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An NMR method to characterize multiple water compartments on mammalian collagen

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Abstract

A molecular model is proposed to explain water ^{1}H NMR spin-lattice relaxation at different levels of hydration (*NMR titration method*) on collagen. A fast proton exchange model is used to identify and characterize protein hydration compartments at three distinct Gibbs free energy levels. The NMR titration method reveals a spectrum of water motions with three well-separated peaks in addition to bulk water that can be uniquely characterized by sequential dehydration. Categorical changes in water motion occur at critical hydration levels h (g water/g collagen) defined by integral multiples $N = 1$, 4 and 24 times the fundamental hydration value of one water bridge per every three amino acid residues as originally proposed by Ramachandran in 1968. Changes occur at (1) the Ramachandran single water bridge between a positive amide and negative carbonyl group at $h_1 = 0.0658$ g/g, (2) the Berendsen single water chain per cleft at $h_2 = 0.264$ g/g, and (3) full monolayer coverage with six water chains per cleft level at $h_3 = 1.584$ g/g. The *NMR titration* method is verified by comparison of measured NMR relaxation compartments with molecular hydration compartments predicted from models of collagen structure. *NMR titration* studies of globular proteins using the hydration model may provide unique insight into the critical contributions of hydration to protein folding.

Keywords: Collagen; Protein hydration; NMR relaxometry; MRI; Proton relaxation; Tendon; Hydration compartments

1. Introduction

The scientific community generally accepts that protein hydration and protein induced water structuring are fundamental sources of protein structural behaviors that support life functions. Collagen is frequently selected as the model protein to elucidate structural and hydration relationships (Leikin et al., 1994, 1995, 1997, 2002; Privalov, 1982). Tendon has high concentration of Type I collagen approaching 100% of the dry biomass in some instances. Molecular tropocollagen crystallizes spontaneously and as a result significant new structural information is available with ever improving accuracy from X-ray diffraction studies of both native and molecular analogues of collagen (Bella and Berman, 1996; Bella et al., 1994, 1995, 1996; Berisio et al., 2002; Fraser et al., 1979; Kramer et al., 1998, 1999, 2000, 2001; Miller and Scheraga, 1976; Okuyama et al., 1977; Ramachandran, 1967; Rich and Crick, 1961; Yonath and Traub, 1969). The more recent, high-resolution studies consistently show a water bridge network surrounding the collagen molecule.

Structural studies provide a molecular model of collagen supporting the existence of three distinct categories of water bridges. The most tightly bound consists of one highly immobilized "*water bridge*" per every three protein residues $h_b = 18$ Da/$(3 \times 91.2$ Da$) = 0.0658$ g water/g protein as originally proposed by Ramachandran (1967), Fullerton and Amurao (2005), and Ramachandran and Ramakrishnan (1968). A second less immobilized water fraction consists of three additional cleft waters per tripeptide unit residing in the three groove-like depressions between the peptide chains of the triple helix $h_c = 3 \times h_b = 0.197$ g water/g. The water bridge and three cleft waters

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complete a chain of four water molecules per tripeptide. These chains form a triple helix of water in the three clefts of the α-protein chains of the collagen triple helix. Cleft water was proposed by Berendsen (1962) and discussed by others (Lim, 1981; Privalov, 1982). Recently our laboratory showed that the remaining water on native collagen (~1.62 g water/g protein) is in the first interfacial monolayer $h_{im} = 5 \times 0.263 = 1.315$ g water/g protein at higher energy relative to the first two compartments but still lower than the free energy of bulk water (Fullerton and Amurao, 2005). As a result of these three water compartments we predict NMR detectable changes in water motion at $h_1 = h_b = 0.0658$, $h_2 = h_b + h_c = 0.264$ and $h_3 = h_b + h_c + h_{im} =$ 1.58 g water/g protein or at $h_i = N_i \times 0.0658$ for $N_i = 1, 4$ and 24.

This study tests the hypothesis that that the NMR spin-lattice relaxation time of water on native collagen is not characteristic of any of the three molecular compartments but a weighted average due to fast exchange between three hydration fractions or water phases on the protein surface (Zimmerman and Brittin, 1957). The relaxation characteristics of all three water phases can, however, be extracted and characterized by sequential NMR measurements using a titration of water content (Fullerton et al., 1982, 1986). Tendon is uniquely suited to identifying the molecular origin of the three hydration fractions that may be typical of proteins in general. The collagen molecule is highly immobilized and aligned in protein fiber structure that suppresses proton NMR signal from the protein due to static dipole coupling. The study confirms that proton NMR signal emanates only from the liquid water that shows the presence of three distinct hydration fractions in fast exchange equilibrium. The compartments extracted using the *NMR titration method* quantitatively confirm the three molecular water bridge environments identified in the preceding paragraph. The correlation of protein NMR titration measurements with a molecular model of water bridges holds promise as a general model to evaluate hydration of both fibrillar and globular proteins.

2. Materials and methods

2.1. NMR titration measurements

Samples of bovine flexor tendon from three animals (age and sex unknown) were obtained from a local slaughter house, dissected on site, diced to 3 mm^3 cubes and placed in separate pre-weighed 1″ diameter NMR sample tubes for each animal. Tubes were immediately sealed to prevent dehydration. The initial sample volume was approximately 6 cc when wet and approximately 3 cc when dry. Preliminary measurements of T_1 relaxation reported here were made at 10.7 MHz on a Praxis II pulsed NMR device with a primary magnetic field strength of 0.25 T. The initial research plan was to use these less accurate but rapid measurements at 10.7 MHz to provide pilot data for designing of more accurate and complete experiments as a function of temperature at 500, 700 and 900 MHz. Results at 0.25 T are, however, sufficient to substantiate the hypothesis and are therefore presented as stand alone experiments. Praxis measurements of T_1 use the saturation recovery (90-τ-90) pulse sequence with a repetition time *TR* exceeding five times the longest T_1 relaxation time for the sample. The 90° pulse is approximately 13 μs (bandwidth in excess of 100 kHz). The sample chamber was equilibrated in the instrument at 22 ± 1 °C for at least 1 h prior to each measurement. Samples were out of the vacuum for approximately 1–4 h. Typical measurement times in the NMR unit were approximately 5–10 min. Random orientation of the tendon with respect to the magnetic field was used to eliminate possibility of residual orientation dependence. The signal after a recovery delay τ was measured as an amplitude of the free induction decay, $A(\tau)$, with the first time point at 26 μs. Each data point samples signal for 1 μs and 10 points following the peak signal were averaged to reduce the measurement noise.

Each relaxation measurement consisted of 31 amplitude measurements with a total of 30 sequential delay times with even increments of ~20 ms each for a maximum delay time of $30 \times \Delta\tau$ as shown in Fig. 1. Although delay times were adjusted to evaluate signal amplitude variation over as much as two orders of magnitude only single exponent T_1 decay was observed in all instances. Measurements were repeated three times to improve the signal-to-noise ratio to 300:1 (0.3% noise) at the initial hydration level. A non-linear regression fit to the data was calculated as shown with a single exponent decay using two fitting constants, $A(\infty)$ and T_1, using the equation $A(\infty) - A(\tau) = A(\infty)(e^{-\tau/T_1})$ and non-linear regression from the Graph Pad program (Motulsky, 2003). The T_1 was measured for the tendon samples over the entire range of hydration from native hydration to completely dry protein using the procedure described below. The tissue mass M_t was measured at each hydration level to allow calculation of $h = M_w/M_p$ following measurement of the dry protein mass M_p (see next paragraph). The mass of salt and other cosolutes was neglected for these calculations.

The original sample weights were approximately 5.4 g of tendon which when fully dehydrated decreased to approximately 2.3 g of dry mass. Initial dehydrations were achieved by placing the open NMR tubes in a vacuum chamber at approximately 0.25 atmospheric pressure. Samples were removed at intervals ranging initially from approximately 1 h to several days to achieve significant reduction in the sample water content. After achieving equilibrium mass at room temperature the temperature of the vacuum oven was increased to 90 °C and the dehydration extended for another 30 days with intermittent measurements of T_1 taken until a new equilibrium and final dry mass for the protein were achieved. The dehydration and T_1 measurement protocol for the tendon extended over a total period of approximately 3 months. An extended drying period was undertaken to assure hydration equilibrium over the entire volume of the tendon. Due to the slow dehydration rate no measurable dependence on the time interval between removal from the vacuum oven and NMR measurement was detectable between 1 and 24 h (data not shown). The measurement process was repeated for all three samples. Results for all three samples were similar but only two are reported as one sample tube broke during the final heating phase of the experiment.

2.2. NMR titration analysis of spin-lattice relaxation data

The water bridge hydration hypothesis identifies three hydration water fractions in addition to a bulk water phase as shown in Fig. 2. For the protein

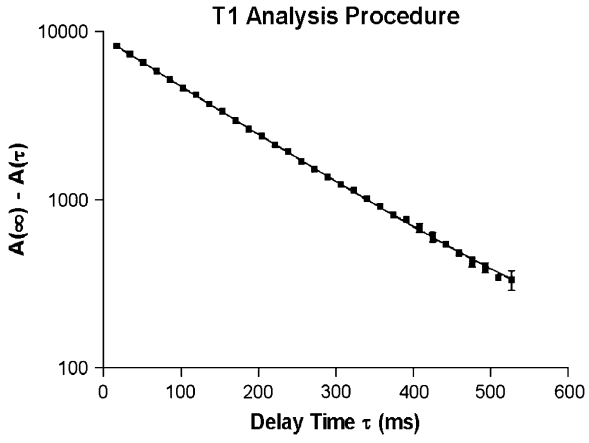

Fig. 1. T_1 analysis of a saturation recovery curve for bovine tendon at the native hydration level is shown here. Solid circles stand for data points. Solid line is a fit to the function $\ln(A(\infty) - A(\tau)) = a\tau$, where τ is a delay time, $A(\tau)_i$ is an FID amplitude after the delay τ and $A(\infty) = A(\tau \gg T_1)$ and a is the regression fit constant $a = 6.7\ s^{-1}$, $T_1 = 1/a = 149 \pm 1$ ms, $R^2 = 0.9992$, signal-to-noise ratio was never lower than 20:1.