Structural Studies of Collagen by Solid State NMR.

Richard J. Wittebort and Anne Marie Clark
University of Louisville, Department of Chemistry,
Louisville, KY 40292 USA

Introduction:

Collagen is one of the most abundant proteins nature and has the same structure and virtually the same composition for diverse species. It provides the organic matrix for teeth and bones, and gives tendons and blood vessels their strength. Collagen has three polypeptide chains, two $\alpha 1$ chains and one $\alpha 2$ chain, coiled together in a 3-1 helix. Each chain is formed from a repeating (Gly-X-Y) triad with Gly always in the first position. The other two positions can have any amino acid but frequently (about 30%) have proline (Pro) and hydroxyproline (Hyp). Hyp always occurs in position 3 while Pro usually occurs in position 2 and only rarely in position 3 in adult animals. The principle difference between the types of chains is their direction of orientation.

Several models have been developed to explain the three dimensional structure of collagen. From steric considerations, it is thought that the collagen triple helix has Gly alpha hydrogens pointing inside. Despite numerous attempts to solve the X-ray structure, collagen's lack of long range order has made it impossible to determine a unique structure from diffraction methods.[1-5]

Collagen is a uniaxially ordered fiber with helical symmetry about the fiber axis. In order to do structural studies by solid state NMR, the fiber axis must be aligned with the magnetic field. Opella used solid state techniques to determine the three dimensional structure of bactreiophage fd coat protein.[6] This experiment requires at least one direction of orientation and the molecular sites of interest must be immobilized and uniformly oriented with respect to the magnetic field. In single crystals, any arbitrary sample orientation can be studied; however, for uniaxially oriented samples the direction of orientation must be along the applied field to obtain single crystal-like spectra. The bacteriophages are well suited for this type of experiment. They spontaneously align with the magnetic field and their large size and rod-like shape immobilize

the protein subunits. The alpha helical structure determined agrees well with X-ray structures of alpha helical proteins.

The three dimensional structure of polypeptides can be described as a series of connected peptide planes. By selectively labelling specific sites and determining their orientation, structural constraints will be added to the three dimensional structure of collagen.

The frequency of resonance lines in solid state NMR spectra depend on the orientation of the local molecular environment relative to the magnetic field H_0. The observed splitting can be used to determine the angle a bond makes with the magnetic field by the following equation, assuming an axially symmetric interaction:

$$\Delta\nu = \nu_{11}(3\cos^2\theta - 1) \qquad \text{Eqn. 1}$$

$\Delta\nu$ is the observed splitting, θ is the angle between the bond and the applied field and ν_{11} is the quadrupolar coupling constant. It has been found that in order to determine the orientation of a peptide plane at least two labels per plane must be examined.

Experimental:

The solenoidal coil geometry typically used in solid state NMR probes is unsatisfactory since a bundle of fibers is only conveniently placed in the coil in the perpendicular orientation. Attempts were made to wrap tendons around several small slides and stack them in the sample holder but satisfactory alignment was not obtained. A different probe design is called for by this experiment. One probe design is based on a small flat coil geometry suggested by Opella.[7] Small, flat frames were made for us upon which to wind the coil. A series of rat-tail tendons were tied together and wrapped around a 1 x 1 cm polycarbonate card. The card was then placed in the coil frame and the coil wound around it. This arrangement consistently gives a 90 degree pulse width of 2.7 μs at 1 kW power. This design has the advantage of allowing the fiber axis to be placed at any angle relative to H_0. Proper turn spacing for good RF field homogeneity and consistent inductance is insured by using a frame with set holes. This also allows the sample to be kept in the center of the coil, away from the edges where the field homogeneity is poor. We obtained satisfactory results with this probe. We were, however, limited to a small sample

size due to the polycarbonate cards and resultant poor filling factor. We had to retain the cards to keep the fibers aligned; otherwise, they shrink during changes in humidity and temperature.

We are principally interested in structural information obtainable from samples with the fiber axis along H_0, that is, the parallel orientation. From that premise, we designed a probe using a Helmhotz coil similar to liquid NMR probes. The probe was constructed based on our circuit for a double resonance design with a 0.5 x 1 cm saddle. The probe has a 90 pulse width of 3.2 microseconds with 400 watts power at the ^2H frequency (38.8 MHz). This probe is capable of holding ten times the sample as the flat probe and has provisions for stretching the sample.

We have labelled selected sites in the collagen molecule by incorporating deuterated amino acid into the rat tail tendon. Torchia incorporated labelled glycine into 1/3 of the Gly positions in rat tail tendon by injecting it into rats.[8] We slightly modified his injection scheme in order to label selected amino acids in rat tail tendon. Given the low natural abundance of deuterium, we were confident that the only signal we would see would be from our covalently bound label.

The first covalently labelled position we tried was alpha-d-Pro. The solutions for injection were 1.3 M in the amino acid and were neutralized with NaOH. Ten rat pups were injected once a day for 21 days and then sacrificed. The rat tail tendon was extracted for use in our experiments. The degree of incorporation of d-Pro in the tendon was determined by GC/MS and found to be 5.4% which agrees well with our NMR results. No incorporation of deuterium in any amino acids other than Pro and Hyp was seen. The oriented fibers were run on the Helmholtz coil probe. A splitting of $\Delta\nu$=117 kHz was observed and the angle of the C-D bond relative to the magnetic field determined.

The glycine amides were labelled by exchange. Samples were heated at 40°C and at constant humidity to completely exchange the labile H's. At 78% relative humidity, exchange reaches a constant level after 24 hours, at 38% relative humidity three days are required to reach a constant level of exchange. The samples were cooled to room temperature at constant humidity for an hour and then back

exchanged in liquid H_2O. Samples that were not heated to label the amide positions showed an extremely rapid loss of the solid echo peak. The heated sample, after back-exchange, had 10% of the solid echo remaining, indicating the exchanged sample has a significant amount of bound water. The doublet splitting of 155 kHz shows that the hydrogen bonded amides are in fact nearly perpendicular to the fiber axis in agreement with the predictions from the X-ray structure. Recent packing studies predict the collagen molecule is tilted 4 to 5 degrees off the fiber axis.

Results and Conclusions:

The asymmetric lineshape of the back exchanged sample indicates a distribution of orientation about θ=89 degrees. The spectral simulation for a Gaussian distribution of orientations $P(\theta) \alpha \exp(-\theta^2/2\sigma)$ with θ=90 and σ=17 matches our experimental data quite well.

We have successfully labelled selected positions and determined their orientation relative to the fiber axis. By carefully choosing our labels, we will be able to determine the orientation of various peptide planes in collagen and gradually build the three-dimensional structure.

Table I. Angle between X-D bond and the fiber axis comparison of solid state NMR results and various models derived from X-ray crystallography data.

Deuteron	Experiment	Fraser's[4] Rich-Crick	Ramachandran's[1] bridging water	Ramachandran's[2] two bonded	Yonath's[5]
Pro C_α-D	90	81.3	73.8	73.1	---
Gly N-D	89	89.4	78.7	78.4	83.1

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