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NMR studies of peptide, protein and mixed-lipid systems

Longo, Paula Alissa, Ph.D.

City University of New York, 1992

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NMR STUDIES OF PEPTIDE, PROTEIN AND MIXED-LIPID SYSTEMS

by

Paula Alissa Longo

A dissertation submitted to the Graduate Faculty in
Chemistry in partial fulfillment of the requirements for the
degree of Doctor of Philosophy, The City University of New
York.

1992

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Abstract

NMR STUDIES OF PEPTIDE, PROTEIN AND MIXED-LIPID SYSTEMS

by

Paula Alissa Longo

Advisors: Professors Ruth E. Stark and Michelle S. Broido

High resolution solution-state and magic-angle spinning NMR spectroscopy have been used to structurally characterize several different types of biological polymers. The amino-terminal fragment of the p21 protein and three analogues (two oncogenic and one not) were found, using two-dimensional NOE spectroscopy, to undergo rapid conformational averaging and to contain no long-lived identifiable structures.

An 11 kD digestive enzyme, porcine pancreatic colipase, was found to exist as a dimer at concentrations suitable for two-dimensional NMR analysis. While this fact precluded a complete conformational study, a possible long-range hydrophobic-hydrophobic dipolar interaction was observed which suggests a foldover of the protein. This foldover has been postulated to bring the two binding sites on the protein close together. Additionally, the results of this study underscore the need for protein size determination using a non-denaturing method. This should be done prior to NMR investigations and at millimolar protein concentrations.

Several model mixtures, representative of the late-stage products of triglyceride digestion, have been analyzed using solution-state and MAS NMR. All mixtures contained a fatty

acid (either C8 caprylic or C18 oleic acid), a monoglyceride (either C8 monocapryoyl-rac-glycerol or C18 monooleoyl-rac-glycerol) and the bile salt taurocholate. C8 mixtures were found, using solution-state NMR spectroscopy, to form mostly mixed micelles. NOESY and ROESY data demonstrate that the BS sidechain has a dual function and location within the aggregate. Additional evidence for a structure accommodating these conditions came from Mn^{2+} titration experiments.

C18 mixtures, which partition into two layers were found to form large aggregates. For C18 top layers, MAS NMR spinning sidebands indicated that the acyl chains are highly ordered. Additional spinning sidebands indicated the presence of surprisingly ordered solvent molecules. These data suggest a bilayer-vesicle aggregate for both C18 top layers. The C18 bottom layers were found to contain either BS monomers or mixed micelles, which were similar to, but larger than, the ones found for C8.

This thesis is dedicated to my family, Barbara, Frank and
Joshua

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List of Abbreviations & Symbols

Å	Angstrom
Ala	alanine
AQ	aquisition time
Arg	arginine
Asp	aspartic acid
B ₀	magnetic field strength (T)
BS	bile salt
C8	Mixture samples with acyl chains having 8 carbons
C18	Mixture samples with acyl chains having 18 carbons
CD	circular dichroism
c-H- <u>ras</u>	cellular Harvey rat sarcoma viral oncogene
CIDNP	photochemically induced dynamic nuclear polarization
CLP	porcine pancreatic colipase
COSY	two-dimensional correlated spectroscopy
CP	cross polarization
¹³ C	spin-½ isotope of carbon
D ₂ O	deuterium oxide
DG	diglyceride
DMSO-d ₆	dimethyl sulfoxide-d ₆
DPMAS	direct-polarization magic-angle spinning
DQF	double-quantum filter
E	energy

ECEPP	empirical conformational energies for peptides and proteins
EDTA	ethylenediaminetetraacetic acid
FA	fatty acid
FID	free induction decay
FT	Fourier transform
Gly	glycine
G-protein	guanine nucleotide binding protein
GDP	guanine diphosphate
Glu	glutamic acid
GTP	guanine triphosphate
h	Planck's constant (J-S)
^1H	spin- $\frac{1}{2}$ isotope of hydrogen
HETCOR	heteronuclear correlated spectroscopy
His	histidine
HMQC	heteronuclear multiple quantum coherence
HOD	water, residual ^1H in D_2O
J	spin-spin coupling constant (Hz)
k	Boltzmann constant (J)
Leu	leucine
Lys	lysine
MAS	magic-angle spinning
MBS	mixed bile salts
MG	monoglyceride
MHz	megahertz
MIL	mixed intestinal lipid

N_α	proton population in lower energy state
N_β	proton population in upper energy state
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser effect
NOESY	two-dimensional nuclear Overhauser effect spectroscopy
PAGE	polyacrylamide gel electrophoresis
Phe	phenylalanine
ppm	parts per million
Pro	proline
QLS	quasi-elastic light scattering
r	internuclear separation
ROE	rotating-frame NOE
ROESY	two-dimensional rotating-frame NOE spectroscopy
SDS	sodium dodecylsulfate
Ser	serine
S/N	signal-to-noise ratio
ssb	spinning sideband
t_1, t_2	time dimensions in 2D NMR
T	temperature (K or °C)
T_1	spin-lattice relaxation time
T_2	spin-spin relaxation time
TC	taurocholate
TDC	taurodeoxycholate
TG	triglyceride
Thr	threonine

TOCSY	two-dimensional total correlated spectroscopy
Tyr	tyrosine
UV	ultraviolet
Val	valine
$\Delta\epsilon_{l-r}$	difference in the extinction coefficient between left and right circularly polarized light
γ_N	gyromagnetic ratio of nucleus N ($s^{-1} T^{-1}$)
σ_{noe}	cross relaxation rate
τ_c	correlation time (ns)
τ_m	mixing time in 2D experiment
ν_1, ν_2	frequency dimensions in 2D NMR
ν_{rot}	spinning speed (kHz)
ω	resonance frequency ($rad s^{-1}$)
1D	one-dimensional
2D	two-dimensional

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Chapter I:

Introduction to Biological NMR

Nuclear magnetic resonance (NMR) spectroscopy has developed into one of the most powerful tools available to study biological molecules. In contrast to most other physical and spectroscopic techniques, NMR can be used to study molecules in both the liquid and the solid state, thereby widening the range of systems to be studied as well as allowing more questions to be addressed. Presented in this chapter is the NMR strategy used in this thesis for studying peptide and protein structure as well as the various NMR techniques used to examine different model mixtures for triglyceride digestion. The chemistry of these systems is discussed individually in the introduction section of each chapter as are specific applications of the experiments which are presented here.

Theoretically, nuclei which possess magnetic dipole moments and spin angular momenta can be observed using NMR spectroscopy (Bloch et al., 1946; Purcell et al., 1946). When spin- $\frac{1}{2}$ nuclei (^1H , ^{13}C etc.) are placed in a magnetic field their magnetic moments will align with or against the field in numbers governed by the Boltzmann distribution, yielding a small net alignment with the field (lower energy state). It is this net magnetization which is manipulated by radiofrequency pulses and delays and finally detected in the NMR spectrum.

The types of pulses and delays used dictate the kind of information that is obtained from the experiment. The inherent disadvantage of NMR is its low sensitivity compared to high-frequency techniques. The net magnetization is roughly one part in 10^5 and therefore very long experimental times can be required to acquire sufficient signal to noise (S/N) for interpretable spectra. Fortunately the advent of pulse and Fourier transform NMR (Ernst and Anderson, 1966) and the availability of high-field spectrometers has made it possible to acquire routine NMR spectra in as little as a few seconds.

Figure I-1 shows the pulse sequence for a typical one-pulse experiment. During the preparation period the nuclei reach an equilibrium population distribution given by Equation 1.

$$N_{\beta}/N_{\alpha} = \exp(-\Delta E/kT) \quad (1)$$

where N_{β} is the population of the upper state,

N_{α} is the population of the lower state,

and $\Delta E = E_{\beta} - E_{\alpha}$.

During the pulse the magnetic moments are perturbed and enter a non-equilibrium state. The return to equilibrium after this perturbation is monitored as a function of time during the time designated t_2 . Fourier transformation of this free induction decay (FID) to a frequency domain results in the

familiar one-dimensional (1D) ^1H spectrum which is shown in Figure I-2. The power of NMR for studies of chemical structure lies in the fact that each proton in the molecule will experience a slightly different electronic environment and will therefore resonate at a slightly different chemical shift. If the magnetic field were strong enough (and therefore the number of Hz/ppm large enough) each proton would theoretically resonate at a different place in the spectrum. For small molecules this behavior may be observed, but for large biological macromolecules with many protons, spectral overlap and multiplicities make this simple description invalid. The problem of overlap is further exacerbated in ^1H NMR by the relatively small chemical shift dispersion of a routine ^1H spectrum (10 ppm). It is, therefore, necessary to find additional ways of differentiating between the various types of protons to obtain complete proton assignments to chemical groups.

The idea of two-dimensional (2D) NMR spectroscopy was first suggested by Jeener (1971), but the experiments did not become generally feasible until a decade later when computers capable of handling the data were available on commercial spectrometers. Basically, the 2D experiment adds another dimension in which to distinguish the nuclei of interest. For example, in CORrelated SpectroscopY (COSY; Marion and Wuthrich, 1983), the resonances are sorted according to their

scalar coupling connectivities as well as being dispersed according to their chemical shifts. The availability of this second dimension allows the overlapping resonances to be spread out and identified definitively with particular structural elements.

The general pulse sequence for a 2D experiment is shown in Figure I-3. The experiments consist of basically four periods: preparation, evolution, mixing and detection. During the preparation period the system comes to equilibrium and a pulse is applied to the spins which creates a non-equilibrium state. This non-equilibrium state evolves during the evolution and mixing periods and is acquired during the detection period.

The major technical difference in the 2D experiment, compared to the 1D experiment, is the introduction of the evolution period, t_1 . Two-dimensional spectra are collected as a series of 1-dimensional spectra, where the evolution period is incremented as a function of time. Figure I-4A shows a partially processed 2D data set for which t_2 has been Fourier transformed to ν_2 and t_1 increases systematically. The data are acquired with a discrete number of points in both time dimensions and a second Fourier transform in the t_1 dimension results in a peak which represents a connection (either scalar or dipolar) between two protons which are located at

frequencies ν_1 and ν_2 (Figure I-4B). Figure I-4C shows a "bird's eye view" through the peak in Figure I-4B which results in a contour. The remainder of the 2D spectra presented herein are shown as contour plots.

In order to obtain structural information it is first necessary to assign many, if not all, of the protons (or nuclei of interest) in the system to be studied. Figure I-5A shows the pulse sequence for the COSY experiment. Provided that the magnitude of the coupling constants exceeds the linewidths, COSY traces out the through-bond spin-spin connectivities in molecules and allows spins which are scalar coupled to be identified. Another correlated experiment which relies on scalar couplings is TOCSY (Total Correlated Spectroscopy; Figure I-5B; Braunschweiler and Ernst, 1983). As opposed to RELAYED-COSY (Eich et al., 1982) which relays scalar information once, TOCSY can theoretically relay information over many protons (in actuality the extent of the relay is dependent upon both the spin-lock mixing time and the spin-spin relaxation time, T_2 , of the molecule). For example, assume spins A,B,C and D belong to the same spin system and are scalar coupled pairwise in alphabetical order. COSY crosspeaks will include A,B; B,C; and C,D. A RELAYED-COSY spectrum will include the following crosspeaks: A,C and B,D; a TOCSY spectrum will include crosspeak A,D as well.

Correlated spectroscopy can be heteronuclear as well as homonuclear. Generally known as HETCOR (HETeronuclear CORrelated), a specific example of this type of experiment is Heteronuclear Multiple Quantum Coherence spectroscopy (HMQC; Bax et al., 1983). The HMQC experiment picks protons which are scalar coupled to carbon and links them spectrally. The sensitivity of this experiment is increased tremendously by the incorporation of indirect detection of ^{13}C (or any low γ heteronucleus). The low γ and natural abundance of ^{13}C make direct detection of this nucleus inherently unfavorable in terms of S/N, but the transfer of the ^{13}C magnetization to ^1H permits the former to be detected with the high sensitivity of ^1H (Maudsley and Ernst, 1977; Bolton, 1985). Specific examples of COSY, TOCSY and $^1\text{H}\{-^{13}\text{C}\}$ HMQC spectra and detailed descriptions of the assignment procedures appear in the individual chapters on peptide, protein and lipid systems of interest.

Once assignments have been made, structural information can be obtained. Whereas $^1\text{H}\text{-}^1\text{H}$ COSY and other correlated experiments (TOCSY etc.) give information about protons which are scalar coupled, $^1\text{H}\text{-}^1\text{H}$ NOESY (2D Nuclear Overhauser Effect Spectroscopy; Figure I-5C; Jeener et al., 1979; Anil-Kumar et al., 1980) gives information about protons which are dipolar coupled and, therefore, close in space. The NOESY experiment, as its name implies, takes advantage of the phenomenon known

as the nuclear Overhauser effect. This is a dipolar cross-relaxation interaction which perturbs the spin state population of protons which are dipolar coupled. This population perturbation is manifested in the NMR spectrum as either an increase or decrease in peak intensity depending on the sign of the cross relaxation rate, σ_{noe} , as shown in Equation 2 where γ is the gyromagnetic ratio, τ_c is the correlation time for molecular motion and ω_0 is the resonance frequency.

$$\sigma_{\text{noe}} = \left\{ \frac{\gamma^4 \hbar^2}{10 r^6} \right\} \left\{ \frac{6\tau_c}{1 + 4\omega_0^2 \tau_c^2} - \tau_c \right\} \quad (2)$$

As can be seen from Equation 2 there is a distance dependence ($1/r^6$) of σ_{noe} , which can be used in macromolecular structure determination. Specific examples of the NOEs observed in peptide, protein and digestive mixture samples will be presented and discussed further in following chapters. The NOESY pulse sequence differs from the COSY pulse sequence in that there is an additional 90° pulse and a mixing period. It is during this mixing time that the NOE is allowed to evolve. Typically NOESY spectra are acquired at several mixing times (100 to 1000 ms, for example), permitting the build-up of the NOE to be monitored. In addition to increasing as inter-proton distance decreases, the NOE will increase as a function of mixing time, until a maximum is reached. By following the NOE

intensity build-up, spin diffusion (indirect transfer of magnetization) can be detected (and avoided) so that NOE data reflect only proximal spin pairs.

The NOE intensity is also a function of τ_c according to the conditions in Equation 3:

<u>NOE</u>	<u>When</u>	
> 0	$\omega\tau_c < 1$	
$= 0$	$\omega\tau_c = 1.2$	(3)
< 0	$\omega\tau_c > 1.$	

For molecules which have a τ_c such that $\omega\tau_c = 1.2$, the expected laboratory-frame NOE intensity will be zero. However, the NOESY experiment can also be performed under "spinlocked" conditions (Rotating frame nuclear Overhauser Effect Spectroscopy; ROESY), where the rotating-frame NOE (ROE) is observed (Bothner-by et al., 1984; Davis and Bax, 1985). Unlike the laboratory-frame NOE, which is observed in NOESY, the ROE is always positive. This feature makes ROESY a powerful experiment for medium-sized molecules that often have NOE intensities near zero (Kessler et al., 1987; Jelicks et al., 1988). The appearance of ROE crosspeaks which are opposite in sign to the diagonal improves spectral resolution and distinguishes proximal dipolar interactions from crosspeaks that arise from spin diffusion and TOCSY-type interactions (Bax and Davis, 1985). In NOESY all crosspeaks are the same sign as the diagonal peaks.

Many interesting biological systems contain particular structural elements which can be used as spectroscopic probes. For example, the protons near the metal in a large protein which contains a paramagnetic heme can be studied because they are characteristically shifted due to the paramagnetic property of the heme (Satterlee and Erman, 1991). While not all biological systems inherently contain these "handles," their information-yielding properties can be exploited in other ways. A common technique used to determine which parts of a micellar or bilayer aggregate are exposed to the solvent is to titrate in a paramagnetic solution and note which resonances are markedly shifted (Gabriel and Roberts, 1987; Mukidjam et al., 1986). Broadening reagents, like Mn^{2+} , can also be used when the systems under investigation are not amenable to conventional paramagnetic shift reagents (Stark et al., 1985).

As noted above, one of the advantages of NMR is its ability to study molecules both in solution and the solid state. The combination of magic-angle spinning (MAS) with cross polarization (CP) and high-power proton decoupling techniques have permitted routine acquisition of narrow-line ^1H and ^{13}C solid-state spectra (Schaefer and Stejskal, 1979). Recent progress has extended these techniques to semisolid samples and employed 2D solid-state NMR to mixed lipid and steroid systems (Oldfield et al., 1987; Forbes et al, 1988;

Halladay et al, 1990; Hamilton et al., 1991). One particular spectral feature which is found in liquid-crystalline and solid sample spectra is spinning sidebands. Additional insight about molecular order can be obtained by monitoring these spinning side bands as a function of the spinning speed (v_{rot}).

Figure I-1. General one-pulse sequence for NMR experiments. Typical 90° pulse width and relaxation delay are 15 μ s and 1 s, respectively.

Preparation

Transmitter



t_2

Receiver



Figure I-2. Typical 1D ^1H 400 MHz mixed lipid spectrum with presaturation to suppress the solvent. The FA/MG acyl chain and BS steroid backbone protons resonate upfield (0.5 - 2.5 ppm) and BS sidechain, BS β and MG glycerol protons resonate downfield (2.8 - 4.2 ppm).

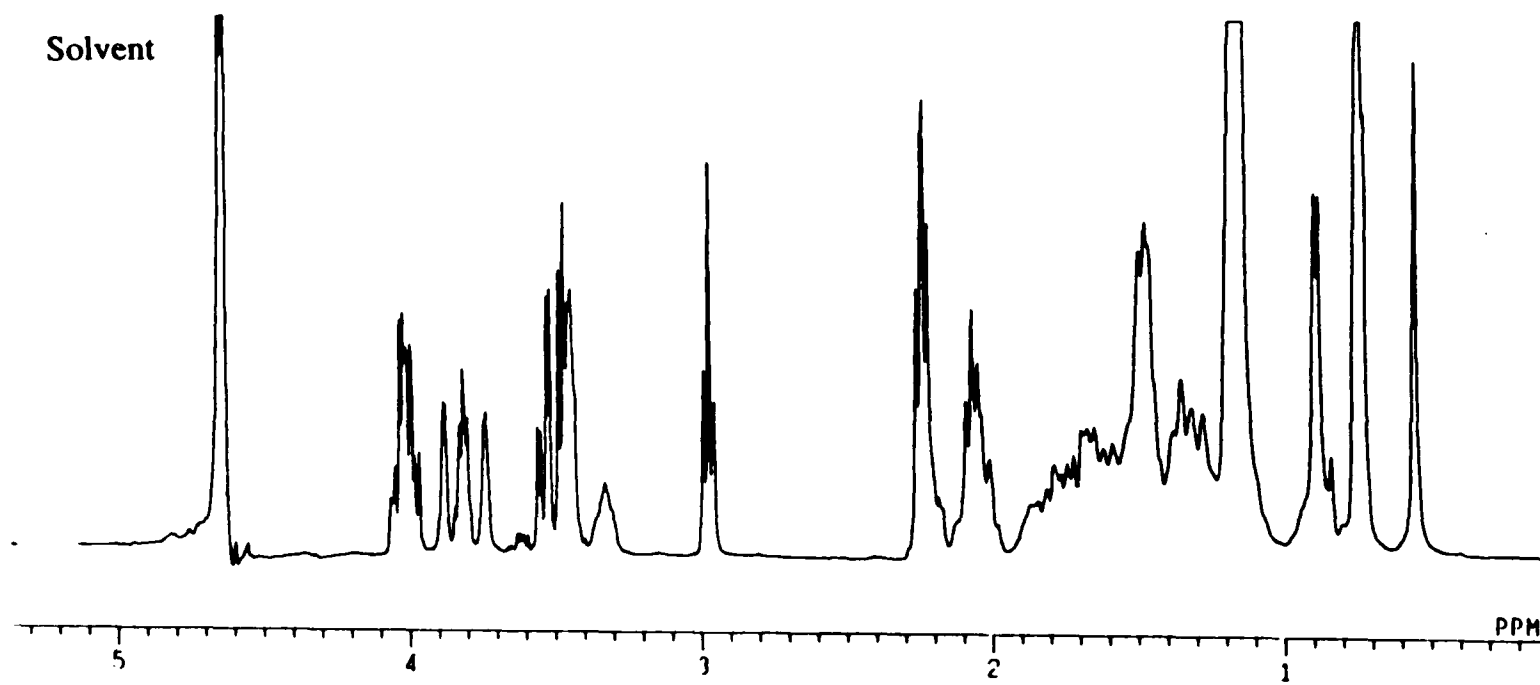


Figure I-3. General scheme for a 2D pulse sequence. Preparation period is about 1 s. The evolution period generally starts at 0.01 ms and increases in increments of 0.250 ms. Mixing periods range from 0 (COSY) to 1 s (NOESY), depending on the experiment. Total acquisition time for 1 scan is typically 1.5 s.

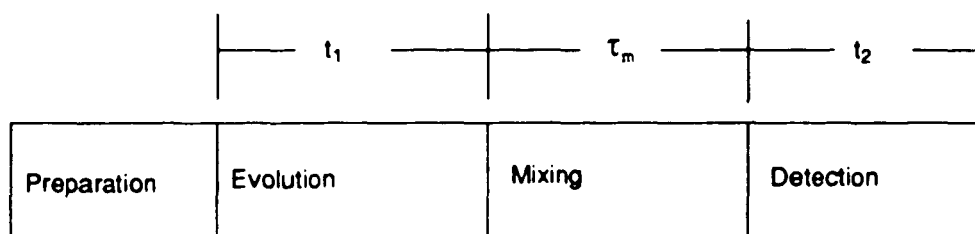


Figure I-4. Various stages of a processed 2D data set. A: Data set after the Fourier transform in the t_2 dimension. B: Stacked plot after double Fourier transform. C: Contour plot which results from "slice" through peak in B.

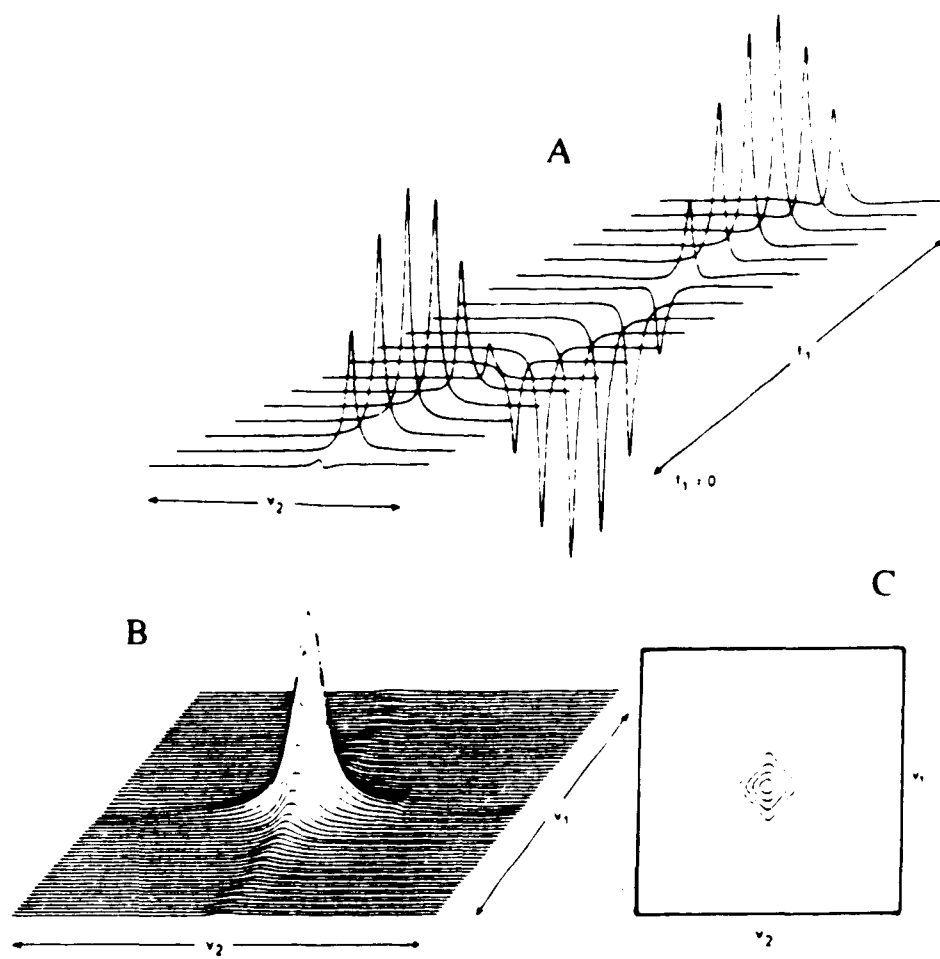
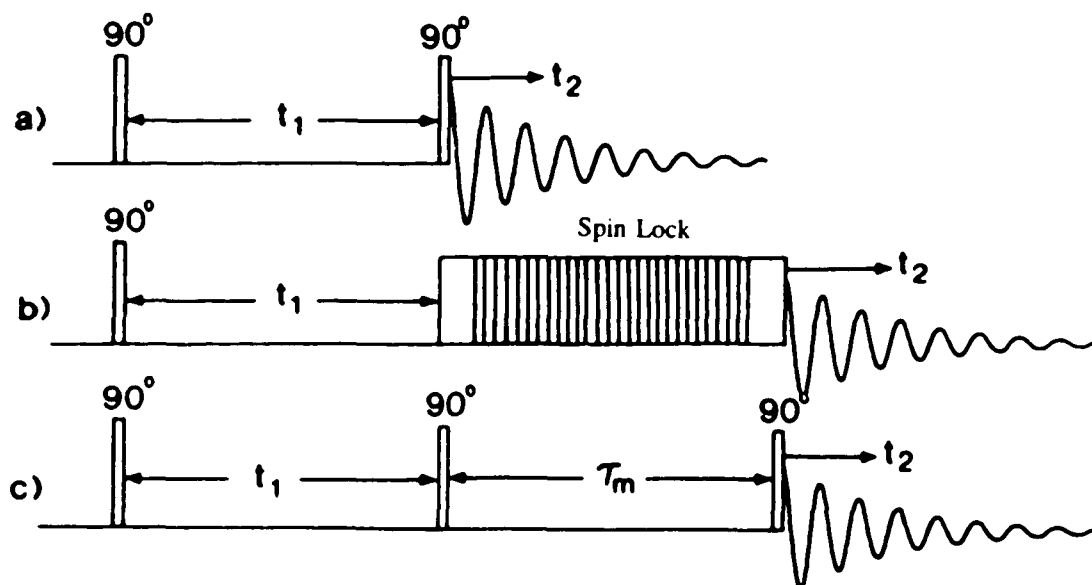


Figure I-5. Pulse sequences for A: COSY; B: ROESY and TOCSY and C: NOESY.



Chapter IIA

Materials and Methods: Sample Preparation

p21 Samples (Longo et al., 1988)

p21 samples were prepared by dissolving 4 mg of synthetically prepared peptide (Asp-12, Arg-12, Pro-12 or Gly-12; from Pincus et al.) in 0.5 ml DMSO- d_6 (100%, Cambridge Isotope Laboratories, Woburn, MA). Samples were contained in Wilmad 528 PP NMR tubes (5 mm).

Colipase Samples (Longo et al., submitted)

Colipase was purified by W.D. Behnke et al. (Univ. of Cincinnati) according to the procedure of Chapus et al. (1981). All initial NMR samples were prepared by dissolving between 11.5 and 14.1 mg of lyophilized, powdered protein in buffers with either H_2O or 99.96 atom% ^2H_2O (Cambridge Isotope Laboratories, Woburn, MA) as solvent (Longo et al., submitted). The starting buffer (pH 5.7) included 150 mM NaCl, 50 mM potassium phosphate, 50 μ M EDTA (to chelate metal ions) and .02% NaN_3 (to prevent bacterial growth). The resulting 2 mM samples were optically clear.

All samples for 2D NMR were 2 mM in CLP and prepared from previously lyophilized protein. Samples for 1D NMR were derived from either lyophilized powder or were concentrated

using an Amicon ultrafiltration cell with a membrane cutoff of 3 kDa. Concentrations of samples used for 1D NMR were calculated from the measured absorbance using an ϵ (280 nm) of 4.2 (Donner et al., 1976).

All dilutions for 1D concentration studies were carried out by adding aliquots of H₂O buffer to a previously prepared 2 mM sample. All pH changes were made by adding μ l aliquots of 100 mM HCl to a previously prepared 2 mM sample.

Model digestive mixtures

Aqueous medium- and long-chain lipid samples were prepared from stock solutions of fatty acid (either (C8) caprylic or (C18) oleic acid), monoglyceride (either (C8) 1-monocapryloyl- or (C18) 1-monooleoyl-rac-glycerol) and bile salt (taurocholate) in methanol (see Figure II-1). Fatty acids and monoglycerides were purchased from Sigma Chemical Company (St. Louis, MO); taurocholate was purchased from Calbiochem (San Diego, CA). Methanolic stock solutions were combined volumetrically in the appropriate molar ratios and were dried under a stream of N₂ followed by evaporation under reduced pressure overnight to remove any remaining solvent. Mixtures were then rehydrated in D₂O buffer (100 mM phosphate, 150 mM NaCl, 0.02% NaN₃, pH 6.5) and were either stored at 5°C (monophasic C8 samples) or at room temperature (C18 samples).

C18 samples were incubated at 37°C for at least 4 days prior to use. All C8 samples were optically clear and monophasic. All C18 samples were biphasic, consisting of an optically clear lower layer and a whitish opalescent upper layer.

Samples had the following molar ratios designated as MBS:MIL/FA:MG where MBS = total bile salt concentration, MIL = total mixed lipid concentration, FA+MG, FA = fatty acid concentration and MG = monoglyceride concentration: 20:80/20:1 and 20:80/1:1.25. The final concentrations of each component in the individual mixtures are listed in Table II-1.

Samples which contained deuterated FA were prepared as described above except that caprylic acid- d_{11} , (Cambridge Isotope Laboratories, Woburn, MA) was used.

Chapter IIB

Materials & Methods: NMR Spectroscopy

All high-resolution NMR spectroscopy was performed on the JEOL GX-400 spectrometer (^1H resonance frequency = 400 MHz) of the CUNY NMR facility which is located at Hunter College. All magic-angle spinning assisted NMR experiments were performed on the Bruker WP-200 spectrometer (^1H resonance frequency = 200 MHz) located at the College of Staten Island. Data acquired at Hunter College were processed off-line using FTNMR (Hare Research, Woodinville, WA) running on a MicroVAX II of the Hunter College RCM Computer Graphics Facility. Data acquired at the College of Staten Island were processed off-line using software from New Methods Research (East Syracuse, NY) running on a SUN SPARC I 1station (SUN Microsystems, Mountain View, CA).

400 MHz High-Resolution NMR Experiments

1D ^1H experiments were performed with solvent suppression (either residual DMSO, H_2O , or HOD) using a one-pulse experiment with solvent irradiation employed during the relaxation delay. High-resolution spectra were acquired with 32 - 64 signal-averaged FIDs each containing 16 - 32 K points. The spectral width varied over the range 2100 - 5500 Hz (5.25 - 13.8 ppm) depending on the sample being examined. A line broadening of 0.5 Hz was usually applied prior to Fourier

transformation. A typical 90° pulse, depending on the sample, was between 13 and 18 μ s.

^{13}C NMR spectra were acquired with broadband decoupling of protons. Typically a spectral width of 25,000 Hz (250 ppm) was used. Due to the lower natural abundance of ^{13}C , overnight accumulation (60,000 FIDs) was required. A line broadening of 10 Hz was typically used in processing these spectra.

Pure-absorption double-quantum filtered correlated spectroscopy (DQF-COSY) and total correlated spectroscopy (TOCSY) spectra were recorded using the methods of States et al. (1982). Resolution enhancement was achieved by using assorted shifted and unshifted sinebell window functions in the two time dimensions prior to the Fourier transformations. Spectra had 32 - 128 acquisitions per t_1 value, 2048 points in the t_2 dimension and 384 - 512 points in the t_1 dimension. The t_1 dimension was zero-filled in all cases to produce 1K X 1K matrices subsequent to the double Fourier transformation. Solvent suppression was achieved by presaturation of the residual DMSO, H_2O or HOD resonance. TOCSY spectra (colipase and mixture samples) were collected under the same conditions as DQF-COSY, using 70 ms mixing times. Typical DQF-COSY and TOCSY experiments required about 12 hours of spectrometer time.

For the determination of amide temperature coefficients (p21; Asp-12) COSY spectra in the absolute value mode were collected. Spectra were collected every 5° from 20°C - 45°C. The temperature was controlled and monitored using the variable temperature unit on the spectrometer. Compressed air was dried and then used in controlling sample temperature. Experimental parameters and processing were as described for phase-sensitive DQF-COSY spectra.

^1H -(^{13}C) (natural abundance) HMQC spectra (mixture samples), acquired with ^1H detection (Bax et al., 1983), had 64 acquisitions per t_1 value, 2048 points in the t_2 dimension and 384 points in the t_1 dimension. Spectra were acquired with broadband ^1H decoupling, but without ^{13}C decoupling. For 3 - 60 mM ^{13}C at natural abundance, this experiment required approximately 55 hours of spectrometer time.

NOESY and ROESY spectra were recorded in the pure absorption mode as previously described for phase-sensitive DQF-COSY spectra. NOESY spectra were acquired with mixing times ranging from 75 to 1000 ms depending on the samples. ROESY spectra were acquired with 100 and 250 ms mixing times depending on the samples. NOESY and ROESY spectra were collected under the same conditions as DQF-COSY spectra except that 32 - 128 acquisitions per t_1 value were used. A typical NOESY or ROESY experiment required 26 hours of

spectrometer time.

Quantitation of the various components in the clear, bottom layer for each C18 mixture was accomplished using a DMSO/ CCl_4 insert. Approximately 25 μl of a DMSO solution in CCl_4 was transferred to a Wilmad coaxial insert (520-3). The DMSO concentration itself was calibrated by placing the insert in solutions of known BS concentration (a 10 mM BS solution and C8 20:80/20:1) and integrating with respect to the narrowest resolved ^1H BS peaks (BS 25 and 18; Figure II-2). The concentrations determined using the above method were averages based on integration of several resonances. Based on the observed spread of concentrations determined in this fashion, quoted values for the C18 mixtures have a precision of $\pm 0.5\%$.

Paramagnetic shift experiments were carried out by titrating μl aliquots of $\text{PrCl}_3 \cdot 6\text{H}_2\text{O}$, $\text{Tb}(\text{NO}_3)_3 \cdot 6\text{H}_2\text{O}$ and $\text{Dy}(\text{NO}_3)_3 \cdot 5\text{H}_2\text{O}$ stock solutions into previously prepared mixture samples and measuring the change in chemical shift (ΔHz) with respect to an internal DMSO reference.

C18 200 MHz Magic-Angle Spinning NMR Experiments

Magic-angle spinning ^1H NMR spectra were obtained on an IBM/Bruker WP-200 spectrometer equipped with a Doty Scientific

probe. For aqueous salt solutions, better spectral sensitivity was obtained if probe matching was optimized at the expense of probe tuning. The 90° pulse used was 44 μ s, with a corresponding spectral coverage of 23,000 Hz. Both 1D- and 2D-MAS ^1H NMR experiments were performed with presaturation of the HOD signal.

1D spectra were acquired with 32 signal-averaged FIDs each containing 16 K points. The spectral width varied over the range 2500 - 6500 Hz (12.5 - 32.5 ppm), depending on the spinning speed used and whether or not spinning sidebands were present. Spinning speeds in separate experiments varied from 0.55 to 2.0 kHz ($\pm$ 0.02 kHz). All ^1H spectra were acquired at 37°C and were referenced to HOD, which resonates at 4.65 ppm at this temperature. Sample temperature was controlled to within 1.0°C using a Bruker Instruments, Inc. B-VT 1000 unit. A line broadening of 1 Hz was applied prior to Fourier transformation, unless stated otherwise.

MAS-assisted phase-sensitive COSY and NOESY spectra (Forbes et al., 1988) were recorded using the method of Marion and Wuthrich (1983). Phase-sensitive NOESY spectra were recorded with the following mixing times: 100, 600 and 800 ms. 2D spectra had 32 acquisitions per t_1 value, 512 points in the t_2 dimension and 256 points in the t_1 dimension with zero-filling resulting in 512 x 512 matrices. Prior to Fourier

transformations various shifted and unshifted sinebell window functions were employed for signal and resolution enhancement. A typical experiment required 4 h of spectrometer time.

Direct-polarization magic-angle spinning (DPMAS) ^{13}C NMR experiments were carried out with auxillary radiofrequency amplifiers under conditions of high-power broadband proton decoupling (50 kHz). Typical conditions included a 5 μs 90° pulse for ^{13}C , 4K points and a recycle delay of 1 s. Overnight accumulation was required to obtain adequate S/N for these spectra.

Chapter IIC

Materials & Methods: Biochemical Techniques

Gel Electrophoresis (Longo et al., submitted)

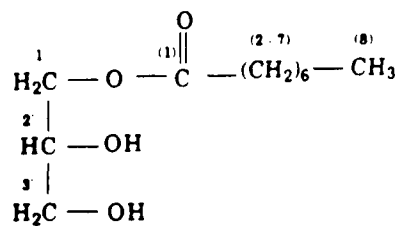
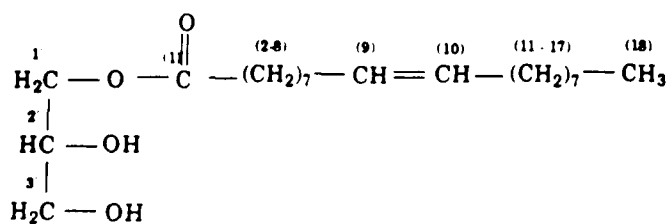
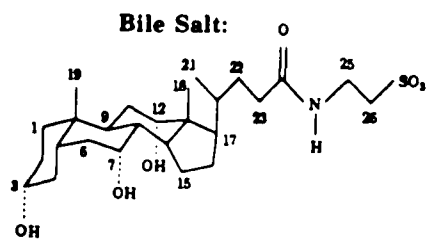
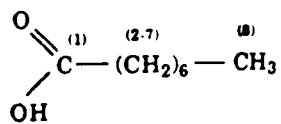
Polyacrylamide gel electrophoresis (PAGE) was carried out under denaturing (SDS) and native conditions. Miniature slab gels (Hoefer Scientific Instruments, San Francisco, CA) were used with 10 - 15% polyacrylamide, a Tris running buffer at pH 8.8 and a Tris stacking buffer at pH 6.8. All PAGE samples, ranging from 0.09 mM to 0.12 mM, were prepared with a Tris buffer containing 5% β -mercaptoethanol (to avoid aggregation through disulfide bridges), 10% glycerol and 0.01% bromophenol blue. Low-molecular-weight markers (14.4 - 94 kDa), purchased from Pharmacia (Piscataway, NJ), were prepared with the same buffer as colipase samples. All samples were run using an Ephortec power supply operating at 110 mV and 15 mA. Gels were stained with Coomassie blue and stored in acetic acid/methanol destaining solution until they were vacuum dried.

Circular Dichroism (Longo et al., submitted)

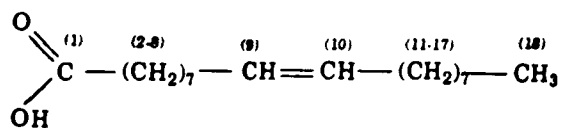
Samples for CD spectra were 0.14 mM or 0.02 mM in colipase and were prepared in starting buffer as described above for NMR samples. Ultraviolet CD was observed with a Jobin-Yvon (ISA) Mark V circular dichograph. Spectra were measured at 25°C in either 1 cm or 5 cm cells, respectively. Circular dichroism results are reported in terms of $\Delta\epsilon_{1-r}$, the difference in the extinction coefficients between left and

right circularly polarized light.

Figure II-1. Structures of monoglycerides, bile salt and fatty acid. Carbons are noted in standard fashion with MG and FA sites denoted by parentheses.

Monoglycerides:**1-monocapryloyl-*rac*-glycerol****1-monooleoyl-*rac*-glycerol****Taurocholate****Fatty Acids:****Caprylic Acid**

(deprotonated at pH 6.5)

**Oleic Acid**

(deprotonated at pH 6.5)

Figure II-2. 1D ^1H 400 MHz NMR spectra used for quantitation of digestive mixtures. A: 10 mM BS solution which was used to calibrate DMSO insert. The labelled peaks (7, 12, 18 and 25) were integrated with respect to DMSO. B: C18 20:80/1:1.25 spectrum with DMSO insert. The extraneous peak at 3.0 is an impurity.

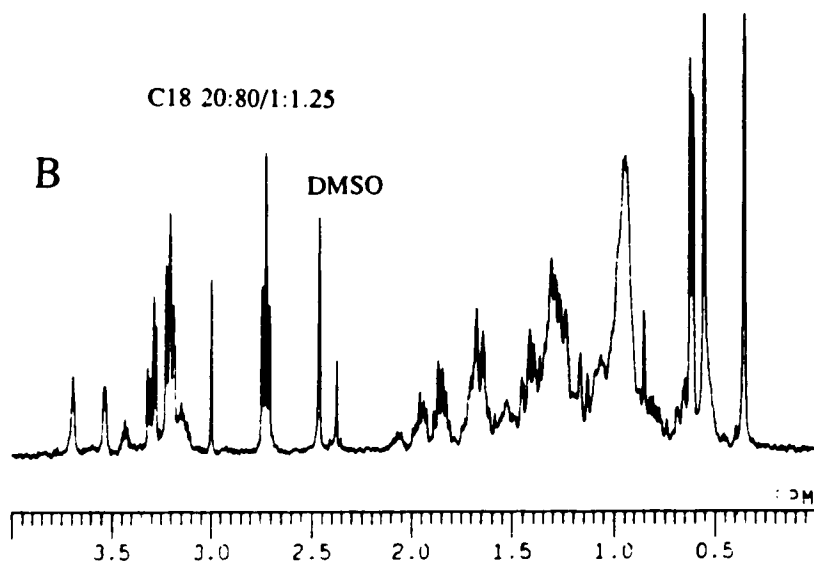
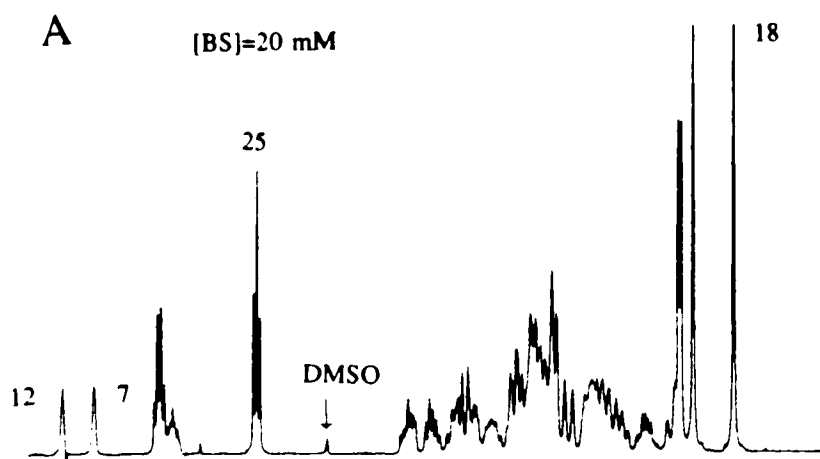


Table II-1 Acyl Chain Length and Concentrations of Components in Digestive Mixture Samples

Sample #	Chain length	MBS:MIL/ FA:MG	[BS]	[FA]	[MG]
23	C8	20:80/ 20:1	20 mM	60 mM	3 mM
27	C8	20:80/ 1:1.25	16 mM	36 mM	28 mM
31	C18	20:80/ 20:1	12 mM	45 mM	2.3 mM
33	C18	20:80/ 1:1.25	11 mM	25 mM	11 mM

Chapter IIIA (Longo et al., 1988)

Introduction to Amino Terminal Segment of the p21 Protein

The 189 amino acid p21 proteins are ras (rat sarcoma viral oncogene) oncogene encoded guanine nucleotide binding proteins whose normal cellular genes code for glycine at residue 12 of the amino acid sequences. Any substitution other than Pro at this position causes malignant transformation of cells. Substitutions at several other locations also result in mutant protein products (Tabin et al., 1982; Reddy et al., 1982; Seeburg et al., 1984; Stacey and Kung, 1984; Santos et al., 1983; Taparowsky et al., 1983).

Although the biological function, including transformation activation, of the ras p21 proteins is not understood in detail, the proteins are postulated to act in a fashion similar to that of the G-proteins (Gilman, 1984; Hurley et al., 1984). G-proteins are guanine nucleotide binding proteins which transduce signals from receptors that have an immediate effect on cellular metabolism; they have significant GTPase activity and are substrates for phosphorylation (Gilman, 1987). G-proteins become activating when the GTP complex is unable to hydrolyze its third phosphate and remains in the bound GTP form, i.e. the biologically active state is the GTP-bound state, the biologically inactive state is the GDP-bound state (Gilman, 1987). The p21 and G-proteins have some sequence homology,

including conservation of glycine at position 12 (Gay and Walker, 1983; Wierenga and Hol, 1983; Kataoka et al., 1985). Additionally, p21 proteins are known to bind with high specificity and affinity to GTP (McGrath et al., 1984; Gibbs et al., 1984; Manne et al., 1985). Based on these similarities, it has been proposed that there are parallels in the mechanisms for GTP hydrolysis and transforming abilities of G- and p21 proteins.

As mentioned above, p21 proteins bind both GTP and GDP. Surprisingly, nucleotide binding constants of both native and mutant proteins are similar (McGrath et al., 1984). Whereas all p21 proteins have GTPase activity, the transforming analogues do show marked decrease in this hydrolytic activity (Gay and Walker, 1983).

X-ray structures of c-H-ras p21 (cellular Harvey rat sarcoma viral oncogene; residues 1 - 171; de Vos et al., 1988) and of Val-12-c-H-ras p21 (residues 1 - 171; Tong et al., 1989) have been reported. Both proteins consist of six β -sheets, four α -helices and nine connecting loops. One of these loops, composed of residues 9 through 18 and hence containing the transforming site at residue 12, is known to participate in phosphate binding of the guanine nucleotides. Of particular interest in the comparison of the structures of the normal and mutant proteins is that the largest differences in structure

between the two proteins occur in this loop (Tong et al., 1989).

Knowledge of the solution structure of the nucleotide hydrolysis site in the p21 proteins, normal and transforming, would serve as a foundation for understanding the mechanism of binding and transforming activity. Since it has been shown that the segment spanning residues 4-21 of p21 binds GTP competitively (only four orders of magnitude less) than the intact protein (Niu et al., 1988) it was postulated that this p21 segment could be independently structured.

The computational programs ECEPP (Empirical Conformational Energies for Peptides and Proteins (Momany et al., 1975) and the chain build-up procedure (Pincus et al., 1982) have been used to determine the different lowest energy conformations for different p21 analogues (Pincus et al., 1983; Pincus and Brandt-Rauf, 1985; Pincus and Brandt-Rauf, 1986). These procedures calculate the lowest free energy conformations of individual amino acids and then "link" the minimized residues and determine the lowest free energy conformation of the polypeptide chain. It was determined using these methods that, for p21 peptide fragments with substitutions at position 12, there are corresponding different lowest-energy conformations (Pincus et al., 1983; Pincus and Brandt-Rauf, 1985; Pincus and Brandt-Rauf, 1986).

Gly-12, the native analogue, was shown to adopt two different low-energy conformations, one of these being inaccessible to the oncogenic analogues. The high degree of flexibility inherent in glycine permitted this peptide to adopt both its lowest-energy conformation (D' conformation, chain-reversal at position 11 and 12) and, to a smaller degree, the lowest-energy conformation found for the analogues (CD' conformation, alpha-helix with a hair-pin turn at residues 12 and 13). Since the analogues are oncogenic (except proline) and cannot adopt the lowest-energy conformation of Gly-12, it has been suggested that these peptides are adopting a "malignancy causing" structure.

Calculated results show that Pro-12 is able to adopt the same D' conformation as Gly-12, thereby remaining non-oncogenic. This is most likely due to the fact that the cyclic proline residue acts as a helix breaker and is more likely than other amino acids to accomodate a chain reversal at position 12. These studies predicted that there should be a lower energy structure which only the non-transforming segments could adopt. This structure was predicted to exist when Gly-12 was in a left-handed conformation (helical structure from residues 6-10 with a hairpin turn at Ala¹¹ which destroys the α -helix) (Pincus and Brandt-Rauf, 1986). Our goal was to determine, using a suitable p21 segment, if the functionally significant conformational differences between

the native and the oncogenic analogues predicted from ECEPP and chain build-up procedure methods could be detected and characterized using NMR spectroscopy.

Chapter IIIB

Results

The results reported in this chapter focus on the synthesized N-terminal fragment of the p21 protein and three analogue peptides which contain a substituted amino-acid at position 12 in the sequence. The fragment chosen for this study spans residues 4 - 20 in the native protein (Tyr⁴-Lys⁵-Leu⁶-Val⁷-Val⁸-Val⁹-Gly¹⁰-Ala¹¹-X¹²-Gly¹³-Val¹⁴-Gly¹⁵-Lys¹⁶-Ser¹⁷-Ala¹⁸-Leu¹⁹-Thr²⁰), where position 12 is glycine (Gly-12). The three analogues contain either aspartic acid (Asp-12), arginine (Arg-12) or proline (Pro-12) at position 12. Asp-12 and Arg-12 are known malignancy causing mutations in the full protein. For the remainder of the text the four peptides are identified as follows: Gly-12, Arg-12, Asp-12 and Pro-12.

The 1D ¹H spectra for the peptides are shown in Fig. III-1. Despite the high S/N and resolution of the spectra it is impossible to assign all the proton resonances due to signal overlap. A possible assignment strategy might include using a modified peptide where one residue is labelled with deuterium. Spectral overlap precludes using this method. Additionally, labelling several residues is a lengthy, expensive and therefore undesirable alternative. It was, therefore, necessary to employ 2D NMR to fully assign the spectra for the peptides.

All proton assignments were made using well established techniques (Wuthrich, 1986). Initial identification of the amino-acid spin systems was made through the scalar coupling patterns in the DQF-COSY spectra. Fig. III-2 shows the NH- α CH "fingerprint" region of the DQF-COSY spectrum for Asp-12, and Fig. III-3 shows an entire DQF-COSY spectrum where the Asp-12 scalar connectivity (resulting in the resonance assignments for this amino-acid) is shown. The repetition of several amino-acids residues requires the use of sequential NOEs (obtained from NOESY spectra) to complete assignments and remove any remaining ambiguities. Between the NOESY and ROESY spectra of each p21 peptide studied all the sequential α CH_i-NH_{i+1} connectivities, which are necessary for assigning replicated residues correctly in the sequence, are observed. For Pro-12, which does not contain an amide proton, the analogous α CH- δ CH connectivity is observed.

Table III-1 compares the chemical shifts for each of the protons in each of the four p21 peptides studied. Chemical shifts are within 0.22 ppm of the corresponding resonances in each p21 analogue peptides. The significance of this result is discussed below.

Several NMR techniques can be employed to determine the presence or absence of secondary structure, with the most informative and specific being NOESY experiments. In addition,

analysis of both amide temperature coefficients and $^3J_{\alpha-NH}$ coupling constants can reveal the presence of strong intramolecular hydrogen bonding. This type of bonding is needed to stabilize the structural features in flexible peptides. Table III-2 shows the temperature coefficients (reported in ppm K⁻¹) experimentally determined for the amide protons of Asp-12. All values were greater than 2×10^{-3} ppm K⁻¹ which indicates a lack of strong intermolecular hydrogen bonding between interresidue p21 protons (Balaram, 1985; Kessler, 1982).

Table III-3 shows the $^3J_{\alpha-NH}$ coupling constants obtained for some of the amide protons in the p21 peptides. The $^3J_{\alpha-NH}$ coupling constants (which were estimated where possible from peak splitting in high-resolution 1D spectra) are all (except glycine, which has a smaller J value when the 2 α protons are non-degenerate) in the range expected (7-9 Hz) for rapid conformational averaging (Wuthrich, 1986; Balaram, 1985).

To determine unequivocally the absence of long-range intramolecular bonding in the peptides, NOESY and ROESY spectra were acquired. NOESY spectra contain information about protons spins which are close in space (generally less than 5Å). Secondary structure is confirmed by the presence of non-sequential NOE crosspeaks. A lack of such features indicates that the molecule is undergoing rapid conformational averaging

and generally means that a long-lived secondary structural feature does not exist.

The rotating-frame 2D NOE experiment, ROESY, is more sensitive to short correlation times modulating interproton dipolar interactions than is the laboratory-frame experiment (Bothner-By et al., 1984; Davis and Bax, 1985). Because linear peptides often have subnanosecond motions (Jelicks et al., 1988), ROESY spectra were obtained for Asp-12, Arg-12 and Gly-12 in order to determine if any interactions are transparent to the NOESY experiment. For example, a "missing" sequential α CH-NH NOE between Gly10 and Ala 11 is "found" in the ROESY spectrum of Asp-12. However, for none of the p21 peptides studied were long-range NOESY or ROESY crosspeaks observed.

Chapter IIIC

Discussion

Evidence from NMR spectroscopic data indicates that the p21 fragment (residues 4 - 20; Gly-12) and its three analogues, Asp-12, Arg-12 and Pro-12, do not have long-lived structural features in DMSO solution. The strongest evidence for this conclusion comes from the NOESY data of the four peptides. None of the spectra collected contain any crosspeaks other than the intra-residue and sequential types expected for peptides which are undergoing rapid conformational averaging. The absence of crosspeaks resulting from long-range interactions indicates a lack of any long-lived ordered secondary structure (e.g. α -helix or β -sheet) (Wright et al., 1988). Specifically, we find no spectroscopic evidence for the loop which was predicted to be found in the oncogenic peptides. The absence of strong intramolecular hydrogen bonding and the presence of rapid conformational averaging is further confirmed by analysis of the amide temperature coefficients (for Asp-12 only, vide infra) and coupling constant data, respectively.

For peptides which are undergoing rapid conformational changes, the average $^3J_{\alpha-NH}$ values are expected to be greater than 9 Hz or less than 7 Hz (Wuthrich, 1986; Balaram, 1985). Almost all coupling constant values for the peptides studied are less than 7 Hz. For Asp-12, the values of the temperature

coefficients indicate a lack of strong hydrogen bonding (Balaram, 1985). Since Asp-12 is postulated to contain a loop structure and we found no spectroscopic evidence to support this, the amide temperature coefficients were not determined for Gly-12 or the remaining two analogues.

The chemical shift of a particular proton is determined not only by the applied magnetic field B_0 , but also by the much smaller local magnetic fields which are generated by surrounding electrons (charges, dipoles, double bonds etc.). The closer a proton is, in general, to a source of electrons the more downfield it is shifted from its "ideal" chemical shift. Conversely, a proton which is "shielded" in some way from electrons will resonate at a chemical shift which will be characteristically located upfield (e.g. vinylic protons will resonate at lower field than methylene protons). In addition to being a way of identifying various types of protons in a molecule, the chemical shift of the same proton in different analogues of a molecule supplies information about any electronic changes which the proton is feeling as a result of a mutation. The p21 peptides studied in this investigation are all chemically equivalent except for the amino acid which is located in position 12. A significant change in chemical shifts of a p21 proton, with respect to the chemical shift of the same proton in a different analogue, could indicate a structural change in the peptide. We find, however, that a

conservation of chemical shifts throughout the series suggests that no gross structural change has taken place. Surprisingly, even the amino acids which are directly next to position 12 show little change in chemical shift (for amide protons) from one analogue to another.

The results presented herein unequivocally show that that the p21 fragment and its analogues do not contain any long lived identifiable secondary structural features in DMSO solution. It seems reasonable to assume then that a larger portion of the macromolecule if not the entire protein must be present for the proper folding to take place. This result is not unreasonable since a loop structure is highly flexible and would not necessarily be expected to contain many intramolecular interactions like its more defined structural counterparts (α -helix, β -sheet and turns).

Figure III-1. 1D ^1H 400 MHz NMR spectra of A: Asp-12, B: Arg-12, C: Gly-12 and D: Pro-12 in DMSO-d_6 at 25°C.

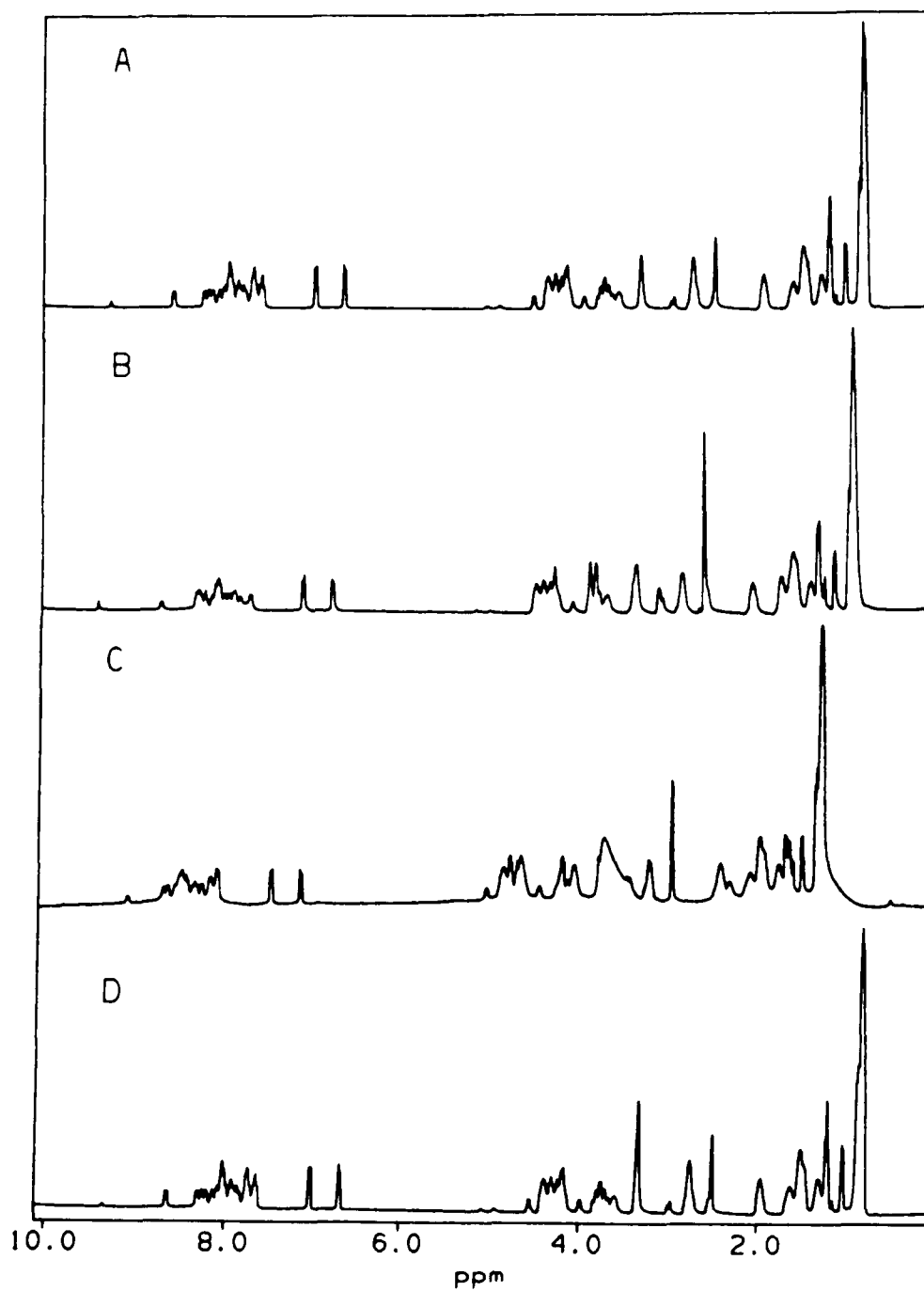


Figure III-2. NH- α CH "fingerprint" region of the DQF-COSY spectrum of Asp-12 in DMSO.

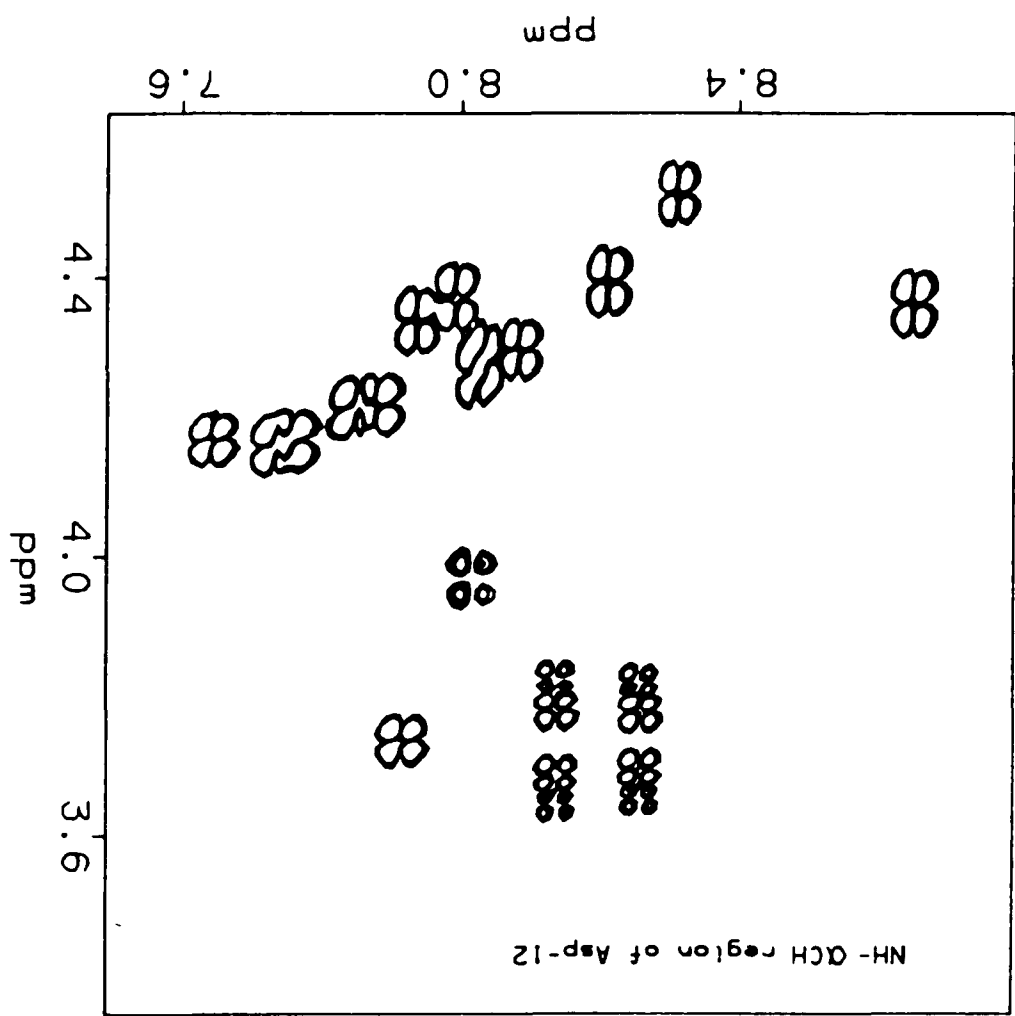


Figure III-3. DQF-COSY spectrum of Asp-12 in DMSO. The complete assignment of the proton resonances for one residue is shown by the solid lines.

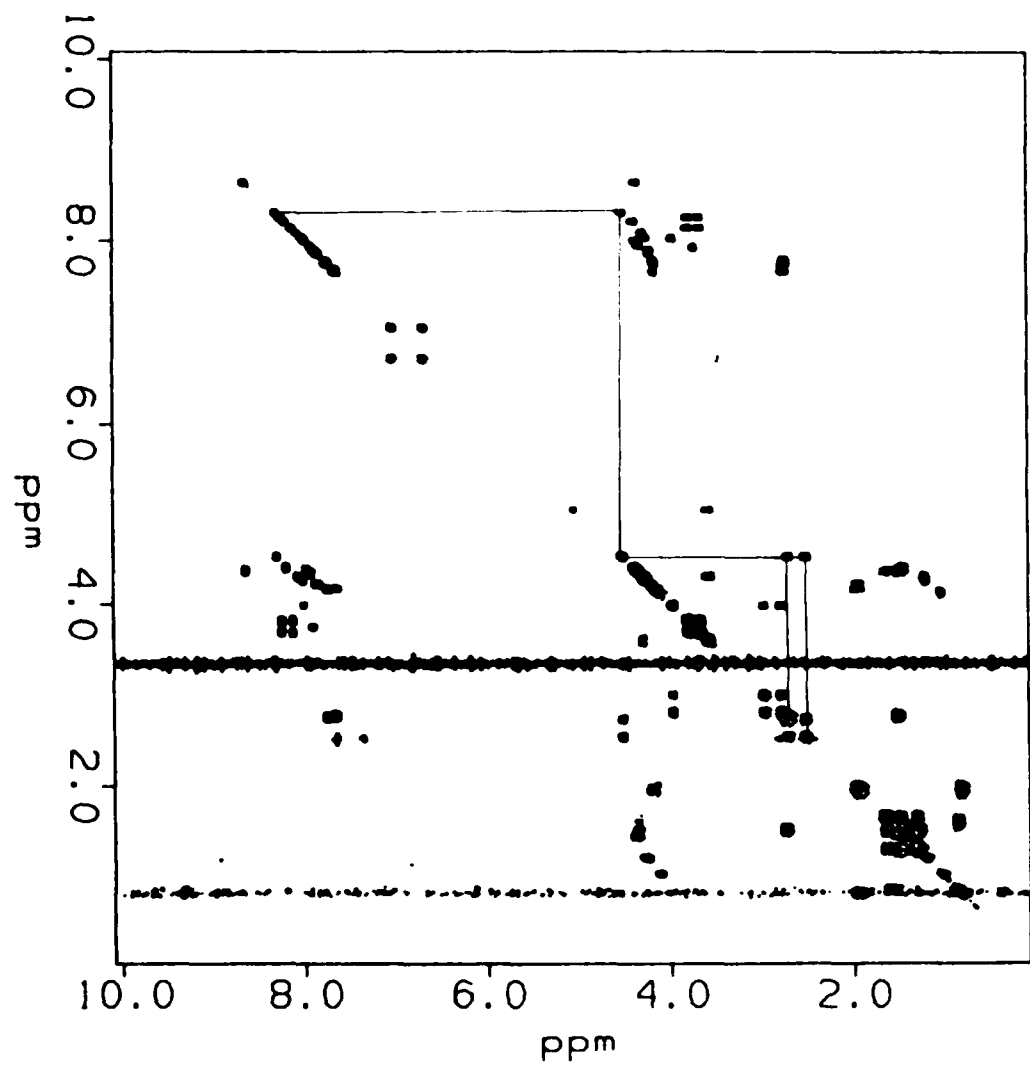


Table III-1. ¹H assignments for p21 peptide and analogues

Residue	Peptide	NH	α CH	β CH	γ CH	Δ CH	other
Tyr 4	Asp-12	8.02	3.99	2.72 2.98			ring 7.04 6.70
Tyr 4	Arg-12	====	3.92	2.75 2.97			7.14 6.78
Tyr 4	Gly-12	===	3.94	2.76 2.96			7.00 6.66
Tyr 4	Pro-12	===	3.94	2.76 2.96			
Lys 5	Asp-12	8.65	4.37	1.65	1.30	1.53	ϵ CH ϵ NH 2.76 7.67
Lys 5	Arg-12	8.59	4.36	1.68	1.30	1.54	2.77 ===
Lys 5	Gly-12	8.61	4.34	1.52	1.41	1.62	2.99 ===
Lys 5	Pro-12	8.61	4.34	1.64	1.28	1.50	2.73 7.00
Leu 6	Asp-12	8.21	4.40	1.49	1.61	0.84	
Leu 6	Arg-12	8.21	4.39	1.45	1.62	0.86	
Leu 6	Gly-12	8.17	4.37	1.43	1.59	0.84	
Leu 6	Pro-12	8.16	4.37	1.44	1.59	0.84	
Val 7	Asp-12	7.88	4.22	1.96	0.82		
Val 7	Arg-12	7.87	4.22	1.94	0.82		
Val 7	Gly-12	7.85	4.20	1.93	0.82		
Val 7	Pro-12	7.84	4.18	1.93	0.79		
Val 8	Asp-12	7.86	4.22	1.96	0.82		
Val 8	Arg-12	7.78	4.15	1.94	0.82		
Val 8	Gly-12	7.79	4.19	1.93	0.82		
Val 8	Pro-12	7.78	4.18	1.93	0.82		

Val 9	Asp-12	7.76	4.17	1.96	0.82		
Val 9	Arg-12	7.87	4.22	1.94	0.82		
Val 9	Gly-12	7.72	4.13	1.93	0.82		
Val 9	Pro-12	7.68	4.14	1.93	0.79		
Gly 10	Asp-12	8.13	3.81 3.68				
Gly 10	Arg-12	8.29	3.75				
Gly 10	Gly-12	8.11	3.75 3.67				
Gly 10	Pro-12	8.08	3.73 3.61				
Ala 11	Asp-12	8.03	4.28	1.22			
Ala 11	Arg-12	7.97	4.29	1.24			
Ala 11	Gly-12	7.98	4.25	1.20			
Ala 11	Pro-12	8.00	4.52	1.17			
Asp 12	Asp-12	8.31	4.54	2.73 2.53			
Arg 12	Arg-12	8.15	4.25	1.71 1.54	1.49	3.09	7.74
Gly 12	Gly-12	8.19	3.69				
Pro 12	Pro-12		4.27	2.00	1.81	3.55	
Gly 13	Asp-12	7.92	3.74				
Gly 13	Arg-12	8.10	3.77				
Gly 13	Gly-12	7.96	3.75				
Gly 13	Pro-12	8.04	3.69				
Val 14	Asp-12	7.73	4.18	1.96	0.82		
Val 14	Arg12	7.84	4.18	1.97	0.82		
Val 14	Gly-12	7.78	4.15	1.93	0.82		
Val 14	Pro-12	7.60	4.14	1.93	0.79		

Gly 15	Asp-12	8.26	3.81 3.68				
Gly 15	Arg-12	8.18	3.78 3.66				
Gly 15	Gly-12	8.22	3.76 3.68				
Gly 15	Pro-12	8.21	3.75 3.66				
Lys 16	Asp-12	7.94	4.34	1.65	1.30	1.53	2.76 7.67
Lys 16	Arg-12	8.00	4.33	1.68	1.30	1.54	2.77 ===
Lys 16	Gly-12	7.89	4.32	1.52	1.41	1.50	2.72 ===
Lys 16	Pro-12	7.88	4.31	1.64	1.28	1.50	2.73 7.00
Ser 17	Asp-12	8.04	4.29	3.58			
Ser 17	Arg-12	7.97	4.29	3.60			
Ser 17	Gly-12	8.03	4.27	3.55			
Ser 17	Pro-12	8.02	4.26	3.56			
Ala 18	Asp-12	8.09	4.30	1.22			
Ala 18	Arg-12	8.06	4.31	1.20			
Ala 18	Gly-12	7.98	4.25	1.20			
Ala 18	Pro-12	7.98	4.25	1.20			
Leu 19	Asp-12	8.00	4.38	1.48	1.62	0.84	
Leu 19	Arg-12	8.15	4.30	1.48	1.62	0.86	
Leu 19	Gly-12	7.95	4.35	1.43	1.59	0.84	
Leu 19	Pro-12	7.94	4.34	1.44	1.59	0.84	
Thr 20	Asp-12	7.65	4.14	4.14	1.04		
Thr 20	Arg-12	7.49	4.07	4.07	0.99		
Thr 20	Gly-12	7.60	4.15	4.10	1.20		

Thr 20	Pro-12	7.60	4.14	4.10	1.02		
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Table III-2. Temperature Coefficients ($\Delta\delta_{\text{NH}}/\Delta T$) for Asp-12 in DMSO

Residue	$\Delta\delta_{\text{NH}}/\Delta T$ ($\times 10^3$) ppm K ⁻¹
Lys 5	5.0
Leu 6	5.0
Val 7	5.0
Val 8	4.0
Val 9	5.3
Gly 10	4.6
Ala 11	5.0
Asp 11	5.0
Gly 13	3.3
Val 14	4.0
Gly 15	4.7
Lys 16	5.0
Ser 17	6.0
Ala 18	5.5
Leu 19	4.0
Thr 20	6.5

Table III-3. $^3J_{\alpha-NH}$ Values for p21 Peptides in DMSO

Peptide	Residue	J (Hz)
Asp-12	Lys 5	8.1
Asp-12	Leu 6	7.7
Asp-12	Gly 10	4.6, 5.3
Asp-12	Asp 12	7.7
Asp-12	Ser 17	7.5
Gly-12	Lys 5	7.6
Gly-12	Val 7	8.8
Gly-12	Val 9	8.4
Gly-12	Gly 10	4.8, 5.0
Gly-12	Gly 12	7.4
Gly-12	Gly 13	7.4
Gly-12	Lys 16	7.7
Gly-12	Thr 20	8.5
Pro-12	Lys 5	8.2
Pro-12	Leu 6	7.8
Pro-12	Val 8	8.9
Pro-12	Val 9	8.4
Pro-12	Val 14	8.1
Pro-12	Gly 15	5.1, 5.4
Pro-12	Thr 20	8.1
Arg-12	Lys 5	7.5
Arg-12	Gly 15	4.6, 5.0
Arg-12	Ala 18	8.2
Arg-12	Thr 20	7.3

Chapter IVA (Longo et al., submitted)**Introduction to Porcine Pancreatic Colipase**

Pancreatic lipase is a digestive enzyme that hydrolyzes triglycerides to alcohols and fatty acids in the duodenal lumen. When the triglyceride substrates become covered with bile-salt (BS) micelles the activity of lipase is inhibited, possibly because of the inability of lipase to anchor itself to the micellar surface (Wieloch and Falk, 1978). The digestive activity of lipase is restored in the presence of a 95 amino-acid cofactor called colipase (CLP) that is secreted from the pancreas (Wieloch and Falk, 1978; Chapus et al., 1981; Borgstrom et al., 1979; Erlanson-Albertsson, 1982). The mechanism for this reactivation is not known at present, but it has been postulated that two hydrophobic patches on colipase are involved (Canioni et al., 1980; Cozzzone et al., 1981). It has been suggested that colipase and lipase form a 1:1 complex that enables lipase to remain anchored to the BS-covered triglyceride interface (Erlanson-Albertsson, 1982).

The importance of the aromatic residues of CLP in the interactions of the protein with bile salts and lipase has been suggested by several types of electronic spectroscopy. Donner et al. (1976) demonstrated in circular dichroism (CD) studies that binding of taurodeoxycholate (TDC) to CLP alters the environment of the tyrosine and phenylalanine residues but leaves the conformation of the polypeptide backbone unchanged.

Additionally, De Caro et al. (1983) showed that the CD spectrum of colipase containing nitrated tyrosine residues is changed markedly by the presence of BS micelles. Involvement of tyrosine in CLP-TDC binding was confirmed by several fluorescence studies. Granon observed spectral perturbations at the tyrosines (55,58,59) and at fluorescent-labeled lysine 60 upon complex formation (Granon, 1986). Using steady-state and time-resolved fluorescence of dansyltyrosine CLP derivatives, McIntyre et al. (1990) showed that Tyr 55 rotates freely in a polar surface-exposed environment. By contrast, Tyr 59 undergoes hindered rotation in a somewhat less polar environment; it rotates more freely upon TDC binding (McIntyre et al., 1990a; McIntyre et al., 1990b). The buried location of Tyr 58 is judged from its low reactivity in chemical modification studies (McIntyre et al., 1990a)

A more detailed view of interactions between BS micelles and CLP was obtained by ¹H NMR and the hybrid method of photo-CIDNP (Cozzzone et al., 1981). Addition of TDC micelles was shown to broaden and shift several of the protein's aromatic-proton NMR signals (two Tyr's and one His) as well as to suppress completely the signals for two tyrosines in the photo-CIDNP experiment. Based on this evidence it was proposed that folding of the protein brings two aromatic regions into close proximity and forms a hydrophobic binding site for TDC micelles. Although all of the

foregoing studies point to the importance of aromatic residues in this enzyme, the involvement of UV-inaccessible amino-acid sidechains in its binding phenomena should not be ruled out.

Multidimensional ^1H NMR (Chazin et al., 1988; Tappin et al., 1989) is a logical next step in structural investigations of CLP, since complete sequential assignments should be feasible for a protein of this size (11.5 kDa) that is reported to be heat stable and monomeric at millimolar concentrations (Borgstrom et al., 1979; Charles et al., 1975). The presence of five disulfide structural constraints should facilitate a determination of the enzyme's three-dimensional structure, which in turn may reveal potential binding sites formed by foldover of the hydrophobic aromatic regions of CLP. This report draws on the results of NMR and fluorescence experiments to revise and extend prior spectral identifications of the aromatic amino acids in porcine pancreatic colipase and to confirm the importance of hydrophobic interactions among these residues. In contrast to prior physical measurements (Borgstrom et al., 1979; Charles et al., 1975) and the reasonable assumptions of earlier NMR work on this protein (Canioni et al., 1980; Cozzzone et al., 1981), it is demonstrated that CLP above 0.09 mM forms dimers as well as monomers over a wide range of temperature and pH. This new finding precludes more extensive conformational studies of CLP in aqueous solution and presents the

possibility that the hydrophobic interactions deduced by us and others (Canioni et al., 1980; Cozzone et al., 1981) may well be intermolecular.

Chapter IVB

Results

Aromatic Proton Spin Systems

The one-dimensional (1D) ^1H NMR spectra of CLP in H_2O and D_2O are shown in Figs. IV-1 and IV-2. While it is possible to make some tentative assignments based on established chemical-shift trends for the constituent amino acids (Wuthrich, 1986), most definitive identifications are precluded by spectral overlap. CLP contains only seven aromatic residues (Fig. IV-3), producing a relatively uncrowded aromatic region (Figs. IV-1 and IV-2). Previous investigations used 1D NMR spectroscopy in conjunction with pH titrations to make assignments for some of these aromatic protons (Cozzone et al., 1981; Canioni et al., 1984; Wieloch et al., 1979; Canioni and Cozzone, 1979). For instance, three of the peaks furthest upfield in the aromatic region (6.61, 6.89 and 7.04 ppm) were assigned previously to the 3,5-orthophenyl protons on the three tyrosine rings. It was assumed that four metaphenyl protons were located under the downfield peaks, with the doublet at 7.18 ppm assigned to the remaining 2,6-metaphenyl protons (*vide infra*).

In the present work, the application of 2D NMR methods makes it possible to assign many of the remaining aromatic protons within each amino-acid spin system. Crosspeaks that are present in the aromatic region of DQF-COSY spectra in both

H₂O and D₂O (Fig. IV-4) arise exclusively from pairwise interactions of the 24 non-exchangeable aromatic protons in CLP (10 from 2 Phe, 12 from 3 Tyr and 2 from 2 His 4H's). For instance, Fig. IV-4 displays scalar couplings between each set of Tyr orthophenyl protons and their corresponding metaphenyl protons. This region of the 2D COSY spectra confirms previous assignments for the 6 orthophenyl protons and 2 of the 6 metaphenyl protons. In addition, it establishes through scalar couplings the first unambiguous assignments of the remaining 4 metaphenyl protons (Table IV-1). Sequential assignments are discussed in the following section. Without 2D NMR methods, no assignments to the individual phenylalanines could be made (Canioni et al., 1984). From the COSY spectra (inset, Fig. IV-4A) it is now possible to assign all ten of the phenylalanine resonances to two spin systems (Table IV-1).

Histidine 2H protons characteristically resonate upfield and appear as sharp singlets. From the 1D spectrum in D₂O (Fig. IV-1B) it is clear that two of the histidine 2H protons in CLP appear as sharp singlets at 8.72 and 8.61 ppm, respectively. DQF-COSY spectra in D₂O reveal one crosspeak (8.72, 7.43 ppm; not shown) attributable to the long-range coupling sometimes observed between the 2H and the 4H ring protons of histidine (DeCelle et al., unpublished observations). Integration of the aromatic region shows that in addition to the 23 assigned resonances, the spectrum

includes the remaining histidine 4H proton and three exchangeable sidechain NH protons. Based on careful integration of the region between 6.5 and 6.95 ppm, the unresolved His 4H proton may be located at 6.90 ppm (Table IV-1). The use of this long-range coupling to make sequential assignments of the His residue is discussed below.

Hydrophobic Interactions

As noted above, it has been postulated that two hydrophobic aromatic regions of CLP are brought into proximity by protein folding. Evidence for through-space interactions between pairs of aromatic residues comes from NOESY experiments run in both H₂O or D₂O. Fig. IV-5 shows that both spectra contain a crosspeak at 7.41, 6.87 ppm. This crosspeak must indicate an interaction between Tyr II and either Phe II or His II, since the exchangeable sidechain protons do not contribute NMR signal intensity at either of these chemical-shift positions. Both of these possibilities are consistent with the suggested interaction between the two binding domains of CLP (Cozzzone et al., 1981). Supporting evidence for hydrophobic interactions that bury some residues within the protein's three-dimensional structure comes from the observation that 20% of the amide protons in this protein do not exchange with solvent, even over a period of several months.

At least 20 α CH- α CH crosspeaks are also observed in the NOESY spectrum of CLP (data not shown). This observation is in accord with the 25% estimate of β -sheet structure made from CD experiments and predictive methods (Canioni et al., 1980).

Self Association of CLP

Fig. IV-6A shows that many of the 95 expected crosspeaks are absent from the crucial NH- α CH "fingerprint" region of the COSY spectrum, suggesting problems with either the NMR setup or the CLP sample. Possible saturation of the α CH protons during irradiation of the H₂O resonance may be ruled out by conducting parallel DQF-COSY experiments in D₂O, for which much more modest radiofrequency power is required. In D₂O, the 18 non-exchanging NH's (found from integration of the spectrum in Fig. IV-1B) still yield only 10 COSY crosspeaks (Fig. IV-6B). In H₂O, systematic variation of the sample pH and temperature gives a maximum of approximately 30 crosspeaks (Fig. IV-6C). Possible difficulties with spectrometer sensitivity or pulse sequences are ruled out by the observation of numerous strong crosspeaks when the DQF-COSY protocol is applied to a 2 mM lysozyme sample.

Could the colipase entity observed by NMR be large enough to produce antiphase cancellation of COSY crosspeaks such as that reported (Veitch et al., 1990; Torchia et al., 1989) for proteins larger than 15 kDa? A molecular weight of 11 kDa has

been determined by SDS-PAGE (Erlanson-Albertsson, 1982); the protein is expected to remain monomeric at 2 mM, judging from prior studies by ultracentrifugation (Charles et al., 1975), 1D NMR (Borgstrom et al., 1979), and neutron scattering (Charles and Semeriva, 1980). Although the CLP solutions are optically clear and EDTA is present to prevent paramagnetic broadening of protein resonances, its ^1H spectral linewidths exceed those of lysozyme (molecular weight 14 kDa), and it exhibits inefficient magnetization transfer in TOCSY experiments (data not shown). Neither the linewidths nor the crosspeak patterns depend on whether the samples are lyophilized, despite reports that CLP may aggregate under these circumstances (Charles and Semeriva, 1980).

SDS-PAGE of CLP yields a molecular weight of 11.5 kDa, in agreement with published reports (Erlanson-Albertsson, 1982). By contrast, the results obtained from gel electrophoresis performed under non-denaturing conditions indicate heavier species (Fig. IV-7). Two bands corresponding to approximate sizes of 14 and 28 kDa, respectively, are present for these and other 0.09 mM CLP samples. Thus the majority of CLP seen by ^1H NMR exists in self-associated form at 2 mM and pH 5.7.

Fig. IV-8 shows the circular dichroism (CD) spectra for 0.02 and 0.14 mM CLP. At the higher concentration a broader peak is observed, indicating enhanced light scattering from a

larger dimeric species (Urry and Krivacic, 1970). The decrease in optical activity between 250 and 300 nm may be attributable to scattering by the larger aggregate, or it may reveal a surprising drop in asymmetry that accompanies self-association of the CLP monomers. A similar trend in CD behavior has been reported for this protein upon complexation with TDC micelles (Donner et al., 1976). Although a detailed interpretation of the spectra in this near-UV region would require knowledge of the separate contributions from Tyr, Phe and disulfide groups, dependence of the CD data on concentration is consistent with the aggregation phenomena deduced from NMR and electrophoretic studies.

Minimization of CLP Aggregation

Non-invasive means such as raising the sample temperature should promote the formation of CLP monomers (and thus avoid cancellation of its COSY crosspeaks). When the solution pH is also lowered to slow the rate of amide exchange with solvent protons, it is possible to triple the number of crosspeaks in DQF-COSY spectra, as noted above in Fig. IV-6C. Nonetheless 95 crosspeaks are expected in this region. Increasing the temperature to 55°C effects no further increase in the number of crosspeaks, and heating above 60°C causes coalescence of the amide proton signals (suggesting some denaturation of the protein).

Previously reported studies indicated that CLP forms a 1:1 complex with TDC micelles (Charles et al., 1975; Charles and Semeriva, 1980). In an effort to break up the CLP aggregates and form a modest-sized CLP:micelle complex, aliquots of taurocholate (TC) solution were added systematically. TC was preferred over other bile salts because of the small primary micelles it forms under the conditions of these experiments (Carey and Small, 1969). TC added at concentrations up to 5 mM broadens some peaks in the CLP spectrum and actually produces a DQF-COSY α CH-NH region with fewer crosspeaks than the previously-collected spectra (data not shown). Thus the CLP aggregates are not broken apart; any TC-CLP complexes appear to be larger in size than CLP dimers.

Given that hydrophobic interactions are thought to be responsible for CLP binding to both lipase and BS micelles (Borgstrom et al., 1979; Donner et al., 1976), it seems reasonable that the aggregation of CLP could also be driven by interactions between the two hydrophobic aromatic regions of the protein. To minimize this hydrophobic interaction a "concentrated" (2 mM) sample of CLP was diluted progressively to 0.09 mM. The dramatic decrease in linewidth and increase in spectral resolution (Fig. IV-9) indeed suggests that dilution diminishes the degree of protein self-association. Nevertheless, gel electrophoresis indicates that at least 50% of CLP still exists as a dimer at this "low" concentration

(Fig. IV-7) .

Chapter IVC

Discussion

Porcine pancreatic colipase would appear to be an ideal candidate for 2D NMR studies of solution-state conformation: it is moderate in size, it is highly soluble in water, it has structural constraints imposed by disulfide bridges, and it is proposed to bind BS micelles at localized regions (Borgstrom et al., 1979; Cozzone et al., 1981; Charles and Semeriva, 1980). Our finding of dimer formation at concentrations above 0.09 mM clearly precludes such NMR investigations, but both the identifiable aromatic amino-acid sites and the self-association itself provide significant insight into the behavior of the CLP protein.

The aromatic region of colipase has been the focus of the majority of the work done to date (Donner et al., 1976; Granon, 1986; McIntyre et al, 1990a; McIntyre et al., 1990b; Wieloch et al., 1979), though not only because this area is most easily probed via UV, CD, fluorescence and NMR spectroscopies. The three tyrosines also represent the only conserved residues in CLP for different species, suggesting an important role for these amino-acid sites in binding to BS, substrates and lipase (Granon, 1986). Thus the aromatic residues of colipase should yield a disproportionate amount of interesting information, if sequential assignments can be deduced from the aromatic protons in the NMR spectrum.

Earlier NMR investigations assigned the 3,5-orthophenyl protons of the three tyrosine residues and the histidine aromatic protons by using pH titration and selective decoupling experiments (Wieloch and Falk, 1978; Canioni and Cozzone, 1979; Cozzone, 1976). However, due to the inherent limitations of the pH titration method and substantial overlap in the 1D NMR spectrum, it was impossible to assign any of the phenylalanine aromatic protons or most of the 2,6-metaphenyl tyrosine protons. With the aid of 2D methods all resonances in the aromatic region have now been assigned to individual spin systems.

Only the His II residue of CLP exhibits a long-range scalar interaction between its 2H and 4H protons (8.72, 7.43 ppm); its well-resolved 2H proton also has a narrower NMR peak than the analogous resonance of His I. It is likely that the linewidths of the His I ring protons exceed their long-range coupling constant and preclude observation of the COSY crosspeak (Wagner, 1990). His I, which thus appears to be immobilized and is probably located in the protein interior, has now been assigned to His 30 (Table IV-2). Prior investigators made the opposite assignment of histidine residues (Canioni and Cozzone, 1979). Although it was argued that the pH dependence of the His II 2H and 4H proton resonances was perturbed by a proximal carboxyl group from Asp 31 (Fig. IV-3), such an effect could also be produced if the

disulfide linkage vicinal to His 88 brings the carboxyl group of Glu 16 into contact with the ring protons of this histidine.

All three of the tyrosine residues in CLP are located in close proximity in the protein sequence (55, 58 and 59). Nevertheless there are obvious environmental differences among these residues, as revealed by the large variations in chemical shift and linewidth of their aromatic resonances. The assignments reported here for the 3,5 tyrosine protons are in agreement with previously published work (Wieloch and Falk, 1978; Canioni and Cozzone, 1979; Cozzone, 1976). It is noteworthy that the aromatic protons of Tyr III have much broader resonances and diminished spectral resolution in comparison to those of Tyr I and II (Fig. IV-2). The COSY crosspeak between the 2,6 and 3,5 protons of Tyr III is also anomalously weak (Fig. IV-4B). Observations of broad linewidths and weak COSY crosspeaks indicate preferential dynamic restrictions for Tyr III and suggest an interior location in the three-dimensional structure of CLP. Since Tyr 58 is inaccessible to chemical modification techniques (McIntyre et al., 1990a), Tyr III has now been assigned to position 58 (Table IV-2). Tyr I and II, with probable surface locations deduced from photo-CIDNP experiments (Canioni et al., 1980; Cozzone et al., 1981), are assigned to positions 55 and 59 (Table IV-2). For Tyr 55 and Tyr 59, locations at or

near the surface were also suggested by fluorescence results (McIntyre, et al., 1990a; McIntyre et al., 1990b). Thus our 2D NMR results support a revision of published assignments for Tyr 55 and Tyr 58 and confirm definitively the complete peak assignments for each of the aromatic amino acid spin systems.

These sequential assignments allow us to evaluate the through-space interactions observed in NOESY spectra in terms of various proposals regarding hydrophobic interactions, " β -clam" structures, and bile-salt binding sites for the CLP protein (Canioni et al., 1980; Cozzone et al., 1981; Sacchetini et al., 1989). The β -clam structural motif, which has been postulated for many hydrophobic ligand-binding proteins (Sacchetini et al., 1989), is given some credence by two overall features of the NMR data. First, the importance of hydrophobic interactions is demonstrated by the fact that 20% of CLP amide protons fail to exchange with solvent, even during many months at neutral pH. Second, the significant density of NOESY crosspeaks in the α CH- α CH region is consistent with the 25% estimate of β -sheet structure deduced from CD experiments and predicted using the methods of Chou and Fasman (1974).

The issue of specific interactions between hydrophobic-aromatic regions of CLP may be addressed through our observation of a significant crosspeak (6.87, 7.41 ppm) in the

NOESY spectrum (Fig. IV-5). It is clear that the through-space interaction involves the 3,5 protons of Tyr 55/59, but the other proton may belong to either Phe II or His 88. If the resonance at 7.41 ppm belongs to His 88, or if Phe II is identified as Phe 84, then the NOESY crosspeak provides direct evidence for proximity of the two hydrophobic-aromatic regions. These amino acids may approach one another in intra- and/or intermolecular fashion, since dimers are present in the solution (vide supra). Judging from the high solution concentrations and similar linewidths reported in prior NMR studies (Donner et al., 1976; Granon, 1986; Wieloch et al., 1979), published hypotheses regarding hydrophobic-aromatic binding sites should be reevaluated given the likelihood that CLP dimers were present.

It should be emphasized that standard SDS-PAGE determinations of protein molecular weight (including our own) give a falsely optimistic prospectus for NMR structural studies, since they are typically conducted in the presence of detergent and with solutions 1-2 orders of magnitude more dilute than used for multidimensional NMR. Despite prior reports of CLP monomers in ultracentrifugation and neutron-scattering studies (Charles et al., 1975; Charles and Semeriva, 1980) and our SDS-PAGE determination of 11.5 kDa for the protein molecular weight, a significant dimer population is evident above 0.09 mM based on native-PAGE, CD and NMR

experiments. The tendency for CLP to form dimers is quite reasonable in retrospect, both because of its hydrophobicity and in light of its re-lacitran folding behavior (Ernst and Behnke, 1991). Its self association demands caution when planning conformational studies of ligand-binding proteins, but two-dimensional NMR nevertheless gives us a preliminary view of the hydrophobic interactions that drive such aggregation behavior.

Figure IV-1: 1D ^1H NMR spectra at 400 MHz of 2 mM porcine pancreatic colipase (CLP) at 20°C and pH 5.7. Samples included 150 mM NaCl, 50 mM phosphate buffer, 50 μM EDTA, and 0.02% NaN_3 ; (A) H_2O ; (B) D_2O . A comparison of the two spectra shows that 20% of the amide protons exchange very slowly with solvent.

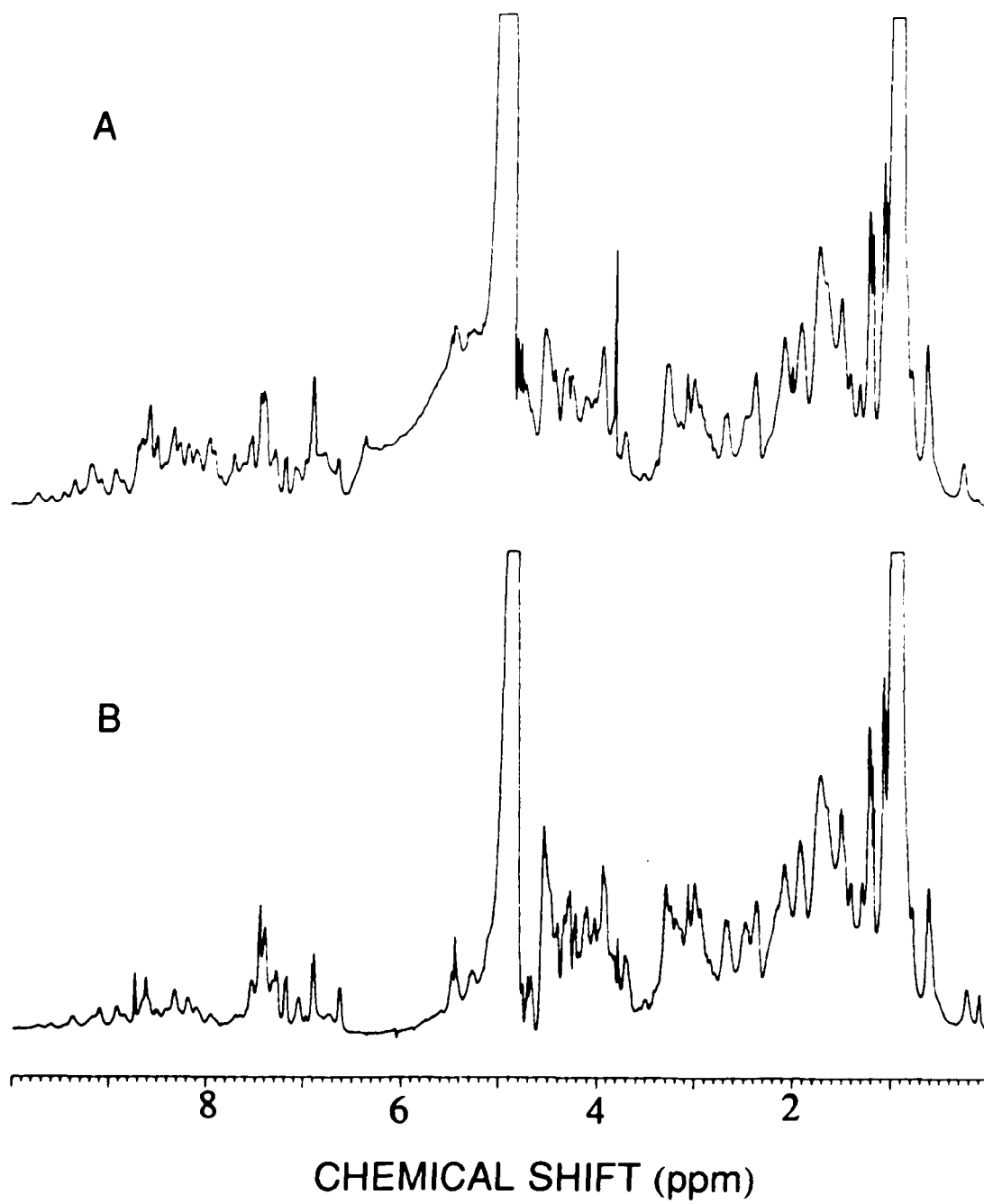


Figure IV-2: Aromatic region of the 1D ^1H NMR spectrum of CLP in D_2O . Sample conditions were as described for Fig. IV-1. Scalar connectivities obtained from DQF-COSY spectra are shown by arrows: Tyr (solid lines); Phe I (dotted lines); Phe II (dashed lines). His ring protons ortho to amide nitrogens (His 4H) also resonate in this region (see text for assignments). The observed spectral peaks match those reported previously (Canioni and Cozzone, 1979; Cozzone, 1976); small differences from published chemical shifts are due to variations in pH.

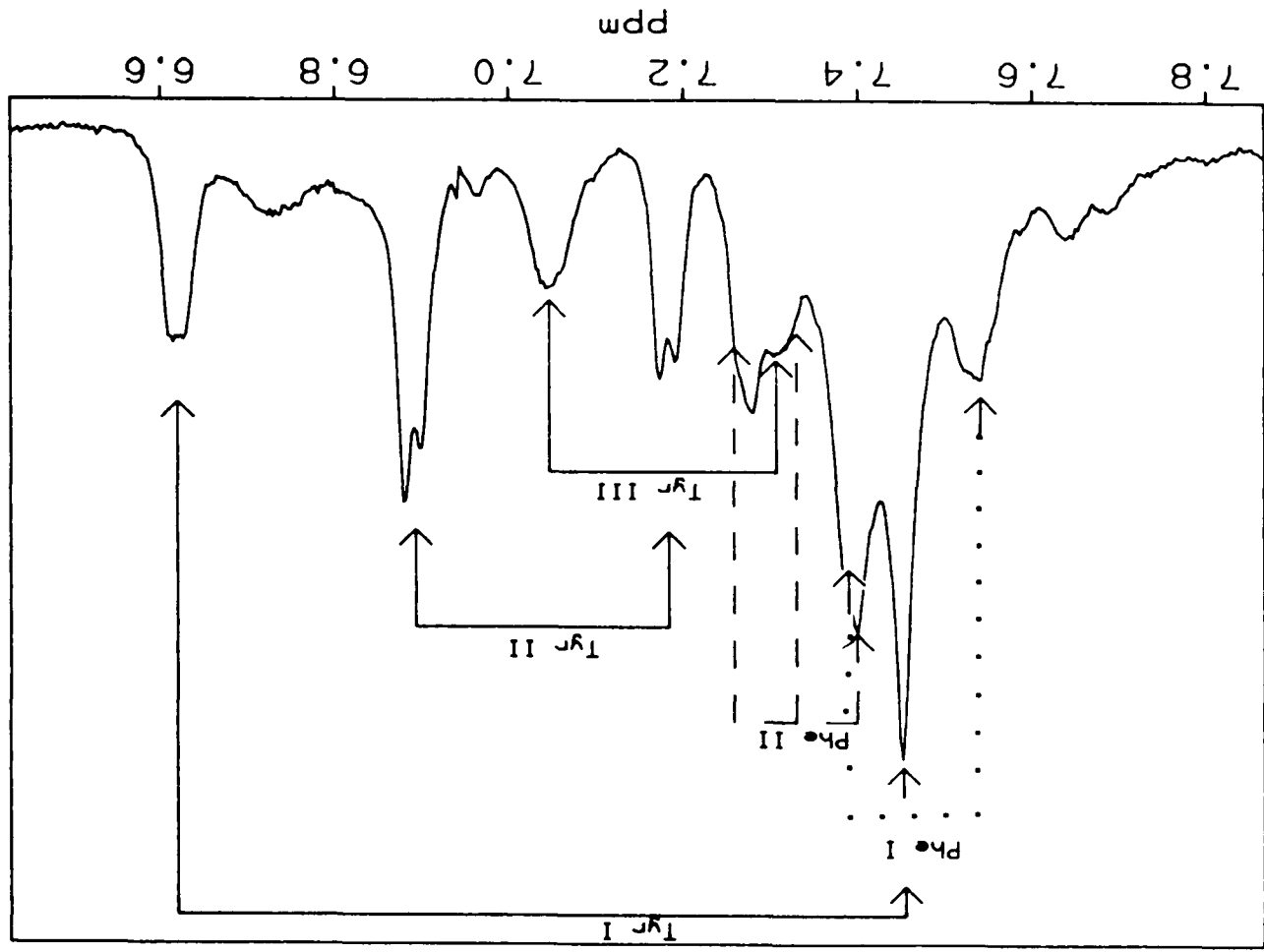


Figure IV-3: Schematic representation of CLP constructed using the distance constraints imposed by the five known disulfide bridges. Adapted from (Borgstrom et al., 1979), with numbering from (Sternby et al., 1984).

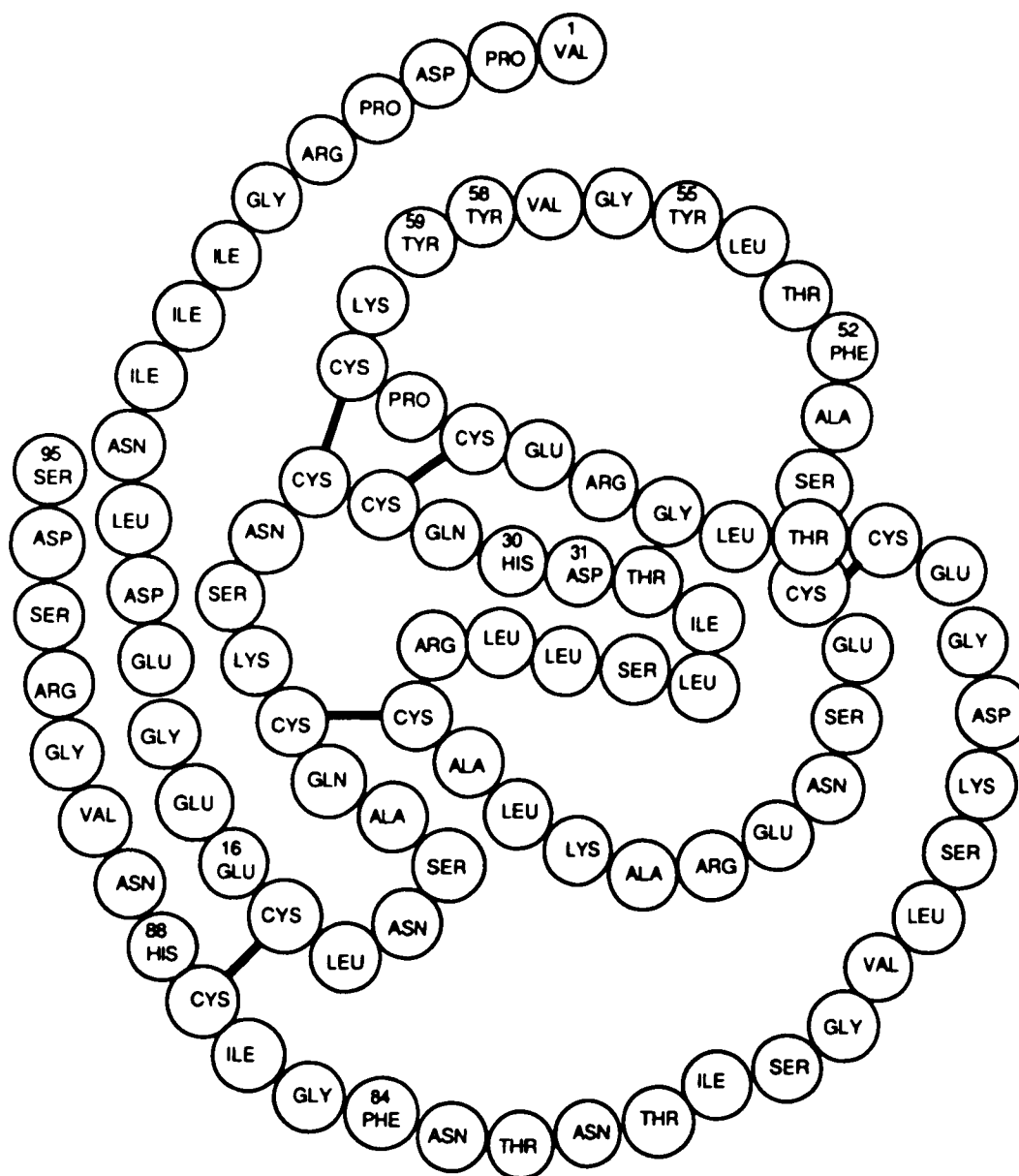


Figure IV-4: Aromatic regions of DQF-COSY spectra of CLP. Sample conditions were as described for Fig. IV-1. (A) H₂O; (B) D₂O. Crosspeaks that are present in both (A) and (B) arise from pairwise interactions of the 24 non-exchangeable aromatic protons in CLP (10 from Phe, 12 from Tyr and 2 from His 4H; Wuthrich, 1976). Inset to (A) shows the connectivities of the 2 Phe residues at a lower contour level.

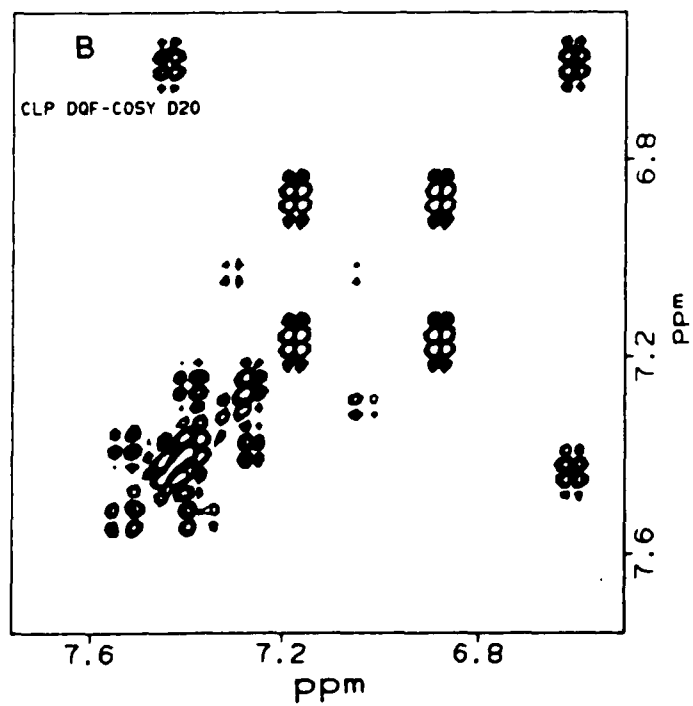
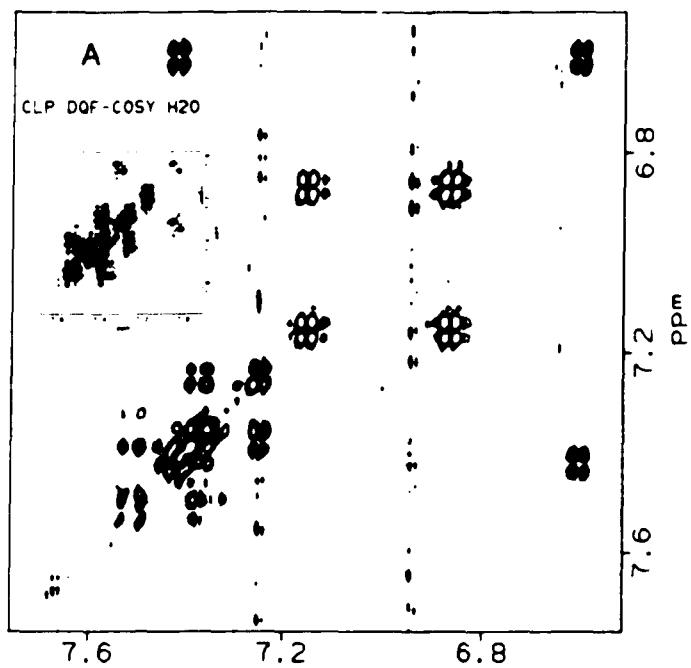


Figure IV-5: Aromatic regions from 150 ms NOESY spectra for CLP in (A) H₂O and (B) D₂O. Sample conditions were the same as described for Fig. IV-1. The starred crosspeak (*) indicates an interaction between Tyr II and either Phe II or His II.

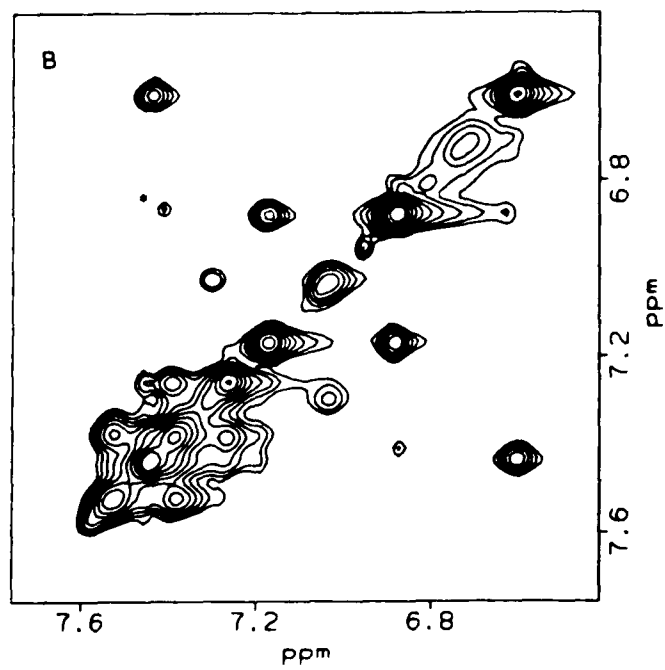
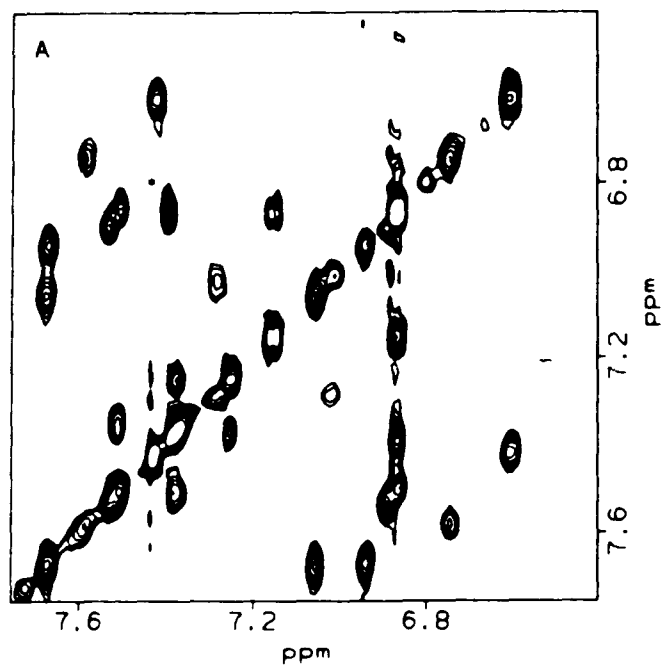


Figure IV-6: NH- α CH regions of DQF-COSY spectra for CLP. (A) and (C) were acquired in H₂O, and (B) was acquired in D₂O. Samples (A) and (B) were as described for Fig. IV-1. For sample (C) the pH was lowered to 3.1 and the temperature was increased to 45°C. Similar COSY results were obtained whether or not CLP was lyophilized.

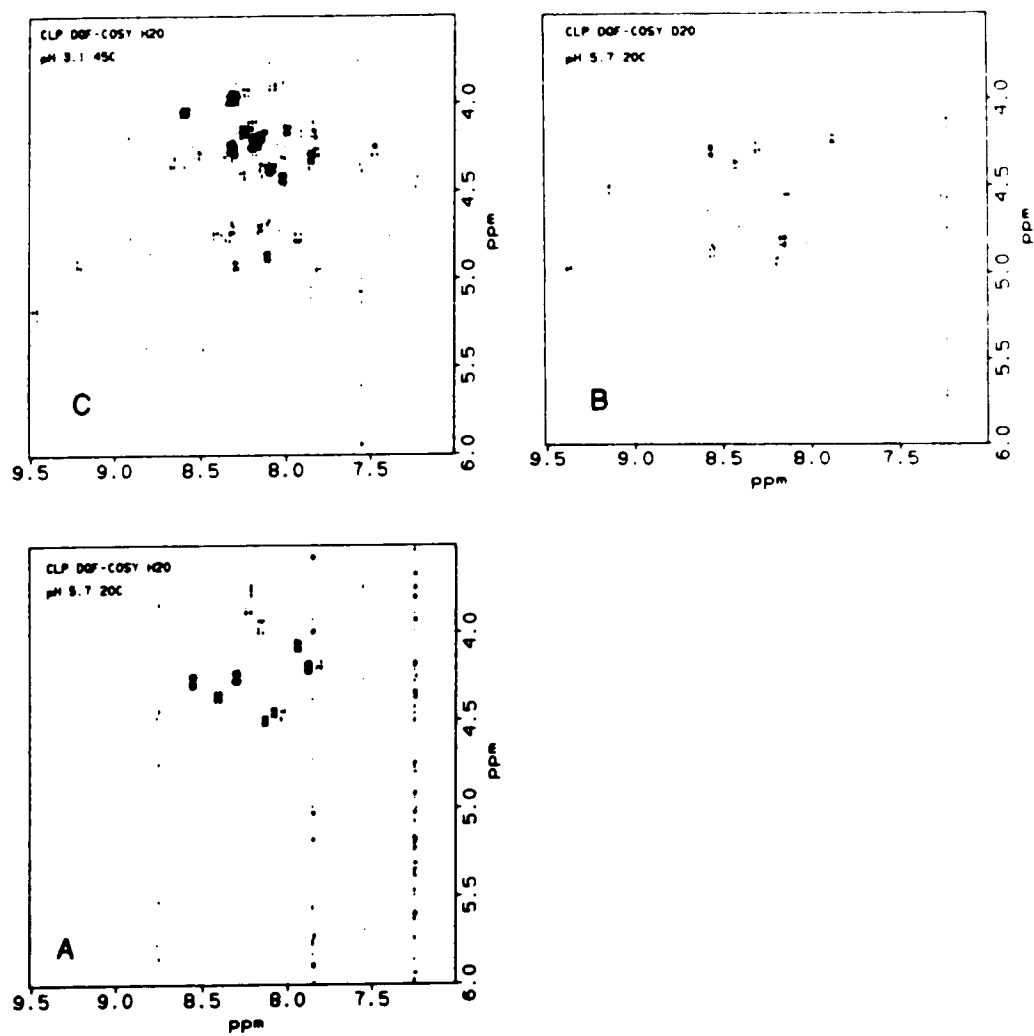


Figure IV-7: Native-PAGE analysis of 0.09 mM CLP. Outer lanes: low-molecular-weight markers; middle lane: CLP. Reagents and conditions are detailed in the Materials & Methods chapter.

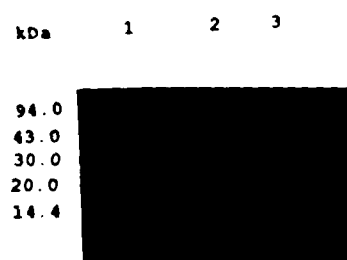


Figure IV-8: The circular dichroism spectra of CLP in the near- ultraviolet region as a function of concentration. (A): 0.14 mM CLP (B): 0.02 mM CLP. Intensities have been normalized to account for concentration differences.

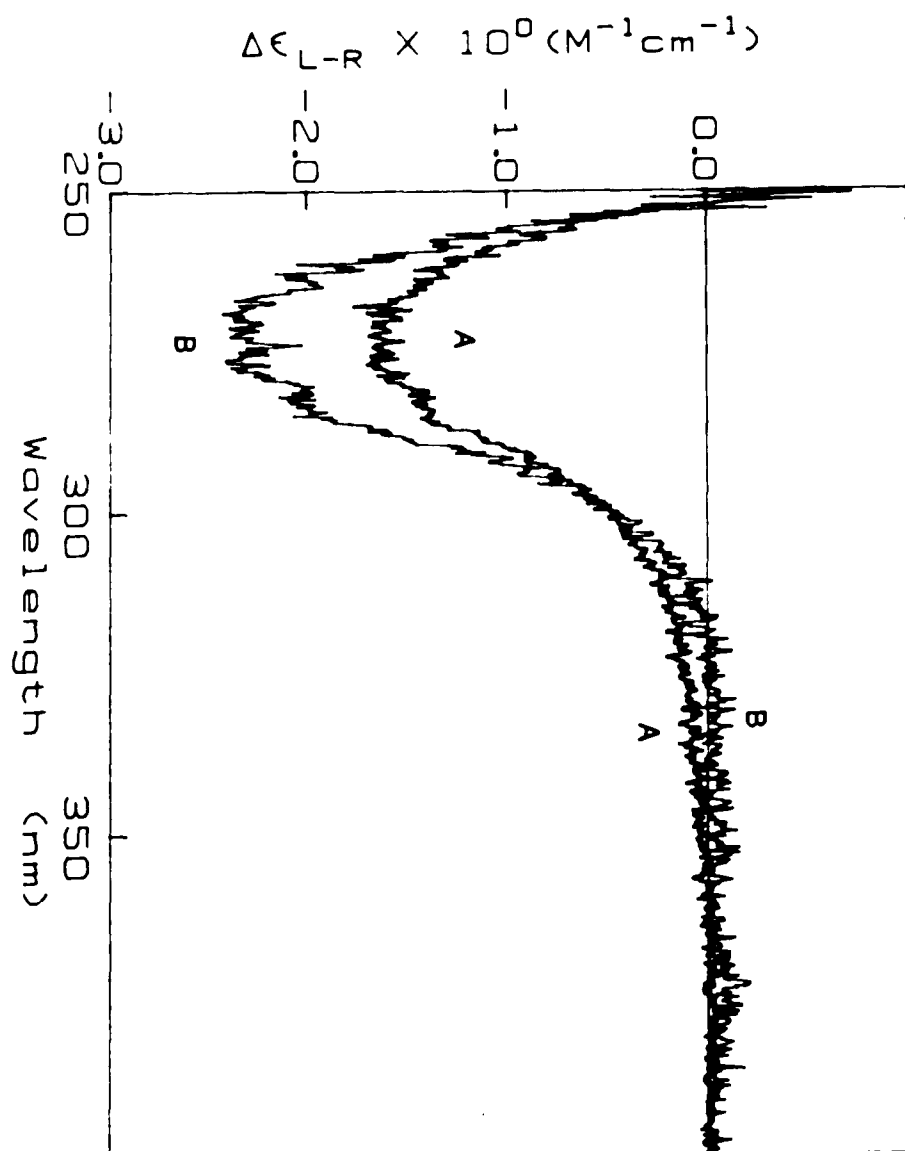


Figure IV-9: ^1H NMR spectra in H_2O as a function of CLP concentration. (A): 2 mM; (B): 1 mM; (C): 0.14 mM; (D): 0.09 mM. Initial sample conditions were the same as described for Fig. IV-1. Sample (A) was systematically diluted to a final concentration of 0.09 mM. (The sharp resonance at 8.50 ppm is an impurity in the dilution buffer.)

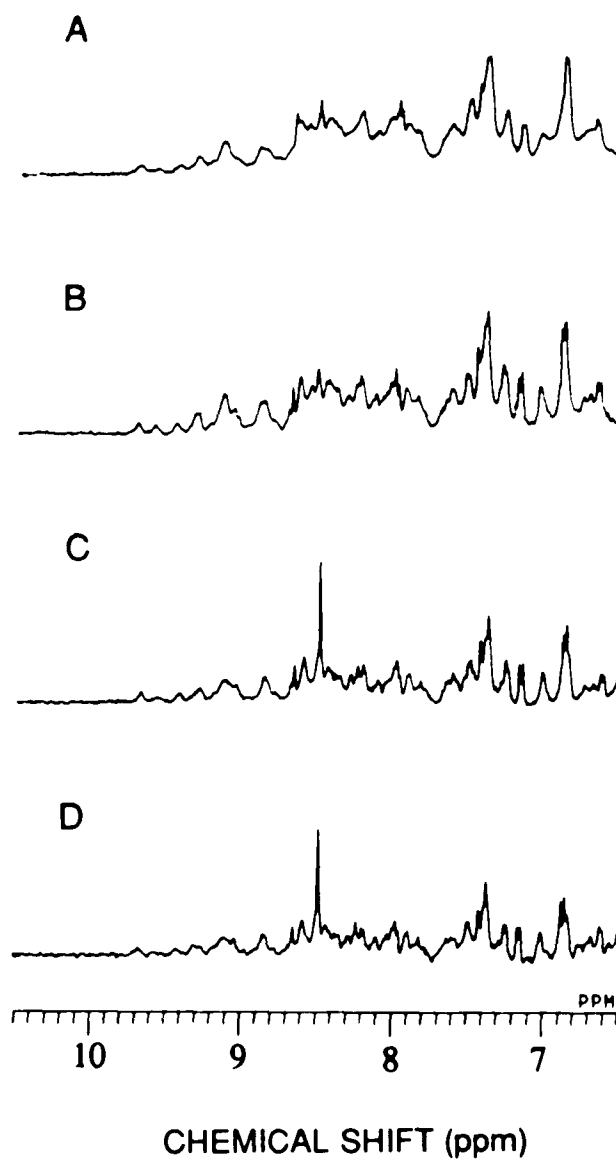


Table IV-1. Resonance Assignments in the Aromatic Region of the ^1H NMR Spectrum of CLP

Amino Acid	Proton	Chem. Shift (ppm)
Tyr I	2, 6H	7.45
	3, 5H	6.61
Tyr II	2, 6H	7.18
	3, 5H	6.89
Tyr III	2, 6H	7.31
	3, 5H	7.04
Phe I	2, 6H	7.37
	3, 5H	7.53
	4H	7.45
Phe II	2, 6H	7.25
	3, 5H	7.39
	4H	7.32
His I	2H	8.61*
	4H	6.65*
His II	2H	8.72
	4H	7.43

* These resonances were assigned from the 1D spectrum

Table IV-2. Sequential Assignments for Several Aromatic Residues of CLP

Amino Acid	Sequential Assignment
Tyr I	Tyr 55 / Tyr 59
Tyr II	Tyr 55 / Tyr 59
Tyr III	Tyr 58
His I	His 30
His II	His 88

Chapter VA

Introduction to Model Digestive Mixtures

Triglycerides are the major storage unit of fats in plants and animals. Of particular importance and interest to chemists and biochemists are the mechanisms behind the digestion of these lipids. The hydrophobicity of triglycerides results in their insolubility in aqueous solution and necessitates the presence of natural detergents (phospholipids and bile salts) for stable solubilization. It is, therefore, of considerable interest how these lipids are digested since a large proportion of them appear to be inaccessible to the aqueous medium of the hydrolytic enzymes lipase and colipase.

While the details of fat digestion are not completely understood, the general scheme is well documented. The initial stage of triglyceride digestion is the emulsification of these fatty substrates. Emulsification is followed by enzymatic hydrolysis of the fatty acid ester linkage. Finally the end products (mono- and diglycerides and fatty acids) are absorbed by the upper small intestine.

Preliminary triglyceride digestion begins in the mouth where triglycerides are acted upon by lingual lipases. Digestion continues in the stomach where the peristaltic action of the pyloric canal results in the aforementioned emulsification of the substrates. Triglycerides alone in an

aqueous environment are known to form unstable emulsion particles. However, in the presence of phospholipids (lecithins), the triglycerides are surrounded by the phospholipid molecules which form a stable monolayer. The initial hydrolysis, which begins here, is completed in the less acidic environment of the lumen and mucosa of the upper intestine where the necessary lipases and detergents are present.

After the triglycerides are organized as stable emulsions with lecithin, those triglyceride molecules which are located at the interface of the emulsion particle are rendered susceptible to enzymatic hydrolysis to fatty acids and mono- and diglycerides. Short- and medium-chain protonated fatty acids, which are soluble at the low pH in the stomach, now leave the emulsion particle and diffuse through the surface to the lumen, the mucosa and eventually the portal vein where they are used directly as sources of energy (Figure IV-1; Carey et al. 1983). However, since the majority of the triglycerides (especially the long-chain species) reside in the core of the emulsion particle this digestive pathway is representative of only a small portion of the substrates.

The remainder of the triglyceride/lecithin emulsion particles are subject to severe physical forces which promote smaller particle formation in the upper small intestine. It is

these smaller particles which bind to various digestive enzymes (lipase, colipase, phospholipase A₂) and become hydrolyzed to the products described previously. The mechanism for binding and hydrolysis of the substrates is not well defined, but it is likely that the supramolecular organization of the substrates and enzymes plays a vital role.

The molecular components of the aggregated digestive products at different stages of glyceride digestion have been extensively studied, however, little direct structural information about their organization has been uncovered. Previous investigations of the C8 analogs have confirmed the presence of mixed aggregates of bile salt, fatty acid and monoglyceride in aqueous solution by first examining several NMR parameters in both aqueous and methanolic solutions. The observation of field dependent ¹³C spin-lattice (T₁) relaxation times and ¹³C{¹H} NOE's made in D₂O, nearly full NOEs in CD₃OD, and a reduction in ¹³C T₁ values in D₂O as compared to CD₃OD all suggest that the fatty mixtures are forming mixed micellar aggregates in D₂O as compared to methanol which contains only monomers of the three components. Additionally, the results of the aforementioned NMR study allowed the carbons to be grouped into the following types: interfacial, hydrophobic and hydrophilic. Detailed analysis of T₁ and NOE data collected at two field strengths provided a dynamic profile consistent with a micellar aggregate where the hydrophobic acyl chains of the

MG and FA are surrounded by a belt of the hydrophilic portions of the BS, FA and MG backbone (Figure V-2; Wang et al., in press).

The studies presented herein focus on model digestive mixtures which are representative of the late-stage products of triglyceride digestion. The mixtures all contain a bile salt (taurocholate), a monoglyceride (acyl chain either C8 or C18) and a fatty acid (acyl chain either C8 or C18; Figure II-1). Both medium (C8) and long (C18) chain FAs and MGs are products of milk and butter fat digestion because both milk and butter fat contain triglycerides with one short or medium chain and two long chains. The long chain products also come from the digestion of animal fats. Additionally the medium chain analogs are typical of the hydrolysis products of nutrient cocktails which are given to patients who cannot absorb fats properly.

Apart from the study of Wang et al., NMR has not been used extensively as a tool for structural characterization of these systems. However, significant work has been done on the equilibria, size and polydispersity for related C18 samples. Quasi-elastic light scattering (QLS) studies (Staggers et al., 1990) have been conducted for mixtures containing C18 mixed intestinal lipids (oleic acid, monoolein, diolein and lecithin) and mixed bile salts (mixture of 10 glycine- and

taurine-conjugated BS) in a molar ratio equivalent to ours (20:80/20:1). These systems are expected to be biphasic (micelles and vesicles) with a mean hydrodynamic radius of at least 1000 Å. To date, no work has been done on mixtures having molar ratios equivalent to our second series (20:80/1:1.25), but extrapolation of the curves presented by Stagers et al. indicates that these aggregates latter (probably mostly vesicles) are likely to be larger than when FA:MG = 20:1.

While it is possible to study the monophasic C8 samples with traditional solution-state NMR techniques, the large size and molecular order of the C18 aggregates precludes the use of this method. Previous investigators have shown that it is possible to successfully study related liquid crystalline biological samples with solid-state NMR techniques (Forbes et al., 1988; Halladay et al., 1990; Stark et al., unpublished results). Specifically Li, Tihal and Stark have used ^1H and ^{13}C MAS NMR to study samples which model the early stages of triglyceride digestion. For example, MAS-assisted 2D NMR has shown that there are intercomponent NOE interactions between triolein and phosphatidylcholine in these systems. We have examined the molecular organization of late-stage C18 model digestive mixtures, at physiologically relevant ratios, through the use of high-resolution and MAS-assisted 1D and 2D NMR.

The paramagnetic property of certain molecules makes them good structural probes to use in NMR spectroscopy. Generally, protons which are accessible to the paramagnetic reagent are preferentially shifted or broadened. Lanthanide salts are frequently used to induce changes in chemical shifts (Meyerhoffer et al., 1992; Gabriel and Roberts, 1987), while ions like Mn^{2+} are used as line-broadening agents (Stark et al., 1985; Burns and Roberts, 1981;). Previous investigators have used this strategy to study which chemical moieties are located at the surface of bilayers and micelles (Meyerhoffer et al., 1992; DeKruyff, et al. 1977). We have used Pr^{3+} , Dy^{3+} , Tb^{3+} and Mn^{2+} solutions to determine which chemical moieties in our systems are exposed to the aqueous environment.

Chapter VB

Results

The results presented herein focus on 1D and 2D high-resolution and MAS-assisted NMR experiments on four late-stage model mixtures for triglyceride digestion. In order to model various physiologically relevant digestive products, mixtures of bile salt [BS], fatty acid [FA] and monoglyceride [MG] were prepared; FA and MG had either a medium (C8) or long (C18) acyl chain. The third component of the mixture was always taurocholate (BS). Our studies define an assignment protocol (^1H and ^{13}C) for these complex samples and employ NOEs to deduce the molecular organization of 3-component aggregates. Additionally, paramagnetic shift reagents are used to deduce which groups are located at the surface of the molecular assemblies.

C8: Assignments

Figure V-3 shows the 400 MHz 1D ^1H NMR spectrum of C8 20:80/20:1 (MBS:MIL/FA:MG where MBS = total bile salt concentration, MIL = total mixed lipid concentration, FA + MG, FA = fatty acid concentration and MG = monoglyceride concentration). While the high field strength gives sufficient spectral resolution to allow various components to be identified (MG, FA or BS), many of the individual protons cannot be assigned due to extensive signal overlap. In the upfield portion of the spectrum, BS signals in particular

complicate the assignments because all of the steroid protons resonate over a very small chemical shift range (2-4 ppm). Other investigators have used both homo- and heteronuclear correlated experiments to assign spectra of simple BS (TC, TDC etc.) micelles (Campredon et al., 1986). These published results as well as taurocholate 2D spectra collected in our laboratory were used to assign spectra of the C8 mixtures. In order to unambiguously assign all the ^1H resonances for three components, a combination of DQF-COSY and TOCSY spectra was required.

An example of the assignment strategy used throughout this work is described here and illustrated using Figures V-4 and V-5. Making resonance assignments in peptides and proteins is somewhat simpler than in steroids because there is only one "direction" in which the scalar connectivity can be made for an amino acid (from amide proton "out" through side-chain protons). As can be seen from Figure II-1 this is not the case for the BS skeleton. Figure V-4 shows the upfield portion of the DQF-COSY spectrum of C8 20:80/20:1 and highlights the crosspeak between BS 5 and BS 4 (2.01, 1.26 ppm). Figure V-5 shows the region of the TOCSY spectrum which can be used to make this assignment unambiguous. The TOCSY crosspeak at 3.45, 1.26 ppm confirms the assignment by showing a transfer of magnetization from BS 7 (with a well-established assignment) to BS 5.

In Figure V-4 the FA crosspeaks are shaded. All assignments for this component could be made from this spectrum (see Table V-1) and confirmed with TOCSY. However, it is expected that the chemical shifts of the acyl chain protons (3-8) for FA and MG will be indistinguishable due to the similarity in their molecular structure. Given this circumstance and the fact that this particular mixture had $[FA]/[MG] = 20$, any MG COSY crosspeaks were expected to be very weak. In order to assign the MG protons, a sample which contained perdeuterated FA was examined (Figure V-6). As expected, the chemical shifts for MG protons 3-8 were almost identical to those from FA. The remainder of the crosspeaks, which were from BS, were identified using a combination of previous assignments for simple BS micelles (Campredon et al., 1987, Stevens et al., 1992) and TOCSY spectra of the mixture (acquired in our laboratory).

Tables V-1 and V-2 list the ^1H resonance assignments for all three components in both C8 20:80/20:1 and 20:80/1:1.25, respectively. The proton resonances for the latter C8 sample (20:80/1:1.25) were assigned using DQF-COSY, with the assignments for the C8 20:80/20:1 mixture as a guide. Although a number of small changes in chemical shift were observed, no clear trends were apparent. Table V-3 lists the COSY and TOCSY interactions which were observed for all three

components for C8 20:80/20:1. Similar COSY results were found in C8 20:80/1:1.25 (data not shown).

Using a combination of 1D ^{13}C spectra (collected at 100 MHz; natural abundance), a $^1\text{H}\{^{13}\text{C}\}$ HMQC spectrum (Figure V-7) and previous assignments from our laboratory (Wang et al., 1992), it was possible to assign all of the ^{13}C resonances for both C8 mixtures (see Table V-4). To determine if there were any gross conformational changes induced upon mixed-aggregate formation, the ^{13}C chemical shifts were compared for simple one- and complex three-component samples. As can be seen from Table V-4, most of the chemical shifts change by a very small amount (< 0.5 ppm), suggesting that the electronic environment of the components does not change drastically. Departures from this trend include positions (1) and (2) of both FA and MG components, in agreement with the results of Wang et al. (1992).

C18 Assignments & Quantitation

While the molar ratios were identical for these long-chain analogs as compared to the C8 samples, the long hydrophobic acyl chain of the FA and MG causes the mixtures to partition into two layers. Both C18 mixtures separate into visually similar biphasic states consisting of a clear bottom layer and a milky, opalescent top layer. By monitoring the 400 MHz ^1H NMR spectrum as a function of time we have concluded

that the equilibration process between the two layers is a rather lengthy one (2 - 4 days). We have assumed here that equilibration is reached when there is no discernible change in the NMR spectrum (data not shown). These results were not unexpected, as previous investigators have remarked on the long equilibration times and biphasic nature of C18 mixtures of this sort (Staggers et al., 1990).

C18 Bottom Layers: Assignments & Quantitation

Figure V-8 shows the 400 MHz ^1H spectra for the two C18 bottom layers. The spectrum of the bottom layer of 20:80/1:1.25 (Figure V-8A) contains primarily sharp peaks from BS. Because there is no significant intensity attributable to the vinylic MG and FA protons (5.18 ppm), it is reasonable to conclude that there is no significant amount of MG or FA present in that layer. Monitoring the layer over several days revealed a peak at 1.18 ppm (FA/MG) whose intensity decreased, after 2 - 4 days, to that expected for the steroid skeleton of pure BS. This peak is attributable to FA/MG which is slowly partitioning into the "fatty" top layer. Since the prominent BS signals from the methyl protons and the protons geminal to the hydroxyl groups (18,19,21 and 3,7,12, respectively) displayed no chemical shift changes compared to C8 mixtures, we assume that chemical shifts for the remainder of the BS

steroid protons follow those reported in Tables V-1 and V-2 for C8 mixtures.

Quantitation of this bottom layer with a DMSO/ CCl_4 insert indicates a BS concentration below 0.1 mM. This is a surprising result, because there is no spectral evidence for BS in the corresponding top layer (vide infra). This result, however, does suggest that BS (and other components) may be present in large aggregates.

The second C18 mixture, 20:80/20:1, has a markedly different ^1H spectrum (Figure V-8B) compared to 20:80/1:1.25. Not only are resonances observed for the acyl chain of FA/MG and for the vinylic protons, but very broad BS peaks are also easily identified in the downfield portion of the spectrum. The increased BS and FA/MG linewidths, for C18 as compared to C8, suggest the presence of much larger mixed aggregates despite the optical clarity of this layer. For analogous positions (for example (2), (3) and terminal CH_3), all the observed resonances for protons in C18 retain the same chemical shifts as those reported for C8 (Table V-5).

Analysis of DQF-COSY spectra of the C18 20:80/20:1 sample (data not shown) confirms the assignments for two BS protons, 25 and 26 based on their chemical shifts, and several acyl chain protons for FA and MG. Quantitation of this layer with

the DMSO/ CCl_4 insert shows that [BS] and ([FA]+[MG]) are 0.7 mM and 0.5 mM, respectively. As for C18 20:80/1:1.25, there is missing BS, FA and MG intensity in these spectra.

C18 Top Layers: Assignments

Figure V-9 shows the 1D ^1H MAS spectra for the two top layers of the C18 samples. While the same spectral features as those shown in Figure V-9 are observed at 400 MHz with traditional z-spinning, seriously distorted baselines and lineshapes are also unavoidable (data not shown). Two-dimensional experiments are therefore extremely difficult to process reliably. Thus all subsequent NMR experiments were acquired on a 200 MHz spectrometer with MAS capabilities.

As can be seen from Figure V-9A, the C18 20:80/1:1.25 sample top layer contains strong resonances from both FA/MG, and one identifiable, weak, broad peak for BS. Figure V-10 shows the MAS-assisted phase-sensitive COSY for this mixture, with scalar connectivities highlighted for MG. The two resonances immediately upfield of HOD may be assigned to MG based on their chemical shifts and the presence of a strong COSY crosspeak (Figure V-10A) between them (it is likely that BS protons 7 and 25 are buried under these strong MG peaks, as they have very similar chemical shifts). BS protons 25 and 26, which could also exhibit such a crosspeak, are expected to resonate further upfield and would be weak in any case based

on their low concentration in this sample. Given the milkiness of the solution and the presence of an extremely broad "hump" at the chemical shift of BS proton 26 (2.90 ppm), any BS in this layer is presumably incorporated into large aggregates with linewidths large enough to preclude observation of COSY crosspeaks and narrow 1D peaks (vide supra). While the observation of ^{13}C peaks could confirm the presence of "missing" BS in this top layer, the low concentration of this component makes this strategy impractical (vide infra).

Oldfield and coworkers have shown, in ^1H NMR studies at 360 MHz, that MAS at 2.6 kHz is not sufficient to narrow the lines if molecular motion and order are solidlike (Forbes et al. 1988). These previous MAS studies on phospholipid multilayer systems show an increase in the order of the lipid hydrocarbon chain upon addition of cholesterol. Such solidlike behavior is also observed for our systems in that the hydrophobic acyl chain of FA/MG seems to rigidify the BS and produce broad spectral lines.

Figure V-9A shows the 1D ^1H MAS spectrum of the top layer of C18 20:80/20:1. Although quite similar in appearance to 20:80/1:1.25 there are two notable differences. The 20:80/20:1 spectrum has better spectral resolution in the upfield acyl chain/steroid region as well as an increase in the intensity of the resonance at 2.9 ppm, compared to the other peaks

immediately upfield of HOD. This "increase" in intensity is due to a decrease in MG concentration (2.3 mM in 20:80/20:1 as opposed to 11 mM in 20:80/1:1.25) in this sample, which makes the BS peaks observable. Additionally the intensity of the peaks at 3.5 and 3.8 ppm decreases compared to the vinylic proton resonance at 5.2 ppm. This decrease is also due to a decrease in MG concentration. The increase in spectral resolution in the 1D spectrum could be due to smaller aggregates, albeit still large ones, which Staggers et al. (1990) reported to be formed when higher FA:MG ratios are used. This spectrum, however, contains only broad BS peaks of low intensity, suggesting that it too is "hiding" them in large aggregates.

As shown in Figure V-10B, the COSY spectrum of 20:80/20:1 contains the same crosspeaks as 20:80/1:1.25 except that the overall intensity of the FA/MG crosspeaks has decreased and the MG:MG crosspeak is missing.

C8: NOESY and ROESY

NOESY spectra of C8 20:80/20:1 were acquired at the following mixing times: 100 ms, 600 ms and 1000 ms. Unlike other biological systems (peptides, proteins etc.) for which the NOE intensity increases rapidly as a function of mixing time, we find that these mixtures have a characteristically

slow build-up of NOE intensity. For mixing times typically used in protein NMR, NOEs of the C8 mixture are generally small, but spin diffusion may also be less of a complication.

NOE interactions for simple BS micelles have been reported previously (Stark et al., 1986). The NOEs observed are all very weak and are between protons geminal to the hydroxyl groups, methyl protons and nearby steroid protons. To our knowledge no published reports exist on NOESY of simple FA and MG systems, but any NOEs observed should be positive based on the sizes of the unassociated FA⁻ and MG monomers. For the mixtures, QLS sizing studies of C8 assemblies suggest that the NOEs observed will be negative (R.E. Stark, H.N. Halladay and A. Al-Bashir, unpublished observations).

While the 100 ms NOESY spectra contain no intercomponent NOE crosspeaks, many intracomponent interactions may be readily identified for both C8 20:80/20:1 and C8 20:80/1:1.25 (Figure V-11). As the mixing time is increased to 600 ms and then 1000 ms these intracomponent interactions increase in intensity for C8 20:80/20:1. For C8 20:80/1:1.25 the NOE intensities increase from 100 ms to 600 ms, but begin to decrease by 1000 ms. Tables V-6 and V-7 list the intramolecular interactions which are observed for C8 20:80/20:1 and C8 20:80/1:1.25, respectively. While spin diffusion cannot be ruled out completely at this point, the

many strong NOEs observed at the relatively short mixing time of 100 ms may be interpreted as interactions between proximal spin pairs.

A traditional method for detecting spin diffusion is analysis of NOESY build up curves where the NOE intensity is monitored as a function of mixing time. Other investigators (Anil-Kumar et al., 1981) have shown the usefulness of this method. While an analysis of this sort has not been done for the mixed lipid systems discussed here, careful employment of this procedure may confirm the absence of spin diffusion in our systems.

Figure V-12 shows the "geminal" and "sequential" NOEs which are observed for taurocholate in C8 20:80/20:1 and C8 20:80/1:1.25. For both C8 mixtures a particularly high density of these interactions is localized on rings A and B, which might be expected to exhibit considerable motional restraint. There are also a large number of non-sequential crosspeaks between BS methyl protons (18, 19, 21) and protons geminal to the hydroxyl groups (3β , 7β , 12β) (see Table V-6). Given the rigidity in the BS skeleton and the tendency for BS dimers to form, it is most likely that these interactions are intermolecular in nature. The appearance of such long-range intracomponent NOE crosspeaks may be attributed to an increase in the motional restraint of these molecules, compared to

component monomers. Such motional behavior has been confirmed upon aggregate formation (Wang et al., in press). Moreover, other investigators studying BS/phospholipid mixed micelles have noted an increase, upon aggregate formation, of both the number and the intensity of such crosspeaks in NOESY spectra (Stark et al., 1986). Further confirmation of aggregated assemblies comes from our observation of positive as well as negative NOEs for FA and MG in these mixtures (see Tables V-6 and V-7). As mentioned, positive NOEs are only expected for unassociated FA/MG monomers. The presence of a large number of non-sequential NOEs and the possibility of dimer formation are extremely useful in terms of constructing a model for these digestive mixtures and are discussed in greater detail later.

In addition to intramolecular NOESY interactions there are several specific intercomponent interactions which are observed at 1000 ms for C8 20:80/20:1 and at 600 ms for C8 20:80/1:1.25. Figure V-13 shows the NOESY interaction between the FA acyl chain and TC 18 (methyl) of C8 20:80/20:1. Tables V-8 and V-9 list the intercomponent interactions which are observed for C8 20:80/20:1 and C8 20:80/1:1.25, respectively. Despite modest qualitative similarities in observed intercomponent NOEs, the difference in the NOE build-up behavior suggests that the correlation times for the aggregated assemblies in the two C8 mixtures are different.

The structural implications of such a difference, as well as the specific intra-component long-range NOEs which are observed, are commented on further in the Discussion.

While it was possible to observe several intercomponent NOEs in these systems, the slow NOESY build-up suggested that a significant number of additional NOEs were too small to be observed at the mixing times we used. Given the considerable evidence for supramolecular organization in these mixtures (Wang et al., in press), the number of NOEs observed was surprisingly and disappointingly small. Nonetheless, motional freedom in these mixtures could well exceed that typical of protein and nucleic acid systems studied by NOESY techniques. The ROESY experiment (Bothner-by et al., 1984; Davis and Bax, 1985), which is more sensitive to short correlation times modulating interproton dipolar interactions, should be able to determine if the absence of NOEs is due to large interproton distances or attenuation of the NOEs by molecular fluidity. A further complication is the differing motional behaviors expected for the hydrophobic, hydrophilic and boundary portions of the aggregate.

To complement the results obtained with NOESY, ROESY spectra were collected with a variety of spin-lock mixing times for C8 20:80/20:1. As mentioned above, ROESY is useful in studying molecules whose laboratory-frame NOE is near zero

because their correlation times meet the condition $\omega\tau_c = 1$. In ROESY spectra, the NOEs are expected to produce signals of opposite phase to the diagonal. Conversely, COSY, TOCSY and spin-diffusion interactions appear as crosspeaks which have signal intensity in phase with the diagonal (Bax and Davis, 1985; Bauer et al., 1990). ROESY is therefore a powerful experiment for discriminating between true NOEs and spin-diffusion effects.

ROESY spectra collected for C8 20:80/20:1 were found to contain both types of crosspeaks, indicating the presence of scalar and dipolar interactions (strong scalar connectivities are observed in the ROESY spectra even when the Hartmann-Hahn condition is not strictly satisfied). However, careful analysis of individual slices of the ROESY spectrum shows modest but clearcut intercomponent NOE interactions. Figure V-14 shows a slice of the ROESY spectrum at the frequency of BS 18. The diagonal peak is phased positive and the rotating-frame NOE interactions are negative; as expected there were no scalar interactions observed for this proton.

In contrast to the picture presented in NOESY experiments, it is possible to observe large numbers of ROEs between BS and FA. In particular, ROEs are seen between BS methyl groups (18, 19 and 21), sidechain (25 and 26), and protons geminal to the hydroxyl groups (7β and 12β) and FA.

While a detailed analysis of the resulting distance constraints is required to develop a structural model for the mixed aggregate, extensive intercomponent association is obvious from these ROE results. Additionally, the appearance of extensive intermolecular ROEs supports the idea that most or all of the molecular regions of the components are motionally restricted upon aggregate formation, with respect to monomers and simple BS micelles. However, the appearance of new dipolar connectivities when ROESY is performed instead of NOESY indicates that compared to proteins, these systems are quite flexible and mobile. Table V-10 lists the interactions which are observed in the ROESY spectrum.

Paramagnetic Reagent Experiments for C8

Preliminary titration experiments using the broadening reagent MnCl_2 support our structural conclusions drawn from NOESY and ROESY experiments. Addition of high concentrations of Mn^{2+} causes the well-resolved sidechain proton 26 in C8 20:80/1:1.25 to be selectively broadened and reduced in intensity compared to other resonances (Figure V-15). MG glycerol backbone protons also appear to broaden more than BS methyl group and FA/MG acyl chain resonances. These results are not surprising since the hydroxylated portion of MG and the hydrophilic BS sidechain would tend to "prefer" a more hydrophilic environment and hence be more readily exposed to the solvent.

Previous titration experiments carried out in our laboratory using the lanthanides Pr^{3+} , Dy^{3+} and Tb^{3+} all resulted in precipitation before any noticeable chemical shift changes were noticed, despite published reports that similar BS systems could be studied using this method (Meyerhoffer et al., 1992). However, this insensitivity to shift reagents is also observed with Mn^{2+} . Relative to previous investigators (Stark et al., 1985; Burns and Roberts, 1981), the concentration of Mn^{2+} required to observe broadening was very high, suggesting that the mixed C8 aggregate may be rather impermeable and tightly packed.

C18 Bottom Layers: NOESY

Due to the presence of only one component (BS) in the bottom layer of C18 20:80/1:1.25 and the fact that the chemical shifts of these BS protons remained basically unchanged with respect to C8 mixtures, no NOESY experiments were done on this layer. It is assumed that simple BS micelles are present and that all FA and MG are located in the top layer.

Table V-11 shows the NOESY results for the bottom layer of C18 20:80/20:1. Only intracomponent NOEs are observed in this layer, with the largest number of crosspeaks seen at a mixing time of 600 ms. In addition to strong, vicinal NOEs several long-range NOEs are observed at the longer mixing

times. As in the C8 case, while spin diffusion can not be ruled out at this point, the presence of many strong NOEs at the relatively short mixing time of 100 ms provides direct confirmation of the presence of aggregate assemblies. Additionally, a weak NOE crosspeak between BS protons 25 and 26 must reflect a decrease in the flexibility of the BS side-chain compared with C8 systems; there is no way that this crosspeak can be attributed to spin diffusion. This result suggests that the C18 bottom layer forms aggregates which are similar to the complex mixed micelles formed by C8. The decrease in sidechain flexibility suggests that this moiety is not just extended outward into the aqueous environment, but is incorporated into an aggregate to some extent.

C18 Top Layers: Spinning Sideband Analysis

Figure V-16 shows the spectra of both top layers at reduced spinning speeds (Figure V-16A: $\nu_{\text{rot}} = 0.61$ kHz and Figure V-16B: $\nu_{\text{rot}} = 0.55$ kHz). At these lower spinning speeds spinning sidebands (ssbs) are observable. Generally, protons which are experiencing a more ordered environment will give rise to ssbs (Forbes et al., 1988). In our case, ssbs arise as a result of incomplete averaging of the chemical shift anisotropy. As shown in Figure V-16, ssbs are observable, in both mixtures, for the two most intense resonances (HOD and $(\text{CH}_2)_n$) as well as several of the weaker ones [(9), (10); (8), (11); (18)].

It is not surprising that ssbs are observed for the bulk methylene protons. Firstly, this peak is extremely intense in both mixture spectra; secondly, it involves a central portion of the acyl chain which is likely to be immobilized and ordered in an aggregated state. Previous investigators have also observed ssbs from bulk methylene protons (Forbes et al, 1988). However, surprising ssbs are observed from both the HOD resonance and the terminal methyl group (18). HOD ssbs suggest that some portion of the solvent molecules experiences an increase in order when the aggregate is formed. In contrast to previous MAS studies on mixed phospholipid-cholesterol aqueous dispersions, we observe ssbs from the terminal methyl group of the hydrocarbon acyl chain (Forbes et al., 1988). The ssbs which are observed are summarized in Tables V-12 and V-13 for C18 20:80/20:1 and 20:80/1:1.25, respectively.

Other investigators (Forbes et al., 1988) have characterized liquid crystalline phospholipid systems using comparable MAS techniques. While it is difficult to make a direct comparison between this previous work and ours, a qualitative one can be quite insightful. The retention of bulk methylene and terminal methyl group ssbs suggests that our mixed lipid samples also exist in the liquid crystalline phase. However, unlike the previous results, the presence of H₂O ssbs suggest a more complicated solvent ordering, around the outside of the aggregate and/or within the core.

C18 Top Layers: NOESY

Tables V-14 and V-15 list the NOEs observed in the 100, 600 and 800 ms NOESY spectra for C18 20:80/1:1.25 and C18 20:80/20:1, respectively. Largely due to the variation of the component concentrations in the mixtures, some discrepancies are observed among the possible intra- and intercomponent NOEs. Figure V-17 compares the 600 ms NOESY spectra for the two C18 mixtures.

For C18 20:80/1:1.25 possible inter- as well as intracomponent interactions are seen (Figure V-17A). Due to the chemical shift degeneracy of the FA and MG acyl-chain protons, all of the interactions listed here can be either intra- (FA;FA or MG;MG) or intermolecular (FA;MG) in nature. Using a sample prepared with perdeuterated FA would identify MG:MG interactions.

While the majority of the NOE interactions listed in Table V-14 involve acyl chain protons, two backbone:acyl chain long-range interactions are observed. These two NOEs involve the glycerol backbone protons (2' and 3') of MG in proximity to the acyl chain protons of either FA or MG. In either case (intra- or intercomponent), since the glycerol backbone and the acyl chain represent the two "ends" of FA/MG, such an NOE interaction would require the presence of an atypical

conformation. Involvement of the acyl chain in these NOEs is not completely surprising, since the presence of strong ssbs suggests that these chain protons are motionally restricted.

For C18 20:80/20:1 additional acyl-chain NOEs are observed which involve the (2) and (3) protons of FA (Table V-15). These interactions are easily observable because the FA is at least three times as concentrated as in C18 20:80/20:1, compared to both MG and BS. These interactions could be either inter- or intramolecular for the same reasons discussed for 20:80/1:1.25. While all 4 of the acyl chain NOE interactions observed for 20:80/1:1.25 are also observed for 20:80/20:1, the low [MG] in the latter mixture would preclude observation of any interactions involving the MG glycerol backbone. As can be seen in Figure V-9, the concentrations of these MG protons are so low that they are not observed in the 1D ^1H spectrum. No NOEs involving the BS component are observed for either mixture.

C18 Top Layers: 1D ^{13}C Spectra

"Missing" BS peaks in the ^1H spectra of these mixtures required another technique for observation of this component. Figure V-18 shows the 1D MAS ^{13}C spectra of the top layers of both C18 mixtures. Unfortunately, the low S/N (even with overnight accumulation) makes it impossible to observe BS peaks in C18 20:80/1:1.25 as well as C18 20:80/20:1. The peaks

which are observed are assigned to FA/MG and are listed in Table V-16.

While several of the C18 20:80/1:1.25 FA/MG carbons are identifiable, the majority of the acyl chain carbons have coincident chemical shifts. Following the same trend that is observed for the ^1H spectra of these top layers, the C18 20:80/20:1 mixture has increased spectral resolution and additional resonances are observed. As listed in Table V-16, we have assigned these resonances to carbons 4 and 7-12.

Chapter VC

Discussion

The three-component system of BS, FA and MG serves as an excellent model for the late-stage products of triglyceride digestion (Staggers et al., 1990). Our studies of such mixtures focus on the structural properties of the molecular assemblies which are formed when the FA/MG acyl chains have either 8 or 18 carbons and BS is the functionalized steroid taurocholate. Prior work on similar systems includes NMR relaxation studies to monitor molecular motion for C8 mixtures (Wang et al., 1992) and QLS studies to probe the size and polydispersity of related C18 mixtures (Staggers et al., 1990). In agreement with these previous studies, we find extensive NMR evidence to support the idea that complex (mixed-component) aggregates are formed. Our results also indicate that the C18 systems form mixed aggregates, some of which are quite large compared to the C8 particles. Through the use of high-resolution and MAS NMR it is possible to confirm and/or refine preliminary structural models for the C8 and the C18 systems, as well as to assess the effects of component concentration and acyl chain length on aggregate organization.

Since limited 2D NMR work has been done on systems similar to these complex mixtures, it was first necessary to define strategies for making structural assignments and to

optimize the conditions for observing dipolar interactions. As stated previously the NOE buildup rate in mixtures of this type has not been well characterized; previous 2D NMR work on mixed micelles has been carried out with a τ_m of at least 500 ms (Stark et al., 1986). To avoid complications from spin diffusion, our preliminary NOESY experiments were conducted with a short mixing time of 100 ms. No intercomponent interactions are observed under these conditions, suggesting that longer mixing times are necessary to detect potential intercomponent interactions. While a full analysis is necessary to confirm the absence of spin diffusion (Ellena, et al., 1985), the crosspeak intensity patterns which we observe strongly suggest that spin diffusion is minimal, if not absent.

All NOESY experiments for C8 20:80/20:1 show a large number of intracomponent NOEs. The most informative of these, in a structural sense, are the long-range (not geminal or vicinal) NOEs. Of the 23 such interactions observed for C8 20:80/20:1, 21 involve either the three protons geminal to the hydroxyl groups ($3\beta, 7\beta, 12\beta$) and/or methyl (18,19,21) protons. Only one of these long-range NOE interactions involves sidechain protons 23, 25 or 26, indicating that neither the methyl group protons nor the protons geminal to the hydroxyl groups are interacting extensively with sidechain protons. For C8 20:80/1:1.25 23 of the 26 interactions involve carbinol

and/or methyl protons. This is not surprising, since the sidechain protons are expected to have an interfacial location, as opposed to an interior one, in a mixed aggregate. It is also likely that the motional flexibility of the sidechain will attenuate NOEs from any spatial interactions which are present. A more complete view of dipolar interactions for this part of the molecule requires ROESY techniques which are more sensitive to short correlation times (vide infra).

Since the BS methyl groups show extensive dipolar relaxation with many steroid protons, it most likely that the two groups are in close proximity. The preliminary model suggested by Wang et al., (1992) permits these types of NOESY interactions, however, it does not predict the high density of methyl:steroid interactions which we observe. Our data also show numerous interactions which suggest a close spatial proximity between hydrophobic methyl groups and the protons geminal to the hydroxyl groups. This latter result further confirms the need for a better three-dimensional model which includes more BS:BS connectivities. A possible refinement is shown in Figure V-19. The refined model in Fig. V-19 accounts for the large number of BS;BS proton interactions by including back-to-back BS dimers within the mixed micelle.

The number of BS:BS interactions which are conserved in

the two C8 mixture spectra is quite high (~85% of the total number of crosspeaks). There are fewer than 10 interactions seen for 20:80/20:1 which are not seen for 20:80/1:1.25 and vice versa (see Tables V-6 and V-7). Several of these are sequential in nature, but a few are long-range and may have structural implications. For C8 20:80/20:1 there are roughly seven such interactions, all involving protons 2, 19 and/or 18. For C8 20:80/1:1.25 there are approximately the same number of interactions, involving protons 12, 18 and/or 21. Given the similar nature of these few long-range interactions, we may conclude that compositional changes produce only subtle variations in the molecular organization of BS. Nevertheless, it is important to note that the relative sizes of the aggregates formed may still be quite different. QLS would help clarify these differences by giving both aggregate size and polydispersity information.

In addition to intramolecular BS NOEs there are strong intramolecular NOEs for the other two components. The presence of these strong negative NOEs confirms the involvement of FA and MG in larger aggregates, as only positive NOEs would be expected for MG/FA monomers. While no long-range FA/MG NOEs were observed in C8 20:80/20:1, long-range FA/MG NOEs are observed between acyl-chain protons in C8 20:80/1:1.25. These long-range C8 20:80/1:1.25 interactions are (2);(4-7) and (2);(8). The numerous chain:chain interactions which we

observe suggest tight "packing" of the hydrophobic chains in the aggregate. This type of interaction would be expected for a BS/FA/MG mixed micelle which has the general appearance of the models in Figures II-1 and V-19.

While the above data and prior QLS studies (Staggers et al., 1990) confirm the presence of larger aggregates, they do not unambiguously indicate the presence of mixed molecular assemblies. At the longer NOESY mixing times, several strong intercomponent NOEs are observed which verify the presence of a mixed aggregate, probably a mixed micelle. Observation of intercomponent NOEs is expected using the model in Fig. V-19. However, while the model does not prohibit any experimental intercomponent NOEs which are observed, it does suggest that a larger number of these intercomponent interactions should be seen. Given this fact, it seems reasonable that a significant number of these NOEs satisfy the requirement in Equation II-3 and approach a value of zero under the conditions used here. This idea is given further support by the increase in intercomponent interactions which is seen in ROESY (vide infra). However, despite the small number of these intercomponent NOESY crosspeaks valuable insight can be gained about the molecular organization of these aggregates and which portions of the individual components are close in space and/or exposed to the aqueous interface.

For C8 20:80/20:1 there are two unambiguous intercomponent NOEs that indicate a close spatial relationship between the hydrophobic FA/MG acyl chain and the hydrophobic BS surface (proton 7 and methyl group 18, both of which point toward the hydrophobic face of taurocholate). This result is not surprising and is quite representative of the type of "back-to-back" hydrophobic association which is observed upon formation of primary BS micelles and mixed phospholipid/BS micelles (Small 1971; Stark et al., 1983). Additionally, it is depicted by the molecular model of Wang et al. (1992).

The lack of intermolecular NOEs involving BS sidechain protons suggests that these protons may be located at the aggregate surface. Preliminary titration experiments using the broadening reagent MnCl_2 support this conclusion. At the high Mn^{2+} concentration of 2.7 mM, selective broadening of the sidechain resonance (26) clearly demonstrates that this proton has increased aqueous accessibility compared to others. Additionally, the preliminary paramagnetic results presented here demonstrate that the hydrophilic glycerol backbone and the taurine sidechain have increased aqueous accessibility in the mixed micellar aggregate compared to the acyl chains and other hydrophobic groups. However, while selective broadening is observed, the high Mn^{2+} concentration required suggests that the micelle is, on the whole, quite impermeable to solvent. This constraint, as shown in Fig. V-19, is satisfied by close

packed BS dimers which surround the micelle. A more detailed picture should be forthcoming from future titration experiments which will monitor the change in broadening reagent more carefully as well as analyze the changes in spectral linewidths more thoroughly. It is important to note that due to the high Mn^{2+} concentration used it is possible that the rather large chemical shift changes, which we observe uniformly across the entire spectrum, are due to a change in the bulk solvent as opposed to specific Mn^{2+} binding.

Similar intercomponent NOE interactions are observed for C8 20:80/1:1.25, again suggesting that changing the relative concentrations of the individual components does not radically change the organization of the aggregate. Clearcut FA/MG:BS hydrophobic interactions are observed between the acyl chains of FA/MG and both 7β and methyl group 18 of BS. Additionally, in C8 20:80/1:1.25 NOEs between the pairs, FA (2);BS 2 and FA (2);BS 19 confirm that the hydrophobic steroid ring and methyl groups of BS and the FA/MG acyl chains are close together. The refined model in Fig. V-19 does not prohibit any on the intercomponent interactions which are observed for either C8 20:80/20:1 or C8 20:80/1:1.25.

Where possible, for C8 20:80/20:1 only, the above results were augmented by running parallel NOESY experiments using perdeuterated FA. Crosspeaks involving FA are then expected to

disappear. For example, the intercomponent crosspeak between FA (2) and BS 19 is not present in a NOESY spectrum of 20:80/20:1 with FA-d₁₅, confirming that FA is a partner in this interaction. Because FA and MG and one BS methyl group have almost identical chemical shifts, use of deuterated MG and BS could potentially be very informative. For example, there are several NOESY interactions involving BS and/or FA (one such example is: FA (4-7) to either FA (8) or BS 19)). Unfortunately, a deuterated FA sample causes the disappearance of the crosspeak without revealing any new information. Use of deuterated BS, however, would result in the disappearance of this crosspeak only if BS were involved in the interaction. On the other hand, the crosspeak would remain if it were due to a FA:FA interaction. Unfortunately, prices are prohibitive for deuterated MG, and heavily deuterated BS is unavailable.

As mentioned above, the ROEs identified in the ROESY spectrum of C8 20:80/20:1 indicate that this experiment can be very useful in conformational analyses of mixtures for which the molecular motions may occur at different rates throughout the aggregate. In contrast to our NOESY results showing an absence of intermolecular interactions involving the BS sidechain protons 25 and 26, ROEs are observed which involve these protons. A particularly large number of ROEs are observed between BS sidechain protons 25 and 26 and FA protons (2), (3) and (4-7). These interactions involve 2 out of the 3

interfacial groups previously defined by Wang et al., (1992). It is not surprising that the third interfacial group (MG 3') is missing as its concentration in this mixture is very low. The structural implications of these results are reasonable because, as the models in Figures II-1 and V-19 show, the interfacial sidechain protons of BS, as well as proton 25, are located near the acyl chain of FA/MG (particularly protons (2) and (3)).

ROEs are also observed between two of the BS methyl groups (18 and 21), and several BS and FA protons. In agreement with our NOESY results, ROEs for 21 and 18 are observed to proton 12 β and methyl group protons. Additionally, our NOESY results are supplemented by ROEs which are observed between BS proton 21 and BS sidechain protons 25 and 26 as well as between 21 and FA protons (2), (3) and (4-7). Other investigators have commented on the necessary presence of the sidechain in order for BS aggregation to occur (Barnes, 1984). Our results confirm the involvement of the BS sidechain in the aggregate as well as its role as a solubilizing detergent. Therefore, while our system is complicated by both the presence of two other components as well as the BS taurine group, there appear to be mechanistic similarities in the forces which drive both simple and complex aggregate formation. Given the short correlation times of the sidechain groupings in our system (compared to other hydrophobic

protons), it appears falsely from NOE data that the presence of the sidechain is not as crucial as in other simple BS systems. While the resulting model would not differ tremendously if ROE data are excluded, significant motional and functional information about the sidechain would be missing. Hence, ROE data are more revealing since they elucidate the dual function and location of the BS sidechain.

Based on T_1 measurements and 1D NOE data, Wang et al., (1992) have determined that there are different motions for particular types of protons, and that these motions depend upon the proton's location in the aggregate (hydrophilic; exterior, interfacial/boundary or hydrophobic; interior). Our observation of both ROEs and NOEs agrees with this classification scheme. Because the ROESY experiment is more sensitive to interactions with shorter correlation times (Davis and Bax, 1985), it is possible to observe interactions which are transparent to NOESY. It is likely that the steroid protons and FA/MG acyl chain protons have a longer correlation time and are more motionally restrained than interfacial BS protons (21, 23, 25 and 26 which are primarily observed in ROESY) whose molecular motions are a function of the aggregate as a whole (Wang et al., 1992).

To supplement qualitative NOESY and ROESY spectral

analysis, it is possible to obtain accurate distance information from NOE crosspeaks through a well-documented and widely-used quantitation procedure (Wuthrich, 1986). This procedure uses the NOE intensity of a particular crosspeak, whose interproton distance is known, as a reference. While this quantitation procedure has been successfully used to determine the tertiary structure of proteins and DNA, the different mobilities in these mixed-lipid aggregates make the choice of one particular reference crosspeak difficult. For this reason, the use of several "reference" NOEs may make this NOE quantitation procedure more reliable.

While the particular intercomponent NOEs which we observe for the two C8 mixtures are not identical, the types of interactions we see are similar (hydrophobic-hydrophobic; except for one hydrophobic-hydrophilic in C8 20:80/1:1.25). However, whereas the NOEs for C8 20:80/20:1 increase in strength up through a mixing time of 1000 ms, the NOEs for C8 20:80/1:1.25 reach a maximum somewhere between 600 and 1000 ms. This trend indicates a longer correlation time for the 20:80/1:1.25 mixture, suggesting larger aggregates despite the optical clarity of both solutions. As mentioned previously, QLS studies could help elucidate these differences.

In summary it seems that changing the relative concentrations of the three components perturbs the

organization of the mixed micelle very modestly. One major difference which we do observe between the two C8 mixtures is a large change in the chemical shift of carbon (1). For both mixtures the acyl chains are motionally restrained and are primarily located on the "inside" of the micelle. The hydrophilic hydroxyl and taurine moieties, however, are much more flexible and are presumably exposed to the aqueous environment. In agreement with Wang et al. (1992), our data support a model including interfacial groups that exhibit different motional behavior than either interior or exterior parts of the micelle.

As a refinement of the preliminary model discussed above, our NOESY and ROESY data support a model where BS dimers exist. Additionally, while the BS sidechain is extremely mobile it is not completely limited to interacting with other hydrophilic groups. The apparent dual functions of the BS sidechain include being a detergent which solubilizes lipids, as well as being a necessary internal promoter of micelle formation.

In comparison to C8, the C18 mixtures typical of dietary fats are very complex. A previous study of the physical-chemical behavior of such C18 systems (Staggers et al., 1990) characterized mixtures of this sort as being biphasic and composed of both micelles and vesicles. Mean aggregate size

was reported, apparently for each mixture as a whole. As expected, both of our C18 mixtures partition into two layers, one which is optically clear and one which is cloudy and opalescent.

Visual examination of the linewidths in the NMR spectra for the clear, bottom layer of C18 20:80/20:1 clearly indicates the presence of molecular species which are larger than either component monomers or C8 aggregates. In contrast, the bottom layer of C18 20:80/1:1.25 contains only sharp resonances and shows the presence of only BS. For C18 20:80/20:1 all resonances are broadened extensively and, while no intercomponent NOE are observed, strong long-range intracomponent NOEs are seen. For example, crosspeaks between (9), (10); (4-7) and (2); (4-7) indicate a tightly packed core of acyl chains similar to that observed for C8 micelles. While it is difficult to comment on the precise molecular organization of this mixture without intercomponent information, it seems plausible, based on the NOEs observed, that these aggregates are organized in a similar fashion to the C8 ones. As in the case of the C8 mixtures, future ROESY experiments may be useful in observing interactions which are transparent to NOESY.

Integration of the C18 20:80/1:1.25 bottom-layer spectra indicates a BS concentration of less than 1.0 mM, suggesting

that most BS is located in the top layer. Given this low concentration of BS, we assume that only BS monomers are present. In this bottom layer the integrated peak intensities translate into the following amounts: ~1% of the total BS and none of the FA/MG.

While the 400 MHz ^1H spectrum for the C18 20:80/20:1 bottom layer is very different in appearance from the one for C18 20:80/1:1.25 (all three components and broad lines are present in the former solution), the two spectra are similar in that only low concentrations of the components are present. In the 20:80/20:1 mixture there is a slightly higher BS concentration of ~6% and a [FA+MG] concentration of ~1%. Therefore, for C18 20:80/20:1 also, most of the components must be located in the top layer (we have assumed that since the bottom layer is optically clear, there are no aggregates which are too large to be observed). While it seems reasonable that the "missing" components are located in the top layer, confirmation of this through quantitation is necessary. Additionally, the sizing ability of QLS could help confirm that large aggregates are present in these mixtures.

Due to the broad lines in the top-layer spectra of both C18 mixtures, 1D and 2D NMR spectra were collected using magic-angle spinning. The presence of ssbs in the 1D ^1H spectra for both C18 top-layer mixtures indicates that chain

protons are highly ordered. Of particular significance are the presence of ssbs from HOD and from the terminal methyl group of the FA/MG acyl chain. In contrast to the mixed micelle model for C8, the presence of ordered water suggests that solvent molecules may be trapped "inside" the aggregate in a fashion typically found for vesicles or microemulsions (Langevin, 1988). Thus a careful confirmation of the HOD ssb's is desirable.

A possible arrangement of this vesicle is shown in Figure V-20. This "bilayer" model includes ordered solvent and tightly packed acyl chains (especially the terminal methyl groups), both of which would result in ssb's. While not shown in Fig. V-20, it is also possible that in addition to the presence of "trapped" water inside the vesicle, solvent may be ordered around the outside of the aggregate as well.

While the NOEs observed for both C18 top-layer mixtures are similar, the higher MG concentration in C18 20:80/1:1.25 makes it possible to observe two long-range NOEs involving the backbone MG protons. These are $3'; [(12-17), (4-7)]$ and $2'; [(12-17), (4-7)]$. The lack of a crosspeak involving MG 1' is surprising. This proton appears to be broadened much more than the other two backbone protons suggesting that it is in a more motionally restrained environment. Due to the chemical-shift degeneracy of many acyl chain protons, it is not possible to

determine unambiguously which chain proton(s) are involved in the two interactions involving MG backbone protons. Using a mixture prepared with deuterated FA could remove this ambiguity. If the crosspeak is intermolecular in nature, it will disappear because the FA chain will be deuterated. If, however, it is an intramolecular MG;MG interaction the crosspeak will be retained. In either case the presence of this interaction supports a model where there is close molecular "packing" between the ends of MG and/or FA. As can be seen from Fig. V-20, the antiparallel orientation of several of the acyl chains would result in these types of chain:chain interactions.

Due to the low MG concentration in C18 20:80/20:1, no crosspeaks involving these resonances are observed. However, this decrease in MG concentration makes it theoretically possible to observe any crosspeaks involving BS protons. While no BS interactions are seen (most likely due to the large linewidths of their resonances), this spectrum does contain 5 crosspeaks which are not seen in the NOESY spectrum of C18 20:80/1:1.25. These 5 interactions all involve acyl chain protons. Because no such long-range crosspeaks are observed for 20:80/1:1.25 and because for 20:80/20:1 they are only observed at the longest mixing time, it is possible that the 20:80/20:1 aggregate is more tightly packed than 20:80/1:1.25. However, given the increased spectral resolution for C18

20:80/20:1 in both its ^1H MAS and ^{13}C DPMAS spectra, this result seems surprising. While unlikely, it is also possible that the long-range interactions are due to spin-diffusion effects and not spatial proximity. Future work with ROESY should help answer these questions and illuminate the nature of these interactions.

Like the two C8 mixtures, the two top-layer C18 aggregates seem on the whole to be structurally similar. There are some differences in the NOESY interactions attributable to either different molecular organization or spin diffusion effects. It is important to note that while the total concentrations of each component were used to construct the model in Fig. V-20, it is possible that the aggregates which we actually observe have different ratios depending upon each component's hydrophobicity and solubility.

In contrast to the top layers, the two C18 bottom layers are extremely different. C18 20:80/1:1.25 contains only BS monomers, whereas C18 20:80/20:1 most likely contains mixed micelles such as the ones observed for the C8 mixtures. For both C8 and C18 it is difficult at this point to reliably determine the absolute numbers of the individual components in each aggregate. Therefore the models drawn in Figs. V-19 and V-20 are based on the relative ratios of these components. It is clear, however, that for C18 the aggregates formed are always larger than the C8 ones. This is expected due to the long, hydrophobic acyl chains in the C18 components.

Figure V-1. A schematic representation of triglyceride digestion, from Carey et al., 1983.

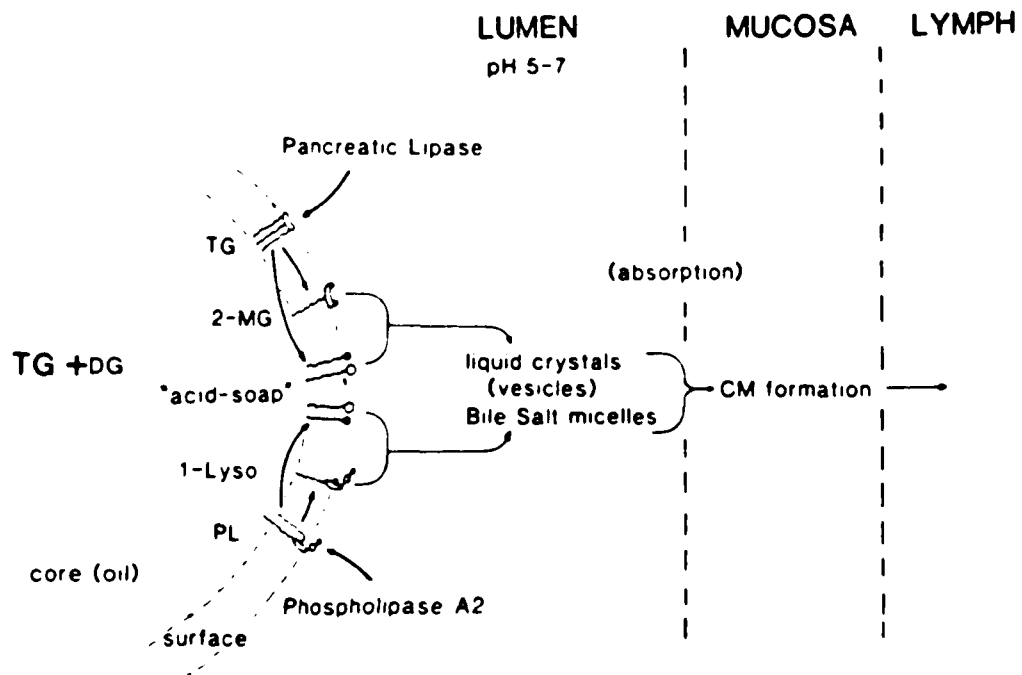


Figure V-2. A possible orientation of the three components in an aggregate assembly. Zigzags denote alkyl chains and closed circles denote interfacial (boundary) carbons, from Wang et al., in press.

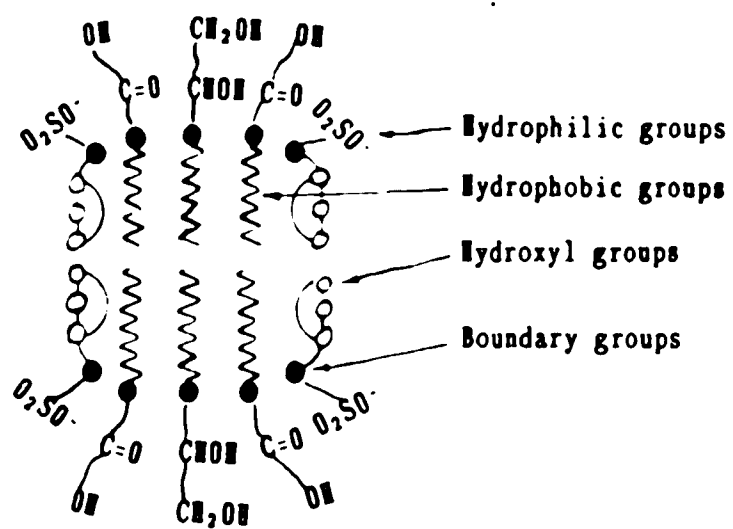


Figure V-3. 400 MHz ^1H NMR spectrum of C8 20:80/20:1, where [BS] = 20 mM, [FA] = 60 mM and [MG] = 3 mM. Numbering as in Fig II-1.

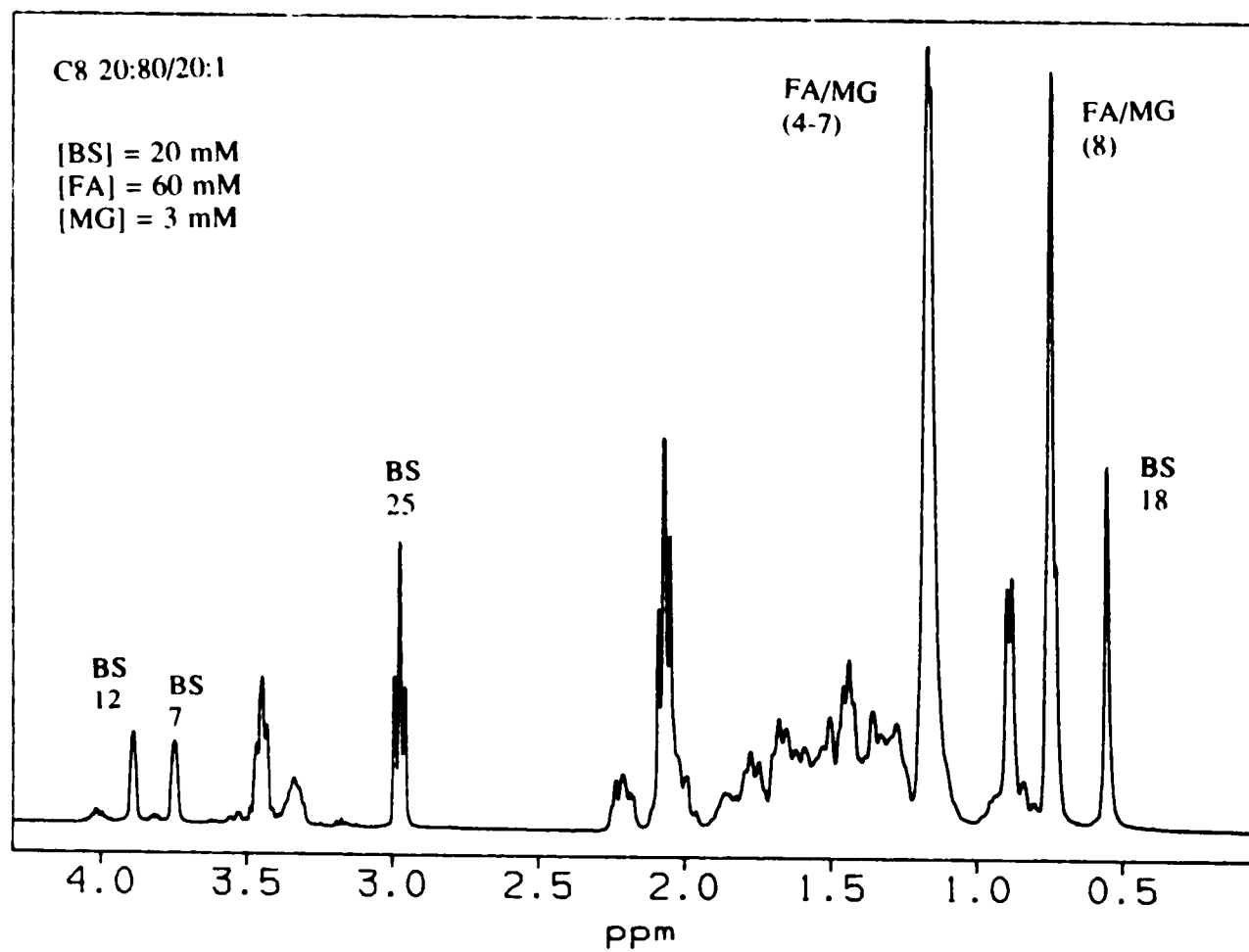


Figure V-4. Upfield region of the DQF-COSY of C8 20:80/20:1, processed with a 45° shifted sine-bell window function. The shaded crosspeaks represent scalar interactions between protons belonging to FA, with possible minor contributions from overlapping MG protons. The labelled crosspeak represents a scalar interaction between BS 5 and BS 4.

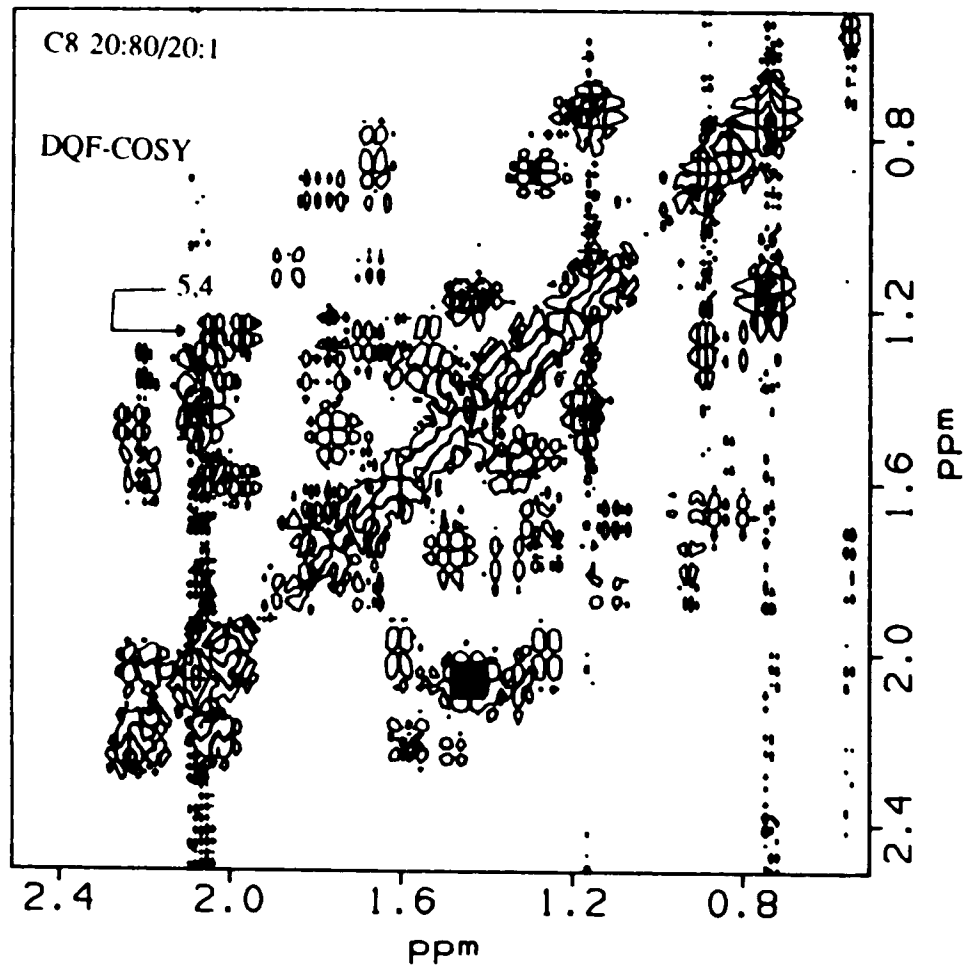


Figure V-5. Expanded region of the 70 ms TOCSY spectrum of C8 20:80/20:1. The crosspeaks involving BS 7 are labelled and the one which confirms the assignment of BS 5 is highlighted with an arrow.

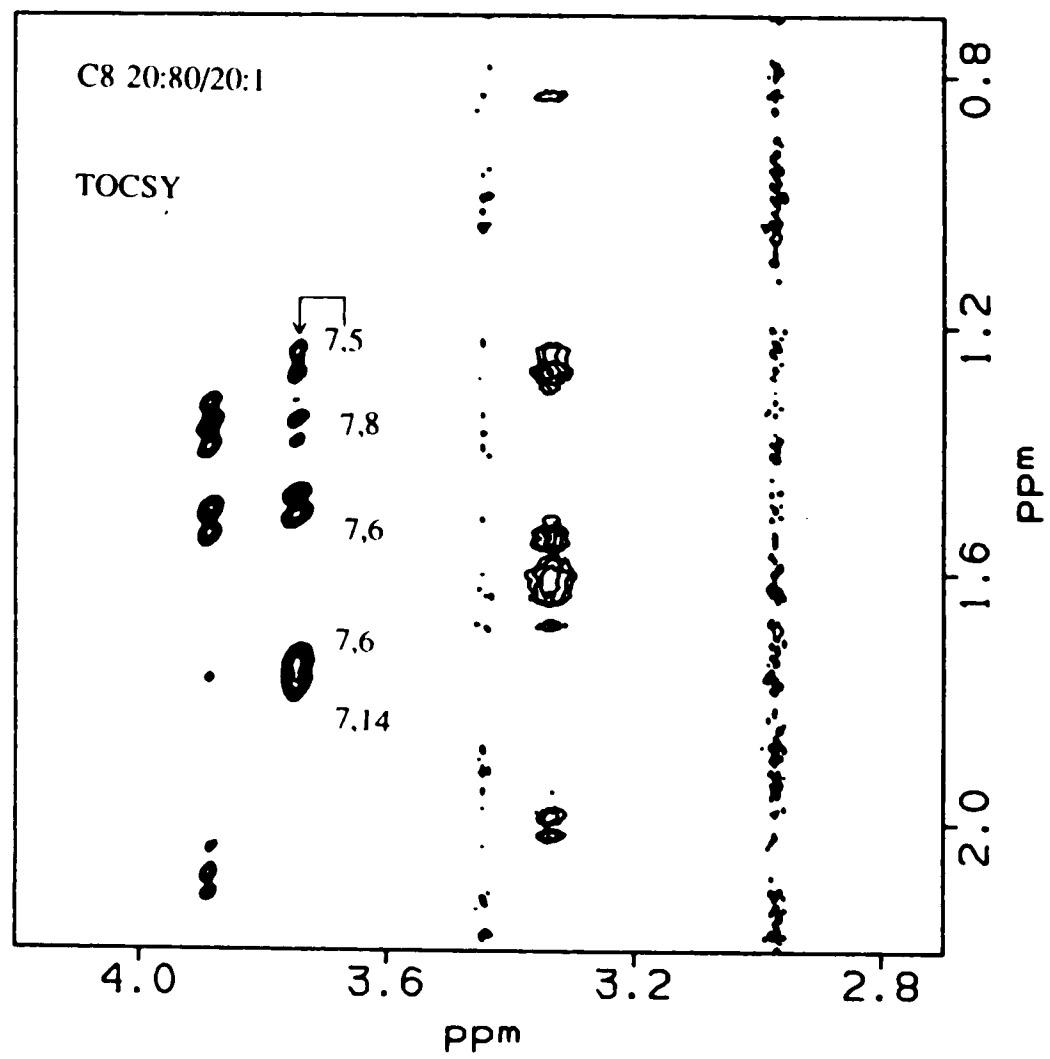


Figure V-6. Upfield region of the DQF-COSY of C8 20:80/20:1 with FA-d₁₅. The expected scalar interactions for MG are labelled.

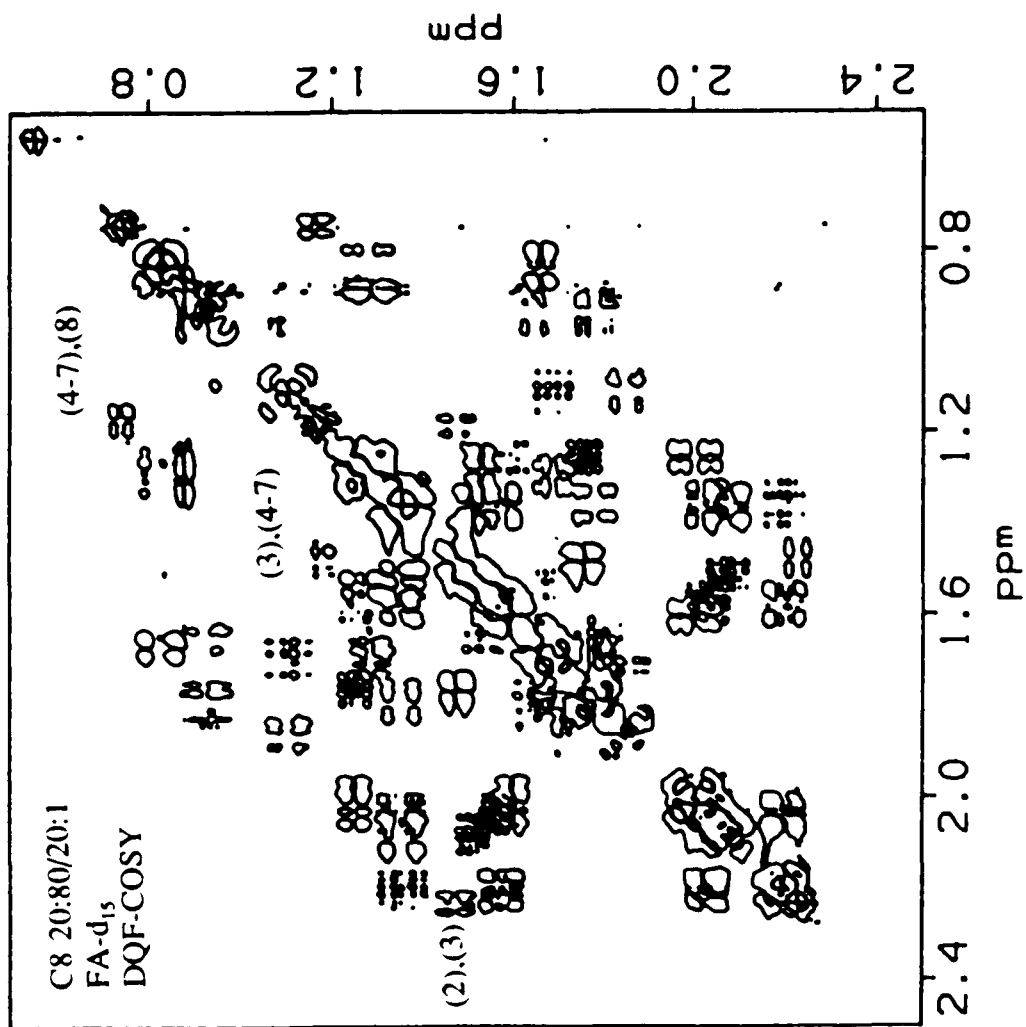


Figure V-7. $^1\text{H}\{^{13}\text{C}\}$ HMQC spectrum for C8 20:80/20:1. Crosspeaks from both FA and BS are labelled. MG crosspeaks were too weak to be detected.

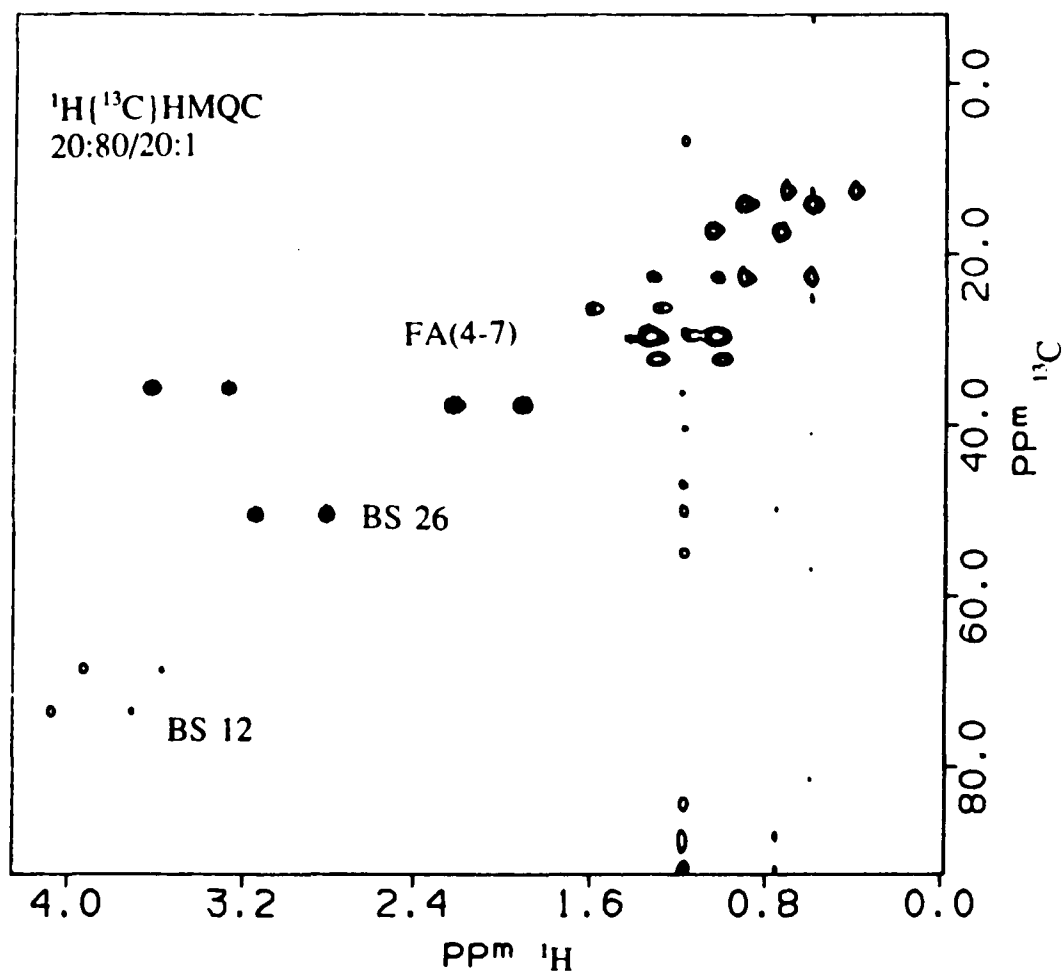


Figure V-8. 400 MHz ^1H spectra of bottom, optically clear, layers of two C18 mixtures. A: C18 20:80/1:1.25 and B: C18 20:80/20:1. In A only BS peaks are observed. A FA/MG peak at 1.18 ppm is highlighted, but this peak decreases in intensity as the FA/MG migrates into the "fatty" top layer over a period of several days. In B the BS, FA/MG and DMSO (which was used to calibrate concentrations of each component) resonances are labelled. Peaks 12, 7, 26, 18 and (9)(10) (not shown) were used for quantitation.

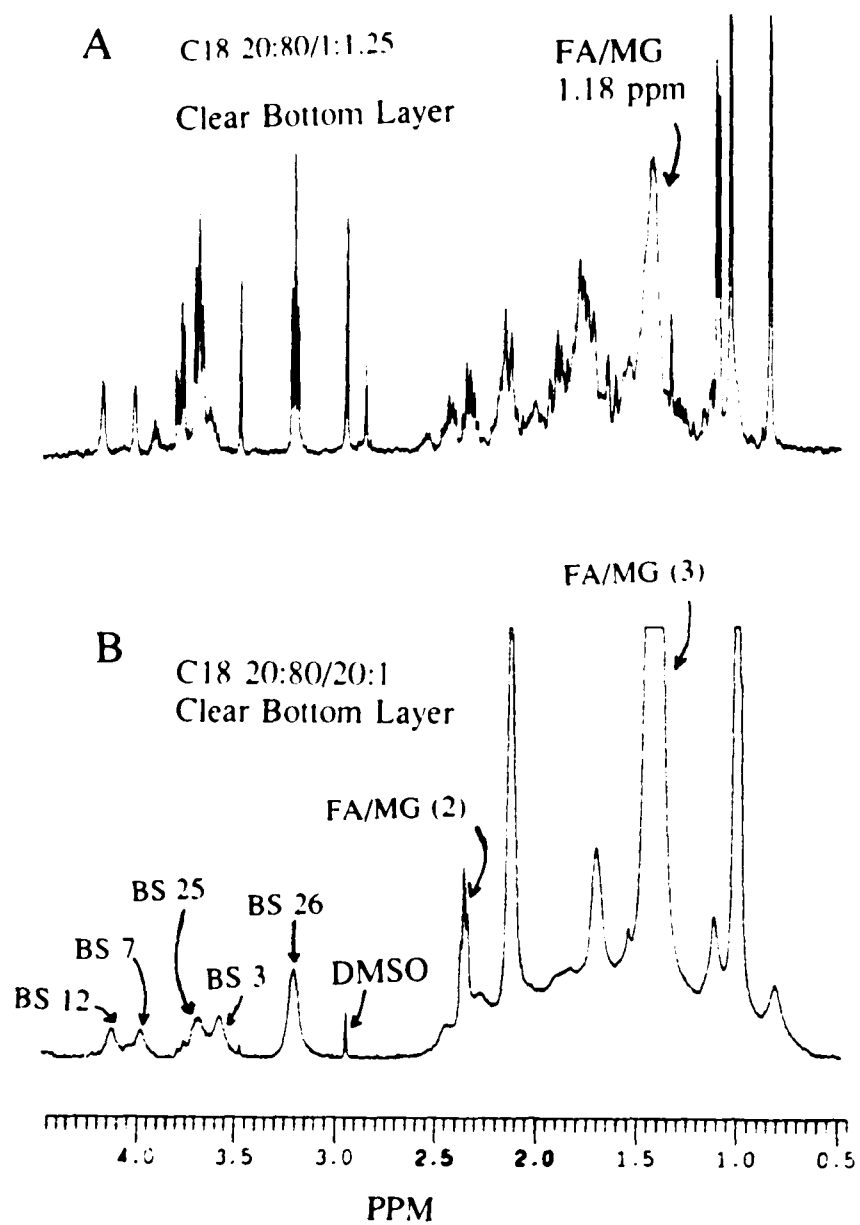
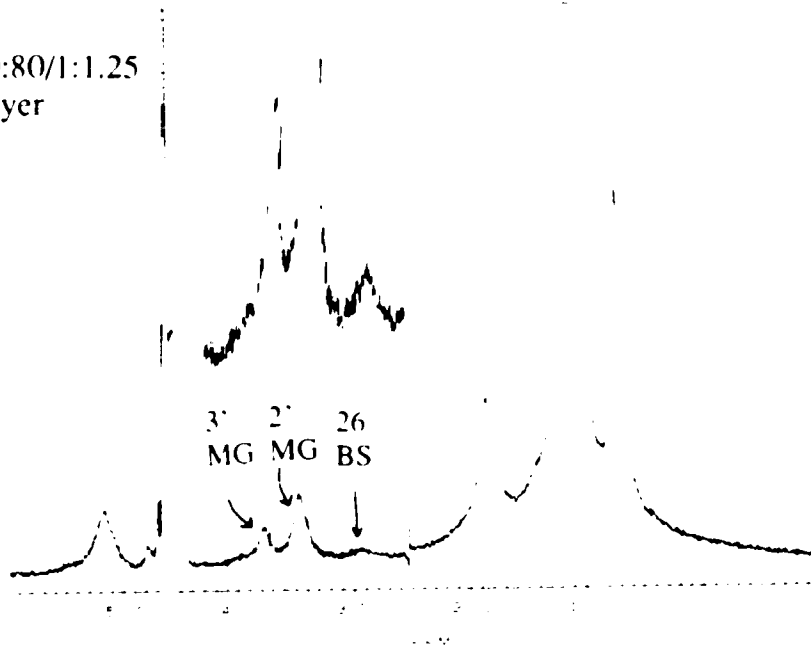


Figure V-9. 1D 200 MHz ^1H MAS spectra of opalescent top layers of two C18 mixtures. A: C18 20:80/1:1.25 and B: C18 20:80/20:1 at $\nu_{\text{rot}} = 1.2$ kHz. Comparable shimming, based on HOD lineshape and linewidth in both samples, supports structural changes as the source of these spectral differences.

A
C18 20:80/1:1.25
Top Layer



B
C18 20:80/20:1
Top Layer

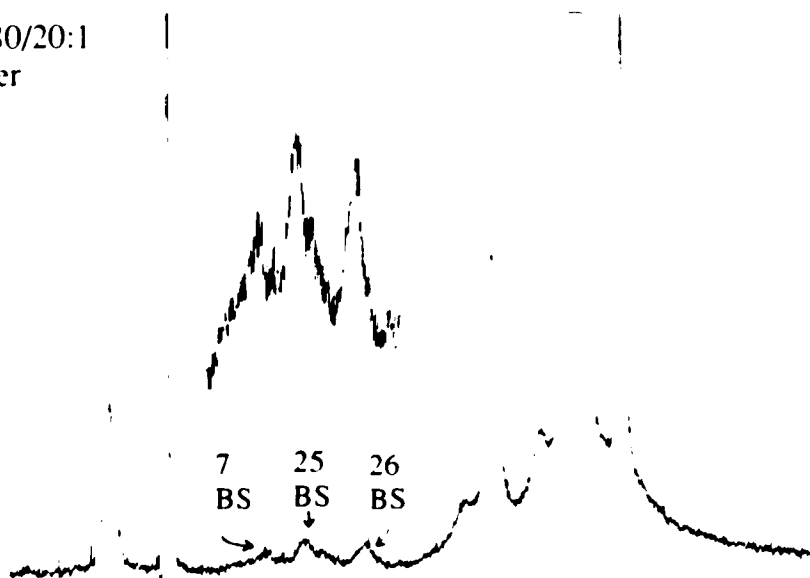
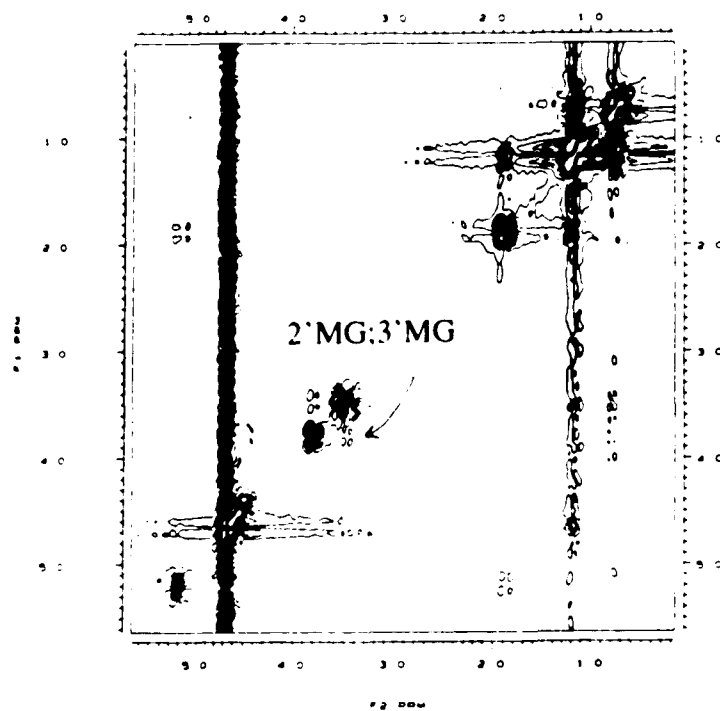


Figure V-10. 200 MHz MAS-assisted phase-sensitive COSY of two C18 mixtures. Unshifted sinebell window functions were used in both dimensions. A: C18 20:80:1:1.25 and B: C18 20:80/1:1.25 ($\nu_{\text{rot}} = 1.2$ kHz in both spectra). Interactions between pairs of FA protons and MG protons are labelled.

C18 20:80/1:1.25
COSY
Top Layer

A



C18 20:80/20:1
COSY
Top Layer

B

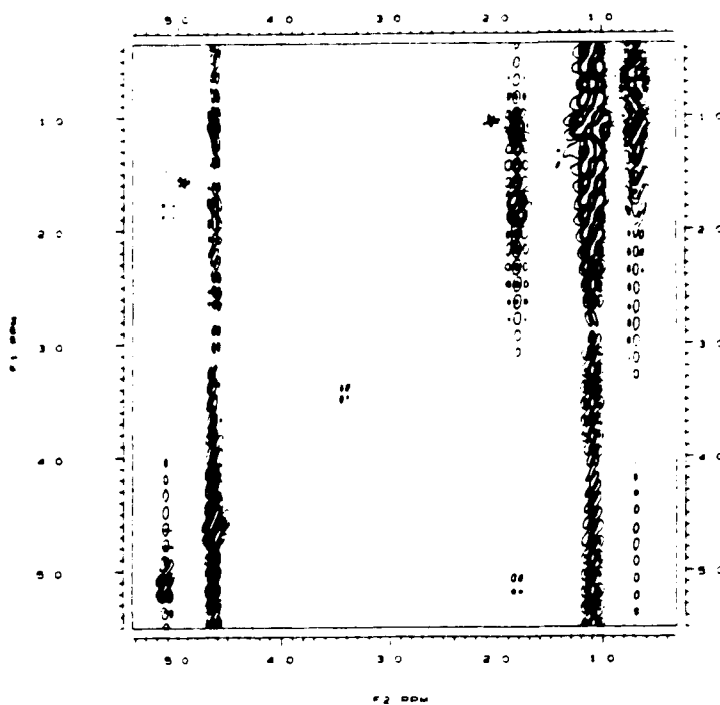


Figure V-11. 100 ms NOESY spectrum of C8 20:80/20:1. At this short mixing time, only intracomponent interactions are observed.

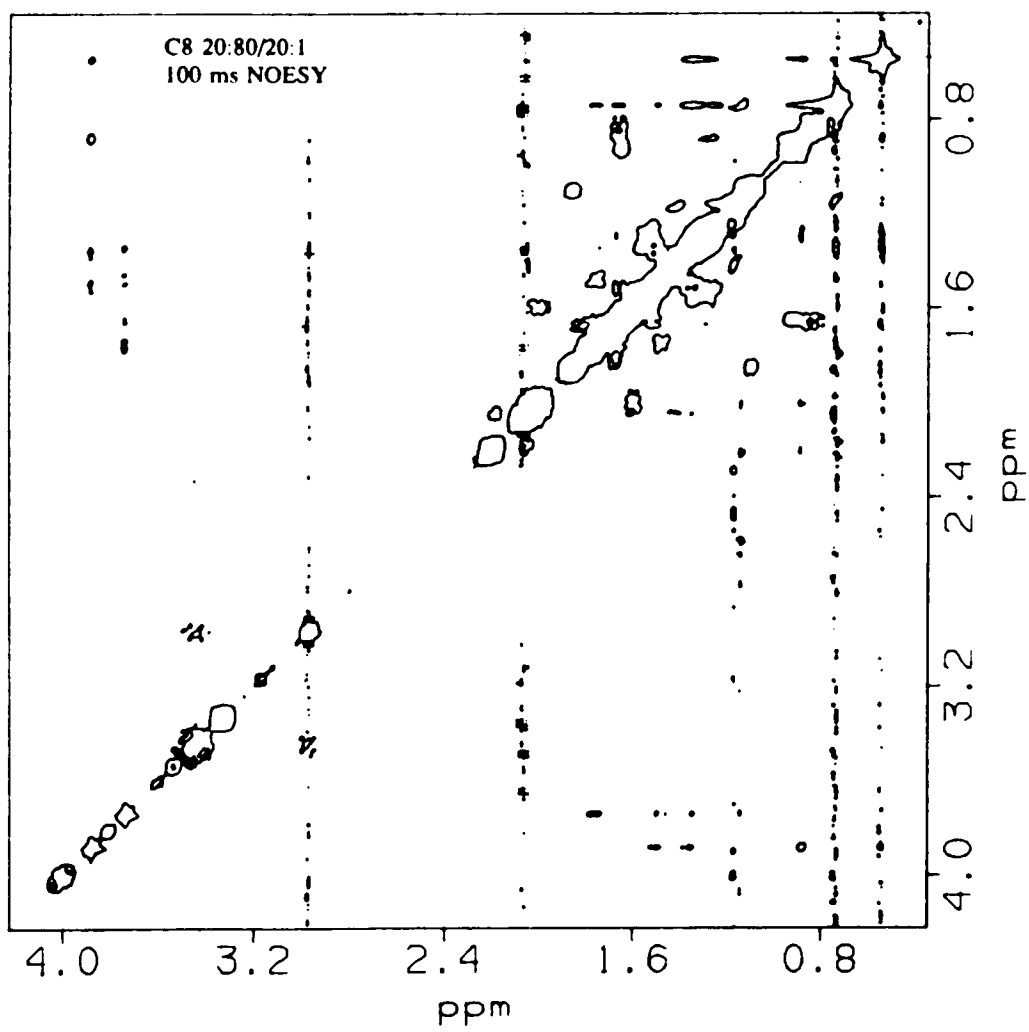
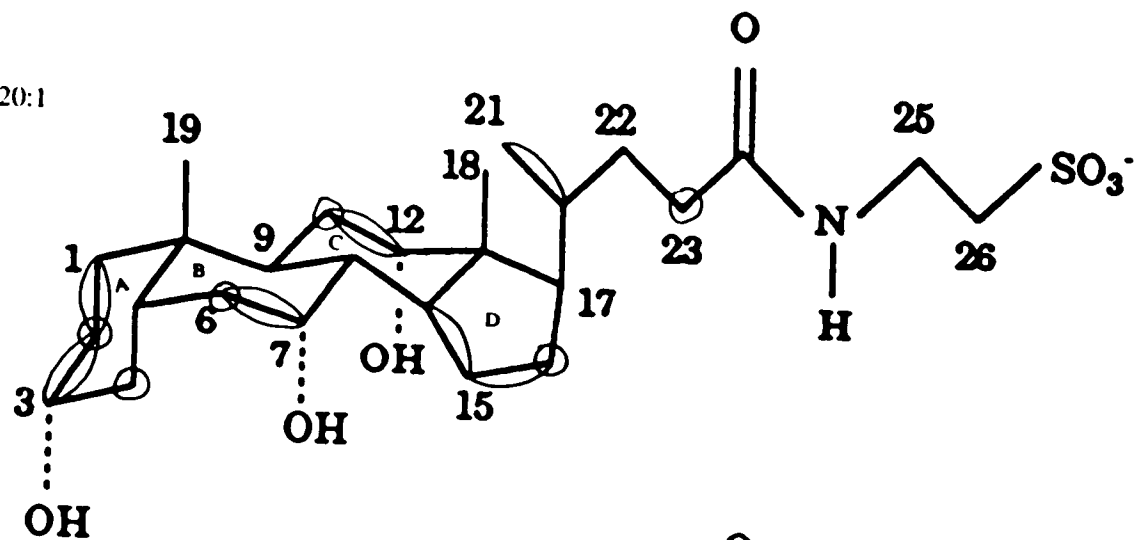


Figure V-12. BS skeleton showing both "geminal" and "sequential" NOEs which are observed for C8 20:80/20:1. Sequential interactions are not identified stereospecifically; two curved lines means that at least two of the four possible sequential interactions are observed (α, α' ; α, β' ; α', β ; β, β'). For further clarification see Table V-6. Geminal interactions are shown by a circle around the carbon to which the protons are attached.

C8 20:80/20:1



C8 20:80/1:1.25

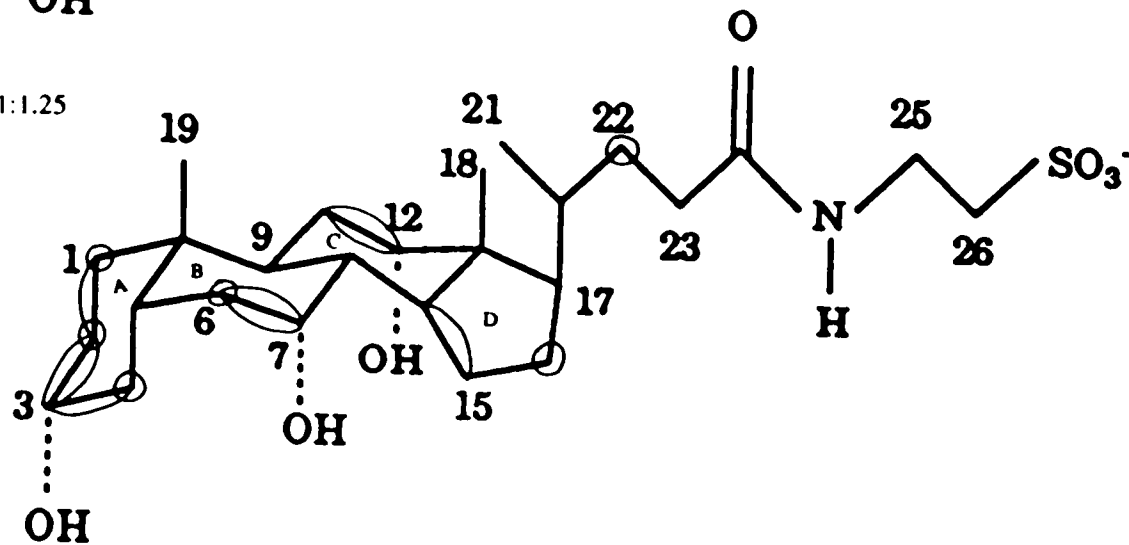


Figure V-13. Upfield portion of the 400 MHz 1000 ms NOESY of C8 20:80/20:1. The intermolecular interaction FA (4-7) and BS 18, also observed at a mixing time of 600 ms, is labelled.

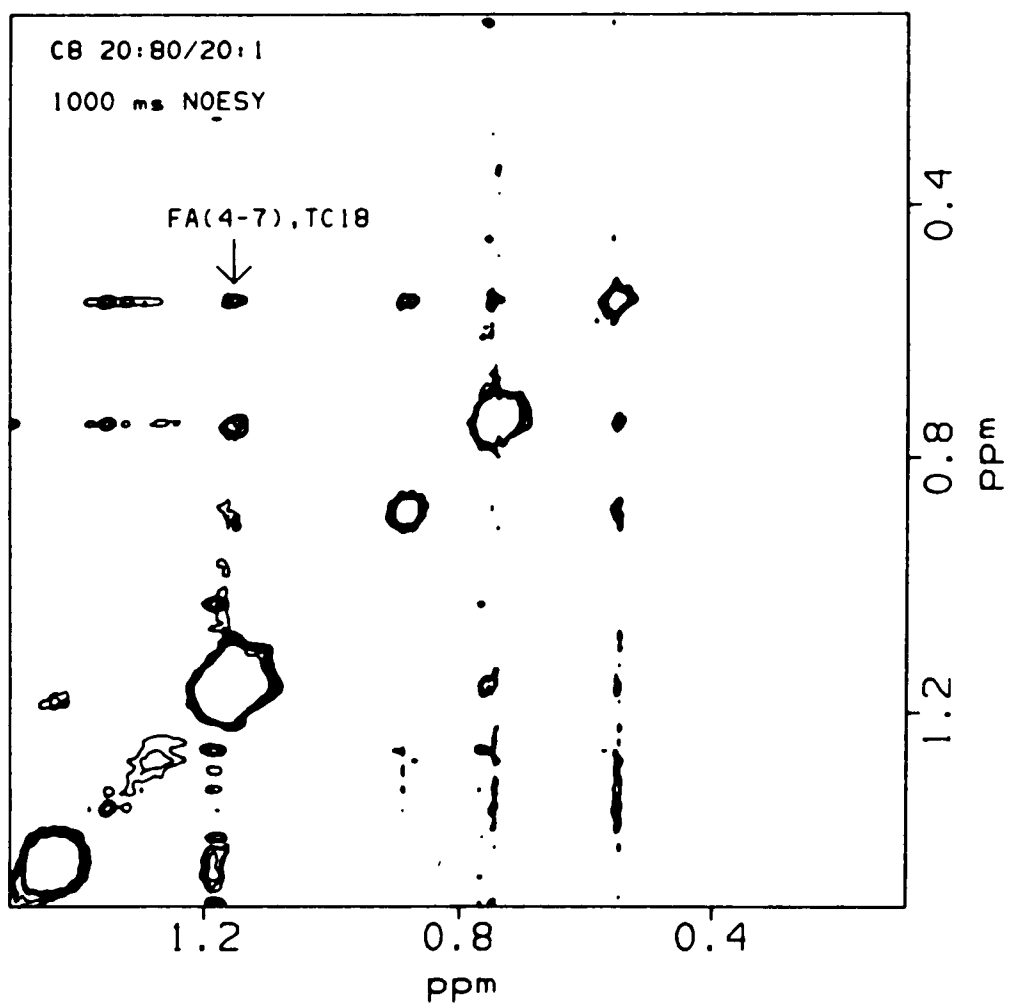


Figure V-14. Slice of 400 MHz 250 ms ROESY spectrum. The slice was obtained by loading a column at the resonance frequency of BS 18. The diagonal peak is phased positive and the ROEs are displayed as negative.

170

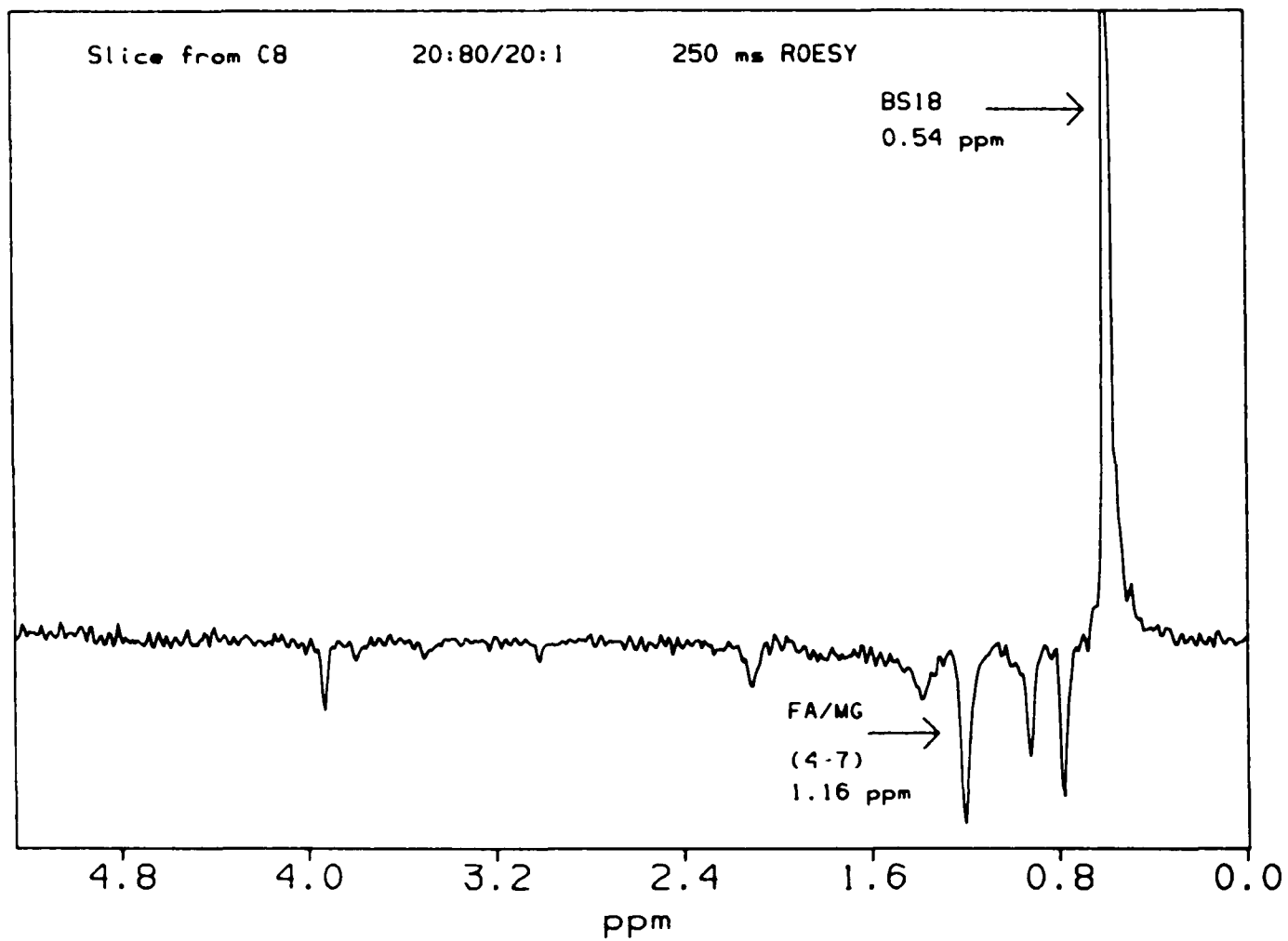


Figure V-15. ^1H spectra for C8 20:80/1:1.25. A: without Mn^{2+} and B: with 2.7 mM Mn^{2+} . BS sidechain proton 26, MG glycerol proton 1' and DMSO are labelled. Spectra were referenced using a DMSO insert.

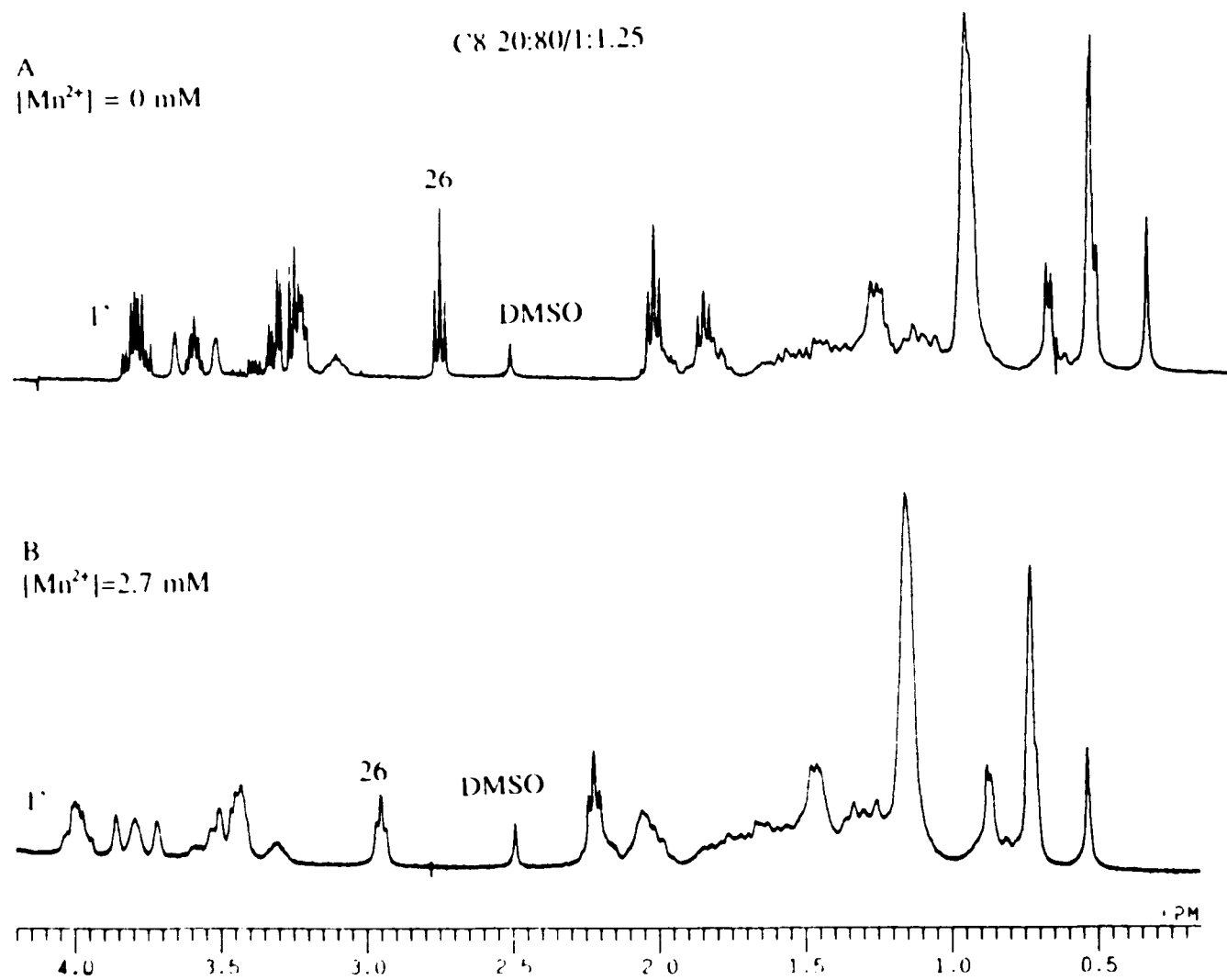


Figure V-16. 200 MHz 1D ^1H MAS spectra, with presaturation of HOD, of two C18 mixtures. Top: C18 20:80/1:1.25 at $\nu_{\text{rot}} = 0.61$ kHz and Bottom: C18 20:80/20:1 at $\nu_{\text{rot}} = 0.55$ kHz. First and second ssbs (ssb1 and ssb2) from HOD and other resonances are labelled. It is clear at present why $\text{ssb2} > \text{ssb1}$. While they are unexpected, future work will verify that no ssbs appear in a spectrum of pure water.

C18 20:80/1:1.25

$\nu_{\text{rot}} = 0.61 \text{ kHz}$

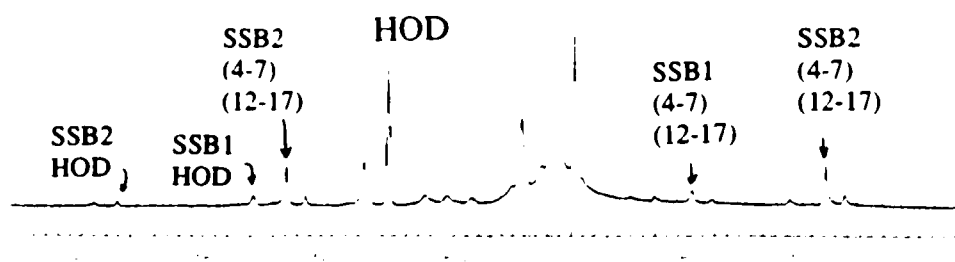
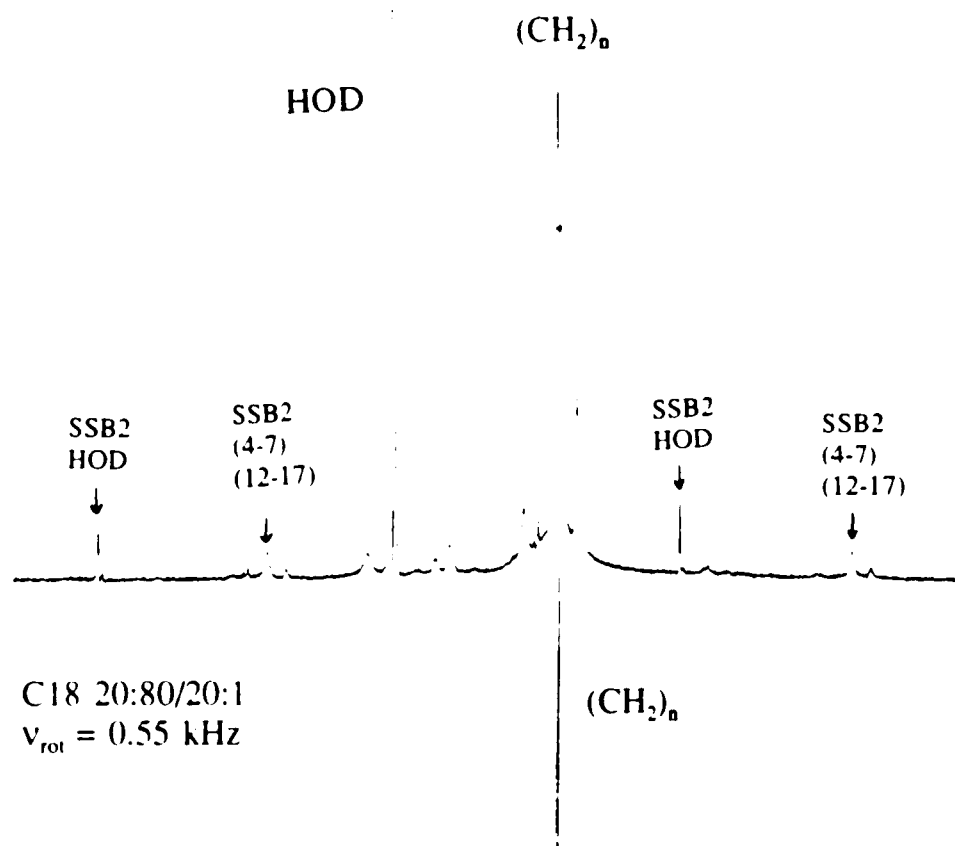
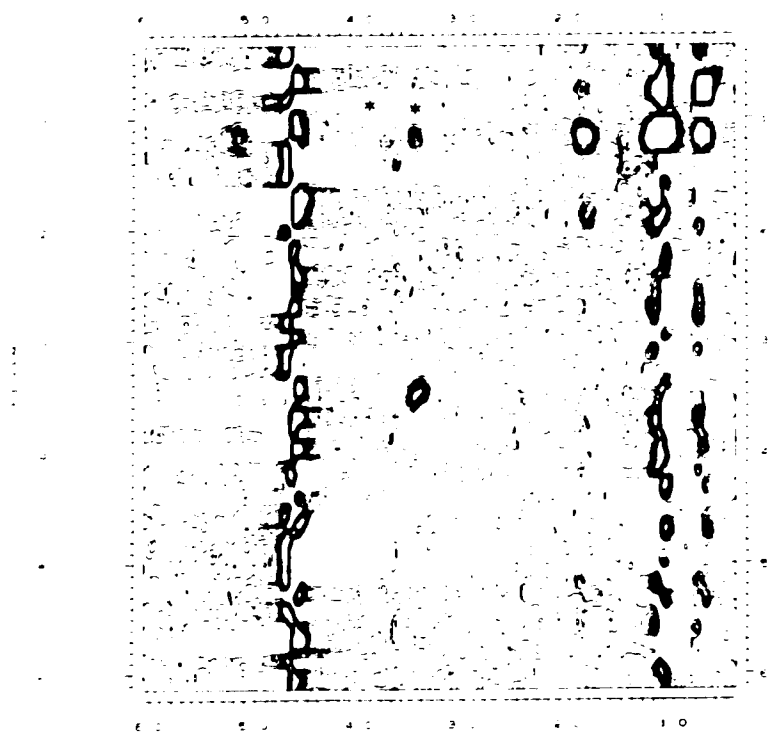


Figure V-17. 600 ms NOESY spectra of A: C18 20:80/1:1.25 and B: 20:80/20:1. In A the labelled crosspeaks (*) indicate long-range and possible intermolecular interactions between MG glycerol backbone protons and FA/MG acyl chain.

C18 20:80/1:1.25

A



C18 20:80/20:1

B

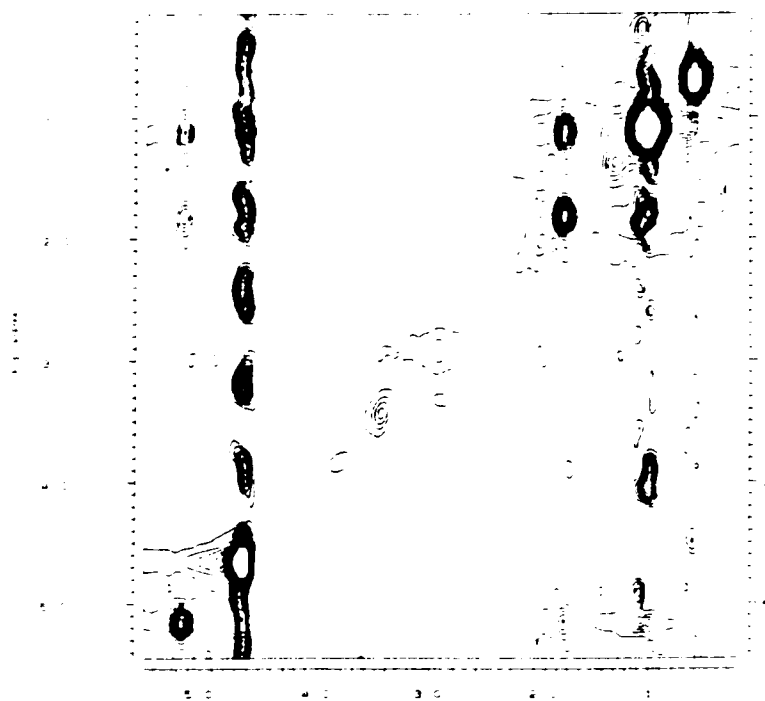
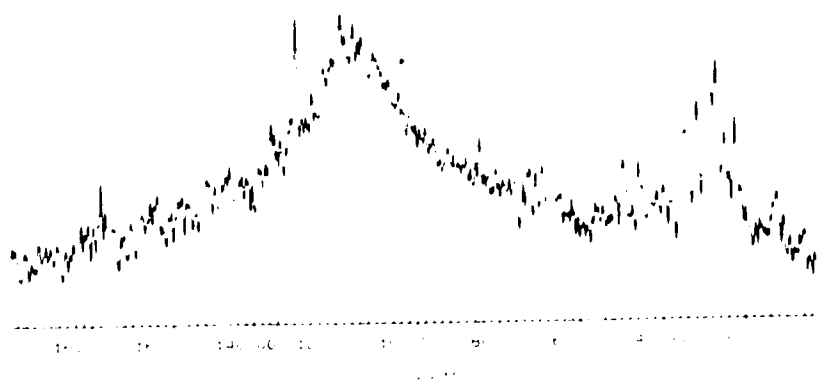


Figure V-18. 1D DPMAS ^{13}C 50 MHz NMR spectra of top layer C18 A:20:80/1:1.25 and B: 20:80/20:1. Due to low [BS] and low S/N no BS resonances are observed. A line broadening of 20 Hz was used for both spectra. The large hump at 110 ppm is due to a probe artifact. Overnight accumulation was required.

A 50 MHz ^{13}C Spectra
C18 Top Layer
20:80/1:1.25

[FA+MG]=36mM



B
C18 Top Layer
20:80/20:1

[FA+MG]=47mM

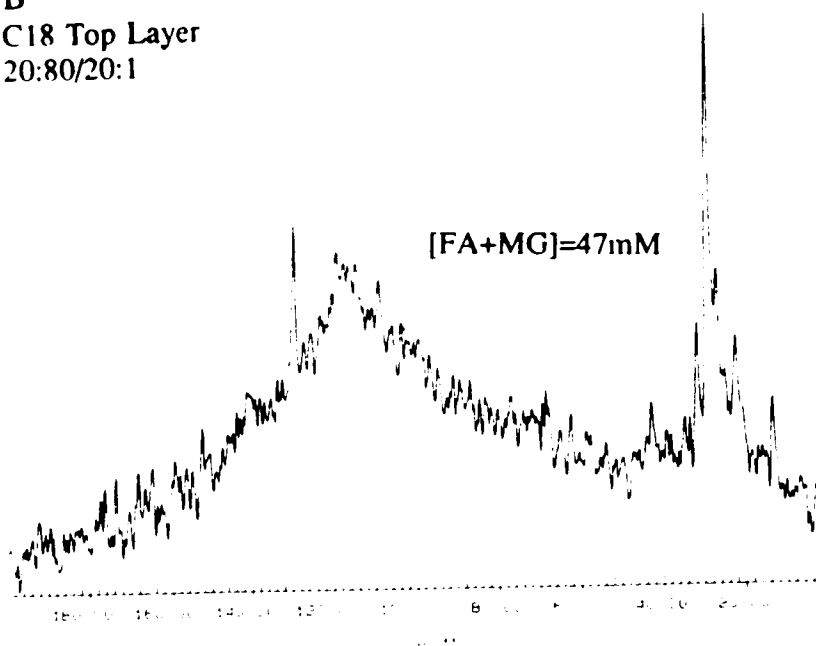
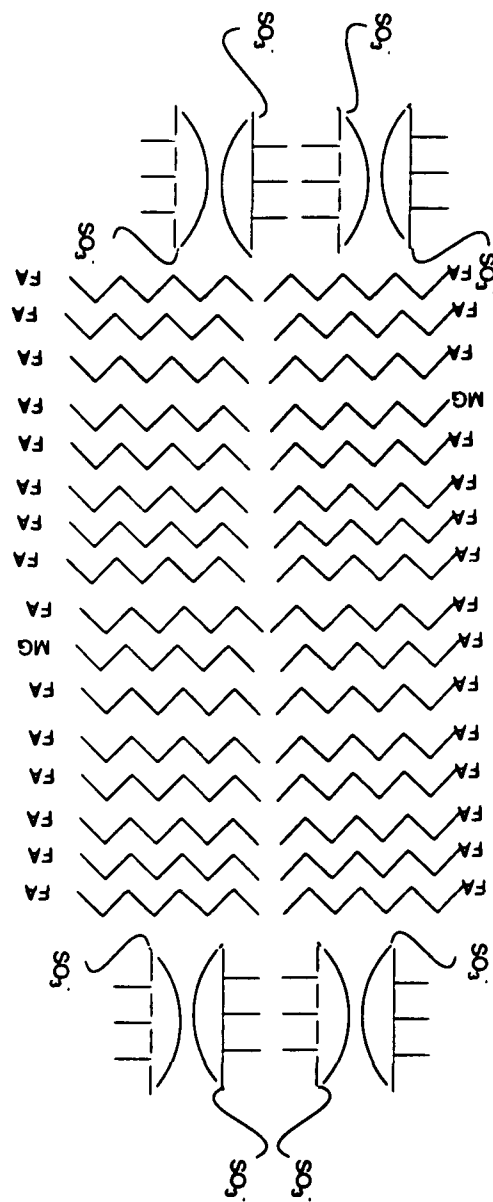
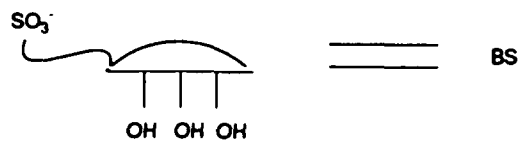


Figure V-19. Model for C8 mixed micelle showing internal and external location of BS sidechain protons and presence of back-to-back BS dimers. The model contains the correct molar ratios for C8 20:80/20:1. For C8 20:80/1:1.25 the MBS:MIL ratio would remain the same, but the FA/MG would be as described in Table II-1.



WHERE:



FA = COO⁻

MG = Glycerol backbone

Figure V-19. C18 bilayer model with correct molar ratios for C8 20:80/20:1 shown. For C18 20:80/1:1.25 MBS:MIL ratio would remain constant, but FA:MG would be as described in Table II-1. This model shows water trapped within the core of the bilayer and the antiparallel orientation of the acyl chains. As discussed in the text, this model assumes that molar ratios of the components remain as in Table II-1 despite the absence of NMR intensity for some components.

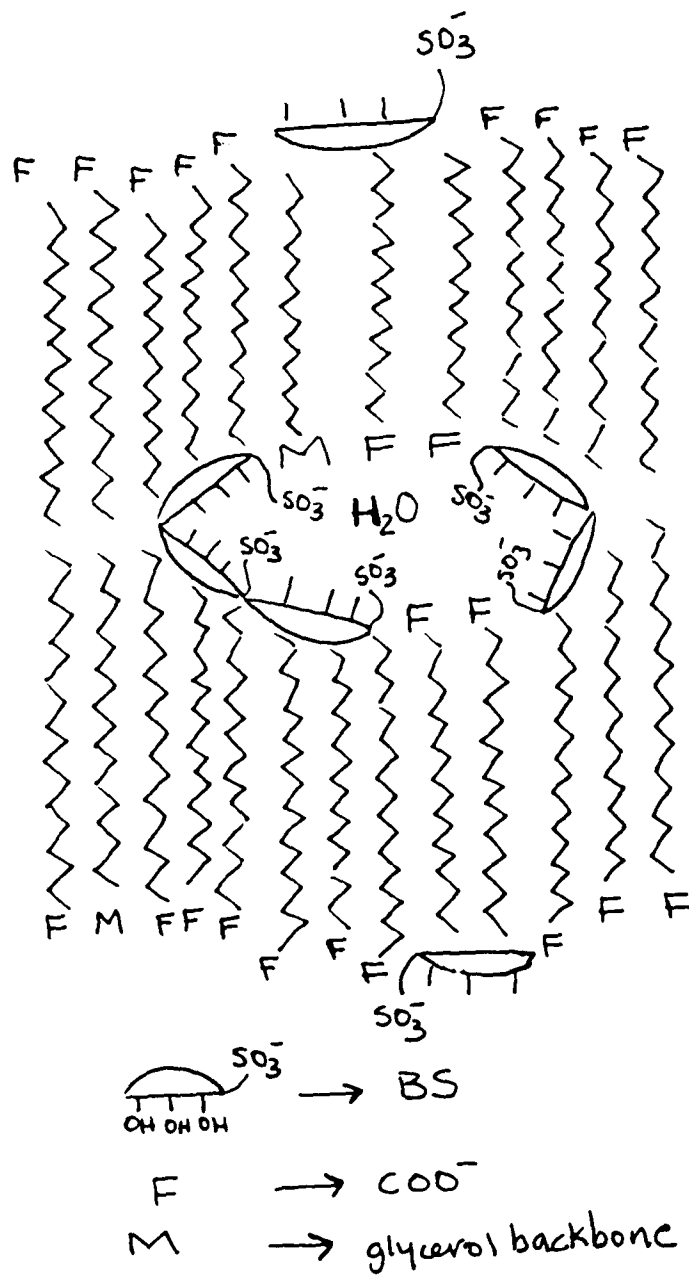


Table V-1 ^1H Chemical Shifts for C8 20:80/20:1*

Proton ^b	Component	Chemical shift ^c
1 (CH_2)	TC	1.67, 0.83
2 (CH_2)	TC	1.27, 1.52
3 (CHOH)	TC	3.34
4 (CH_2)	TC	1.61, 2.00
5 (CH)	TC	1.26
6 (CH_2)	TC	1.49, 1.76
7 (CHOH)	TC	3.75
8 (CH)	TC	1.34
9 (CH)	TC	2.10
11 (CH_2)	TC	1.36, 1.52
12 (CHOH)	TC	3.89
14 (CH)	TC	1.78
15 (CH_2)	TC	0.93, 1.68
16 (CH_2)	TC	1.11, 1.86
17 (CH)	TC	1.68
18 (CH_2)	TC	0.54
19 (CH_3)	TC	0.71
20 (CH)	TC	1.29
21 (CH_3)	TC	0.87
22 (CH_2)	TC	1.57, 1.35
23 (CH_2CO)	TC	2.22, 2.03
25 (CH_2)	TC	3.46
26 (CH_2)	TC	2.98
(2) (CH_2COOH)	FA	2.06
(3) (CH_2)	FA	1.43
(4), (5), (6) & (7) (CH_2) _n	FA	1.16
(8) (CH_3)	FA	0.73

1' (CH ₂ OH)	MG	4.01
2' (CHOH)	MG	3.82
3' (CH ₂ OC=OR)	MG	3.46, 3.54
[2] (COCH ₂)	MG	2.23
[3] (CH ₂)	MG	1.48
[4], [5], [6] & [7] (CH ₂) _n	MG	1.18
[8] (CH ₃)	MG	0.74

^aSpectra were collected at 400 MHz with HOD presaturation under the following conditions: [BS] = 20 mM; [FA] = 60 mM; [MG] = 3 mM; pH = 6.5; 37°C; 0.1 M phosphate buffer; 40 mM NaCl; 50 μM EDTA; 0.02% NaN₃

^bNumbering scheme and molecular structures are as shown in Figure II-1.

^cReferenced to HOD at 4.65 ppm.

Table V-2 ^1H Chemical Shifts for C8 20:80/1:1.25*

Proton ^b	Component	Chemical shift ^c
1 (CH_2)	TC	1.67, 0.82
2 (CH_2)	TC	1.29, 1.52
3 (CHOH)	TC	3.33
4 (CH_2)	TC	1.60, 2.03
5 (CH)	TC	1.26
6 (CH_2)	TC	1.49, 1.76
7 (CHOH)	TC	3.74
8 (CH)	TC	1.35
9 (CH)	TC	2.09
11 (CH_2)	TC	1.36, 1.52
12 (CHOH)	TC	3.88
14 (CH)	TC	1.81
15 (CH_2)	TC	0.92, 1.67
16 (CH_2)	TC	1.10, 1.87
17 (CH)	TC	1.71
18 (CH_2)	TC	0.57
19 (CH_3)	TC	0.76
20 (CH)	TC	1.31
21 (CH_3)	TC	0.89
22 (CH_2)	TC	1.58, 1.35
23 (CH_2CO)	TC	2.20, 2.04
25 (CH_2)	TC	3.45
26 (CH_2)	TC	2.97
(2) (CH_2COOH)	FA	2.08
(3) (CH_2)	FA	1.45
(4), (5), (6) & (7) (CH_2) _n	FA	1.18
(8) (CH_3)	FA	0.75

1' (CH ₂ OH)	MG	4.02
2' (CHOH)	MG	3.80
3' (CH ₂ OC=OR)	MG	3.47, 3.54
[2] (COCH ₂)	MG	2.25
[3] (CH ₂)	MG	1.49
[4], [5], [6] & [7] (CH ₂) _n	MG	1.20
[8] (CH ₃)	MG	0.76

^aSpectra were collected at 400 MHz with HOD presaturation under the following conditions: [BS] = 16 mM; [FA] = 36 mM; [MG] = 28 mM; pH = 6.5; 37°C; 0.1 M phosphate buffer; 40 mM NaCl; 50 μM EDTA; 0.02% NaN₃

^bNumbering scheme and molecular structures are shown in Figure II-1.

^cReferenced to HOD at 4.65 ppm.

Table V-3 COSY and TOCSY peaks observed for C8 20:80/20:1

Proton	COSY	TOCSY
1 α	1 β , 2 α	
1 β	1 α	
2 α	2 β , 1 α , 3 β	
2 β	2 α , 3 β	
3 β	2 α , 2 β , 4 α , 4 β	
4 α	4 β , 3 β	
4 β	4 α , 3 β , 5	
5	4 β , 6 β	6 β , 7 β
6 α	6 β , 7 β	
6 β	6 α , 5, 7 β	5
7 β	6 α , 6 β , 8	5
8	7 β , 9, 14	
9	8, 12 β	14
11 α	11 β , 12 β	
11 β	11 α , 12 β	
12 β	11 α , 11 β	
14	15 α , 15 β , 8	9
15 α	15 β , 14	
15 β	15 α , 14, 16 α , 16 β	
16 α	16 β , 15 β , 17	
16 β	16 α , 15 β , 17	
17	16 α , 16 β , 20	21
20	17, 21	
22 α	22 β , 23 β	23 α
22 β	22 α , 23 β	
25 α	25 β , 26 α , 26 β	
25 β	25 α , 26 α , 26 β	
26 α	26 β , 25 α , 25 β	
26 β	26 α , 25 α , 25 β	

The following COSY interactions were not observed:

1 β , 2 α ; 1 β , 2 β ; 1 α , 2 β ; 5, 6 α ; 9, 11 α ; 9, 11 β ; 15 α , 16 α ; 15 α , 16 β ; 20, 22 α ; 20, 22 β and 22 β , 23 α

For all assignments at least 1 sequential expected COSY interaction was observed and all expected geminal interactions were observed.

Table V-4 ^{13}C Chemical Shifts for C8 20:80/20:1, C8 20:80/1:1.25 and components in D_2O

Carbon	Chem. Shift (ppm) ^a 20:80/20:1	Chem. Shift (ppm) ^b 20:80/1:1.25	Chem. Shift (ppm) ^c
25	50.7 ^d	50.7	50.7
26	35.8 ^d	35.8	35.0
12	73.6 ^d	73.6	73.5
7	68.8 ^d	68.8	68.7
3	72.2 ^d	72.2	72.1
18	12.9 ^d	12.9	13.0
21	17.5 ^d	17.5	17.5
1	30.1 ^f	30.1	30.0
2	33.1 ^f	33.1	32.1
4	39.2 ^f	39.2	39.2
5	42.2 ^f	42.2	42.0
6	35.1 ^f	35.1	33.2
8	40.4 ^f	40.4	40.1
9	27.2 [*]	27.1	27.0
11	28.2 [*]	28.1	27.9
14	42.2 [*]	42.2	42.0
15	23.9 ^f	23.9	23.6
16	28.3 [*]	28.8	28.5
17	47.0 ^f	47.0	46.8
19	22.9 ^d	23.1	22.9
22	36.0 ^f	36.0	35.7
23	35.3 ^f	35.3	34.7
C=O	177.91	177.88	177.7
(1)	183.13	175.66	183
(2)	37.9 ^d	37.9	38.6

(3)	26.5 ^d	26.6	26.8
(4)	32.6 ^a	32.2	31.9
(5)	29.8/29.5 ^a	29.9	29.1/29.7
(6)	29.8/29.5 ^a	29.9	29.1/29.7
(7)	22.9 ^a	23.1	22.8
(8)	14.3 ^d	14.3	14.3
C1'	66.0 ^f	66.0	65.5
C2'	70.5 ^f	70.4	71.1
C3'	63.5 ^f	63.6	64.0
[2]	----- ^g	37.8 ^d	34.9
[3]	----- ^g	26.6 ^d	25.9
[4]	----- ^g	32.3 ^a	32.8
[5]	----- ^g	29.9 ^a	30.1
[6]	----- ^g	29.9 ^a	30.1
[7]	----- ^g	23.1 ^a	23.6
[8]	----- ^g	14.3 ^d	14.3

^a C8 20:80/20:1 referenced to (8) at 14.3 ppm (Wang et al., 1992)

^b C8 20:80/1:1.25 referenced to (8) at 14.3 ppm (Wang et al., 1992)

^c Components alone: TC and FA in D₂O; MG in CH₃OH (data from Wang et al., 1992 because no buffer was used). MG and FA referenced to (8) at 14.3 ppm (Wang et al., 1992) ; TC referenced with 18 at 13.0 ppm (Wang et al., 1992)

¹³C resonances were assigned using the following sources:

^d ¹H-¹³C HMQC spectra

^e Wang et al., 1992

^f Campredon et al., 1986

^g MG peaks were too weak to be detected in the presence of a much higher concentration of FA protons.

Table V-5 ^1H NMR Chemical Shifts of Top and Bottom Layers of C18 20:80/20:1 and 20:80/1:1.25

Proton	Sample	Component	Layer	Chemical shift ^c (ppm)
1-26	20:80/1:1.25	BS	bottom	same as Table V-1
(2)	20:80/1:1.25	FA/MG	top	not obsv.
(3)	20:80/1:1.25	FA/MG	top	not obsv.
(4-7), (12-17)	20:80/1:1.25	FA/MG	top	1.18
(8), (11)	20:80/1:1.25	FA/MG	top	1.90
(9), (10)	20:80/1:1.25	FA/MG	top	5.20
(18)	20:80/1:1.25	FA/MG	top	0.76
2'; 7	20:80/1:1.25	MG; BS ^a	top	3.75
3'; 25	20:80/1:1.25	MG; BS ^a	top	3.45
26	20:80/1:1.25	BS	top	2.90
(2)	20:80/20:1	FA/MG ^b	top	2.05
(3)	20:80/20:1	FA/MG	top	1.40
(4-7), (12-17)	20:80/20:1	FA/MG	top	1.15
(8), (11)	20:80/20:1	FA/MG	top	1.80
(9), (10)	20:80/20:1	FA/MG	top	5.10
(18)	20:80/20:1	FA/MG	top	0.61
7	20:80/20:1	BS	top	3.75
26	20:80/20:1	BS	top	2.90
25	20:80/20:1	BS	top	3.42
(2)	20:80/20:1	FA/MG	bottom	2.12
(3)	20:80/20:1	FA/MG	bottom	1.46
(4-7)	20:80/20:1	FA/MG	bottom	1.18

(8), (11)	20:80/20:1	FA/MG	bottom	1.88
(12-17)	20:80/20:1	FA/MG	bottom	1.17
(18)	20:80/20:1	FA/MG	bottom	0.76
(9), (10)	20:80/20:1	FA/MG	bottom	5.18
12	20:80/20:1	BS	bottom	3.88
7	20:80/20:1	BS	bottom	3.74
26	20:80/20:1	BS	bottom	3.44
3	20:80/20:1	BS	bottom	3.34
25	20:80/20:1	BS	bottom	2.97
18	20:80/20:1	BS	bottom	0.57
21	20:80/20:1	BS	bottom	0.87

* Low concentration BS peaks are buried underneath high concentration MG peaks.

^b MG peaks were too weak to be detected in the presence of a much higher concentration of FA protons.

^c Referenced to HOD at 4.65 ppm.

Table V-6 Intramolecular NOEs observed for C8 20:80/20:1

Chem. Shift (ppm)	Long Range	Assignment	100 ms	600 ms	600 ms FA-d ₂	1000 ms
3.89, 1.67''	*	TC 12, TC (1 α , 15 β , 17)	w	x	x	x
3.89, 1.51		TC 12, TC 11 β	x	x	x	x
3.89, 1.36		TC 12, TC 11 α	x	x	x	x
3.89, 0.89	*	TC 12, TC 21	x	x	x	x
3.89, 0.75	*	TC 12, TC 19	x	x	x	x
3.89, 0.56	*	TC 12, TC 18	x	x	x	x
3.75, 0.56	*	TC 7, TC 18	x	x	x	x
3.75, 0.75	*	TC 7, TC 19	x	x	x	x
3.75, 1.35	*	TC 7, TC 11 α	x	x	x	x
3.75, 1.51		TC 7, TC 6 α	x	x	x	x
3.75, 1.67	*	TC 7, TC (1 α , 15 β , 17)	x	x	x	x
3.75, 1.77		TC 7, TC (14, 6 β)	x	x	x	x
3.34, 1.27		TC 3, TC 2 α	w	x	x	x
3.34, 1.54		TC 3, TC 2 β	x	x	x	w
3.34, 1.61		TC 3, TC 4 α	x	x	x	x
3.34, 2.01''		TC 3, TC 4 β	- --	x	x	w
2.20, 2.06		TC 23 β , TC 23 α	x	x	x	x
2.01, 1.61		TC 4 β , TC 4 α	x	x	x	---
1.49, 1.76		TC 6 α , TC 6 β	x	x	x	w

1.68, 1.79		TC 15 β , TC 14	X	X	X	X
0.75, 0.56		TC 19, TC 18	X	X	X	X
0.88, 0.56	*	TC 21, TC 18	X	X	X	X
1.35, 0.56	*	TC 22 α , TC 18	X	X	X	**
1.35, 0.75	*	TC 22 α , TC 19	X	X	X	X
1.26, 0.75	*	TC 5, TC 19	X	X	X	X
1.16, 0.75		FA (4)-(7), FA(8); TC 19	X	X	---	X
1.43, 1.18		FA (3), FA (4)- (7)	X	X	---	X
3.55, 3.52		MG 3', MG 3'	X	X	X	X
1.29, 0.89''		TC 20, TC 21	X	X	X	X
3.75, 1.27''	*	TC 7, TC (2 α ,5)	- --	W	X	X
3.34, 0.76''	*	TC 3, TC 19	- --	W	X	X
1.52, 1.67		TC 2 β , TC 1 β	X	X	X	X
1.79, 0.56''	*	TC 14, TC 18	- --	X	X	X
1.68, 0.56		TC 17, TC 18	W	X	X	X
1.50, 0.56	*	TC 6 α , TC 18	W	X	X	X
1.50, 0.76	*	TC (11 β ,2 β), TC 19	X	X	X	X
1.67, 0.76''	*	TC (1 β , 15 β ,17), TC 19	X	X	X	X
1.77, 0.76	*	TC (6 β ,14), TC 19	X	X	X	X
2.07, 0.76''	*	TC 9, TC 19	**	X	X	---
1.67, 0.84		TC 2 β , TC 1 α	X	X	X	W
1.84, 1.68''		TC 16 β , TC 15 β	X	X	X	X

1.84, 1.12		TC 16 β , TC 16 α	w	x	x	---
1.53, 0.84		TC 2 β , TC 1 α	x	x	x	w
1.50, 1.36		TC 11 β , TC 11 α	x	x	x	w
1.27, 1.61''	*	TC 2 α , TC 4 α	x	x	x	x
1.29, 1.54	*	TC 20, TC 11 β	**	x	x	x
1.52, 1.28		TC 2 β , TC 2 α	**	x	x	x
0.76, 1.37	*	TC 19, TC 11 α	x	x	x	x
1.34, 0.89''		TC 22 α , TC 21	- --	x	x	w
1.30, 0.89''		TC 20 β , TC 21	x	x	x	w

* These interactions are not sequential or geminal

** These crosspeaks were buried under the diagonal or in t₁ noise.

· These interactions are not observed in C8 20:80/1:1.25

'' These crosspeaks are not seen in C8 20:80/1:1.25

Table V-7 Intramolecular NOEs observed for C8 20:80/1:1.25

Chem. Shift (ppm)	Long Range*	Assignment	100 ms	600 ms	1000 ms
3.47, 3.54		MG 3' α , MG 3' β	X	X	---
3.88, 1.32''	*	TC 12, TC 20	X	X	---
3.88, 1.35		TC 12, TC [11,8]	X	X	X
3.88, 1.52		TC 12, TC 11 β	X	X	---
3.88, 1.49''	*	TC 12, TC 6 α	X	X	---
3.73, 1.32	*	TC 7, TC 20	X	X	---
3.73, 1.35		TC 7, TC [8,11]	X	X	X
3.73, 1.50		TC 7, TC 6 β	X	X	W
3.73, 1.66	*	TC 7, TC [1,15]	X	X	X
3.73, 1.76		TC 7, TC 6 α	X	X	X
3.73, 0.55	*	TC 7, TC 18	---	X	---
3.88, 0.55	*	TC 12, TC 18	X	X	X
3.88, 0.75	*	TC 12, TC 19	---	X	---
3.74, 0.75	*	TC 7, TC 19	---	X	---
3.88, 0.89	*	TC 12, TC 21	X	X	X
3.74, 0.89''	*	TC 7, TC 21	---	X	---
3.33, 1.27		TC 3, TC 2 α	W	X	---
3.33, 1.53		TC 3, TC 2 β	W	X	---
3.33, 1.60		TC 3, TC 4 α	W	X	---
1.75, 1.48		TC 6 α , TC 6 β	X	X	---
1.80, 1.68		TC 14, TC 15 β	W	X	X
1.60, 2.02		TC 4 α , TC 4 β	X	X	X
2.05, 2.21		TC 23 α , TC 23 β	X	X	X
1.32, 0.75		TC 22, TC 19	X	X	---
1.32, 0.55	*	TC 22, TC 18	X	X	X
1.35, 0.55''	*	TC 8, TC 18	X	X	X
1.35, 0.75	*	TC 8, TC 19	X	X	---
1.38, 0.55	*	TC 11, TC 18	X	X	X
1.38, 0.75	*	TC 11, TC 19	X	X	X
1.27, 0.75	*	TC 5, TC 19	---	X	---
1.15, 0.75		FA (4-7), FA (8)	X	X	N'

0.55, 0.89	*	TC 18, TC 21	W	X	X
0.55, 0.75		TC 18, TC 19	W	X	---
1.29, 1.53		TC 2 α , TC 2 β	X	X	---
1.50, 0.55	*	TC 6, TC 18	X	X	X
1.69, 0.55	*	TC [1,15,17], TC 18	---	X	X
1.77, 0.75	*	TC 6, TC 19	W	X	---
1.69, 0.89''	*	TC [1,15,17], TC 21	X	X	W
1.49, 0.75	*	TC 6 β , TC 18	---	X	---
1.49, 0.88''	*	TC 6 β , TC 21	---	X	X
2.07, 1.17	*	FA (2), FA (4-7)	---	X	N
2.07, 0.75	*	FA (2), FA (8)	---	X	---
2.99, 3.89''	*	TC 25, TC 12	X	X	---
1.10, 1.87		TC 16 α , TC 16 β	X	---	---
1.67, 1.53		TC 1 α , TC 2 β	X	---	---
1.35, 1.50		TC 11 α , TC 11 β	---	X	X
1.67, 0.83		TC 1 α , TC 1 β	X	---	---

* These interactions are neither sequential or geminal.

' t₁ noise was too severe to determine if crosspeak was present.

'' these interactions are not observed for C8 20:80/20:1

Table V-8 Intermolecular NOEs observed for C8 20:80/20:1

Chem. Shift (ppm)	Assignment	100 ms	600 ms	600 ms FA-d ₁₅	1000 ms
1.16, 0.56	FA (4)-(7), TC 18	---	X	---	X
1.16, 0.75	FA (4)-(7), FA (8); TC 19	X	X	---	X
3.75, 1.48	TC 7, MG 3	---	---	---	X

Table V-9 Intermolecular NOEs observed for C8 20:80/1:1.25

Chem. Shift (ppm)	Assignment	100 ms	600 ms	1000 ms
3.73, 1.46	TC 7, FA (3)	X	X	---
2.26, 1.46	MG [2], MG/FA (3)	---	X	---
1.16, 0.55	FA (4-7), TC 18	---	X	---
2.07, 1.29	FA (2), TC 2	---	X	---

Table V-10 250 ms ROESY results for C8 20:80/20:1 (Columns*)

Proton A	Type of Interaction	Proton B
BS 26	TOCSY	BS 25
BS 26	ROE	BS 4 FA/MG (2) FA/MG (3) FA/MG (4) - (7) FA/MG (8)
BS 25	TOCSY	BS 26
BS 25	ROE	FA/MG (2) FA/MG (4) - (7)
FA (2)	TOCSY	FA (3) FA (4) - (7)
FA (2)	ROE	FA (8)/BS 19 BS 25 BS 26
FA (3)	TOCSY	FA (2) FA (4) - (7)
FA (3)	ROE	BS 18 BS 25 BS 26
FA (4) - (7)	TOCSY	FA (2) FA (3) FA (8)
BS 21	TOCSY	BS 20
BS 21	ROE	BS 12 BS 15 BS 18 BS 23 BS 25 BS 26 FA (2) FA (3) FA (4) - (7)
FA (8)	TOCSY	FA (2) FA (4) - (7)
FA (8)	ROE	FA (2)

BS 18	ROE	BS 12 BS 19/FA (8) BS 21 BS 25 BS 26 FA (2) FA (3) FA (4) - (7)

* Data were obtained by loading a column at the resonance frequency of individual protons.

Table V-11 NOEs Observed in C18 Bottom Layer 20:80/20:1

Protons	Chemical Shift (ppm)	100 ms	600 ms	1000 ms
(9), (10); (8), (11)	5.18, 1.88	X	X	X
(9), (10); [(4) - (7)]	5.18, 1.19	X	X	X
(2); [(4) - (7)]	2.12, 1.19	X	X	X
(8), (11); [(4) - (7)]	1.88, 1.19	X	X	X
(3); [(4) - (7)]	1.46; 1.19	X	X	X
26; 25	3.43; 2.97	---	X	---
(2); (8), (11)	2.12; 1.88	---	X	X
(2); (3)	2.12; 1.46	---	X	---
(8), (11); (3)	1.88; 1.46	---	X	X
(18); [(12) - (17)]	0.76; 1.15	---	X	---

Table V-12 Spinning Sidebands observed for top layer C18
20:80/20:1 at $\nu_{\text{rot}} = 0.56$ kHz

Proton	Chemical Shift	SSB ^a 1	SSB ^a 2
(9), (10)	5.18 ppm	---	---
HOD	4.65 ppm	X ^b	X
(8), (11)	1.89 ppm	---	---
(4) - (7), (12) - (17)	1.18 ppm	X ^b	X
(18)	0.76 ppm	X ^c	X

^a ssbs are symmetrical unless otherwise specified

^b One ssb buried under peak in spectrum

^c One ssb is buried and one ssb is missing

Table V-13 Spinning Sidebands observed for top layer C18
20:80/1:1.25 at $\nu_{\text{rot}} = 0.61$ kHz

Proton	Chemical Shift	SSB ^a 1	SSB ^a 2
(9), (10)	5.20 ppm	---	---
HOD	4.65 ppm	X'	X
(8), (11)	1.90 ppm	---	---
(4)-(7), (12)-(17)	1.18 ppm	X	X
(18)	0.76 ppm	---	X

^a ssbs are symmetrical unless otherwise specified

^b One ssb buried and one ssb observed

^c One ssb is buried and one ssb is missing

Table V-14 NOEs observed for top layer C18 20:80/1:1.25

Protons	Chemical Shifts (ppm)	100 ms	600 ms	800 ms
(9), (10); (8), (11)	5.2; 1.90	X	---	---
(9), (10); [(12-17), (4-7)]	5.2; 1.18	X	X	X
(8), (11); [(12-17), (4-7)]	1.90; 1.18	X	X	X
[(12-17), (4-7)]; (18)	1.18; 0.76	X	X	X
[(12-17), (4-7)]; MG 3'	1.18; 3.45	X ^a	X ^a	W ^a
[(21-17), (4-7)]; MG 2'	1.18, 3.75	---	X ^a	W ^a

^a Represents a possible intercomponent interaction

Table V-15 NOEs observed for Top Layer C18 20:80/20:1

Proton	Chemical Shift (ppm)	100 ms	600 ms	800 ms
(9), (10); (8), (11)	5.10; 1.80	X	X	---
(9), (10); [(12-17), (4-7)]	5.10; 1.15	X	X	W
[(12-17), (4-7)]; (8), (11)	1.15; 1.80	X	X	W
[(12-17) - (4-7)]; (18)	1.15, 0.61	---	X	X
[(12-17), (4-7)]; (2)	2.05, 1.15	---	---	X
(9), (10); (3)	5.10; 1.40	---	---	X
(2), (3)	2.05; 1.40	---	---	W
(8), (11); (3)	1.80; 1.40	---	---	X
[(12-17); (4-7)]; (3)	1.40, 1.15	---	---	X

Table V-16 ^{13}C Chemical Shifts for C18 Top Layers 20:80/20:1 and 20:80/1:1.25

Carbon	Chem. Shift (ppm) * 20:80/20:1	Chem. Shift (ppm) * 20:80/1:1.25
2	35	33
3	27	27
4	31	28.5
7;12	22	28.5
(4-6); (13-17)	29	28.5
8;11	25	23
9;10	129	129
18	14	14

* Chemical shifts are referenced to ^{13}C standard

Chapter VI

Conclusions

Various types of NMR spectroscopy are used to study the different kinds of biological polymers of interest in physiology and medicine. For studying smaller molecule, peptides for example, 2D NMR has become the most routinely used method. In the work presented here COSY and NOESY were used to fully assign and structurally characterize the amino terminal segment of the p21 protein and three analogues. The functionally relevant nucleotide binding site is located in this segment. Our results show that, despite high GDP binding, this segment contains no long-lived identifiable structure. The loop located in this region must, therefore, be stabilized by long-range interactions from other areas of the protein.

Although much larger than peptides, some proteins can be structurally analyzed using ^1H 2D NMR. However, due to the larger linewidths which proteins generally have, this method is limited to biopolymers which have a molecular weight no larger than 12 kD. Our study of the 11 kD enzyme porcine pancreatic colipase was impeded by the fact that this protein exists, despite published reports to the contrary, as a dimer in solution at millimolar concentrations. Our NOESY results, however, do lend support to a colipase β -clam structure. Given the hydrophobicity of the two binding sites, which could be brought together by either a β -clam foldover or formation of

a dimer, it is not surprising that this protein aggregates at the "biologically" high concentrations required for NMR.

In an attempt to study the digestive processes from the substrates' point of view, model mixtures of late-stage triglyceride digestive products were prepared. These model mixtures were comprised of monoglyceride (C8 or C18), fatty acid (C8 or C18) and bile salt (taurocholate). To investigate the digestion of various types of fats, mixtures of different component ratios as well as different acyl chain lengths were selected (C8 or C18).

Mixtures containing C8 components were studied using high-resolution, solution-state ^1H and ^{13}C 1D and 2D NMR. In contrast to QLS methods used previously, NMR spectroscopy allows specific sites in these mixtures to be studied. As shown by NOESY, ROESY and Mn^{2+} data, these C8 mixtures exist as mixed micelles where the BS sidechain has only modest aqueous accessibility. Contrary to previous models (Wang et al., in press) for similar systems we find, using NOESY and ROESY, that BS sidechains are located within the aggregate as well as outside. In agreement with T_1 relaxation studies (Wang et al., 1992) our results support an aggregate where different protons are undergoing different motions and may be grouped as hydrophilic, hydrophobic and interfacial.

Due to the long hydrophobic acyl chain, mixtures containing C18 components partition into two layers. The optically clear bottom layers were studied using solution-state NMR and were found to exist in solution as either BS monomers or mixed micelles. These mixed micelles were similar to, but larger than, the ones formed with C8. Due to their broad resonances, the opalescent C18 top layers required 1D and 2D MAS NMR. Spinning sideband analyses of these spectra show that there is considerable orientational order in these aggregates. In addition to strong ssbs observed for the bulk methylene chain protons, the presence of solvent ssbs support the presence of lipid bilayers for both C18 top-layer mixtures.

For all mixtures studied the change in FA/MG seemed to have only a modest effect on the aggregates formed. Assuming that the structures presented here are realistic representations of these aggregates, it seems likely that a variance in the most amphiphilic component, BS, may result in more drastic changes. Future NMR experiments with varying MBS:MIL ratios will help determine the extent of these changes.

When conducted along with QLS assessment of the average radius, NMR techniques provide direct information on motional and molecular organization at physiologically relevant

concentrations. Our finding of different aggregate arrangements for C8 and C18 suggests a structural basis for the different ways in which digestive products are processed physiologically.

Chapter VII

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