Thermodynamic Linkage of Ion Binding and Hydration in Myofibrillar Protein Solutions Determined from Multinuclear Spin Relaxation Studies

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SUMMARY: The mechanisms for the anionic and cationic interactions with myofibrillar proteins in aqueous solutions were investigated by nuclear magnetic resonance over a wide range of salt concentration. Markedly nonlinear dependeces of the ^{17}O and ^{23}Na NMR transverse relaxation rates on salt concentration were analyzed with a thermodynamic linkage model of salt-dependent solubility and hydration (ligand-induced association model), according to Wyman's theory of linked functions. Nonlinear regression analysis of both ^{17}O and ^{23}Na NMR data suggested cooperative, reversible binding of hydrated ions to myofibrillar proteins. Both ions and water were found to exchange fast, on the NMR timescale, between the binding sites of the myofibrillar proteins and the aqueous solution. At sodium chloride concentrations higher than about 0.1 grams salt/gram water, ion activities have marked effects upon the NMR relaxation rates of both ions and water. A salt activity model allowed quantitative fitting of the NMR data at high salt concentrations. The effect of neglecting the ion activity in solutions of myofibrillar proteins was also estimated and compared with the ligand-induced, cooperative association model for myofibrillar proteins. The comparison between the ^{17}O and ^{23}Na results strongly suggests that water is exchanged as the hydrated ion species between the myofibrillar protein binding sites and the bulk, aqueous solution.

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Introduction

Equilibrium sedimentation and light-scattering studies of dilute solutions of purified vertebral skeletal myosin A suggested a strong tendency of myosin A to associate even in high-salt solutions where myosin was claimed to be in a rapidly reversible monomer-dimer equilibrium[1,2]. The myofibrils in muscle consist of myosin, actin, troponin, tropomyosin and other minor proteins such as α-actinin, nebulin, c- and M- proteins.

Myosin is one of the principal contractile proteins, and appears to have significant affinity for both cations and anions (e.g., Cl^-)[3,4,5,6]. The understanding of muscle protein interactions with ions is important in the study of *chemomechanical energy transduction* in muscle.

The strength of long-range protein-protein interactions is determined by the net protein charge, the ionic strength and the dielectric constant of the medium. Previous reports have shown that the protein self-association is **thermodynamically linked** to the ion-induced solubility in the case of milk proteins[7,8,9,10]. A complex nonlinear dependence of the ^{17}O and ^{23}Na NMR relaxation rates on the myofibrillar protein concentration in aqueous solutions, with or without salt, was previously reported[11]. Nonidealities of protein solutions are often related to the presence of significant protein-protein interactions in solution, or to protein "activity." In such cases, protein activities can be determined by nonlinear regression analysis of the experimental data (such as NMR relaxation date, for example[12,13]).

In this report, we aim to present a detailed quantitative analysis of protein-protein, protein-water and protein-salt interactions for myofibrillar proteins in solution. An attempt is also made to determine the molecular dynamics of water and ions in this system and to characterize by NMR the association of myofibrillar proteins.

Methods

A. NMR Measurements

The ^{17}O (I = 5/2) and ^{23}Na (I = 3/2) NMR measurements for myofibrillar proteins in solution, suspensions or hydrated powders in the presence of salt were previously reported[11,14]; details of the sample preparation and NMR measurements were also given in ref. 11.

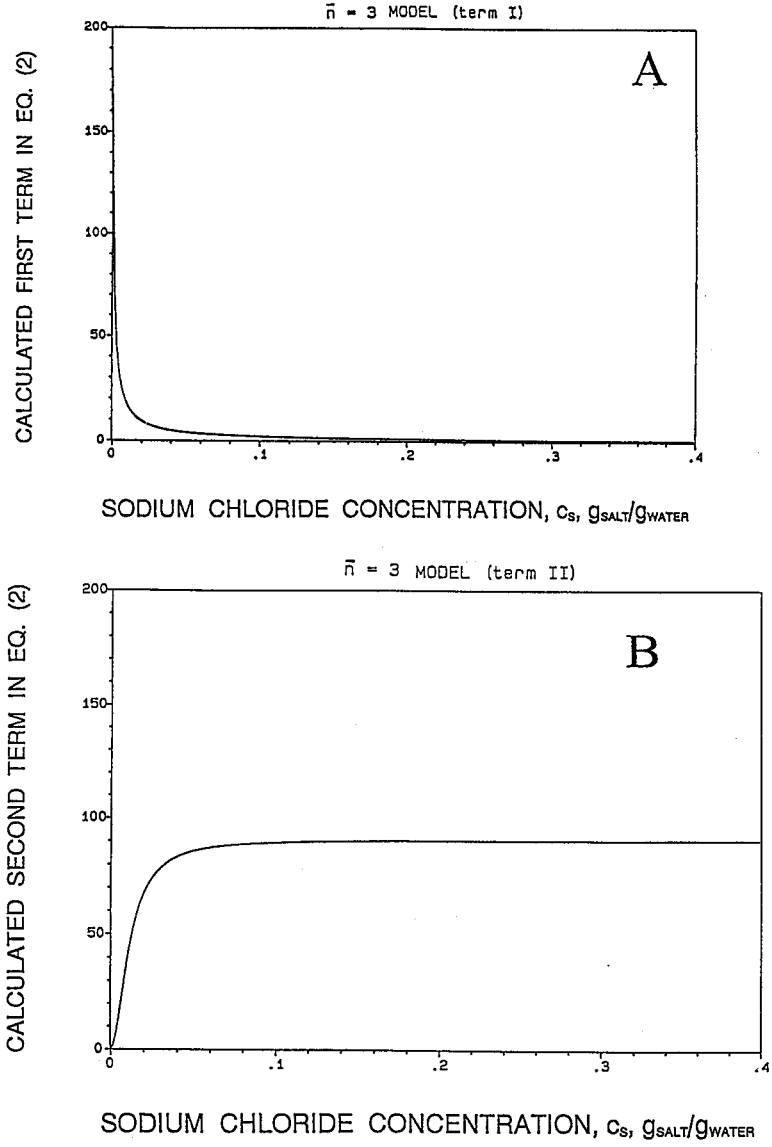

Figure 1. Graphical representation of the two terms in equation (2):
A) The simple binding isotherm $k_{1S}/1+k_{1S} \cdot C_S$)
B) The cooperative binding, second term in equation (2)

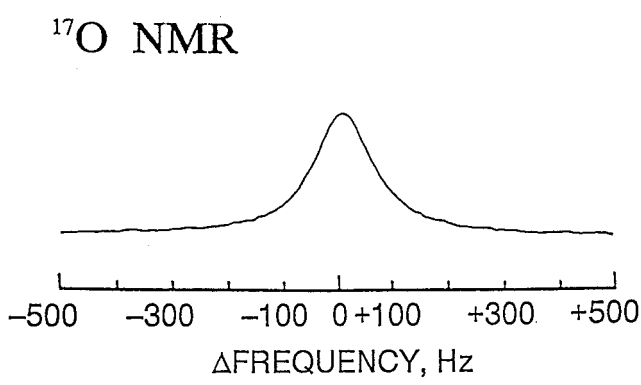

Figure 2. Single Lorentzian ^{17}O NMR peak of water in myofibrillar protein solutions

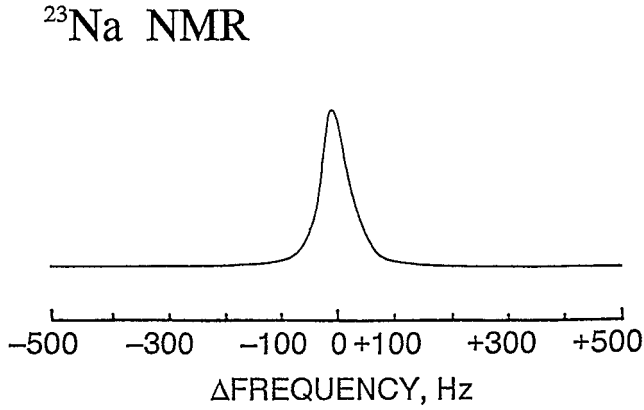

Figure 3. Single Lorentzian ^{23}Na NMR peak of sodium ions in aqueous solutions of myofibrillar proteins with salts (0.1 M NaCl at pD 7.4, 20 °C)

B. NMR Relaxation and Myofibrillar Protein Hydration

Nuclei with a spin quantum number I > 1/2 possess an electric quadrupole moment which interacts with electric field gradients at the nucleus. If not all of the orientations of the nuclear quadrupole moment of a bound sodium ion with respect to the protein were equally probable, a quadrupole splitting of the resonance signal would be observed. Such a splitting would indicate that sodium ions are oriented by their binding to the protein surface. The magnitude of the splitting would depend on the electric field gradient, on the degree of anisotropy, and on the fraction of sodium ions bound to the surface. On the other hand, fast reorientation of the ions at the binding site, and the exchange between bound and free ions, would cause a marked narrowing of the NMR absorption peak of the ions associated with the protein.

In the **extreme narrowing limit** ($\omega_o \bar{\tau}_c \ll 1$), the ^{17}O (I = 5/2) NMR transverse relaxation rate, R_{2B}, of water "bound" to proteins is related in a first approximation[15] to an apparent (isotropic) correlation time $\bar{\tau}_c$ as follows:

$$R_2 = (12\pi^2/125) \cdot (e^2qQ/h)^2 \bar{\tau}_c (1 + \eta^2/3 + S^2) \qquad (1)$$

where: $R_{2B} = (T_{2B})^{-1}$, $K(^{17}O) = (12\pi^2/125)$, e^2qQ/h is the nuclear quadrupole coupling constant in Hz, $\bar{\tau}_c$ is the average (apparent) correlation time of "bound" water, η is the asymmetry parameter for the electric field gradient at the nucleus, and S is the **order parameter** which describes the anisotropic orientation of water molecules near the protein surface. Under **extreme-narrowing conditions** (e.g., $\omega_o \cdot \bar{\tau}_c \ll 1.0$) the relaxation processes are mainly affected by the **fast** motions of water molecules or ions. Therefore, one can estimate the apparent correlation time associated with such fast motions of the water molecules or ions using Equation 1[14] for water, and appropriately modified equations for the ions.

Theoretical calculations of the nuclear spin relaxation for nuclei with half-integral spin larger than I = 1 show that the nuclear magnetization decays as a weighted sum of (I + 1/2) exponentials rather than a single exponential[16]. For the same binding strength and dynamics, the higher the nuclear spin, the smaller will be the expected deviation from a

single exponential decay or Lorentzian lineshape. Although the motions of sodium ions bound to protein molecules would be sufficiently slow to make the ^{23}Na relaxation nonexponential at low fields, single-quantum ^{23}Na NMR at high fields monitors primarily **weakly** "bound" ions which are apparently coupled with **fast water motions**. We have utilized results[16] to consider possible first-order corrections related to nonexponentiality of the ^{23}Na NMR relaxation rates of Na^+ bound to myofibrillar proteins. The values of the ^{23}Na NMR relaxation rates did not, however, require significant corrections since the experimental values were obtained in the extreme narrowing limit where the relaxation becomes almost exponential, as in the case of ^{17}O NMR. It is relevant in this context that weak water binding to myofibrillar proteins and very fast dynamics of water were previously reported from ^{17}O NMR studies of myofibrillar proteins in water, in the absence of salt ($\bar{\tau}_c$ - 17.5 ps[13]). Such fast motions of water molecules were also present when 0.67 M NaCl was added.

B. Protein Activity and the Thermodynamic Linkage Model

Since proteins are polyampholites they are expected to exhibit repulsive and/or attractive interactions in solution[17]. Binding of Na^+ to proteins was shown to increase the electrostatic free energy resulting in increased repulsive forces between proteins[18]. Therefore, a model which takes into account such interactions must be considered for the analysis of the NMR data for myofibrillar proteins in solutions with salt.

Interactions of small ions in solution with the ionized groups of polyelectrolytes are important because of their effects on the thermodynamic, conformational, electrical and hydrodynamic properties of polyelectrolytes, such as proteins. In recent years, a series of ion-binding studies were carried out[7,8,9] which indicated that ion binding by proteins is thermodynamically linked to protein solubility. It was also previously shown that characteristic changes in protein hydration occur as a function of increasing salt concentration[10, 11, 14, 19, 20].

For the case when the variation of NMR relaxation rates of water ^{17}O or ions with added salt is measured at a **constant** protein concentration, a relatively simple ligand-induced association model[9, 21] should be considered:

$$R_{2obs} - R_{2FR\cdot SALT} = (R_{2Bound1} - R_{2FR\cdot SALT})\, k_{1S}/ (1+k_{1S}\cdot C_S) +$$

$$+ (R_{2Bound2} - R_{2FR\cdot SALT}) \cdot k_{2S} C_S^{\,n} \cdot / [1+k_{2S}C_S^{\,n} \cdot] \qquad (2)$$

where $R_{2obs} = 1/T_{2obs}$ is the measured transverse relaxation rate, C_S is the salt concentration, k_{1S} is an apparent average salt binding constant for individual ion binding (that is, non-cooperative) to protein, and k_{2S} is the apparent, **average** salt binding constant for cooperative binding of n moles of salt ions to protein; $R_{2Bound1}$ and $R_{2Bound2}$ are the transverse relaxation rates of bound ions (or water, respectively), and $R_{2FR,SALT}$ is the concentration-independent; ^{23}Na transverse relaxation rate in water at infinite dilution, without any protein added. Furthermore, $\bar{n}$ is here defined as the **average number** of moles of sodium ions that bind cooperatively to myofibrillar proteins. In the case of ^{17}O NMR measurements, $R_{2FR\cdot SALT}$ is replaced by R_2 (D_2O), the ^{17}O NMR transverse relaxation rate of free D_2O.

The two terms on the right hand side of equation (2) are represented, respectively, in Figures 1A and 1B.

Results

A. A Fast-Exchange Model of Myofibrillar Protein Hydration and Ion Binding

If the exchange between "bound" and "free" (or bulk) water is **fast** on the time scale of the NMR measurements, that is, if the exchange rate is greater than the value of R_{2Bound}, the observed relaxation times will be the weighted average of those for the "bound" and "free" water populations[12,22,23]. Single Lorentzian ^{17}O and ^{23}Na NMR lineshapes were previously reported for both water and Na^+ ions in myofibrillar solutions (Figs. 2 and 3, respectively), consistent with a **fast exchange** between the "bound" and "free" water, and "bound" and"free" Na^+ ions, respectively. The lineshapes of the ^{17}O and ^{23}Na NMR peaks were fitted by nonlinear regression analysis and found to be single Lorentzians to a very good approximation ($\geq$ 98%).

The variations of ^{17}O and ^{23}Na NMR transverse relaxation rates, (R_{2obs} - $R_{2FR\cdot SALT}$), of myofibrillar protein solutions with salt concentration are illustrated in Figs. 4A, B and Figs. 4C, D, respectively.

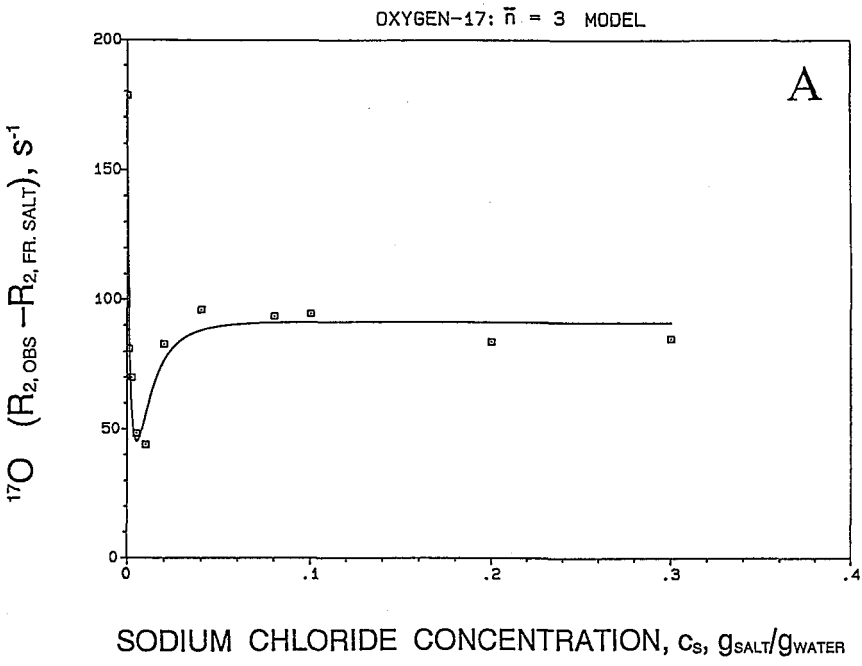

Figure 4A. Comparison of water ^{17}O NMR and ^{23}Na NMR transverse relaxation data (open squares) with cooperative ion-binding, n-mer models: nonlinear regression analysis of ^{17}O NMR data with the $\bar{n} = 3$ model

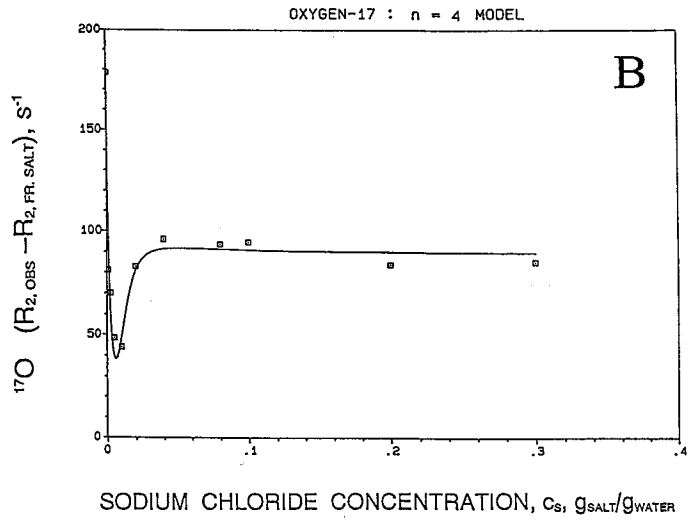

Figure 4B. Comparison of water ^{17}O NMR and ^{23}Na NMR transverse relaxation data (open squares) with cooperative ion-binding, n-mer models: nonlinear regression analysis of ^{17}O NMR data with the $\bar{n} = 4$ model

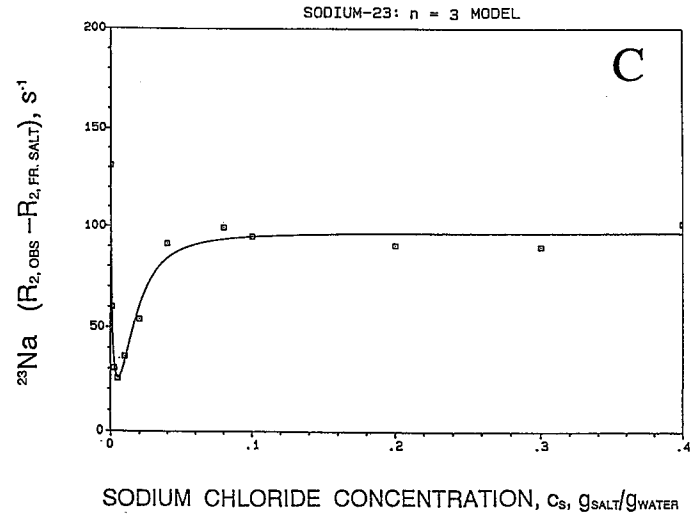

Figure 4C. Comparison of water ^{17}O NMR and ^{23}Na NMR transverse relaxation data (open squares) with cooperative ion-binding, n-mer models: nonlinear regression analysis of ^{23}Na NMR data with the $\bar{n} = 3$ model

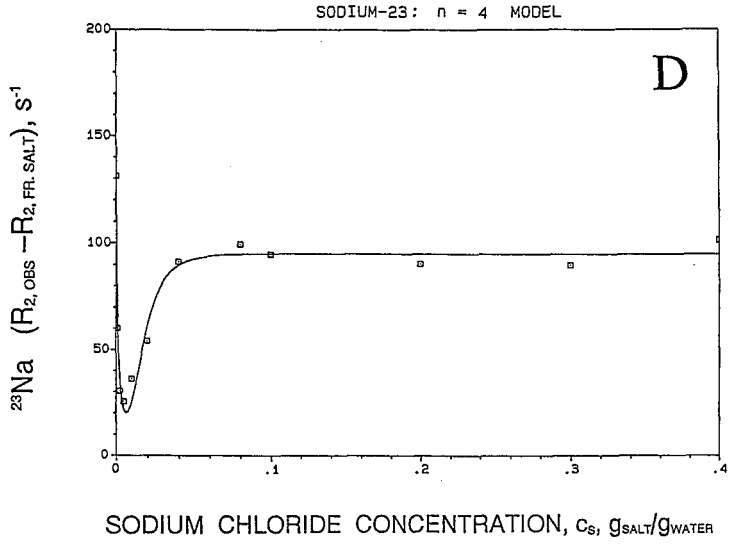

Figure 4D. Comparison of water ^{17}O NMR and ^{23}Na NMR transverse relaxation data (open squares) with cooperative ion-binding, n-mer models: nonlinear regression analysis of ^{23}Na NMR data with the $\bar{n} = 4$ model

The lines drawn in Figs. 4A and 4B were calculated by nonlinear regression analysis with Equation 2 by fitting the experimental ^{17}O NMR data with either an $\bar{n}=3$ model (that is, cooperative binding of three salt ions to a protein molecule; Fig. 4A) or an $\bar{n}=4$ model (Fig. 4B). The corresponding results for such cooperative ion-binding, n-mer models in the case of ^{23}Na NMR are shown, respectively, in Figs. 4C (for $\bar{n}=3$) and 4D (for $\bar{n}=4$).

We have also carried out calculations with Equation 2 for $\bar{n}=2$, 5 and 6. Figs. 5A and 5B illustrate the dependence of such salt variation curves on $\bar{n}$.

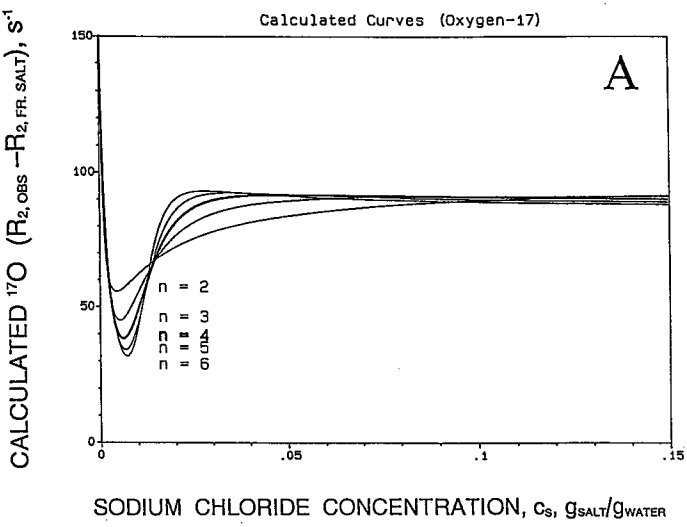

Figure 5A. Dependence of the calculated salt variation curves for myofibrillar proteins: calculated curves for the ^{17}O NMR transverse relaxation data, with $\bar{n} = 2, 3, 4, 5$ and 6

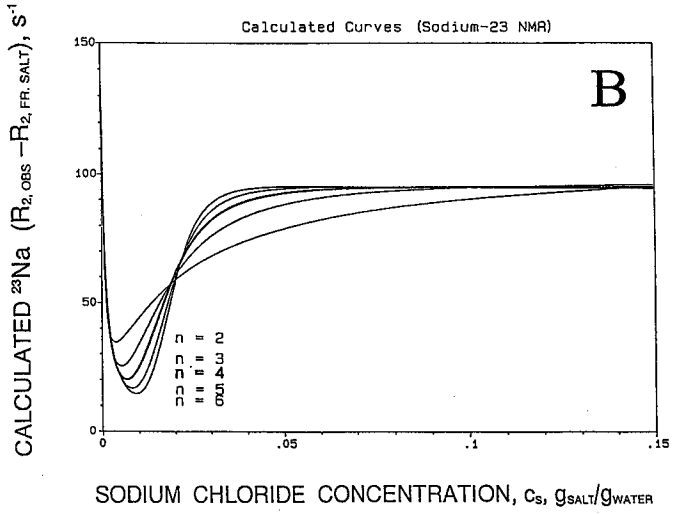

Figure 5B. Dependence of the calculated salt variation curves for myofibrillar proteins: calculated curves for the ^{23}Na NMR transverse relaxation data, with $\bar{n} = 2$ to 6

Curves with $\bar{n}$=2 and $\bar{n}$=3 are somewhat broader and shallower than the measured salt concentration dependence of ^{23}Na or ^{17}O NMR relaxation rates, whereas those with $\bar{n}$=5 and 6 are deeper than the experimental minima, and the minima for $\bar{n}$=5 or 6 occur at higher salt concentrations than those found in the NMR experiments (for both ^{17}O and ^{23}Na). The best fits to the experimental NMR relaxation data were provided by the $\bar{n}$=4 cooperative ion-binding model (Figs. 4B and 4D, for ^{17}O and ^{23}Na NMR data, respectively), although one cannot completely exclude the possibility of $\bar{n}$=3 (Fig. 4C), within the experimental errors associated with the NMR measurements. For salt concentrations higher than 0.1 g_{salt}/g_{water} one needs also to consider the possibility of salt-mediated interactions and protein activity coefficients that modulate the NMR relaxation behavior. Such an alternative "*salt-activity*" model would be described by the following equation:

$$R_{2obs} - R_{2FR\cdot SALT} = (R_{2BOUND1} - R_{2FR\cdot SALT})\, C_S / (1+K_{1S} \cdot C_S) +$$
$$+ (R_{2BOUND2} - R_{2FR\cdot SALT}) \cdot n_S \cdot c_P \cdot \exp(-s^2/C_S + B_1 \cdot \sqrt{C_S} + B_2 \cdot C_S^2) \qquad (3)$$

where $S = (\partial m_3/\partial m_2)$ is the preferential binding coefficient for salt, C_P is the (fixed) protein concentration, n_S is the average number of salt ions bound to each protein at saturation; B_1 and B_2 are virial coefficients of salt activity, and other notations are as specified in Equation 2. Nonlinear regression analysis of both ^{17}O and ^{23}Na NMR transverse relaxation data with the salt activity model described by Equation 3 provides the best fitting to the experimental data with varying salt, especially at salt concentrations $C_S \geq 0.1$ g_{SALT}/g_{WATER}, as illustrated in Figs. 6A to 6D.

The results obtained with the salt activity model by fitting the ^{17}O NMR relaxation data are compared in Fig. 6A with those obtained with the $\bar{n}$=4 model; in Fig. 6B, the results obtained with Eq. 3 from the ^{17}O NMR data are compared with those obtained by neglecting the virial coefficients of salt activity (interrupted line in Fig. 6B). Similarly, the results obtained with the salt activity model by fitting the ^{23}Na NMR relaxation data are compared in Fig. 6C with those obtained with the $\bar{n}$=4 model (Eq. 2; $\bar{n}$=4); in Fig. 6D the results obtained with Eq. 3 from the ^{23}Na NMR data are compared with those obtained by neglecting the virial coefficients (B_1 and B_2) of salt activity (interrupted line in Fig. 6D). The latter fittings in Figs. 6B and 6D are substantially worse than those obtained with the $\bar{n}$=4 model (Eq. 2) and the complete, salt activity model (Eq. 3).

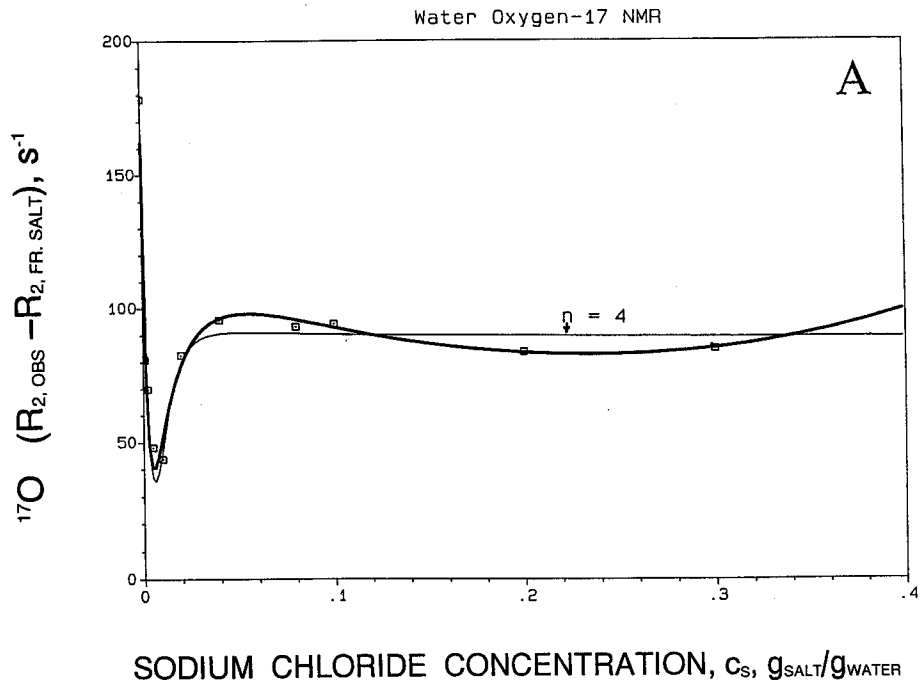

Figure 6A. Comparison of the fitted curves with the salt activity model (Eq. 3) with those obtained by employing the simpler, $\bar{n} = 4$ model (Eq. 2), or by neglecting the virial coefficients B_1 and B_2 in Eq. 3: Salt-variation curves determined by ^{17}O NMR salt activity model versus $\bar{n} = 4$ model

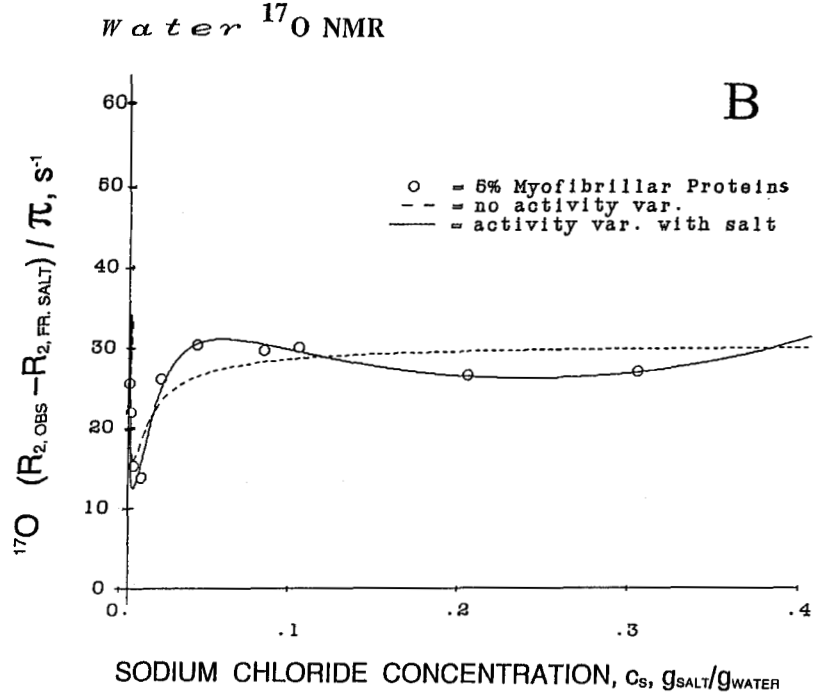

Figure 6B. Comparison of the fitted curves with the salt activity model (Eq. 3) with those obtained by employing the simpler, $\bar{n} = 4$ model (Eq. 2), or by neglecting the virial coefficients B_1 and B_2 in Eq. 3: Salt-variation curves determined by ^{17}O NMR salt activity model (Eq. 3; continuous line), compared with a preferential complete binding model that neglects B_1 and B_2 in Eq. 3 (interrupted line

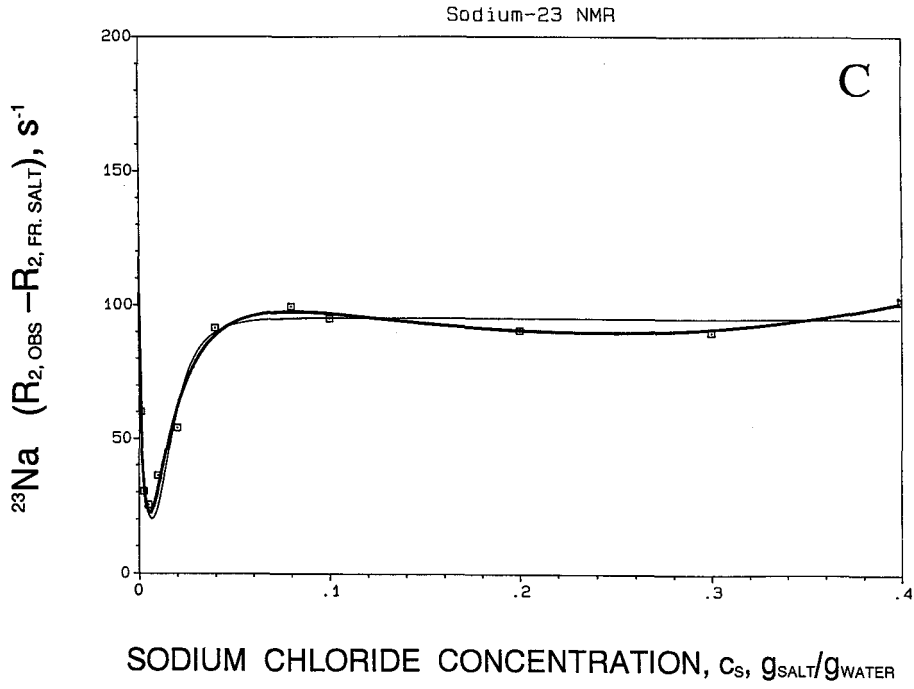

Figure 6C. Comparison of the fitted curves with the salt activity model (Eq. 3) with those obtained by employing the simpler, $\bar{n} = 4$ model (Eq. 2), or by neglecting the virial coefficients B_1 and B_2 in Eq. 3: Salt-variation curves determined by ^{23}Na NMR salt activity model (Eq. 3) versus $\bar{n} = 4$ model

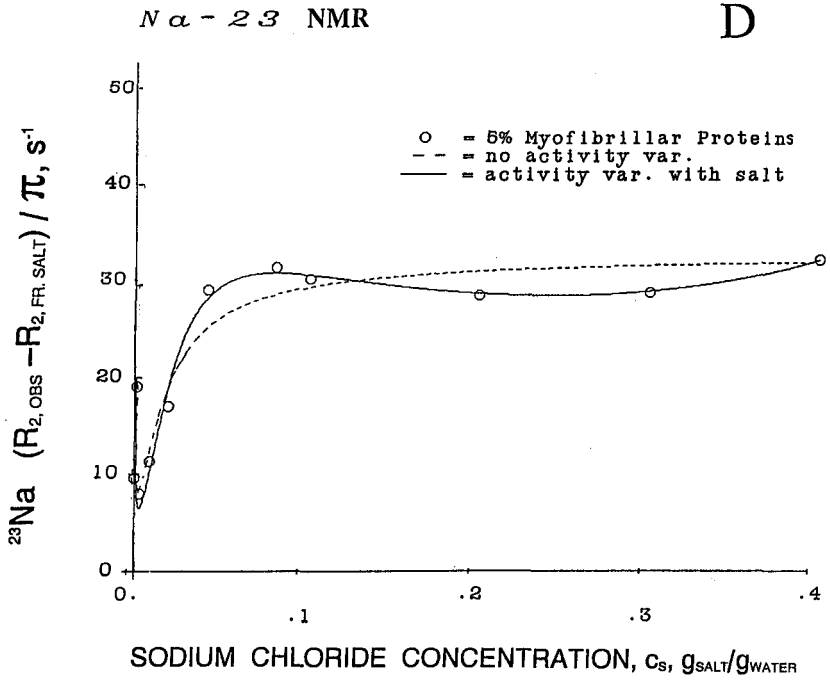

Figure 6D. Comparison of the fitted curves with the salt activity model (Eq. 3) with those obtained by employing the simpler, $\bar{n} = 4$ model (Eq. 2), or by neglecting the virial coefficients B_1 and B_2 in Eq. 3: Salt-variation curves determined by ^{23}Na NMR complete salt activity model (continuous line), compared with a preferential binding model that neglects the virial coefficients of salt activity B_1 and B_2

The parameters determined with these models, as well as the ones obtained from the best fittings with Eq. 3, are summarized in Tables 1 and 2.

Table 1. Parameters derived by nonlinear regression analysis of nuclear spin relaxation data for myofibrillar protein solutions with cooperative ion-binding, n-mer models (Eq. 2)

	Sodium-23				Oxygen-17			
	n=3 Model	SD[a]	n=4 Model	SD[a]	n=3 Model	SD[a]	n=4 Model	SD[a]
R_{2B1}, S^{-1}	141	7	140.6	8	390	10	399	9
R_{2B2}, S^{-1}	109.5	3	107	3	304	5	303	4
k_{1S}	1269	239	1136	233	936	180	823	142
k_{2S}	14.5	1.1	19.9	1.6	19.3	2.5	26.5	2.8

[a]SD = standard deviation

Table 2. Parameters derived by nonlinear regression analysis of nuclear spin relaxation data for myofibrillar protein solutions with NaCl using Eq. 3 for the salt-activity model.

	A. Preferential binding only (B_1=B_2=0.0)				B. Model with salt activity coefficients B_1 and B_2			
	Na-23 NMR	SD[a]	O-17 NMR	SD[a]	Na-23 NMR	SD[a]	O-17 NMR	SD[a]
R_{2B1}, S^{-1}	133	14	270	3	128	10.4	390	9
$(R_{2B2\text{-}R2F})x \cdot x \cdot n_S \cdot c_P$	103	5	96	6.6	160	18	153	23
k_{1S}	987	250	979	236	840	100	858	159
S	0.11	0.04	0.088	0.05	0.14	0.009	0.120	0.015
B_1	0	-	0	-	-3.8	1	-4.5	1.5
B_2	0	-	0	-	6.9	2	8.8	4.0

[a]SD = standard deviation

Preferential binding of Na^+ ions to the myofibrillar proteins increases the electrostatic free energy of the system, and the repulsive forces between these proteins (the B_1 coefficient in Eq. 3).

Previous work that utilized ion selective electrodes has neglected the effect of ion activity on the process of ion binding to muscle proteins[4]. Equation 3 takes into account such effects, as well as the protein activity.

The ^{23}Na NMR transverse relaxation rates of myofibrillar protein solutions and suspensions in D_2O for varying salt concentrations at a fixed protein concentration (5% w/v), were analyzed by assuming that **both** ion **binding and chemical exchange** contribute to the ^{23}Na nuclear spin relaxation.

An interpretation of the data is here proposed in terms of an **association-dissociation** process which is similarly manifested in both the ^{17}O and ^{23}Na NMR dependencies for myofibrillar proteins solutions with varying salt, at a fixed protein concentration (5% w/v).

Discussion

The analysis of ^{17}O and ^{23}Na relaxation data for myofibrillar protein solutions with varying salt concentration was carried out using Wyman's theory of thermodynamic linkage with a ligand-induced association model. Such an analysis yielded parameters that characterize both the hydration and ion binding to myofibrillar proteins. A notable feature of this analysis is that the values of the preferential ion binding coefficient $(\partial m_3/\partial m_2)^2$ obtained from ^{17}O and ^{23}Na NMR (0.029 and 0.022 ml/g, respectively) are very close to each other (within the experimental error). The preferential ion binding coefficient monitors the changes in the free energy of the system induced by the addition of salt[18]. Myofibrillar protein **dissociation**, which was observed at low salt concentrations (Figs. 4B and 4D), is linked to the ion binding process. The agreement between the ^{17}O and ^{23}Na NMR-derived values of the preferential binding coefficients obtained from myofibrillar proteins with varying salt indicates that the hydration water is exchanged as the hydrated ion species in myofibrillar protein solutions.

Another parameter obtained from this thermodynamic linkage analysis is k_{1S}, the apparent **ion binding constant**. The value of k_{1S} is only ~ 15% higher when determined from the ^{17}O NMR data than in the case of ^{23}Na NMR, suggesting that **only ion binding directly linked to hydration** is involved, and that the binding process may involve the apparent association constant (k_{1S}) of Na^+ for carboxyl groups of myofibrillar proteins.

In most protein solutions the electrostatic interactions are suppressed at about 0.1 to 0.15 M salt concentrations[24]; at higher concentrations lyotropic effects of the ions on protein conformation may also occur that also involve hydrophobic interactions. The decrease in the electrostatic repulsion between the nonpolar amino acid residues of proteins may, therefore, induce the formation of n-mer protein aggregates. In the case of myofibrillar proteins in the presence of high NaCl concentrations, repulsive interactions are still present and such

interactions are found to influence both the myofibrillar protein hydration and their preferential ion binding properties.

Conclusions

^{17}O and ^{23}Na NMR transverse relaxation rates of myofibrillar protein solutions and suspensions exhibit complex nonlinear dependencies on salt concentration; such variations, are, however, modeled at all salt concentrations by a relatively simple model for the activity of myofibrillar proteins and ion binding to them. In this model, myofibrillar protein hydration is linked to salt binding in the sense of Wyman's theory of linked functions[25,26]. Ligand(ion)-induced association is highly cooperative and involves binding of $\bar{n}$=4 moles of ions to myofibrillar protein sites. The net average charge of myofibrillar proteins and the preferential binding of ions to myofibrillar proteins make, respectively, important contributions to the B_1 and B_2 virial coefficients of salt activity. Good fits of both ^{17}O and ^{23}Na NMR relaxation data for myofibrillar proteins were obtained with Equation 3.

Furthermore, the values of the preferential ion binding coefficient, $(\partial m_3/\partial m_2)^2$ obtained from the ^{17}O NMR data (Table 1) are close to those obtained from ^{23}Na NMR (Table 2), suggesting that hydration water is exchanged as the hydrated ion species in myofibrillar protein solutions.

It would be reasonable to compare the results for myofibrillar proteins with those for myosin, without expecting their salt variations to be the same; the main reason for making such a comparison is the large proportion (~55%) of myosin found in our myofibrillar protein preparations. Therefore, it is interesting that the salt-induced protein association-dissociation process observed with myofibrillar proteins in electrolyte solutions (as derived from Figs. 4B and 4D) involves the cooperative binding of 4 moles of ions to myofibrillar protein sites. This average $\bar{n}$ value does not exclude, however, the possibility of different n-values for the individual protein species being present in the myofibrillar protein system, as illustrated in Figs. 5A and 5B. Additional experiments with purified myosin may allow one to check the latter possibility and further refine the ligand-induced protein association model that was here presented. ^{35}Cl NMR measurements of chloride binding to myofibrillar proteins would allow one to further specify this thermodynamic linkage model that relates the hydration of myofibrillar proteins to cooperative ion binding.

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