apparent inflection occurring at approx. 70°C, the temperature at which secondary structural denaturation was observed in the far-UV CD spectrum (Fig. 4). The sharp increase in fluorescence of the protein-ANS complex paralleled a drastic increase in protein aggregation (data not shown) which occurred as a result of protein conformational changes that increased the exposure of hydrophobic domains.

Stabilization of Partially-Folded States

Because structural studies of several toxins implicate the formation of intermediate structures as a step in membrane insertion (19,20), we investigated whether liposomes would interact with the exposed hydrophobic domains of folding intermediates, thus avoiding protein-protein association and subsequent precipitation.

A solution of KP6 β was heated to 70°C to form partially unfolded intermediates, and liposomes were added prior to cooling to 25°C. As controls, two additional samples were prepared; in one, the protein was heated and cooled in the absence of liposomes. In the second, the liposomes were added to the protein at 25°C without heating, to determine the interaction of liposomes with the native structure. The near-UV CD spectrum and Trp fluorescence were observed as a means to probe the conformational state of the protein.

For the native protein at 25°C, a well-defined Trp emission peak was observed, with a maximum at approximately 345 nm (Fig. 6A, curve a). The fluorescence spectrum was not changed by the addition of liposomes (data not shown), suggesting little spontaneous interaction of native protein with the ePC membranes.

For the sample of KP6 β heated and cooled in the absence of liposomes, a quenched Trp fluorescence spectrum was observed at 25°C, resembling the spectrum of the protein at high temperature (data not shown; cf. Fig. 3B). Quenching of Trp fluorescence most likely resulted from the observed self aggregation of the protein; when the protein in its partially folded conformation is cooled to refold, a fraction of the protein is lost as aggregates due to the exposure of hydrophobic domains to the aqueous environment.

For KP6 β heated and cooled in the presence of liposomes, an increase in fluorescence intensity was observed (Fig. 6A, curve b) relative to that of the protein in its native (unheated)

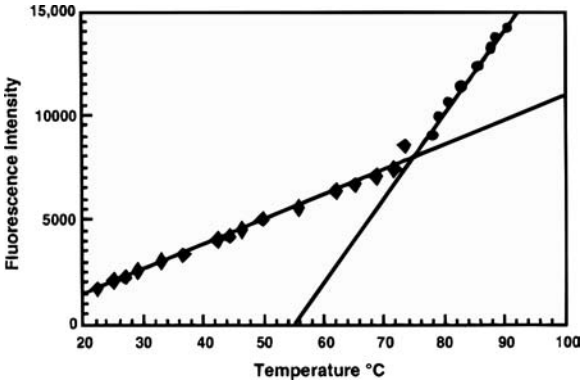

Fig. 5. Exposure of hydrophobic domains of KP6 β probed by ANS complex formation. ANS was dissolved at high concentration in water and a small volume was added to a solution of 10 μM KP6 β, to a final probe concentration of 0.3 μM. The samples were excited at 380 nm and the emission was monitored at 482 nm. Each data point is average of three experiments. A.U.: arbitrary units of fluorescence.

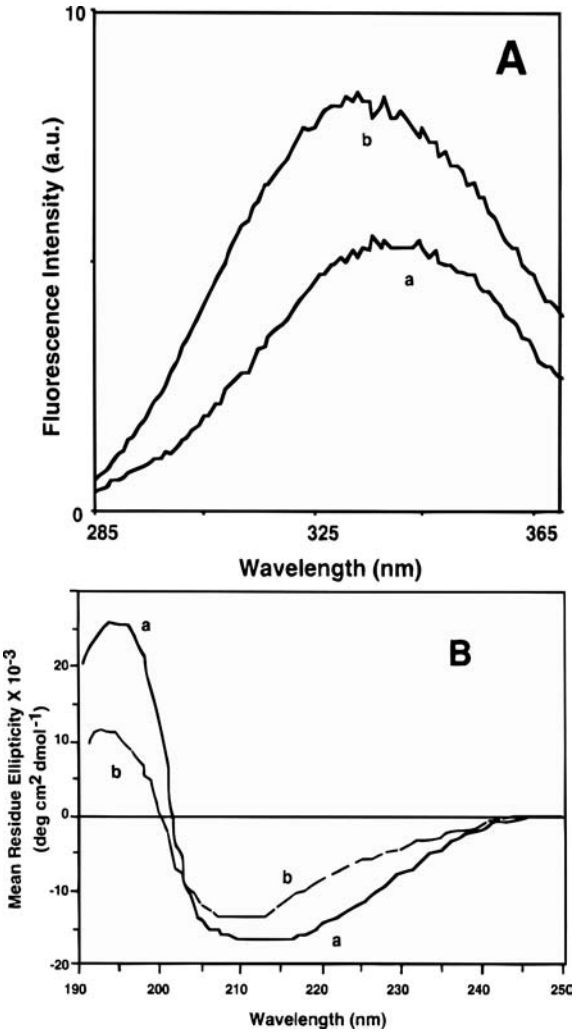

Fig. 6. Effect of liposomes on the secondary and tertiary structure of KP6 β. Trytophan fluorescence and far-UV CD spectra of KP6 β were acquired in the presence and absence of small unilamellar liposomes (SUV) composed of egg phosphatidylcholine. The protein concentration was 30 μM and the protein/lipid ratio was 1:100. In each panel, the curve marked "a" represents native (unheated) protein in the absence of liposomes, and the curve marked "b" represents protein that was heated to 70°C in the absence of liposomes and then cooled to 25°C in the presence of egg phosphatidylcholine SUV. (Panel A) Tryptophan fluorescence spectra acquired at 25°C. The samples were excited at 290 nm and the emission was scanned in the range of 300–400 nm, using a 1 cm pathlength quartz cuvette and emission/excitation slits of 4 nm. (Panel B) CD spectra acquired over the range 260–190 nm using a 1 mm path length quartz cuvette. Sample preparation is described in the text.

state (Fig. 6A, curve a). In addition, the Trp emission spectrum was blue-shifted relative to that of the native protein. Based on the general solvent effect on fluorophores (16), the observed spectral changes are consistent with the preservation of a hydrophobic location for the Trp fluorophore, most likely as a result of interaction with the liposomal membrane. In the presence of liposomes, the intermediate structures may interact with liposomes via exposed hydrophobic domains, thereby avoiding aggregate formation. Acrylamide and iodide ions did not quench the fluorescence of KP6 β that was heated and cooled in the presence of liposomes (data not shown), suggesting a shielding of the fluorophore from the external environment by interaction with the liposome membrane.

KP6 β conformation was also examined by far-UV CD following association with liposomes (Fig. 6B, curve B); the CD spectrum of protein in its native conformation is given for comparison (Fig. 6B, curve A). In the native conformation, the protein exists predominantly in beta sheet conformation, but with detectable helical content (cf. Fig. 1). The CD spectrum of liposome-associated KP6 β displayed a less intense negative band at 215 nm and a less intense positive band at 195 nm. Analysis of the structural content of this spectrum by Convex Constraint Analysis suggested that the protein exists almost entirely in beta sheet conformation. The decrease in spectral intensity around 220 nm as the protein binds to liposomes may result from the loss of helical domains; CCA analysis calculated that the helical content decreased from 18% (±6%) to 6% (±2%) after heating and cooling in the presence of liposomes.

DISCUSSION

The formulation of protein and peptide pharmaceuticals is largely an unsolved problem. Both chemical and physical instabilities can interfere with activity. Chemical instabilities such as deamidation, hydrolysis, beta elimination, and incorrect disulfide bridges are well known to occur, as are physical instabilities such as aggregation and precipitation.

Although a variety of approaches exist to reduce chemical instability, the elimination of water by lyophilization has been a frequent approach for many formulations. However, the freeze-drying process may also create problems; physical instability can arise through the formation of ice crystals, and the freeze-concentration and crystallization of buffer salts may cause radical changes in pH. In addition, the removal of water also eliminates solvent contributions that encourage the retention of the native state, thereby permitting conformational relaxation. Upon re-addition of aqueous media during reconstitution, the protein pharmaceutical must regain its native conformation, refolding through a possible series of intermediates. During the refolding process, misfolding, disulfide exchange, and aggregation are potential problems that can result in physical instability and protein inactivation. The process of refolding, the role of transient and equilibrium intermediates, and the methods to encourage proper refolding are poorly understood.

Thermal denaturation studies of KP6 β were carried out to dissect the process of protein unfolding and physical destabilization into discrete steps. In the temperature range of 20 to 60 °C, small changes were observed in the secondary structure, coupled with greater changes in tertiary structure, suggesting a transition of the native structure to a structured intermediate state (18). Such changes may be sufficient to cause inactivation of proteins or their aggregation.

The conformation of the native state elucidated by CD and fluorescence spectroscopy is consistent with a 3D structure for KP6 β obtained by molecular modeling, in which homologous regions of KP6 β were aligned with corresponding regions of KP6 α, for which a high-resolution crystal structure has been obtained (21). In this model, the protein structure has four loops that connect α helices and β strands, most of the amino acid residues are incorporated into secondary structural elements, and all Cys residues are involved in disulfide bridges that link these secondary structural elements into a compact domain (W. Duax, personal communication). Therefore, the native structure may be stabilized by several influences, including secondary structural elements, hydrogen bonds, disulfide bridges, and Van der Waals forces. Upon warming to the temperature range of 20–60°C, changes would be expected in the conformation of more flexible structural elements such as side chains, but not necessarily in the secondary structure.

In the temperature range of 20–60°C, fluorescence studies also showed that the structured intermediate unfolded states had progressively exposed hydrophobic domains, and these intermediate structures underwent aggregation. Our formulation strategy was aimed at the stabilization of the intermediate structures and encouraging the proper refolding of the protein. The fluorescence studies performed with KP6 β heated and cooled in the presence of liposomes indicated that liposomes stabilize the intermediate structures against aggregation, presumably through protein intercalation into the bilayer membrane. The protein in the liposome-bound form regained its global fold and activity, but small conformational differences from the native structure were observed.

Based on these observations, we suggest a formulation strategy for protein pharmaceuticals that form partially folded structures; this formulation strategy exploits the properties of the intermediate structures. The first step is to form "structured" intermediate states; in this case, the intermediate states were formed by thermally unfolding the protein, although other methods of unfolding are possible. Conditions would be controlled carefully, enabling the exposure of domains that permit interaction with the excipient. The second stage is to add the stabilizing excipient (in this case, pre-formed unilamellar liposomes), to bind to the intermediates.

In this process, the liposomes may play several roles. First, they may associate with the intermediate states without altering the refolding appreciably, exerting a beneficial effect through stabilization of the intermediate states or inhibiting progression to conformations that lead to other physical instabilities, such as aggregation. Alternatively, the liposomes may act as chaperones, assisting the protein to refold to a state that resembles more closely the native structure. Finally, liposomes may guide the protein refolding to unique intermediate structures that are stabilized and active, yet different from the folding intermediates that would exist in the absence of the liposomes. The results here indicate clearly that liposomes stabilize the protein against aggregation and may assist the refolding of the protein. The interaction of liposomes with folding intermediates, rather than the native protein, suggest the potential to achieve specificity. Further work is in progress to investigate the structure of the transient intermediates in the refolding pathway and the release of active protein under biological conditions.

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