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Effect of lipids on the thermal stability and conformational changes of proteins: ribonuclease A and cytochrome c

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Abstract

Lipids have been increasingly used as carriers for delivery of proteins and peptides. In this study, thermal stability and conformational properties of two basic proteins, ribonuclease A (RNase A) and cytochrome c (cyt. c), incorporated in lipid membranes made with dipalmitoylphosphatidylglycerol (DPPG), a negatively charged lipid, was studied by differential scanning calorimetry (DSC) and Fourier transform infrared (FT-IR) spectroscopy. DSC studies showed that when incorporated in DPPG at concentration <4 mol%, these two proteins were stabilized by DPPG binding, and >4 mol%, a destabilizing effect was observed. Such a decrease in thermal stability of cyt. c or RNase A suggested a strong intermolecular protein–protein interaction, because of the relatively low lipid to protein ratio. When cyt. c bound to membranes made of a mixture of DPPG and DPPC (dipalmitoylphosphatidylcholine), the extent of structural perturbation depended on the surface density of the negatively charged lipid head groups; perturbation became smaller with increasing acidic phospholipid in the membrane. FT-IR studies showed a shift of the major amide I component band from 1653 to 1649 cm^{-1} for cyt. c and from 1639 to 1633 cm^{-1} for RNase A after incorporation into DPPG membranes. However, from the quantitative determination of Fourier self-deconvoluted spectra, only slight perturbation of the secondary structure was observed after DPPG binding. These reductions, along with the shifts to lower frequencies of the main component bands, suggested that some rearrangement within the α helices/β sheets and/or the loosening of the protein tertiary structure existed, resulting in a stronger hydrogen bonding accessibility after their binding to DPPG membranes. © 1998 Elsevier Science B.V.

Keywords: Ribonuclease A; Cytochrome c; Dipalmitoylphosphatidylglycerol; Differential scanning calorimetry; Fourier-transform infrared spectroscopy

Abbreviations: CUS, cooperative units; Cyt. c, cytochrome c; DPPC, dipalmitoylphosphatidylcholine; DPPG, dipalmitoylphosphatidylglycerol; DSC, differential scanning calorimetry; FSD, Fourier self-deconvolution; FT-IR, Fourier transform infrared; RNase A, ribonuclease A; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; T_d, denaturation temperature; TLC, thin layer chromatography; ΔH_{cal}, calorimetric enthalpy change; ΔH_{vH}, van't Hoff enthalpy change.

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1. Introduction

Among formulation issues for protein drugs, prevention of protein inactivation during the manufacturing process and storage is often one of the most difficult problems to be solved. In cases where lipid carriers are involved, studies on the conformational properties and the thermal stability of the protein molecules within the protein/lipid complex are essential for solving such a problem, yet to date little attention has been paid to studies of this nature.

DSC is a useful tool to study the thermal denaturation, tertiary structure (domain structure and interaction) and thermodynamic properties of proteins (Privalov, 1979, 1982, Lepock et al., 1992). The thermal stability of proteins after interacting with liposomes can be judged by following their thermal-induced folding–unfolding process. However, DSC, while useful for macroscopic thermodynamic characterization of materials, yields no detailed information about the molecular structure and dynamics of proteins.

Advanced by its sensitivity, specificity and accuracy, FT-IR has been widely used to study the secondary structure of polypeptides and proteins in solution. Combined with computerized Fourier transform instrumentation and powerful mathematical band-narrowing techniques that enhance spectral resolution—namely Fourier self-deconvolution (FSD) and second derivative analysis, FT-IR spectroscopy permits the quantitative determination of different secondary structure components in proteins (Surewicz and Mantsch, 1988; Arrondo et al., 1993). From the band changes in conformation-sensitive amide I region, the liposome-induced changes of protein secondary structure could be detected. In addition, the frequency of the amide I bands reveals the strength of the hydrogen-bonding, and is thus, useful to judge the stability of the proteins after liposome binding.

The combination of DSC with FT-IR would provide some of the basic thermodynamic data and conformational properties necessary for the development and refinement of the theoretical models of protein–lipid interactions in a general term. This combination would enable us to study the effects of varying the lipid–protein ratio, to examine the system when the lipids are either in the gel or the liquid–crystalline state and finally to monitor any changes in protein conformation after membrane binding. In this study, the effects of DPPG on the thermal stability and conformational properties for two hydrophilic proteins, RNase A and cyt. c, were investigated using DSC and FT-IR.

RNase A is a chain-cutting enzyme. Bovine RNase A contains a single polypeptide chain of 124 residues and a molecular weight of 13 700. This protein has a hydrophobic core on one side of the active site crevice and the hydrophilic residues are exposed outside (Blackburn and Moore, 1982). X-ray diffraction studies have shown that the secondary structure of RNase A includes seven short α-helical segments, one major β-sheet composed of three roughly antiparallel β-strands and several turns (Kartha et al., 1967).

Cyt. c is an electron transporter of the respiratory chain in mitochondria. It is consisted of a single polypeptide chain of 104 residues. The X-ray diffraction studies have shown that this protein contains only two α-helical segments and no β-structure (Dikerson et al., 1971). The hydrophobic amino acid side chains are buried inside the molecule, while the polar, positively charged chains are on its surface (Vanderkooi and Erecinska, 1976).

The basic physical parameters for these two model proteins are summarized in Table 1.

2. Materials and methods

2.1. Materials

DPPG ($>99.5\%$ purity) was purchased from Avanti Polar Lipids (Alabaster, AL). The purity of this lipid was tested by TLC with satisfaction and was used without further purification. Cyt. c (bovine heart) was purchased from Sigma (St. Louis, MO), and RNase A (bovine pancreas) was purchased from Calbiochem-Behring (La Jolla, CA). Purity of these two proteins have been monitored by SDS-PAGE with satisfaction and was used without further purification.

Table 1
Some physical parameters of the model proteins[a]

Protein	Molecular weight[b]	pI	Dimensions (nm)	No. of S–S bonds	% Non-polar residues[b]	No. of α-helices	No. of β-strands
RNase A	13 700	9.0	$3.8\times2.8\times2.2$	4	51	3	7
Cyt. c	12 400	10.6	$3.8\times4.0\times2.8$	0	45	2	0

[a] Source: protein data bank and Margoliash and Schejter (1966), Dikerson et al. (1971), Richards and Wyckoff (1971), Vanderkooi and Erecinska (1976) and Blackburn and Moore (1982).
[b] Calculated from the amino acid composition.

2.2. Methods for DSC

2.2.1. Preparation of protein–lipid complexes for DSC: By the classical film method

RNase A or cyt. c was dissolved in 600 mM sodium chloride solution to a final protein concentration of 2 mg/ml. The solution was then warmed to 45°C and mixed with dry lyophilized lipids. All samples were annealed for 4 h at 45°C, vortexed for 4 min, degassed for 30 min and then subjected to DSC thermal analysis by heating from 10 to 105°C at a rate of 10°C/h (or 0.167°C/min). The size distribution of lipid vesicles was determined by dynamic light scattering using a Nicomp 370 submicron particle sizer. The lipid concentration was determined by phosphorus assay (Chen et al., 1956).

2.2.2. DSC measurement

Measurements of protein denaturation were made on a Microcal MC2 Ultrasensitive Scanning Calorimeter, which has a sensitivity of 5 μcal/deg. Data were analyzed by Origin™ 2.9 Program. All samples were prepared in triplicate and were scanned separately. Results shown were the averages of three measurements.

2.2.3. DSC data analysis

The function, Baseline: Progress Baseline, provided by the Origin™ 2.9, was used to correct the increase in heat capacity (ΔC_p) upon denaturation, by employing a correction factor proportional to the extent of the transition completed.

2.2.4. Protein denaturation parameters recorded in DSC

(a) *Denaturation temperature*, T_d. Temperature at which denaturation is 50% completed.

(b) *Calorimetric Enthalpy*, ΔH_{cal}. The actual heat absorbed during the protein denaturation, usually normalized per mol or per unit weight.

$$\Delta H_{cal} = \int_{T_1}^{T_2} C_{p,ex}\, dT \quad (3)$$

where $C_{p,ex}$ is the excess molar heat capacity.

(c) *van't Hoff enthalpy*, ΔH_{vH}. The heat change per cooperative unfolding unit

$$\Delta H_{vH} = 4RT_d^2 C_{p,ex,m}/\Delta H_{cal} \quad (4)$$

(Sturtevant, 1974; Privalov and Khechinashvili, 1974) where $C_{p,ex,m}$ is the excess molar heat capacity at T_d.

(d) *Cooperative Units* (CUS). The ratio of the van't Hoff enthalpy to the calorimetric enthalpy, $\Delta H_{vH}/\Delta H_{cal}$, is called the cooperative units. This ratio is a measure of the validity of the assumption that studied protein denaturation process is a two-state transition.

2.3. Methods for FT-IR

2.3.1. Preparation of protein–lipid complexes for FT-IR

Protein–lipid complexes for FT-IR were prepared according to the methods modified from Fabian et al. (1993). Protein solution in D_2O (10 mg/ml if 50 μm spacer was used) was added to the dry lipid films of the required composition, so that a lipid-to-protein molar ratio of $\approx$26:1 was achieved. The mixture was vortexed for 2 min. Hydrogen–deuterium exchange was achieved by incubating the sample at 55 and 65°C for 3 h for RNase A and cyt. c, respectively. This led to

Table 2
Thermodynamic parameters of RNase A denaturation before or after incorporation into DPPG liposomes

System	RNase A (mol%)	T_d (°C)	ΔH_{cal} (kJ/mol)	ΔH_{vH} (kJ/mol)	$\Delta H_{vH}/\Delta H_{cal}$ (CUS)
NaCl solution (600 nM)	100	62.02 ± 0.02	439.3	418.4	0.95
DPPG	1.5	64.67 ± 0.01	502.1	346.0	0.69
DPPG	4	62.91 ± 0.03	485.3	405.4	0.84
DPPG	9	61.97 ± 0.02	405.4	456.1	1.12
DPPG	14	61.79 ± 0.01	347.3	472.8	1.36

complete H/D exchange as judged by the absence of the amide II band. The protein solution was placed between demountable liquid flow cell with CaF_2 windows.

2.3.2. FT-IR measurement

A Nicolet Magna-IR™ 550 spectrometer equipped with a liquid- nitrogen-cooled mercury-cadmium-telluride (MCT) detector was used to record the FT-IR spectra. The spectrometer was continuously purged with dry air for 30 min to eliminate interference from water vapor and 128 scans were recorded at 2 cm^{-1} resolution. Spectral contributions from residual water were eliminated using a set of water vapor spectra measured under identical conditions (Fabian et al., 1993).

2.3.3. FT-IR data analysis

The software used for data collection and analysis included OMNIC software version 1.2 from Nicolet (Madison, WI) and Grams/386™ from Galactic (Salem, NH). The position of component peaks in the amide I band was determined using second derivative and Fourier self-deconvolution (FSD). For quantitative analysis, the spectra were Fourier deconvoluted using half-width at half-height (HWHH) = 15 and a resolution enhancement factor of $k = 2.4$. These values were adopted to avoid sidelobes and preserve the constancy of band areas. Deconvoluted spectra were fitting using Grams/386™ program. The procedures suggested by Byler and Susi (1986) were then applied here. Two assumptions were made: (1) The deconvoluted spectra can fit well with Gaussian lineshapes; and (2) the absorptivities of the different band components are alike. The general procedures were as follows:

1. HWHH were assumed to be ~ 4 cm^{-1} and were fixed. The FSD was initially fit with a linear baseline and all other parameters free. When the reconstructed spectrum was indistinguishable from the actual FSD spectrum (determined by a minimum root mean square (RMS) error for intensities), iterations were stopped.
2. All frequencies and intensities were fixed at the obtained values and all widths were iterated.
3. All frequencies were iterated and other variables kept constant.
4. All cycles were repeated until a satisfactory fit was obtained.

We found that there was < 1 cm^{-1} change in the peak position during this final stage. The fit was not improved by increasing the number of iterations.

3. Results and discussion

3.1. Thermal stability studies of RNase A and cyt. c by DSC

3.1.1. Positively charged, hydrophilic protein RNnse A in DPPG

Table 2 showed the thermodynamic parameters of RNase A as a function of RNase A mol% in DPPG obtained by DSC. The excess heat capacity versus temperature curve for RNase A in a NaCl solution (600 mM) showed a major endothermic transition at 62.02°C with ΔH_{cal} equal to 439.3 kJ/mol. The ratio of the integrated calorimetric enthalpy to the van't Hoff enthalpy (CUS) was close to 1 (Battistel et al., 1991; Straume and Freire, 1992), indicating that this thermotropic

Table 3
Thermodynamic parameters of Cyt. c denaturation before or after incorporation into DPPG or DPPG/DPPC liposomes

System	Cyt. c	T_d (°C)	ΔH_{cal} (kJ/mol)	ΔH_{vH} (kJ/mol)	$\Delta H_{vH}/\Delta H_{cal}$ (CUS)
NaCl solution (600 mM)	100	76.63 ± 0.02	497.9	447.7	0.90
DPPG	1	77.50 ± 0.02	523.0	481.2	0.92
DPPG	3	77.23 ± 0.01	514.6	477	0.93
DPPG	4	77.18 ± 0.01	502.1	481.2	0.96
DPPG	6	76.50 ± 0.02	407.5	506.3	1.24
DPPG	8	76.20 ± 0.03	292.0	502.1	1.72
DPPG	11	75.83 ± 0.02	282.4	552.3	1.96
DPPG:DPPC (1:1)	4	76.50 ± 0.02	346.9	514.6	1.48
DPPG:DPPC (1:3)	4	76.00 ± 0.01	309.2	531.4	1.72

event could be approximated by a simple two-state model (Privalov and Khechinashvili, 1974; Privalov, 1979; Sturtevant, 1987). As shown in Table 2, when the mol% of RNase A in DPPG was equal to 1.5, T_d and ΔH_{cal} of RNase A increased to 64.67°C and 502.1 kJ/mol, respectively. The increase of RNase A mol% to 4, T_d and ΔH_{cal} decreased to 62.91°C and 485.3 kJ/mol, respectively, but both parameters were still larger than those of free protein, indicating that this protein is stabilized by DPPG binding. Above 4 mol%, T_d and ΔH_{cal} of RNase A were lower than those of the corresponding free protein, suggesting a destabilizing effect. CUS increased from 0.69 to 1.36 with increasing RNase A concentrations.

3.1.2. Positively charged, hydrophilic protein cyt. c in DPPG

The thermodynamic parameters for the DSC traces of cyt. c with increasing concentrations of cyt. c in DPPG are listed in Table 3. From Table 3, in the absence of DPPG, the folding–unfolding transition of cyt. c showed a major endothermic transition at 76.63°C with ΔH_{cal} equal to 497.9 kJ/mol. This transition confirmed closely to a two state transition (CUS = 0.9). This was consistent with that reported by Muga et al. (1991). When the mole percent of cyt. c in DPPG was equal to 1, T_d and ΔH_{cal} of cyt. c increased to 77.5°C and 523 kJ/mol, respectively. Increasing cyt. c mol% to 3, T_d and ΔH_{cal} of cyt. c decreased to 77.23°C and 514.6 kJ/mol. Further increase of mol% to 4, T_d and ΔH_{cal} decreased slightly to 77.18°C and 502.1 kJ/mol, respectively, but both parameters were still larger than those of free protein, indicating that this protein was stabilized by DPPG binding. Above 4 mol%, the T_d and ΔH_{cal} of cyt. c decreased to a lower level than those of the corresponding free protein, suggesting a destabilizing effect. CUS showed an increasing trend from 0.9 to 1.96 (approaching 2) as cyt. c concentration increased.

3.1.3. Positively charged, hydrophilic protein cyt. c in DPPG/DPPC mixture

To investigate the effects of charge density of lipid head groups on the thermal stability of cyt. c, this protein was incorporated into liposomes made with pure DPPG, or a mixture of DPPG and DPPC, either in 1-to-1 ratio or 1-to-3 ratio (cyt. c mol% in lipids is 4). From Table 3, free cyt. c showed T_d at 76.63°C with ΔH_{cal} equal to 497.9 kJ/mol. Upon binding of cyt. c to liposomes containing DPPG as the sole lipid component (cyt. c mol% is 4), both T_d and ΔH_{cal} of cyt. c increased slightly compared with those parameters of free protein. After cyt. c was incorporated into liposome containing DPPG and DPPC in 1-to-1 ratio, T_d decreased to 76.5°C and ΔH_{cal} reduced to 346.9 kJ/mol. As the proportion of negatively charged lipid reduced from DPPG to DPPC in 1-to-3 ratio, T_d further decreased to 76°C and ΔH_{cal} down to 309.2 kJ/mol. This implied that the stability of cyt. c in lipid bilayers depended on the surface density of negatively charged lipid head group; it decreased as the proportion of acidic DPPG in the membrane decreased. The electro-

static interactions between the negatively charged lipid head groups and the basic protein residues (e.g. lysines, arginines) were important for cyt. c's overall stability. As the negative surface potential decreased (and the proportion of DPPC increases), this stabilization force weakened and thus, the stability of cyt. c decreased. As shown in Table 3, CUS of cyt. c increased from 0.9 to 1.7 as the negative charge density of lipid lessened.

For many small compact globular proteins, such as lysozyme, RNase A, cyt. c and α-chymotrypsin, CUS was 0.96 ± 0.02 (Privalov and Khechinashvili, 1974; Privalov, 1979). The deviation from 1.0 in most cases did not exceed 6%. This meant that the intermediates in the denaturation of compact globular proteins were highly unstable thermodynamically and the population was rather small. It followed that the denaturation of these proteins could be considered in a first approximation as a simple transition between two discrete macroscopic states, the native and the denatured, that was, as an 'all-or-none' process. For RNase A, it was shown in Table 2 that after its incorporation into DPPG liposomes for lower mol%, such as 1.5 and 4, CUS was far below 1 (exceed 6% deviation from 1). The most probable explanation for this reflection from the two-state model was that this protein consisted of two quite independent and equal cooperative regions. This was not surprising, since the X-ray diffraction study of the RNase A structure had shown that it was possible to observe the presence of two structural domains in RNase A, which were separated by a deep cleft (Kartha et al., 1967). For free protein, two structural domains may strongly interact with each other and couple to give a single transition (Battistel et al., 1991). After binding to DPPG at lower concentrations (≤ 4 mol%), RNase A showed the distinguished two independent transitions corresponding to two separate domains. However, at RNase A mol% higher than 4, CUS was larger than 1, indicating multimolecular interactions (interactions between molecules, such as aggregations). An irreversible aggregation process, presented schematically below, resulted in an increase of the van't Hoff enthalpy and thus the CUS.

$$N \underset{k_2}{\overset{k_1}{\rightleftarrows}} U \overset{k_3}{\rightarrow} D \quad (5)$$

where N is the initial native state, U is the unfolded state, and D is the aggregated state. The reaction $U \rightarrow D$ results in an irreversible loss of the active molecules participating in the equilibrium $N \rightleftarrows U$.

In the case of cyt. c, for free protein or its incorporation into DPPG at lower concentrations (≤ 4 mol%), CUS was close to 1, indicating an approximately two-state model. As cyt. c mol% increased, this ratio approached 2. This meant that the cooperative unit of this protein at elevated temperature was not a monomolecule, but at least two molecules and the two-molecule unit underwent only a single coupled transition. It was clear from this that ΔH_{cal} referred to the heat change per mol, while ΔH_{vH} was heat change per unfolding unit (called the cooperative units). CUS was thus a measure of the degree of the intermolecular cooperation. When cyt. c was bound to the mixture of DPPG and DPPC, CUS also approached 2 in the case of a smaller proportion of negatively charged density of lipid. This suggested that cyt. c formed a two-molecule unit to undergo a single transition. The formation of aggregates was thus possible at this protein mol% and low negatively charged lipid proportion.

The increase in thermal stability of RNase A or cyt. c at protein/DPPG mol% less than 4 implied that these two proteins were stabilized. This may be because the electrostatic interactions between the negatively charged lipid head groups and the basic protein residues stabilized these proteins' internal stabilizing forces. However, the decrease in thermal stability of cyt. c or RNase A at protein/DPPG mol% higher than 4 may suggest a strong intermolecular protein–protein interaction (and likely self-association) because of the relatively low lipid to protein ratio. This was confirmed by the fact that CUS was larger than 1 for protein/DPPG mol% larger than 4, indicating intermolecular aggregation of proteins. This self-association of proteins may weaken the internal stabilizing forces within the proteins and also decreased the electrostatic stabilization due to interactions between the basic protein residues and negatively charged lipid head groups. Thus, the stabilization by DPPG was no longer observed.

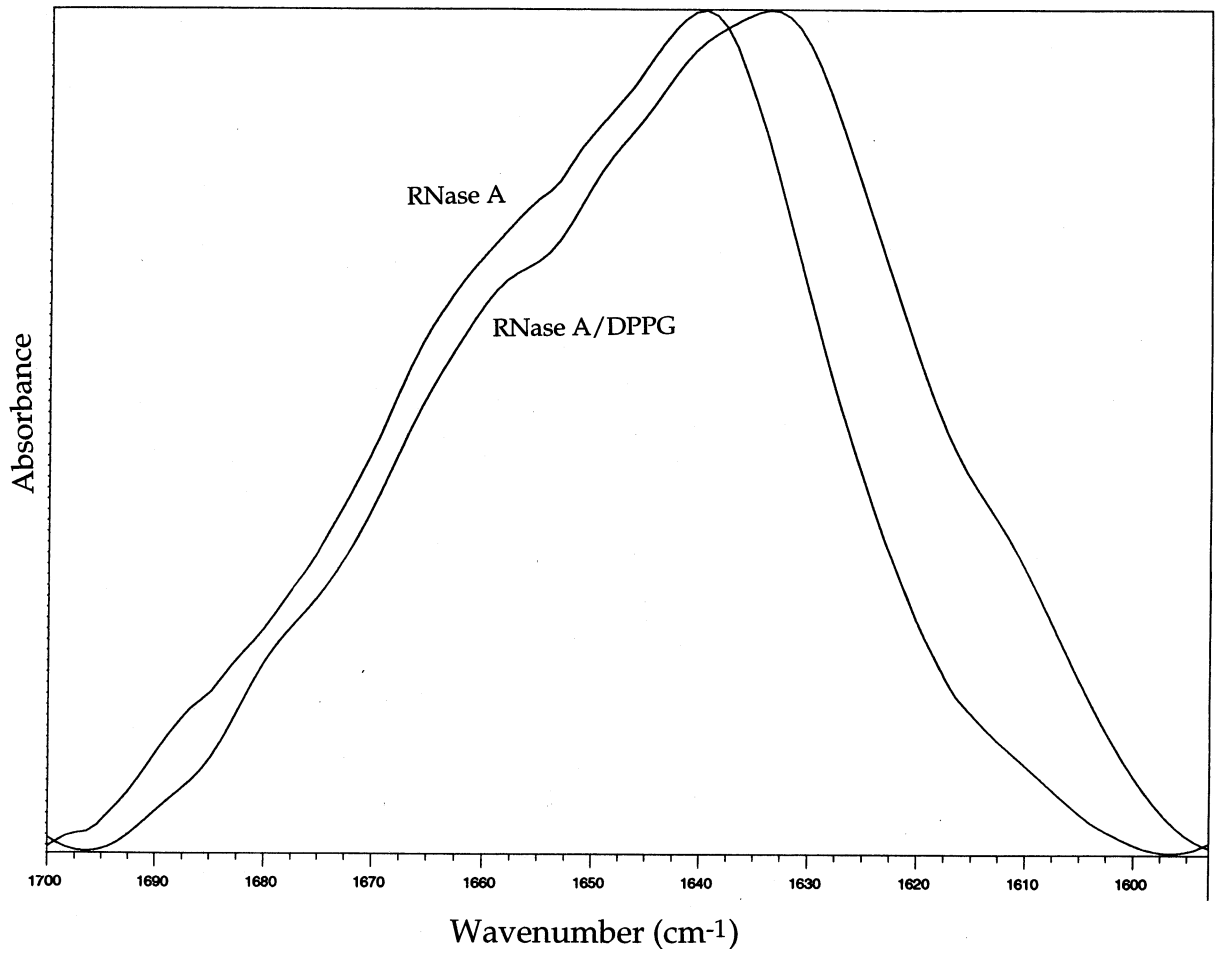

Fig. 1. Original infrared spectra in the amide I′ region of RNase A in solution or complexed with DPPG.

3.2. FF-IR studies of conformation of RNase A and cyt. c

3.2.1. RNase A

The original FT-IR spectra of RNase A in 600 mM NaCl solution (in D_2O) or complexed with DPPG were compared in Fig. 1. The peak position, relative area and band assignment for all the bands of amide I′ region of FSD spectra of RNase A in solution and complexed with DPPG was given in Table 4. The secondary structure components of FCD FT-IR spectra from RNase A in solution or complexed with DPPG were also shown in Table 4. The amide I′ band of the D_2O solution of RNase A exhibited a maximum at 1639 cm^{-1}, together with some shoulders (Fig. 1). The deconvoluted spectrum of RNase A solution (Fig. 2) showed two dominant bands at 1641 and 1631.5 cm^{-1}, which were assigned to the β-sheet components of the protein backbone amide groups. Upon binding to DPPG, we observed changes in the amide I′ band contour from 1639 to 1633 cm^{-1} (Fig. 1). The deconvoluted spectrum of the DPPG-associated RNase A showed two dominant bands at 1637 and 1631 cm^{-1}. However, the band fitting analysis of the deconvoluted spectra showed that the secondary structure of the protein was mainly conserved. The 45% β-sheet content of RNase A in D_2O was in agreement with the previous X-ray diffraction

Table 4
Peak position (in cm^{-1}) and relative area (in %) of the amide I′ component of the Fourier self-deconvoluted FT-IR spectra from RNase A[a]

Secondary structure[b]	RNase A in D_2O		RNase A/DPPG complex	
	cm^{-1}	% Area	cm^{-1}	% Area
Turn	1690.0	2.5	1689.6	2.5
Turn	1681.1	4.3	1680.0	4.7
β-sheet	1673.5	6.5	1673.3	5.9
Turn	1665.8	8.8	1665.0	9.2
α-helix	1659.1	11.5	1658.1	11.6
Unordered	1649.4	13.4	1649.2	13.3
Turn	1641.0	14.7	1637.0	15.3
β-sheet	1631.5	28.8	1631.3	27.6
β-sheet	1621.7	9.5	1621.5	10.0
Total turn		30.3		31.7
Total β-sheet		44.8		43.5
Total α-helix		11.5		11.6
Total unordered		13.4		13.3

[a] In D_2O solution or in complexes with DPPG at 25°C.
[b] The secondary structure band assignments were based on Byler and Susi (1986) and Surewicz and Mantsch (1988).

study of the crystalline protein (Levitt and Greer, 1977).

3.2.2. Cyt. c

The original FT-IR spectra of cyt. c in 600 mM sodium chloride solution (in D_2O) or complexed with DPPG were compared in Fig. 3. The peak position, relative area and band assignment for all the bands of amide I′ region of FSD spectra of cyt. c in solution or complexed with DPPG is given in Table 5. The secondary structure components of FSD FT-IR spectra from cyt. c in solution or complexed with DPPG is also shown in Table 5. The amide I′ band of the D_2O solution of cyt. c exhibited a maximum at 1653 cm^{-1}, together with some shoulders in the low wavenumber and high wavenumber. The deconvoluted spectrum of cyt. c solution showed one dominant bands at 1653 cm^{-1}, which was characteristic of the α-helix of the protein backbone amide groups. Upon binding to DPPG, we observed changes in the amide I′ band contour from 1653 to 1649 cm^{-1}. The deconvoluted spectrum of the DPPG-associated cyt. c showed one dominant band at 1650 cm^{-1}. However, the band fitting analysis of the deconvoluted spectra showed that only slight perturbation of the secondary structure was observed; there was no more than 3.5% in individual secondary structure content upon binding of cyt. c to DPPG. The 35.5% α-helix content of cyt. c in D_2O was in agreement with the previous circular dichroism measurements (Manavaian and Johnson, 1987) and FT-IR study of cyt. c in solution (Heimburg and Marsh, 1993).

For both RNase A and cyt. c, we observed the frequency shift of the major amide I band component to lower wavenumber. Currently, the physical basis of this particular observation was unclear, but we proposed the following hypothesis:

It has been reported by George and Veis (1991), LaBrake et al. (1993), and Zhang et al. (1992a,b) and Zhang et al. (1995) that the frequency shifts observed for a highly α-helical protein under different experimental conditions could be correlated with the changes in helical lengths due to changes in hydrogen bond strength. This could be explained by the distinction between the ordered and disordered helices (Dousseau and Pezolet, 1990). Van Wart and Scheraga (1978) have suggested that the vibrational frequencies of amide group in the center of helical segments were dif-

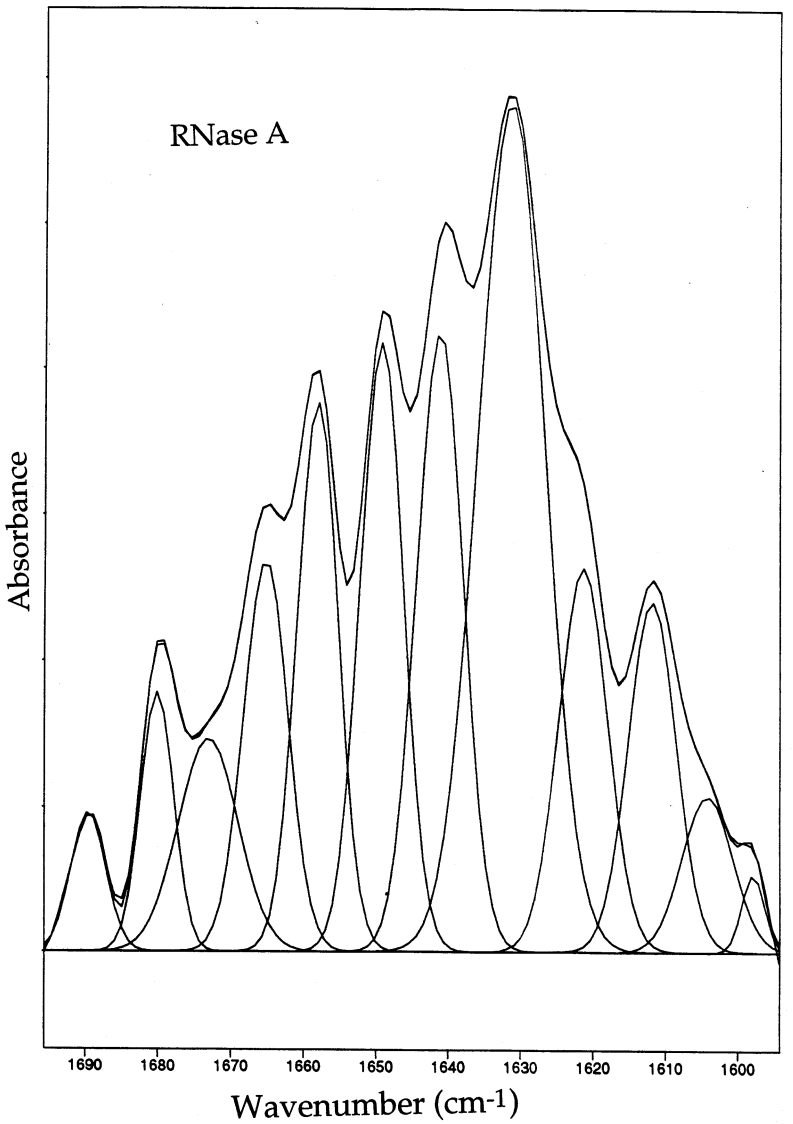

Fig. 2. Band fitting of FSD FT-IR spectra in the amide I′ region of RNase A in solution.

ferent from those at the end. They divided end-helical amides into two classes: $\alpha(-,+)$, when the amide NH group was hydrogen-bonded to a third neighbor amide in the helix while the amide CO was not hydrogen-bonded and $\alpha(+,-)$, when the CO group was hydrogen-bonded to a third neighbor amide group while the amide NH was not hydrogen-bonded. These end helical groups were more disordered and most likely to hydrogen-bond to water and thus, had characteristic amide frequencies different from those of the center $\alpha(+,+)$ amide groups. For one protein under different experimental conditions, the condition with low amide I band frequency had a large fraction of ordered helix as compared to disordered helix and thus, had a long helical length. In addition, small distortion of α-helical conformation due to mismatch between peptide hydrophobic length and bilayer hydrophobic thickness also resulted in frequency shifts (Zhang

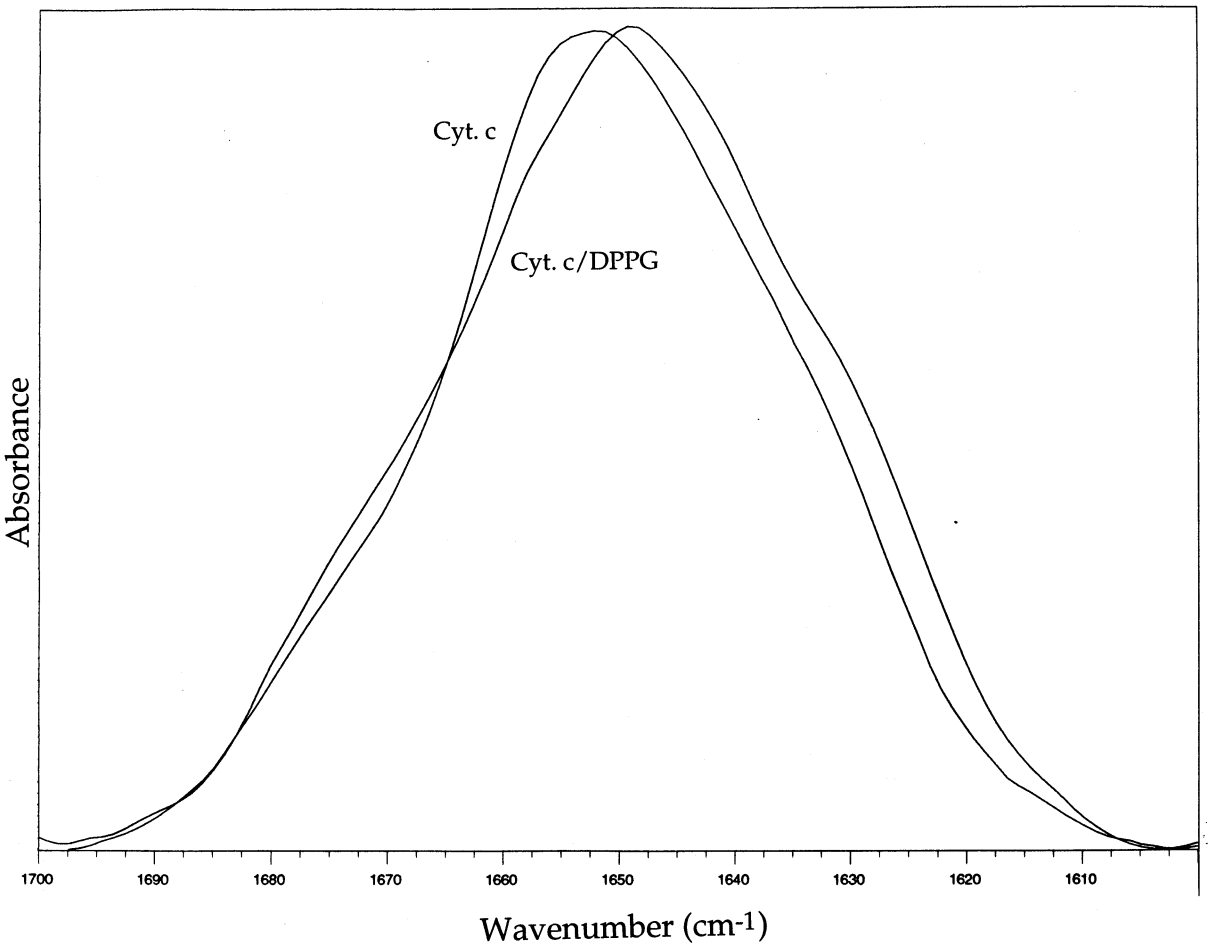

Fig. 3. Original infrared spectra in the amide I′ region of cyt. c in solution or complexed with DPPG.

et al., 1992a,b, 1995). For cyt. c, the 1653 cm^{-1} band in the spectrum of the free protein was attributed to the residues in the cyt. c helix which formed weaker hydrogen bonds (more disordered helix and shorter helical length), and the frequency was shifted to 1649 cm^{-1} upon binding to DPPG to give stronger hydrogen bonds (thus more ordered helix and longer helical length).

For RNase A, the possible explanation for the downshift of the frequency from 1639 to 1633 cm^{-1} was the changes in the hydrogen bond strength in the β-sheets. Dousseau and Pezolet (1990) have suggested that the differences between parallel and antiparallel β-sheets were due to the length of the hydrogen bonds, which were longer in the parallel pleated sheet conformation. Although X-ray diffraction studies have shown that RNase A contained three antiparallel β-strands (Kartha et al., 1967), it was possible that upon binding to DPPG, RNase A could have had part of the peptide chain hydrogen-bonded with a neighboring chain in a parallel pleated sheet and thus, had stronger hydrogen bonding and longer hydrogen bonds. However, some other part of the peptide chain may still remain hydrogen-bonded with a neighbor in an antiparallel pleated sheet.

From the quantitative determination of the deconvoluted spectra of these proteins, only minor changes in the main secondary structural content were observed. These small reductions in major

Table 5
Peak position (in cm^{-1}) and relative area (in %) of the amide I′ component of the Fourier self-deconvoluted FT-IR Spectra from Cyt. c[a]

Secondary structure[b]	Cyt. c in D_2O		Cyt. c/DPPG complex	
	cm^{-1}	% Area	cm^{-1}	% Area
Extended chains	1679.0	5.5	1678.8	6.0
Extended chains	1670.9	13.9	1670.0	15.9
β-turn	1660.0	14.3	1659.2	12.6
α-helix	1653.0	35.5	1650.0	33.7
Extended chains	1640.5	10.6	1640.2	8.4
Extended chains	1629.9	20.2	1629.5	23.2
Total α-helix		35.5		33.7
Total β-turn		14.3		12.6
Total extended chains		50.2		53.5

[a] In D_2O solution or in complexes with DPPG at 25°C.
[b] The secondary structure band assignments were based on Byler and Susi (1986) and Surewicz and Mantsch (1988).

secondary structural content, along with the shifts to lower frequencies of the main component bands of RNase A and cyt. c suggested that some rearrangement within the helices/sheets, possibly rearrangements of the relative amounts of different order groups and/or the overall loosening of the protein tertiary structure existed, resulting in a stronger hydrogen bonding accessibility after their binding to DPPG liposomes. Recent resonance Raman (Hildebrandt et al., 1990) and magnetic resonance spectroscopy (Soussi et al., 1990) indicated that binding of cyt. c to anionic lipids affects the conformation and immediate environment of the heme group and resulted in the alterations in the coordination pattern of the heme iron and a partial opening of the heme crevice. Their results suggested that the perturbation of the prosthetic group was a local effect, but our results suggested a more extensive change in the conformation and thermodynamic state of the protein backbone.

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