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Multiple-quantum filtered ^{17}O and ^{23}Na NMR analysis of mitochondrial suspensions

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Abstract

The fraction of strongly- and weakly-bound water molecules within mitochondrial suspensions, determined using three-quantum filtered ^{17}O NMR relaxation analysis, was found to be large in comparison with that in erythrocytes and concentrated solutions of bovine serum albumen. It is suggested that bound water, together with regulation of mitochondrial matrix volume, may be an important controlling factor in the modulation of enzymic activity in the matrix. A spin $I = 5/2$ Jeener–Broekaert experiment and a four-quantum filtration experiment were used to demonstrate the absence of orientationally ordered water molecules within the mitochondrion. In contrast, the mitochondrial sodium environment was shown to be highly ordered using a spin $I = 3/2$ Jeener–Broekaert experiment.

Keywords: ^{17}O NMR; ^{23}Na NMR; Mitochondria; Erythrocytes; Multiple-quantum filled NMR

1. Introduction

The mitochondrion occupies a central role in the synthetic activity and energy metabolism of the cell. Knowledge of the physical properties of the mitochondrial environment and how these properties affect processes occurring within it are therefore of central importance toward achieving a more complete understanding of whole cell metabolism in vivo.

The mitochondrial matrix protein concentration has been estimated at more than 50% (w/v) [1]. In comparison, protein crystals used for X-ray diffraction measurements are typically ~40% (w/v) protein [2]. Water in high protein environments such as this can be readily divided into bulk water (osmotically active) and bound water (osmotically inactive) on the basis of osmotic studies of cell and organelle suspensions [3,4].

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This simple classification is usually extended to a three-phase system describing weakly-bound (vicinal), strongly-bound, and bulk or free water [5,6]. Strongly-bound water is hydrogen bonded to macromolecules and, therefore, exhibits long rotational correlation times between 8 and 25 ns [7]. Weakly-bound water molecules are generally associated with surfaces within the solution and have rotational correlation times ranging from 9 to 25 ps [7].

Multiple-quantum filtration NMR experiments provide non-invasive, non-destructive and extremely selective information on the molecular environment of quadrupolar nuclei within the cellular milieu [6,8–15]. In previous work, we quantified weakly- and strongly-bound fractions of water in protein solutions using a regression procedure to analyse the triexponential transverse relaxation profile of the three-quantum filtered signal [6,15]. Here, we extend this procedure to the analysis of mitochondrial and (for comparison) erythrocyte suspensions; the potential biochemical consequences of these results are discussed. In the preceding paper, two NMR pulse sequences have been designed for selective excitation of orientationally ordered $I = 5/2$ nuclei. These experiments have been used to show that ordered ^{17}O-enriched water can be detected in collagen and cartilage samples [16]. Using these methods, we determined whether the three- or five-quantum filtered ^{17}O NMR signal observed in mitochondrial and erythrocyte samples results primarily from triexponential relaxation or from residual quadrupolar splittings. In addition, the mitochondrial sodium environment was investigated using a similar experimental procedure and the results compared with those for human erythrocytes.

2. Procedure for estimation of strongly- and weakly-bound water fractions

The measured three-quantum filtered ^{17}O NMR relaxation decay profile was fitted with a theoretical decay function as described previously [6,15]. This method of fitting requires an estimate of the correlation time (τ_c) of the strongly-bound water in solution and assumes a value for the quadrupolar coupling constant. In our earlier work, we showed that the interpretation of the relaxation profile of protein solutions is affected in a similar fashion by variations in the specified τ_c of the bound water or in the fraction of the strongly-bound water (p_{sb}) [6]. Based on the assumption that strongly-bound water molecules in a heterogeneous system possess a finite range of correlation times and for the purpose of illustration, fitting was performed using a variety of values of τ_c.

Under the conditions used in these experiments, the contribution of vicinal water to the multiple-quantum filtered NMR signal should be insignificant, but it will increase the relaxation rate of the free water considerably. This fact can be used to estimate the fraction of weakly-bound water associated with the cell suspension, as described by Torres et al. [6].

3. Materials and methods

3.1. Materials

Bovine serum albumen (BSA) (Fraction V) was obtained from Sigma Chemical Co., St. Louis, MO, USA. ^{17}O-enriched water (20 at.%) was purchased from CK Gas Products Ltd., Finchampstead, UK. All other reagents were analytical grade.

3.2. Mitochondrial sample preparation

Mitochondria were isolated using differential centrifugation [17]. Australian white rats (three-quantum filtered ^{17}O NMR experiments) and guinea pigs (all other experiments) were sacrificed and their livers removed and homogenised in buffer composed of 250 mM sucrose, 25 mM HEPES, 1 mM EDTA and 0.1% (w/v) BSA, pH 7.4. Cellular debris and erythrocytes were removed from the crude homogenate by centrifugation (3 min at $2500 \times g$). The supernatant was then pelleted and washed twice more by centrifugation (10 min at $10\,000 \times g$). The final suspension was used immediately and tested for respiratory control both before and after the NMR experiment according to the method of Estabrook

[18]. Protein estimation was performed using the Biuret assay [19]. A series of enzymic assays were performed as described by Hovius et al. [20]. The purity of the mitochondrial preparations was assessed visually using transmission electron microscopy. Samples were prepared for electron microscopy according to standard techniques described elsewhere [21]. On the basis of enzyme activity assays and electron micrographs, contamination of the mitochondrial preparation by other organelles was minimal (results not shown).

3.3. Erythrocyte and erythrocyte ghost preparation

Erythrocytes were isolated from whole blood by centrifugation and prepared for the NMR experiment by washing three times in isotonic saline. Erythrocyte ghosts were prepared according to the method of Nash et al. [22]. Erythrocytes were resuspended at 12.5% (v/v) and stored at 0°C. They were then lysed by suspending the erythrocyte solution at a ratio of 1:30 in 'lysing medium' consisting of 7 mM KH_2PO_4, 4 mM $MgSO_4$, 1 mM ATP, plus penicillin and streptomycin (100 U ml^{-1} each) at 0°C and pH 7.4. After 2 min, this mixture was further diluted with a quarter volume of lysing medium and kept at 0°C for a further 5 min. The cells were incubated at 37°C for 1 h, collected (10 min at $20\,000 \times g$), and washed once in saline. The integrity of the ghosts was assessed visually with a light microscope and using a sucrose gradient.

3.4. NMR experiments

^{17}O and ^{23}Na NMR experiments were performed at 298 K on a Bruker AM 400 WB or a MSL 400 spectrometer. Sufficient ^{17}O-enriched water was added to metabolically active samples in order to keep the signal-to-noise ratio high while still retaining the stability of the cells or organelles. Typically, this represented an abundance of approx. 0.7%, which is ~ 20 times the natural abundance for the ^{17}O isotope (0.037%). Respiratory control ratio values were initially greater than four and declined to no less than two during the experiment. Multiple-quantum filtered ^{17}O NMR experiments were performed using pulse sequences described previously [6,9,16].

4. Results and discussion

4.1. Estimation of strongly- and weakly-bound water fractions

In erythrocytes, where effectively all the protein present is haemoglobin, and in prepared protein solutions, the slow motion of bound water may be characterised by a single value of τ_c. The mitochondrial matrix contains no one dominant protein, therefore the proteins in this environment, and the strongly-bound water molecules associated with them, possess a variety of different correlation times. This prevents precise calculation of p_{sb} in mitochondrial suspensions but, because τ_c generally ranges from 8 to 25 ns [7], we have estimated p_{sb} using two values of τ_c (10 and 25 ns) to provide an upper and lower limit, respectively, for p_{sb}, as well as for a value of τ_c equal to 15 ns for convenient comparison with a BSA solution. Fig. 1 shows a typical fit to a three-quantum filtered ^{17}O relaxation profile. The data shown is fitted using a value of 25 ns, but other values of τ_c gave a similarly good fit to the data.

Table 1 shows the fraction of strongly-bound water in erythrocytes, in a BSA solution, and in mitochondrial suspensions, calculated using a variety of values of τ_c. (It is important to stress that, as the nature of the binding sites is not always known, these assumed τ_c values are speculative and represent a probable range only.) For a given value of τ_c, the mitochondrial suspensions have nearly twice the bound water per mg of protein compared with a 30% (w/w) BSA solution. This result is significant given that BSA has a large number of strongly-bound water molecules compared with other proteins that have been measured [6]. No three-quantum filtered signal was detected from the erythrocyte ghost suspension. Since the erythrocyte membrane is composed of 49% protein [23], this means that either the membrane proteins do not generally have strong water binding sites or the three-quantum filtered exper-

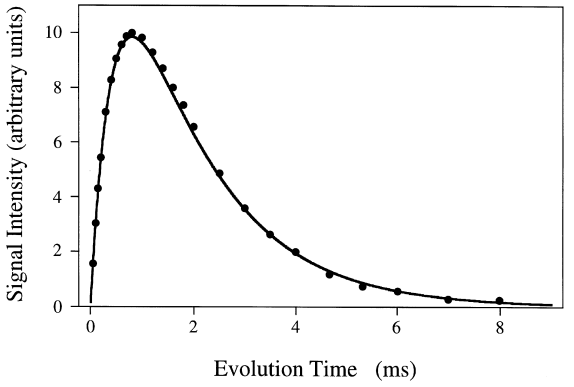

Fig. 1. Curve fit to the three-quantum filtered ^{17}O NMR relaxation profile of a sample of mitochondria suspended at 33 mg ml^{-1}. The free precession interval (evolution time) τ in the three-quantum filtration pulse sequence was increased from 50 μs to 8 ms. Experimental transverse three-quantum filtered signal intensities (●) are plotted as a function of this evolution time. The curve fit (———) was obtained by restricted direct iterative fitting with τ_c equal to 25 ns and a T_1 spin-lattice relaxation rate of 330 s^{-1}. For this sample, p_{sb} was calculated as 0.0986%. This value is then used to obtain the fraction of bound water per mg protein and as a percentage of matrix volume. Similar curves were obtained with the rotational correlation time, τ_c, set to 15 and 10 ns.

iment does not detect the water associated with these proteins. In rat liver mitochondria, 68% of the total mitochondrial proteins are present in the matrix in a soluble form, plus a further 6.4% in intercristal and peripheral spaces; the remaining 25.6% is associated with the inner and outer membranes [24]. Since the membrane components in the mitochondrial preparation will presumably not contribute to the observed three-quantum filtered ^{17}O NMR water signal, the fraction of bound water (calculated per mg of total protein) is likely to be an underestimate of the fraction per mg of soluble protein.

In mitochondrial samples, the fraction of strongly-bound water (Table 1), expressed as a percentage of the matrix volume, ranges from approx. 1 to 2% of the total matrix water volume within the likely range of τ_c values. Similar calculations for a 30% (w/w) BSA solution and for erythrocytes yield values that are roughly between two and six times lower than this. The binding of four oxygen molecules to a haemoglobin molecule is associated with the binding of 60 additional water molecules, yielding an estimated 33.5 kJ mol^{-1} haemoglobin [25,26]. Similar energetic changes could occur in proteins within the matrix if strongly-bound water molecules were displaced or new sites created as a result of protein conformational changes or through association and dissociation reactions. Owing to matrix conformational changes that occur in the course of normal metabolic function, the matrix can occupy between 50% and nearly 100% of the total mitochondrial volume [24]. This would affect the fraction of bound water as a percentage of volume and alter the matrix protein concentration, introducing a concomitant change in the degree of 'non-ideality' in this environment. In numerous cases, 'non-ideality' has been shown to effect the function of enzymes through changes in conformation and association. Generally, increased 'non-ideality' through macromolecular crowding will favour more compact protein structural conformation and also the formation of enzyme complexes; detailed treatments on this subject are available elsewhere [27,28]. In view of the points raised above, it seems reasonable to suggest that mitochondrial matrix volume and bound water may be important controlling factors in regulating enzymic activity in vivo. Scalettar et al. have made similar suggestions based on demonstrated changes in rotational diffusion anisotropy of the fluorescence probe carboxyfluorescein and in effective viscosity due to respiration state and osmolarity [29].

The calculated fraction of weakly-bound water as a percentage of cell volume in erythrocyte ghosts was $7.7 \pm 0.8\%$ compared with $33.0 \pm 4.0\%$ in unaltered erythrocytes. Clearly, weakly-bound water is associated with both macromolecules and biological membranes. Table 1 shows the calculated fractions of weakly-bound water per mg of protein in mitochondrial suspensions compared with erythrocytes and with 30% (w/w) BSA. The large difference in these values is presumably a reflection of the massive membrane surface area present in the folded cristae of the mitochondrial structure (40 m^2 g^{-1} [30]).

If developed and refined further, relaxation analysis of three-quantum filtered ^{17}O NMR sig-

Table 1
Estimation of strongly- and weakly-bound fractions of water using restricted iterative regression of three-quantum filtered ^{17}O NMR relaxation data and *assumed* correlation times

Sample and *assumed* correlation time, τ_c	Strongly-bound water[a] (nmol mg^{-1} protein)	Strongly-bound water[a,b] (% matrix water)	Weakly-bound water[c] (μmol mg^{-1} protein)
Mitochondria ($\tau_c = 10$ ns)	1396 ± 296	2.05 ± 0.54	150 ± 50
Mitochondria ($\tau_c = 15$ ns)	939 ± 204	1.40 ± 0.37	158 ± 51
Mitochondria ($\tau_c = 25$ ns)	568 ± 120	0.85 ± 0.22	166 ± 52
Erythrocytes ($\tau_c = 25$ ns)	202 ± 12	0.27 ± 0.02	19 ± 1
Erythrocyte ghosts	0.0	0.0	—
30% (w/v) BSA ($\tau_c = 15$ ns)	484 ± 15	0.42 ± 0.02	41.2 ± 0.8

[a]A value of 1.1 μl mg^{-1} was assumed for the matrix in situ volume [32]. It was assumed that the solid fraction in the mitochondrion occupies 1.6 μl mg^{-1} protein [33].
[b]This calculation assumes that virtually all the bound water exists in the matrix as the intermembrane space contains only 8.6% of the total soluble protein [24].
[c]The water exchange time for erythrocytes at 25°C is larger than the T_1 of ^{17}O under the conditions of these experiments (13 vs. 5 ms) [34]. For the mitochondrion, the water exchange time is estimated at less than 100 μs [33]. For this reason the internal volume of the erythrocyte was treated as the total volume of solution in the calculation of the percentage of weakly-bound water. The mitochondrial suspension was assumed completely mixed on an NMR timescale.

nals could be used to correlate possible changes in bound water fractions with mitochondrial function. A logical progression of this work would be to demonstrate that the fraction of strongly-bound water molecules is altered from dilute enzyme solutions to conditions of self-association or with the assembly of mixed enzyme complexes.

4.2. Multiple-quantum filtered analysis of sodium in mitochondria and erythrocytes

The existence of sodium ions in anisotropic or ordered environments has been demonstrated previously in several other systems [10,11,13]. In accordance with these observations, a strong ^{23}Na NMR signal was obtained from the mitochondrial sample using the $I = 3/2$ Jeener–Broekaert experiment [11]. Fig. 2 shows three-quantum filtered and Jeener–Broekaert ^{23}Na NMR spectra from mitochondrial and erythrocyte samples. Allowing for the difference in relative sodium concentrations in each sample, the signal in the Jeener–Broekaert experiment is stronger from the mitochondrial preparation than from the erythrocyte suspension. Navon and co-workers showed that this latter signal was largely due to membrane association but was highly dependent on the presence of structural proteins. The large mitochondrial signal is therefore likely to be a reflection of both the massive membrane area of the mitochondrion and of the remarkable degree of cytoskeletal structure within the mitochondrion [10,13,31].

4.3. Detection of ordered water in biological systems

The experiments described in the preceding paper offer a further tool for investigating water structure in vivo. The four-quantum filtration experiment and the $I = 5/2$ Jeener–Broekaert experiment were both applied to mitochondrial suspensions containing ^{17}O-enriched water with no signal observed in either case. This result rules out the possibility that the large signal observed in the three-quantum filtered experiments resulted from the efficient generation of triple-quantum coherence due to residual quadrupolar splittings. The absence of a significant ordered

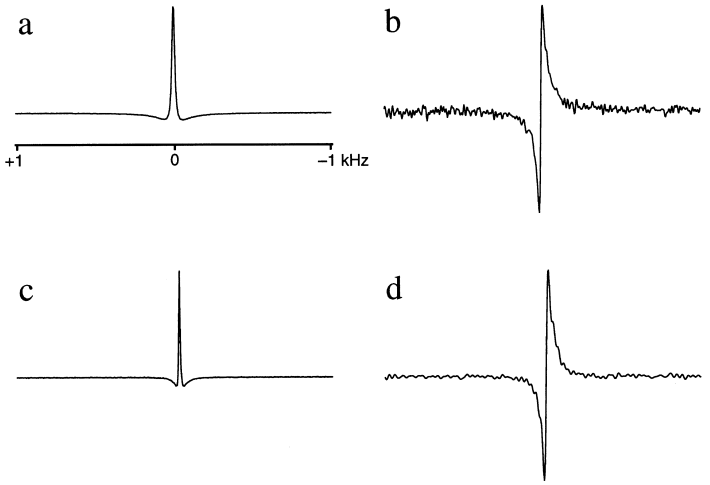

Fig. 2. (a,c) Three-quantum filtered and (b,d) $I = 3/2$ Jeener–Broekaert ^{23}Na NMR spectra of suspensions of (a,b) mitochrondria and (c,d) erythrocytes. Acquisition parameters: 105.8 MHz Larmor frequency, 2016 Hz spectral width; (a) 18432, (b) 18432, (c) 10224 and (d) 10240 transients co-added; (a,b) 17.3 μs and (c,d) 17.7 μs 90° pulse length; (a) $\tau = 3.28$ ms, (b) $\tau = 3.28$ ms, (c) $\tau = 4.00$ ms and (d) $\tau = 3.20$ ms free precession interval.

fraction of water also validates one of the assumptions inherent in the fitting procedure used here, which assumes triexponential relaxation only. However, although the residual ^{17}O quadrupolar splittings appear to be completely quenched, it is possible that they may still contribute to ^{17}O transverse relaxation and this is something that is not as yet included in our three-site exchange model.

5. Conclusions

The fractions of strongly- and weakly-bound water within mitochondrial suspensions were determined using three-quantum filtration NMR relaxation analysis of ^{17}O-enriched water. In comparison with erythrocytes and with concentrated solutions of BSA, the number of strongly- and weakly-bound water molecules was found to be very high. It was suggested that changes in the volume of the mitochondrial matrix may regulate enzyme function thermodynamically by altering the relative fractions of water within this environment. A spin $I = 5/2$ Jeener–Broekaert experiment and a four-quantum filtration experiment were both used to demonstrate the absence of ordered water molecules within the mitochondrion. In contrast, the sodium environment of the mitochondrion was shown to be highly ordered using a spin $I = 3/2$ Jeener–Broekaert experiment.

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