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Oxygen-17 and Sodium-23 Nuclear Magnetic Resonance Studies of Myofibrillar Protein Interactions with Water and Electrolytes in Relation to Sorption Isotherms

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An NMR and water activity study of the interactions of myofibrillar proteins with sodium chloride and water is presented. Sorption isotherms are compared with high-field NMR data in an attempt to identify and quantitate different fractions of water. Salt "bound" to myofibrillar proteins in suspensions changed markedly the sorption isotherm; upon addition of 4% salt, the "monolayer" coverage, N_I, increased from 1.55 to 1.66 g of H_2O/100 g of solids; the total amount of "bound" water ($N_I + C$) increased from 22.6 to 27.2 g of water/100 g of solids. The fraction of Na^+ bound to the proteins increases with myofibrillar protein concentration up to a maximum of 500 mol of NaCl/10^6 g of protein. Above 50% (w/v) protein, the water activity decreases rapidly whereas the ^{17}O NMR line width increases only slightly with increasing concentration of myofibrillar proteins. Such data are interpreted in terms of protein activity that becomes dominant above 20% (w/v) myofibrillar protein. The effects of ion binding and charge fluctuations on myofibrillar protein–protein interactions are briefly discussed.

Per capita consumption of red meat, poultry, and fish in America has been in excess of 100 kg/year for the past decade (Meat Facts, 1986), hence the importance of research on meat and meat components. Current consumer trends indicate that more meat is being consumed in the form of further processed meat items; also, more meat items are being prepackaged and marketed with longer shelf life. This means that a better understanding of factors controlling meat shelf life is becoming increasingly important to the meat industry. Because meat contains approximately 75% water and 18–22% protein, the interactions between these two meat components are important in determining shelf life. Over half of the proteins in skeletal muscle are classified as myofibrillar or contractile proteins. The hydrated myofibrillar proteins are essentially those responsible for functional properties associated with meat. Binding and trapping of water in meat is of special interest in this context as the mechanism by

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which water is held affects directly the functional properties and can alter the sensory (organoleptic) characteristics of the meat product. Also, water associated with the myofibrillar proteins is of economic importance because most meat products have high-moisture contents. Finally, the water "activity" of meat can be modulated, for example, by the addition of salt, and this alters the storage properties of meat.

We have previously investigated the relationship between protein hydration and water activity in a model system (hydrated lysozyme) over the entire concentration range (Baianu et al., 1985; Lioutas et al., 1986a,b, 1987) by nuclear magnetic resonance (NMR) and sorption equilibration techniques. A major component of the lysozyme sorption isotherm (component III) was assigned to the water trapped between lysozyme molecules, whose translational motions are restricted more by diffusion barriers than by "binding" to the protein. This interpretation is consistent with a D'Arcy and Watt analysis (1970) of protein sorption isotherms, with our NMR relaxation data (Lioutas et al., 1987) and with an improved sorption isotherm model reported recently (Asbi and Baianu, 1986). The latter model gives the best fit, so far, for the component III (trapped water) of the sorption isotherms in a wide variety of food systems, including 20 food proteins (Asbi and Baianu, 1986).

We are presenting here the results of a similar approach that we employed previously for the hydrated lysozyme model system (Lioutas et al., 1986b, 1987), but with greater emphasis on the effects of salts on water activity and myofibrillar protein interactions with water and ions, both in solution and aggregated form, or hydrated powders. Our NMR measurements are concerned primarily with the observation of an average over the water and cation populations, respectively. Therefore, the conformations and stability properties of the myofibrillar proteins are only *indirectly* probed by the oxygen-17 (^{17}O), sodium-23 (^{23}Na), and lithium-7 (^{7}Li) NMR observations. To address directly the question of myofibrillar protein conformations and stability as a function of ionic strength and pH, additional carbon-13 (^{13}C), deuterium (^{2}H), and proton (^{1}H) NMR observations at high field would be required; such measurements are the subject of further research. The latter techniques may also encounter difficulties caused by the high molecular weight of the myofibrillar proteins, which may limit the spectral resolution and information content. Nevertheless, the choice of an appropriate model for the analysis of our ^{17}O, ^{7}Li, and ^{23}Na NMR data may allow indirect derivation of conformational, as well as solubility, salt-binding and charge fluctuation characteristics of the myofibrillar proteins.

The complex three-dimensional structure of a protein is the result of the various repulsive, attractive, and hydrophobic interactions of the protein chain with itself and with the surrounding solvent (Berendsen, 1975). In the case of normal striated muscle, such interactions are compounded by the complexity of the three-dimensional matrix formed within the muscle fibers. The myofibrillar proteins have been implicated in many of the properties of muscle, such as heat gelling, hardening, texture changes, and binding of water (Woeff, 1982). This group includes proteins such as myosin, actin, tropomyosin, troponin, and other less abundant proteins. Myofibrillar proteins are soluble in solutions containing strong electrolytes, such as sodium chloride. Interactions of these proteins with water and ions determine their functional properties; these protein–solute–water interactions are also very important in determining the myofibrillar protein conformation and stability in various solvents (Price and Schweigert, 1971).

Because of the difficulties mentioned above, there are relatively few reports concerned with the detailed mechanisms of these interactions (Lioutas et al., 1985a; Palnitkar and Heldman, 1971). Methods routinely used for other proteins such as casein, paracasein (Gal, 1975), or bovine serum albumin (Bull and Breese, 1968) are not useful for myofibrillar proteins because of their higher molecular weight and diversity. On the other hand, recent studies have proved that nuclear spin relaxation measurements by pulsed nuclear magnetic resonance (NMR) techniques can, in principle, provide information as to both structural and binding effects of water molecules, or ions, on the charged (or polar) groups of the myofibrillar proteins. Proton NMR studies have been reported on the interactions of myofibrillar proteins, or whole striated muscle, with water (Bratton and Hopkins, 1965; Hazelwood et al., 1969; Belton et al., 1972, 1973; Belton and Packer, 1974). Other nuclei such as ^{43}Ca and ^{23}Na have been used to study the interactions of these ions with purified muscle proteins by NMR techniques (Andersson et al., 1981).

A purely electrostatic model, such as the simple theory of Melander and Horvath (1977a,b), predicts that the solubility of a protein should increase at low salt concentration because of the electrostatic free energy contributions. According to this electrostatic model, at higher salt concentrations a salting-out free energy would dominate the solubility due to the increased surface tension of the electrolyte solution and the solvent-exposed hydrophobic groups of the protein. The opposite was, however, observed in both native and heat-denatured, high molecular weight soybean proteins (Shen, 1981); that is, the soy protein isolate solubility decreased with added salt to a minimum value and then increased to a constant value at a salt concentration of approximately 1 M. Shen (1981) suggested that these results can be explained by an increase in the hydrophobic surface area of the protein due to protein *self-association* upon adding salt, followed at higher concentrations by a salting-in process caused by an increased dipole moment of the protein induced by a "nonspecific solvation effect" that dominates at higher salt concentrations. However, Shen's model (1981) neglects completely the binding of ions to soy proteins and could only qualitatively relate to the observed behavior. Furthermore, Steinhardt and Reynolds (1969) have previously shown that both Na^+ and NH_4^+ cations bind significantly to proteins and, therefore, a model that includes ion binding must be considered. Recently, a *quantitative* fit of Shen's experimental data for soy protein isolates was obtained with an *ion-binding* model based on Wyman's theory of linked functions (Wyman, 1964), in conjunction with a computer program for nonlinear regression analysis (Kumosinski, 1987). The applicability of this ion-binding model to our NMR data for myofibrillar proteins will be considered, and its implications for the conformations, charge fluctuations, and stability of myofibrillar proteins will be briefly discussed. The previous results of the ion-binding model (Kumosinski, 1987) strongly suggest that ion binding to protein charged residues can have large effects on the protein solubility even for small binding constants of the order of 5–50 L/mol.

The objective of our work is to further investigate the mechanism of the interactions of skeletal muscle myofibrils (MFP) with water and Na^+ ions in the three-component system MFP–water–NaCl by applying both NMR and water sorption techniques.

EXPERIMENTAL SECTION

Preparation of Dialyzed Skeletal Muscle Myofi-

Table I. Composition of Myofibrillar Proteins (MFP) and Salt Mixtures for Sorption Experiments and Their Water Activity[a]

sample no.	water, %	solids total, %	protein (MFP),[a] %	NaCl (S),[b] %	S/MFP[b]	NaCl in aq phase,[c] %	a_w[d]
MP	14.26	85.74	85.69	0.05	0.0	0.0	
1	95.62	4.38	4.28	0.095	0.0222	0.10	0.9970
2	95.50	4.50	4.32	0.240	0.0555	0.25	0.9960
3	95.26	4.74	4.31	0.480	0.1113	0.50	0.9921
4	94.80	5.20	4.25	0.950	0.2234	1.00	0.9850
5	93.89	6.11	4.20	1.900	0.4521	2.00	0.9720
6	92.06	7.94	4.13	3.81	0.9220	4.00	0.9610
7	86.57	13.43	3.88	9.55	2.4606	10.00	0.9150
8	90.00	10.00	0.0	10.00			
9	0.20	99.80	0.0	99.80			

[a] Values are expressed on percent wet-weight basis. [b] This value is expressed as total NaCl plus KCl content on total weight basis from atomic absorption analysis. [c] Samples are calculated on the basis of salt added. [d] Water activity values were measured with the modified Landrock and Proctor (1951) method.

brils. Our preparation of bovine skeletal muscle myofibrils was based on the isolation method reported by Schollmeyer (1978). Freshly ground bovine skeletal muscle was added to a Waring blender and blended for 1 min in 10 volumes of standard salt solution (SSL). The composition of SSL was 100 mM KCl, 20 mM K_3PO_4, 2 mM $MgCl_2$, 2 mM EDTA, and 0.1 mM NaN_3 at pH 6.8. The blended myofibrils were centrifuged at 100*g* for 10 min. The supernatant was discarded, and the sediment was resuspended in 6 volumes (v/w) of SSL, blended for 1 min, and centrifuged at 1000*g* for 10 min. The resuspension–blending–centrifugation procedure was repeated four more times, with the addition of 1.0% (v/w) Triton X-100 to SSL. After the final sediment was resuspended in 8 volumes (v/w) of SSL by stirring vigorously the suspension was centrifuged at 1000*g*. The sediment was then suspended in a 100 mM KCl solution with a Waring blender and centrifuged at 1500*g* for 10 min, and the process was repeated four times. Examination of the myofibril preparation with a low-power microscope did not reveal appreciable amounts of connective tissue or contaminating particulate material. Finally, the suspension was dialyzed versus cold water at 4 °C for 72 h, with three changes, and then lyophilized. The moisture content of the lyophilized material was 14.3% (w/w). The sodium and potassium chloride contents of the dialyzed and lyophilized myofibrils were determined to be, respectively, 520 and 985 ppm by atomic absorption analysis.

Preparation of Samples for NMR and Sorption Measurements. A set of samples containing MFP, sodium chloride, and water was prepared by keeping the protein to water ratio at 5% (w/w) and by increasing the addition of sodium chloride from 0.10 to 40.0% (w/w) (Table I). (All w/w percentages for the NMR samples are expressed in terms of mass of protein ratioed to mass of water, rather than to the total mass.) The water activity (a_w) of these samples was measured by the modified Landrock and Proctor (1951) method (Table I). A duplicate set of these samples was prepared with D_2O instead of H_2O, so that ^{17}O and ^{23}Na NMR measurements could be made without proton-exchange broadening (Lioutas et al., 1986b).

MFP samples were vapor equilibrated either against dilute sucrose or against saturated salt solutions as shown in Table II; the equilibrium moisture values listed were used for plotting a complete sorption isotherm for each sample.

A second set of samples was prepared by adding incremental amounts of myofibrillar proteins from 0.1 to 96.0% (w/w) to a constant ratio of salt to water, S/W, of 4% (w/w). The water activity of these samples was also measured as described above. The values were used for the D'Arcy and Watt (1970) analysis as further explained in the text.

Table II. Water Activity Values for MFP-D_2O and MFP-NaCl-D_2O at 20 °C

% MFP in aq phase (w/w)	N, g H_2O/g solids	a_w	
		MFP, no salt	MFP, 4.0% NaCl
10.0	9.00	0.996 ± 0.003	0.965 ± 0.004
20.0	4.00	0.994 ± 0.003	0.961 ± 0.004
35.0	1.857	0.990 ± 0.003	0.951 ± 0.004
50.0	1.00		
51.0	0.960	0.985 ± 0.003	
58.06	0.722	0.976 ± 0.003	
60.00	0.667		0.910 ± 0.004
66.67	0.500	0.946 ± 0.003	
70.40	0.420		0.875 ± 0.004
74.77	0.338	0.910 ± 0.003	
75.20	0.330		0.833 ± 0.004
77.52	0.290	0.890 ± 0.003	
78.10	0.280		0.794 ± 0.004
80.00	0.250		0.750 ± 0.004
80.97	0.253	0.851 ± 0.003	
82.03	0.219		0.695 ± 0.004
82.64	0.210	0.813 ± 0.003	
84.18	0.118	0.755 ± 0.003	
85.40	0.171	0.699 ± 0.003	
86.00	0.163		0.591 ± 0.004
87.95	0.137	0.591 ± 0.004	
90.60	0.104		0.331 ± 0.002
92.00	0.087	0.331 ± 0.002	
95.70	0.045		0.113 ± 0.003
96.20	0.039	0.113 ± 0.003	

^{17}O and ^{23}Na NMR Measurements. Nuclear magnetic resonance measurements were carried out on a laboratory-assembled NSF-250 NMR spectrometer operating at 5.875 T. Samples for ^{17}O NMR were placed in standard 10-mm NMR tubes, and ^{17}O NMR measuements were carried out at 33.94 MHz. In the case of solids, the samples were packed as homogeneously as possible. Single-pulse experiments were carried out at 20 °C, and the spectra were stored in an 8K point array that provided adequate resolution. Measurements were carried out over the whole range of concentration—whenever the ^{17}O NMR signal-to-noise permitted—for accumulation times up to 1 h per sample. ^{23}Na NMR single-pulse measurements were carried out at 66.17 MHz on the same samples.

RESULTS

Water Sorption by the Myofibrillar Protein–Sodium Chloride System. The effect of sodium chloride on the hydration of myofibrillar proteins was investigated by parallel sorption equilibration and nuclear magnetic resonance (NMR) measurements. Sorption isotherms of myofibrillar proteins in the rehydration mode, with and without added NaCl, are presented in Figures 1 and 2, respectively. In Figure 1 sorption isotherms are shown for varying concentrations of MFP from 0 to 97%, at various levels of NaCl added from 0.5% to 10%. The compositions

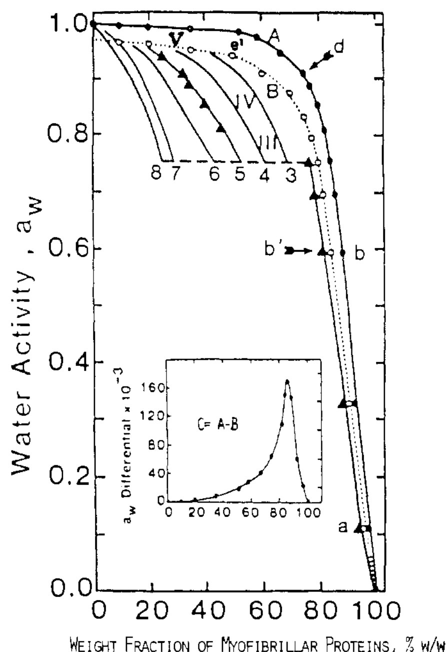

Figure 1. Dependence of water activity, a_w, on the myofibrillar protein concentration (%, w/w) at 20 °C: (A) myofibrillar protein-water system, the family of isotherms labeled 3-8 representing isotherms for the myofibrillar protein-NaCl-water system where the ratio of NaCl to myofibrillar protein was kept constant for every sample examined: (B) myofibrillar protein-NaCl-water, with NaCl constant at 4% (w/w); (C) water activity difference between curves A and B (inset).

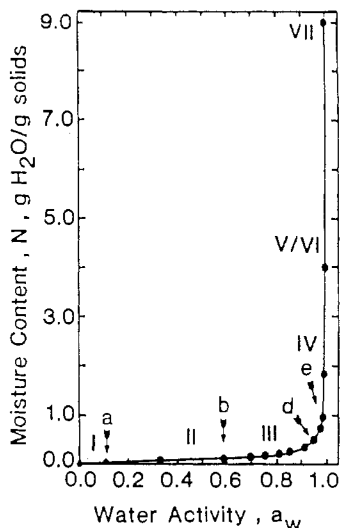

Figure 2. Water sorption isotherms for the MFP-NaCl-water system at 20 °C. Regions I-VII correspond to different sorption processes. Point a represents the BET monolayer limit. Point b represents the upper limit of the BET model.

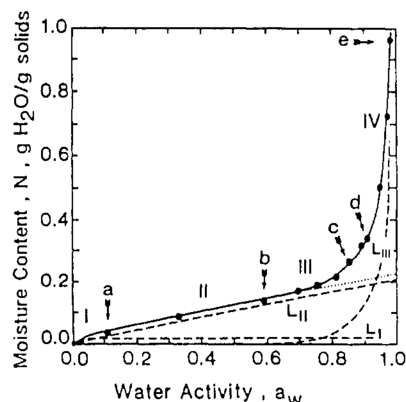

Figure 3. D'Arcy-Watt modified analysis of the sorption isotherm of myofibrillar proteins at 20 °C. The dashed curves L_I-L_{III} represent the three components in this theory: monolayer, weakly bound, and multilayer water, respectively. Point d is the upper a_w limit of applicability of the D'Arcy-Watt theory.

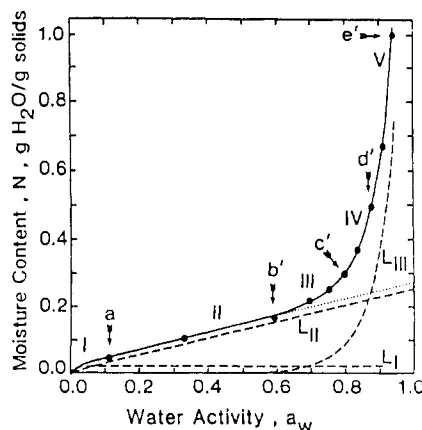

Figure 4. D'Arcy-Watt modified analysis of the myofibrillar protein-NaCl-water system at 20 °C. The NaCl content was kept constant at 4.0% (w/w). The dashed curves L_I-L_{III} correspond to the same components as in Figure 3. Points a, b', d', and e' are equivalent to the points marked on Figures 2 and 3.

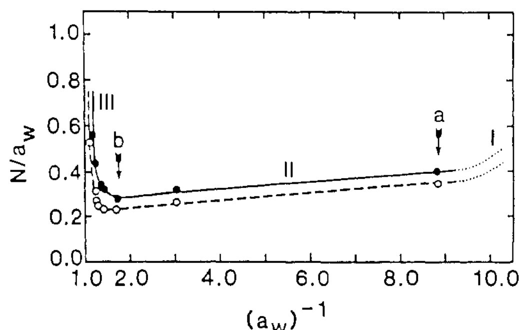

Figure 5. Linear plot of the weakly bound water component of the D'Arcy-Watt theory for the myofibrillar protein-water (open circles) and myofibrillar protein-NaCl-water (filled circles) systems at 20 °C. Standard least-squares fit coefficients for the linear segment are 0.9944 and 0.9919, respectively. Point a is the upper a_w limit of the BET monolayer, and point b is the upper a_w limit of the BET model.

of the various MFP-NaCl-water samples that were generated are given in Table I, with the exception of curve A (at 0% NaCl) and curve B (which is for 4.0% NaCl added at all MFP concentrations), whose compositions are specified in Table II. The sorption isotherms obtained with a fixed salt to water ratio (curve B, Figure 1; last column, Table II) resemble the sorption isotherm of the system without salt (curve A, Figure 1; third column, Table II) and are continuous throughout the whole range of MFP concentrations. In contrast, the family of sorption isotherms obtained with constant salt to MFP (S/MFP) ratios (curves 3-8, Figure 1; Table I) show a discontinuity at a_w = 0.76, corresponding to the saturation limit of NaCl solutions. Below a_w = 0.75 all sorption isotherms of this family (curves 3-8) fall on a family of curves resembling isotherms A and B in this region. The inset in Figure 1 presents a plot of the a_w difference, between A and B, which increases up to a concentration of 86% (w/w) MFP and then decreases sharply to reach the zero level at 100% MFP.

Increasing the ratio S/MFP causes the curves of the isotherms (curves 3-to 8, Figure 1) to be displaced to the left, toward the sorption isotherm of NaCl solutions (curve

Table III. Calculated Values of the Parameters in the D'Arcy-Watt Three-Component Equation $N = N_I + Ca_w + [K_3K_4a_w/(1 - K_4a_w)]$ for the MFP-D_2O and MFP-NaCl-D_2O Systems (Note the Modified Treatment of the D'Arcy-Watt Equation Used Here as in Lioutas et al. (1987))

system	D'Arcy-Watt parameters	value
MFP-D_2O	N_I	0.0155 g of H_2O/g of solid
	C	0.21022 g of H_2O/g of solid
	K_3	4.5×10^{-3}
	K_4	1.0622
MFP-D_2O-NaCl	N_I'	0.0166 g of H_2O/g of solid
	C'	0.2549 g of H_2O/g of solid
	K_3'	1.26×10^{-2}
	K_4'	1.0600

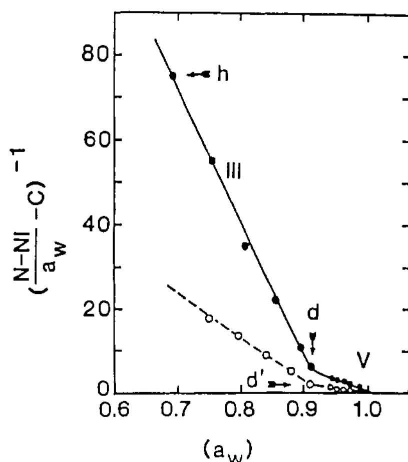

Figure 6. Linear plot of the multilayer component of the D'Arcy-Watt theory for myofibrillar protein-water (filled circles) and myofibrillar protein-NaCl-water (open circles). Points d and d' represent the upper a_w limit of this theory. (The coefficients for the least-squares fit of the linear segment are 0.995 and 0.997, respectively.)

8, Figure 1), as expected. The "traditional" plots of moisture contents, N (g of H_2O/g of MFP) versus water activity, corresponding to the data in Figure 1A,B, are presented in Figures 3 and 4, respectively. Analyses of these sorption isotherms according to the theory of D'Arcy and Watt (1970) with the modifications described by Asbi and Baianu (1986) are also shown in Figures 2 and 4, respectively, and in Table III. The sorption isotherms of MFP without NaCl and with 4.0% NaCl added have the three distinct components L_I–L_{III} predicted by the theory of D'Arcy and Watt (1970) (as indicated in Figures 3 and 4); in addition, two solution components, IV and V, are also present, as previously shown for hydrated lysozyme (Lioutas et al., 1986b). Component L_{III} becomes significant at $a_w = 0.59$, in the absence of added NaCl. The linear plot of L_{III} (Figure 6) breaks down at point d ($a_w = 0.91$), which is also the break point of L_{III} in the presence of 4.0% NaCl (Figure 6, point d), while both plots extrapolate to zero ordinate at $a_w \approx 0.94$.

Each isotherm originated from one sample in the set for which the protein (MFP) to water ratio was kept constant with increasing salt addition (Table I).

^{17}O and ^{23}Na NMR Investigations of Myofibrillar Protein Interactions with Sodium Chloride and Water. ^{17}O NMR spectra of MFP suspensions in D_2O with added NaCl (Table I), and without added NaCl, were recorded at 33.94 MHz; an example is shown in Figure 7. For comparison, the ^{17}O NMR spectra of D_2O (Figure 7A) and 10.0% NaCl (w/w) in D_2O (Figure 7B) are also shown. The 66.17-MHz ^{23}Na NMR spectra of NaCl solutions

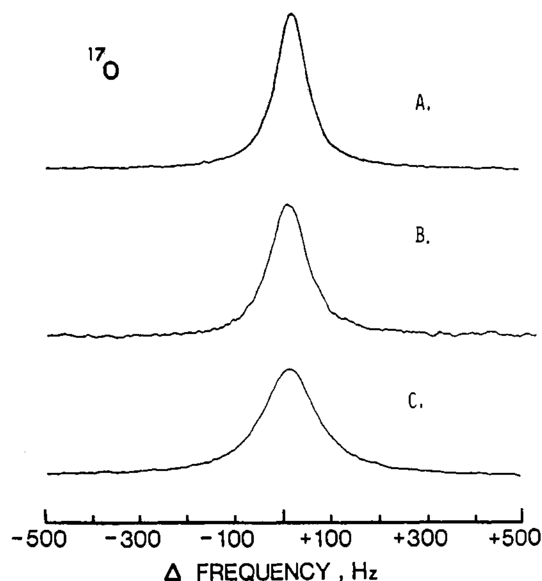

Figure 7. ^{17}O FT NMR spectra of D_2O and myofibrillar protein-NaCl-water at 20 °C: (A) 99.8 (w/w) D_2O; (B) 10.0% (w/w) NaCl in D_2O; (C) 5.0% (w/w) myofibrillar protein + 10.0% NaCl in D_2O.

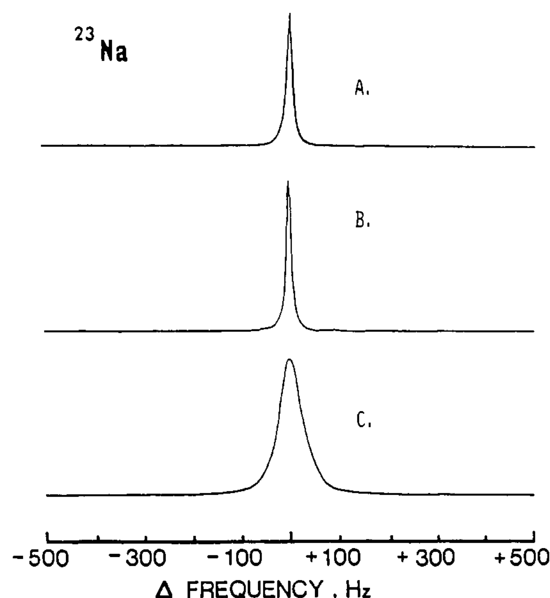

Figure 8. ^{23}Na FT NMR spectra of 5% myofibrillar protein-NaCl-water at 20 °C: (A) with 99.8 (w/w) D_2O + 0.2% NaCl; (B) with 10.0% (w/w) NaCl in D_2O; (C) with 5.0% (w/w) myofibrillar proteins + 10.0% NaCl in D_2O.

(Figure 8A,B) and MFP suspensions with 4.0% NaCl added (Figure 8C) are also presented. In all cases a single Lorentzian line shape is observed at 20 °C for both ^{17}O and ^{23}Na. NMR line widths measured at half-height increased with increasing NaCl, above 5% NaCl (w/w) and MFP concentrations above 1.5 (w/v) (data not shown). In order to determine the extent of hydration of MFP, a plot of the ^{17}O NMR line broadening, $\Delta\nu = \Delta\nu_{obsd} - \Delta\nu_{free}$, is required as a function of the molar ratio of MFP to water, r^{-1}; such a plot is shown in Figure 9, curve B, for MFP without added salt, together with a plot of the same data versus MFP concentrations (% w/v) (bottom scale, solid circles in Figure 9, curve A, for the whole range of concentrations). The addition of 4% NaCl causes a marked change in the dependence of ^{17}O NMR line broadening on the MFP concentration (Figure 10, curve B). Below 1.5% MFP (w/v), the ^{17}O line broadening decreases with increasing

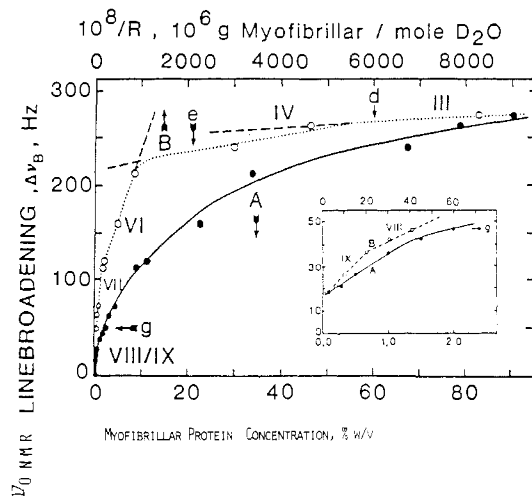

Figure 9. Dependence of ^{17}O NMR line broadening, $\Delta\nu_B$, on myofibrillar protein concentration (% w/v in curve A) and protein to water molar ratio (r^{-1} in curve B) at 20 °C. Inset shows the region below point g enlarged.

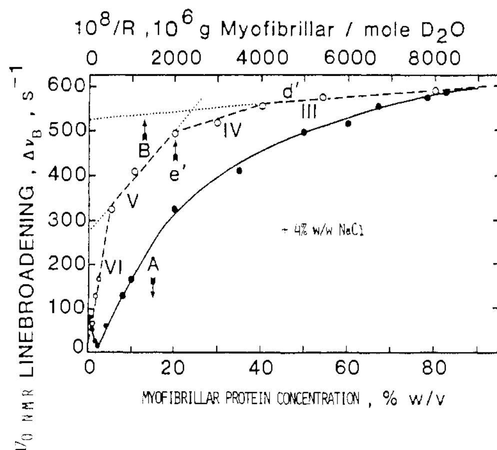

Figure 10. ^{17}O NMR line broadening, $\Delta\nu_B$, as a function of myofibrillar protein concentration at 20 °C in the presence of 4.0% (w/w) NaCl. Curve A is expressed in percent (w/v), and curve B is expressed as protein to water molar ratio. Regions III–IV correspond to the sorption regions of the isotherm (Figure 2A).

MFP concentration in the presence of salt, whereas above 1.5% MFP (w/v) the ^{17}O line broadening increases dramatically with increasing MFP concentration (Figure 10, curve A); the line broadening continues to increase throughout region III of the sorption isotherm at 20 °C (Figure 4). A plot of ^{17}O NMR line widths as a function of NaCl concentration for 5% (w/w) MFP suspensions is presented in Figure 11.

^{23}Na NMR line widths for samples containing 5% MFP, and with varying NaCl concentrations from 0.1% to 50% (Figure 12), show a dependence on NaCl concentration similar to that presented in Figure 11, curve A, for ^{17}O NMR data at NaCl concentrations lower than 0.5% (w/w). Above 0.5% NaCl (w/w), the ^{23}Na NMR line broadening shows only two linear regions of distinct slopes in the r^{-1} plot (Figure 12). Similar observations were made by ^{7}Li NMR for MFP with LiCl, but at lower LiCl concentrations than NaCl (data not shown).

The relationship between water activity and the transverse NMR relaxation time of aqueous solutions, as measured by ^{17}O NMR, is illustrated in Figure 13. As can be readily noted in this figure, the presence of salt decreases markedly the T_2 of myofibrillar protein–salt–water system, but it changes to a much lesser extent the water activity until the threshold of 50% (w/w) is reached when

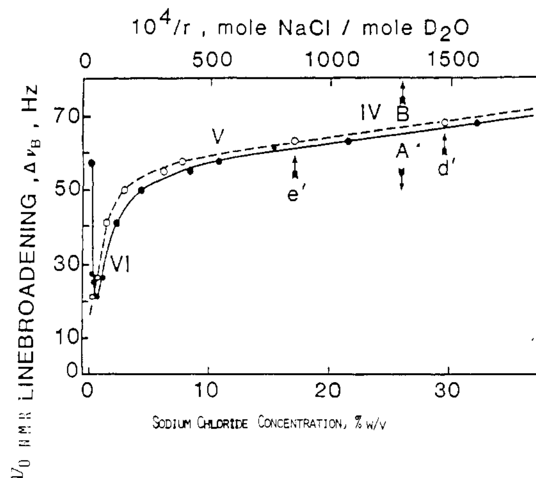

Figure 11. Dependence of ^{17}O NMR line broadening, $\Delta\nu_B$, on NaCl concentration in the presence of 5.0% (w/w) myofibrillar protein. The NaCl concentration is expressed in percent (w/v) (curve A) and as the molar ratio to water (curve B) at 20 °C. Regions IV and VI correspond to different sorption regions of the family of sorption isotherms (Figure 1).

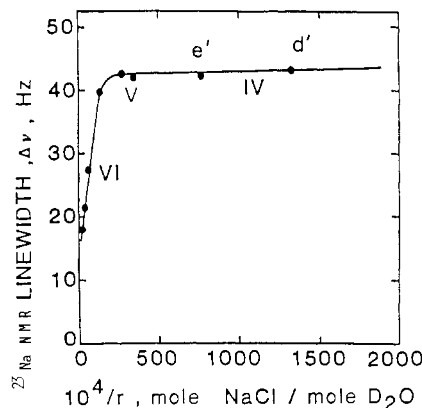

Figure 12. ^{23}Na NMR line widths as a function of NaCl concentration at 20 °C in the presence of 5.0% (w/w) myofibrillar proteins. Concentrations are expressed as salt to water molar ratios, r^{-1}.

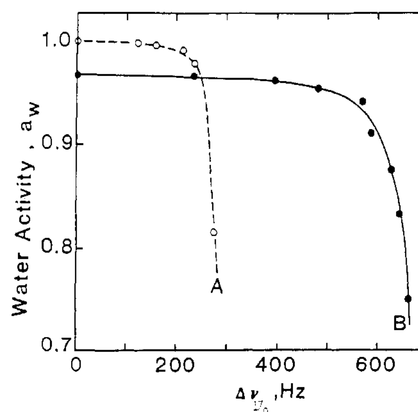

Figure 13. Dependence of the ^{17}O NMR line width, $\Delta\nu_B$, on the water activity for the system myofibrillar protein–water (A) and in the presence of 4.0% (w/w) NaCl (B) at 20 °C.

a_w drops rapidly, whereas T_2 varies slowly.

A. Sorption Isotherms. The addition of 4.0% (w/w) sodium chloride to MFP suspensions modifies significantly the sorption isotherm (Figure 1B compared with Figure 1A). The monolayer coverage, N_I (limit of region I), in Figure 3 is increased by the addition of 4.0% NaCl from

1.55 to 1.66 g of H_2O/100 g of solids (Figure 4; Table III). This corresponds to 4.85 mol of D_2O/mol of NaCl, which is twice as large compared to the value obtained previously for NaCl bound to hydrated lysozyme at the limit of region I (Lioutas et al., 1985a). This large difference may be accounted for by the lack of Na^+ bridging between carboxyl groups in MFP because the carboxyls are likely to be widely separated. This can be seen from the relatively low total number (425) of the carboxyl groups per myosin molecule (MW ≈500 000; Arakawa and Timasheff, 1982). The total amount of bound water (N_I + C) is also increased from 22.6 to 27.16 g of H_2O/100 g of solids as a result of MFP interactions with Na^+ and Cl^- that result in additional bridging of water to MFP. As previously observed for lysozyme solutions in the presence of NaCl, the sorption isotherms (Figure 3B) is shifted toward lower water activities (Figure 4) by the presence of NaCl (Lioutas et al., 1985a).

The amount of salt bound to MFP can be estimated from the extrapolation of the linear region (Figure 1B) at high water activities (a_w > 0.9) to 100% solids. The decrease in water activity in this region (labeled V in Figure 1B), in comparison with the system in the absence of NaCl (Figure 1A), is faster down to point e′ (Figure 1B), possibly because of Na^+-bridging and charge-screening effects (Canfield, 1963) that increase water binding to MFP. As the MFP concentration increases—at a fixed NaCl to water ratio (4%, w/w)—the fraction of bound Na^+ increases up to a maximum of 456 mol of NaCl/10^6 g of MFP. At higher MFP concentrations, this fraction decreases because the number of MFP binding sites for Na^+ (carboxyl groups) is in excess of the number of Na^+ ions available (inset C, Figure 1). This number reached the value of 11.4 mol of NaCl/10^6 g of MFP at the limit of region I (point a, Figure 1B). Our interpretation is supported by the fact that the ^{7}Li NMR line broadening also increased linearly at high water activities (corresponding to region V in Figure 1B), at the same time as water activity decreased linearly with increasing lysozyme concentration (Lioutas et al., 1985a). Such observations indicate that the fraction of bound Li^+ or Na^+ increased with increasing protein concentration in region V, causing also a marked decrease in water activity.

B. Fast-Exchange NMR Relaxation Models. The single Lorentzian line shapes of the ^{17}O and ^{23}Na NMR peaks in MFP samples (Figures 7 and 8) are consistent with a model of fast exchange of water and ions between the free and bound states, that is, dynamically associated with the MFP. The presence of only two major water populations, bound and free, would yield a linear dependence of the NMR relaxation rates on the protein concentration, according to eq 1, where $\Delta\nu_{obsd}$ is the observed NMR line

$$\Delta\nu_{obsd} = P_{free}\Delta\nu_{free} + P_{bound}\Delta\nu_{bound} \quad (1)$$

width determined at half-height for MFP suspensions, $\Delta\nu_{bound}$ is the line width of the bound water or ions, and $\Delta\nu_{free}$ is the line width of free water or ions; P is the fraction of bound water or ions, and $\Delta\nu_{free}$ is the ^{17}O line width of water or the ^{23}Na NMR line width of an NaCl solution of the same concentration as in the corresponding MFP–NaCl–water system. The ^{17}O ($I = {}^5/_2$) transverse relaxation time of the bound water is related to the value of the line width as in eq 2.

$$T_2^{-1} = \pi\Delta\nu_{bound} = R_2 = (12\pi^2/125)(e^2qQ/h)^2\tau_c(1 - \eta^2/3 + S^2) \quad (2)$$

The linear dependence of the NMR line widths on concentrations predicted by eq 1 is observed only in the very dilute range (≈1.5% w/v; region IX in the inset of Figure 9 and region VII in Figure 11); this is not surprising in view of the nonideal character of MFP solutions.

The addition of Na^+ to a 5% MFP suspension increases substantially the ^{23}Na NMR line width dependence on the concentration of NaCl (James and Noggle, 1969). In solutions of 5% MFP this dependence is linear up to 78 mol of D_2O/mol of NaCl. Above 78 mol of D_2O/mol of NaCl, the ^{23}Na NMR line width continues to increase with a much lower slope in comparison with the previous region (Figure 12), presumably because of the saturation of the carboxyl groups of the MFP with Na^+ ions, at a ratio of 28 000 mol of NaCl in solution per 10^6 g of MFP; a sharp transition is readily discernible at this point in Figure 12. The transition is not, however, present for NaCl solutions in D_2O, in the absence of MFP. Such a transition is also seen in ^{23}Na NMR data for lysozyme (Lioutas et al., 1986a), but is is less sharp than in the case of myofibrillar proteins.

From the plot in Figure 12 one can obtain the correlation time τ_c of Na^+ ions bound to MFP, as described by Lioutas et al. (1986b). We find surprisingly that the τ_c of Na^+ bound to MFP is ≈28 ps, which is approximately 3 times more mobile than Na^+ bound to lysozyme ($\tau_c \approx$ 76 ps), indicating a significant difference between salt binding by myofibrillar proteins and lysozyme.

Saturation of the MFP carboxyls with Na^+ ions (Figure 11) has a very different effect on water mobility in comparison with the effects of Na^+ ions at a point immediately before saturation (Figure 11); the value of the ^{17}O correlation time of water reaches a maximum limiting value of about 6.8 ps in the presence of a saturated solution of Na^+ and Cl^-, which is very close to the minimum average value of ≈4.2 ps occurring at 0.5% NaCl (w/w), in the presence of 5.0% MFP (w/w). Above the saturation point, even at 90.0% NaCl (w/w), the ^{17}O NMR line width continues to increase, and at this concentration the corresponding average is about 32.7 ps, which is comparable to that found for bound water (31.2 ps) in lysozyme without any added salt at pH 7.0 (Lioutas et al., 1986b). Binding of Na^+ and Cl^- ions below the saturation points of Na^+ and Cl^- binding sites increases the average correlation times of water as a result of an increasing degree of MFP aggregation with increasing MFP concentration in comparison with that of suspensions in the absence of added NaCl (Figure 10). For example, at the highest MFP concentration measured (82.0%, w/v), the average ^{17}O NMR correlation time of bound water is twice as large in the presence of 4.0% NaCl as with the very low-salt, hydrated MFP ($\tau_c \approx$ 17.5 ps).

A possible explanation of the strongly nonideal character of the MFP suspensions in the absence of salt, or at very low salt concentrations, is provided by the charge fluctuation mechanism (Kirkwood and Shumaker, 1952; Kumosinski and Pessen, 1982); in this case, a plot of the NMR relaxation should be made against protein activity rather than concentration (Kumosinski and Pessen, 1982). In the case of β-lactoglobulin such a plot against protein activity was linear up to ≈8–10% protein (w/v). The determination of the protein activities in a system as complex as the mixture present in our myofibrillar protein suspensions requires involved calculations and a number of assumptions; a substantially more detailed model of the MFP activities will be presented in a subsequent report. We note that both the ^{17}O and ^{23}Na NMR data (Figure 11) exhibit two distinct behaviors at low and high NaCl concentrations, respectively. The rapid decrease of the line broadening, $\Delta\nu_B = \Delta\nu_{obsd} - \Delta\nu_{free}$, at low NaCl concentrations (region VII, Figures 11 and 12) can be understood in terms of the two-site, fast-exchange model; as more NaCl

is added, the population of free ions increases because the Na-binding sites on MFP are already filled. The rapid decrease of the ^{17}O and ^{23}Na NMR line broadening implies that the fraction P_B of bound ions and water is small and corresponds to strong binding at low concentrations of salt. The observed behavior at salt concentrations higher than about 0.5% (w/v) (or MFP concentrations higher than $\approx$1.5% (w/v)) is however strikingly different and cannot be simply explained only in terms of weak binding of water and ions by a different group of sites of MFP; such a process would yield a low, negative slope in Figure 11, according to eq 1, for concentrations higher than about 0.5% (w/w) NaCl. A more complex process is, therefore, taking place in the higher concentration range of salt in MFP suspensions; such a process is likely to involve the formation of Na^+ bridges between myofibrillar proteins, as shown by the decrease in the mobility of the Na^+ ions and water bound to the MFP complex. Both ^{17}O ($I = {}^5/_2$) and ^{23}Na ($I = {}^3/_2$) are quadrupolar nuclei whose NMR relaxation is dominated by the quadrupole relaxation mechanism, that is, by the interactions of the nuclear quadrupole moment (eq) with the electric field gradient (eQ) at the nucleus. The binding strength is actually related to the quadrupole coupling constant, QCC = e^2qQ/h, that can be also determined by NMR and, in most solids, by nuclear quadrupole resonance (NQR). The quadrupole relaxation is, therefore, expected to be quite sensitive to charge fluctuations at the surface of myofibrillar proteins, in the absence of salt as we have indeed observed.

The ion-binding model (Kumosinski, 1987) and the more complex, charge fluctuation model do not, however, take into account the contributions of hydrophobic interactions to the myofibrillar protein stability and conformations; the latter are difficult to treat analytically without recourse to thermodynamics or statistical mechanics. With the ion-binding model, which is perhaps an oversimplification, the mechanism of the processes illustrated in Figures 10–13 involves binding of the cations Na^+ or Li^+ to the net *negatively* charged MFPs at pH 7.0, as described for the soy proteins (Kumosinski, 1987), which exhibit similar behavior (Shen, 1981; Figures 1 and 2 of Kumosinski (1987)) to that of MFP's with increasing salt concentrations. Furthermore, soy proteins without salt also appear to have a net *negative* charge at neutral pH (Kumosinski, 1987). At the higher salt concentrations in Figure 11, on the right-hand side of the minima, a partial unfolding, or swelling, of the MFP may occur, exposing a new set of weaker binding sites for salt and water (Figure 11), consistent with the sequential ion-binding mechanism at two different types of sites, postulated from a thermodynamic analysis of salt binding to soy proteins (Kumosinski, 1987). Further experiments by chlorine, bromine, and iodine NMR are needed to confirm this mechanism and to obtain a detailed explanation of the observed behavior of myofibrillar proteins in salt solutions.

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