NATURAL ABUNDANCE ^{13}C NMR SPECTRA OF INTACT MUSCLE

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1. Introduction

Much attention has been focused during the past years on the NMR of intact muscle. ^{31}P NMR of muscle [1,2] was the frontrunner of a large number of studies on intact tissue by the non-destructive NMR technique [3–7]. Application of ^{13}C NMR to muscle came somewhat later; in the proton decoupled, natural abundance ^{13}C spectra of mouse muscle at 25.2 MHz, Fung observed 3 peaks which centered around 30,130 and 175 ppm (from tetramethylsilane) and were assigned to aliphatic, aromatic and carbonyl carbons, respectively [8]. Natural abundance ^{13}C spectroscopy at 15 MHz was used subsequently to identify taurine and betaine in adductor muscles of molluscs [9]. Recently, the oxidation of acetate in the Krebs cycle was followed by perfusing heart with [^{13}C]acetate labeled in the methyl carbon [10].

We wish to report the first high resolution natural abundance ^{13}C spectra of intact muscle at 90.5 MHz. The resonances could be assigned to carbons located in phospholipids, proteins and soluble organic compounds of muscle. We also observed that below 10°C in intact muscle the methylene carbons of fatty acids of phospholipids were largely immobilized, but they gained considerable freedom of motion upon raising the temperature of muscle.

2. Materials and methods

2.1. *Preparation of muscle and muscle fractions*

The pectoralis muscle of chicken was excised, cleaned of all visible fat and kept on ice until use. For NMR experiments with intact muscle two muscle pieces, ~1.5 g each, were pressed into the bottom of the 12 mm sample tube. No fluid was added to the muscle for recording the spectrum.

Microsomes were prepared from the pectoralis muscle by homogenization in a solution containing 0.1 M KCl, 0.01 M KP_i (pH 7.0). After centrifugation at 8000 × *g*, the supernatant was centrifuged at 200 000 × *g* and the pellet containing the microsomes was washed once. The soluble proteins of the muscle were prepared from the 200 000 × *g* supernatant. The supernatant was exhaustively dialyzed against distilled water to remove soluble organic non-protein carbon compounds and it was concentrated by freeze drying. The perchloric acid extract of muscle was prepared by homogenization of the pectoralis muscle in 5% $HClO_4$. After centrifugation, the supernatant was neutralized with KOH, and after removal of the potassium perchlorate precipitate, it was freeze dried. A solution of 0.10 M KP_i (pH 7.0) in 25% D_2O was used to suspend the microsomes, or to dissolve the soluble proteins and the perchloric acid extract of muscle for NMR analysis.

2.2. *NMR spectra*

^{13}C NMR spectra were recorded on a Nicolet NT-360 instrument operating in the Fourier transform mode. The instrument was operated in the quadrature mode with an irradiation frequency of 90.546 MHz and a sweep width of ± 10 000 Hz. Radiofrequency pulses of 7–10 μs pulse width (30–50°) and free induction decays of 8000 data points each were used, with 1.2 s recycling time. Dioxane dissolved in D_2O was placed in a capillary and used as an external standard and assigned a value of 67.40 ppm. The D_2O in the reference capillary served as the lock agent. For all spectra bilevel broad-band decoupling was employed with ~8 W decoupling power during the time of pulsing

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and data acquisition and 0.5 W during the delay period. The decoupler irradiation point was set 2.5 ppm downfield relative to tetramethylsilane at 0 ppm (150 Hz square wave modulation on the decoupler). For all spectra the line broadening was 10 Hz.

The temperature of the sample was controlled by flowing nitrogen, which was first cooled with aceton–dry ice and then heated with a heater in the probe, past the tube in the probe. The temperature of the sample was measured by a hand thermometer after scanning was completed and it was found to be ~8–10°C higher than the temperature of the controlled nitrogen flow (22°C) for all samples except the microsome sample in which it was 18°C higher.

3. Results and discussion

Natural abundance ^{13}C spectra of intact chicken pectoralis muscle at 90.5 MHz in 12 mm sample tubes are shown in fig.1. Reasonable spectra may be collected with ~1800 scans (upper and middle parts of fig.1); it takes 35 min for recording these spectra. When the pectoralis muscle was scanned 45 010 times in an overnight experiment, no new peaks appeared, although the resolution of the resonances was greatly improved, especially in the 115–185 ppm region (bottom part of fig.1).

3.1. *Assignment of the resonances*

In order to identify the peaks in spectra of intact muscle, we recorded the spectra of various subfractions of muscle (fig.2). Microsomes were used as a fraction of muscle containing a large amount of phospholipids. The soluble proteins were chosen as a pure protein fraction free of any other substance. The low M_r organic compounds of muscle were represented in the perchloric acid extract. These muscle fractions were used in concentrations which are 10-fold higher than those existing in muscle, thus only major resonances of any of these fractions could contribute appreciably to peaks observed in muscle.

The aliphatic region of the muscle spectrum, 10–75 ppm, has contributions from carbons of lactic acid, phospholipids, and proteins. Two major resonances at 21 and 69 ppm correspond to the methyl and alcoholic carbons of lactic acid, respectively, as identified by the spectrum of pure lactic acid. These resonances are also present in the perchloric acid extract of muscle (bottom part of fig.2) but they are absent from the

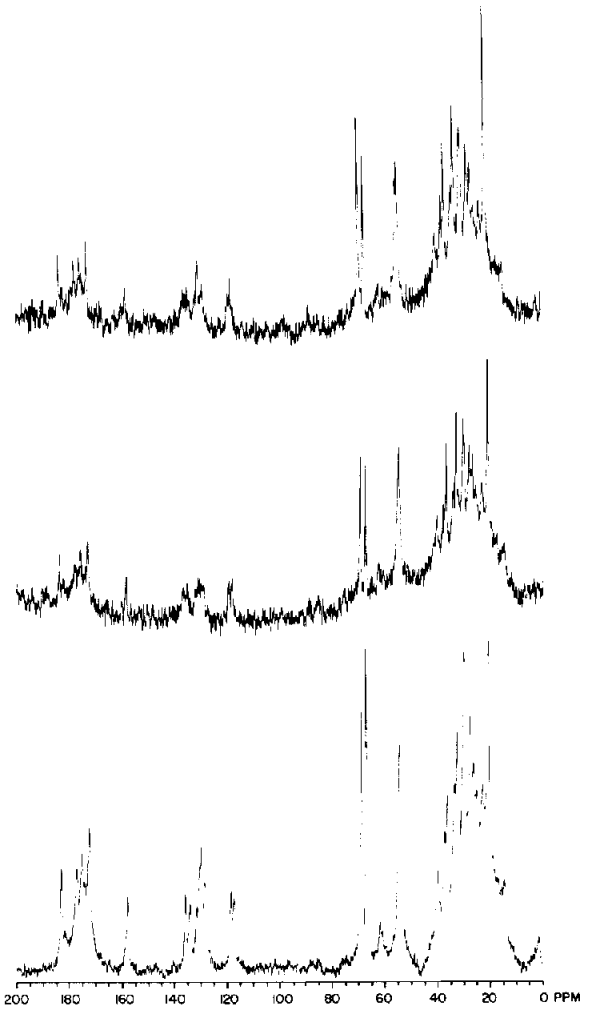

Fig.1. Proton-decoupled 90.546 MHz ^{13}C NMR spectra of intact chicken pectoralis muscle: upper, 1776 scans at 17°C; middle, 1800 scans at 28°C; bottom, 45 010 scans at 31°C. The peak at 67.40 ppm corresponds to that of the dioxane standard.

microsomes (upper part of fig.2). The pure protein fraction of muscle contains a significant peak around 21 ppm (middle part of fig.2). Thus, it appears that the 21 ppm peak in muscle spectrum is the coresonance of lactic acid methyl carbon and the carbons of amino acid side chains of proteins. The γ- and δ-carbons of several amino acids are known to resonate at this frequency [11].

A major peak in the muscle spectrum at 30.0 ppm (fig.1) is almost exclusively due to the $(CH_2)_n$ repeating unit of the fatty acids [12,13] since this peak is

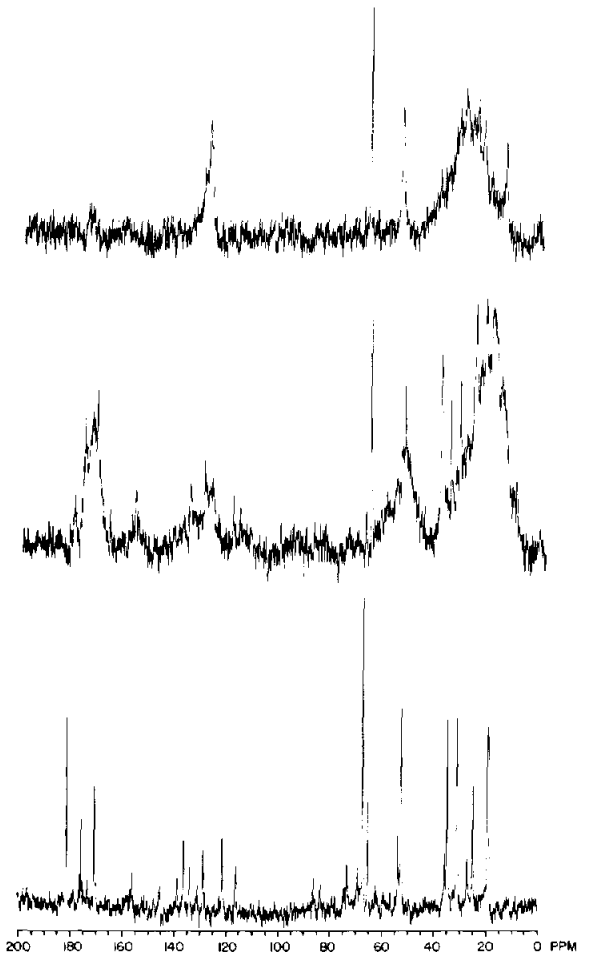

Fig.2. Proton-decoupled 90.546 MHz ^{13}C NMR spectra of various subfractions of chicken pectoralis muscle: upper, microsomes (~100 mg/ml), 2416 scans, 40°C; middle, soluble proteins (~250 mg/ml), 2000 scans, 30°C; bottom, perchloric acid extract (10 g muscle/ml), 1000 scans, 32°C. The peak at 67.40 ppm corresponds to that of the dioxane standard. Note, in the spectrum of perchloric acid extract, the dioxane peak is much smaller than the lactic acid peak at 69.4 ppm.

pronounced in the microsomal fraction, virtually absent in the pure protein fraction and not detectable in the perchloric acid extract (fig.2). In contrast, the muscle peaks at 36.5, 38.0 and 40.0 ppm are derived from proteins or small peptides, i.e., the β-carbons of several amino acid side chains [11]; these peaks are missing from the microsomes, but they are present in the protein fraction and in the perchloric acid extract.

The major peak in the spectrum of muscle which resonates around 54.7 ppm is shared by the methyl carbons of choline [14] and creatine (verified by the pure compounds). The 115–140 ppm region of the spectrum contains several individual peaks (bottom part of fig.1). Out of these peaks, one, the 130 ppm, is a major peak in the microsomal spectrum (fig.2), the other, at 128.5 ppm, appears as a shoulder in the same spectrum. These peaks correspond to single and double bonded allylic carbons in fatty acids [12,13]. Since the characteristic 130–128.5 ppm doublet is not observable either in the soluble proteins or in the perchloric acid extract (fig.2), phospholipids must be the major source for these peaks in muscle. The γ- and δ-carbons of histidine, phenylalanine, tyrosine and tryptophan residues make up the other resonances observed in the 115–140 ppm region of the muscle spectrum [11,15]. The guanidino carbon in phosphocreatine and creatine and arginine residues gives rise to the 158 ppm peak, whereas the peaks in the 170–185 ppm region of the muscle spectrum belong to the carbonyl carbons in the peptide bonds of proteins [11], with the exception of the carboxylic carbon of lactic acid at 183 ppm.

3.2. *Mobility of phospholipids*

Throughout this work we observed consistently that the 30.0 ppm peak, corresponding to the methylene carbons of fatty acids in phospholipids of intact muscle, decreased when the muscle temperature was decreased. The extreme case of this effect was when the muscle spectrum was recorded below 10°C, this lowered the 30.0 ppm peak to the bottom of the aliphatic envelope. On the contrary, raising the temperature of the muscle in the sample tube invariably resulted in the elevation of the 30.0 ppm peak. In the extreme case, the muscle was heated to 60°C in the spectrometer by using the decoupler without temperature control; the 30.0 ppm peak appeared much higher than any other peak including those of lactic acid. A qualitative comparison of the intensity of the 30.0 ppm peak, relative to its neighboring 28.0 and 33.0 ppm peaks, is illustrated in fig.1. At 17°C, the 30.0 ppm peak intensity is between the 28.0 and 33.0 ppm peaks. At 28°C, the 30.0 ppm peak approaches that of the 33.0 ppm, and at 31°C, the 30.0 ppm peak is greater than that of the 33.0 ppm.

A semiquantitative demonstration of the temperature effect was provided by the following experiment. The ^{13}C spectrum of the same pectoralis muscle was recorded at 24, 27.5, 31 and 40°C. The integral values of the 30.0 ppm peak, the 69.4 ppm lactic acid

peak, and the dioxane standard were measured by cutting out and weighing these peaks from Xerox copies of the original spectra. The ratio of the lactic acid peak integral to that of the dioxane standard integral remained constant from 24–40°C. The ratio of the 30.0 ppm peak integral to that of the dioxane standard increased continuously from 24–40°C; the value at 40°C was about twice the value at 24°C. These results suggest that at lower temperature the long fatty acid chains are largely immobilized in the muscle membranes, but upon rise in temperature the restrictive molecular interactions are minimized, thus permitting the increased motion of the aliphatic chains.

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